

High molecular weight hyaluronic acid inhibits advanced glycation endproduct-induced NF- κ B activation and cytokine expression

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Abstract Advanced glycation endproducts (AGEs), which accumulate on long-lived proteins and protein deposits (amyloids), induce the expression of proinflammatory cytokines through NF- κ B-dependent pathways. Hyaluronic acid with a molecular weight above 1.2 MDa (HMW-HA) inhibits the AGE-induced activation of the transcription factor NF- κ B and the NF- κ B-regulated cytokines interleukin-1 α , interleukin-6 and tumor necrosis factor- α . Since the molecular weight of hyaluronic acid in humans decreases with age and under conditions of oxidative stress, it is likely that the protective effect of HMW-HA against AGE-induced cellular activation is lost at sites of chronic inflammation and in older age.

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Key words: Hyaluronic acid; Signal transduction; Advanced glycation endproduct

1. Introduction

Advanced glycation endproducts (AGEs) are sugar- or sugar degradation product-derived protein modifications which irreversibly crosslink long-lived proteins and thus contribute to the formation of insoluble protein deposits. AGE formation begins with the reaction of nucleophilic protein side chains, i.e. of lysine and arginine, with reducing sugars or sugar-derived carbonyl and dicarbonyl compounds. This modification, termed non-enzymatic glycosylation, glycation or Maillard reaction, leads, through subsequent rearrangements, dehydrations and oxidations, to the formation of a heterogeneous group of mostly fluorescent and brown products, the so-called advanced glycation endproducts [1,2] (Fig. 1).

AGE-modified proteins elicit an acute-phase response in phagocytotic cells, including microglia and macrophages, and lead to the subsequent generation of free radicals like superoxide and NO and the upregulation of pro-inflammatory cytokines, such as interleukin (IL) 1 α , IL-6 and tumor necrosis factor- α (TNF- α) [3,4]. AGEs exert their cellular effects by interacting with specific cell surface receptors, the best characterized of which is the receptor for AGEs (RAGE)[5]. AGEs have been shown to activate two major signal transduction pathways: a redox-sensitive pathway involving NADPH oxidase and the transcription factor NF- κ B [6,7]

and a mitogenic pathway involving the small G-protein Ras, protein kinases further downstream including ERK-2 and the transcription factor AP-1 [8–10]. These two pathways converge at the transcriptional level to induce the expression of specific pro-inflammatory cytokines [5,11,12].

AGEs have been suggested to be inducers of chronic inflammation and acute-phase responses in a variety of diseases. In the brain of Alzheimer disease patients activated microglia are found in close colocalization with AGE-modified β -amyloid plaques [13–17].

Pharmacological interference with AGE-induced signal transduction is considered to be a promising treatment strategy for these inflammatory conditions. A key second messenger in AGE-activated signal transduction is the superoxide anion and hydrogen peroxide which can be scavenged by intracellularly acting antioxidants including 17-estradiol or thiocetic acid [7,18]. However, as yet no physiological antagonist or inhibitor of AGE-induced cell activation has been described. Two observations suggested that hyaluronic acid (HA), a glycosaminoglycan polymer of the extracellular matrix consisting of repeat units of disaccharide, *N*-acetyl-D-glucosamine and *N*-acetyl-D-glucuronic acid, might serve this purpose. For example, HA-positive neurons have been shown to be less prone to cell death in Alzheimer's disease brain, suggesting that these neurons are more resistant to the pathological processes of AD, particularly to oxidative stress [19]. Secondly, application of synthetic HA preparations is beneficial in chronic inflammatory conditions associated with rheumatoid arthritis and osteoarthritis. For example, injection of HA reduces total amounts of leukotrienes, prostaglandins and IL-1 α in synovial fluid of the knee and the temporomandibular joint [20,21]. In contrast, some groups show that a pro-inflammatory response can be induced by HA, i.e. an increase in IL-1, TNF- α , and IL-8 [22] or an increase in the expression of ICAM-1 and VCAM-1 [23]. However, a growing body of evidence suggests that the chemical properties of HA, such as its molecular weight, determine its pro- or anti-inflammatory potential.

