Glycosaminoglycans and glucose prevent apoptosis in 4-methylumbelliferone treated human aortic smooth muscle cells

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† This paper is dedicated to the memory of our wonderful friend Professor Marco Ramoni, who suddenly passed away on June 8, 2010.
Running title: TLR4 and CD44 mediates anti-apoptotic effect of HA on SMC.

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Smooth muscle cells (SMCs) have a pivotal role in cardiovascular diseases and are responsible for hyaluronan (HA) deposition in thickening vessel walls. HA regulates SMC proliferation, migration and inflammation, which accelerates neointima formation. We used the HA synthesis inhibitor 4-methylumbelliferone (4-MU) to reduce HA production in human aortic SMCs and found a significant increase of apoptotic cells. Interestingly, the exogenous addition of HA together with 4-MU reduced apoptosis. A similar antiapoptotic effect was observed also by adding other glycosaminoglycans and glucose to 4-MU treated cells. Further, the anti-apoptotic effect of HA was mediated by Toll Like Receptor 4 (TLR4), CD44 and PI3K, but not by ERK1/2.

Hyaluronan (HA) is one of the most abundant glycosaminoglycans (GAGs) in extracellular matrices (ECM) and is composed of linear, unsulfated repetitions of D-glucuronic acid and N-acetylglucosamine. In mammals, two specific HA synthases (HAS1, and 2) produce high molecular weight HA (HMW-HA), in the range of millions of Da, whereas the other isoenzyme (HAS3) synthesizes HA of lower molecular mass, in the range of several thousands of Da (1). The size of HA depends also on specific degrading enzymes (i.e., hyaluronidases) that can produce bioactive HA oligosaccharides. Therefore, in vivo, HA chains can greatly vary in lengths and can differently regulate cell behavior through interactions with several receptors, including CD44, Receptor for HA-mediated motility (RHAMM), Lymphatic Vessel Endothelial Receptor 1 (Lyve-1), HA Receptor for Endocytosis (HARE), and Toll Like Receptor 4 (TLR4) (2).

In cardiovascular pathologies, HA accumulates during neointima formation and alters smooth muscle cell (SMC) behavior (3). In some pathological conditions, contractile SMCs dedifferentiate to form a synthetic phenotype characterized by a high production of ECM components, including HA and versican, by synthesis of ECM modifying metalloproteinases (4), and by increased rates of proliferation and migration. Therefore, SMCs acquire the capability to invade the vascular tunica intima, thereby contributing to vessel wall thickening. HMW-HA is involved in the modulation of SMC migration and proliferation through interaction with CD44 (5-7), which can mediate a signaling cascade inside the cell that activates different pathways, including PI3K, AKT and ERK1,2 (8).

We previously demonstrated that human aortic SMC (AoSMC) migration is strictly dependent on HA-CD44 signaling and recently reported that the HA synthesis inhibitor 4-methylumbelliferone (4-MU) reduced proatherosclerotic properties of AoSMCs by...
decreasing cell migration and proliferation, and by inhibiting monocyte binding to the HA-rich ECM that contributes to inflammation (9,10). Moreover, we found that the simultaneous addition of HMW-HA to 4-MU treated AoSMCs restored cell proliferation to the levels of controls. Therefore, the aim of this study was to investigate at the molecular level the effects of HMW-HA after 4-MU treatment of AoSMCs and the pathways involved in its effects on the cells.

