

Hyaluronan Fragments Activate an NF- κ B/I- κ B α Autoregulatory Loop in Murine Macrophages

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Summary

Macrophages play an important role in the acute tissue inflammatory response through the release of cytokines and growth factors in response to stimuli such as lipopolysaccharide (LPS). Macrophage inflammatory effector functions are also influenced by interactions with the extracellular matrix (ECM). Such macrophage-ECM interactions may be important in regulating chronic inflammatory responses. Recent evidence has suggested that hyaluronan (HA), a glycosaminoglycan (GAG) component of ECM can induce inflammatory gene expression in murine macrophages. HA exists in its native form as a large polymer, but is found as smaller fragments under inflammatory conditions. The NF- κ B/I- κ B transcriptional regulatory system has been shown to be a critical component of the host inflammatory response. We examined the effects of high molecular weight HA and lower molecular weight HA fragments on NF- κ B activation in mouse macrophages. Only the smaller HA fragments were found to activate NF- κ B DNA binding activity. After HA stimulation, I- κ B α mRNA was induced and I- κ B α protein levels, which initially decreased, were restored. The induction of I- κ B α expression was not observed for other GAGs. The time course of I- κ B α protein regeneration in response to HA fragments was consistent with an autoregulatory mechanism. In support of this mechanism, *in vitro* translated murine I- κ B α inhibited HA fragment-induced NF- κ B DNA binding activity. The NF- κ B DNA binding complex in HA-stimulated extracts was found to contain p50 and p65 subunits. Activation of the NF- κ B/I- κ B system in macrophages by ECM fragments may be an important mechanism for propagating the tissue inflammatory response.

Monocytes/macrophages are an essential cellular component of the inflammatory response due to their ability to debride damaged tissues and initiate reparative processes through the selective release of proteases, lipid mediators, reactive oxygen, and nitrogen species, cytokines and growth factors (1). Studies have shown that under circumstances where tissue macrophage function is impaired, the appropriate repair process does not occur (2). Conversely, states of chronic inflammation have been associated with excessive macrophage activation (3, 4). Extensive investigations have established the role of LPS as a potent macrophage activating stimulus in the host response to gram-negative infections (5). However, several macrophage-derived cytokines that are induced by LPS such as TNF α , and members of the chemokine family such as IL-8 and MIP-1 α , have also been shown to be produced at sites of chronic inflammation where LPS is not thought to be present (6–8). Thus, the mechanisms involved in regulating macrophage activation in states of chronic inflammation are not well characterized. In addition to microbes and soluble media-

tors such as cytokines, inflammatory cell function may be regulated by components of the inflammatory milieu such as the extracellular matrix (ECM) (9, 10). An ECM component that has been shown to influence cell behavior is the glycosaminoglycan (GAG) hyaluronan (HA) (11). HA is a polymer consisting of repeating sugar residues D-glucuronic acid and N-acetyl-D-glucosamine. HA exists in its native form in sites such as the joint space in a high molecular weight form usually in the range of 1–6 $\times 10^6$ Da (12). However, under inflammatory conditions such as arthritis, HA has been shown to be more polydisperse, with a significant fraction of lower molecular weight fragments (12). The molecular weight reduction of HA may be the result of depolymerization by oxygen-derived free radicals, enzymatic cleavage and/or an altered pattern of synthesis. Several studies have suggested that different biologic effects are observed with high and lower molecular weight HA (13, 14).

The NF- κ B/I- κ B transcriptional regulatory system exhibits an essential role in transducing signals leading to the expression of a number of genes involved in the host in-

flammatory response (15). We have previously shown that HA induces the expression of IL-1 β and TNF α , both of which are regulated, at least in part, by NF- κ B (16–18). In this study we demonstrate that defined HA fragments, but not the larger native molecule, activate an NF- κ B/I- κ B α autoregulatory mechanism in macrophages.

Materials and Methods

Cells, Mice, and Cell Lines. The mouse macrophage-like cell line RAW 264.7 and the mouse alveolar macrophage-derived cell line MH-S (19) were obtained from the American Type Culture Collection (Rockville, MD). Mouse bone marrow-derived macrophages were isolated from female C3H/HeJ, LPS hyporesponsive mice obtained from the Jackson Laboratory (Bar Harbor, ME) as previously described (16). To exclude effects of contaminating LPS on experimental conditions, cell stimulation was carried out in the presence of polymixin B (10 μ g/ml).

