The effects of 4-methylumbelliferone on hyaluronan synthesis, MMP2 activity, proliferation, and motility of human aortic smooth muscle cells

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Received on July 18, 2008; revised on January 30, 2009; accepted on February 3, 2009

Extracellular matrix remodeling after proatherosclerotic injury involves an increase in hyaluronan (HA) that is coupled with vascular smooth muscle cell (SMC) migration, proliferation, and with neointima formation. As such events are dependent on HA, in this study we assessed the effects on SMC behavior of 4-methylumbelliferone (4-MU). As previously described in other cell types, 4-MU reduced HA in cultures of primary human aortic SMCs (AoSMCs) as well as the cellular content of the HA precursor UDP-glucuronic acid. We found that SMCs increased UDP-glucuronyl transferase 1 enzymes, which can reduce the cellular content of UDP-glucuronic acid confirming that the availability of the UDP-sugar substrates can regulate HA synthesis. Interestingly, we reported that 4-MU reduced the transcripts coding for the three HA synthases as well as UDP glucose pyrophosphorylase and dehydrogenase. As HA synthase transcript reduction is common to other cell types, the 4-MU effect on gene expression may be considered a mechanism for HA synthesis inhibition. Moreover, we showed that 4-MU strongly inhibits AoSMCs migration, which was restored by the addition of exogenous HA indicating that the rescuing depends on the interaction of HA with its receptor CD44. Besides the decrease in HA synthesis and cell migration, 4-MU reduced AoSMCs proliferation, indicating that 4-MU may exert a vasoprotective effect.

Keywords: atherosclerosis/neointima/vascular pathology/UDP-sugars

Introduction

Vascular pathologies are among the most frequent causes of death in western countries. In atherosclerosis or restenosis after angioplasty, vessel thickening is determined by complex mechanisms that involve remodeling of the extracellular matrix (ECM) (Newby 2006). Hyaluronan (HA) is an ECM component that is strongly remodeled after vessel injury. HA is a linear, unsulfated, and unbranched glycosaminoglycan (GAG) composed of glucuronic acid (GlcUA) and N-acetylgalactosamine (GlcNAc). The length of the GAG chain can vary greatly up to 2 × 10⁴ kDa, and its size is important to modulate its biological effects (Stern et al. 2006). HA synthesis occurs at the plasma membrane where three HA synthase (HAS1, 2, and 3) enzymes are located. UDP-GlcUA and UDP-GlcNAc are the substrates of HASs, and the synthesis of the nucleotide sugar substrates occurs in the cytosol. UDP glucose pyrophosphorylase (UGPP) and UDP glucose dehydrogenase (UGDH) are critical enzymes for synthesis of the HA precursors (Magee et al. 2001; Vigetti et al. 2006). Recently, it was shown that synthesis of UDP-N-acetylgalactosamines can regulate HA synthesis (Jokela et al. 2008).

In vascular pathologies, HA has a critical effect on the initiation of vessel wall thickening. After injury, HA accumulates in the tunica media of arteries and induces smooth muscle cells (SMC) proliferation, migration, and neointima formation. Neointima formation is a complex mechanism determined by several factors. Among them, matrix metalloproteinases (MMPs) are known to have a critical role in cardiovascular diseases (Newby 2006). In such pathologies, MMP2 and 9 are critical enzymes as they have wide substrate specificities for native type IV, V, VII, and X collagen, as well as for denaturated collagens, proteoglycans, and elastin (Li et al. 1999; Sternlicht and Werb 2001). Moreover, MMP-mediated ECM degradation can release signal molecules bound to specific ECM structures (i.e., FGF-2 bound to proteoglycans), which can stimulate cells to proliferate and migrate (Li et al. 1999). Regulation of their activity can be achieved at both transcriptional and post-translational levels by activation of latent zymogen forms (pro-MMP2 and 9). Moreover, their activities can be inhibited by endogenous inhibitors, including TIMPs (i.e., tissue inhibitors of MMP) (Lambert et al. 2004). Moreover, ECM components can modify MMP expression and activation, as recently outlined by lida and
collaborators (Iida et al. 2007). In this context, HA was shown to induce MMP activity in fibroblasts (Isnard et al. 2001), lung carcinoma cells (Zhang et al. 2002), and chondrocytes (Takahashi et al. 1999; Ohno et al. 2006).

