Hyaluronan oligosaccharides induce cell death through PI3-K/Akt pathway independently of NF-κB transcription factor

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Several studies indicate that hyaluronan oligosaccharides (oHA) are able to modulate growth and cell survival in solid tumors; however, no studies have been undertaken to analyze the effect of oHA on T-lymphoid disorders. In this work we showed that oHA were able to induce apoptosis in lymphoma cell lines. Since PI3-K/Akt and nuclear factor-κB (NF-κB) are major factors involved in cell survival and anti-apoptotic pathways in lymphoma cells, we hypothesized that oHA could induce apoptosis through inhibition of these pathways. oHA were identified by a method which allows characterization of length using a high pH anion exchange chromatography with pulse amperometric detection (HPAEC-PAD). oHA inhibited PIP3 production (principal product of PI3-K activity) and reduced Akt phosphorylation levels, similarly to the specific inhibitor wortmannin. However, treatment with either oHA or wortmannin failed to inhibit constitutive NF-κB activity and modulate 1xBz protein levels, suggesting that PI3-K and NF-κB signaling pathways are not related in the cell lines used. Cell behavior differed using native hyaluronan (HA), which induced PIP3 production, Akt phosphorylation, and NF-κB activation, although not related with cell survival since treatment with native HA showed no effect on apoptosis. Our results suggest that oHA induce apoptosis by suppression of PI3-K/Akt cell survival pathway without involving NF-κB activation, through a mechanism that differs from the one mediated by native HA.

Key words: apoptosis/HPAEC-PAD/hyaluronan oligomers/NF-κB/PI3-K/Akt

Introduction
Extracellular matrix (ECM) components have been shown to influence tumor cell behavior with respect to migration, metastasis, angiogenesis, and survival (Bissell and Radisky, 2001). Hyaluronan (HA) is a large, linear polysaccharide with a molecular weight ranging from 10^5 to 10^7 Da. It is the major glycosaminoglycan found in all types of mammalian ECM and influences the hydration and physical properties of tissues. Interaction of HA with other ECM components such as proteoglycan, aggrecan, or versican allows the assembly of several tissues. Besides, interaction with CD44 and RHAMM cell surface receptors modulates cell behavior (Tammi et al., 2002; Ponta et al., 2003). HA is present at high levels in many cancers and plays an important role in tumor malignancy (Heldin, 2003). The biological effects of HA appear to vary depending upon its average mass. In addition, fragments of HA have been reported to have biological activities, which are not associated with the whole molecule (Camenisch and McDonald, 2000; Toole, 2004), promoting angiogenesis (Lokeshwar et al., 2001) or migration (Sugahara et al., 2003). However, some studies have also shown that HA oligosaccharides (oHA) are able to inhibit in vivo tumor growth (Zeng et al., 1998), induce apoptosis (Ghatak et al., 2002), reverse drug resistance in cancer cells (Misra et al., 2003), and stimulate dendritic cell maturation activating the immune response against tumors (Termeer et al., 2000). Therefore, an insightful study on the signaling pathways activated by oHA would allow elucidation of the molecular events that direct the effect of oHA on tumor cells.

Growth balance, which is determined as the ratio between the rate of cell proliferation and apoptosis incidence, is out of control in cancer cells, and an increase in abnormal growth may cause tumor invasion and metastasis (Kim et al., 2002). Several studies indicate that both PI3-K/Akt and NF-κB signaling pathways may be related, and after activation are crucial for tumor development. Besides, it has been reported that Akt directly phosphorylates IKK kinase leading to the activation of NF-κB, which in turn activates the transcription of a variety of substrates including anti-apoptotic genes (Karin et al., 2002; Paez and Sellers, 2003).

