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Research Article

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Hyaluronan (HA) Fragments Induce Chemokine Gene Expression in Alveolar Macrophages

The Role of HA Size and CD44

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Abstract

Hyaluronan (HA) is a glycosaminoglycan constituent of extracellular matrix. In its native form HA exists as a high molecular weight polymer, but during inflammation lower molecular weight fragments accumulate. We have identified a collection of inflammatory genes induced in macrophages by HA fragments but not by high molecular weight HA. These include several members of the chemokine gene family: macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, cytokine responsive gene-2, monocyte chemoattractant protein-1, and regulated on activation, normal T cell expressed and secreted. HA fragments as small as hexamers are capable of inducing expression of these genes in a mouse alveolar macrophage cell line, and monoclonal antibody to the HA receptor CD44 completely blocks binding of fluorescein-labeled HA to these cells and significantly inhibits HA-induced gene expression. We also investigated the ability of HA fragments to induce chemokine gene expression in human alveolar macrophages from patients with idiopathic pulmonary fibrosis and found that interleukin-8 mRNA is markedly induced. These data support the hypothesis that HA fragments generated during inflammation induce the expression of macrophage genes which are important in the development and maintenance of the inflammatory response. (*J. Clin. Invest.* 1996. 98:2403–2413.) Key words: extracellular matrix • interleukin-8 • inflammation • proteoglycan • pulmonary fibrosis

Introduction

Hallmarks of chronic inflammation and tissue fibrosis are the increased synthesis and degradation of components of the extracellular matrix (ECM).[1] The dynamic turnover of ECM results in the net deposition of matrix in the interstitium of the affected tissues. An additional consequence of the degradation of ECM components is the generation of fragments from larger precursor molecules. In the cases of collagen and fibronectin, fragments generated as a result of proteolytic cleavage exhibit biological activities not attributable to the intact precursor (1, 2). A major component of the ECM that undergoes dynamic regulation during inflammation is the glycosaminoglycan (GAG) hyaluronan (HA). HA is a nonsulfated, linear GAG consisting of repeating units of (β,1→4)-D-glucuronic acid-(β,1→3)-*N*-acetyl-D-glucosamine (3). In its native state, such as in normal synovial fluid, HA exists as a high molecular weight polymer, usually in excess of 10^6 D (3). However, under inflammatory conditions HA has been shown to be more polydisperse, with a preponderance of lower molecular weight forms (4). The accumulation of lower molecular weight forms of HA has been postulated to occur by a variety of mechanisms including depolymerization by reactive oxygen species, enzymatic cleavage, and de novo synthesis of lower molecular weight species (3, 5–8). Several studies have suggested that high and lower molecular weight HA may exhibit different biological effects on cells and in tissues (9, 10).

HA has been suggested to play an important role in a number of biological processes including wound healing (11), embryonic development (12), and tumor growth (13) by providing a provisional matrix for supporting cellular migration and adherence (3). Further evidence has suggested that certain HA functions are mediated by interactions with the recently described HA receptor, CD44 (13, 14). Recent work has provided evidence that, in addition to serving as a structural scaffold, HA may function as a cellular signaling molecule under certain circumstances (15–17). The observation that HA stimulated the expression of the cytokines interleukin-1β and tumor necrosis factor-α raised the possibility that HA may have a role in macrophage activation (18).

Products of activated macrophages play an important role in initiating and maintaining the chronic inflammatory state that is characteristic of disorders such as rheumatoid arthritis and pulmonary fibrosis (19–23). Chemokines represent a

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1. *Abbreviations used in this paper:* BAL, bronchoalveolar lavage; crg-2, cytokine-responsive gene-2; ECM, extracellular matrix; F-HA, fluorescein-labeled Healon GV hyaluronan; GAG, glycosaminoglycan; HA, hyaluronan; HAM, human inflammatory alveolar macrophages; ICAM-1, intracellular adhesion molecule-1; LFA-1, lymphocyte function-associated antigen-1; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; MIP-1β, macrophage inflammatory protein-1β; RANTES, regulated on activation, normal T cell expressed and secreted; RHAMM, receptor for HA-mediated motility.

newly described family of small peptides, many of which are derived from macrophages, which are potent modulators of immune cell function (24). Recent evidence has suggested that several members of the chemokine gene family are expressed at sites of chronic inflammation (20, 25–29) and that many of these are derived from activated macrophages. The mechanisms responsible for macrophage activation in the chronic inflammatory state, however, have not been well characterized. Lipopolysaccharide (LPS) is a potent macrophage activator that mediates acute inflammation but is not generally present at sites of chronic inflammation. Interferon-γ is another major macrophage activating factor which, while present in certain chronic inflammatory conditions, is generally associated with the generation of macrophage killing activity, a property important in the host response to infection. However, recent evidence has suggested that interferon-γ may down-regulate genes that are involved in mediating chronic inflammation and tissue fibrosis (30, 31). We have investigated the hypothesis that fragments of HA generated at sites of inflammation may serve to activate macrophages. The purpose of this study was threefold: (*a*) to identify genes induced in macrophages by HA; (*b*) to determine the role of HA size in mediating gene expression; and (*c*) to determine the role of CD44 in mediating HA-induced gene expression in mouse alveolar macrophages. Using the technique of differential screening we have identified a subset of genes that are induced by both LPS and HA. These include four members of the chemokine gene family: macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP-1β), cytokine-responsive gene-2 (crg-2 [interferon-inducible protein-10]), and murine regulated on activation, normal T cell expressed and secreted (RANTES). Purified high molecular weight HA does not induce chemokine gene expression, but smaller fragments generated in several ways are capable of inducing gene expression in a murine alveolar macrophage cell line (MH-S). We also demonsrate the secretion of MIP-1α and β, RANTES, JE, and KC into the media of HA-stimulated MH-S cells. In addition, we found that HA fragments induce the expression of interleukin-8 (IL-8), another chemokine, in primary alveolar macrophages isolated from patients with idiopathic pulmonary fibrosis (IPF). HA fragments as small as hexamers are able to induce gene expression. Fluorescein-labeled high molecular weight HA, while unable to induce gene expression, does bind to cultured mouse alveolar macrophage cells. This binding is blocked by the active HA fragments and inhibited by anti-CD44 monoclonal antibody. HA-induced chemokine gene expression is inhibited in the presence of anti-CD44 monoclonal antibody but not control antibodies. These results support the hypothesis that HA fragments induce genes whose functions are important in regulating the chronic inflammatory response.

Methods

Sequencing of cDNA clones. The sequence of one strand of each clone was determined by the dideoxy-chain termination method (32) with reagents purchased from United States Biochemicals (Cleveland, OH). Sequence comparisons were made with the EMBL/GenBank/DDBJ data base.

Cells and cell lines. The murine alveolar macrophage cell line MH-S (33) and the human monocytic leukemia cell line THP-1 (34) were purchased from the American Type Culture Collection, Rockville, MD. Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated low-LPS fetal bovine serum and 1% penicillin-streptomycin/1% glutamine (all from Biofluids, Rockville, MD) at 37°C under 5% CO_2. All experiments were carried out in the presence of the LPS inhibitor polymixin B (10 μg/ml) unless stated otherwise in order to exclude the effects of contaminating LPS on inflammatory gene expression.