**Experimental procedures**

**Cell culture and treatments** - Human aortic SMCs (AoSMCs) were purchased from Lonza and were grown for 2-6 passages in complete SmGm2 culture medium (Lonza) supplemented with 5% FBS. As we previously reported (9), a final concentration of 1 mM 4-MU (Sigma) in DMSO (final concentration 0.1%) inhibited HA synthesis ~40% and reduced cell viability ~25%. In some experiments, AoSMCs were grown in the presence of 25 µg/ml HMW-HA (~4x10^6 Da) (Healon, Abbott Medical Optics), or 25 µg/ml of chondroitin 4 sulfate (C4S, Seikagaku), or 25 µg/ml of chondroitin 6 sulfate (C6S, Seikagaku), or 25 µg/ml of dermatan sulfate (DS, Seikagaku), or 25 µg/ml of keratan sulfate (KS, Sigma), or 25 mM (final concentration) of glucose, or 25 mM (final concentration) of 2-deoxyglucose (2DG) or 25 mM (final concentration) of sorbitol, or of 10 µM U0126 (Sigma), or of 10 mM NH4Cl (Sigma), or of 5 µM LY294002 (Sigma), or of 5 µg/ml of anti-CD44 Hermes-1 monoclonal antibody (Development Studies Hybridoma Bank), or of 5 µg/ml of anti-CD44 BRIC-235 monoclonal antibody (International Blood Group Reference Laboratory, Bristol, UK.), or of 5 µg/ml of anti-tubulin monoclonal antibody (Sigma), or of 25 µg/ml of purified, low endotoxin 34-mer HA oligosaccharide (o-HA) (6.8 kDa) (Glycoscience Laboratories) (11), or of 1 µg/ml of anti-TLR4 monoclonal antibody (MTS510, Santa Cruz), or of 15 ng/ml of E5564 (eritoran, Eisai Inc.), a pharmacological inhibitor of TLR4 signaling (12). Such treatment concentrations were determined after preliminary dose-response experiments.

**Microarray** - Total RNA was extracted from 3 independent cultures of AoSMCs treated for 24 h with 1 mM 4-MU, and from 3 independent untreated cell cultures using a commercial kit (Ambion). Bioanalyzer (Agilent) was used to quantify RNA, and only RNA samples with an RNA integrity number greater than 7.5 were used. Two µg of total RNA were used to generate cDNA and digoxigenin-labeled cRNA. Ten µg of the cRNA were hybridized to a human genome survey microarray (Applied Biosystems, Foster City, CA). The signal was developed using a chemiluminescent detection kit (Applied Biosystems), and chips were scanned by using a 1700 chemiluminescent microarray analyzer (Applied Biosystems). The intensity distributions of the microarrays were highly similar, so normalization was not required. Probes whose Flags value exceeded 5000 in more than 4 (of the 6) arrays were filtered out. The data were assessed on the log2 scale, and differential expression analysis between the two groups was done using the limma package (13). Enrichment of KEGG pathways was computed by submitting the identified probes to DAVID (14) and using all human genes as background.

**Quantitative RT-PCR** - Quantitative RT-PCRs were done with an Abi Prism 7000 Real Time instrument (Applied Biosystems) using the Taqman Universal PCR Master Mix and Human pre-developed TaqMan gene expression assays for p53, p21, CDK2, p16, CD44, and β-actin (Applied Biosystems). The relative quantification of gene expression levels was determined by comparing ΔCt values as previously described (15-17).

**DNA content by cytofluorimetry** - AoSMCs were resuspended in phosphate-buffered saline (PBS) containing 1% Igepal for membrane permeabilization. Cell pellets obtained after centrifugation were resuspended in 1 ml of PBS with propidium iodide and RNase. The DNA contents of the cells were quantified by using a FACSCanto cytofluorimeter (Becton Dickinson).

**HA quantification** - Polyaacrylamide gel electrophoresis of fluorophore labelled saccharides (PAGEFS) and HPLC were used to measure the amounts of unsaturated HA disaccharides in the conditioned cell culture media as previously described (18-20).