We describe high molecular weight HA (HMW-HA) acting extracellularly to inhibit BSA-AGE-induced cell activation in macrophages via NF- κ B and, further downstream, IL-1 α , IL-6 and TNF- α . Since the molecular weight of HA decreases with age and in regions of high radical load, we suggest that HA may lose its protective properties under these conditions, allowing a vicious circle of chronic inflammation, oxidative stress and radical-induced HA depolymerization.

2. Materials and methods

2.1. Materials

HA (MW: 1.2 MDa, 0.72 MDa and 0.6 MDa) LMW-HA (MW: 0.2–0.5 MDa) were purchased from Lifecore and Sigma, respectively.

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Abbreviations: AGE, advanced glycation endproduct; IL-1 α , interleukin 1 α ; IL-6, interleukin 6; TNF, tumor necrosis factor; HA, hyaluronic acid; LMW, low molecular weight; HMW, high molecular weight

D-Glucuronic-acid, N-acetylglucosamine and chondroitin sulfate were from Sigma, the anti-p50 antibody was supplied by Santa Cruz Biotechnology. Cell culture reagents were from Gibco, PCR primer pairs specific for murine β -actin, TNF- α , IL-6 and IL-1 α from Eurogentec.

2.2. Activation of murine macrophages

J774 (ATCC TIB 67) mouse macrophages were grown in 75 cm² tissue culture flasks for 24–36 h until confluence in RPMI 1640, 10% FCS, supplemented with penicillin/streptomycin (100 U/ml, 100 μ g/ml) and L-glutamine (2 mM) at 37°C in 5% CO₂. Macrophage activation was induced by the addition of 2 μ M BSA-AGE. The negative control contained 2 μ M BSA and/or medium only. HA was added at a concentration of 4 mg/ml 15 min before the start of the experiment. The monoclonal anti-CD44 antibody was used at a concentration of 0.48 μ g/ml and added 15 min before addition of HA.

2.3. Production of AGEs

BSA-AGEs were produced by incubation of 1 mM BSA with 1 M glucose in PBS, pH 7.4 at 50°C for 6 weeks and the 2w-BSA-AGE under the same conditions for 2 weeks. Since a steady and almost linear increase in both fluorescence (370 nm excitation/440 nm emission) and absorbance at 400 nm was observed after 6 weeks and the increase thereafter was marginal, the BSA was assumed to be maximally modified by AGEs. Unbound sugars were removed by extensive dialysis with distilled water. Model AGEs were lyophilized and resuspended in PBS (pH 7.4). Protein content was determined by the Bradford assay, using BSA as a standard. AGE content was estimated by measuring the optical density at 400 nm and the fluorescence at 440 nm. Although bacterial growth should be negligible under these conditions, LPS content of the BSA-AGE preparation was further minimized by passing it over a polymyxin column (Pierce). These BSA-AGE preparations contained no endotoxin as judged by a negative result in the Limulus amoebocyte lysate (LAL) assay (Bio-Whittaker, Inc.).

2.4. Preparation of nuclear protein extracts

Nuclear protein extracts (for determination of nuclear NF- κ B by electrophoretic mobility shift assay) were prepared essentially as described by Hauf et al. [24]. Briefly, PBS-washed macrophages were scraped from the culture plate and collected by centrifugation. Pellets were resuspended in 0.4 ml of hypotonic lysis buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). After 15 min on ice, Nonidet P40 was added to a final concentration of 0.5%. The cells were mixed for 10 s and then centrifuged for 30 s at

12000 \times g (4°C). The pellets, containing the nuclei, were resuspended in 140 μ l buffer containing 20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF and mixed for 30 min at 4°C. After centrifugation (5 min, 4°C, 12000 \times g), the supernatant (= nuclear extract) was stored at -80°C.

