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Hyaluronan Fragments Induce Nitric-oxide Synthase in Murine Macrophages through a Nuclear Factor κB-dependent Mechanism*

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Activated macrophages play a critical role in controlling chronic tissue inflammation through the release of a variety of mediators including cytokines, chemokines, growth factors, active lipids, reactive oxygen, and nitrogen species. The mechanisms that regulate macrophage activation in chronic inflammation are poorly understood. A hallmark of chronic inflammation is the turnover of extracellular matrix components, and recent work has suggested that interactions with the extracellular matrix can exert important influences on macrophage effector functions. We have examined the effect of low molecular weight fragments of the extracellular matrix glycosaminoglycan hyaluronan (HA) on the induction of nitric-oxide synthase (iNOS) in macrophages. We found that HA fragments induce iNOS mRNA, protein and activity alone, and markedly synergize with interferon-γ to induce iNOS gene expression in murine macrophages. In addition, we found that resident tissue alveolar macrophages respond minimally, but inflammatory alveolar macrophages exhibit a marked induction in iNOS expression in response to HA fragments. Finally, we demonstrate that the mechanism of HA fragment-induced expression of iNOS requires activation of the transcriptional regulator nuclear factor κB. These data support the hypothesis that HA may be an important regulator of macrophage activation at sites of chronic tissue inflammation.

Nitric oxide ($NO^{\cdot}$)[1] mediates a number of the host defense functions of activated macrophages, including antimicrobial and tumoricidal activity (1–7). $NO^{\cdot}$ and its metabolites have also been implicated in the pathogenesis of the tissue damage associated with acute and chronic inflammation (8–12). Macrophages generate $NO^{\cdot}$ from the guanido moiety of L-arginine through a reaction catalyzed by the inducible form of nitric-oxide synthase (2). In contrast to the constitutive, calcium-dependent form of the enzyme found in the central nervous system and endothelial cells, iNOS can be induced by numerous immune stimuli. Maximal, synergistic iNOS induction occurs in response to the combination of a priming stimulus, such as IFNγ, and a triggering stimulus, examples of which include LPS, tumor necrosis α, and interleukin-2 (6, 13, 14).

Hallmarks of chronic inflammation include the accumulation of activated macrophages and of macrophage-derived mediators. However, the mechanisms of macrophage activation in this setting have not been clearly defined. The ECM undergoes increased degradation and turnover during inflammation, and fragments of ECM molecules have been found to possess biological activities distinct from their parent compounds (15–17). It has therefore been proposed that ECM fragments may be responsible for activating macrophages that infiltrate chronically inflamed tissues (18). We have recently demonstrated that fragments of the ECM component HA can bind to macrophages and induce the expression of a number of inflammatory genes (32), suggesting that HA fragments may be capable of activating macrophages at non-infectious sites of inflammation.

HA is a high molecular weight, nonsulfated linear glycosaminoglycan composed of repeating units of (β,1-→4)-D-glucuronic acid-(β,1-→3)-*N*-acetyl-D-glucosamine (19). Increased concentrations of HA have been found at sites of chronic inflammation (20, 21), and when the size of this HA has been studied the molecular weight range has been found to include lower molecular weight species (22, 23). Chronically inflamed tissues also contain elevated levels of the soluble, chemotactic cytokines known as chemokines (24–31), many of which are major products of activated macrophages. We have found that HA fragments of a size comparable with that found in inflammation, but not HA in the physiologic, higher molecular weight size range, induce the expression of chemokine genes in macrophages through a mechanism involving the cellular HA receptor, CD44 (32). We have also demonstrated that HA fragments induce activation of the transcriptional regulator NF-κB in murine macrophages (41). Since NF-κB mediates the expression and regulation of numerous inflammatory genes, including LPS-induced iNOS (33–38), the ability of HA fragments to induce this transcriptional regulator provides further evidence that HA fragments are likely to play a role in the inflammatory response.

The purpose of the present study was to investigate the hypothesis that HA fragments induce the expression of iNOS in inflammatory macrophages and to characterize the molecular mechanism of this induction. We report here that murine macrophages produce iNOS in response to HA fragments and that this induction is synergistically enhanced by IFNγ. Normal alveolar macrophages respond to HA fragments with only minimal iNOS gene expression, but inflammatory AM produce significant levels of iNOS gene products when stimulated with HA fragments. Additionally, our findings indicate that HA

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[1] The abbreviations used are: $NO^{\cdot}$, nitric oxide; iNOS, inducible nitric-oxide synthase; IFNγ, interferon γ; LPS, lipopolysaccharide; ECM, extracellular matrix; HA, hyaluronan; BMDMs, murine bone marrow-derived macrophages; AM, alveolar macrophages; BAL, bronchoalveolar lavage; NF-κB, nuclear factor κβ; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; PDTC, pyrrolidinedithiocarbamate.

fragment-dependent iNOS gene induction occurs through an NF-κB-dependent mechanism.