HA also induces the migration of several cell types (Toole 2004), and recently it was reported that in SMCs, HA is involved in cell migration through CD44-mediated signaling (Vigetti et al. 2008). Based on these observations, it is possible that the reduction of HA levels could decrease CD44 signaling and reduce MMP activation. Therefore, 4-methylumbelliferone (4-MU), a modified coumarin (7-hydroxy-4-methylcoumarin) that is known to inhibit HA synthesis when added to cell cultures (Nakamura et al. 1995), is a useful tool. In vivo, liver rapidly catabolizes 4-MU, and when it is administered in mice, HA reduction is evident mainly in this tissue (Yoshihara et al. 2005).

The effect of 4-MU on MMPs is complex. It has been reported that 4-MU induces MMP2 activation in human skin fibroblasts (Nakamura et al. 2002) whereas it reduces MMP9 activation in human lymphoma cell lines (Nakamura et al. 2007).

Although the mechanisms of 4-MU action are complex, we hypothesize that its effect on HA synthesis may be mainly by downregulating the expression of HASs and by depleting the HA precursor UDP-GlcUA by activation of UDP-glucuronyltransferases (UGTs) (Kakizaki et al. 2004). UGTs are enzymes involved in the detoxification of many lipophilic xenobiotics and endobiotics. They are divided into two main families, named UGT1 and 2, that are expressed primarily in the gastrointestinal tract. UGTs catalyze the glucuronidation of a variety of compounds (including 4-MU) by using UDP-GlcUA as the donor substrate (Tukey and Strassburg 2000).

In this work, we report that HA synthesis by human aortic SMCs (AoSMCs) is inhibited by 4-MU and that its effect is mainly due to a downregulation of HAS2 expression. In addition, the finding that AoSMCs express UGT1A isozymes may explain the UDP-GlcUA depletion that we found in the cells, confirming that the availability of the UDP-sugar precursors may have an important role in regulating HA synthesis. Moreover, we report that 4-MU does not modify the activity of MMP2, but decreased dramatically SMCs migration, indicating that HA is a critical molecule for controlling cell movement in vitro by CD44 receptor signaling.

Results and discussion

4-MU inhibits HA synthesis of AoSMCs

We treated AoSMCs with 4-MU, a well-known inhibitor of HA synthesis already described for other cell types (Nakamura et al. 1995, 1997, 2002, 2007; Kosaki et al. 1999; Sohara et al. 2001; Kudo et al. 2004; Rilla et al. 2004; Morohashi et al. 2006). A clear dose-dependent decrease of HA released into the culture medium was confirmed in AoSMCs after 24 h of treatment with 0.5, 1, and 2 mM of 4-MU by means of PAGEFS (Figure 1A) and HPLC (Figure 1B) quantification methods. At these concentrations of 4-MU, we did not detect a significant reduction of the total amount of chondroitin sulfate (Figure 1A, ΔCS bands) although a slight alteration of chondroitin sulfate 4S/6S/8S ratio was appreciable. Under the same treatment conditions, we also quantified heparan sulfate on the cell surface as well as into the conditioned culture medium without finding any differences (results not shown) indicating that 4-MU is a specific inhibitor of HA synthesis as observed in a previous study (Nakamura et al. 1995). Moreover, we confirmed in AoSMCs that 4-MU also reduced (0.5 mM) and essentially eliminated (1 and 2 mM) the pericellular matrix of AoSMCs as shown in Figure 1C by hyaluronidase digestion before the addition of erythrocytes. Original magnification 40×. (D) Agarose gel electrophoresis of HA secreted in the AoSMC culture medium in untreated (0) and 48 h 4-MU-treated cells at the indicated concentration. To highlight HA secreted in the cell culture medium, some samples were digested with hyaluronidase prior to electrophoretic separation. HA size distributions were analyzed by 0.8% TAE-agarose gel electrophoresis stained with Stains-All. Lane M shows the separation of HA markers of defined mass (Hyalose).

