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Abstract

The intra-articular injection of high molecular weight hyaluronic acid (HA) has been reported to be an effective treatment for pain of osteoarthritis of the knee. However, the mechanism by which HA exerts its effect is unknown. To explore HA's influence on the growth of U937 human macrophages, cells were incubated for 168 h with three concentrations, 1, 0.1 and 0.01 mg/mL, of Hyalgan®, a high molecular weight HA preparation. At 24-h increments, the cells were examined for proliferation, cell cycle distribution as well as the number of apoptotic and dead cells. Exposing macrophages to 1 mg/mL Hyalgan® significantly reduced the rate of cellular proliferation and altered the cell cycle distribution to yield decreased proportions of G0/G1 cells but increased S and G2/M cells. Concomitantly, a 10-fold increase in apoptotic cells and a 12-fold increase in dead cells were observed. The population doubling time (PDT) for cells treated with 1.0 mg/mL Hyalgan® increased from 23.6 to 52.9 h. By contrast, the two lower Hyalgan® concentrations significantly promoted macrophage proliferation in a dose-dependent manner. They also increased the proportion of G2/M cells, but had no effect on the number of apoptotic or dead cells. The PDTs of 21.5 and 22.2 h were less than the control time of 23.6 h. These results demonstrate that Hyalgan® concentrations have a differential effect on macrophage growth dynamics and suggest an anti-inflammatory effect at high HA concentrations.
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Keywords: Hyalgan®; Hyaluronic acid; Macrophage; Proliferation; Apoptosis; Osteoarthritis

Introduction

The intra-articular injection of hyaluronic acid (HA) has been approved by the United States Food and Drug Administration as a treatment for the pain of osteoarthritis (OA) of the knee which is refractory to conservative non-pharmacologic therapy and to simple analgesics [31]. This joint fluid therapy, sometimes termed viscosupplementation, typically requires multiple weekly injections of high molecular weight HA. Injectable HA is available in the United States as Hyalgan® (Sanofi, New York, NY), Synvisc® (Genzyme/Biomatrix™, Ridgefield, NY), or Supartz® (Smith & Nephew, Memphis, TN), all of which are purified from rooster comb HA. The efficacy of joint fluid therapy was first reported in 1974 [30] when a statistically significant reduction in pain and improved activity in patients with OA were noted. Subsequent studies of OA patients have reported transient analgesia and improved activity following joint fluid therapy [1,24,29,43]. More recently, HA injections have been reported to be an effective conservative treatment for rotator cuff tears [35] and an adjunct to arthrocentesis for temporomandibular joint internal derangements [3]. Widespread use of HA in OA patients, however, merits additional investigation due to the reported high placebo effect associated with joint fluid therapy as well as a lack of uniform, cost-effective benefits [4,10,42].

The mechanism of HA's action is poorly understood. Joint fluid therapy originally was based on the observations that the viscosity and elasticity of synovial fluid are reduced in human arthritis [7] and that these fluid features result from the unique rheological properties of HA [6]. Also, HA concentration and molecular size are reduced in human and equine arthritis [7]. The clinical benefits of exogenous HA therefore were thought to result from restoration of synovial fluid's viscosity and elasticity. With the observation that HA's mean intra-synovial half-life is comparatively short, less than 24 h in animal studies [22], restoration of rheological properties

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alone could not account for joint fluid therapy's long-term benefit.

More recently, HA was shown to directly affect cellular proliferation or apoptosis. At low concentrations, HA of high molecular weight, similar to that of Hyalgan®, generally was reported to stimulate cellular proliferation [18,19] while high concentrations, such as those in synovial fluid, inhibited proliferation [12,15,27]. In separate studies, Hyalgan® reduced anti-Fas-mediated apoptosis [23] while HA directly reduced apoptosis [37]. Both proliferative and apoptotic effects are likely mediated by the binding of HA to CD44, the major receptor for HA on cell surfaces [5], since receptor cross-linking with specific antibody enhances both cellular activities [5,14,37].

In diseases like OA that are associated with cellular, cartilaginous, crystal, and particulate debris, synovial macrophages are important for removing debris and for mediating inflammation. Synovial macrophages exist in an environment that normally contains a high concentration of high molecular weight HA [7], but both concentration and size may be reduced in acute inflammation or chronic disease due to hyaluronidases and oxygen radicals [21,28]. HA's influence on several cell types has been reported, but its influence on macrophages has not been studied previously. Further, HA's influence on cellular growth, a dynamic that additively combines the net effects of cellular proliferation and cellular apoptosis, has not been reported previously.

