Inhibition of Hyaluronan Synthesis Accelerates Murine Atherosclerosis: Novel Insights Into the Role of Hyaluronan Synthesis

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Background—Hyaluronan is thought to mediate neointimal hyperplasia but also vasoprotection as an integral component of the endothelial glycocalyx. The present study addressed for the first time the effects of long-term pharmacological inhibition of hyaluronan synthesis on vascular function and atherosclerosis.

Methods and Results—Four-week-old apolipoprotein E–deficient mice on a Western diet received orally an inhibitor of hyaluronan synthesis, 4-methylumbelliferone (4-MU; 10 mg/g body wt), resulting in 600 nmol/L 4-MU in plasma. As a result, aortic plaque burden was markedly increased at 25 weeks. Furthermore, acetylcholine-dependent relaxation of aortic rings was decreased and mean arterial blood pressure was increased in response to 4-MU. However, hyaluronan accelerated the hypertensive effect of 4-MU without inhibiting the proatherosclerotic effect. A photothrombosis model revealed a procoagulant state that was not due to increased platelet activation or increased thrombin activation as monitored by CD62P expression and the endogenous thrombin potential. Importantly, increased recruitment of macrophages to vascular lesions was detected after 2 and 21 weeks of 4-MU treatment by immunohistochimistry, by intravital microscopy, and in a peritonitis model. As a potential underlying mechanism, severe damage of the endothelial glycocalyx after 2 and 21 weeks of treatment with 4-MU was detected by electron microscopy of the innominate artery.

Conclusions—The data suggest that systemic inhibition of hyaluronan synthesis by 4-MU interferences with protective function of the endothelial glycocalyx, thereby facilitating leukocyte adhesion, subsequent inflammation, and progression of atherosclerosis. (Circulation. 2010;122:2313-2322.)

Key Words: atherosclerosis • inflammation • glycocalyx • hyaluronan

Hyaluronan is a ubiquitous constituent of the extracellular matrix. The synthesis is mediated through 3 hyaluronan synthase (HAS) isoforms (HAS1, HAS2, and HAS3) that assemble UDP-glucuronic acid and UDP-N-glucosamine at the plasma membrane, forming a high-molecular-weight glycosaminoglycan chain. Hyaluronan is unbranched and is not further modified, in contrast to sulfated glycosaminoglycans such as heparan sulfate. Extensive research on the hyaluronan system in tumor biology, reproductive biology, lung injury, and cardiovascular pathology has contributed to an understanding of the physiological and pathophysiological role of hyaluronan in vitro and in vivo.

In the healthy arterial vessel wall, hyaluronan is positioned at 2 strategic positions: the endothelial glycocalyx and the adventitia. However, during atherosclerosis, hyaluronan is produced by activated vascular smooth muscle cells (VSMC) in the neointima. Extensive evidence from studies on atherosclerosis and restenosis shows that hyaluronan promotes VSMC proliferation and migration and that hyaluronan accumulates during neointimal hyperplasia in association with proliferating smooth muscle cells. From these studies, it was hypothesized that interstitial hyaluronan in the plaque matrix will promote neointimal expansion because of the extracellular accumulation of hyaluronan and phenotypic activation of VSMC. These results may recommend the HAS enzymes as therapeutic targets to inhibit atherosclerosis and...
neointimal expansion. However, it must be taken into consideration that hyaluronan is an important constituent of the endothelial glycocalyx, which protects the vessel wall from leukocyte and platelet interactions and is crucial for endothelial function. Therefore, it might be assumed that hyaluronan has dual roles. Specifically, the apical hyaluronan-rich glycocalyx of endothelial cells (EC) may be vasoprotective, whereas the intimal VSMC-associated hyaluronan matrix may promote atherosclerosis.

The HAS2 knockout is lethal during embryonic development because of malformation of the heart and the atriocentral outflow tract. The HAS1 and HAS3 knockouts are apparently vital but have not been used for experimental studies in the cardiovascular field. Therefore, until now, direct experimental evidence has not been available on the question of whether inhibition of hyaluronan synthesis will affect and possibly reduce atherosclerosis.