2.5. Electrophoretic mobility shift assay (EMSA)

The oligonucleotides representing the consensus sequence for NF- κ B (5'-AGC TTC AGA GGG GAC TTT CCG AGA GG-3' and 5'-TCG ACC TCT CGG AAA GTC CCC TCT GA-3') were hybridized to form a double-stranded probe; and the ends were ³²P-labeled with Klenow polymerase. The binding reactions were performed on ice in a volume of 15 μ l, containing 5 μ l 3 \times binding buffer (60 mM HEPES (pH 7.9), 1 mM DTT, 3 mM EDTA, 150 mM KCl and 12% Ficoll), 20000 cpm of ³²P-labeled DNA probe, 7 μ g nuclear proteins, and 2 μ g poly dIdC (Boehringer-Mannheim). After 30 min on ice, the complexes were separated on a native 5% polyacrylamide gel and scanned. For the supershift assays, the anti-p50 antibody was included at a concentration of 1 mg/ml in the EMSA standard reaction mixture 20 min before the addition of the labeled oligonucleotides.

2.6. RT-PCR for detection of IL-6 and TNF- α expression

Cells were grown in cultured medium in 75cm² tissue culture flasks (1 \times 10⁶ cells per flask) and incubated with BSA (2 μ M) or BSA-AGEs (2 μ M) for 24h, then lysed with guanidinium thiocyanate. Total RNA was prepared by phenol-extraction and reverse transcribed with Moloney murine virus reverse transcriptase (20U/ μ l). The primers used for cytokine-specific PCR have been described recently [25]. PCR was performed with an initial denaturation step of 3min at 91°C; then 30 cycles were performed as follows: 1min of denaturation at 91°C, 1min of annealing at 60°C and 1min extension at 72°C. The reaction products, IL-1 α (308bp), IL-6 (154bp), β -actin (348bp) and TNF- α (307bp) were visualized by electrophoresis of 15 μ l of the reaction mixture in a 2% agarose gel containing 0.5 μ g/ml ethidium bromide.

3. Results

AGEs have been shown to activate the transcription factor NF- κ B and to upregulate various NF- κ B-controlled genes in various cell types [7,26]. This can be seen in the clonal mouse macrophages line J774 too, where addition of BSA-AGE activates NF- κ B, as demonstrated by the translocation of NF- κ B subunits into the nucleus. Binding of the NF- κ B subunits