The phosphatidylinositol 3-kinase (PI3-K) is formed by heterodimeric lipid kinases that catalyze the phosphorylation of inositol-containing lipids, known as phosphatidylinositol (PtdIns), allowing the conversion of phosphatidylinositol-3,4-biphosphate (PtdIns-P_{2}) to phosphatidylinositol-3,4,5-triphosphate (PtdIns-P_{3}). The latter is absent or undetectable in resting cells and although PI3-K activity in normal cell is tightly regulated, it is deregulated in a wide spectrum of tumors.
Akt is a serine/threonine protein kinase that mediates various of the downstream effects of PI3-K, it plays a central role in signaling by the PI3-K pathway regulating many biological processes such as proliferation, apoptosis, and cell growth, thus it may also be involved in PI3-K mediated tumorigenesis. Akt controls a variety of mechanisms that inhibit apoptosis and prolong cell survival, exerting a positive effect on NF-κB function (Osaki et al., 2004; Paez and Sellers, 2003, Ozes et al., 1999).

NF-κB is a dimeric transcription factor that is present in the cell cytoplasm in association with inhibitory proteins termed IκB. The predominant form in the cells is the p50/p65 heterodimer, which is associated with IκB. After activation, IκB proteins become phosphorylated and degraded resulting in nuclear translocation of NF-κB. As an inhibitor of programmed cell death, NF-κB activates the transcription of several target genes that block apoptosis induction. Therefore, prevention of apoptosis increases the proportion of genetically altered cells, which raise the transformed progeny (Baldwin, 2001).

It has been reported that HA is able to promote an intracellular signaling cascade that leads to activation of Rac1, Ras, PI3-K, and NF-κB in several types of cancer (Toole, 2004). However, there are few studies on lymphoma tumors regarding the signaling pathway modulated by either HA or oHA, especially those related to cell survival.

Previously we showed that NF-κB, as a p50/p65 heterodimer, was constitutively activated in the murine T-lymphoma cell lines LBLa and LBLc. This activity was inhibited by BAY-117082 and modulated by high as well as low molecular weight forms of HA (Alaniz et al., 2004). As PI3-K/Akt has been reported to activate NF-κB, we investigated the effect and interrelationship of HA and oHA on these pathways. On the basis of our previous reports we also analyzed the effect of HA and oHA on cell survival as well as the pathways involved in their effect. Despite enhanced PI3-K/Akt and NF-κB activity, we found that native HA did not affect cell survival, whereas oligosaccharides, although failing to modulate NF-κB activity, induced inhibition of PI3-K activity and consequently inhibited PtdIns-P3 (PIP3) production and decreased Akt phosphorylation. Besides, oHA were able to induce apoptosis in both tumor cell lines, showing for the first time that oHA modulate T-cell lymphoma survival by decreasing activation of PI3-K/Akt, independently of the NF-κB signaling pathway.

Results

Generation and analysis of oHA

Enzymatic digestion from a preparation of recombinant HA, either high- (HMW) or low-molecular weight (LMW), free of ECM contaminants, (as indicated in the certificate of quality) was carried out; oHA length was determined using a high pH anion exchange chromatography with pulse amperometric detection (HPAEC-PAD). Conditions were optimized for the analysis of charged oligosaccharides, which rendered fragments ranging from HA4 tetrasaccharides (2 disaccharide units in length) to HA14 oligosaccharides (7 disaccharide units in length) (Figures 1A and B). Similar oligosaccharide fragments pattern obtained by hyaluronidase incubation have already been described (Mahoney et al., 2001; Prebyl et al., 2003). For this study whole fraction obtained by enzymatic digestion with testicular hyaluronidase from either HMW-HA or LMW-HA were used since both of them rendered similar fragments.

Modulation of NF-κB activity and IκBα by HA

The T-lymphoma cell lines LBLa and LBLc, which differ in invasive capacity and migration towards HA as determined in our laboratory (Alaniz et al., 2002), were used to analyze the effect of HA and oHA on NF-κB binding activity and PI3-K/Akt activity, as well as on the effect on cell survival related with these signaling pathways.