Chemicals and Reagents. Healon GV and Healon (sterile sodium hyaluronate for intraocular use) were a generous gift of Kabi Pharmacia (Upsala, Sweden). Hyaluronic acid was from ICN (HA-ICN) Biomedicals, Inc. (Costa Mesa, CA). The HA-ICN preparation is free of chondroitin sulfate (<3%) and free of protein (<2%). Chondroitin sulfate, heparin sulfate, and E. Coli 0111:B4 LPS prepared by the Westphal method were purchased from Sigma Chem. Co. (St. Louis, MO). Polymixin B was purchased from Calbiochem Novabiochem (La Jolla, CA). Stock solutions of reagents were tested for LPS contamination using the Limulus amoebocyte assay (Sigma).

Preparation and Molecular Weight Determination of HA Fragments. Purified preparations of hyaluronan (Healon, Healon GV, or HA-ICN) were subjected to sonication using a Branson Sonifier for varying periods of time. The output was set at the micro tip limit. Purified human umbilical cord hyaluronan was provided by J. Yannariello-Brown (University of Texas, Galveston; 20). Molecular weight determination of HA was performed as described (21). In brief, HA samples were electrophoresed on a 0.5% agarose gel and then detected using the cationic dye Stains-All (3,3'-dimethyl-9-methyl-4,5,4',5'-dibenzothiacarbocyanine). Densitometric scans were performed with a scanning densitometer (model 800; Kontes, Vineland, NJ). Calibration was performed with HA fragments of known sizes.

Isolation and Sequencing of Full-length Murine I- κ B α cDNA. A cDNA library was prepared from poly(A)⁺ RNA isolated from RAW 264.7 cells that were stimulated for 3 h with LPS (1 μ g/ml) in the presence of cycloheximide (10 μ g/ml) as described (22). The library was screened by differential plaque hybridization using cDNA probes that were prepared from RNA harvested from RAW 264.7 cells that were either stimulated with LPS + CHX, or CHX alone (22). Plaques that hybridized with only the stimulated probe were purified and partial sequences obtained. To identify mRNAs that were induced by HA fragments, total RNA was prepared from mouse bone marrow-derived macrophages stimulated with HA fragments and Northern analysis performed with the differentially expressed cDNA probes. Partial sequence analysis of one cDNA (L318) suggested homology with human I- κ B α (MAD-3) (23). A full-length cDNA was then obtained by further screening the original cDNA library. The cDNA was sequenced (in both directions) using exonuclease III (Erase-a-Base). The sequence data has been submitted to EMBL/GenBank/DDBJ and the accession number is U36277.

In Vitro Transcription and Translation of Murine I- κ B α Protein. The full-length mI- κ B α cDNA subcloned into Bluescript KS was used for in vitro transcription and translation performed with the TNT T3 coupled reticulocyte lysate and wheat germ lysate systems (Promega Corp., Madison, WI). A 37-kD product was identified on 6% polyacrylamide gels (not shown).

Northern Analysis. The extraction, purification, electrophoresis, and transfer of the total RNA to nylon filters were carried out as described (16). The filters were hybridized with 10⁶ dpm/ml of ³²P-labeled mI- κ B α cDNA using the random prime method (Amersham Corp., Arlington Heights, IL). Selected blots were rehybridized with a GAPDH cDNA for purpose of comparing loading amounts of RNA.