The purpose of the present study was to determine the net effect of varying concentrations of high molecular weight HA on macrophage population growth by measuring the individual influences of proliferation and apoptosis. To avoid potential cellular activation due to phagocytosis of oncotic cells and/or cellular debris, U937 macrophages, which are not phagocytic [32], were used. The results demonstrate that HA at a high concentration negatively influences cell proliferation while inducing apoptosis. Lower HA concentrations failed to induce these effects. These findings show that HA has a dose-dependent regulatory influence on macrophages, and suggest an anti-inflammatory effect at high HA concentrations.

Materials and methods

Cell culture

The U937 human macrophages cell line (American Type Culture Collection, Manassas, VA) was utilized in all experiments. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in RPMI-1640, pH 7.2, supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY), 2 mM L-glutamine and 1 mM sodium pyruvate. Stock U937 cultures were maintained in the exponential growth phase, below 5.0×10^5 cells/mL. For experiments cells were seeded in 24-well plates (Corning Costar, Cambridge, MA) in 900 µL of medium plus 100 µL of Hyalgan® (Sanofi Synthelabo, New York, NY) or phosphate buffered saline (PBS), pH 7.2 diluted in medium to the appropriate

Table 1
Plating concentrations of U937 macrophages for cell growth experiments

Hyalgan® exposure time (h)	Plating concentration (cells/mL)
0	1,000,000
24	640,000
48	320,000
72	160,000
96	80,000
120	40,000
144	20,000
168	10,000

At eight times (Hyalgan® exposure time) cells were seeded in 24-well plates at varying concentrations (plating concentration) and maintained in culture for the duration of the experimental period. The concentrations were calculated to yield 10^6 cells at the end of the 168-h period based on a PDT of approximately 24 h.

concentrations. Cell numbers and population doubling time (PDT) were determined by counting with a hemacytometer. Viability was assessed by trypan blue dye exclusion.

Experimental design

U937 macrophages were seeded at 24-h intervals during a 168-h period using replicates for each of four assay types: proliferation, cell cycle distribution, apoptotic cells, and dead cells. To perform all analyses at one time, increasing concentrations of cells were seeded at each interval and maintained in culture for the duration of the experiment (Table 1). Seeding densities were chosen to provide a final concentration of 10^6 cells/mL by the end of the experimental period assuming a PDT of approximately 24 h [9]. Experimental wells contained 1, 0.1, and 0.01 mg/mL Hyalgan®. Control wells contained PBS, the diluent for Hyalgan®.

Flow cytometric analyses

Analysis of proliferation, cell cycle distribution, apoptotic and dead cells was performed using a FACSort flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 488 nm excitation laser. For each sample, a minimum of 15,000 whole cell or nuclei events was captured using exclusionary gating of the forward vs. side scatter plots. Data acquisition was performed using the FL-1 green fluorescence (530 nm) or the FL-2 red fluorescence (>600 nm) detection channel with Cell Quest data acquisition software (Becton Dickinson, San Jose, CA).

Cell proliferation analyses

Immediately before culture U937 macrophages were labeled with the green fluorescent dye, carboxyfluorescein diacetate succinimidyl ester (CFDA, SE, Molecular Probes, Eugene, OR) as previously described [25]. Non-fluorescent CFDA, SE spontaneously crosses the cell membrane, is converted to a green fluorescent succinimidyl ester (CFSE) by intracellular esterases, and forms stable, long-term bonds with intracellular proteins. A 5 mM CFDA, SE stock in dimethylsulfoxide was diluted 1/100 in PBS, and 110 µL was added with rapid mixing per 1 mL of cells suspended in PBS. After a 5 min incubation at room temperature, the cells were washed three times with PBS containing 5% FBS, and resuspended in RPMI-1640 at the required seeding density. After incubation, the cells were washed twice in PBS containing 2% FBS and 0.1% sodium azide. They were analyzed for CFSE fluorescent intensity by flow cytometry.

Cell cycle distribution analyses

The cell cycle distribution of U937 macrophages was determined by quantifying DNA in isolated nuclei using propidium iodide (PI) [20]. PI staining measures the aneuploidy of each cell and can be translated into three distinct cycling phases: G0/G1, S, and G2/M [20]. U937 cells

were washed twice with PBS containing 2% FBS and 0.1% sodium azide. To isolate and stain nuclei, cells were resuspended in 1 mL of a modified Vindelov buffer [41] containing 50 mg/mL PI, 0.7 U/mL RNase, and 0.1% Igepal CA-630. After a 10 min incubation at room temperature, flow cytometry was performed using the FL-2 detection channel. Further analysis utilized Modfit LT software (Verity Software House, Topsham, ME).