**Cell vitality and motility assays** - Apoptotic cells were detected by using the Annexin-V-FITC kit (Roche Diagnostic), and necrotic cells were detected by staining with propidium iodide as described by the manufacturer. To quantify apoptosis and necrosis, green (apoptotic) and red (necrotic) cells were counted in 10 independent fields under a fluorescent microscope (Olympus). In experiments with C4S, C6S, DS, KS, ammonium chloride, anti-CD44 antibody, anti-
TLR4 antibody and inhibitors, the numbers of viable cells were counted in a Burker’s chamber by using Trypan Blue. To measure cell motility, confluent AoSMCs were treated either with 1 mM 4-MU, or with 25 μg/ml HMW-HA, or 1 μg/ml of anti-TLR4 monoclonal antibody, or 15 ng/ml of E5564, and scratched by pipette tip. Migration was quantified after 24 h of incubation as previously described (9).

**CD44 silencing** - Small interfering RNA (siRNA) was used to reduce expression of CD44 in AoSMCs. CD44 siRNA (s2681) and scramble negative control siRNA #1 kit (code 4611) were both purchased from Ambion. The transfections were done using a Nucleofector apparatus (Amaxa) as previously described (4,21). After 48 h of incubation, cells were treated with 4-MU and HMW-HA and cell viability measured.

**Statistical analyses** - Statistical analysis of the data was done using analysis of variance (ANOVA), followed by post hoc tests (Bonferroni) using Origin 7.5 software (OriginLab). Probability values of $P < 0.01$ or 0.05 were considered statistically significant. Experiments were repeated three times each time in duplicate, and data are expressed as mean ± standard error (SE).

**Results and Discussion**

In vascular pathologies, vessel thickening is a very common problem and is determined by complex mechanisms that involve remodelling of the ECM. SMCs are primarily responsible for arterial wall ECM production, and when SMCs dedifferentiate to become atherosclerotic prone cells, they synthesize large amounts of specific ECM molecules, including HA (22). HA is known to accumulate in neointima and to induce SMC migration, which increases progression of lesions by the formation of a highly hydrated ECM that facilitates cell movements, and by triggering cell receptor signaling. Recently, we showed that the pro-atherosclerotic properties of AoSMCs are reduced by treating them with 4-MU, a well known HA synthesis inhibitor. At 1 mM 4-MU, we found a clear reduction of mRNA coding for HASes and decreased UDP-glucuronic acid levels, which decreased production of HA. Further, cell migration and proliferation were also reduced (9). Interestingly, the addition of 25 μg/ml of exogenous HMW-HA to 4-MU treated cells restored AoSMC proliferation and motility (9). The rescue of cell migration by HMW-HA was clearly mediated by CD44, whereas the rescue of cell proliferation was not investigated.

In this study, cellular pathways altered by 4-MU were investigated by whole genomic expression profiling by using a microarray approach to compare 10 μg of cRNA prepared from untreated and 4-MU treated AoSMCs (at a concentration of 1 mM for 24 h). Bioinformatic analyses identified 107 probes (Supplemental Table 1) with a False Discovery Rate (FDR) below 5%, and these yielded two enriched pathways: the cell cycle pathway (p-value <0.001) and the p53 signalling pathway (p-value 0.012). The complete dataset of the microarray experiment is reported in Supplemental Table 2. As HA is known to regulate proliferation in endothelial cell types, it was not surprising to find that inhibition of HA synthesis alters cell cycle genes. On the other hand, the link between HA and p53 is not known, although recently, it was reported that reduction of an HA/versican ECM induced senescence and p53 accumulation in fibroblasts (24).

We previously showed that the addition of exogenous HMW-HA to 4-MU treated AoSMCs rescued cell viability (9). Therefore, we measured the expression of several transcripts coding for cyclins, cyclin-dependent kinases, p53, BCL2 and several other proliferation related genes in untreated AoSMCs, and in AoSMCs treated for 24 h with 1 mM 4-MU, and with 1 mM 4-MU + 25 μg/ml of HMW-HA by means of quantitative RT-PCR (results not shown). Interestingly, among the tested genes, only p21 mRNA responded to HMW-HA by returning to the level of untreated AoSMCs as shown in Figure 1.

