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Pro-inflammatory activity of contaminating DNA in hyaluronic acid preparations

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Abstract

Hyaluronic acid (HA), an abundant non-sulfated glycosaminoglycan component of the extracellular matrix, has applications in drug delivery, tissue engineering and as an ingredient in cosmetics. HA preparations containing high-molecular-weight polymers are also used in the treatment of inflammatory disorders such as arthritis and interstitial cystitis. Low-molecular-weight fragments derived from HA have been reported to induce pro-inflammatory cytokines such as IL-12 and TNF-α, and could therefore potentially exacerbate existing inflammation. We therefore examined the pro-inflammatory activity of HA preparations, since inflammatory reactions are known to occur following administration of HA. We tested low-molecular-weight fragments obtained from seven different HA preparations, either by sonication ($\approx 3 \times 10^5$ Da) or by hyaluronidase digestion ($\approx 1 \times 10^4$ Da), for the ability to induce the synthesis of IL-12 and TNF-α by human monocytic cells. We found that two of the seven HA preparations tested stimulated the synthesis of IL-12 and TNF-α by human monocytic cells. We unexpectedly found that the induction of IL-12 and TNF-α by these HA preparations was not due to their degradation to low-molecular-weight fragments, since their native high-molecular-weight forms possessed the same ability to stimulate IL-12 and TNF-α synthesis, but was due to the presence of contaminating DNA. Treatment of these two HA preparations with deoxyribonuclease I abrogated or reduced the induction of IL-12 and TNF-α. It is clear from this study that HA preparations can induce the synthesis of pro-inflammatory cytokines by monocytes. The ability of HA to act as a pro-inflammatory mediator may not, however, be related to the presence of low-molecular-weight HA fragments, but to the presence of DNA. The presence of pro-inflammatory DNA in HA preparations should be evaluated before its use, not only for the treatment of patients with inflammatory disorders, but also before many other applications.

Introduction

Hyaluronic acid (HA), also called hyaluronan, hyaluronate or sodium hyaluronate, is one of the major non-structural elements of the extracellular matrix present in all connective tissues (Engström-Laurent 1997). In its native form, HA exists as a high-molecular-weight polymer (10^6–10^7 Da) (Laurent & Fraser 1992). HA preparations containing high-molecular-weight polymers ($> 0.5 \times 10^6$ Da) have been reported to decrease inflammation in a number of diseases. The efficacy of intra-articular HA administration has been demonstrated in patients with osteoarthritis (Namiki et al 1982; Lussier et al 1996) and rheumatoid arthritis (Matsuno et al 1999). HA has also been reported to decrease the inflammation of irritated bladder wall of patients having interstitial cystitis (Morales et al 1996). Several hypotheses have been

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postulated to explain the mechanism of action of HA: reconstruction of the deficient glycosaminoglycan layer (Morales et al 1996); supplementation of the viscous and elastic properties of pathologic synovial fluid (Balazs & Denlinger 1993); inhibition of lymphocytes proliferation (Anastassiades & Robertson 1984); and inhibition of synovial cell proliferation (Goldberg & Toole 1987). However, although pharmaceutical-grade high-molecular-weight HA has been shown to reduce inflammation in a number of diseases, it has recently been reported that inflammatory reactions occur following intra-articular administration of high-molecular-weight HA preparations (Puttick et al 1995; Adams 1996; O'Hanlon 1996; Kirwan & Rankin 1997).

Low-molecular-weight HA fragments ($< 5 \times 10^5$ Da) have been shown to be present at abnormally high levels in the joints of patients with rheumatoid arthritis (Grootveld et al 1991), in bronchoalveolar lavage fluid of patients with pulmonary fibrosis (Brejmer et al 1989), in the intestinal lumen of patients with Crohns's disease (Ahrenstedt et al 1992) and in the urine of patients with interstitial cystitis (Erickson et al 1998). Low-molecular-weight HA fragments are generated during the course of inflammation by de-novo synthesis of low-molecular-weight fragments (Prehm 1989) or by digestion by hyaluronidase or depolymerization by free-radical species of the native high-molecular-weight HA present in the extracellular matrix (McNeil et al 1985; Sampson et al 1992). Several studies have suggested that high- and low-molecular-weight HA exhibit differential biological effects on cells and tissues. While high-molecular-weight HA are components of the extracellular matrix and substrates for cell adhesion, it has been shown that low-molecular-weight fragments are sentinel molecules which alert the immune system to significant tissue damage occurring at sites of inflammation (Aruffo 1996).