Quantifying apoptotic and dead cells

Viable, apoptotic, and dead cells were measured by flow cytometry after staining with YO-PRO-1/PI (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. For gating calibration of the flow cytometer, apoptotic and dead cells were prepared by exposing U937 macrophages to 20 μM camptothecin (Sigma-Aldrich, St. Louis, MO) for 4 h. The green fluorescent dye YO-PRO-1 selectively crosses the membranes of apoptotic, but not viable cells. The red fluorescent dye PI does not cross the membranes of live or apoptotic cells, but easily enters dead cells and strongly binds to nucleic acids. With YO-PRO-1/PI staining, apoptotic cells exhibit green fluorescence, dead cells display both green and red fluorescence, and live cells show little or no fluorescence.

Analyses for CD44 and HA binding

To demonstrate that the macrophages used in the present study possessed surface CD44 receptors capable of binding HA, cells were stained with fluorescent anti-CD44 antibody and with fluorescein (FITC)-conjugated HA. 10^6 macrophages were washed and incubated with 20 μL fluorescein-conjugated anti-CD44 antibody (Immunotech, Marseille, France) at 4 °C for 20 min. A mouse IgG1 antibody (Becton Dickinson, San Jose, CA) was used as an isotype control. After washing the cells were resuspended in 200 μL of PBS containing 1% bovine serum albumin (BSA) and 1% paraformaldehyde. To detect HA binding, an additional 10^6 cells were incubated with 1 mg/mL fluorescein-conjugated HA (mean molecular weight 10^6 Da, CarboMer, Inc., Westborough, MA) at 4 °C for 30 min. In inhibition studies, 10^6 cells treated with conjugated HA were pre-incubated with 0.5 ug/uL rat anti-CD44 IgG (Zymed Laboratories, San Francisco, CA) diluted in PBS containing 1% BSA at 4 °C for 30 min. Negative control cells were treated only with the unconjugated anti-CD44 antibody. All cell preparations were examined by flow cytometry for green fluorescence using the FL-1 detection channel.

Statistical analyses

Cell enumeration data were expressed as means with error bars indicating one standard deviation. Mean values from each time point using experimental cells cultured with HA were compared to control cells cultured without HA using a Student's *t*-test assuming unequal variances. A probability of $p < 0.05$ was considered to be statistically significant.

Results

Hyalgan® has a differential effect on macrophage proliferation

To determine Hyalgan®'s effect on cellular proliferation, macrophages were labeled with the green fluorescent dye, CFDA, SE and incubated with three concentrations of Hyalgan® for a maximum of 168 h. Fluorescent intensity was measured by flow cytometry at 24-h intervals during the experimental period. The results (Fig. 1) demonstrated that the 1 mg/mL Hyalgan® concentration inhibited macrophage proliferation, compared to control cultures, as evidenced by increased fluorescence beginning at 72 h. By comparison, cells
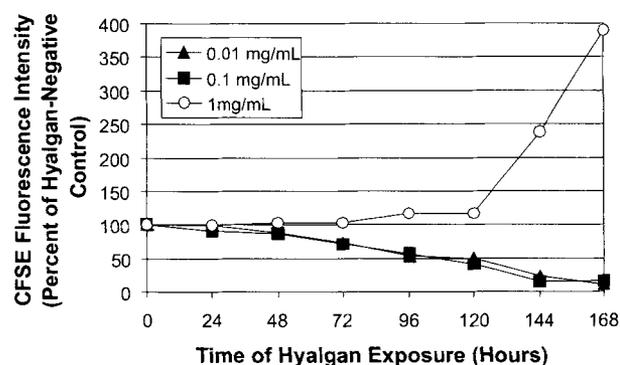

Fig. 1. Effect of Hyalgan® concentration on macrophage proliferation. U937 cells were labeled with CFDA, SE which spontaneously permeates cells and is converted to an ester, CFSE, that is retained within the cells. As cells divide, the fluorescent dye is divided between the daughter cells. Therefore, CFSE fluorescent intensity is inversely proportional to the number of cellular divisions. Cells were cultured with various concentrations of Hyalgan® or PBS (control) for eight 24-h periods ranging from 0 to 168 h. At the end of each culture period, a minimum of 15,000 cells from each culture was analyzed by flow cytometry for CFSE fluorescent intensity. Percent of control was calculated as (CFSE intensity of Hyalgan®-treated cells)/(CFSE intensity of control cells) × 100.

incubated with the two lower Hyalgan® concentrations, 0.01 and 0.1 mg/mL, exhibited less fluorescence and therefore increased proliferation. The difference between these inhibitory and stimulatory effects was substantial. At 168 h, the fluorescence of cells incubated with 1 mg/mL Hyalgan® was 381% of control cells. However, cells exposed to the two lower Hyalgan® concentrations displayed only 11% and 15% of control-cell fluorescence. Together these data demonstrate that Hyalgan® has a differential, dose-dependent effect on the rate of macrophage proliferation.