Because p21 is strictly related to cell cycle arrest, we measured the DNA content in AoSMCs after 4-MU or 4-MU + HMW-HA treatments by means of cytofluorimetric analyses (Fig. 2). Untreated cells were 50.9% in G1, 21.5% in S, and 27.6% in G2. After 4-MU treatment the cells showed a clear G1 arrest (84.1% in G1, 10.8% in S, and 5.1% in G2). Interestingly, in addition to the G1 peak, another sharp peak appeared after 4-MU treatment, which is similar to the extra peak that has been associated with apoptosis in other cell types (25). Further, in AoSMCs treated with 4-MU + HMW-HA the extra peak disappeared, even though these cells continued to be blocked in G1 (84.2% in G1, 9.0% in S, and 6.9% in G2). These results indicate that 4-MU inhibits cell growth through a G1 block that is probably mediated through p21 or cyclin D1 as previously observed (7). Moreover, the cytofluorimetric analyses indicate the possibility...
that apoptosis could occur after 4-MU treatment, which can be prevented in the presence of HMW-HA. This would be consistent with the results of the microarray experiment that identified the p53 pathway and cell cycle as the most affected cellular functions, which fit well with cell growth arrest and apoptosis induction. Further, p53 is known to induce apoptosis through mitochondrial outer membrane permeabilization and other mechanisms (26). Interestingly, during the preparation of this manuscript, Lokeshwar and colleagues published that 4-MU induced apoptosis in prostate tumor cells probably by activating the extrinsic pathway of apoptosis (27).

To confirm the induction of apoptosis in 4-MU treated AoSMCs, we used a commercial kit to detect phosphatidylserine in the outer leaflet of the plasma membrane and found that the percentage of apoptotic cells increased ~8-fold in 4-MU treated AoSMCs compared to untreated AoSMCs (Fig. 3A). Further, the population of apoptotic cells in AoSMCs treated with 4-MU + HMW-HA was ~10% and not statistically different from untreated AoSMCs (Fig. 3A). There were no significant differences in the percentage of necrotic AoSMCs in the 3 treatments as measured by propidium iodine staining (data not shown). We also have measured viable cells after 4-MU treatment by means of trypan blue staining and found a reduction of ~40% of live cells whereas in 4-MU+HMW-HA, vitality was similar to controls (Fig. 3B). Interestingly, the reduction of ~40% of viable cells after the 4-MU treatment quantified by trypan blue correlates well with the ~40% increment of apoptotic cells determined with Annexin-V kit (Fig. 3A) suggesting that trypan blue staining could be conveniently used to evaluate apoptosis in our conditions. To better demonstrate the effects of 4-MU and HMW-HA on cell viability, we treated AoSMCs with 0.5, 1, and 2 mM 4-MU and 25 μg/ml of HMW-HA finding a clear 4-MU dose-dependent reduction of cell proliferation (Supplemental Figure 1A). Similarly, apoptosis also had the same trend (Supplemental Figure 1B). The apoptotic process after 4-MU treatment was substantiated by showing that the cleaved poly (ADP-ribose) polymerase (PARP) protein, a marker for apoptosis, was only present in AoSMCs treated with 4-MU (Western blot, Fig. 3C). The protective effect of HA against apoptosis has been reported for other cell types than vascular cells (28-32). However, it has been reported that HA induced apoptosis in dendritic cells via inducible nitric oxide synthase (33).

The transcripts for anti-apoptotic Bcl-2 protein and pro-apoptotic genes (Noxa, Puma, Bax, and Gadd45) that are known to be transcriptionally regulated by p53 (34-36) were measured by quantitative RT-PCR in the 3 AoSMC culture treatments. However, the expression analyses of these genes did not show any differences (results not shown) indicating that a different mechanism is involved for activation of apoptosis by 4-MU and rescue by HMW-HA.