Low-molecular-weight fragments derived from HA by either sonication or digestion with hyaluronidase have been reported to induce pro-inflammatory cytokine gene expression in mast cells, monocytes and macrophages, and could potentially exacerbate inflammation in patients with inflammatory disorders. The inflammatory mediators up-regulated by low-molecular-weight HA fragments include IL-1β, IL-12, TNF-α, MIP-1α, MIP-1β, MCP-1 and RANTES (Noble et al 1993; McKee et al 1996; Hodge-Dufour et al 1997). IL-12 and TNF-α play a key role in tissue damage during inflammation (Feldman 1998; Trinchieri 1998). Monocytes have been shown to be present in the joints of patients with osteoarthritis (Dodds et al 1999) and rheumatoid arthritis (Lioté et al 1996) and in the bladder wall of patients having interstitial cystitis (Christmas & Bottazzo 1992). We therefore tested whether low-molecular-weight fragments obtained from seven different HA preparations by sonication ($\approx 3 \times 10^5$ Da) or by hyaluronidase digestion ($\approx 1 \times 10^4$ Da) have the ability to induce expression of IL-12 and TNF-α by monocytes.

Materials and Methods

HA preparations

The characteristics of the HA preparations used in this study are summarized in Table 1. Samples A, B, C, D, E and F were utilized for therapeutic use in human or animal diseases. Sample G was obtained from a chemical company. All HA preparations were tested for endotoxin content using a colorimetric *Limulus* amoebocyte lysate assay with a sensitivity of 0.01 endotoxin units mL^{-1} (QCL-1000, BioWhittaker, Walkersville, MD), and for protein content by a micro protein determination kit (Sigma-Aldrich Canada, Oakville, ON) using Coomassie Brillant Blue G (Sedmak & Grossberg 1977). Low-molecular-weight HA was obtained by digestion with hyaluronidase type IV-S derived from bovine testes (Sigma-Aldrich Canada) for 60 min at 37°C or by sonication on ice using a Fisher model 550 sonifier (Fisher Scientific, Nepean, ON) for 20 min at maximal intensity. The molecular-weight distribution of HA was analysed by electrophoresis in 0.5% agarose gels prepared in TAE buffer (40 mM Tris, 20 mM acetic acid and 2.0 mM EDTA, pH 7.9) for 3 h at 100 V (Lee & Cowman 1994) and visualized with the cationic dye Stains-All (Sigma-Aldrich Canada). HA molecular weight was evaluated after gel photo scanning using 1D software (Advance American Biotechnology, Fullerton, CA).

Table 1 Characteristics of HA preparations used in this study.

Preparation	Molecular mass (Da)	Source
A	$0.5–0.8 \times 10^6$	*Streptococcus* sp.
B	$0.6–2.0 \times 10^6$	*Streptococcus* sp.
C	$0.5–1.0 \times 10^6$	*Streptococcus* sp.
D	$0.5–0.8 \times 10^6$	Rooster comb
E	$0.6–2.0 \times 10^6$	Rooster comb
F	$> 2.0 \times 10^6$	Rooster comb
G	$0.5–1.2 \times 10^6$	Bovine trachea

HA preparations were provided or purchased from several manufacturers.

Evaluation of IL-12 and TNF-α synthesis

Human monocyte THP-1 cells were obtained from the American Tissue Culture Collection (ATCC; Rockville, MD). THP-1 monocytes were incubated in six-well flat-bottomed tissue culture plates at 1×10^6 cells mL^{-1} with various concentrations of the HA preparations in 1 mL of RPMI-1640 supplemented with 10% fetal bovine serum (Gibco-BRL, Burlington, ON) for 48 h at 37°C in an atmosphere of 5% CO_2. The amount of IL-12 and TNF-α released into the supernatant was measured using commercial ELISA kits (BioSource, Camarillo, CA).