To further explore Hyalgan®'s effects on proliferation, cell numbers were determined by microscopic examination. The number in Hyalgan®-treated cultures was compared to the corresponding number of control cells, and both numbers were compared to the initial seeding density to determine the PDT. The results (Fig. 2(A)) confirmed that 1 mg/mL Hyalgan® decreased cell number while the two lower Hyalgan® concentrations stimulated proliferation. Notably, as shown by comparing seeded and 1 mg/mL Hyalgan® numbers, cells exposed to the high Hyalgan® concentration still proliferated. However, the rate was substantially slowed (compare the control and 1 mg/mL cell numbers). The calculated PDTs supported these observations. Control macrophages at 168 h had a baseline PDT of 23.6 h. Hyalgan® at 1 mg/mL slowed the PDT to 52.9 h while the 0.1 and 0.01 mg/mL concentrations yielded PDTs of 21.5 and 22.2 h, respectively. Hyalgan®'s differential effect was readily apparent when cell counts were expressed as a percent of Hyalgan®-negative control (Fig. 2(B)). Over the 168-h experimental period, the 1 mg/mL
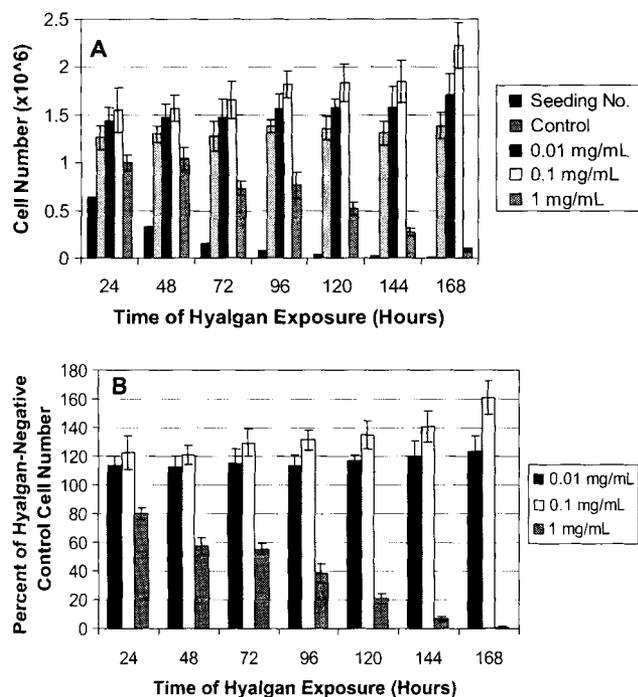
Fig. 2. Effect of Hyalgan® concentration on macrophage population growth. U937 cells were cultured with various concentrations of Hyalgan® or PBS (control) for seven periods ranging from 24 to 168 h. At the end of each period, cells were stained with trypan blue and counted using a hemacytometer. (A) The number of Hyalgan®-treated cells as compared to the number of Hyalgan®-negative control cells and the initial seeding number at each time point. (B) The number of Hyalgan®-treated cells is shown as a percent of the corresponding number of control cells at each time point. Each point is the mean of 16 replicates. Error bars show one standard deviation. The number of Hyalgan®-treated cells at all concentrations and at all time points was significantly different from the number of control cells ($p < 0.05$).

Hyalgan® concentration progressively reduced the number of macrophages in culture to less than 1% of the control level. This inhibition was statistically significant at all time points ($p < 0.01$). The two lower Hyalgan® concentrations stimulated macrophage proliferation by 161% and 123%, respectively. These increases were statistically significant at all intervals ($p < 0.01$). At all concentrations, Hyalgan®'s effects were apparent within 24 h and progressed in a time-dependent manner.

