A 340 kDa hyaluronic acid secreted by human vascular smooth muscle cells regulates their proliferation and migration

E.Papakonstantinou, G.Karakiulakis, O.Eickelberg 1, A.P.Perruchoud 1, L.-H.Block 2 and M.Roth 1,3

Department of Pharmacology, School of Medicine, Aristotle University, 54006 Thessaloniki, Greece, 1Department of Research, University Hospital of Basel, 4031 Basel, Switzerland and 2Department of Internal Medicine IV, University Hospital Vienna, 1091 Vienna, Austria

Received on December 3, 1997; revised on February 13, 1998; accepted on February 17, 1998

The formation of atherosclerotic lesions is characterized by invasion of vascular smooth muscle cells (VSMC) into the tunica intima of the arterial wall and subsequently by increased proliferation of VSMC, a process apparently restricted to the intimal layer of blood vessels. Both events are preceded by the pathological overexpression of several growth factors, such as platelet-derived growth factor (PDGF) which is a potent mitogen for VSMC and can induce their chemotaxis. PDGF is generally not expressed in the normal artery but it is upregulated in atherosclerotic lesions. We have previously shown that PDGF-BB specifically stimulates proliferating VSMC to secrete a 340 kDa hyaluronic acid (HA-340). Here, we present evidence regarding the biological functions of this glycan. We observed that HA-340 inhibited the PDGF-induced proliferation of human VSMC in a dose-dependent manner and enhanced the PDGF-dependent invasion of VSMC through a basement membrane barrier. These effects were abolished following treatment of HA-340 with hyaluronidase. The effect of HA-340 on the PDGF-dependent invasion of VSMC coincided with increased secretion of the 72-kDa type IV collagenase by VSMC and was completely blocked by GM6001, a hydroxamic acid inhibitor of matrix metalloproteinases. HA-340 did not exert any chemotactic potency, nor did it affect chemotaxis of VSMC along a PDGF gradient. In human atheromotic aortas, we found HA-340 expression with a negative concentration gradient from the tunica media to the tunica intima and the atherotic plaque. Our findings suggest that HA-340 may be linked to the pathogenesis of atherosclerosis, by modulating VSMC proliferation and invasion.

Key words: atherosclerosis/hyaluronic acid/migration/proliferation/vascular smooth muscle cells

Introduction

In human arteries, vascular smooth muscle cells (VSMC) are mainly located within the tunica media in a contractile state, expressing differentiation specific genes that are important for VSMC functions, such as regulation of vessel tone and blood pressure (Munro and Cotran, 1988; Tyberg et al., 1990; Ross, 1993). Under pathological conditions, such as vessel injury or atherosclerotic plaque development, VSMC become exposed to certain growth factors and cytokines, such as platelet-derived growth factor (PDGF), that induce a transformation from the contractile to a synthetic state (Ross et al., 1990; Ross, 1991, 1993; Jawien et al., 1992). Only in the latter state, VSMC acquire the ability to proliferate and accumulate within the intimal layer of blood vessels under the influence of chemotaxis-inducing growth factors. During migration, VSMC have been shown to digest major extracellular barriers such as basement membranes (BM), interstitial collagens, and proteoglycans (Sperti et al., 1992; Pauly et al., 1994). This enzymatic breakdown of extracellular matrix (ECM) components is mediated by the tightly regulated activity of proteases (Quigley et al., 1990). Within this group of proteases, the family of matrix metalloproteinases (MMPs) are essential for the digestion of ECM components such as collagens, gelatins, or proteoglycans (Matrisian, 1990; Woessner, 1991). As shown earlier, VSMC in vivo and in vitro predominantly secrete the 72 kDa type IV collagenase (MMP-2) (Galis et al., 1994), the expression of which can be stimulated by PDGF-BB (Roth et al., 1996a).

Extensive in vitro studies revealed that PDGF-BB stimulates the proliferation of VSMC, endothelial cells and fibroblasts (Majeski et al., 1990; Okazaki et al., 1992; Ross, 1993). However, recent data suggest that this growth factor is only a weak mitogen for VSMC in vivo (Ferns et al., 1991; Jawien et al., 1992; Jackson et al., 1993) but it induces their invasion from the tunica media into the tunica intima (Munro and Cotran, 1988; Ross, 1993). This in vivo effect of PDGF may be attributed to the enhanced expression of MMP-2 (Pauly et al., 1994; Roth et al., 1996a). We have recently reported that PDGF-BB specifically stimulates proliferating VSMC to secrete a 340 kDa hyaluronic acid (HA-340) (Papakonstantinou et al., 1995). In order to assess the biological functions of this glycan, we investigated its effects on proliferation and motility of human VSMC. We show that HA-340 significantly inhibits the PDGF-BB-induced VSMC proliferation and enhances their invasion through basement membrane (BM) along a PDGF-BB gradient by increasing the secretion of MMP-2. We also demonstrate that HA-340 is expressed with a negative concentration gradient from the tunica media to the tunica intima and the atheromatous plaque, indicating that HA-340 may modulate the state of dedifferentiation of VSMC associated with atherogenesis.