EXPERIMENTAL PROCEDURES

Cells, Mice, and Cell Lines—The mouse alveolar macrophage cell line MH-S (39) and the mouse macrophage-like cell line RAW 264.7 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 (Biofluids, Rockville, MD) at 37 °C under 5% CO_2. Mouse bone marrow-derived macrophages were isolated as described previously (40) from female C3H/HeJ, LPS hyporesponsive mice purchased from the Jackson Laboratory (Bar Harbor, ME). Bleomycin experiments were performed using male Harlan Sprague Dawley rats obtained from Harlan (Indianapolis, IN). All experiments were carried out in the presence of the LPS inhibitor polymyxin B (10 μg/ml) unless stated otherwise in order to exclude the effects of contaminating LPS on inflammatory gene expression.

Chemicals and Reagents—Purified HA fragments from human umbilical cords were purchased from ICN Biomedicals, Inc., Costa Mesa, CA. The HA-ICN preparation is free of protein (<2%) and free of chondroitin sulfate (<3%), and we have previously determined its peak molecular size to be approximately 200,000 Da (41). *Escherichia coli* 011:B4 LPS prepared by the Westphal method, the antioxidant pyrrolidinedithiocarbamate (PDTC), and the serine protease inhibitor *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were obtained from Sigma. Polymyxin B was purchased from Calbiochem. Recombinant mouse IFNγ (specific activity, 3.0×10^5 units/ml; endotoxin level less than 0.2 ng/μg) was from Genzyme Corp., Cambridge, MA. The proteasome inhibitor MG132 was a generous gift of MyoGenics, Inc., Cambridge, MA. Bleomycin sulfate and chondroitinase ABC from *Proteus vulgaris* were purchased from Sigma.

Bleomycin Administration and Bronchoalveolar Lavage—Bleomycin was administered to male Harlan Sprague Dawley rats according to published methods (42). Rats were anesthetized using inhaled Isofluorane. Following tracheostomy, 500 μl of sterile normal saline with 1 unit of bleomycin sulfate was instilled into the lungs through a 25-gauge needle inserted between the cartilagenous rings of the trachea. Control animals received saline lavage alone. The tracheostomy site was sutured, and the animals were allowed to recover until the time of BAL. Rats were killed with a lethal injection of sodium pentathol (Ampro Pharmaceutical, Arcadia, CA) at specified time points following intratracheal instrumentation.

BAL was performed by cannulating the trachea and instilling and retrieving approximately 50 ml of sterile normal saline in 5-ml aliquots. The entire lavage volume was centrifuged, and the cell pellet was resuspended in RPMI supplemented with fetal bovine serum and antibiotics. A differential cell count performed on the lavage fluid by Wright-Giemsa stain prior to centrifugation and repooling of cells consistently showed at least 95% macrophages. Cell viability was determined using trypan blue exclusion. Alveolar macrophages were purified by adherence to plastic tissue culture dishes for 1 h at 37 °C in RPMI without serum or antibiotics under 5% CO_2; nonadherent cells were removed by aspiration, and adherent macrophages were washed once in 1 × sterile phosphate-buffered saline. All subsequent experiments were performed in RPMI without serum or antibiotics.

Northern Analysis of mRNA Expression—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 (40). Ten-fifteen μg of total RNA was electrophoresed under denaturing conditions through a 1% formaldehyde-containing agarose gel, and RNA was transferred to Nytran (Schleicher & Schuell) or Zetaprobe (Bio-Rad) hybridization filters. Blots were briefly rinsed in 5 × SSC, and RNA was cross-linked to the filter by UV cross-linking (Stratagene, La Jolla, CA). Northern blots were hybridized overnight with 10^6 cpm/ml of the iNOS cDNA (43) labeled with [^{32}P]dCTP by the random prime method (Amersham Corp.). Following hybridization, blots were washed once in 2 × SSC, 0.1% SDS at room temperature for 30 min with shaking and 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 or aldolase (kindly provided by Dr. M. Shin, University of Maryland School of Medicine, Baltimore). Densitometric scanning was performed using a Molecular Dynamics Personal Densitometer SI (Sunnyvale, CA).

Western Analysis—Western blot analysis was performed as described (44). Briefly, 200 μg of macrophage total cell lysate was fractionated by SDS-polyacrylamide gel electrophoresis (10%), transferred to a nylon membrane, blocked and washed, incubated with polyclonal anti-iNOS antibody (45) at a dilution of 1:2500, and developed with a chemiluminescent system according to the manufacturer's instructions (Amersham Corp.).