Hyalgan® differentially influences the cell cycle distribution

Hyalgan®'s influence on cellular proliferation suggested that it might also affect the cell cycle distribution of macrophages. To test this possibility, nuclear DNA from control and experimental cells was labeled with PI and analyzed by flow cytometry. The results showed that, in general, the highest Hyalgan® concentration affected the cell cycle distribution differently than the two lower concentrations. In cultures with 1 mg/mL
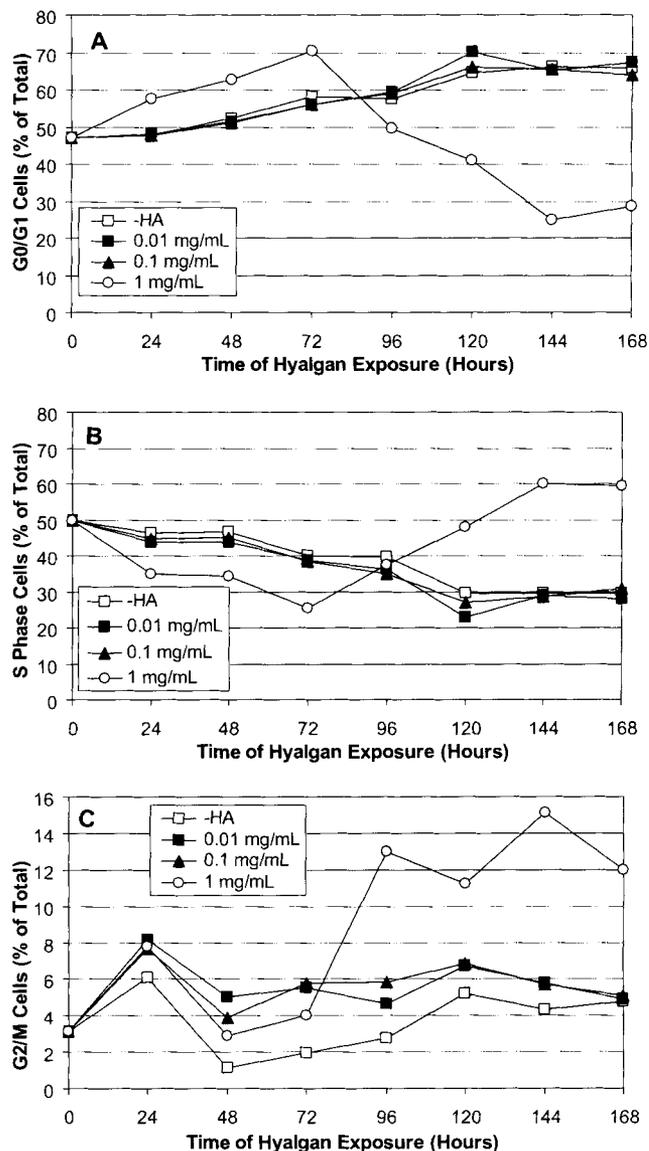

Fig. 3. Influence of Hyalgan® concentration on macrophage cell cycle distribution. Cells were cultured with medium containing various concentrations of Hyalgan® or PBS (control) for eight periods ranging from 0 to 168 h. At the end of each period, the cell cycle distribution of at least 15,000 nuclei stained with PI was determined by flow cytometry. (A) Cells in the G0/G1 phase. (B) Cells in the S phase. (C) Cells in the G2/M phase. Each point is the percent of total Hyalgan®-treated or control cells at that time period.

Hyalgan®, the proportion of cells in the G0/G1 phases progressively increased through the first 72 h of incubation (Fig. 3(A)). Thereafter, the proportion decreased. After 72 h the proportions in the S phase (Fig. 3(B)) and the G2/M phases (Fig. 3(C)) also increased. These data suggest that Hyalgan® at a high concentration promotes cell cycle transit from G0/G1 to the S and G2/M phases, or that cells in the G0/G1 phase are eliminated, thereby leaving a higher proportion of cells in the S and G2/M phases.

The cells exposed to the two lower Hyalgan® concentrations exhibited G0/G1 and S phase distributions similar to those of control cells (Fig. 3(A) and (B)). The exception was the increased proportions in the G2/M phases throughout the 168-h time course (Fig. 3(C)). This observation is consistent with a dose-dependent proliferative effect.

Hyalgan® has a differential effect on apoptosis and cell death

Hyalgan®'s negative influence on cellular proliferation and G0/G1 phase distribution suggested that it might induce apoptosis and cell death. To test that possibility, a flow cytometric method using fluorescent dyes was employed to directly enumerate apoptotic and dead cells. Hyalgan® at 1 mg/mL stimulated an immediate increase in apoptotic cell number, which peaked at 8.5% of the total cell population at 120 h (Fig. 4(A)). Similarly, Hyalgan® stimulated a progressive increase in the proportion of dead cells beginning at 72 h, which at its maximum accounted for 30.9% of all remaining intact cells (Fig. 4(B)). Thus, Hyalgan® at 1 mg/mL induced a 10-fold increase in apoptotic cells and a 12-fold

increase in dead cells. By contrast, cells treated with 0.1 or 0.01 mg/mL Hyalgan® demonstrated no increase in the proportion of apoptotic or dead cells throughout the experiment (Fig. 4(A) and (B)). These results show that Hyalgan® at a high concentration induces cell death via apoptosis. Overall, the reduced cell numbers following Hyalgan® exposure result from both reduced proliferation and induced cell death.