Results

Time course of the PDGF-induced VSMC proliferation and HA-340 secretion

We have previously shown that PDGF-BB stimulates proliferating VSMC to secrete HA-340. This glycan was isolated, purified and characterized from the culture media of human pulmonary VSMC which were collected at different time points (0, 12, 24, 36, 48, and 72 h) after pulse stimulation with PDGF-BB (10 ng/ml), as described previously (Papakonstantinou et al.,...
Fig. 1. PDGF-induced HA-340 secretion in relation to PDGF-induced de novo synthesis of DNA by human VSMC. Quiescent subconfluent VSMC (80% cell density, starved for 48 h at 0.1% FCS) were stimulated with a single pulse of PDGF-BB (10 ng/ml) in the presence of [3H]-thymidine and incubated at 37°C. The incorporation of the radioligand into newly synthesized DNA and the secreted amounts of HA-340 were determined, respectively, at the time points indicated. Unstimulated cells were used as controls for each time point. Results are presented as percentage of [3H]-thymidine incorporation or secretion of HA-340 over controls. Each data point represents the mean ± SE of triplicate determinations from three independent experiments performed using at least four different cell lines of human primary VSMC.

Effect of HA-340 on the spontaneous and the PDGF-BB-induced proliferation of VSMC

The addition of HA-340 (1 µg/ml) to quiescent, subconfluent VSMC cultures (80% cell density, starved for 48 h at 0.1% FCS) resulted after 36 h of incubation at 37°C in ~60% inhibition of the spontaneous VSMC proliferation, as indicated by the incorporation of [3H]-thymidine (Figure 2A). Pulse stimulation with PDGF-BB (10 ng/ml) induced, as expected, VSMC proliferation, which was ~600% after 36 h of incubation (Figure 2B). Under the same conditions, HA-340 significantly inhibited the PDGF-induced proliferation of human VSMC. Maximal inhibition of the PDGF-induced VSMC proliferation was obtained at 1.0 µg/ml of HA-340 (35 ± 6.5%; p < 0.001) (Figure 2B). The concentrations of HA-340 used are within the effective concentration range at which HA-340 can be measured in the culture media of PDGF-stimulated VSMC (Papakonstantinou et al., 1995).

Effect of HA-340 on the chemotaxis of VSMC

The chemotactic potency of HA-340 on VSMC was studied using a Boyden chamber system equipped with cell permeable filter membrane (pore size 8 µm). As expected, PDGF-BB (10 ng/ml) was a strong chemoattractant, forcing human VSMC to migrate through the filter membrane along its concentration gradient (Figure 3). HA-340, when added to the lower chamber compartment, caused a considerable increase in VSMC invasion, which HA-340 can be measured in the culture media of PDGF-stimulated VSMC (Papakonstantinou et al., 1995).

Effect of HA-340 on the invasion of VSMC through reconstituted BM

The effect of HA-340 on the spontaneous and the PDGF-induced VSMC invasion was studied using the Boyden chamber-system described above for the chemotaxis experiments, modified so that the filter membranes were precoated with reconstituted BM. As expected, PDGF-BB (10 ng/ml) significantly enhanced the PDGF-induced invasion of VSMC, in a dose-dependent manner, which was set to 100% (Figure 4). HA-340 (1 µg/ml) did not affect the spontaneous invasion of VSMC when added to the lower compartment. However, HA-340 significantly enhanced the PDGF-induced invasion of VSMC, in a dose-dependent manner, which was set to 100% (Figure 4). At the highest concentration of HA-340 used (1 µg/ml), the PDGF-induced invasion of VSMC was enhanced by 42 ± 5.7% (p < 0.001). The above described effect of HA-340 on the PDGF-induced invasion could not be observed when HA-340 was added to the lower compartment together with PDGF (Figure 4).
Hyaluronic acid modulates human VSMC differentiation

Fig. 3. Effect of HA-340 on the chemotaxis of VSMC. The effect of HA-340 on the spontaneous and the PDGF-induced chemotaxis of human primary VSMC was assessed using a modified Boyden chamber cell culture system. Cells (10^6/ml) were seeded into the upper compartment of the Boyden chamber and brought to quiescence (0.1% FCS, 48 h). Stimuli where then added to the lower compartment, as indicated. Chemotactic activity was assessed 24 h after the addition of each stimulus, as described under Materials and methods. PDGF-BB (10 ng/ml) -induced chemotaxis was regarded as 100%. Each bar represents the mean ± SE of triplicate determinations from two independent experiments using at least four different primary VSMC lines. Student’s t-test (unpaired, two sided) was used for statistical analysis. Control, 0.1% FCS.