Assay for NO· Production—Production of NO· was determined by measurement of NO_2^-, a stable product of the reaction of NO· with molecular oxygen, in culture supernatants as described previously (6, 46). In brief, 100 μl of supernatant was mixed with 100 μl Greiss reagent (0.5% sulfanilamide, 0.05% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% H_3PO_4) and incubated at 22 °C. Optical densities of samples were determined at 5 and 30 min at 540 nm. NO_2^- concentrations were determined by comparison with a standard curve prepared with $NaNO_2$.

Electromobility Gel Shift Assays—Nuclear extracts were prepared from macrophage monolayers stimulated in the absence of serum using the technique of Andrews and Faller (47). Confluent macrophage monolayers were stimulated on 10-cm Falcon tissue culture dishes in the absence of serum, rinsed once in cold 1 × phosphate-buffered saline, scraped, resuspended in 400 μl of Buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 M phenylmethanesulfonyl fluoride), and incubated on ice for 10 min. Nuclei were sedimented by centrifugation, resuspended in a suitable volume of Buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 M phenylmethanesulfonyl fluoride), and incubated on ice for 20 min. The protein concentration of the extracts was determined using the BCA method (Pierce). Extracts were kept at −70 °C.

Gel shift assays were performed as described previously (41). Five μg of nuclear extract in 2–3 μl was incubated for 5 min at room temperature with 1 μl of 1 mg/ml bovine serum albumin in AP-1 buffer (10 mM HEPES-KOH, pH 7.5, 16% glycerol, 20 mM NaCl, 4 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol, 2 mM spermidine (48)) for 5 min at room temperature with 2 μg of poly(I·C) (Pharmacia Biotech Inc.) and for 15 min at 37 °C with 1 μl of 10 × TBE (890 mM Tris base, 890 mM boric acid, 20 mM EDTA, pH 8.0) plus 1 μl of ^{32}P-labeled (50,000 cpm) or 2 μl of unlabeled double-stranded oligonucleotide containing the proximal NF-κB site from the murine iNOS promoter. The double-stranded oligonucleotide containing the κB site extending from −85 to −76 was made by annealing the following two oligonucleotides: 5′AACTGGGGACTCTCCCTTTG3′ and 3′CAAAGGGAGAGTCCCCAGTT5′. The double-stranded oligonucleotide containing the mutated κB site was made by annealing the following two oligonucleotides, AACTGG**AA**ACTCTCCCTTTG and CAAAGGGAGAGT**TT**CCAGTT, with the mutated bases in boldface. The mixture was electrophoresed at 4 °C on a 6% polyacrylamide gel in 0.4 × TBE at 200 V. Gels were dried and exposed to XAR Kodak film at −70 °C. To identify the protein components of the NF-κB complex, 1 μl of specific antibodies to p50, p65, or c-REL (Santa Cruz Biotechnology, Santa Cruz, CA) was added prior to addition of labeled oligonucleotide and incubated on ice for 10 min.

iNOS Promoter Analysis—The transcriptional regulation of iNOS was assayed using a reporter plasmid containing 1700 base pairs of the iNOS promoter region placed immediately upstream of the *luc* cDNA in the pGL2-Basic vector (Promega, Madison, WI) (46). Another iNOS promoter-luciferase construct was also used in which the κB site was mutated by site-directed mutagenesis (Transformer Kit, Clontech) so that base pairs −83 to −84 were changed from GG to AA. For liposomal mediated transfection, 0.5 μg of iNOS reporter plasmid and 0.5 μg of pSV-β-galactosidase control vector (Promega) were mixed with the 15 μl of Lipofectin (Life Technologies, Inc.), brought to a volume of 200 μl with Opti-MEM (Life Technologies, Inc.), incubated for 20 min at 22 °C, brought to 1 ml with Opti-MEM, and then added to aspirated 6-well plates of subconfluent RAW 264.7 macrophages. After 16 h incubation, the transfected macrophages were stimulated with HA, cultured for 24 h, and then harvested in lysis buffer (Promega). Luciferase activity was assayed in 40 μg of protein lysate in a luminometer following the manufacturer's recommendation (Promega) and standardized by dividing by β-galactosidase activity also measured in 200 μg of protein lysate (Promega).