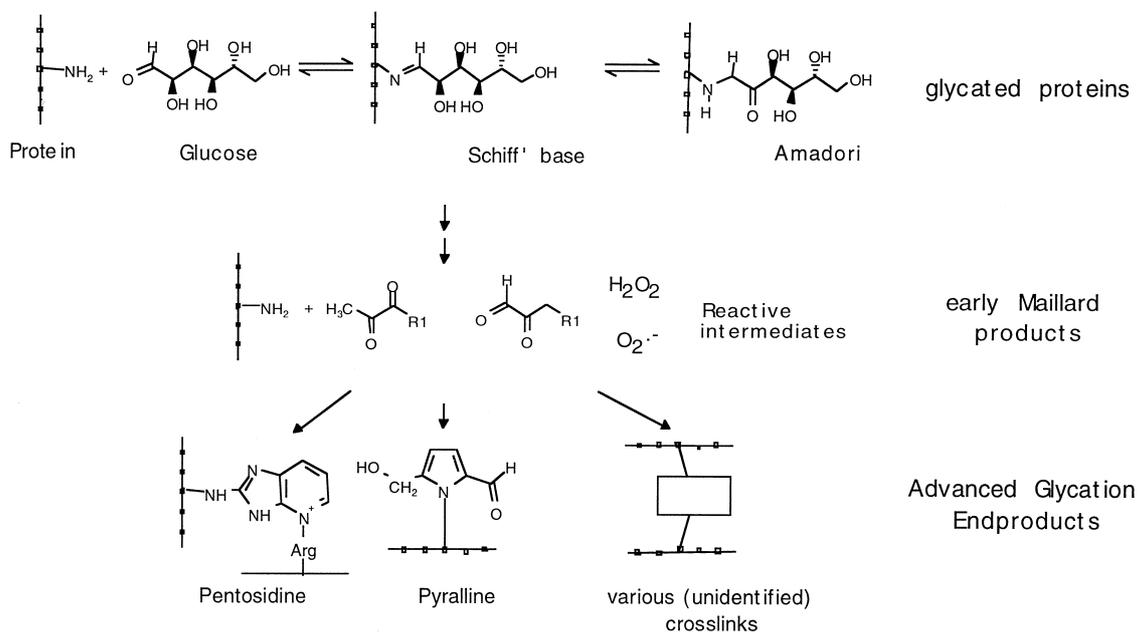


Fig. 1. Chemical reactions leading to the formation of advanced glycation endproducts.

to the labeled oligonucleotide containing the NF-κB consensus sequence was specific, as it could be inhibited by the addition of excess unlabeled oligonucleotide (Fig. 2, lanes 4–6). The activated NF-κB complex contains p50, as demonstrated by a supershift assay with an anti-p50 antibody (Fig. 2, lane 1). The 2w-BSA-AGE was less effective in activating NF-κB than BSA-AGE (Fig. 2, lanes 2, 3).

BSA-AGE leads to a strongly active NF-κB complex after 6 h of incubation, whereas 15 min preincubation of cells with HMW-HA (MW: 1.2×10^6 Da) at a concentration of 4 mg/ml completely inhibited the BSA-AGE-induced activation of NF-κB (Fig. 3, lanes 2, 3). One of the major cell surface receptors for hyaluronic acid is CD44, therefore an anti-CD44 antibody was used to inhibit the binding of HA to the macrophage CD44 receptors. This restored the BSA-AGE-induced translocation of the activated NF-κB complex into the nucleus (Fig. 3, lane 5), with a somewhat different pattern of the activated NF-κB complex. The anti-CD44 antibody, HMW-HA and BSA showed no NF-κB-activating effects (Fig. 3 lanes 6, 4 and 1).

The protective effect of the HA diminished with decreasing molecular weight of HA from 1.2 MDa to 0.3–0.5 MDa (Fig. 4A). At a size below 0.5 MDa the HA not only lost its protective effect, it became NF-κB-activating itself. This was only slightly reduced by the anti-CD44 antibody (Fig. 4B, lanes 3, 4). The activating effect seemed to be equivalent to that of the BSA-AGE, whereas the two activators did not act synergistically (Fig. 4B, lanes 1, 2). Other glycosaminoglycans like D-glucuronic acid, N-acetylglucosamine and chondroitin sulfate were not able to protect the macrophages from BSA-AGE-induced NF-κB activation (Fig. 4B, lanes 6–8), as the resulting NF-κB complex was nearly as prominent as that of BSA-AGE (lane 5). These three glycosaminoglycans did not activate NF-κB (data not shown), an effect already described for the cell line RAW 264.7 [27].

HMW-HA interference with NF-κB-mediated signaling induced by AGEs was investigated further downstream by analyzing the expression of the NF-κB-regulated pro-inflamma-

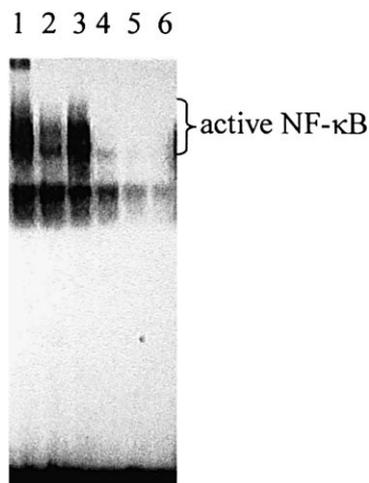


Fig. 2. Addition of 2 μM BSA-AGE (lane 3) leads to a strong activation of NF-κB in J774 macrophages, with 2w-BSA-AGE the activation was lower (lane 2). The binding of the NF-κB complexes to the radiolabeled NF-κB probe was inhibited by 1-, 3- and 7-fold excess of non-radioactive oligonucleotide (lanes 4, 5, 6). Addition of p50 antibody supershifted the NF-κB complex (lane 1).