Detection and treatment of DNA

The presence of DNA in the HA preparations was determined by electrophoresis in agarose gels containing 0.7% agarose and 0.5 $\mu g\ mL^{-1}$ ethidium bromide at 100 V for 3 h. DNA was visualized using a UV transilluminator (wavelength: 302 nm). HA preparations containing DNA were incubated with 10 U of deoxyribonuclease I (DNase I) (Gibco-BRL) for 1 hour at 37°C in 20 mM Tris-HCl (pH 8.4), 2 mM magnesium chloride and 50 mM potassium chloride. EDTA was then added to the reaction mixture (final concentration 2.5 nM) which was heated for 10 min at 65°C to inactivate the DNase I. Digestion of the DNA was confirmed by electrophoresis in agarose gels as described above. For experiments with inactivated DNase I, DNAse I was inactivated as described above before its addition to HA. HA preparations treated with DNase I or with inactivated DNase I were incubated with THP-1 cells and IL-12 and TNF-α synthesis was evaluated as described in the previous section.

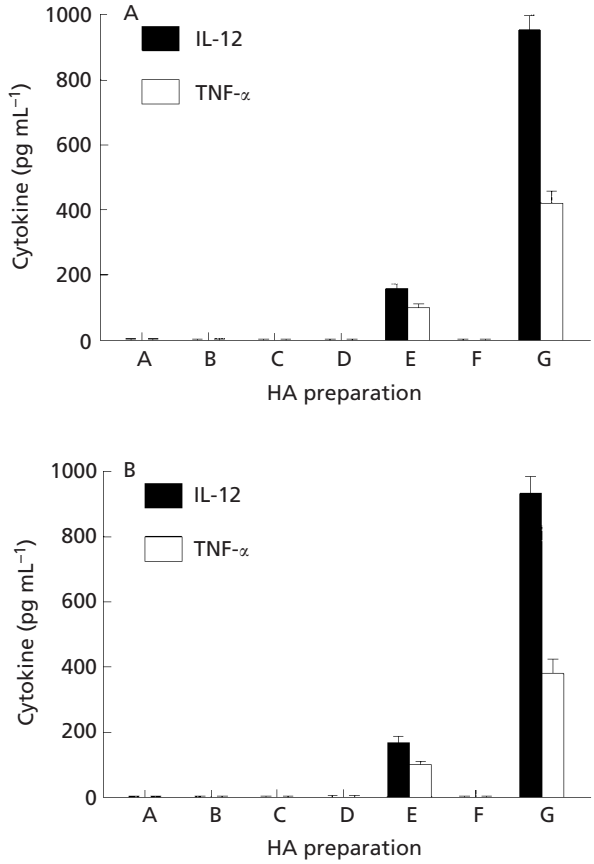

Figure 1 Induction of IL-12 and TNF-α synthesis by monocytes in response to low-molecular-weight fragments of HA preparations A–G. THP-1 monocytes were incubated at 1×10^6 cells mL^{-1} with 100 $\mu g\ mL^{-1}$ of low-molecular-weight HA obtained by hyaluronidase digestion (approximately 1×10^4 Da) (A) or with low-molecular-weight HA obtained by sonication (approximately 3×10^5 Da) (B), for 48 h at 37°C, 5% CO_2. IL-12 and TNF-α released into the supernatant were measured after 48 h by ELISA. Data are expressed as the mean ± s.d. of three independent experiments.

Results

It has been previously shown that low-molecular-weight fragments derived from HA are able to induce the expression of a number of pro-inflammatory cytokines including IL-12 and TNF-α (Noble et al 1993; McKee et al 1996; Hodge-Dufour et al 1997). The generation of low-molecular-weight HA fragments following intravesical or intra-articular administration could be due to the presence of inflammation-related hyaluronidase. Levels of hyaluronidase are higher in patients with cystitis than in healthy individuals (Pham et al 1997). In patients with rheumatoid arthritis, hyaluronidase activity in the synovial fluid varies from undetectable to very high (Fiszer-Szafarz et al 1995). We have determined whether low-molecular-weight HA fragments could induce the synthesis of the pro-inflammatory cytokines IL-12 and TNF-α by human monocytes. As shown in Figure 1A, low-molecular-weight HA fragments obtained from two out of seven HA preparations by hyaluronidase digestion ($\approx 1 \times 10^4$ Da), preparations E and G, had the ability to induce the synthesis of IL-12 or TNF-α by human monocytes. Similar results were obtained using HA degraded by sonication ($\approx 3 \times 10^5$ Da) (Figure 1B). We unexpectedly found that native high-molecular-weight HA preparations E and G were also able to stimulate the synthesis of significant levels of IL-12 and TNF-α in a dose-dependent manner (Figure 2A and B) while other native high-molecular-weight preparations showed no activity (data not shown). No significant difference, in terms of