RESULTS

HA Fragments Induce iNOS Expression in MH-S Cells—We first investigated the ability of HA fragments to induce iNOS in the mouse alveolar macrophage-like cell line MH-S. MH-S cells are resident alveolar macrophages that have been transformed with SV40 (39), and when stimulated with HA fragments of

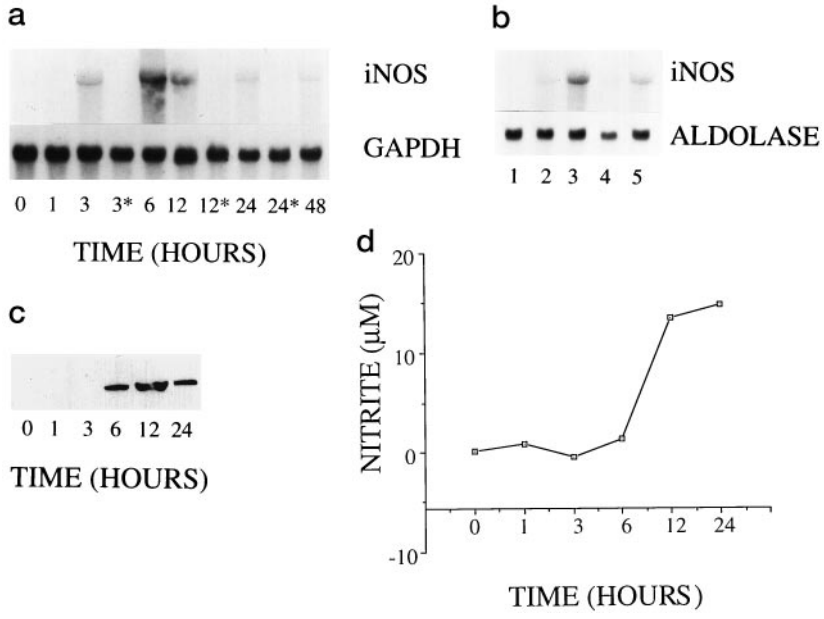

FIG. 1. **HA fragments induce iNOS expression.** *a*, MH-S cells were stimulated with purified HA fragments (100 μg/ml) for the indicated periods. All medium contained the LPS inhibitor polymyxin B (10 μg/ml). Lanes marked with an * indicate cells stimulated with LPS (100 ng/ml) in the presence of polymyxin B. Following stimulation, total RNA was collected, and Northern analysis was performed as described under "Experimental Procedures." *b*, MH-S cells were incubated for 18 h with either medium alone (*lane 1*), 10 units chondroitinase ABC (*lane 2*), purified HA fragments (*lane 3*, 60 μg/ml), LPS (*lane 4*, 100 ng/ml, in medium without polymyxin B), or HA fragments (*lane 5*) previously treated with 10 units of chondroitinase ABC for 12 h. RNA was then collected and Northern analysis was performed as described. *c*, MH-S cells were stimulated with purified HA fragments (100 μg/ml) for the indicated periods. Cell lysates were then prepared, and Western analysis was performed as described. *d*, MH-S cells were stimulated with purified HA fragments (100 μg/ml) for the indicated periods. Following stimulation, cell supernatants were collected, and nitrite production was measured by the Greiss reaction. Results represent the average of two separate measurements. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

peak molecular size of 200,000 Da, these cells expressed iNOS gene products in a time-dependent fashion (Fig. 1*a*). Maximal mRNA expression was observed at 6 h. Related glycosaminoglycans, including chondroitin 4-sulfate, dermatan sulfate, chondroitin 6-sulfate, and heparan sulfate all failed to induce iNOS gene expression in MH-S cells, as did the individual saccharide components of HA (data not shown). We have previously demonstrated that HA disaccharides are unable to induce inflammatory gene expression in murine macrophages (32), and Fig. 1*b* shows that HA fragment-dependent iNOS gene induction was significantly reduced following digestion of HA fragments to disaccharides with chondroitinase ABC (49). High molecular weight HA (with a peak molecular size of approximately 6×10^6 Da) also failed to induce iNOS mRNA expression (data not shown). HA fragment-induced iNOS mRNA expression in MH-S cells was accompanied by the production of iNOS protein, as shown in Fig. 1*c*. The time course of HA fragment-dependent nitrite production in MH-S cells is shown in Fig. 1*d*, demonstrating that HA fragments induced functional NOS enzyme activity in these cells.

Synergy Between HA Fragments and IFNγ in the Induction of iNOS Gene Expression in MH-S Cells—Next we determined the effect of the addition of IFNγ on HA fragment-dependent iNOS induction in MH-S cells. IFNγ alone induced little or no iNOS mRNA in these cells, as seen in Fig. 2*a*. However, HA fragment-induced iNOS mRNA expression was dramatically increased by the addition of IFNγ. Fig. 2*b* demonstrates that addition of IFNγ allowed detection of iNOS gene expression at doses of HA fragment as low as 1 μg/ml. IFNγ similarly enhanced HA fragment-dependent iNOS protein production, shown in Fig. 2*c*. These results suggest that HA fragments function as a triggering stimulus for iNOS production in macrophages, acting together with the priming stimulus IFNγ to induce iNOS expression.