Although we found constitutive NF-κB binding activity in both cell lines, under similar protein concentration LBLa cell line displayed significantly higher NF-κB binding activity (4.6 ± 2.04-fold) than LBLc cell line, evaluated by electrophoretic mobility shift assay (EMSA). It was considered that signaling obtained from binding of NF-κB dimers with specific DNA oligonucleotides was correlated with NF-κB activity in this model. Both species of HA were able to enhance NF-κB binding activity (1.8 ± 0.15 HMW-HA, 1.2 ± 0.05 LMW-HA for LBLa, and 3.0 ± 0.75 HMW-HA, 1.5 ± 0.05 LMW-HA for LBLc); however, oHA failed to do so (Figures 2A and B). Stimulation of LBLc by HA was remarkably increased, suggesting that NF-κB activity in this cell line is far from saturation levels.

To confirm that the results obtained by EMSA were due to nuclear translocation of NF-κB, which occurs after
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proteolytic degradation of the IκBα inhibitory subunit, an
immunoblotting was carried out to analyze the levels of this
subunit after treatment with either HA or oHA. After treat-
ment with native HA (HMW) IκBα degradation was
found; however, no effect was observed with oHA, con-
fiming the results obtained by EMSA (Figures 2C and D).
As control of NF-κB inhibition, such activity and IκBα
degradation were also analyzed in the presence of the IκB-
kinase inhibitor BAY 11–7082, which does not allow IκBα
phosphorylation, prevents its degradation and inhibits NF-
κB nuclear translocation. Treatment of both cell lines with
3.5 and 5 μM BAY 11–7082 failed to inhibit NF-κB bind-
ing activity, while significant inhibition was detected when
7.5 and 10 μM were used (Figures 3A and B). A dose-
derpendent accumulation of cytosolic IκBα was observed
after treatment with the above concentrations of BAY 11–
7082, as determined by immunoblotting assays (Figures 3C
and D).

We ruled out contamination in HA or oHA solutions
since HA was prepared by the manufactures by fermenta-
tion of Streptococcus equi subsp. zooepidemicus bacterial
strain in selecting production medium, then HA was puri-
fied by repeated ultrafiltration, and specificity of product
obtained was tested by IR and UV spectrum (Contipro,
Ústí nad Orlicí, Czech Republic). Besides, solutions were
boiled to discard the effect of unknown contaminants, as
stability and activity of preparations were not affected by
heating (enzymatic reaction was stopped by treatment at
90°C for 5 min, as indicated in Materials and Methods).
Control was done using hyaluronidase tested alone to rule
out possible effects of the enzyme on the cells.

Treatment with oHA induced apoptosis by PI3-K/Akt
signaling pathway

Since modulation of NF-κB activity in these tumor cell
lines is related to apoptosis and as HA enhances NF-κB
activity, we analyzed if treatment with native HA or with
oligomers affected apoptosis levels in LBLa and LBLc cell
lines. Acridine orange and propidium iodide stain (Figures 4A
and B) showed that native HA did not induce significant
effect on apoptosis levels when compared to untreated cells
(-1.19 ± 3.01% and 2.97 ± 2.6% for LBLa and LBLc, respec-
tively). Treatment with mAb anti-CD44 (IM7.8.1) or iso-
type control (rat IgG 2b) had no significant effect on cell
survival. However, oHA were able to induce apoptosis in
both cell lines (22.16 ± 5.85% for LBLa and 19.74 ± 1.13%
for LBLc) (Figure 4A), although they were unable to modu-
late NF-κB binding activity or IκBα levels (Figure 2).

Apoptosis levels were also analyzed by Annexin V and pro-
pidium iodide stain, Figure 4C shows the percentage of
apoptotic cells found for LBLa. Similar results were
obtained using LBLc cell line (data not shown).