Fig. 1. 4-MU effects on HA synthesis of AoSMCs. (A) PAGEFS analysis of the AoSMC culture medium in untreated (CNT) and 24 h treated cells with the indicated concentrations of 4-MU. ΔHA and ΔCS indicates HA and chondroitin disaccharides, respectively, while ΔCS4S and ΔCS6S indicate chondroitin 4 and 6 sulfate disaccharides, respectively. The arrow indicates an artifact due to AMAC degradation (Vigetti personal observation). (B) HPLC quantification of HA secreted into the AoSMC culture medium after 24 h treatment with 4-MU. Results are expressed as mean ± SE. ∗P < 0.001 control (CNT) versus 4-MU. (C) Particle exclusion assay performed on AoSMCs untreated (control) and after 24 h from 4-MU addition. To highlight the HA pericellular matrix, some cells were treated with Streptomyces hyaluronidase before the addition of erythrocytes. Original magnification 40×. (D) Agarose gel electrophoresis of HA secreted in the AoSMC culture medium in untreated (0) and 48 h 4-MU-treated cells at the indicated concentration. To highlight HA secreted in the cell culture medium, some samples were digested with hyaluronidase prior to electrophoretic separation. HA size distributions were analyzed by 0.8% TAE-agarose gel electrophoresis stained with Stains-All. Lane M shows the separation of HA markers of defined mass (Hyalose).
of HA. This issue is critical to verify that the 4-MU effects are actually due to a complete inhibition of HA synthesis and not to the presence of short HA oligosaccharides due to altered synthesis or degradation products.

The mechanism of 4-MU inhibition of HA is complex and involves changes in gene expression as well as an alteration in the cellular content of UDP-GlcUA (Kakizaki et al. 2004), a critical precursor of the synthesis of HA and other GAGs with the exception of keratan sulfate. In fact, it has been reported that UGTs, by using UDP-GlcUA can convert 4-MU to 4-MU-glucuronide (4-MUG) and compete with HASs for this UDP-sugar substrate (Kakizaki et al. 2004). To demonstrate whether 4-MU treatment could alter the UDP-GlcUA content, we measured the UDP-GlcUA:UDP-Glc ratio in untreated and in 1 mM 4-MU-treated AoSMCs by means of capillary zone electrophoresis and found the ratio value of 1.8 and 1.2, respectively. A quantitative analysis revealed that the UDP-GlcUA content in untreated cells and 1 mM 4-MU-treated cells was 1.3 nmol/cell and 0.8 nmol/cell (P = 0.016), respectively. These values clearly indicated that the 4-MU treatment reduced the cellular content of UDP-GlcUA. In a previous study on the same cells, we demonstrated that the depletion of UDP-GlcUA obtained by silencing UDPH inhibited HA release into the culture medium (Vigetti et al. 2006) indicating the critical role of UDP-sugar precursors in HA synthesis. The UDP-GlcUA reduction induced by 4-MU is previously described in rat epidermal keratinocytes (Jokela et al. 2008) and fibroblasts (Kakizaki et al. 2004) suggesting that this 4-MU effect can be generalized to other cell types.

The reduction in the UDP-GlcUA content could be mediated by UGTs. As these enzymes are typical of digestive tract (Tukey and Strassburg 2000), we asked whether they were expressed and active in AoSMCs. By RT-PCR and quantitative RT-PCR, we have shown that UGT1s, but not UGT2s, are expressed in our cells (Figure 2A and C). cDNA from a human liver carcinoma cell line (HepG2) was used as a positive control both for UGT1 and UGT2 RT-PCRs (result not shown). Among the UGT1 family, the most expressed isoforms are A3, A4, A5, A7, A8, and A10 (Figure 2B), whereas A1, A6, and A9 were not detected (result not shown). Interestingly, 4-MU treatment induced a slight increase in the expression of UGT1 (Figure 2A–C) even if not statistically significant. Notably, this result was obtained by using quantitative PCR with primers able to amplify all UGT1 isoforms while the semi-quantitative RT-PCR shown in Figure 2B highlighted a slight increase of UGT1A5 and A8 transcripts. Although isoform A4 was not active toward coumarins, all the other members of UGT1 family are known to have a strong activity toward such compounds (Tukey and Strassburg 2000) indicating that the 4-MU inhibitory effect in AoSMCs can be, at least in part, ascribed to a reduced HA precursor availability due to UGTs. To support this issue and to demonstrate the presence of an UGT activity in AoSMCs, we were able to identify the product of the UGT reaction with 4-MU (i.e., 4-MUG) in the medium of AoSMCs treated with 4-MU by means of HPLC (Figure 2D). To rule out the possibility that the 4-MU preparation could contain 4-MUG contaminations, we performed the experiments assessing the presence of 4-MUG in 4-MU-treated AoSMCs immediately after the 4-MU addition or after an incubation of 24 h. The presence of 4-MUG only in 24 h incubated samples indicates that the UGTs are not only transcribed, but also active and can compete with HASs for UDP-GlcUA. Under these separation conditions, the 4-MU peak appeared at the end of the runs at a retention time of about 50 min (result not shown). As UGTs have typical detoxification properties, it is noteworthy to find these enzymes in cells not belonging to the gastroenteric tract, where UGTs are abundantly expressed. However, UGTs have been found in the circulatory system of birds and fishes although their physiological role is still debated (Bond et al. 1980; Yang et al. 1986; Leaver et al. 2007).