Electrophoretic Mobility Shift Assays (EMSA). Nuclear extracts were prepared from macrophage monolayers that were stimulated in the absence of serum using the technique of Andrews (24). 10⁷ macrophages were stimulated in serum-free conditions on 10-cm tissue culture dishes (Falcon, Becton Dickinson, Lincoln Park, NJ), rinsed once in cold PBS, scraped, and resuspended in 400 ml of buffer A (10 mM Hepes-KOH, pH 7.9 at 4°C, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 M PMSF) for 10 min. Nuclei were sedimented by centrifugation and resuspended in buffer C (20 mM Hepes-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 M PMSF) and incubated on ice for 20 min. The protein concentration of the supernatants was determined by the BCA method (Pierce Chemical, Rockford, IL) and kept frozen at -70°C. For electrophoretic mobility shift assays (22), 5 to 7.5 μ g of nuclear extract in 2–3 μ l was incubated for 5 min at room temperature with 1 mg/ml of BSA in AP-1 buffer (10 mM Hepes, pH 7.5, 16% glycerol, 0.1 mM EDTA, 20 mM NaCl, 4 mM MgCl₂, 2 mM dithiothreitol, 2 mM spermidine), for 5 min with 2 μ g of poly-(I:C) (Pharmacia LKB Biotechnology, Piscataway, NJ) at room temperature and then for 15 min at 37°C with 1 μ l of unlabeled or ³²P-end-labeled κ B oligonucleotide. The κ B oligonucleotide used was the consensus NF- κ B site from the κ light chain promoter: 5' AGTTGAGGGGACTTTCCCAGGC 3'. The mutated κ B oligonucleotide was purchased from Santa Cruz Biotechnology (Santa Cruz, CA): 5' GGCGACTTTCCC 3'.

To identify the protein components of the NF- κ B complex specific polyclonal antibodies to p50, p65, and c-rel were purchased from Santa Cruz Biotechnology. 1 μ l of Ab was added after the nuclear extract was incubated with labeled oligonucleotide. Incubation was for 1 h at 4°C. The mixture was then electrophoresed on 6% polyacrylamide gels in 0.4 \times TBE at 200 V at 4°C.

Western Blot Analysis. Cell cytosol extracts (50 μ g/lane) were electrophoresed through a 12% SDS-polyacrylamide gel and then transferred onto a nitrocellulose membrane in an aqueous methanol buffer. The membrane was then blocked with 5% BSA in TBS-T, incubated with rabbit anti-human I- κ B α at 1:250, washed, incubated with biotinylated goat anti-rabbit antibody (Vector Labs. Inc., Burlingame, CA), washed, the signal was amplified with streptavidin-horseradish peroxidase (Vector Labs.), and then it was washed again. The signal was developed by a chemiluminescent system (Amersham).

Results

HA Fragments Induce Murine I- κ B α mRNA Expression. We have previously provided evidence that HA could induce the expression of IL-1 β , TNF α , and IGF-1 in mouse

bone marrow-derived macrophages (16). The HA for those studies was prepared from bovine trachea and carried a molecular mass of ~40–80 kD (Sigma). Further experiments using the purified HA preparation Healon (Kabi Pharmacia) showed no induction of inflammatory cytokines (data not shown). This led us to suspect that there may be an HA size requirement for induction of inflammatory genes in macrophages. Fig. 1 shows the relationship between HA size and induction of *mI- κ B α* mRNA in the mouse alveolar macrophage cell line MH-S in response to soluble HA. As demonstrated in Fig. 1, the purified Healon (HA-1) has a peak molecular mass of 6×10^6 Da (Fig. 1 A) and does not induce *mI- κ B α* mRNA expression (Fig. 1 B). However, when the same Healon preparation is sonicated, lower molecular mass fragments are generated that induce *mI- κ B α* gene expression. In addition, the cells were incubated in serum-free media containing polymyxin B (10 μ g/ml). As shown, LPS (1 μ g/ml) did not induce *I- κ B α*

mRNA expression. This excludes contaminating LPS as a potential stimulus. In the absence of polymyxin B, LPS induced *mI- κ B α* mRNA in a similar manner to HA (data not shown). Fig. 1 C shows the time course for *mI- κ B α* mRNA expression in response to HA fragments in primary mouse bone marrow-derived macrophages. Peak mRNA expression is seen at 3 h and almost completely gone at 12 h. Identical results were obtained with the purified HA (molecular mass 400 kD) provided by J. Yannariello-Brown (University of Texas, Galveston), and the purified human umbilical cord HA (molecular mass 210 kD) purchased from ICN (not shown). Data related to the specificity of the HA fragment effect on macrophages are shown in Fig. 1 D. As shown in Fig. 1 D, neither the individual sugars nor the combination are able to induce *mI- κ B α* gene expression. Similarly, neither heparan sulfate nor chondroitin 4-sulfate induced gene expression in MH-S cells. We also tested heparin, chondroitin 6-sulfate, and derma-