Treatment with BAY 11–7082 induced a dose-dependent
increase in the percentage of apoptotic cells. Concentra-
tions of BAY 11–7082 of 7.5 and 10 μM, induced apoptosis

Fig. 2. (A) Effect of HA and oHA on NF-κB activity evaluated by EMSA. Four micrograms of nuclear proteins from LBLa and LBLc cells were
incubated with a 32P-labeled NF-κB probe and NF-κB binding activity was analyzed in cells without treatment (lane 1), treated with LMW-HA (lane 2),
HMW-HA (lane 3), or oHA (lane 4). Results are representative of four independent experiments. (B) Densitometry analysis of the bands showed in (A).
The results are expressed as Index (untreated cells/treated cells), determined from four independent experiments and normalized to untreated cells. (C)
Immunoblotting analysis of IκBα proteolysis from cytoplasmatic extracts. LBLa and LBLc cells were treated with native HA or oHA and analyzed by
SDS–PAGE and immunoblotting (upper panel); without treatment (lane 1) and treated with HMW-HA (lane 2) or oHA (lane 3). Expression of β-actin
was used as invariant control (lower panel). Results are representative of three independent experiments. (D) Densitometry analysis of the bands observed
in (C). Results are expressed as Index (untreated cells/treated cells), determined from three independent experiments and normalized to untreated cells.
Bars represent mean ± SE, * indicates p < 0.05; ns: non-significant.
levels of 36.67 ± 7.45% and 53.8 ± 8.66% in LBLa cell line. Moreover, in the LBLc cell line, which displayed low constitutive NF-κB binding activity compared to LBLa, only treatment with 10 μM of BAY 11–7082 was able to induce apoptosis (18.5 ± 3.84%) (Figure 5A), suggesting less dependence of NF-κB activity for survival; higher activity render LBLa cell line more susceptible to apoptosis induced by inhibition of NF-κB activity. Flow cytometry analysis of apoptotic cells (by Annexin V and propidium iodide stain) showed similar results to those obtained by microscopy (Figure 5B). In this case, untreated control cells displayed higher basal apoptosis level, probably due to cell manipulation associated with the methodology.

As oHA failed to modulate NF-κB binding activity, we also investigated PI3-K/Akt cell survival signaling pathway. First we analyzed basal PI3-K/Akt activity and its modulation by either native HA or oHA. Increased basal production of PtdIns-P_3, the major product of PI3-K activity, was found in both cell lines while differences were observed between them. LBLa showed higher basal levels than LBLc (2.16-folds). Besides, native HA preparations increased PtdIns-P_3 (PIP_3) production in LBLa and LBLc with respect to the basal (1.67- and 1.2-folds respectively). Treatment with oHA reduced PIP_3 production similarly in both cell lines, and comparable to inhibition levels obtained after treatment with 200 nM of the specific PI3-K inhibitor wortmannin (Figures 6A and B). These results correlate with those observed for Akt phosphorylation levels, since HA improved Akt phosphorylation and treatment with oHA induced a significant inhibition of Akt phosphorylation in both cell lines, similarly to the effect induced by wortmannin (Figures 6C, D, E, and F). Inhibition of Akt phosphorylation by different doses of either wortmannin or oHA was not followed by accumulation of IkBα, or inhibition of NF-κB binding activity (data not shown).

Treatment with 100 or 200 nM wortmannin induced significant apoptosis levels in both cells (25.34 ± 0.1 and 24.51 ± 0.2 for LBLa, 23.4 ± 0.01 and 28.7 ± 0.28 for LBLc) (Figure 7), similar to that described for oHA.