As far as gene expression is concerned, we exposed AoSMCs to 0, 0.5, 1, and 2 mM 4-MU for 24 h, purified total RNA, used it to prepare cDNAs, and used quantitative RT-PCR to measure the expression of HAS2 transcript finding a clear dose-dependent reduction of its abundance (Figure 3A). This gene codes for the main HAS isoform and its 4-MU-dependent downregulation fits well with the HA reduction observed by using the same 4-MU concentrations (Figure 1). As 4-MU reduced HAS2 transcript also in rat fibroblasts (Kakizaki et al. 2004), this effect on gene expression may be considered common to other cell types. As AoSMCs treated with 1 mM 4-MU showed a significant reduction of HAS2 transcript, we also quantified the expression of other genes involved in HA synthesis under this condition. As shown in Figure 3B, 4-MU treatment decreased transcripts
HAS2 abrogation reduces HA synthesis in AoSMCs

AoSMCs express all of the three HAS isoforms (Vigetti et al. 2008). Although 4-MU reduced mainly HAS1 and HAS2 transcripts, we determined the contribution of each HAS isoform on HA synthesis in AoSMCs. By using siRNA approach, we silenced the three HASs and, after 48 h from siRNA transfections, we assayed the gene silencing efficiency by quantitative RT-PCR and quantified the amount of synthesized HA by HPLC. Figure 4A–C shows that the reduction of the transcripts was comparable, in fact their expression was decreased from about 80% for HAS1 and 3 mRNAs to about 85% for HAS2 messenger. As HASs show high degrees of similarity, in each case we also quantified the two HAS transcripts not subjected to siRNA and found only minor nonspecific and not statistically significant reduction of expression with respect to the controls (data not shown). The synthesis of HA was significantly reduced after HAS1, 2, and 3 siRNA treatments whereas it was not decreased after the transfection of a scramble siRNA (Figure 4D). These results indicated that all the three HASs contributed to HA production and their decrease after 4-MU treatment correlated well with the observed HA reduction. However, a major HA reduction was obtained by HAS2 silencing suggesting a pivotal role of this enzyme.

The crucial importance of HAS2 was highlighted by Camenisch and coworkers showing that HAS2 knockout mice died early during gestation by accumulating serious heart defects (Camenisch et al. 2000). In SMcs, HAS2 silencing by siRNA decreased cell proliferation (van den Boom et al. 2006). Moreover, HAS2 expression was modulated by pharmacological treatments (Sussmann et al. 2004), indicating a fine control of the expression of this enzyme. Moreover, HAS2 has a critical role in vascular diseases as demonstrated in transgenic mice that overexpress HAS2 (Chai et al. 2005). By using an in vitro aging model, we found HA accumulation and HAS2 overexpression in senescent AoSMCs (Vigetti et al. 2008) highlighting the crucial role of HAS2 in this particular cell type.

MMP2 activity is not affected by 4-MU in AoSMCs

MMP2 is a key enzyme in remodeling ECM (Newby 2006). By degrading a variety of substrates, including basement membranes, MMP2 increases cell migration (Vigetti et al. 2006) and, therefore, promotes the development of several vascular pathologies. Because it has been reported that 4-MU induces MMP2 activation in skin fibroblasts (Nakamura et al. 2002), we have studied whether such activation could be detected in AoSMCs. We treated the cells with 1 mM 4-MU and, by means of gelatin zymography, visualized the 72 kDa inactive pro-MMP2 as well as the 66 kDa active MMP2 bands. Previously, we demonstrated that AoSMCs did not have MMP-9 (Vigetti et al. 2006). No changes in bands intensities were found after 4, 6, and 24 h of incubation with 4-MU indicating that 4-MU was not able to induce MMP2 activation in AoSMCs. Similar results were obtained using 0.5 and 2 mM coding for HAS1, HAS3, UGPP, and UGDH whereas CD44 messenger was not affected. Interestingly, because 4-MU decreased HA synthesis, we added exogenous HMW-HA to 4-MU treated cells and compared their expression of UGPP, UGDH, HASes, and CD44 without finding any significant change (result not shown). These results indicate that the expression of all the genes involved in HA synthesis can be affected by 4-MU treatment. Previously, we reported that UGDH downregulation by siRNA reduced the HA production in AoSMCs (Vigetti et al. 2006). Further, the elevated expression of UGPP was associated with augmented HA accumulation in hypertrophic chondrocytes (Magee et al. 2001). These findings strongly suggest the presence of an unknown mechanism that coordinates the expression of the genes involved in HA synthesis probably by sensing the concentration of some crucial metabolites, including UDP-sugar precursors. As the treatment with 4-MU-reduced UDP-GlcUA content, we can speculate that this sugar nucleotide could be critical not only in HA synthesis but also directly or indirectly, at the nuclear level to control the transcription of specific genes. To support this hypothesis, we performed experiments demonstrating that an increase in UDP-GlcUA induced an upregulation of HAS2 and 3 transcripts in AoSMCs (Vigetti et al. 2006). Another possibility is UDP-N-acetylglucosamine (UDP-GlcNac). This sugar nucleotide was recently discovered to be critical for HA synthesis (Jokela et al. 2008) and, moreover, belongs to the hexosamine biosynthetic pathway that is known to control several cellular functions by O-GlcNac glycosylation (Hart et al. 2007). In our conditions, UDP-GlcNac slightly decreased after 4-MU treatments (data not shown) as previously reported in rat epidermal keratinocytes treated with 4-MU (Jokela et al. 2008) suggesting that also this latter control mechanism could have role in HA gene regulation even if this point will require more specific analyses.
4-MU inhibits HA synthesis in AoSMCs