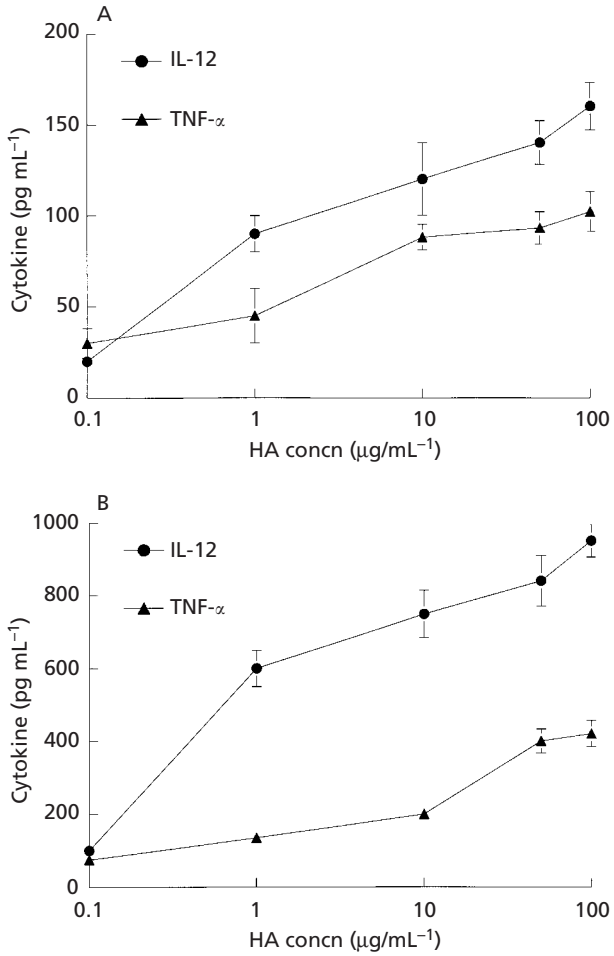

Figure 2 Induction of pro-inflammatory cytokine IL-12 and TNF-α synthesis by monocytes in response to native high-molecular-weight HA. THP-1 monocytes were incubated at 1×10^6 cells mL^{-1} with 0.1, 1.0, 10.0, 50.0 and 100.0 μg mL^{-1} of HA preparation E (A) or 0.1, 1.0, 10.0, 50.0 and 100.0 μg mL^{-1} of HA preparation G (B) for 48 h at 37°C, 5% CO_2. IL-12 and TNF-α released into the supernatant were measured after 48 h by ELISA. Data are expressed as the mean $\pm$ s.d. of three independent experiments.

IL-12 and TNF-α produced by monocytic cells in response to 100 μg mL^{-1} of HA, was observed between low-and high-molecular-weight HA for preparations E and G (Figure 1 and 2).

The ability of high-molecular-weight HA to induce the synthesis of IL-12 and TNF-α by monocytes suggested that contaminating molecules could be responsible for the induction of these cytokines. Endotoxin contamination has been shown to be able to induce pro-inflammatory cytokines such as IL-12 and TNF-α by monocytes and to cause septic shock in mammals (Astiz et al 1996; Jansen et al 1996). None of the HA preparations evaluated contained endotoxin (*Limulus* amoebocyte lysate assay; sensitivity: 0.01 endotoxin units mL^{-1}), or protein as determined using the method of Sedmak & Grossberg (1977) (sensitivity: $\geqslant 1.0$ μg).

Table 2 Presence of DNA in HA preparations.

Preparation	Quantity of DNA associated with HA (mg DNA per mg HA)
A	undetectable
B	0.05 ± 0.02
C	undetectable
D	undetectable
E	0.03 ± 0.01
F	undetectable
G	0.15 ± 0.04

The quantity of DNA present in HA preparations was evaluated on agarose gels containing ethidium bromide. Data are expressed as the mean $\pm$ s.d. of three independent experiments.