Discussion

Both PI3-K/Akt and the transcription factor NF-κB pathways are involved in the development of leukemia and lymphomas and may be associated with prevention of cell death or apoptosis (Baldwin, 2001; Karin et al., 2002; Paez and Sellers, 2003; Turco et al., 2004). Several reports showed that PI3-K activation and its downstream target Akt result in IkB degradation and activation of NF-κB (Ozes et al., 1999; Manna and Aggarwal, 2000), moreover, Akt activation suppressed apoptosis by stimulating NF-κB (Madrid et al., 2000). In contrast, other research including ours described that PI3-K/Akt signaling is not involved in mediating NF-κB activation and that interrelationship of both pathways would depend on...
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Several studies demonstrated that HA, through its cell surface receptors CD44, RHAMM, the lymphatic-vessel endothelial HA receptor 1 (LYVE1) and TLR-4, is able to induce intracellular signaling pathways that influence cell proliferation, regulation of multidrug resistance, motility, and survival. A number of molecules are involved in these processes, such as tyrosine kinases, protein kinase C, PI3-K, RAS, cytoskeletal components, or NF-κB (Toole, 2002; Toole, 2004). These biological effects are dependent on HA molecular size. Furthermore, it has been reported that oHA are able to inhibit the signaling pathways that native HA is able to induce in tumor cells (Zeng et al., 1998; Ghatak et al., 2002; Misra et al., 2003).

We have previously shown that native HA enhanced NF-κB activity in murine lymphoma cell lines and that this activity was related to activation of MMP-9 (Alaniz et al., 2004), suggesting that the effect may be correlated with cell survival. Despite of HA ability to improve PI3-K/Akt and NF-κB activities, we did not find any effect on survival after treatment with HA. However, when cells were treated with oHA (which did not modify NF-κB binding activity or its cytoplasmatic inhibitor IkBα levels) apoptosis was induced. These results indicate that apoptosis levels obtained with oHA may be due to a decrease in PI3-K/Akt activity, as our results clearly showed that oHA diminished constitutive PI3-K/Akt activity and that this pathway was not related to NF-κB signaling. These findings are in line with those of other authors who found that oHA inhibit tumor growth and anchorage-independent cell survival in solid tumors by suppressing the PI3-K/Akt pathway (Ghatak et al., 2002).

Inhibition experiments carried out herein support such hypothesis, since treatment with the PI3-K/Akt specific inhibitor wortmannin did not modify NF-κB activity, and induced apoptosis in similar percentages to those obtained with oHA. Besides, as we have previously shown, the cell lines used in this work have different constitutive NF-κB activity, with LBLa cell line displaying higher activity than LBLc. Despite these differences, apoptosis levels reached with oHA were similar in both cell lines.

In this study we report for the first time that oHA interacts in vitro with T-lymphoma cell lines inducing apoptosis. These results also agree with previous studies in solid tumors which showed that either in vitro or in vivo treatment with oHA, or transfection with hyaluronidase suppressed tumor development and induced reversal of drug resistance (Croix et al., 1996; Zeng et al., 1998; Ghatak et al., 2002; Jacobson et al., 2002; Misra et al., 2003). However, there seems to be some discrepancies between our results and those of other authors who suggested that HA

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**Fig. 4.** Detection of apoptosis in LBLa and LBLc cell lines treated with HA and oHA. (A) Analysis by fluorescence microscopy of the cells stained by acridine orange and ethidium bromide. Data are expressed as mean ± SE (n = 5), * indicates p < 0.05. (B) Photograph of LBLa cell line treated with oHA presenting morphological features of apoptotic cells: the upper left (single) cell appeared orange and showed condensed chromatin and nuclear fragmentation, and the lower right (two) cells appeared green and showed preserved nuclear structure. (C) Left panel shows FACS analysis of LBLa cell line untreated or treated with oHA; late-stage apoptotic cells are labeled by dual positivity with Annexin V and PI (right quadrant), percentage of positive cells are indicated in each quadrant. Right panel shows the results expressed as mean ± SE (n = 3). Total percentage of apoptotic cells was calculated as subtraction of spontaneous apoptosis from induced apoptosis (% apoptosis of treated cells–% apoptosis of untreated cells).
degradation products play an important role in other kind of tumors, promoting development and invasion of pancreatic carcinoma (Sugahara et al., 2003), angiogenesis and growth in colon carcinoma, melanoma, and glioblastoma cell lines (Liu et al., 1996; Lokeshwar et al., 2001). Further studies are necessary to solve these apparent contradictions.