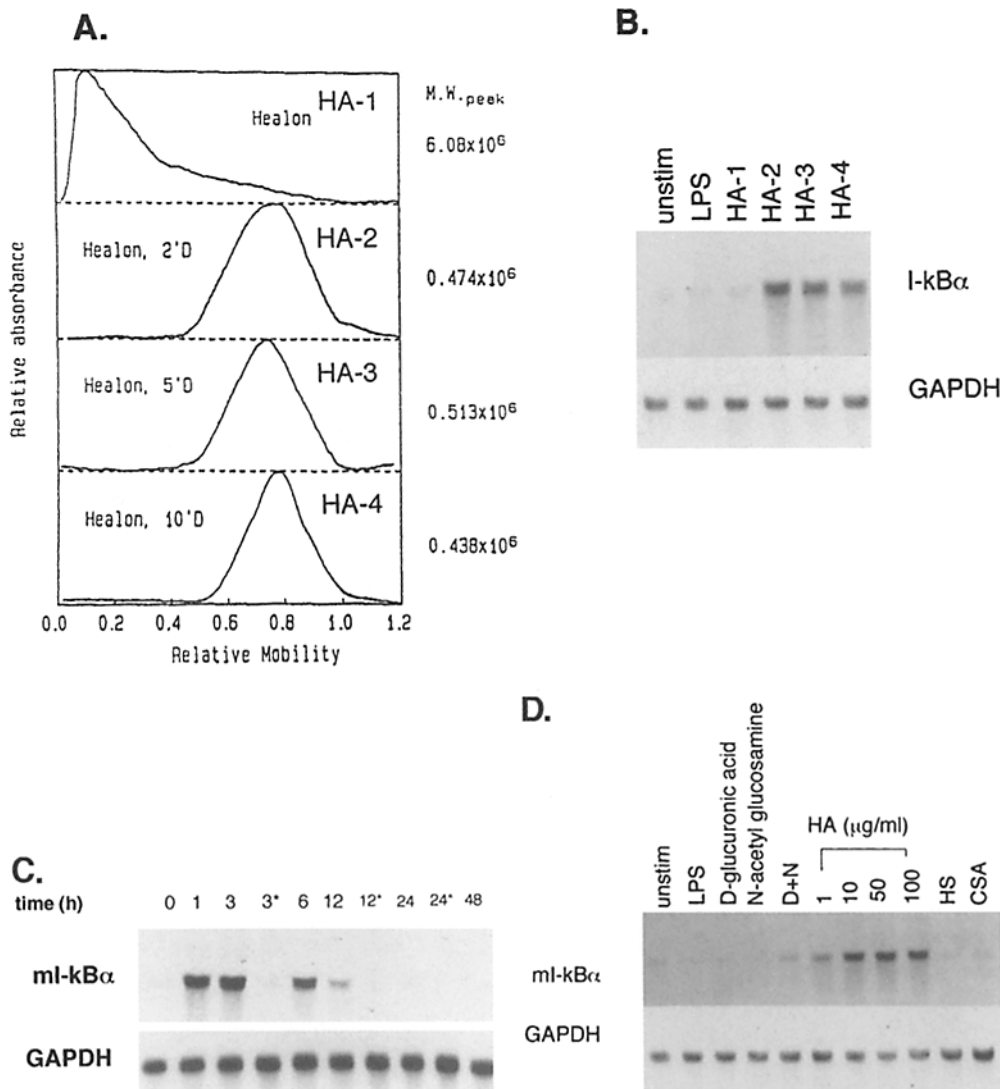


Figure 1. Hyaluronan fragments induce *mI- κ B α* mRNA expression in mouse macrophages. (A) Purified HA (Healon) was sonicated for the indicated times as described in Material and Methods. The molecular weight of the samples was then determined relative to known HA sizes as described. (B) 10^7 MH-S cells were stimulated under serum-free conditions in the presence of 10 μ g/ml of polymyxin B with soluble HA fragments of known size at a concentration of 1 mg/ml for 4 h. The RNA was then isolated and Northern analysis performed with 32 P-labeled *mI- κ B α* cDNA as described. The blots were also probed with GAPDH. This is a representative example of 4 similar experiments. (C) Time course of HA fragments induced *mI- κ B α* mRNA expression in mouse bone marrow-derived macrophages. Primary bone marrow-derived macrophages were cultured from C3H/HeJ mice for 7 d in 10% L cell conditioned media. The macrophages were stimulated with purified HA fragments as described in Material and Methods. The time points marked with * indicate stimulation with LPS (1 μ g/ml). (D) HA fragment-induced *mI- κ B α* mRNA expression requires intact HA and is not induced by related glycosaminoglycans. MH-S cells