Effects of HA-340 on the PDGF-induced VSMC proliferation and invasion following hyaluronidase treatment

HA-340 was treated with Streptomyces hyaluronidase (0.25 units per µg of HA-340), for 6 h at 60°C and enzyme activity was stopped by boiling for 5 min. Digestion of HA-340 completely abolished both: (1) the inhibitory effect of HA-340 on the PDGF-induced VSMC proliferation (Figure 5A) and (2) the stimulatory effect of HA-340 on the PDGF-dependent invasion of VSMC across an artificial BM (Figure 5B). Inactivated hyaluronidase (0.25 units, boiled for 5 min) did not affect the spontaneous or the PDGF-induced VSMC proliferation or invasion (data not shown). These results indicate that the oligosaccharides produced by hyaluronidase treatment of HA-340 do not retain the activity of the intact fraction. Furthermore, our data exclude the possibility of any contaminants copurified with HA-340 interfering with the effects attributed to this glycan.

Effect of HA-340 on the secretion of MMP-2 by VSMC

We tested the possibility that the stimulatory effect of HA-340 on the PDGF-dependent invasion of VSMC may be associated with enhanced degradation of the BM barrier by MMPs. The effect of HA-340 on the spontaneous and the PDGF-induced secretion of MMPs by VSMC was studied using gelatin zymography analysis. Unstimulated primary human VSMC (80% cell density, starved for 48 h at 0.1% FCS) secreted in the culture medium gelatinase activity with molecular mass corresponding to 72 kDa (Figure 6A, lane 1), indicating MMP-2 activity. The secretion of MMP-2 by VSMC could be significantly stimulated by PDGF-BB (10 ng/ml) (Figure 6A, lane 2, and Figure 6C). HA-340 enhanced, in a dose dependent manner, the spontaneous (Figure 6A, lanes 3 and 4; Figure 6B) and the PDGF-induced (Figure 6A, lanes 5 and 6; Figure 6C) secretion of MMP-2 by VSMC.

The effect of HA-340 on the PDGF-dependent invasion of VSMC is inhibited by the hydroxamic acid GM6001

GM6001 was shown to be a specific inhibitor of MMPs (Grobelny et al., 1992). Using gelatin zymography analysis we confirmed that GM6001, at 10^{-6} M, inhibits completely the activity of commercial, purified MMP-2 as well as VSMC-secreted MMP-2 (data not shown). The effect of GM6001 on VSMC invasion was studied using the modified Boyden chamber system, as described above. The stimulatory effect of HA-340 on the PDGF-dependent invasion of VSMC through a reconstituted BM towards PDGF-BB (10 ng/ml) (Figure 7Ac,7B), was significantly inhibited by GM6001 (10^{-6} M) (Figure 7Ad) in a dose dependent manner (Figure 7B), indicating that the effect of HA-340 on VSMC invasion requires MMP-2 activity. At the concentrations used, GM6001 did not affect VSMC viability, as defined by trypan blue exclusion (data not shown). Furthermore, VSMC growth determined by thymidine incorporation was unaffected by GM6001 at concentrations 10^{-8} to 10^{-6} M (data not shown). Thus, the inhibitory effect of GM6001 on HA-340-induced VSMC invasion could not be attributed to nonspecific cytotoxicity.

Differential distribution of HA-340 in the layers of human aortas

To assess the in vivo expression of HA-340 in human aortas, we separated the atheromatic plaque and the different layers (tunica
adventitia, tunica media, tunica intima) of human atheromatic aortas and determined the amount of HA-340 (µg glycan/g of tissue) in each vessel compartment. We found that, among other glycosaminoglycans (GAGs), HA is present as a single molecular size species in all described layers of human aortas. Biochemical characterization of the HA isolated from each layer revealed that it has an average molecular weight similar to the previously purified HA-340 from human VSMC (Papakonstantinou et al., 1995). The human aorta HA-340 was differentially distributed within the different layers of human aortas (Table I). The highest concentration of HA-340 was found in the tunica media (124.2 ± 24 µg/g tissue) with a notable decline in the neighboring compartments (tunica intima: 39.8 ± 6.8 µg/g tissue; tunica adventitia: 30.9 ± 5.2 µg/g tissue). Within the atheromatic plaque, the quantity of HA-340 (26.1 ± 3.9 µg/g tissue) was the lowest as compared to the other aortic compartments.
Table I. Distribution of HA-340 in the layers of atheromatic aortas