Hyalgan®'s ability to induce cell death also was apparent microscopically. Macrophages exposed to 1 mg/mL Hyalgan® exhibited reduced size, increased granularity, budding, and fragmentation (Fig. 5), morphological features consistent with apoptosis. The appearance of cells incubated with the lower concentrations of Hyalgan® was indistinguishable from that of control cells (data not shown).

U937 cells have surface receptors that bind hyaluronic acid

To document that U937 macrophages possessed surface CD44 receptors capable of binding HA, cells were incubated with a FITC-conjugated anti-CD44 antibody or with FITC-conjugated HA and examined by flow cytometry. The results (Fig. 6) demonstrated a 128-fold increase in fluorescent intensity following exposure to the anti-CD44 antibody or a 217-fold increase after incubation with the conjugated HA (Fig. 6). Prior incubation of cells with an anti-CD44 antibody reduced the FITC-HA binding by 94%. These results demonstrate CD44's presence on U937 macrophages as well as its ability to bind HA, thereby suggesting one mechanism whereby Hyalgan® could influence cellular proliferation and apoptosis.
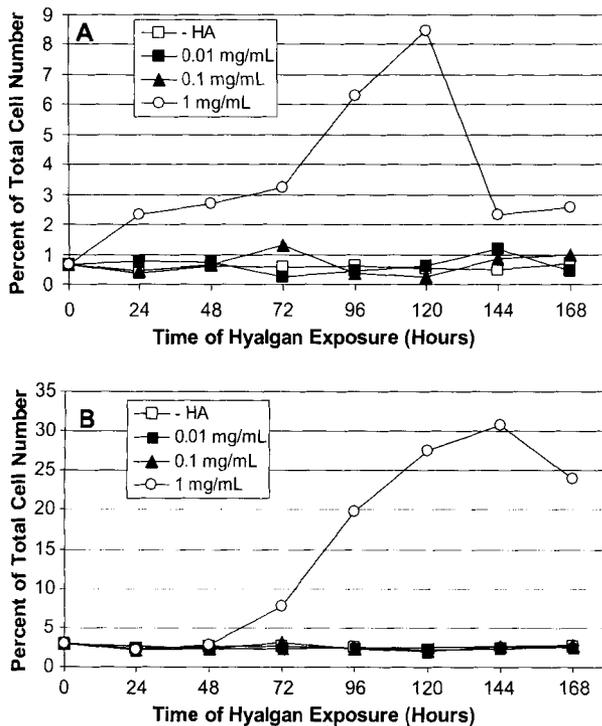

Fig. 4. Influence of Hyalgan® concentration on the induction of apoptosis and cell death. Cells were cultured with various concentrations of Hyalgan® or PBS (control) for eight periods ranging from 0 to 168 h. At the end of each period, cells were stained with the fluorochromes YO-PRO-1 and PI which can be used to detect apoptotic and dead cells by flow cytometry. (A) YO-PRO-1-stained apoptotic cells. (B) PI-stained dead cells. Each point is the percent of total Hyalgan®-treated or control cells at that time period.