Because 4-MU alters the cellular content of UDP-glucuronic acid (9), we hypothesized that the inhibition of 4-MU-induced apoptosis by HA could be mediated by a metabolic effect. Although the inhibitory effect of 4-MU was specific for HA synthesis, we added other polysaccharides usually present in the ECM to 4-MU treated AoSMCs to test for possible rescue from apoptosis: 25 μg/ml of each of the commercial GAGs: C46, C6S, DS, KS. Moreover, in order to check the metabolic hypothesis in this process, we also used glucose and 2DG. We also treated AoSMCs with sorbitol as osmotic control and, it did not show any antiapoptotic property. To check the purity of the GAG preparations, we verified absence of HA in sulfated GAG solutions by PAGEFS and HPLC (results not shown). As shown in Figure 4A, among these compounds, only 2DG was not able to inhibit cell mortality induced by 4-MU supporting the metabolic hypothesis. In fact, 2DG is known to induce ATP depletion and energetic stresses in treated cells, which would be somewhat facilitative toward apoptosis. As far as glucose is concerned, we did not further investigate neither its anti-apoptotic mechanism nor if it could trigger specific signals from HA receptors as CD44 or TLR4 (see below), in neurons and cancer cells it was elegantly demonstrated that glucose can protect from apoptosis regulating glutathione and cytochrome c metabolism (37). The anti-apoptotic role of such GAGs after 4-MU treatment was also confirmed by detecting Annexin V FITC positive cells (Supplemental Figure 2). Interestingly, among the GAGs, only KS does not contain glucuronic acid suggesting that UDP-glucuronate is not critical in the anti-apoptotic effect, whereas it has a pivotal role to control HA synthesis (21).

However, GAGs would have to be degraded by the cells to furnish intermediate metabolites (i.e., UDP-sugars or energy) through the action of several lysosomal glycosydases. To test this possibility, we treated AoSMCs with NH4Cl, a well known inhibitor of lysosomal enzymes. As shown in Fig. 4B, NH4Cl alone did not decrease viability of the cells, and it did not
alter the effect of HMW-HA to prevent the decrease in viability in the presence of 4-MU. Further, NH_{4}Cl alone did not prevent the decrease of viability in the presence of 4-MU. To verify the effectiveness of ammonium chloride treatment, we measured an increment of about 50% in the content of HA after NH_{4}Cl treatment of AoSMCs by PAGEFS demonstrating the inhibition of HA degrading enzymes (result not shown). Therefore, the blocking of lysosomal enzymes necessary to catabolize HA and the other polysaccharides is not involved in the anti-apoptotic effect of HMW-HA suggesting that the metabolic hypothesis is not critical in this process.

Previous studies have demonstrated the central role of the HA receptor CD44 in regulating AoSMC behavior (5,10), and have reported a link between p53 and CD44 (38). Moreover, another study with chondrocytes showed that the HA anti-apoptotic effect was due to CD44 (29). CD44 has several variants derived from alternative splicing events at the RNA maturation level. As CD44 interacts with many ECM components (i.e., collagen, fibronectin, laminin, HA, DS and CS) (39,40), such CD44 isoforms could be involved in receptor-ligand recognition, thereby explaining the evidence that other GAGs as CS and DS inhibited the 4-MU-induced apoptosis. To test this hypothesis, we inhibited the HA-CD44 interaction by using the CD44-blocking Hermes-1 or BRIC235 monoclonal antibodies, and by attenuating HA-CD44 signaling with a 34-mer o-HA as we previously showed in AoSMCs (10). As shown in Figure 5A, neither Hermes-1 or BRIC235 nor the o-HA were able to inhibit the rescuing effect of HMW-HA.