were stimulated for 4 h with LPS (1 μ g/ml), D-glucuronic acid (100 μ g/ml), N-acetyl-D-glucosamine (100 μ g/ml), the two combined (D+N) (1 mg/ml), increasing concentrations of HA fragment, heparan sulfate (1 mg/ml), or chondroitin 4-sulfate (chondroitin sulfate A-CSA) (1 mg/ml). Total RNA was isolated and Northern analysis performed.

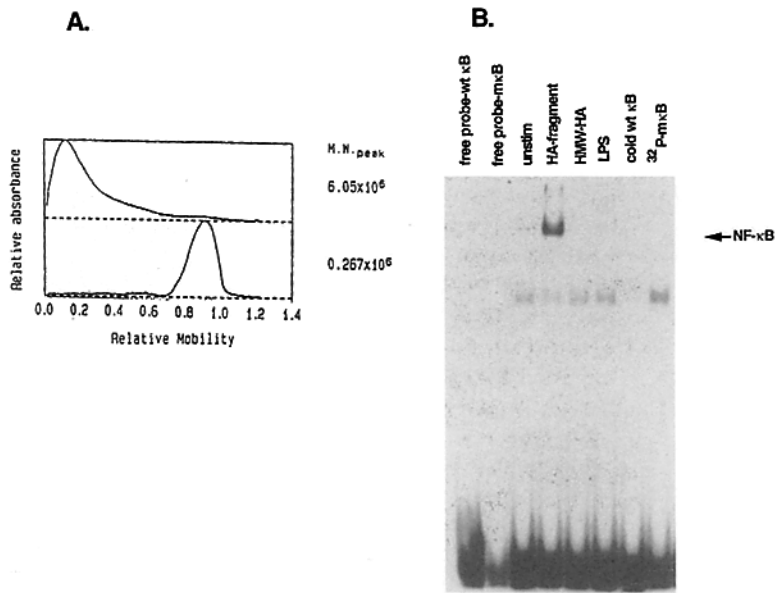


Figure 2. Purified HA fragments but not HMW-HA induced NF- κ B DNA binding activity in MH-S cells. (A) Densitometric scanning demonstrating the molecular weights of HMW-HA (top), and purified HA fragments (bottom). (B) Electrophoretic mobility shift assay of nuclear extracts prepared from MH-S cell stimulated for 2 h with either serum-free media containing polymyxin B (unstim), HMW-HA (100 μ g/ml), HA-fragment (HA-ICN) (100 μ g/ml), or LPS (1 μ g/ml), all with polymyxin. 5 μ g protein/lane was incubated with 32 P-labeled κ B oligonucleotide as described in Materials and Methods. Demonstration of NF- κ B DNA binding specificity. HA fragment-induced NF- κ B DNA binding activity at 2 h is competed by unlabeled κ B oligonucleotide (cold wt κ B) and the shifted band is not observed in the presence of 32 P-labeled mutated κ B oligonucleotide (32 P-m κ B).