<table>
<thead>
<tr>
<th>[A] Vessel compartment</th>
<th>HA-340 (µg/g tissue; mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atheromatic plaque</td>
<td>26.1 ± 3.9</td>
</tr>
<tr>
<td>Tunica intima</td>
<td>39.8 ± 6.8</td>
</tr>
<tr>
<td>Tunica media</td>
<td>124.2 ± 24.0</td>
</tr>
<tr>
<td>Tunica adventitia</td>
<td>30.9 ± 5.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>[B] Vessel compartment</th>
<th>Tunica intima</th>
<th>Tunica media</th>
<th>Tunica adventitia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atheromatic plaque</td>
<td>0.039</td>
<td>0.0025</td>
<td>0.027</td>
</tr>
<tr>
<td>Tunica intima</td>
<td>—</td>
<td>0.0047</td>
<td>0.145</td>
</tr>
<tr>
<td>Tunica media</td>
<td>0.0047</td>
<td>—</td>
<td>0.003</td>
</tr>
</tbody>
</table>

[A], Quantitative analysis of HA-340 in isolated human aortic layers and in atheromatic plaques. Data represent mean ± SE of triplicate determinations from four individuals. [B], P values: statistical analysis (Student’s t-test, unpaired) comparing the expression of HA-340 between different vessel layers and the atheromatic plaque. P < 0.05 indicates statistically significant differences.

Discussion

GAGs are a group of complex macromolecules that are expressed in a wide range of tissues and compose an essential part of the ECM (Jackson et al., 1991; Knudson and Knudson, 1993). They have been shown to play a pivotal role in the formation of the ECM and thus directly contribute to the control of cell function (Jackson et al., 1991; Scott, 1992; Knudson and Knudson, 1993). For example, it has been shown that the interaction of free HA with cells modulates cell proliferation and migration (Laurent and Fraser, 1992). Furthermore, it has been shown that the initial phase of the ECM remodelling that occurs during the development of fibroproliferative lesions is characterized by elevated amounts of HA which could facilitate biological processes, such as cell migration (Riessen et al., 1996).

We have recently reported that PDGF-BB specifically stimulates proliferating VSMC to secrete a 340 kDa HA (Papakonstantinou et al., 1995). The PDGF-induced secretion of HA-340 was restricted to VSMC, since neither fibroblasts, epithelial, or endothelial cells upregulated the synthesis of HA-340 in response to PDGF (Papakonstantinou et al., 1995). In the present study, we demonstrated that HA-340 exerts a regulatory biological activity on proliferation and invasion of human VSMC. We found that synthesis and secretion of HA-340 is increased in proliferating VSMC. This observation is in agreement with the enhanced synthesis and secretion of HA into the pericellular microenvironment of proliferating cells which is generally believed to facilitate the process of mitosis and cell growth (Laurent and Fraser, 1992).

However, it has also been reported that when HA is added directly to mitogen-activated cells, it exerts antiproliferative effects on mesenchymal derived human cell types such as fibroblasts (Laurent and Fraser, 1992; Bodo et al., 1993; Mast et al., 1993). In our experiments, HA-340 significantly inhibited the PDGF-induced thymidine incorporation in human VSMC. Since the maximal PDGF-induced secretion of HA-340 that we observed occurred with a considerable time lag, as compared to the phase of maximal de novo DNA synthesis, we may conclude that VSMC in culture increase the synthesis of HA-340 in a late stage of the cell cycle and thereby mobilize antiproliferative mechanisms to counterbalance the effects of mitogenic growth factors.

One might hypothesize that, in a physiological environment, the synthesis and release of HA-340 functions as a regulatory mechanism to regain homeostasis and to counterbalance an ongoing state of cell proliferation.

The available data regarding the impact of GAGs and especially of HA on invasion of different human cell types, at present, remains conflicting. Depending on the cell type used, commercial HA has been reported to exert either a stimulatory effect on the invasion of glioma cell lines (Koochekpour et al., 1995) or an inhibitory effect on the invasion of macrophages (Levesque et al., 1994). Here, we present evidence that HA-340 did not affect the PDGF-induced chemotaxis of VSMC, but it significantly increased the PDGF-dependent invasion of VSMC through an artificial BM, towards PDGF.

Both the inhibitory effect of HA-340 on the PDGF-induced proliferation of VSMC and the stimulatory effect of HA-340 on the PDGF-dependent invasion of VSMC across an artificial BM were abolished following exhaustive digestion of HA-340 by Streptomyces hyaluronidase. These data indicate that the ensuing oligosaccharides do not retain the activity of the intact HA-340 preparation and furthermore exclude the possibility of interference by any contaminants copurified with HA-340. It remains to be clarified if the above described biological effects of HA-340 on VSMC are size dependent. Evidence in support of this theory are: (1) the fact that commercial HA with molecular mass of 225 kDa was about four times less potent than HA-340 on the above described biological effects on VSMC (unpublished observations) and (2) that the oligosaccharides produced after treatment of HA-340 with hyaluronidase did not have any effect on the PDGF-induced VSMC proliferation or invasion. The average molecular mass of HA-340, as calculated using HPLC gel filtration analysis, was 340 kDa but the fraction was spreading from 250 to 380 kDa, which is in agreement with the inherent polydispersity of HA preparations (Papakonstantinou et al., 1995). It is possible that endogenous mechanisms exist where by tightly controlled synthesis of HA species of particular molecular mass is employed as means of regulating cellular responses.