In conclusion, we demonstrated that HA oligomers exert their action on T-lymphoma cell lines by suppressing the PI3-K/Akt pathway and independent of NF-κB cell survival pathway, suggesting that other signaling pathways may also be relevant in the development of T-cell lymphoma. We also showed that although modulation of PI3-K/Akt activity by either oHA or HA is not related with NF-κB pathway, both pathways are important for T-lymphoma cells survival, since inhibition by specific NF-κB inhibitor such as BAY 11–7082 or PI3-K/Akt by either oHA or wortmannin also produced apoptosis on these cell lines.

Materials and Methods

Preparation and analysis of oHA

Oligomers were generated after digestion of recombinant high (HMW 1.5–1.8 × 10^6 Da) and low (LMW 1–3 × 10^5 Da) molecular weight HA (5 mg/mL) (CPN spol.s.r.o Czech Republic kindly supplied by Farmatrade, Buenos Aires, Argentina) with bovine testicular hyaluronidase (Sigma Aldrich, Buenos Aires, Argentina) employing 500 U/mg of HA, incubating at 37°C for 24 h. The reaction was stopped by boiling for 5 min. The size of oHA was determined by HPAEC-PAD. The reaction mixture was diluted with 200 μL of distilled water and then passed through a Millipore filter (MW c.o. 5 kDa). The filtered solution was diluted to 625 μL, and 20 μL were injected into the HPAEC system. Analysis by HPAEC-PAD was performed using a Dionex DX-300 HPLC system equipped with a pulse amperometric detector (PAD) with a CarboPac PA-100 ion exchange analytical column (4 × 250 mm) and a PA-100...
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The following conditions were used: (a) A linear gradient over 80 min from 400 to 700 mM C$_2$H$_3$NaO$_2$ in 150 mM NaOH at a flow rate of 1.0 mL/min at room temperature (data not shown), (b) A linear gradient over 80 min from 300 to 700 mM C$_2$H$_3$NaO$_2$ in 150 mM NaOH at a flow rate of 1.0 mL/min at room temperature (Figures 1A and B). As standard, 1.6 μg of HA$_4$ (HA tetrasaccharide, 2 disaccharide units in length) in 20 μL of distilled water was used. The HA$_4$ was kindly provided by Dr. Anthony J. Day from Oxford University.

Nuclear extract preparation and EMSA

LBLa and LBLc cell lines (1 × 10$^7$ cells), were treated with HA (HMW or LMW) or oHA at a concentration 200 μg/mL or BAY 11–7082 (IκB phosphorylation inhibitor) at different concentrations (3.5, 5, 7.5, and 10 μM) for 2 h. Nuclear extracts were prepared as previously described (Alaniz et al., 2004). Briefly, cells were incubated with 400 μL of hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride, and 0.1% Nonidet P-40) for 15 min on ice and centrifuged at 11,000 × g for 10 min. Nuclear pellets were resuspended in 80 μL of nuclear lysis buffer (20 mM HEPES, 1.5 mM MgCl$_2$, 420 mM NaCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 mM ethylenediaminetetraacetic acid [EDTA], and 25% glycerol) and incubated at 4°C for 15 min, followed by centrifugation at 13,000 × g for 15 min. NF-κB binding activity was examined by EMSA as previously described (Alaniz et al., 2004). Autoradiography images obtained with a digital camera (Olympus, Camedia, D-510 ZOOM) were subjected to densitometry analysis using Image Scion software and the densitometry was expressed as Index: treated cells/untreated cells.