Chemicals and reagents. Healon GV and Healon (sterile sodium hyaluronate for intraocular use) were a generous gift of Kabi Pharmacia (Upsala, Sweden). Purified hyaluronic acid fragments from human umbilical cords were purchased from ICN Biomedicals, Inc. (Costa Mesa, CA) and also kindly provided by J. Yannariello-Brown (University of Texas, Galveston, TX). The HA-ICN preparation is free of protein (< 2%) and free of chondroitin sulfate (< 3%). Chondroitin 4-sulfate, dermatan sulfate, chondroitin 6-sulfate, heparan sulfate, and *E. Coli* 011:B4 LPS prepared by the Westphal method were obtained from Sigma Chemical Co. (St. Louis, MO). Polymixin B was purchased from Calbiochem (La Jolla, CA). Stock solutions of reagents were free of LPS contamination as determined by the Limulus amoebacyte assay (Sigma Chemical Co.).

Purified rat anti–mouse monoclonal antibodies to CD44 (KM201 [35]), Mac 3 (isotype-matched control antibody for KM201), intracellular adhesion molecule-1 (ICAM-1), and lymphocyte function-associated antigen-1 (LFA-1) were the gift of E. Puré (Wistar Institute, Philadelphia, PA). KM201 hybridoma supernatants were a gift of J. Lesley (Salk Institute, La Jolla, CA), and rabbit anti–rat polyclonal antibody to the receptor for HA-mediated motility (RHAMM) was provided by E. Turley, University of Manitoba (36). The cDNA probe for *JE* (the murine homolog of monocyte chemoattractant protein-1 (MCP-1) [37]) was purchased from the American Type Culture Collection, and the cDNA probe for human IL-8 was a gift of J. Vilcek (New York University Medical Center, New York) (38).

Preparation and purification of HA fragments. Hyaluronan disaccharides were purchased from Sigma. A 35-kD molecular weight HA fragment was provided by J. Yannariello-Brown. HA hexamers were provided by C. Knudson (Rush-Presbyterian-St. Luke's Medical Center, Chicago, IL) (39). Sonication was performed on purified preparations of Healon HA using a Branson Sonifier for 2 min on ice with the output set at the micro tip limit (40). Analysis of the molecular weight of HA preparations was performed as described (41). Briefly, samples were separated by 0.5% agarose gel electrophoresis and visualized with the cationic dye Stains-All (3,3′-dimethyl-9-methyl-4,5,4′,5′-dibenzothiacarbocyanine; Bio-Rad Laboratories, Hercules, CA). A Kontes model 800 scanning densitometer (Kontes, Vineland, NJ) was used for densitometric scanning and HA fragments of known sizes were used for calibration.

Bronchoalveolar lavage (BAL). BAL was performed as previously described (20) in two patients with IPF confirmed by biopsy. A total of 240 ml sterile normal saline at room temperature was instilled in 60-ml aliquots through the bronchoscope and recovered immediately by gentle hand suction applied to the instilling syringe. A differential count was performed on the lavage fluid by Wright-Giemsa stain before centrifugation and repooling of cells and showed 95% macrophages. Pooled lavage fluid was centrifuged at 1500 rpm at 4°C for 10 min, the cell pellet was resuspended in RPMI 1640 without serum or antibiotics. Cell viability was determined using trypan blue exclusion. Alveolar macrophages were purified by adherence to plastic tissue culture dishes for 30 min at 37°C in RPMI 1640 without serum or antibiotics under 5% CO_2, nonadherent cells were removed by aspiration, and adherent macrophages were washed once in 1× sterile PBS. All subsequent experiments were performed in RPMI 1640 without serum or antibiotics.

Northern analysis of mRNA production. RNA was extracted from confluent cell monolayers using 4 M guanidine isothiocyanate and purified by centrifugation through 5.7 M cesium chloride for 12–18 h at 35,000 rpm as described (18). 15 μg of total RNA was electrophoresed under denaturing conditions through a 1% formaldehyde-containing agarose gel and RNA was transferred to Nytran (Schleicher and Schuell, Keene, NH) or Zetaprobe (Bio-Rad Laboratories) hy-

bridization filters. Blots were briefly rinsed in 5 × SSC, RNA was crosslinked to the filter by UV crosslinking (Stratagene, La Jolla, CA), and blots were hybridized overnight with 10^6 cpm/ml of ^{32}P-labeled DNA labeled by the random prime method (Amersham, Arlington Heights, IL). After hybridization, blots were washed once in 2 × SSC/0.1% SDS at room temperature for 30 min with shaking, then washed twice in 0.1 × SSC/0.1% SDS at 50°C with shaking (30 min each wash). Blots were exposed at −70°C against Kodak XAR diagnostic film. Differences in RNA loading were documented by hybridizing selected blots with ^{32}P-labeled cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Densitometric scanning was performed using a Molecular Dynamics Personal Densitometer SI (Sunnyvale, CA).

Determination of chemokine protein production. MH-S cells were plated at a densitiy of 5 × 10^6/ml and after stimulation with HA (200,000 D) supernatants were collected after 18 h. ELISA measurements were performed as previously described (42, 43).

Flow cytometry. Healon GV hyaluronan was labeled with fluorescein according to published methods (42). HA (10 mg) was dissolved in 8 ml ddH_2O and added to 4 ml DMSO. A mixture of acetaldehyde (5 μl)/cyclohexylisocyanide (5 μl)/fluoresceinamine (5 mg; all from Aldrich, Milwaukee, WI) in 0.3 ml DMSO was added to the HA and the mixture was incubated at room temperature with stirring for 5 h. This was added to 160 ml cold 100% EtOH and precipitation was performed with ~2 ml saturated NaCl. Precipitates were centrifuged at 3000 rpm for 2 min, pooled in 10 ml ddH_2O, and added to 100 ml cold 100% EtOH. Centrifugation was repeated and precipitates were resuspended in 5 ml ddH_2O and dialyzed exhaustively against PBS/azide. Titration of the product was done experimentally using MH-S cells and a dilution of 1:3200 was determined to be half maximal.

The presence of CD44 on MH-S cells and CD44 and CD14 on THP-1 cells was detected as described (43, 44). Cells were washed in PBS, triturated to disrupt aggregates, and suspended in 10% normal goat serum in PBS for a final concentration of 2 × 10^6 cells/ml. 100 μl cells were added to 100 μl of either control solution (RPMI for MH-S cells, 20 μg/ml mouse IgG1 isotype-matched control antibody for THP-1 cells) or antibody (25 μg/ml of KM201 for MH-S cells, H4C4 hybridoma supernatant containing anti-CD44 monoclonal antibody or 20 μg/ml of the anti-CD14 monoclonal antibody 63D3 for THP-1 cells) (44) and incubated on ice for 45 min. Cells were washed twice with 2 ml cold PBS/0.1% BSA/0.1% sodium azide (PBS/BSA/AZ), stained with 100 μl FITC-rabbit anti–rat serum (20 μg/ml, Vector Labs, Burlingame, CA [MH-S cells]) or FITC-F(ab)′$_2$ goat anti–mouse (20 μg/ml) (THP-1 cells; provided by J. Hildreth, Johns Hopkins University, Baltimore, MD) in 10% normal goat serum in PBS, and placed on ice for 45 min. Cells were then washed with 2 ml cold PBS/BSA/AZ and suspended in 0.5 ml PBS. 5,000 cells per run were analyzed on an automatic flow cytometer (FAScan®; Beckman Instruments, Inc., Fullerton, CA). The negative gate was set using control cells for which primary antibody was omitted.

Binding of fluorescein-labeled HA (F-HA) to cells was assessed in an analogous fashion. Cells were prepared as above, then 100 μl F-HA was added and incubated on ice for 45 min. Cells were washed with 2 ml PBS/BSA/AZ, resuspended and analyzed by flow cytometry exactly as described above. The negative gate was set using fluorescence of cells treated with all but F-HA.