DNA from numerous species has been shown to induce pro-inflammatory cytokines, including IL-12 and TNF-α (Halpern et al 1996; Krieg 1996; Neujahr et al 1999). We therefore determined whether the different HA preparations contained DNA. We found that three out of the seven HA preparations tested contained significant levels of DNA in the range 0.03–0.15 mg of DNA per mg of HA (Table 2) with molecular weights ranging from 500 to $>$ 20000 base pairs (data not shown). Treatment of the HA preparations with DNase I, an endonuclease which digests both single- and double-stranded DNA molecules to small oligodeoxyribonucleotides, resulted in the abolition of IL-12 and TNF-α induction by HA preparation E (Figure 3A). Treatment of HA preparation G by DNase I also resulted in a reduction of IL-12 and TNF-α synthesis by monocytes, of 77% and 32%, respectively (Figure 3B). The results obtained with preparation G correlated with an incomplete digestion of the DNA by the DNAse I (data not shown). Inactivated DNase I had no effect on the cytokine-inducing activity of HA preparations E and G (Figures 3A and B). The DNase I used in these studies did not possess hyaluronidase activity (data not shown). HA preparation B contained DNA (Table 3), but did not induce IL-12 and TNF-α (Figure 1).

Discussion

Pharmaceutical HA preparations containing high-molecular-weight polymers have gained popularity for

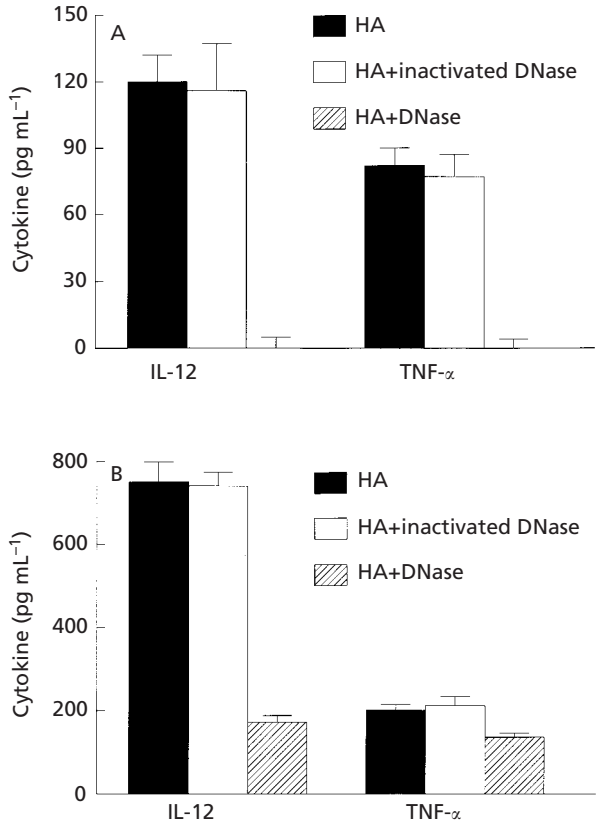

Figure 3 Induction of pro-inflammatory cytokine IL-12 and TNF-α synthesis by HA preparations is due to contaminating DNA. DNA present in HA preparations E and G was removed by treatment with DNase I. Inactivation of DNase I was carried out as described in the Material and Methods section. THP-1 monocytes were incubated at 1×10^6 cells mL^{-1} for 48 h at 37°C, 5% CO_2 with 10 μg of HA preparation E, 10 μg of HA preparation E treated with DNase I or 10 μg of HA preparation E treated with inactivated DNase I (A) or 10 μg of HA preparation G, 10 μg of HA preparation G treated with DNase I or 10 μg of HA preparation G treated with inactivated DNase I (B). IL-12 and TNF-α released into the supernatant were measured after 48 h by ELISA. Data are expressed as the mean $\pm$ s.d. of three independent experiments.

the treatment of inflammatory disorders such as osteoarthritis and rheumatoid arthritis (Namiki et al, 1982; Lussier et al 1996; Matsuno et al 1999) and interstitial cystitis (Morales et al 1996). We therefore felt it prudent to examine the pro-inflammatory activity of HA preparations used either for the treatment of arthritis or interstitial cystitis, since a number of inflammatory reactions have been reported following intra-articular administration of HA (Puttick et al 1995; Adams 1996; O'Hanlon 1996; Kirwan & Rankin 1997). We unexpectedly found that some HA preparations had the ability to induce the synthesis of the pro-inflammatory cytokines IL-12 and TNF-α, and could therefore potentially exacerbate ongoing inflammatory reactions.