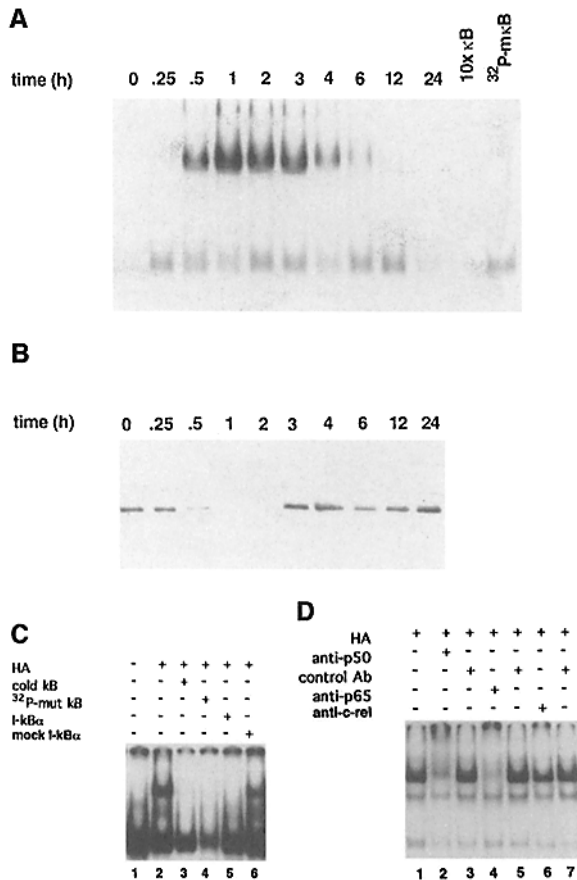


Figure 3. HA fragments activate NF- κ B DNA binding activity and regenerate functional mI- κ B α protein. (A) Time course of HA fragment-induced NF- κ B DNA binding activity. MH-S cells were stimulated over time with HA fragments (HA-ICN) (50 μ g/ml) and electrophoretic mobility shift assays performed as described in Fig. 2. (B) mI- κ B α protein expression following stimulation of MH-S cells with HA fragments (50 μ g/ml). 10^7 MH-S cells were stimulated with HA fragments over time and cytosol extracts were run on a 12% polyacrylamide gel and Western blot performed with polyclonal rabbit anti-human I- κ B α Ab as

tan sulfate and all were inactive (not shown). The ability to induce mI- κ B α mRNA in adherent macrophages appears to be a property of soluble HA fragments.

HA Fragments Induce NF- κ B DNA Binding Activity in Murine Macrophages. The identification of mI- κ B α mRNA expression in response to the structural ECM component HA led us to determine whether soluble HA fragments induced NF- κ B DNA binding activity. As shown by electrophoretic mobility shift analysis in Fig. 2, A and B, high molecular mass HA is unable to induce NF- κ B DNA binding to an oligonucleotide containing the κ B light chain binding site. However, a purified HA fragment from human umbilical cord of peak molecular mass 267 kDa induced a new shifted band (Fig. 2, B). The specificity of this DNA binding activity is also demonstrated in Fig. 2 B. The shifted band in the HA-stimulated lane is competed by unlabeled κ B oligonucleotide (cold weight κ B). The lower band is constitutive and is also competed by the unlabeled κ B oligonucleotide. Importantly, the shifted band that appears only in the HA-stimulated extracts is not present when the nuclear extracts are incubated with 32 P-labeled mutated κ B oligonucleotide (32 P-m κ B). The constitutive band, however, is still present.

Evidence for an HA Fragment-activated NF- κ B/I- κ B α Autoregulatory Loop. Regeneration of I- κ B α following NF- κ B activation has been proposed by several laboratories as the mechanism for turning off NF- κ B activity in response to

described in Materials and Methods. (C) mI- κ B α inhibits HA fragment-induced NF- κ B DNA binding activity in MH-S cells. HA fragment-induced DNA binding activity (lane 2) was inhibited by in vitro translated mI- κ B α (lane 5), but not mock translated protein (lane 6). (D) HA fragment-induced NF- κ B DNA binding complex includes NF κ B1 and Rel A. HA fragment-induced NF- κ B DNA binding activity (lane 1) was inhibited in the presence of anti-NF κ B1 Abs (p50) (lane 3), and anti-Rel A (p65) (lane 4), but not control Abs (lanes 3 and 5), or anti-c-Rel (lane 6).