Results

Identification of HA-inducible genes in murine alveolar macrophages. The observation that HA induced the expression of interleukin-1β and tumor necrosis factor-α suggested there may be overlap between HA-inducible and LPS-inducible genes (18). We have previously described the cloning of the murine *RANTES* gene by differential screening (45). In brief, a cDNA library was prepared from total RNA isolated from the murine macrophage-like cell line RAW 264.7 after stimulation for 3 h with 100 ng/ml of LPS in the presence of cycloheximide (10 μg/ml). Differential plaque hybridization was performed using cDNA probes prepared from RAW 264.7 cells stimulated for 3 h with LPS in the presence of cycloheximide as described above and from RAW 264.7 cells incubated for 3 h in cycloheximide alone. As described (45), 32 immediate early genes were cloned. To identify the subset of mRNAs encoding these clones which were also inducible by HA, we prepared total RNA from the murine alveolar macrophage cell line MH-S stimulated with HA fragments for 3 h and performed Northern analysis with the LPS-inducible cDNAs as probes. Four of the cDNA clones that were sharply induced were sequenced and homology searches were done. Matches were found between the HA-inducible mRNAs and MIP-1α, MIP-1β, crg-2, and RANTES. In addition, having determined that four members of the chemokine family were induced by HA, we obtained the probe for murine MCP-1 from the American Type Culture Collection and found that this gene was also induced by HA. The time course for induction of chemokine mRNA expression in response to human umbilical cord HA are shown in Fig. 1, *a* and *b.* HA-induced chemokine mRNA synthesis is not inhibited in the presence of cycloheximide, suggesting that these are primary response genes (not shown). The effect of increasing concentrations of HA on chemokine mRNA synthesis is shown in Fig. 1 *b.* The concentration yielding a maximum response was 100 μg/ml. The effect of structurally related GAGs on chemokine gene expression is shown in Fig. 1 *c.* No other GAG, including heparin and heparan sulfate, induced chemokine gene expression in MH-S cells or RAW 264.7 cells (Fig. 1 *c* and data not shown). In addition, neither of the individual saccharide moieties D-glucuronic acid or *N*-acetyl-D-glucosamine, either separately or in combination, induced chemokine mRNA synthesis (Fig. 1 *c* and data not shown). It is important to point out that the MH-S cells, which are simian virus 40 transformed normal resident murine alveolar macrophages, are adherent. Adherence alone has been shown to induce cytokine gene expression (46). However, as shown in Fig. 1 *a*, the constitutive expression of chemokine gene products is low. Thus this system allows for the study of gene induction by a soluble stimulus on macrophages which are already adherent as distinct from adhesion-induced gene expression.

The role of HA size in the stimulation of inflammatory gene expression. We investigated various preparations of HA for activity in inducing chemokine gene expression and found that not all were active. Opthalamic Healon, Healon GV, and HA derived from rooster comb all failed to induce chemokine gene expression, whereas HA derived from human umbilical cord was active. Characterization of the different preparations revealed a marked difference in molecular weight. The inactive Healon preparations had a molecular weight in excess of 10^6 D, whereas the active preparations had molecular weights less than 5 × 10^5 D. Active preparations included a purified and well characterized HA preparation from umbilical cord provided by J. Yannariello-Brown (47). These results suggested that HA size may be an important contributor to bioactivity. To investigate this possibility further and exclude the possibility of gene induction by a contaminant present only in the umbilical cord HA preparations, we depolymerized purified, high molecular weight Healon HA by sonication and determined the molecular weight of the resulting fragments. As shown in

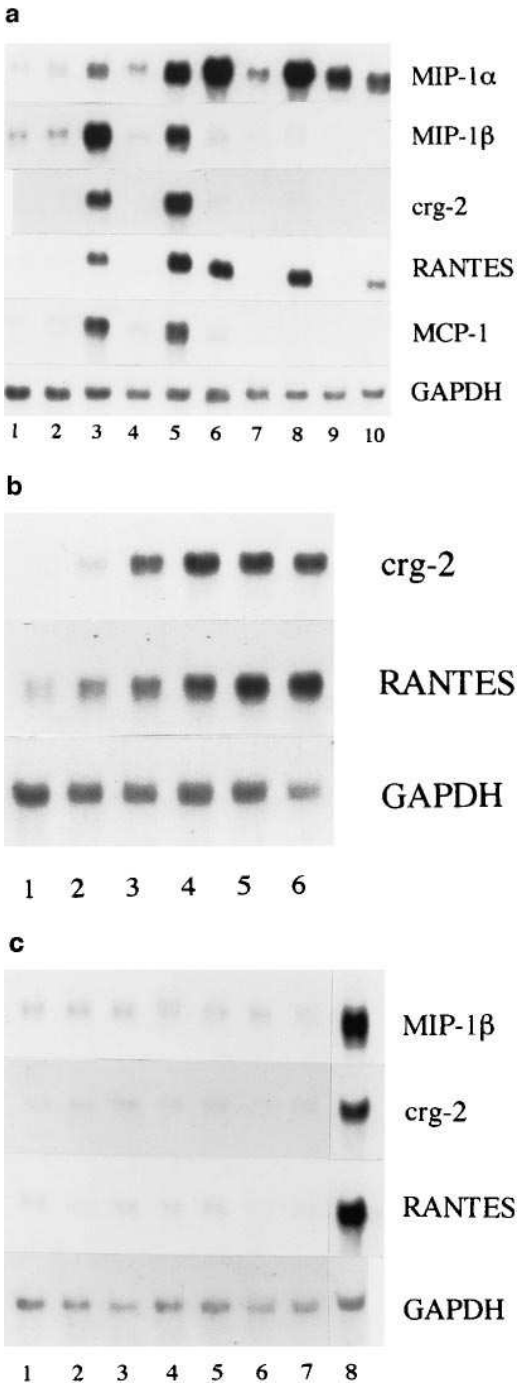

Figure 1. HA-induced chemokine gene expression in MH-S cells. (*a*) Time course of HA-induced chemokine gene expression in MH-S cells. MH-S cells were stimulated with purified HA fragments (100 μg/ml) at 37°C for 0 h (lane *1*), 1 h (lane *2*), 3 h (lane *3*), 6 h (lane *5*), 12 h (lane *6*), 24 h (lane *8*), and 48 h (lane *10*). Lanes *4*, *7*, and *9* represent cells stimulated with LPS (100 ng/ml) for 3, 12, and 24 h, respectively (all medium contained the LPS inhibitor polymixin B [10 μg/ml]). Total RNA was isolated and Northern analysis performed as described in Methods. (*b*) Dose response of chemokine gene induction by HA fragments. MH-S cells were stimulated with purified HA fragments at concentrations of 0 μg/ml (lane *1*), 10 μg/ml (lane *2*), 25 μg/ml (lane *3*), 50 μg/ml (lane *4*), 100 μg/ml (lane *5*), and 1000 μg/ml (lane *6*) for 4 h at 37°C. Total RNA was isolated and Northern analysis performed as described in Methods. (*c*) Structurally related GAGs do not induce chemokine gene expression in MH-S cells. MH-S cells were stimulated with medium alone (lane *1*), LPS (100 ng/ml, lane *2*), or 1 mg/ml of either D-glucuronate (lane *3*), *N*-acetyl glucosamine (lane *4*), chondroitin 4-sulfate (lane *5*), dermatan sulfate (lane *6*), chondroitin 6-sulfate (lane *7*), or purified HA fragments (lane *8*). All conditions were incubated for 4 h at 37°C, then total RNA was isolated and subjected to Northern analysis as described.

Fig. 2 *a*, the original Healon preparation had a molecular weight of 6×10^6 D, whereas the sonicated Healon was reduced in size to a peak molecular weight of 4.7×10^5 D. In addition, a purified umbilical cord HA preparation (HA-ICN) was sized at 2.8×10^5 D.