We determined whether the inhibition of HA synthesis can reduce cell migration. AoSMC monolayers were scratched with a tip and either left untreated (control) or treated with different concentrations of 4-MU and the number of migrated cells measured after 6, 24, and 48 h of incubation. As shown in Figure 5A, 4-MU strongly reduced AoSMCs motility in a time- and dose-dependent fashion. As 4-MU decreased HA synthesis, we assessed whether exogenous added HA could rescue AoSMCs migration. As shown in Figure 5B, the addition of HMW-HA to 4-MU-treated cells restored the migration to levels similar to control AoSMCs whereas HMW-HA digested to disaccharides did not, indicating the specificity of the HMW-HA.

Because CD44 has a critical function in neointima formation, we determined whether exogenous HMW-HA could act through CD44. AoSMCs were treated with 4-MU and HMW-HA in the presence or absence of the monoclonal antibody BRIC295, which specifically inhibits the interaction between HA and CD44 (Liao et al. 1995). The treatment with the antibody prevented the migration of 4-MU+HMW-HA-treated cells.

Fig. 4. HASs silencing effect on HA synthesis. Quantification of transcripts coding for HAS1 (A), HAS2 (B), and HAS3 (C) in AoSMCs treated with siRNA against HAS1, HAS2, and HAS3 by quantitative RT-PCR, respectively. Cells were left untreated (control, black bars), transfected with 50 μM of scramble siRNA (scramble, dotted bars) or transfected with 50 μM of each HAS siRNA (HAS siRNA, white bars). The mRNA expression of each gene in untreated samples was set to 100%. β-Actin was used as a reference gene. Results are expressed as mean ± SE. *P < 0.003 control versus siRNA. (D) Quantification of HA secreted in the cell culture medium after siRNA transfections by HPLC. AoSMCs were left untreated (control), transfected with 50 μM of scramble siRNA (scramble) or transfected with 50 μM of each HAS siRNA and, after 48 h, HA was quantified. Data are expressed as mean ± SE. *P < 0.0001 control versus HAS siRNA.

4-MU concentrations (results not shown). We also quantified the MMP-2 secreted into the conditioned cell medium with a commercial ELISA kit. Untreated cells contained 14.6 ± 2.8 ng/mL whereas 1 mM 4-MU-treated cells contained 10.8 ± 1.1 ng/mL after 24 h of incubation, which was not statistically significant.

Because 4-MU reduced HA released into the culture medium, we added exogenous HMW-HA to 4-MU-treated samples and assayed MMP2 activity by gelatin zymography. No appreciable differences were evident after 4-MU as well as after 4-MU+HA treatments (results not shown). Quantification of MMP-2 activity by ELISA (15.7 ± 5.2 ng/mL) confirmed that the HA addi-
Fig. 5. 4-MU effects on AoSMCs migration. (A) Cell migration assays using scratch wounding. Confluent AoSMCs were growth arrested by serum deprivation and scratched with a blue tip. The cell culture medium was replaced with fresh SmGm2 medium with 0 (CNT, black bars), 0.5, 1, 2, and 4 mM 4-MU (white bars) and incubated for 6, 24, and 48 h. Cells were photographed under an inverted microscope immediately after wounding and at the reported time of incubation. Images were analyzed by using NIH Image software, and the numbers of migrated cells into the scratched areas were counted. Data are expressed as mean ± SE in three different experiments. *P < 0.05 versus CNT. (B) Cell migration was assayed as described above by treating the cells with 1 mM 4-MU, 25 μg/mL HMW-HA, 25 μg/mL digested HA, 4.8 μg/mL CD44 blocking antibody (BRIC235), and 4.8 μg/mL of a control antibody (anti-α-tubulin) after 24 h of incubation. Data are expressed as percentage with respect to untreated samples in three different experiments (*P < 0.05 versus untreated).