HA Fragments Synergize with IFNγ to Induce iNOS Gene Expression in Primary Bone Marrow-derived Macrophages—The *in vivo* macrophage response to an immune stimulus involves the interaction of multiple macrophage populations. Inflammation triggers the recruitment and activation of peripheral monocytes, which differentiate into tissue macrophages and become activated *in situ*. We investigated the ability of HA fragments and IFNγ to induce iNOS in primary BMDMs which, when cultured for 5 to 7 days in the presence of colony stimulating factor-1, represent a mature macrophage population capable of responding to inflammatory stimuli. The time course of HA fragment-dependent iNOS gene expression in these cells is shown in Fig. 3*a*, with maximal mRNA levels seen at 3 h. IFNγ alone induced very low levels of iNOS gene expression in BMDMs (Fig. 3*b*), but the combination of HA fragments and IFNγ induced gene expression in a synergistic fashion. These data demonstrate that HA fragments synergize with IFNγ to induce iNOS in primary BMDMs.

HA Fragments Induce iNOS Gene Expression in Inflammatory Alveolar Macrophages—We next asked whether tissue inflammation affects the capacity of macrophages to produce iNOS in response to HA fragments. To address this question we collected AM by BAL from normal rats and from rats that had received intratracheal bleomycin, which induces a series of well-characterized inflammatory changes in the lungs of experimental animals (50, 51). Normal AM expressed minimal iNOS mRNA in response to HA fragments (Fig. 4) and were much less responsive than either the alveolar cell line MH-S or primary BMDMØ. However, AM isolated 5 days after treatment with bleomycin responded dramatically to HA fragments. In fact, the response to HA fragments alone was equal to that of HA fragments plus IFNγ, suggesting that the inflammatory AM were already maximally primed. This response persisted at day 9 following treatment. These results suggest that the presence of an inflammatory stimulus such as HA fragments is necessary but not sufficient to induce production of iNOS by

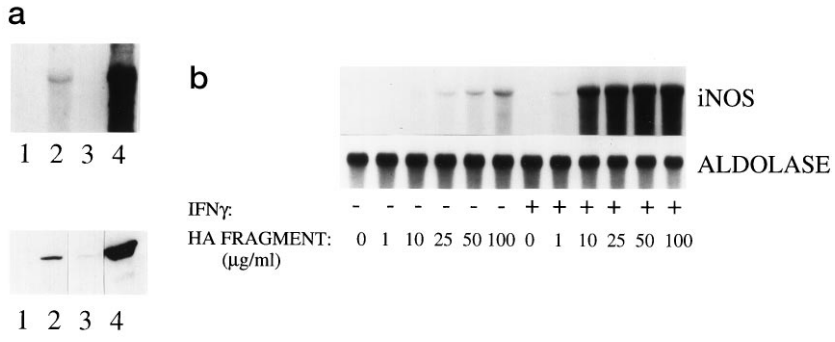

FIG. 2. **HA fragments and IFNγ induce iNOS gene expression in a synergistic fashion.** *a*, MH-S cells were stimulated with medium alone (*lane 1*), purified HA fragments (*lane 2*, 100 μg/ml), IFNγ (*lane 3*, 1000 units/ml), or HA fragments plus IFNγ (*lane 4*). *Top panel*, following stimulation for 4 h, total RNA was collected, and Northern analysis was performed. *Bottom panel*, following stimulation for 6 h, cell lysates were collected, and Western analysis was performed. *b*, MH-S cells were stimulated for 4 h with the indicated concentrations of purified HA fragments with or without IFNγ (300 units/ml). Total RNA was then collected and subjected to Northern analysis as described under "Experimental Procedures."

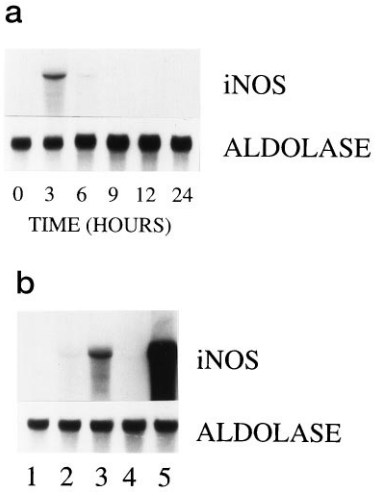

FIG. 3. **HA fragments and IFNγ synergistically induce iNOS mRNA expression in primary BMDMs.** *a*, primary BMDMs were stimulated with purified HA fragments for the indicated periods. Total RNA was extracted, and Northern analysis was performed. *b*, BMDMs were stimulated for 4 h with medium alone (*lane 1*), IFNγ (*lane 2*, 300 units/ml), purified HA fragments (*lane 3*, 100 μg/ml), LPS (*lane 4*, 100 ng/ml), or HA fragments plus IFNγ (*lane 5*) in the absence of polymyxin B. Following stimulation total RNA was collected and subjected to Northern analysis.