Discussion

This study of Hyalgan®'s effects on overall cell growth has three major conclusions: (1) 1 mg/mL Hyalgan® slows the rate of proliferation, beginning as early as 24 h, but does not arrest cell division, while low Hyalgan® concentrations stimulate proliferation without altering the cell cycle (Figs. 2 and 3); (2) the high Hyalgan® concentration induces apoptosis and cell death, which further reduces cell number, but lower Hyalgan® concentrations fail to induce apoptosis (Fig. 4); (3) the progressive increase in cell mortality following exposure to 1 mg/mL Hyalgan® coincides with an abrupt change in the cell cycle distribution after 72 h in which the majority of the cells shift from the G0/G1 phases to the S and G2/M phases (Fig. 3). Additional experiments suggest that Hyalgan®'s effects are likely due to the interaction of HA with the cell surface via a CD44 receptor (Fig. 6), in agreement with previous results [5,14,37].
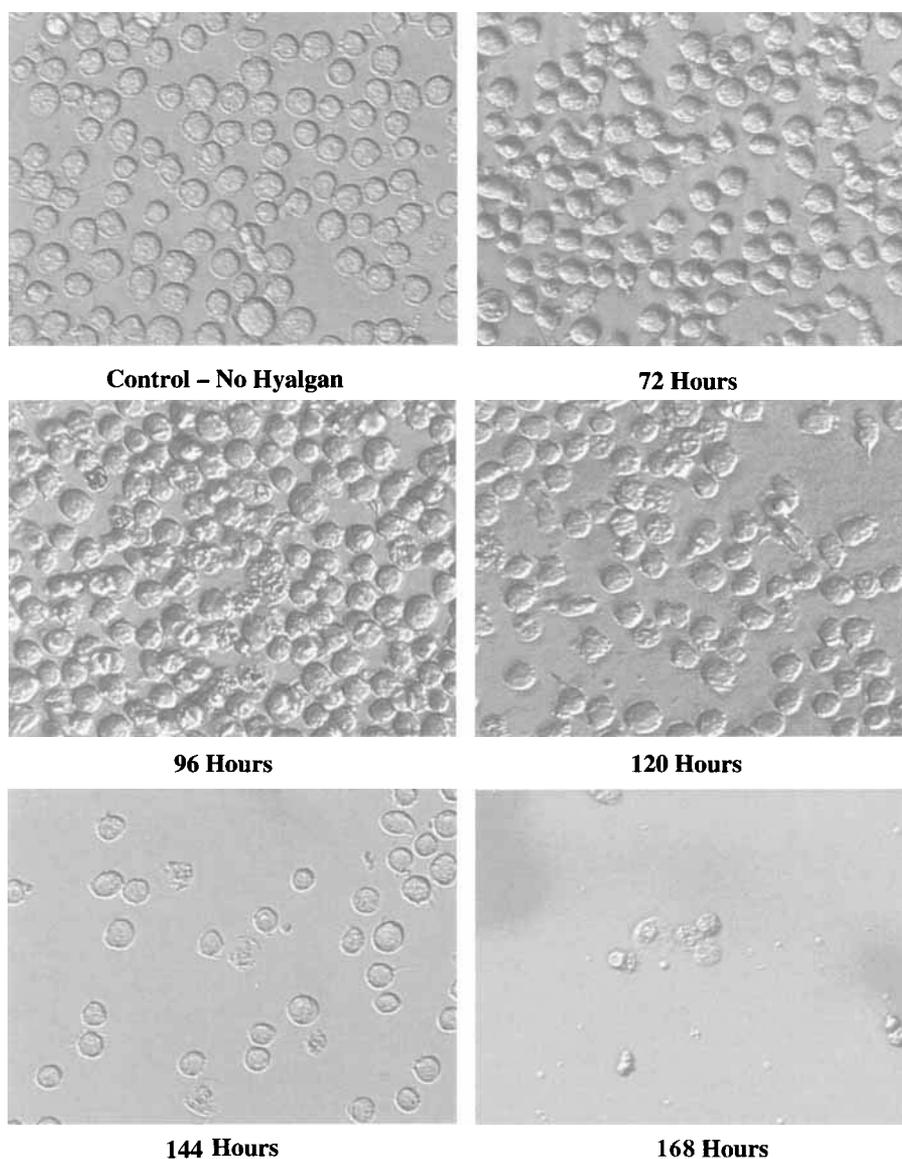
Fig. 5. Microscopic appearance of U937 macrophages exposed to a high concentration of Hyalgan® for 72–168 h. Cells were cultured with 1 mg/mL Hyalgan® (experimental) or PBS (control). Beginning at 72 h, increasing numbers of experimental cells exhibited reduced size, increased granularity, budding, and fragmentation, morphological features consistent with apoptosis. Cells were imaged using Hoffman phase contrast optics and a Sony 390 digital camera.

Hyalgan®, as shown in this study, has regulatory influences on the growth dynamics of U937 macrophages by differentially affecting both cellular proliferation and cell death. However, the time course of Hyalgan®'s inhibitory effect on proliferation differs from the time of its effect on apoptosis. The Hyalgan®-induced slowing of proliferation was immediate and progressive. Hyalgan® most likely slowed early cell cycle progression by preventing G1 to S cell cycle transition. It also increased S to G2 phase transition and reduced mitotic activity. Notably, only proliferation was affected during the first 72 h of Hyalgan® exposure whereas both proliferation and cell death were influenced thereafter. After 72 h, marked decreases in the number of G0/G1 cells ac-

companied substantial increases in the numbers of S and G2/M cells. This temporal delay suggests that apoptosis and cell death, but not slowing of proliferation, result from mechanisms which require at least 72 h to complete. These mechanisms probably involve gene-expression and protein synthesis, events consistent with ligand-induced apoptosis [26,34].