To assess biological activity, we stimulated macrophages with these various preparations and analyzed gene expression by Northern analysis. As shown in Fig. 2, *b* and *c*, Healon HA was inactive whereas the sonication-derived Healon HA fragments and increasing concentrations of HA-ICN were able to induce chemokine mRNA synthesis. The role of HA size is further characterized in Fig. 2 *c*, in which MH-S cells were stimulated with HA fragments of increasing size and total RNA was analyzed for chemokine gene expression. The experimental medium contained polymixin B, and as shown, LPS failed to induced chemokine mRNA synthesis. This excludes contaminating LPS as a stimulus. HA disaccharides were unable to induce gene expression, but HA hexamers derived from rooster comb (supplied by C. Knudson, Rush Medical School, Chicago, IL) stimulated chemokine gene expression in a dose responsive manner. A 35-kD HA fragment prepared by acid hydrolysis of umbilical cord HA (provided by J. Yannariello-Brown) was also active. Of note, the 280-kD HA-ICN preparation was more potent than comparable concentrations of the smaller fragments.

Hyaluronan fragments induce chemokine gene expression in inflammatory human alveolar macrophages. We sought to extend our observation that HA fragments induce chemokine gene expression in a mouse alveolar macrophage-like cell line and primary bone marrow-derived macrophages to human inflammatory alveolar macrophages (HAM). IPF is a progressive interstitial lung disease characterized by the persistence of activated macrophages in the alveolar space (48). Several studies have shown that alveolar macrophages from IPF patients obtained by BAL express increased levels of IL-8 (20, 49). We examined the effects of high molecular weight HA and HA fragments on HAM expression of IL-8 mRNA from two patients with lung biopsy proven IPF. As shown in Fig. 3, there is baseline expression of IL-8 mRNA in IPF HAM after adherence to plastic culture dishes. High molecular weight HA has no effect on this baseline level, but HA fragments induce a marked increase in IL-8 gene expression.

Soluble HA binds to MH-S cells. To investigate the relationship between HA binding and signaling we labeled high molecular weight Healon HA with fluorescein and studied its interaction with MH-S cells. Labeled Healon HA, like unlabeled Healon HA, does not induce synthesis of chemokine mRNA in MH-S cells (data not shown). Using flow cytometry we determined that soluble Healon HA binds to MH-S cells in a dose-dependent fashion (Fig. 4 *a*). Binding could be competed away completely by unlabeled, biologically active HA fragments from human umbilical cord (Fig. 4 *b*), or by unlabeled Healon HA (data not shown). These data show that even though high molecular weight Healon HA does not engage the

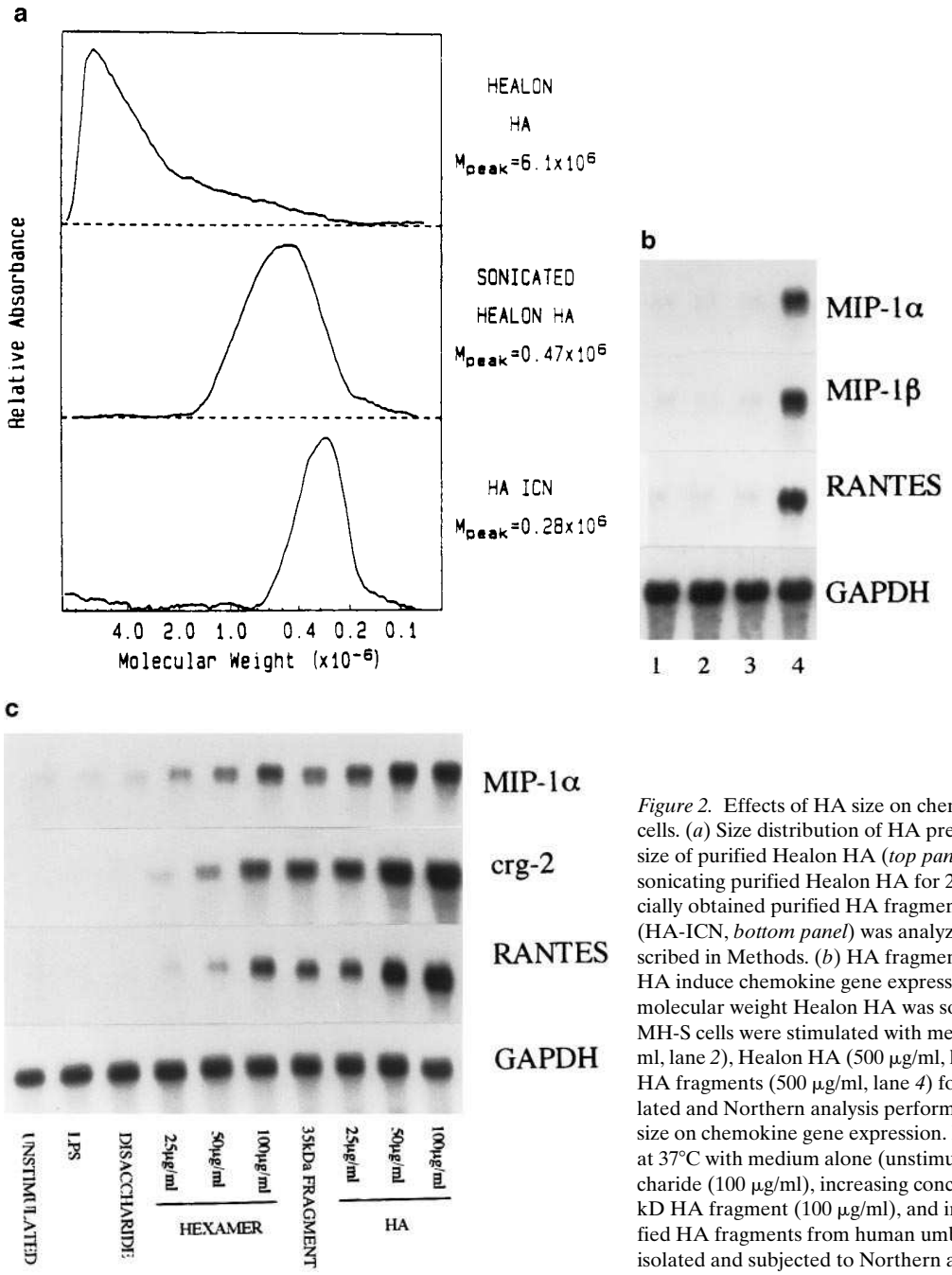

Figure 2. Effects of HA size on chemokine gene expression in MH-S cells. (*a*) Size distribution of HA preparations. The molecular weight size of purified Healon HA (*top panel*), HA fragments generated by sonicating purified Healon HA for 2 min (*middle panel*), and commercially obtained purified HA fragments from human umbilical cord (HA-ICN, *bottom panel*) was analyzed by gel electrophoresis as described in Methods. (*b*) HA fragments but not high molecular weight HA induce chemokine gene expression in MH-S cells. Purified high molecular weight Healon HA was sonicated as described in Methods. MH-S cells were stimulated with medium alone (lane *1*), LPS (100 ng/ml, lane *2*), Healon HA (500 μg/ml, lane *3*) or the sonication-generated HA fragments (500 μg/ml, lane *4*) for 4 h at 37°C. Total RNA was isolated and Northern analysis performed as described. (*c*) Effect of HA size on chemokine gene expression. MH-S cells were stimulated for 4 h at 37°C with medium alone (unstimulated), LPS (100 ng/ml), HA disaccharide (100 μg/ml), increasing concentrations of HA hexamer, a 35-kD HA fragment (100 μg/ml), and increasing concentrations of purified HA fragments from human umbilical cord (HA). Total RNA was isolated and subjected to Northern analysis as described.

signal transduction pathway required to induce gene expression, it is not because of an inability to bind MH-S cells. The ability of active HA to compete away inactive Healon suggests that they recognize the same macrophage cell surface receptor(s).