The stimulatory effect of HA-340 on the PDGF-dependent invasion of VSMC through an artificial BM appears to be mediated via proteolytic degradation of the BM barrier, since we observed that the enhanced invasion of VSMC in response to HA-340 coincides with an increased secretion by VSMC of MMP-2, which specifically degrades collagen type IV (Matri-sian, 1990; Woessner, 1991), the major component of BM (van der Rest and Garrone, 1991). This is supported by the fact that the hydroxamic acid analog GM6001, which is a well characterized and specific inhibitor of MMPs (Grobelny et al., 1992; Leppert et al., 1995), inhibited the effect of HA-340 on VSMC invasion.
in a dose dependent manner. The HA-340-induced MMP-2 proteolytic activity was evident in PDGF-stimulated cells as well as in unstimulated cells. In the latter case, although VSMC secreted increased amounts of MMP-2, they did not invade through the BM barrier due to the lack of a chemoattractant stimulus.

The inhibitory effect of HA-340 on the PDGF-induced proliferation of VSMC may be attributed to the binding of PDGF by the glycan since it has been reported that GAGs, in particular heparin, can bind to and sequester growth factors, such as PDGF (Ross, 1993). This possibility and in fact the molecular mechanism of action of HA-340 is currently being investigated. However, based on the available evidence HA-340 does not appear to act by binding to PDGF since (1) HA-340 could not inhibit the PDGF-induced chemotaxis or invasion of VSMC when added in the lower compartment of the Boyden chamber together with PDGF and (2) the PDGF-induced secretion of MMP-2 was further stimulated in the presence of HA-340.

HA is considered to be involved in some of the key events that lead to the formation of atherosclerotic lesions (Jackson et al., 1991) and changes in the expression pattern of HA during the development of atherosclerosis have been observed compared to nonatherosclerotic vessels (Wasty et al., 1993; Heickendorff et al., 1994; Levesque et al., 1994). In this context, our observation that HA-340 is expressed with a negative concentration gradient from the tunica media to the tunica intima to the atheromatous plaque in human atheromatous aortas is of great interest, particularly if one takes into account the in vivo effect of PDGF on human VSMC. Even though PDGF is an established in vitro mitogen, studies with infused PDGF, as well as studies with anti-PDGF antibodies, have shown that this molecule is only a weak mitogen for VSMC in the media of injured arteries (Ferns et al., 1991; Jawien et al., 1992; Jackson et al., 1993; reviewed by Schwartz et al., 1995). This apparent discrepancy for the weak mitogenic activity of PDGF in the media of arteries may be explained by our observations that: (1) HA inhibits the PDGF-induced proliferation of VSMC and (2) HA is found in highest concentration in the tunica media of the aorta.

During the progression of many vascular diseases, such as atherosclerosis, VSMC undergo a transition from a quiescent and differentiated state to a dedifferentiated, proliferating and motile phenotype (Ross, 1993). Relating the differential distribution of HA-340 in the human atheromatous aorta that we observed here and the above described in vivo effects of PDGF on human VSMC, one might postulate the following hypothesis. Vessel injury at the endothelial cell layer results in the release of growth factors, such as PDGF, which stimulates VSMC proliferation, chemotaxis, and invasion. HA-340, depending on its concentration and probably on the molecular weight predisposes VSMC and their microenvironment in a way that permits either the invasive process towards the chemoattractants or the proliferative process to be manifested. Thus, the relative abundance of HA-340 or related molecules in the tunica media functions to direct VSMC towards dedifferentiation, but still enables them to move in contractile locomotion and to rearrange their positions during conditions like shear stress. Proliferative responses are inhibited, although migratory responses are submitted and VSMC are directed to migrate from the tunica media into the tunica intima along chemotactic gradients. In the tunica intima and especially in the atheromatous plaque, the initially inhibitory signal of HA-340 on VSMC proliferation is diminished and the functional response of VSMC to PDGF is now directed towards proliferation. In conclusion, the PDGF-induced synthesis and secretion of HA-340 by VSMC and the ability of this glycan to inhibit VSMC proliferation and enhance VSMC invasion may represent a further example of the common concept, according to which mitogen-induced changes in the composition of the ECM serve as a key point of cell dedifferentiation (Lin and Bissell, 1993), which in the particular case may be linked to the pathogenesis of atherosclerosis.