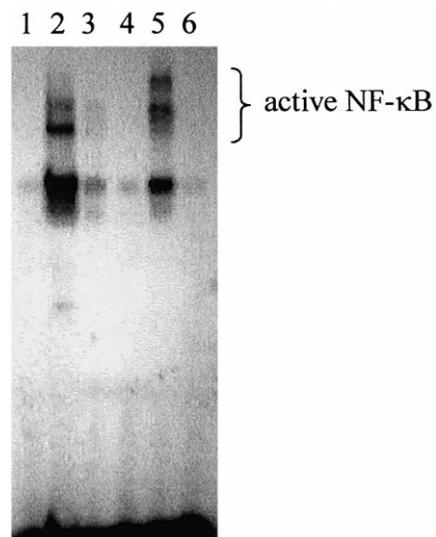
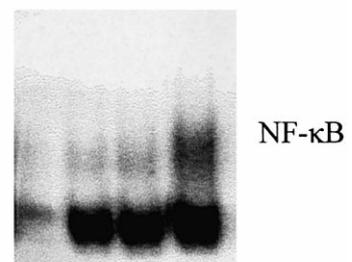


Fig. 3. J774 macrophages exhibited an activation of NF-κB after 6 h by BSA-AGE (lane 2), a process attenuated by HMW-HA (lane 3). This protective effect was abolished by anti-CD44 antibodies (lane 5). BSA, HMW-HA or anti-CD44 antibody had no effect on the translocation of NF-κB (lanes 1, 4 and 6).

A

BSA-AGE	+	+	+	+
HA 1,2 MDa	+	-	-	-
HA 0,7 MDa	-	+	-	-
HA 0,6 MDa	-	-	+	-
HA 0,3-0,5 MDa	-	-	-	+



B

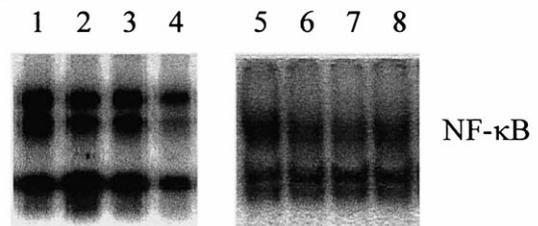


Fig. 4. A: The protective effect of the HA decreased with shrinking size from 1.2 MDa via 0.7 MDa, 0.6 MDa down to 0.3–0.5 MDa. B: LMW-HA activated NF-κB to a similar extent as BSA-AGE (lanes 3, 1) but showed no synergistic effects together with BSA-AGE (lane 2). The anti-CD44 antibody reduced the active NF-κB complex (lane 4) in parts. The glycosaminoglycans D-glucuronic acid, N-acetylglucosamine and chondroitin sulfate (lanes 6, 7 and 8) were not able to inhibit BSA-AGE-activated NF-κB, so that the NF-κB activation was comparable to that of BSA-AGE (lane 5).