HA binding and induction of chemokine gene expression occurs in part through CD44 in MH-S cells. We investigated the role of CD44 in mediating HA binding and signaling in MH-S cells. MH-S cells express abundant cell surface CD44 (Fig. 5 *a*), and an anti-CD44 monoclonal antibody (KM201) completely inhibits the binding of fluorescein-labeled HA to MH-S cells (Fig. 5 *b*). Binding is not inhibited by isotype matched control antibody (anti-Mac 3). Polyclonal antibodies to another well-characterized HA cell surface receptor, RHAMM (50, 51), also fail to reduce binding between soluble HA and MH-S cells (Fig. 5 *c*). The adhesion molecule ICAM-1 has recently been described as having HA binding activity (52), and we examined its role as well as that of the receptor for ICAM-1, LFA-1, in our system. As shown in Fig. 5 *c*, antibodies to ICAM-1 and LFA-1 have no effect on HA binding to MH-S cells. Since β_2 integrins have been shown to mediate adhesion-induced gene expression (53), this suggests that the signal transduction pathway induced by HA is distinct from that induced by adhesion.

The effect of anti-CD44 monoclonal antibody on HA fragment-induced gene expression is shown in Fig. 6, *a* and *b.* HA-

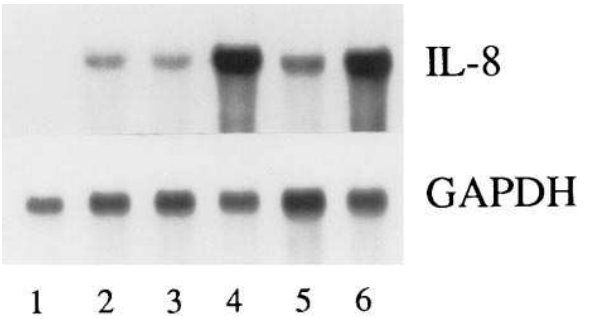

Figure 3. HA fragments but not high molecular weight HA induce chemokine gene expression in human inflammatory alveolar macrophages (HAM). HAM were obtained by BAL from two patients with IPF as described in Methods. Total cellular RNA was extracted from HAM obtained from patient 1 (4×10^6 cells per experimental condition) before plating onto plastic tissue culture dishes (lane *1*) and following a 5.5-h stimulation at 37°C with medium alone (lane *2*), Healon GV HA (100 μg/ml; lane *3*), or purified HA fragments (100 μg/ml; lane *4*). Total cellular RNA was extracted from HAM obtained from patient 2 (2.4×10^6 cells per experimental condition) following a 6-h stimulation at 37°C with medium alone (lane *5*) or purified HA fragments (100 μg/ml; lane *6*). Northern analysis was performed as described.

induced chemokine mRNA synthesis was significantly reduced in the presence of the anti-CD44 monoclonal antibody KM201 but not in the presence of isotype matched control antibody (anti-Mac 3), monoclonal antibodies to ICAM-1 or LFA-1, or polyclonal antibodies to RHAMM (data not shown). Inhibition of HA-dependent chemokine gene expression by anti-CD44 monoclonal antibodies was further enhanced in the presence of high molecular-weight HA (Fig. 6 *c*). It is interesting to note that in addition to potentiating the CD44-specific inhibition observed with monoclonal antibody, high molecular weight HA alone appears to block chemokine gene expression in a nonspecific manner. It is important to point out that HA-induced gene expression is not completely inhibited by antibody to CD44, suggesting that additional receptor(s) may be involved in mediating HA signaling in MH-S cells. In fact, at higher concentrations of KM201 (up to 100 μg/ml) there is a loss of inhibition (data not shown).

HA-induced chemokine gene expression occurs independently of CD14 expression. We sought to determine if CD14, the LPS receptor, was involved in mediating the HA effect since LPS is capable of inducing all of the chemokine genes studied here. CD14 was originally described as a differentiation antigen on monocytes, but it has since been recognized as the receptor for the complex of LPS with lipopolysaccharide binding protein (54, 55). The human monocytic leukemia cell line THP-1 possesses CD44 but expresses essentially no CD14 as demonstrated by flow cytometry in Fig. 7 *a.* We incubated THP-1 cells with LPS and purified HA fragments in the absence of polymixin B and found that chemokine gene expression was induced in response to HA but not in response to LPS (Fig. 7 *b*). Thus, CD14 is not required for HA-induced gene expression in murine macrophages.

HA fragments stimulate chemokine protein secretion in MH-S cells. To determine if HA fragment stimulation of chemokine gene expression resulted in protein secretion, MH-S cells were stimulated with HA fragments for 18 h and supernatants were collected and chemokine prtotein measurements determined by ELISA. As shown in Fig. 8, MIP-1α and β, RANTES, JE (MCP-1), and KC protein levels were detected at 18 h.

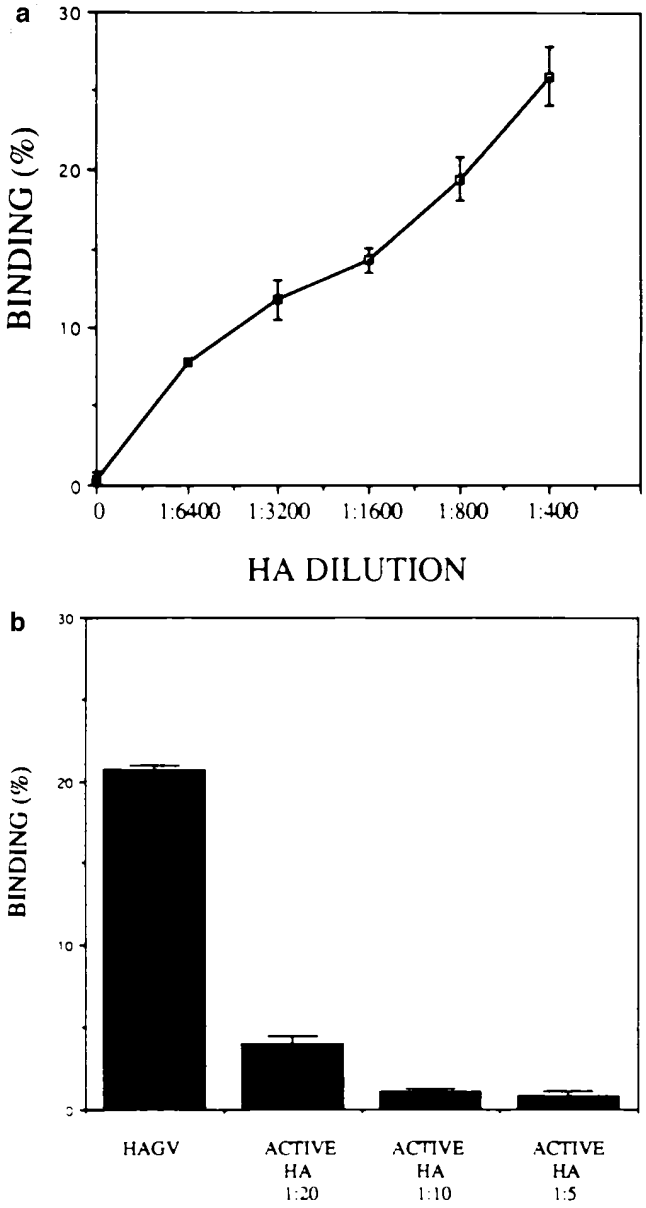

Figure 4. Soluble HA binds to MH-S cells. (*a*) Soluble HA binds to MH-S cells in a dose dependent fashion. Healon GV HA was labeled with fluorescein as described in Methods. MH-S cells were incubated with increasing concentrations of labeled Healon GV HA (initial concentration of labeled HA was estimated to be 1 mg/ml, assuming a yield of 50% from HA labeling) and binding was determined by flow cytometry as described in Methods. Shown are results representative of three separate experiments. (*b*) Unlabeled HA fragments compete away binding of labeled HA to MH-S cells. MH-S cells were incubated with fluorescein-labeled Healon GV HA at a dilution of 1:3200 either alone or in the presence of increasing concentrations of unlabeled purified HA fragments from human umbilical cord (Active HA; initial concentration 5 mg/ml). Binding was determined by flow cytometry as described.