HA can be also recognized by other receptors (2) and, among these, TLR4 could be a good candidate to mediate the rescuing process. TLRs mediate immune responses by sensing bacterial structures, such as LPS, viral RNA, and endogenous molecules released by damaged host cells such as heat shock proteins (41). Notably, TLR4 has been described to interact with other polyanionic molecules, including heparan sulfate (42); and thereby could be involved also in the anti-apoptotic mechanism of the other GAGs. HA has been proposed to regulate TLR4, thereby modulating inflammation and apoptosis in mouse lung (2). To verify whether TLR4 was involved in the anti-apoptotic effect of HMW-HA, we treated AoSMCs with 4-MU, 4-MU + HMW-HA, 4-MU + HMW-HA + TLR4 blocking antibody, or with eritoran, a TLR4-directed endotoxin antagonist (12). Figure 5B shows that both the blocking antibodies and eritoran prevented the rescuing effect of HMW-HA, thereby supporting the critical function of TLR4 in the anti-apoptotic effect mediated by HA in AoSMCs. Anti-TLR4 and eritoran alone (Fig. 5B) or in combination with 4-MU (data not shown) were not statistically significant from control cells. Interestingly, LPS, the main ligand of TLR4, at 1, 10 or 100 ng/ml was not able to reduce the mortality induced by 4-MU (Supplemental Figure 3), suggesting a specific response when HA reacts with TLR4. Although the direct binding of HA to TLR4 has never been demonstrated, it was shown that TLR4, CD44 and MD-2 form a complex that cooperates in HA recognition (43).

Our data obtained with anti-CD44 antibodies and HA oligosaccharide prevented HA-CD44 interaction and signalling, but no information is available as to whether this treatment interferes with TLR4-CD44 complex formation, signalling or stability. Therefore, we decided to abrogate CD44 expression by means of siRNA and verify whether or not the presence of CD44 protein was necessary for the HA antiapoptotic effect. After the silencing, by quantitative RT-PCR we measured the residual CD44 expression that ranged from 15 to 20% respect to control cells. As shown in Figure 5C, the CD44 silencing alone did not influenced cell viability whereas the lack of CD44 inhibited the rescuing effect of HWM-HA after 4-MU treatment indicating that CD44 is critical for HMW-HA the antiapoptotic effect. The specificity of such data was confirmed by a scramble siRNA treatment that maintained the rescuing properties of HMW-HA as untreated sample. The controversy between the anti-CD44 antibodies and CD44 silencing can be explained taking into consideration the fact that CD44 can form a complex with TLR4 (43,44) and the beneficial effect of HMW-HA requires both the receptors. We can speculate that HA could be recognized by TLR4 but, for the antiapoptotic effect, the entire TLR4/CD44 complex is necessary for a survival signaling.

Although it is generally accepted that HA binds to TLR4 or TLR-4-MD-2 (myeloid differentiation factor 2) complex as the polyanionic nature as well as the di-saccharide backbone with β-glycosidic bond of known TLR4 agonist (LPS) and antagonist (Eritoran) (44,45), our results highlighted a central role of CD44 to regulate a specific TLR4 signalling triggered by HA (probably different form that evoked by LPS) as previously reported Taylor and collaborators (43).
We have also studied whether other GAGs as C6S and C4S could abrogate 4-MU induced apoptosis through TLR4. As shown in Supplemental Figure 4, the blocking of the receptor with antibodies or the treatment with the antagonist did not prevent the rescuing indicating a different anti-apoptotic mechanism that could involve physical phenomena such as the "surface screening effect" theory (Gouy-Chapman-Stern theory) (46).

TLR4-mediated signaling leads to rapid activation of PI3K (47), one of a family of kinases involved in regulation of cell growth, apoptosis, and motility. As the PI3K-AKT signaling pathway is strictly related to cell survival, we evaluated whether this kinase was involved in the anti-apoptotic effect of HMW-HA. We used 5 μM of LY294002 to inhibit PI3K and 10 μM of U0126 to block ERK1/2, which is also involved in apoptosis (48). Figure 5D shows that the two inhibitors alone had little or no effect on AoSMCs viability. However, the number of viable cells in 4-MU+HMW-HA treated AoSMCs decreased significantly only after LY264002 addition. This indicates that the PI3K pathway, but not the ERK1/2 pathway, is crucial for the rescuing effect mediated by HMW-HA.