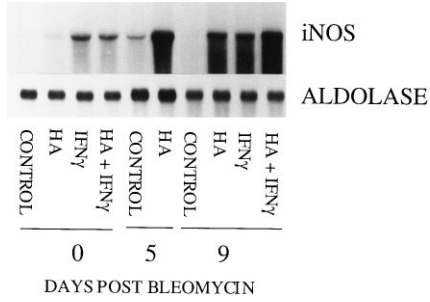

FIG. 4. **HA fragments induce iNOS gene expression in inflammatory alveolar macrophages.** AM were collected by BAL from rats on the indicated days following intratracheal instillation of bleomycin as described under "Experimental Procedures." Cells were then stimulated for 4 h with medium alone (control), purified HA fragments (100 μg/ml), IFNγ (300 units/ml), or HA fragments plus IFNγ. Total RNA was collected, and Northern analysis was performed as described.

macrophages and that macrophages acquire the capacity to respond to HA fragments in the setting of inflammation.

Inhibitors of NF-κB Block HA Fragment-induced NF-κB DNA Binding Activity and iNOS Gene Expression—Recent evidence indicates that the induction of iNOS by LPS, and by LPS plus IFNγ, is dependent upon NF-κB (34, 36–38). Since HA fragments induce NF-κB DNA binding activity (41), we investigated whether this transcriptional regulator might also be involved in HA fragment-dependent iNOS gene induction. Using an oligonucleotide probe containing the proximal NF-κB site from the iNOS promoter, we performed electromobility shift assays with nuclear extracts obtained from MH-S cells stimulated with HA fragments. Fig. 5*a* demonstrates that HA fragments induced NF-κB DNA binding activity in 1 h. In order to characterize the composition of the NF-κB complex, we performed super-shift assays using antibody to the p50 and p65 subunits of NF-κB (Fig. 5*b*). p50 and p65 constituted the majority of the HA fragment-induced DNA binding activity, whereas the related family member c-REL appeared to contribute to this binding activity only minimally.

We then utilized three distinct classes of NF-κB inhibitors in order to determine the effect of NF-κB inhibition on HA fragment-dependent iNOS induction. The serine protease inhibitor TPCK and the ubiquitin-proteasome inhibitor MG132 both block activation of NF-κB by stabilizing the inhibitor I-κBα (34, 52–54). The antioxidant PDTC inhibits NF-κB activity by a mechanism that is not fully understood but that is believed to be related to the scavenging of oxygen radicals (55, 56). Both HA fragment-dependent NF-κB DNA binding activity and iNOS mRNA levels were reduced or eliminated by all three of these inhibitors (Figs. 5, *c* and *d*). HA fragment-induced NF-κB DNA binding activity and iNOS gene expression were unaffected by the control serine protease inhibitor leupeptin (data not shown). Interestingly, HA fragment-induced NF-κB DNA binding activity was not enhanced by the addition of IFNγ (Fig. 5*c*), indicating that the synergistic induction of iNOS mRNA by HA fragments and IFNγ is not due to increased NF-κB binding at the proximal κB site. These data are in accordance with published work demonstrating that IFNγ regulates iNOS transcription through interferon regulatory factor-1 (57, 58).

Mutation of the Proximal NF-κB Binding Site Blocks HA Fragment-induced iNOS Promoter Activity—In order to investigate more directly the effect of HA fragments on iNOS promoter activity, we transiently transfected a construct consisting of 1700 base pairs of the iNOS promoter, which contains two NF-κB binding sites, upstream of a luciferase reporter gene into the mouse macrophage-like cell line RAW 264.7. As shown in Fig. 6, HA fragments induced a greater than 3-fold increase over base line in wild-type promoter activity. However, HA fragment-dependent increases in promoter activity were no longer observed when the proximal NF-κB binding site was