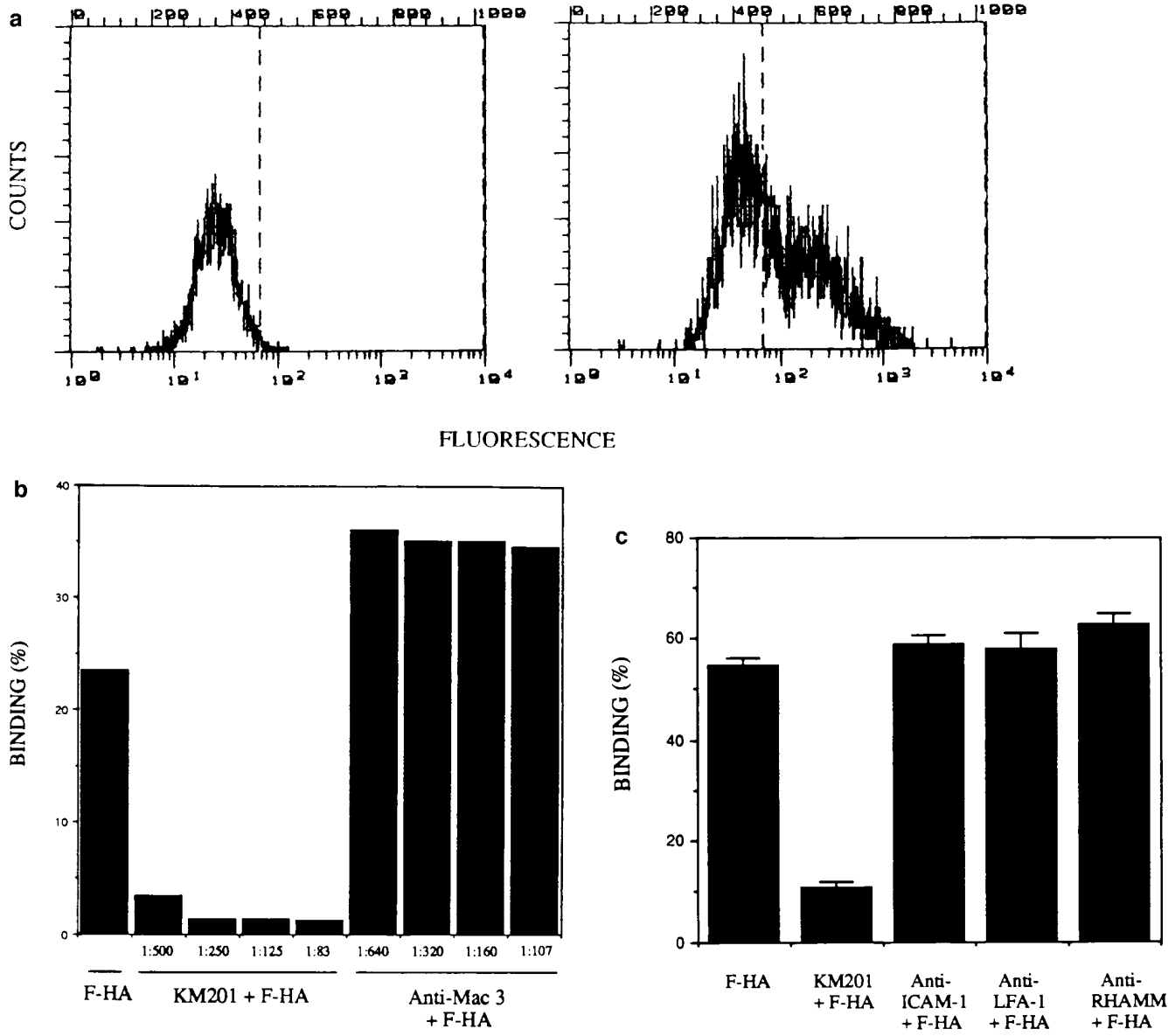

Figure 5. Binding of HA to MH-S cells occurs via CD44. (*a*) MH-S cells express CD44 on their cell surface. As described in Methods, MH-S cells were incubated for 45 min with either medium alone (*left panel*) or the anti-CD44 monoclonal antibody KM201 (25 μg/ml, *right panel*). Cells were stained with fluorescein-labeled rabbit anti–rat serum (20 μg/ml) and fluorescence was detected by flow cytometry. (*b*) Binding of HA to MH-S cells is blocked by monoclonal antibody to CD44. MH-S cells were incubated with fluorescein-labeled Healon GV HA (F-HA) at a dilution of 1:800 either alone or in the presence of the indicated dilutions of KM201 or isotype matched control antibody (anti-Mac 3). The initial concentration of F-HA was estimated to be 1 mg/ml, assuming a yield of 50% from HA labeling, and initial concentrations of KM201 and anti-Mac 3 were 2.5 and 3.2 mg/ml, respectively. Binding was determined by flow cytometry as described in Methods. (*c*) Binding of HA to MH-S cells is unaffected by antibodies to the adhesion molecules ICAM-1 and LFA-1 and to the HA receptor RHAMM. MH-S cells were incubated with either F-HA alone at a dilution of 1:160, F-HA plus 25 μg/ml of KM201 or monoclonal antibodies to ICAM-1 or LFA-1, or F-HA plus a 1:10 dilution of polyclonal antibody to RHAMM. Binding was determined by flow cytometry as described.

Discussion

We provide evidence here supporting the hypothesis that fragments of the ECM component HA are capable of activating macrophages and inducing the expression of genes whose functions are relevant to chronic inflammation. The dynamic nature of the ECM, particularly during stages of tissue development and inflammation, is just beginning to be appreciated, and a great deal of recent research has focused on the effects of ECM on effector cell function (56–58). One general concept which has emerged from these studies is that fragments or degradation products of ECM components may acquire activity not possessed by their larger, precursor molecules (1, 2, 10). This process allows for the recruitment of biologically active ECM fragments from the intact ECM when needed, for example during periods of tissue differentiation or inflammation. As with other ECM components, HA turnover and degradation increase during inflammation and lower molecular weight spe-