indicating that HMW-HA promoted cell movements through a mechanism involving CD44. On the other hand, the same treatment using an unrelated control antibody (against α-tubulin) did not show any change in cell migration.

As AoSMCs proliferation has a critical role in vascular diseases, we assessed whether 4-MU treatment could affect cell proliferation and used the CellTiter 96 AQueous kit (Promega) and trypan blue vital staining to quantify cell viability. As shown in Figure 6A, 4-MU inhibited the proliferation of AoSMCs in a dose-response fashion. Surprisingly, by counting live AoSMCs after 4-MU treatments, we found a significant decrement of viable cells only at high 4-MU concentrations (Figure 6B). The discrepancy between such two results could imply that the MTS assay used in the Promega’s kit is linked to the cellular reducing power and 4-MU could have modified mitochondrial respiration although no changes in pH were observed after 4-MU treatments (data not shown). Such results highlighted the possibility that the reduction of HA synthesis can also be ascribed to some cytotoxic effects that 4-MU has on AoSMCs besides the effects on the UDP-GlcUA concentration and HA metabolic enzymes expression. A similar 4-MU reduction in proliferation has also been observed in other cell types (Rilla et al. 2004; Mahaffey and Mummert 2007).

Further experiments confirmed the crucial role of HA to induce cell proliferation. As shown in Figure 6C, by adding exogenous HMW-HA to 4-MU-treated cells we observed an increase in AoSMCs viability. Similar data were obtained by counting live cells using trypan blue (results not shown).

As HA affects immune cells adhesion to SMCs (de la Motte et al. 2003; Majors et al. 2003) and this could be critical for atherogenesis, we investigated whether 4-MU treatment could influence the adhesiveness of monocytes on AoSMCs. As shown in Figure 6C, 4-MU reduced the number of adherent U937 monocytes on treated AoSMCs. As control, we treated AoSMCs with a well-known pro-inflammatory cytokine (IL-1β) and found a statistically significant increase in monocytes adhesion on treated cells. Interestingly, the AoSMCs–monocytes interaction was mediated by HA as the enzymatic digestion with hyaluronidase significantly reduced monocytes adhesion. Similar results were obtained by inhibiting HA synthesis with 4-MU. It is worth to note that the 4-MU without IL-1β stimulation reduced the monocytes adhesion more efficiently, indicating that the cytokine treatment affected the adhesiveness not only through HA but also inducing the expression of adhesion molecules such as VCAM and ICAM (Vigetti, unpublished results). These results could suggest an anti-inflammatory activity of 4-MU.

SMCs are decisive in neointima formation, and HA was shown to have a critical role to induce SMCs dedifferentiation and migration (Cuff et al. 2001). Therefore, a reduction of HA could diminish SMC activation and limit vessel thickening. In this work we provide evidence showing that inhibition of HA synthesis in AoSMCs is related with reduced cell mobility and viability, indicating that HA is critical to control AoSMCs migration and proliferation in an in vitro model.
system. Overall, these findings suggest that 4-MU could have new vasoprotective effects. The safety of 4-MU use in humans has already been evaluated, and it is used clinically in the treatment of hepatobiliary disease because it has cholangic activity (Fontaine et al. 1968; Stacchino et al. 1983). 4-MU is also used in therapies against Helicobacter pylori infections (Kawase et al. 2003), and in hepatitis B and C therapies (NIH phase II clinical trial no. NCT00225537). However, orally administered 4-MU is quickly removed from the bloodstream by the liver, which limits the bioavailability to the other human body compartments (Yoshihara et al. 2005). As one of the main problems in vascular surgery is restenosis after angioplasty, drug-eluting stents, which release sirolimus or paclitaxel, which arrest SMC cell cycle progression and migration, were introduced in clinical practice (Parry et al. 2005). In this context, by reducing SMC motility and viability, 4-MU could represent a new molecule with potential pharmacological effects in vivo.