Cell motility is crucial in atherogenesis. As HA interaction with TLR4 can regulate cell viability, we wondered whether this receptor is involved also in motility control. To address this issue, we repeated previously reported migration assays in which we demonstrated that HMW-HA enhanced AoSMC motility through CD44 (9). As shown in Figure 6, after 24 h from the wound, the effect of HMW-HA to induce cell movement was abolished by treating AoSMCs with TLR4 blocking antibodies as well as by adding the TLR4 antagonist eritoran, whereas it was unaltered by unrelated antibodies or placebo. Additional control experiments with anti-TLR4 and eritoran alone (and in combination with 4-MU) without added HMW-HA did not show statistically significant differences from untreated cells (results not shown) clearly showing that TLR4 is able to participate to the modulation of AoSMC migration in vitro. As 4-MU reduced the number of vital cells by 40%, the delayed wound healing response may reflect the problem in proliferation rather than in migration. To exclude this issue, we repeated the experiments quantifying migration after 6 h from the wound finding comparable results (Supplemental Figure 5) suggesting a role of TLR4 in motility. Similar results were previously obtained in melanoma cells where the abrogation of TLR4 by short interference RNA inhibited the motility induced by short HA oligosaccharides (49). Another HA receptor (i.e., RHAMM) was shown to control SMC migration in response to HA (50). All these results highlight the importance of HA in the fine tuning of cell movement.

Overall, our results provide strong evidence that the apoptosis induced in AoSMCs in the presence of 4-MU can be blocked through the ability of HMW-HA and other GAGs to induce a PI3K anti-apoptotic signaling pathway through interaction with TLR4-CD44 complex. Therefore, the role of CD44 in TLRs signaling is becoming critical in light of recent literature reporting the modulation of NF-κB pathway throughout not only HA (15), but also other proinflammatory secreted molecules as tumor necrosis factor-inducible gene 6 (49).

References

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Abbreviations: HA, hyaluronan; GAG, glycosaminoglycan; RT-PCR, reverse transcriptase-polymerase chain reaction; HAS, HA synthase(s); HMW-HA, high molecular weight HA; AoSMCs, aortic smooth muscle cells; 4-MU, 4-methylumbelliferone; ECM, extracellular matrix; C4S, chondroitin 4 sulfates; C6S, chondroitin 6 sulfates; DS, dermatan sulfate; KS, keratan sulfate; TLR-4 Toll Like Receptor 4; PAGEFS, Polyacrylamide gel electrophoresis of fluorophore labelled saccharides. AoSMC, aortic smooth muscle cells; LPS, lipopolysaccharide.

Figure legends

Fig. 1. Relative expression of p21 mRNA in untreated AoSMCs, or after 24 h of treatment with 1 mM 4-MU alone, or with 1 mM 4-MU and 25 μg/ml of HMW-HA by quantitative RT-PCRs. The lowest p21 expression in 3 different untreated samples was set at 1, and the standard error is shown on each bar. *p < 0.01 control versus treated samples. Relative expression is in arbitrary units.

Fig. 2. Cytofluorimetric analyses of DNA content in untreated AoSMCs (control), or after 24 h of treatment with 1 mM 4-MU, or after 24 h of treatment with 1 mM 4-MU and 25 μg/ml HMW-HA. The arrow indicates the extra G1 peak that has been associated with apoptosis. The peak is in 4-MU treated samples but absent in 4-MU+HMW-HA treated cells.