cellular activation (25–27). Evidence for activation of an NF- κ B/mI- κ B α autoregulatory loop in response to HA fragments is provided in Fig. 3. Fig. 3 A shows the time course for NF- κ B DNA binding activity in MH-S cells after stimulation with HA fragments. Peak NF- κ B DNA binding activity is seen at 2 h and diminished by 6 h. Fig. 3 B shows the effect of HA fragment stimulation on mI- κ B α protein expression. mI- κ B α protein is present in unstimulated cells, consistent with its role in sequestering the NF- κ B complex in the cell cytoplasm. After stimulation with HA fragments, the protein levels fall by 30 min and are then regenerated at 3 h. The time course for regeneration of mI- κ B α suggested it may be involved in inhibiting NF- κ B DNA binding activity. This is supported by the data shown in Fig. 3 C. In vitro translated mI- κ B α blocks the binding of the HA fragment-stimulated NF- κ B DNA binding activity in extracts of MH-S cells. Inhibition was not seen with mock-translated protein.

To determine the subunit components of the NF- κ B DNA binding complex induced by HA fragments, EMSA was performed on HA fragment-stimulated nuclear extracts from MH-S cells using NF- κ B subunit-specific polyclonal antibodies. Fig. 3 D demonstrates the effect of polyclonal antibodies to NF κ B1 (p50), Rel A (p65), and c-Rel on HA fragment-induced NF- κ B DNA binding activity in MH-S cells. There is an inhibition of DNA binding activity in the presence of anti-NF κ B1 and Rel A antibodies, much less so with anti-c-Rel Abs, and no inhibition with control antibodies.

Discussion

We have demonstrated that HA fragments generated by sonication of a purified, high molecular mass precursor from rooster comb, or lower molecular mass fragments purified from human umbilical cord, activate the transcriptional regulatory complex NF- κ B/I- κ B α in mouse macrophages. An important aspect of this study is that we have carefully defined the characteristics of HA required for activating macrophages. By using highly purified, LPS-free HA preparations and characterizing the sizes of the active fragments, new information has been obtained regarding the interaction of an ECM component with macrophages. We

have shown that following stimulation with HA fragments, mI- κ B α protein levels rapidly fall and are then regenerated. Furthermore, in vitro translated mI- κ B α protein was shown to inhibit the HA fragment induced NF- κ B DNA binding activity. Thus, HA fragments activate the NF- κ B/I- κ B α system in macrophages. Among the HA fragments examined, there were differences in activity which may relate to molecular size, or as-yet-undetected covalent structural differences.

A variety of soluble stimuli and microbes have been shown to activate NF- κ B DNA binding in macrophages, including TNF α , IL-1 β , PMA, and viruses, but we believe this is the first report of an ECM fragment of defined size that has been shown to activate the NF- κ B/I- κ B system in macrophages. Recent evidence has suggested that heparan sulfate can activate NF- κ B DNA binding in mouse peritoneal macrophages (28). We failed to see induction of mI- κ B α mRNA in either bone marrow-derived macrophages or the alveolar macrophage-like cell line MH-S by heparan sulfate. Adhesion alone activates NF- κ B DNA binding activity in human monocytes with peak activity occurring within 15 min (29). The time course in the alveolar macrophage cell line in these data was prolonged in comparison to adherent monocytes with NF- κ B DNA binding activity first seen at 30 min and peaking at 2 h. A similar time course for NF- κ B DNA binding activity was seen with another adherent mouse macrophage cell line, RAW 264.7 cells, in response to LPS (30). These data suggest that there may be differences in the mechanism of NF- κ B activation between already adherent, mature macrophages exposed to a soluble stimulus, and fresh monocytes that adhere to substratum.

The hallmarks of chronic inflammation are the unremitting recruitment of monocyte/macrophages, neutrophils and T cells to tissue sites resulting in continued damage and increased turnover of ECM. We have addressed the hypothesis that one mechanism for the activation of macrophages at sites of inflammation may be through the interaction with components of the ECM that are produced as a consequence of ECM turnover. Data presented in this study show that HA fragments of a size range that have been shown to exist in vivo (31, 32) can activate an NF- κ B/I- κ B α autoregulatory loop in macrophages.

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