Materials and methods
Cell cultures and characterization of VSMC
Primary cultures of human pulmonary VSMC were established as previously described (Nauck et al., 1997) and subcultured in Dulbecco’s minimal essential medium (DMEM, Seromed/Fako-lia) supplemented with 15% colostrum (Chemie Brunschwig), 15% fetal bovine serum (Gibco AG), 20 mM HEPES, and 8 mM L-glutamine (SeroMed). Subconfluent cultures of human VSMC were growth arrested by serum starvation (DMEM + 0.1% fetal bovine serum, 0.1% colostrum, 20 mM HEPES, and 8 mM L-glutamine) for 48 h, followed by stimulation with human recombinant PDGF-BB (10 ng/ml, Gibco AG). VSMC were characterized by immunohistochemistry using monoclonal antibodies specific to human VSMC (Boehringer Mannheim) as positive staining and monoclonal antibodies against factor VIII or keratin as negative staining (Boehringer Mannheim), as described earlier (Roth et al., 1996b; Nauck et al., 1997).

Biopsies of human aortas
Biopsies of human aortas (upper thoracic level and its adjacent part of the aortic arch), from male adults (n = 4, age 30–35 years) were obtained at autopsy within 6 h following death by accident and were kindly provided by the Department of Forensic Medicine, School of Medicine, Aristotle University of Thessaloniki, Greece. The medical history of the individuals was free from diseases, such as hypertension and diabetes, but autopsy revealed that there was significant plaque development in their aorta. The atheromatous plaque was dissected away and the vessels were separated in their respective layers: tunica intima (endothelium and subendothelial layer), tunica media, and tunica adventitia, weighed, and homogenized three times for 5 sec in 10 ml of ice-cold 100 mM Tris-HCl, pH 8.0, also containing 1 mM CaCl₂.

Fig. 7. (opposite) Effect of GM6001 on the HA-340-induced invasion of VSMC. Invasion of VSMC was assessed using a Boyden chamber system containing a cell-permeable filter membrane coated with reconstituted BM. Cells were seeded into the upper compartment of the Boyden-chamber and brought to quiescence (0.1% FCS, 48 h). Stimuli were then added to the upper or lower compartment, as indicated. Chambers were incubated for additional 24 h (37°C, 5% CO₂) before the filters were removed. Cells adherent to the upper side were removed and cells on the lower side of the membranes were fixed with 0.1 ml of 4% paraformaldehyde in PBS and stained with Hoechst dye for 2 min. Invasion was assessed by counting the cells that appeared on the lower side of the filters by immobilofluorescence microscopy. In all cases, three randomly selected areas (1 x 1 mm²) were counted per filter. (A) Immunofluorescent microscopy (field x100): (a) control/control; (b) control/PDGF; (c) HA-340/PDGF; (d) HA-340 + GM6001 (10⁻⁶ M)/PDGF. (B) Number of cells invaded to the lower side of the filter. Each bar represents the mean ± SE of triplicate determinations from three independent experiments using at least four different primary VSMC lines. Student’s t-test (unpaired, two sided) was used for statistical analysis; * indicates levels of statistical significance: ***, p < 0.001. Control, 0.1% FCS; PDGF-BB, 10 ng/ml; HA-340, 1 µg/ml.
Isolation and purification of HA-340

Total glycans were isolated and purified from cell culture medium of VSMC after stimulation with PDGF-BB (10 ng/ml), or from the above biopsy specimens, as previously described (Papakonstantinou et al., 1995). In brief, lipids were extracted with 4 volumes of 1:2 (volume/volume) chloroform/methanol as described by Svennerholm and Fredman (1980), thereby inactivating all hydrolytic enzymes. The mixture was centrifuged at 3200 × g for 20 min at 4°C and the organic solvents were removed from the resulting pellet by the addition of 10 ml of ethanol. The mixture was centrifuged at 3200 g for 20 min at 4°C, the supernatant was removed and the pellet was dried for 4 h at 40°C. The dried pellet was resuspended in 1 ml of 100 mM Tris HCl, pH 8.0, containing 1 mM CaCl₂ and subjected to protein digestion with 0.1 kallikrein units (KU) of pronase (protease from Streptomyces griseus, Calbiochem). The pronase solution was preincubated for 30 min at 60°C to eliminate any glycosidase activity. Digestion was carried out for 72 h at 60°C by adding equal amounts of pronase at 24 h intervals. The sample was then adjusted to 150 mM NaCl and 10 mM MgCl₂, and DNA digestion was accomplished by incubating the sample with 400 KU of DNase I (EC 3.1.21.1, Boehringer Mannheim) for 16 h at 37°C. The concentration of CaCl₂ was adjusted to 1 mM and the reaction was stopped by adding 0.1 KU of pronase for 24 h at 60°C. The pH was adjusted to 10.0–11.0 by adding 10 mM NaOH and glycans were subjected to elimination reaction in the presence of 1 M NaBH₄ for 16 h at 45°C (Roden et al., 1972). Samples were then neutralized with 50% (volume/volume) acetic acid. Glycans were separated from degradation products by gel filtration (Sephadex G-25 column, 0.6 × 25 cm) and eluted with 10 mM pyridine acetate, pH 5.0. Fractions of 0.5 ml were collected and analyzed for neutral hexoses (Shields and Burnett, 1960) and uronic acids (Bitter and Muir, 1962) content.