Fig. 3.
Induction of apoptosis and reduction of cell viability after 4-MU treatment and anti-apoptotic effect of HMW-HA. A. 5x10^5 AoSMCs were plated in the absence or in the presence of 1 mM 4-MU alone, or with 1 mM 4-MU + 25 µg/ml HMW-HA for 24 h. Annexin-V-FITC was used to mark apoptotic cells, and green fluorescent cells in 10 independent microscopic fields were counted. *p < 0.01 control versus treated samples. B. AoSMCs were treated and incubated as in A, but stained with Trypan Blue. Viable cells were counted in 10 independent microscopic fields. *p < 0.01 control versus treated samples. C. Western blots of 50 µg of protein extracted from untreated (control) or treated AoSMCs as described above using anti cleaved PARP (active) antibody. In the figure each band represents a different extract from a replicate culture.

Fig. 4.
Effects of sugars and lysosomal enzymes on inhibition of 4-MU induced apoptosis. A. 5x10^5 AoSMCs were plated for 24 h in the absence or in the presence of 1 mM 4-MU alone, or with 1 mM 4-MU + 25 µg/ml HMW-HA, or with 1 mM 4-MU + 25 µg/ml of C4S, or 25 µg/ml of C6S, or 25 µg/ml of KS, or 25 µg/ml of DS, or 25 mM (final concentration) of glucose, or 25 mM of 2DG, or 25 mM sorbitol as osmotic control. Cells were stained with Trypan Blue, and viable ones were counted in 10 independent microscopic fields. *p < 0.01 control versus treated samples. B. Cells were plated as described above and treated with 1 mM 4-MU alone, or with 1 mM 4-MU + 25 µg/ml HMW-HA, or with 1 mM 4-MU + 25 µg/ml HMW-HA + 10 mM NH₄Cl. After 24 h of incubation, the numbers of viable cells were quantified by using Trypan Blue staining. *p < 0.01 control versus treated samples. Note that there was no statistically significantly difference between cultures treated with 4-MU alone and 2DG.

Fig. 5.
Mechanisms of anti-apoptotic effect of HMW-HA. A. 5x10^5 AoSMCs were plated in the absence or in the presence of 1 mM 4-MU alone, or with 1 mM 4-MU + 25 µg/ml HMW-HA, or with 1 mM 4-MU + 25 µg/ml HMW-HA + 25 µg/ml of the 34-mer HA oligosaccharide (o-HA), or with 5 µg/ml of two monoclonal antibody against CD44 (Hermes-1 and BRIC235), or with an irrelevant control antibody (anti tubulin). B. Cells were plated as described above and treated with 1 µg/ml of a monoclonal antibody against TLR4, or with an irrelevant control antibody (anti tubulin), or with 15 ng/ml of eritoran, or with a placebo. C. 5x10^5 AoSMCs were nuclefected with siRNA against CD44 (siCD44) or with a scramble siRNA (siSCR), or subjected only to the electrical protocol and nucleofection reagent. After 24 h of incubation cells were treated with 4-MU alone or 4-MU + HMW-HA as described above. D. Cells were plated as described above and treated with 10 µM of U0126, or with 5 µM (final concentration) of LY294002. After 24 h of incubation, the numbers of viable cells were quantified by using Trypan Blue staining. *p < 0.01 control versus treated samples.

Fig. 6.
TLR4 affects AoSMCs motility. Confluent AoSMCs were left untreated or treated with various combination of 1 mM 4-MU, 25 µg/ml HMW-HA, 1 µg/ml of anti-TLR4 monoclonal antibody, 1 µg/ml of unrelated antibody (anti-α-tubulin), 15 ng/ml of E5564, or placebo, scratched with a yellow tip, incubated for 24 h, and photographed under an inverted microscope. Images were analyzed by using NIH Image software, and the numbers of migrated cells into the scratched areas were counted. Relative data are expressed as mean ± SE in three different experiments. *P < 0.05 versus untreated sample.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

A

B

C

D

Figure 5
Figure 6
Glycosaminoglycans and glucose prevent apoptosis in 4-methylumbelliferone treated human aortic smooth muscle cells
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