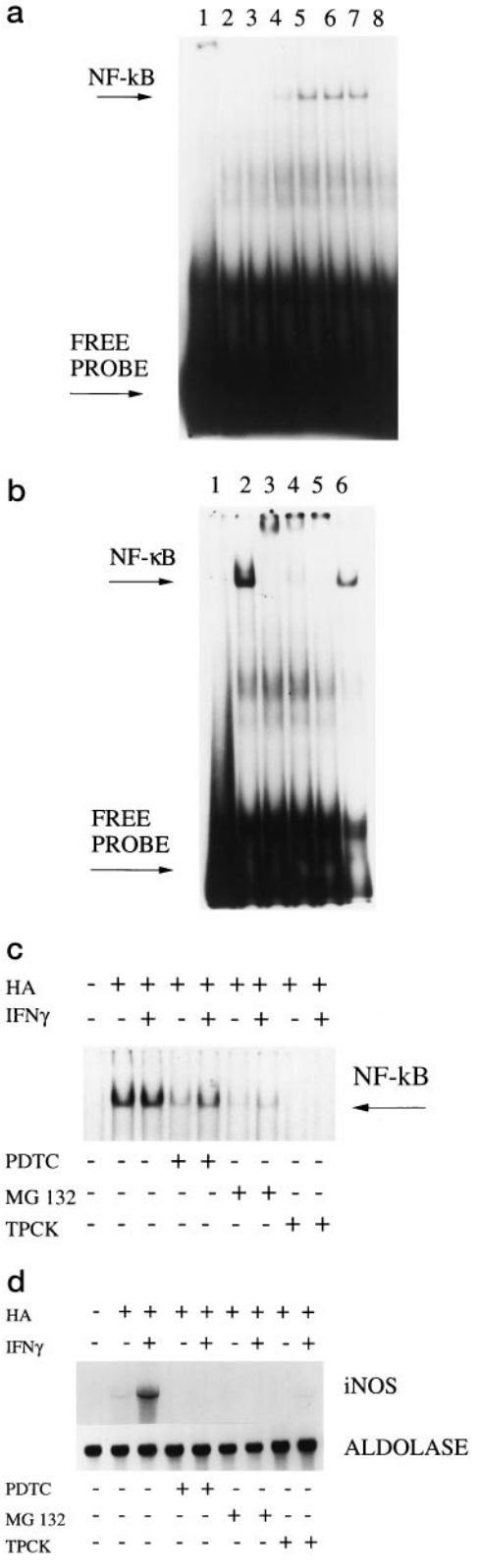

FIG. 5. **HA fragment-induced NF-κB DNA binding activity and iNOS gene expression are blocked by inhibitors of NF-κB.** *a*, electromobility shift assays were performed using nuclear extracts prepared from MH-S cells after 1 h stimulation with medium alone (*lane 2*) or purified HA fragments at concentrations of 1 μg/ml (*lane 3*), 10 μg/ml (*lane 4*), 25 μg/ml (*lane 5*), 50 μg/ml (*lane 6*), and 100 μg/ml (*lane 7*). Nuclear extracts were incubated with ^{32}P-labeled oligonucleotide containing the proximal NF-κB binding site of the murine iNOS promoter as described under "Experimental Procedures." *Lane 1* represents radiolabeled oligonucleotide probe alone, and *lane 8* represents HA fragments (100 μg/ml) plus radiolabeled oligonucleotide in the presence of 10 × excess unlabeled oligonucleotide. *b*, electromobility shift assays were performed using nuclear extracts prepared from MH-S cells after 1 h stimulation with purified HA fragments alone (*lane 2*, 50 μg/ml) or HA fragments in the presence of antibody to p50 (*lane 3*), p65 (*lane 4*), p50 and p65 (*lane 5*), and c-REL (*lane 6*). *Lane 1* represents radiolabeled oligonucleotide probe alone. *c*, nuclear extracts were prepared from MH-S cells after 1 h stimulation with purified HA fragments (50 μg/ml) or HA fragments plus IFNγ (1000 units/ml), with or without PDTC (100 μM), MG132 (15 μM), or TPCK (20 μM). Electromobility shift assays were performed as described. *d*, MH-S cells were stimulated for 4 h with purified HA fragments (100 μg/ml) or HA fragments plus IFNγ (1000 units/ml) with or without PDTC (100 μM), MG132 (15 μM), or TPCK (20 μM). Total RNA was collected, and Northern analysis was performed.

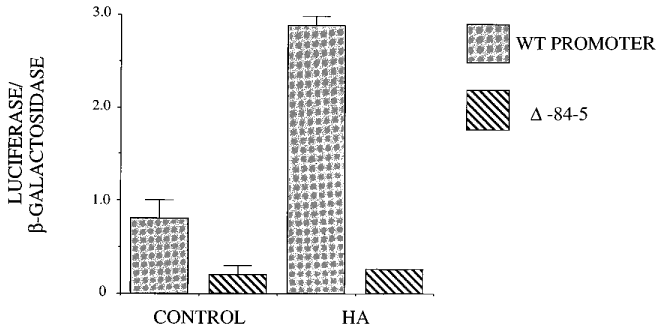

FIG. 6. **HA fragment-induced iNOS promoter activity is blocked by mutation of the proximal NF-κB binding site.** RAW 264.7 cells were transiently transfected with a 1700-base pair wild-type iNOS promoter construct and a construct with the proximal NF-κB site mutated as described under "Experimental Procedures." Cells were stimulated for 24 h with HA fragments, and luciferase activity was measured.

mutated. This indicates that the proximal NF-κB binding site is critical for HA fragment-dependent iNOS gene expression.