HPLC analysis

Fractionation of glycans was performed by HPLC (Bio-Rad) with a Bio-Gel TSK 40XL gel filtration column (300 × 7.8 mm). Elution was carried out with 7 mM Tris, pH 7.4, containing 200 mM NaCl at a flow rate of 0.3 ml/min. The apparent molecular weight of the glycans was estimated by using the following molecular weight markers: HA, 225 kDa (Sigma); chondroitin sulfates, 19.7–101.6 kDa (Sigma); and heparins (bovine intestinal mucosa), and heparin (all from Sigma) were treated with all the above mentioned GAG-degrading enzymes following appropriate incubation procedures. Substrates incubated separately with their respective buffers served as controls. Digestion was initially evaluated by PAGE as previously described (Papakonstantinou and Misevic, 1993) and then by HPLC gel filtration with continuous recording of the absorbance at 206 and 232 nm and of the refractive index. Digestion (percent) was calculated from the ratios of the area under the curve of enzyme treated substrates over the area under the curve of buffer treated substrates of (1) the absorbance at 206 nm, (2) the absorbance at 232 nm, and (3) the refractive index. All glycosaminoglycan-degrading enzymes used could completely digest their respective specific substrates. However, 3% of HA and 2% of chondroitin sulfate A could not be digested by their respective specific enzymes, which may be attributed to impurities.

Proliferation assay

Quiescent subconfluent VSMC (80% cell density starved for 48 h at 0.1% FCS) were pretreated for 2 h with various concentrations of HA-340. PDGF-BB (10 ng/ml) and [³H]-thymidine (0.5 µCi/ml) were then added simultaneously to the culture medium and mitogenic cell activation was assessed after 36 h of further incubation by measuring the amount of [³H]-thymidine incorporated into newly synthesized DNA, as described previously (Roth et al., 1996b). Unstimulated cells were used as controls for each time point.

Chemotaxis, migration, and invasion assays

Cells (10⁵ cells/ml) were seeded onto cell-permeable filters (pore-size 8 µm) of invasion chambers (Boyden-chamber-systems; Collaborative Biolabs, Becton Dickinson), and incubated for 24 h at 37°C prior to the addition of the stimuli. PDGF-BB (10 ng/ml) and/or various concentrations of HA-340 or HA (225 kDa, Sigma) were added either to the lower or the upper compartment. Chambers were incubated for additional 24 h (37°C, 5% CO₂) before the filters were removed. Cells adherent to the upper side were removed and cells on the lower side of the membranes were fixed with 0.1 ml of 4% paraformaldehyde in PBS and stained with Hoechst dye for 2 min. Chemotaxis or migration were assessed by counting the cells that appeared on the lower side of the filters by immunofluorescence microscopy (100×). In all cases, three randomly selected areas (1 × 1 mm²) were counted per filter. For the invasion studies, the same approach was used with the modification that the filters were precoated with a reconstituted BM (Matrigel, Collaborative Biolabs).

Characterization of HA-340 with GAG-degrading enzymes

Lyophilized HA-340 (10 µg of uronic acids) were incubated in a final volume of 20 µl as follows: (1) heparinase: samples were dissolved in 100 mM sodium acetate, pH 7.0, containing 3 mM CaCl₂ and incubated with 4 × 10⁴ units of heparin Iyase (EC 4.2.2.7, Flavobacterium heparinum, Seikagaku) for 15 h at 35°C; (2) heparitinase: samples were dissolved as above and incubated with 4 × 10⁴ units of heparan sulfate Iyase (EC 4.2.2.8, Flavobacterium heparinum, Seikagaku) for 16 h at 43°C; (3) chondroitinase ABC: samples were dissolved in 100 mM Tris HCl, pH 8.0, containing 50 mM sodium acetate and incubated with 5 × 10⁴ unit of chondroitin ABC Iyase (EC 4.2.2.4, Proteus vulgaris, Sigma) for 16 h at 37°C; (4) keratanase: samples were dissolved in 50 mM Tris HCl, pH 7.4, and incubated with 0.1 units of keratan sulfate endo-1,4,β-galactosidase (EC 3.2.10.3 Pseudomonas species, Sigma) for 16 h at 37°C; and (5) hyaluronidase: samples were dissolved in 20 mM sodium acetate, buffered with acetic acid to pH 5.0, and incubated with 5 units of hyaluronate Iyase (EC 4.2.2.1, Streptomyces hyalurolyticus, Sigma) for 6 h at 60°C. Incubation times and enzyme concentrations used were those required for the complete degradation of their respective standard substrates, as estimated by preliminary investigation. In this preliminary study, the standard GAGs (50 µg) chondroitin sulfate A (bovine trachea), chondroitin sulfate B (porcine skin), chondroitin sulfate C (shark cartilage), HA (bovine trachea), keratan sulfate (bovine cornea), heparan sulfate (bovine intestinal mucosa), and heparin (all from Sigma) were treated with all the above mentioned GAG-degrading enzymes following appropriate incubation procedures. Substrates incubated separately with their respective buffers served as controls. Digestion was initially evaluated by PAGE as previously described (Papakonstantinou and Misevic, 1993) and then by HPLC gel filtration with continuous recording of the absorbance at 206 and 232 nm and of the refractive index. Digestion (percent) was calculated from the ratios of the area under the curve of enzyme treated substrates over the area under the curve of buffer treated substrates of (1) the absorbance at 206 nm, (2) the absorbance at 232 nm, and (3) the refractive index. All glycosaminoglycan-degrading enzymes used could completely digest their respective specific substrates. However, 3% of HA and 2% of chondroitin sulfate A could not be digested by their respective specific enzymes, which may be attributed to impurities.
Gelatin zymography