Material and methods

Cell culture

Human aortic SMCs (AoSMCs) were purchased from Lonza and were grown for 2–6 passages in the complete SmGm2 culture medium (Lonza, Basel, CH) supplemented with 5% FBS. The cultures were maintained in an atmosphere of humidified 95% air/5% CO2 at 37°C. 4-MU was dissolved in DMSO. The final concentration of DMSO in the medium in both 4-MU-treated and control cultures was 0.1% as previously described (Nakamura et al. 1995). After preliminary experiments, a final concentration of 1 mM 4-MU was used. In some experiments, cells were grown in the presence of high-molecular-weight HA (HMW-HA, average 4 × 106 kDa) prepared by dilutions of the ultra pure surgical HA solution (Healon, GE Healthcare, Chalfont, UK).

Glycosaminoglycans determinations

HA and chondroitin sulfate released into the culture medium were quantified by polyacrylamide gel electrophoresis of fluorophore-labeled saccharides (PAGEFS) and by HPLC analyses as previously described (Karousou et al. 2004). Heparan sulfates were extracted from the culture medium and cells and quantified by HPLC (Viola et al. 2008).

Pericellular HA matrices were visualized by using a particle exclusion assay (Knudson W and Knudson CB 1991). Briefly, AoSMCs were incubated with 1 × 106/mL paraformaldehyde-fixed human red blood cells that were allowed to settle for 15 min, and then viewed by phase-contrast microscopy. The dependence of the pericellular matrix on HA was shown by treating the cultures with 2 units of HA-specific Streptomyces hyaluronidase (Seikagaku Corporation, Tokyo, Japan) in medium for 1 h at 37°C before visualization with the particle exclusion assay. To avoid hyaluronidase inhibition by serum components, in some control experiments, the cell culture medium was replaced with the fresh serum-free medium before enzyme addition. Representative cells were photographed at a magnification of ×40.

NHI Image software was used to quantify matrices and cellular areas (Simpson et al. 2002).

Agarose gel electrophoresis, as described by Lee and Cowman (1994), was used to assess the molecular weight distributions of synthesized GAGs after 4-MU treatments. Proteins were removed from culture media by proteinase K (Finnzyme, Espoo, Finland) digestion, and GAGs were precipitated by adding 4 volumes of ethanol at 4°C (Karousou et al. 2005). The presence of HA in cell culture media of some samples was determined by digestion with 20 μL of hyaluronidase SD (Seikagaku Corporation, Tokyo, Japan). The samples were then loaded on 0.8% TAE agarose gels. HA standard molecular weight markers were from Hyalose. Stains-all (Sigma, Milan, Italy) was used to visualize GAG distributions on the gels.

UDP-sugar determinations

To assess the cellular content of UDP-GlcUA, we extracted UDP-sugars from untreated and from 24 h AoSMCs treated with 1 mM 4-MU and quantified the sugar nucleotides by using capillary zone electrophoresis as previously reported (Vigetti et al. 2006) by means of pure standards (Sigma, Milan, Italy).

Gene expression determinations by RT-PCR

Total RNA samples were extracted from AoSMCs with Trizol (Invitrogen, Milan, Italy). For quantitative RT-PCR, RNA samples that were prior digested with Turbo DNase (Ambion, Monza, Italy) were retrotranscribed using the High Capacity cDNA synthesis kit (Applied Biosystems, Monza, Italy) and amplified on an ABI Prism 7000 instrument (Applied Biosystems, Monza, Italy) using the Taqman Universal PCR Master Mix (Applied Biosystems) following the manufacturer’s instructions. The following human TaqMan gene expression assays were used: HAS1 (Hs00155410_m1), HAS2 (Hs00193435_m1), HAS3 (Hs00193436_m1), UGDH (Hs0163365_m1), UGGP (Hs00198879_m1), CD44 (Hs00174139_m1), MMP-2 (Hs00234422_m1), TIMP-1 (Hs00171538_m1), TIMP-2 (Hs00234278_m1), MT1-MMP (Hs00237119_m1), and β-actin (Hs99999903_m1). The relative quantification of gene expression levels was determined by comparing ΔΔ Ct (Vigetti et al. 2006). For semi-quantitative RT-PCR analyses of UDP-glucuronyl transferases (UGTs), cDNAs were amplified using primers detecting all UGT1 as well as all UGT2 isoforms (Congiu et al. 2002) and by specific primers were able to discriminate each of the several UGT1 isoforms (Strassburg et al. 1997). The same primers detecting all isoforms of both UGT1 and UGT2 were also used for quantitative RT-PCR determinations with SYBR Green dye (Applied Biosystems, Monza, Italy) following the manufacturer’s instructions.