Low-molecular-weight HA fragments generated during the course of inflammation have been shown to induce the expression of monocyte and macrophage genes which are critical to both the initiation and the maintenance of the inflammatory response (Noble et al 1993; McKee et al 1996; Hodge-Dufour et al 1997). Monocytes and macrophages have been shown to be present in the joints of patients with osteoarthritis and rheumatoid arthritis (Lioté et al 1996, Dodds et al 1999), and in the bladder wall of individuals with interstitial cystitis (Christmas & Bottazzo 1992). We therefore felt that it was important to determine whether low-molecular-weight HA fragments derived from pharmaceutical preparations could induce the expression of pro-inflammatory cytokines by monocytes. We found that degradation, either by sonication or by hyaluronidase treatment, did not result in the induction of IL-12 or TNF-α synthesis for five out of seven HA preparations, indicating that the use of these preparations would not exacerbate inflammation. However, we found that two out of the seven had the ability to induce IL-12 and TNF-α synthesis by human monocytes. We unexpectedly found that the induction of IL-12 and TNF-α by these HA preparations was not due to their degradation to low-molecular-weight fragments since their native high-molecular-form possessed the same ability to stimulate the synthesis of IL-12 and TNF-α by monocytes, but was due to the presence of contaminating DNA. Treatment of the HA preparations with DNase I abrogated or reduced the induction of IL-12 and TNF-α by monocytes. DNA purified from HA by phenol extraction and ethanol precipitation retained its activity to induce IL-12 and TNF-α synthesis (data not shown).

DNA from numerous species has been associated with the induction in-vitro of pro-inflammatory cytokines by either monocytes, macrophages, B lymphocytes or T lymphocytes (Klinman et al 1996; Krieg 1996; Neujahr et al 1999). The synthesis of pro-inflammatory cytokines by DNA has been reported to be due to the presence of unmethylated CpG dinucleotide flanked by two 5′ purines and two 3′ pyrimidines (CpG motifs) (Krieg et al 1995). In-vivo, DNA purified from either Gram-positive or Gram-negative bacteria has been shown to cause septic shock in D-galactosamine-sensitized mice (Sparwasser et al 1997). Administration of bacterial DNA, as well as synthetic oligonucleotides, into the lungs of mice has been found to cause severe inflammation in the lower respiratory tract (Schwartz et al 1997). Furthermore, intra-articular injection of DNA containing CpG motifs has been shown to induce ar-

thritis in mice (Deng et al 1999). The presence of DNA in HA preparations could be explained by association or contamination during the isolation and purification of HA from rooster comb or from bovine trachea. One HA preparation isolated from streptococci also contained DNA, but despite having comparable levels of DNA did not elicit a cytokine response from monocytes. It would therefore appear that the nature of the DNA is important. Experiments are underway to determine the sequence motifs present in DNA associated with HA which are responsible for the induction of cytokines.

Reduction of HA molecular mass by sonication also leads to a reduction in DNA molecular weight. We found that the DNA present in HA preparations E and G ($>$ 500 base pairs) was reduced to small DNA fragments of $>$ 20 base pairs following sonication treatment (data not shown). Nevertheless, no difference in terms of IL-12 and TNF-α synthesis by monocytes was observed after sonication (Figures 1 and 2), suggesting that DNA fragments as small as 20 base pairs are able to modulate pro-inflammatory cytokines. In fact, it has recently been shown that oligonucleotides as small as eight bases have the ability to induce the synthesis of IL-6, IL-12 and interferon-γ by spleen cells (Klinman et al 1996). Furthermore, we found that sonication had the ability to release DNA associated with HA (data not shown). These results could explained why some HA preparations will induce cytokines only after sonication or digestion by hyaluronidase. Experiments are underway to test this hypothesis.

In summary, we found that two out of the seven HA preparations examined had the ability to induce pro-inflammatory cytokines. The ability of HA to induce these cytokines was not related to the presence of low-molecular-weight HA fragments, but rather to the presence of contaminating DNA. HA preparations should be used with caution to treat inflammatory disorders since they have the potential to induce or exacerbate inflammation. It is perhaps advisable to determine whether pro-inflammatory DNA is present in HA preparations before its use, not only for the treatment of patients with inflammatory disorders, but also before studying the modulation of pro-inflammatory cytokines or inflammation in animal models.

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