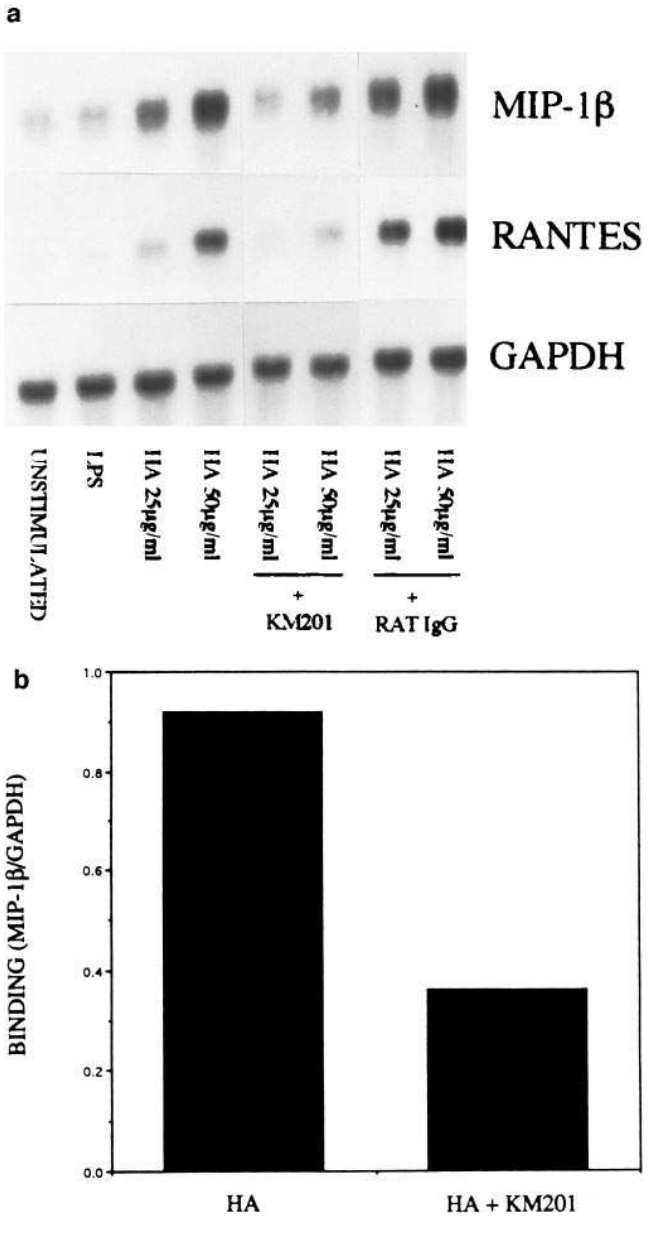

Figure 6. Role of CD44 in HA-induced chemokine gene expression by MH-S cells. (*a*) HA-induced chemokine gene expression is inhibited by monoclonal antibody to CD44. MH-S cells were stimulated for 4 h at 37°C with medium alone (unstimulated), LPS (100 ng/ml), the indicated concentrations of purified HA fragments alone, HA fragments plus the anti-CD44 monoclonal antibody KM201 (25 μg/ml), or HA fragments plus nonspecific rat IgG (25 μg/ml). Cells which received antibody were preincubated with 25 μg/ml KM201 or rat IgG in 1× PBS for 45 min at 4°C before stimulation with HA fragments. Total RNA was then isolated and Northern analysis was performed as described in Methods. (*b*) MH-S cells were stimulated with 25 μg/ml purified HA fragments either alone or in the presence of KM201 (25 μg/ml) for 4 h at 37°C. Cells which received antibody were preincubated with 25 μg/ml KM201 in 1× PBS for 45 min at 4°C before stimulation with HA fragments. Total RNA was recovered and Northern analysis performed as described. Results are expressed as a ratio of MIP-1β expression to GAPDH expression as determined by densitometric scanning. Shown is the average of the results of four separate experiments. (*c*) Evidence for a CD44-independent component of HA fragment-induced gene expression. MH-S cell were stimulated with HA fragments (50 μg/ml) for 4 h in the presence of high molecular weight Healon HA and KM201 anti-CD44 mAb (25 μg/ml) or isotype matched control. Total RNA was then isolated and Northern analysis was performed as described in Methods. Media alone (lane *1*); HA fragments (lane *2*); HA fragments + Healon HA (1 mg/ml) (lane *3*); Healon HA alone (lane *4*); HA fragments + control Ab (lane *5*); HA fragments + KM201 (lane *6*); HA fragments + Healon HA + KM201 (lane *7*).

cies of HA accumulate. Importantly, this accumulation is detected prior to the influx of inflammatory cells and deposition of collagen (59, 60).

Lower molecular weight HA may accumulate through several mechanisms. For example, HA can be depolymerized by reactive oxygen species (5), and while it is not known if this occurs in vivo it has been suggested as a mechanism for generating polydisperse HA in inflammatory arthritis (61). Degradation by hyaluronidase may generate HA fragments as well, since this enzyme is an intracellular glycosidase which requires low pH and could be released upon cell death at sites of inflammation. Additionally, lung fibroblasts have been shown to release hyaluronidase after stimulation with interferon-γ and tumor necrosis factor-α (8). Enzymatic cleavage by secreted acid hydrolases such as β-glucuronidase and hexoseaminadase, both of which can cleave HA, is another potential mechanism, as is de novo synthesis of low molecular weight HA. Several studies have shown that fibroblasts and smooth muscle cells synthesize HA in response to stimulation with cytokines and growth factors, and molecular weight analysis shows that the newly synthesized HA is in the size range that we have found to be biologically active (8, 62).

Macrophages are believed to be crucial to the development and maintenance of chronic inflammation, and among the mediators produced by activated macrophages are the chemokines MIP-1α, MIP-1β, crg-2, MCP-1, IL-8, and RANTES. Considerable evidence has accumulated in recent years implicating members of the chemokine family in the pathogenesis of inflammatory disorders. For example, increased expression of MIP-1α, MIP-1β, MCP-1, and IL-8 has been demonstrated in inflammatory lung diseases and rheumatoid arthritis (20, 25–29, 49). Chemokines are generally believed to serve primarily as chemoattractants for subpopulations of leukocytes (63). However, recent studies have provided evidence that in addition, chemokines may modulate leukocyte functions and under certain circumstances promote or inhibit angiogenesis (64, 65).

The mechanisms that regulate the expression of chemokines in chronic inflammation have not been well described. Our results suggest that one previously uncharacterized mechanism of regulation may involve the generation of biologically active fragments of HA. High molecular weight HA is inactive, but lower molecular weight fragments of the size range found at sites of inflammation acquire the capacity to induce the expression of chemokine genes in both a murine alveolar macrophage cell line as well as in primary alveolar macrophages isolated from patients with IPF. The ability of HA frag-