The results of this study suggest that differences in HA-receptor binding and cross-linking may underlie Hyalgan®'s differential effects on cell growth. In this study a high dose of high molecular weight HA inhibited proliferation, in agreement with previous studies [12,15,19], and induced apoptosis whereas lower doses induced proliferation without apoptosis. These
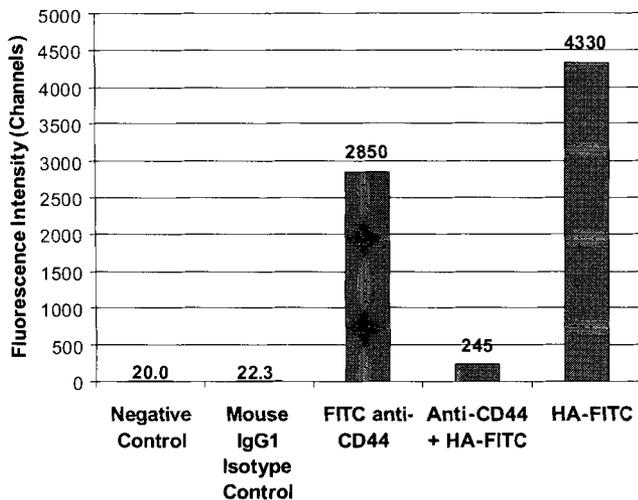
Fig. 6. Macrophage binding of HA via surface CD44 receptors. U937 cells were incubated with a FITC-conjugated anti-CD44 antibody or with a FITC-conjugated mouse IgG1 antibody as an isotype-negative control. Additional cells were incubated with a FITC-conjugated HA or with an anti-CD44 antibody followed by washing and incubation with the FITC-conjugated HA. Relative fluorescent intensity was measured by flow cytometry.

differential effects suggest that HA binding to CD44 without receptor cross-linking, as might occur with a high dose of HA, are required for inhibition of proliferation and apoptosis induction. Support for this mechanism is derived from previous reports showing that treatment with anti-CD44 antibody alone induced cellular apoptosis [14,17,39] or inhibited proliferation [11]. Conversely, extensive receptor cross-linking, as would occur with low HA concentrations or with small HA molecules, would be expected to induce proliferation, as has been shown previously [33,40].

Significance

The efficacy of intra-articular Hyalgan® injections as a treatment for the pain of OA is likely due, in part, to the dramatic reduction of inflammatory macrophages via reduced proliferation and induced apoptosis, as presented in this study. Chronic inflammatory reactions, as in OA, are characterized by the accumulation and proliferation of inflammatory cells, including macrophages, in connective tissue where their chronic activation is perpetuated and leads to severe tissue degradation. Therefore, one mechanism underlying Hyalgan®'s anti-inflammatory effect is a reduction in inflammatory cell number. As shown in the present study, inhibition of proliferation by 1 mg/mL Hyalgan® began soon after HA exposure, within 24 h (Fig. 2(B)), and continued throughout the 168-h experimental period (Fig. 2(A)).

By contrast, the effects of Hyalgan® on apoptosis required at least 72 h to be detected. Following an intra-articular Hyalgan® injection, at least two mechanisms might contribute to increased apoptosis, in view of HA's relatively short half-life. First, the detection of apoptotic cells lags behind apoptosis induction by 72 h [8,16]. Thus, in a clinical setting apoptosis might be induced soon after HA exposure, but apoptotic cells might not be detectable for an additional 72 h. Apoptosis induction could continue as the intra-synovial HA concentration declines due to its relatively short half-life. Second, the concentrations of commercial HA preparations are considerably higher than 1 mg/mL. Even allowing for dilution with synovial fluid and disappearance due to its half-life, the HA concentration after 72 h could be 1 mg/mL or higher. This sustained high concentration of Hyalgan® could inhibit macrophage proliferation and induce apoptosis for several days.

Other studies have shown that high molecular weight HA at a high concentration also inhibits macrophage phagocytic activity [13] as well as the secretion of the prostaglandins [2], reactive oxygen species [36], and nitric oxide [38]. Consequently, another mechanism for Hyalgan®'s anti-inflammatory effect is reduced inflammatory activity. Together these two mechanisms support the efficacy of intra-articular injections of high molecular weight HA as a long-term treatment for the pain of OA.

The present study was performed using a single preparation of HA with a defined size range. While the use of a single preparation was advantageous for the present study, the results cannot be extended to other HA formulations of different size and/or composition. Future studies will need to examine the effects of these multiple parameters.

Acknowledgements

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