The gelatinolytic activity of secreted MMP-2 was determined using gelatin zymography analysis under denaturing but nonreducing conditions, as described previously (Roth et al., 1996a). In brief, quiescent subconfluent cells were challenged with PDGF-BB (10 ng/ml), HA-340, HA (225 kDa, Sigma), or a combination of one of the glycans with PDGF. Cell culture media were collected at 24 h after addition of the respective stimuli, and aliquots (10 µl) were run on a 8% SDS/polyacrylamide gel containing 0.1% gelatin (25 mA, 2 h, room temperature). Gels were then equilibrated in 2.5% Triton X-100 for 1 h and subsequently incubated in enzyme buffer (50 mM Tris-HCl, pH 7.3, containing 200 mM NaCl, 5 mM CaCl₂, and 0.02% Brij 35) for 18 h at 37°C. Bands of enzymatic activity were visualized by negative staining with standard Coomassie brilliant blue dye solution. Molecular sizes of bands displaying enzymatic activity were characterized by comparison to prestained standard proteins (Bio-Rad) and to purified MMP-2 (Anaqua Trading, Wangen).

MMP-2 inhibitor

The ability of VSMC to invade reconstituted BM was also studied in the presence of the didepptide analog hydroxyacid, HONHCOCH₂CH(i-Bu)CO-L-Trp-NHMe (GM6001), a specific MMP inhibitor (Grobelny et al., 1992), which was a gift from Dr. David Leppert, Department of Research, Kantonsspital Basel. GM6001 was dissolved in PBS and sterilized by filtration. Cells were incubated for 30 min with 10⁻⁸ to 10⁻⁶ M GM6001 from Dr. David Leppert, Department of Research, Kantonsspital Basel. GM6001 was dissolved in PBS and sterilized by filtration. The ability of VSMC to invade reconstituted BM was also studied using gelatin zymography analysis under denaturing but nonreducing conditions, as described previously (Roth et al., 1996a). In brief, quiescent subconfluent cells were challenged with PDGF-BB (10 ng/ml), HA-340, HA (225 kDa, Sigma), or a combination of one of the glycans with PDGF. Cell culture media were collected at 24 h after addition of the respective stimuli, and aliquots (10 µl) were run on a 8% SDS/polyacrylamide gel containing 0.1% gelatin (25 mA, 2 h, room temperature). Gels were then equilibrated in 2.5% Triton X-100 for 1 h and subsequently incubated in enzyme buffer (50 mM Tris-HCl, pH 7.3, containing 200 mM NaCl, 5 mM CaCl₂, and 0.02% Brij 35) for 18 h at 37°C. Bands of enzymatic activity were visualized by negative staining with standard Coomassie brilliant blue dye solution. Molecular sizes of bands displaying enzymatic activity were characterized by comparison to prestained standard proteins (Bio-Rad) and to purified MMP-2 (Anaqua Trading, Wangen).

Statistical analysis

Means ± SE were calculated on the basis of at least four different VSMC cell lines and of all biopsy specimens obtained. Determinations were always made in triplicate. Statistical analysis of thymidine incorporation, invasion, chemotaxis, and HA-340 expression was performed using Student’s unpaired t-test.

Acknowledgments

Dr. E. Papakonstantinou was supported by a fellowship from the European Commission (ERBFMBICT-961785).

Abbreviations

BM, basement membrane; ECM, extracellular matrix; GAGs, glycosaminoglycans; HA, hyaluronic acid; HA-340, 340 kDa hyaluronic acid; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; VSMC, vascular smooth muscle cells.

References


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