Cytoplasmatic extracts and immunoblotting assay

Cells were treated with HA (HMW or LMW), oHA or BAY 11–7082 as described above, and with wortmannin (50, 100 y 200 nM) during 2 h. After treatment, cells were lysed with a cytoplasmatic lysis buffer (20 mM Tris pH 8.0, 150 mM NaCl, 100 mM NaF, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 40 μg/mL leupeptin, 20 μg/mL aprotinin, 1 mM sodium orthovanadate) for 30 min at 4°C. After centrifugation, equal amounts of protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane. After blocking with Tris-buffered saline, 2% glycine and 3% nonfat dried milk overnight at 4°C, the membrane was washed and incubated with specific antibodies to IκB-α, p-Akt, Akt and actin (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C followed by incubation with a horseradish peroxidase- labeled...
secondary antibody for 1.5 h at 37°C. The reaction was developed using a chemiluminescent detection system (western blotting Luminol Reagent, Santa Cruz Biotechnology, Inc). Gel images obtained with a digital camera were subjected to densitometric analysis and processed as mentioned above.

**PtdIns-P (PIP) production and lipid analysis**

Untreated and treated cells with HA (HMW or LMW), oHA, or wortmannin (100 and 200 nM) were loaded with 50 μCi (32P)Pi at 37°C during 12 h. The cells were centrifuged, the reaction was stopped by addition of 2 mL de CHCl₃: methanol (2:1 v/v). Lipids were extracted by adding methanol/chloroform (1:1) and 1 M HCl (Catz and Sterin-Speziale, 1996).

**Apoptosis detection**

Apoptotic cells were detected using the Annexin V binding assay. Briefly, cells were treated with 200 μg/mL of HA (high or low molecular weight), 200 μg/mL oHA, 10 μg/mL mAb anti-CD44 IM7.8.1 antibody or IgG 2b rat as isotype control, BAY 11–7082 at different concentrations (3.5, 5, 7.5, and 10 μM) or wortmannin (50, 100 y 200 nM) in the same conditions used to prepare nuclear and cytoplasmatic extracts. Cells were resuspended in binding buffer and a mixture of Annexin V-FITC and propidium iodide (PI) was added. Samples were analyzed using a FACScan flow cytometer (Ortho Cytron Absolute, Raritan, NJ) and acquired data were analyzed using WinMDI 2.8 software (Scripps Institute, La Jolla, CA). The apoptotic cells percentage was determined by adding the percentage of cells present in the right two quadrants. Morphological features associated with apoptosis were also analyzed by acridine orange and ethidium bromide staining (Lopes et al., 2001). Briefly, after 24 h of treatment, pellets were resuspended in the dye mixture (100 μg/mL acridine orange and 100 μg/mL ethidium bromide in phosphate-buffered saline) and visualized by fluorescence microscopy (Zeiss, Berlin, Germany). A minimum number of 200 cells were counted and the number of fragmented nuclei, enlarged cytoplasm, and condensed chromatin were determined. The percentage of apoptotic cells (apoptotic index) was calculated as:

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\text{Apoptotic cells (\%)} = \left( \frac{\text{total number of cells with apoptotic nuclei}}{\text{total number of cells counted}} \right) \times 100.
\]

**Statistical analysis**

Apoptosis data were analyzed by one way-ANOVA and Tukey's test. Densitometry results from EMSA and immunoblotting were analyzed by the nonparametric Mann-Whitney U-test, using the Prism software (Graph Pad, San Diego, CA). p values of 0.05 or less were considered statistically significant.

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Conflict of interest statement
None declared.

Abbreviations
DTT, dithiothreitol; ECM, extracellular matrix components; EMSA, electrophoretic mobility shift assay; HA, hyaluronan; HMW-HA, high-molecular weight hyaluronan; HPAEC-PAD, high pH anion exchange chromatography with pulse anomerometric detection; LMW-HA, low-molecular weight hyaluronan; NF-κB, nuclear factor-κB; oHA, hyaluronan oligosaccharides; PI3-K, phosphatidylinositol 3-kinase; PtdIns-P₃ (PIP₃), phosphatidylinositol 3,4,5-trisphosphate.

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