DISCUSSION

We present data supporting the hypothesis that fragments of the ECM component HA induce iNOS expression in murine macrophages through an NF-κB-dependent mechanism and that IFNγ synergistically enhances the HA fragment-dependent induction of iNOS. These findings expand our understanding of the role of HA fragments in macrophage activation and suggest that low molecular weight HA may function as a macrophage triggering stimulus in the setting of non-infectious inflammation. Together with our previous observation that HA fragments induce the production of chemokines in macrophages, the current findings support the hypothesis that HA fragments participate in the development of a complex inflammatory milieu characterized by the production of multiple macrophage-derived inflammatory mediators that can in turn recruit additional macrophages and directly influence effector cell functions.

Since the *in vivo* response to an inflammatory stimulus involves complex interactions among distinct populations of macrophages, we examined the ability of HA fragments to induce iNOS expression in several different types of macrophages. We found that cells from the transformed cell line MH-S, which are derived from resident alveolar macrophages, and primary BMDMs both readily expressed iNOS mRNA when stimulated with HA fragments. However, we observed significant differences in the response to HA fragments between normal AM and from bleomycin-exposed inflammatory AM. Normal AM responded minimally, whereas inflammatory AM stimulated with HA fragments exhibited a marked induction of iNOS mRNA. The dramatic induction of iNOS mRNA in inflammatory AM was not further enhanced by IFNγ, suggesting that these cells were already maximally primed. These

findings suggest that the response to HA fragments is dependent on the state of macrophage activation. Additionally, these results suggest a role for HA fragments in bleomycin-induced lung injury. HA levels are increased in the lungs of experimental animals following treatment with bleomycin (51), and although bleomycin-exposed AM have been shown to express higher levels of iNOS at base line than normal (59), our data indicate that HA fragments induce further elevations in the levels of iNOS that may contribute to the pulmonary toxicity observed with this antineoplastic agent.

Our results indicate that HA fragment-dependent iNOS induction in murine macrophages occurs through an NF-κB-dependent mechanism. The role of NF-κB in LPS-induced macrophage iNOS production has been well established (36–38). However, what role if any NF-κB plays in the IFNγ-dependent synergistic induction of iNOS is not clear. There is evidence that NF-κB participates in the IFNγ-mediated enhancement of iNOS induction by LPS in epithelial cells (60), but with rare exceptions (37) the studies reported to date in macrophages do not specifically address mechanistic distinctions between induction of iNOS by LPS and by LPS plus IFNγ. We found that the addition of IFNγ did not increase HA fragment-dependent NF-κB DNA binding activity in MH-S cells, and inhibitors of NF-κB were more effective in blocking HA fragment-induced DNA binding activity and iNOS gene expression than in blocking binding activity and gene expression induced by HA fragments plus IFNγ. These findings suggest that the synergistic enhancement of HA fragment-dependent iNOS induction observed with IFNγ is likely to be mediated by alternative transcriptional regulator(s). These data are consistent with the results demonstrating a critical role for interferon regulatory factor-1 in mediating the IFNγ-induced synergy with LPS on the expression of iNOS (57, 58). Induction of iNOS, through the generation of HA fragments at sites of inflammation, may prove to be an important mechanism for the local production of nitric oxide in inflamed tissues. Given the ability of HA fragments to activate the NF-κB/IκB system and induce the production of chemokines by macrophages, HA fragments may play an important part in propagating the inflammatory response. HA fragments may also contribute to the pathological development of chronic inflammation through the induction of $NO^{\cdot}$ and other reactive nitrogen intermediates. Peroxynitrite, for example, is a product of the reaction of $NO^{\cdot}$ and superoxide. This highly reactive species has been implicated in protein nitrosylation and tissue damage (8, 61) and can degrade high molecular weight HA to smaller fragments. It is intriguing to speculate that HA fragments generated during the course of inflammation may indirectly induce peroxynitrite production, which could in turn generate more HA fragments and lead to the development of an ongoing inflammatory state. Through their ability to activate macrophages and induce inflammatory mediators and reactive intermediates, HA fragments may be instrumental in establishing the pathological cycle of inflammation that ultimately results in chronic inflammatory disorders.

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