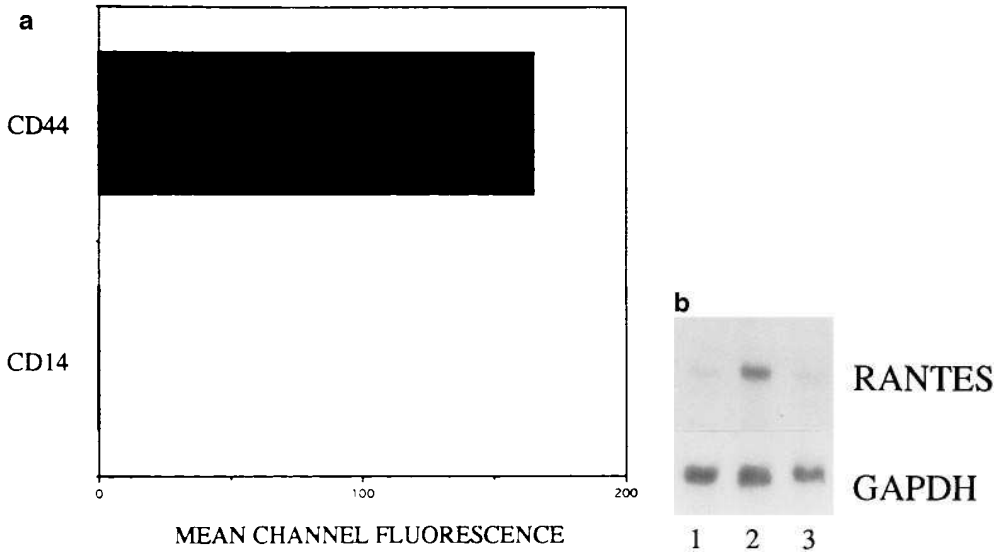

Figure 7. HA-induced chemokine gene expression in THP-1 cells is independent of CD14 expression. (*a*) The presence of the LPS receptor CD14 was measured on the surface of THP-1 cells by flow cytometry. THP-1 cells were incubated with either 20 μg/ml of the anti-CD14 monoclonal antibody 63D3, 100 μl of hybridoma supernatant containing the anti-CD44 monoclonal antibody H4C4, or 20 μg/ml of isotype matched control antibody (not shown). Cells were then stained with fluorescein-labeled rabbit anti–rat serum (20 μg/ml) and analyzed by flow cytometry as described in Methods. (*b*) THP-1 cells synthesize chemokine gene products in response to HA fragments but not LPS. THP-1 cells were stimulated with medium alone (lane *1*), purified HA fragments (100 μg/ml, lane *2*), or LPS (100 ng/ml, lane *3*) for 4 h at 37°C in the absence of polymixin B. RNA was isolated and subjected to Northern analysis as described.

ments to stimulate IL-8 gene expression in IPF alveolar macrophages is particularly interesting, since this provides a potential mechanism for the initiation of the inappropriate inflammatory response characteristic of this disorder. Previous studies have demonstrated increased levels of HA in BAL fluid from IPF patients (66), but the HA found in these studies has not been sized. The lack of biological activity of the high molecular weight HA cannot be explained by an inability to bind to macrophages. Fluorescein-labeled high molecular weight HA binds to macrophages and is displaced by the lower molecular weight forms, suggesting that the two forms recognize the same receptor(s). One possible explanation for the failure of high molecular weight HA to induce gene expression is that the high molecular weight forms may bind to cells in such a way as to prevent receptor crosslinking. Analysis of lower molecular weight fragments demonstrates that fragments as small as hexamers are able to induce gene expression. Previous studies with hexamers have shown them to be the smallest size HA fragment capable of competing away binding between HA and HA receptors such as CD44 (39). Our studies do not exclude the possibility that structural differences between various preparations of HA fragments, including differences in secondary or tertiary structure and alterations in the reducing ends of HA, may contribute to differences in biological activity. However, until more is known about the physical chemistry of HA fragments generated at sites of inflammation, it will be difficult to determine which factors in addition to molecular weight size are important in conferring biological activity.

It is clear from both the binding studies and the studies of gene expression presented here that CD44 participates in the interaction between HA and MH-S cells, but the exact nature of this interaction remains to be elucidated. We found that

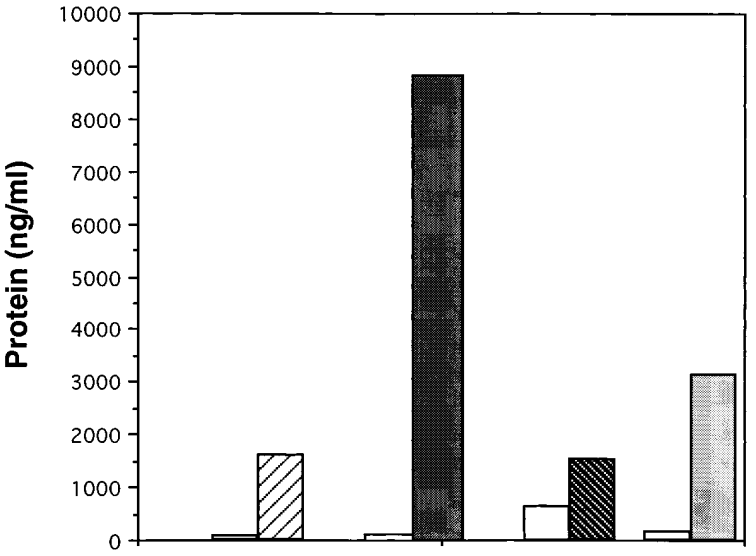

Figure 8. HA fragments stimulate secretion of chemokine proteins in MH-S cells. MH-S cells were plated 10^7/10 cm dish in serum-free media and stimulated for 18 h with HA fragments (100 μg/ml). The supernatants were collected and ELISA measurements performed as described in Methods.

binding between HA and MH-S cells is dose-dependent and is likely mediated almost entirely through CD44. However, while HA binding was completely inhibited in the presence of anti-CD44 antibody, HA-mediated gene expression was not completely blocked. The combination of high molecular weight Healon HA and anti-CD44 monoclonal antibody resulted in near complete inhibition of HA fragment signaling. This suggested the possibility that HA may utilize other receptors in addition to CD44. Since the integrin family of adhesion molecules mediates interactions between macrophages and the ECM, and the integrin adhesion molecule ICAM-1 has been implicated as a receptor for HA on liver endothelial cells (52), ICAM-1 was a likely candidate for involvement in HA-dependent gene induction as a second receptor. We found, however, that neither binding between HA and MH-S cells nor HA-inducible chemokine gene expression was influenced by monoclonal antibodies to ICAM-1. Binding and gene expression were also unaffected by monoclonal antibodies to the β_2 integrin LFA-1, which is an important mediator of adhesion-dependent cellular functions and activation. These results suggest that chemokine gene expression seen in response to HA fragments is distinct from gene induction associated with integrin-mediated adhesion. Nonetheless, we cannot rule out the possibility that HA and LFA-1 may bind to different receptor sites on the ICAM-1 molecule and monoclonal antibodies to ICAM-1 and LFA-1 therefore block epitopes unrelated to HA-dependent gene induction. We also investigated the potential role of the HA receptor RHAMM in mediating HA-induced chemokine gene expression, since RHAMM is involved in a number of HA-dependent processes including locomotion and chemotaxis (51). Polyclonal antibodies to RHAMM, however, failed to inhibit both binding between HA and MH-S cells and HA-dependent chemokine gene induction. We have not excluded the possibility that Fc receptor complexing may induce signal transduction pathways that serve a partial agonist function. One potential limitation to our method of studying binding between HA and MH-S cells is the use of soluble HA, since in vivo the interaction between HA and macrophages is likely to be more complex. Only a fraction of the biologically active molecule may actually be soluble in vivo, with the remainder structurally associated with other components of the ECM.

An additional question addressed in this study is how HA-induced gene expression relates to LPS-dependent induction of inflammatory genes. The majority of chemokine genes which HA has been shown to activate, and all of those discussed here, are also inducible by LPS and it is therefore possible that HA might use the same pathway for gene expression. However, we found that HA-induced chemokine gene expression does not depend on the presence of the LPS receptor CD14 and that gene induction by HA occurs in the absence of an LPS response. Nonetheless, the overlap in gene expression between HA and LPS suggests there may be common postreceptor signal transduction mechanisms.

While it has been recognized that HA plays a role in inflammation, this role has generally been characterized as a structural one, providing a scaffolding for cell–matrix interactions that promote cellular migration and differentiation (3). HA has previously been suggested to function in a signaling capacity in lymphocytes, but the size characteristics of the HA have not been described (15, 17). We provide evidence that HA fragments within a specific size range are capable of inducing chemokine gene expression in macrophages, supporting the hypothesis that HA fragments generated in the context of the inflammatory milieu may function as important signaling molecules and induce the expression of genes whose functions are critical to the maintenance of the inflammatory response. Elucidating the molecular mechanisms of HA-induced gene expression in macrophages may lead to the development of new strategies to interrupt the exhuberant inflammatory response characteristic of chronic inflammatory disorders such as pulmonary fibrosis and rheumatoid arthritis.

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