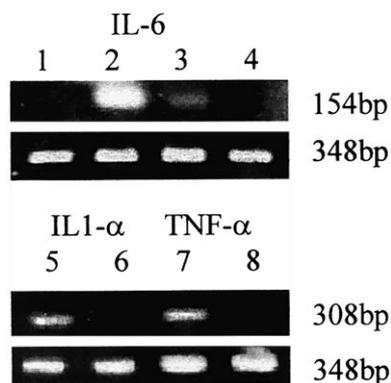


Fig. 5. Addition of BSA-AGE to J774 macrophages leads to an up-regulation of IL-6, IL-1 α and TNF- α mRNA expression (lanes 2, 5, 7), which was inhibited by HMW-HA (lanes 3, 6, 8). BSA-AGE or HMW-HA caused no IL-6 mRNA expression (lanes 1 and 4). The lower panels show the β -actin control (348 bp).

tory cytokines IL-1 α , IL-6 and TNF- α at the mRNA level. Addition of HMW-HA inhibited the AGE-induced increase in expression of IL-1 α , IL-6 and TNF- α after 24 h as detected by RT-PCR, consistent with its effects on NF- κ B (Fig. 5).

4. Discussion

Glucose has been implicated in the ageing process by its ability to react non-enzymatically with long-lived proteins to produce AGEs, which increase in tissues as a function of time (e.g. with age) and sugar concentration [28]. AGEs induce permanent abnormalities in extracellular matrix component function, stimulate cytokine and reactive oxygen species production through AGE-specific receptors, and modify intracellular proteins. AGEs are thus suggested to be promoters of chronic inflammation and to cause an imbalance of proliferation and degeneration as described for diabetic retinopathy, nephropathy, neuropathy, arterial abnormalities, as well as in Alzheimer's disease and rheumatoid arthritis.

No extracellular physiological counterparts ('AGE antagonists') able to attenuate AGE-induced pro-inflammatory signal transduction have been described to date. Our results show that HMW-HA with a molecular weight above 1.2 MDa is an inhibitor of AGE-induced signal transduction, whereas with decreasing size of HA this inhibition is gradually reduced. In addition to their diminished protection smaller HA fragments (<0.5 MDa) are pro-inflammatory. This dependence of the pro-inflammatory effects of HA on its molecular weight might also explain the conflicting reports in the literature concerning the anti-inflammatory potential of HA. Recently other reports regarding the opposing effects of LMW-HA and HMW-HA have appeared in the literature. For example, small HA fragments (MW: 3.5×10^4 Da), but not physiological native HA polymers (MW: 2.2×10^6), were shown to induce prostanoid production via increased COX-2 expression [29]. In mouse cortical tubular (MCT) cells fragmented intermediate HA, but not HMW-HA markedly increased ICAM-1 and VCAM-1 expression. Upregulation of ICAM-1 and VCAM-1 by LMW-HA was preceded by a marked increase in NF- κ B and AP-1 activation [23]. These data also explain the beneficial effects of injections of synthetic HMW-HA preparations on chronic inflammatory conditions in joints, such as the knee and the mandible [30].

Although our results indicate that the protective effect of HMW-HA could be mediated through CD44, it remains to be elucidated whether CD44 signal transduction pathways, such as the src family protein tyrosine kinases lck and fyn, interfere with AGE signal transduction [31] or whether HMW-HA inhibits AGE/RAGE binding by steric effects.

There are two possible physiological causes for the decrease of the molecular weight of HA in vivo: aging and radical-induced depolymerization. The average size of the HA polymers decreases with age, as measured in human cartilage (where the molecular weight drops from >2 MDa in newborns to about 0.3 MDa at the age of 80) and in aqueous humor and vitreous body of rabbit and cattle [32,33]. The age-related decrease in HA size might also be a reason for the increased susceptibility of the elderly to (AGE-induced) chronic inflammations. High radical burden, e.g. at sites of inflammation, also leads to a degradation of HA by released oxidants such as $O_2^-/H_2O_2/HO^\bullet$ and $HOCI/ClO^-$ [34]. These data also explain the observed high levels of HA at sites of inflammation, which are most probably LMW-HA fragments released by radical-induced depolymerization.

Since the molecular weight of HA decreases with age and in regions of high radical load, we suggest that HA loses its protective properties under these conditions leading to a vicious circle of chronic inflammation, oxidative stress and radical-induced HA depolymerization.

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