Analysis of 4-MU glucuronide (4-MUG) by HPLC

HPLC separation of 4-MU and 4-MUG was done using a Jasco-Borwin chromatograph system equipped with a MD-910 diode array multiwavelength detector. Chromatographic separation was done with a Nucleosil® C18 analytical column (4.6 mm i.d., 25 cm length; Macherey-Nagel, Düren, Germany) at room temperature, as described by Zimmerman et al. (1991), with minor modifications. The column was equilibrated with a KH2PO4 50 mM methanol solution (80/20; v/v), filtered through a 0.22 μm membrane filter. The flow rate was 1 mL/min. The separation was obtained under isocratic conditions detecting the absorbance at 313 nm. In order to determine the purity of peaks during preliminary separations, chromatographic runs were made by measuring the absorbance from 200 nm to 400 nm using the diode array scanning. From the absorbance profile, the

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maximum peak at 7 min was 313 nm according to a previous study (Zimmerman et al. 1991) and was identified as 4-MUG.

**Human HAS2 silencing**

Small interfering RNA (siRNA) was used to reduce the expression of human HASs in AoSMCs. HAS1 siRNA (ID116975, 5′-CCUCUCAUGGCUAGCUAUGtt-3′), HAS2 siRNA (ID117326, 5′-GCUGCUUAUAGUGUGCUAtt-3′), HAS3 siRNA (ID119476, 5′-CCUCUCCGUAUGCUAcAtt-3′), and negative control siRNA #1 kit (scramble, code 4611) were purchased from Ambion. The transfections were done using a Nucleofector apparatus (Lonza) and the Human AoSMC Nucleofector kit as previously described (Vigetti et al. 2006). After 48 h of incubation, conditioned cell media were assayed for HA contents by PAGEFS and HPLC analyses, and the silencing efficiency was quantified by quantitative RT-PCR measuring HASs transcripts.

**MMP2 activity**

MMP-2 activity was measured by gelatine zymography as previously described (Vigetti et al. 2006) as well as by the MMP2 ELISA kit (Calbiochem, Milan, Italy) following the manufacturer’s protocol.

**Cell mobility and viability assays**

Confluent AoSMCs were growth arrested by culture in DMEM with 0% FBS for 2 days. The cells were then scratched by blue tip (0 h) and then cultured in the complete SmGm2 medium in the absence or presence of several concentrations of 4-MU and, at different times of incubation, the scratched monolayer cultures were photographed using an inverted microscope (Olympus, Segrate, Italy). Quantification was performed by measuring the number of pixels in each wound closure area using the NIH Image, and data were expressed as number of migrated cells per mm². In another set of experiments, the migration of growth arrested AoSMCs were assayed after 24 h of incubation with 1 mM 4-MU, 25 μg/ml of HMW-HA, 4.8 μg/ml Anti-CD44 (BRIC235 from National Blood Service, UK) monoclonal antibody, and of the same amount of an unrelated isotype antibody used as a control (anti-α-tubulin monoclonal antibody, clone DM1A, Sigma-Aldrich). As a further control, HA was digested to disaccharides by adding 30 mU chondroitinase ABC (Seikagaku Corporation, Tokyo, Japan). After PBS washing, the numbers of adherent cells were assessed under a fluorescent microscope (Olympus, Segrate, Italy).

**Statistical analyses**

Unpaired Student’s t-tests were done for statistical analyses. Probability values of P < 0.05 were considered statistically significant. Experiments were done in triplicate, and data are expressed as mean ± standard error (SE).

**Funding**

FAR and Fondazione Comunitaria del Varesotto-ONLUS to DV, AP, MV, and GDL.

**Acknowledgements**

The authors wish to thank Dr. Paola Moretto for her help during cell culturing, Professor Stefano Banfi, Dott. Enrico Caruso, Pierangelo Borroni for 4-MUG analyses and Dr. Frances Spring (National Blood Service, UK) for Bric235 monoclonal antibodies. The authors gratefully acknowledge the “Centro Grandi Attrezzature per la Ricerca Biomedica” Università degli Studi dell’Insubria, for instruments facility.

**Conflict of interest statement**

None declared.

**Abbreviations**

- 4-MU, 4-methylumbelliferone
- 4-MUG, 4-methylumbelliferone glucuronide
- ΔCS-4S, chondroitin 4-sulfate disaccharide
- ΔCS-6S, chondroitin 6 sulfite disaccharide

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