In the cancer field, it has been demonstrated that pharmacological inhibition of hyaluronan synthesis can inhibit tumor progression dramatically in various tumor entities. In these studies, a prototypic small-molecule inhibitor of HAS synthesis, 4-methylumbelliferone (4-MU), was used. Because of these findings, the "hyaluronan system," including the synthases, hyaluronan receptors, and hyaluronidases, may indeed be developed into therapeutic strategies to treat cancer. Of note, 4-MU is already used in patients as a musculotropism smooth muscle relaxant to treat nonspecific abdominal pain and as a cholagogue (Cholspasmin). Furthermore, 4-MU is advertised in the United States and Europe as a prescription-free "neutraceutical" product (Heparvit) for cancer patients. 4-MU reduces the available intracellular pool of UDP-glucuronic acid because 4-MU itself serves as an acceptor molecule for glucuronic acid from UDP-glucuronic acid and thus inhibits hyaluronan synthesis irrespective of the HAS isoform involved. The reduction of UDP-glucuronic acid pools may, to a lesser extent, also affect the synthesis of other glycosaminoglycan chains at high concentrations of 4-MU.

The aim of the present study was to address for the first time the net effect of long-term pharmacological inhibition of hyaluronan synthesis on atherosclerosis with the use of oral 4-MU treatment in apolipoprotein E (apoE)–deficient mice. The findings will contribute significantly to knowledge about the vascular functions of hyaluronan. Furthermore, this study is relevant with respect to cardiovascular effects that might occur if hyaluronan synthesis inhibitors were indeed developed for cancer therapy.

Methods

Animals

Female apoE–/– mice (Taconic M&B, Lille Skensved, Denmark) received a Western diet (21% saturated fat and 0.15% cholesterol) or Western diet containing 4-MU (10 mg/g body wt) or hydralazine in the drinking water (500 µg/mL) to lower blood pressure or a combination of both drugs. Animals were treated for 2, 4, 11, or 21 weeks beginning at 4 weeks of age (Figure 1A). An additional group received 4-MU from 15 to 25 weeks. 4-MU was pelleted into the Western diet by ssniff (Soest, Germany). Mice were euthanized by asphyxiation at the end of treatment. All experiments were performed according to the guidelines for the use of experimental animals as given by Deutsches Tierschutzgesetz and according to the guidelines for the use of experimental animals as given by Deutsches Tierschutzgesetz.
Thrombosis

Thrombosis of the right carotid artery was induced by photochemical injury with the use of rose bengal and a green light laser as described. During the flow measurement, 3 parameters were defined: time to first occlusion, time to stable occlusion, and frequency of flow recovery. The first time the blood flow declined to zero was defined as time to first occlusion. A stable occlusion was defined as a complete cessation of blood flow, stable for at least 10 minutes. The frequency of flow recovery was determined between first and stable occlusion. An increase of blood flow to >0.09 mL/min was defined as an event of flow recovery.

Platelet Activation and Endogenous Thrombin Potential

Platelet activation was determined in platelet-rich plasma by fluorescence-activated cell sorter (FACS) analysis of CD62P as described. The extent of maximal platelet activation was determined after addition of convulxin to platelet-rich plasma. Endogenous thrombin potential was measured in platelet-poor plasma with the use of a modified thrombinoscope method. A detailed description of these methods is provided in the online-only Data Supplement.

Electron Microscopy of the Glycocalyx

Animals were perfused at 105 mm Hg with 0.3% ruthenium red solution according to Luft to preserve the glycocalyx. As control, hyaluronidase (Streptomyces hyalurolyticus; Sigma, St Louis, Mo) 20 IU/mL was infused before fixation, revealing only minute remnants of the glycocalyx. A limitation of the ruthenium red fixation is that the glycocalyx collapses almost completely onto the apical surface of EC, whereas other fixation methods may lead to a partial recovery of the native dimensions of the glycocalyx.

Analysis of the Inflammatory Response

Intraperitoneal macrophages were isolated 5 days after intraperitoneal injection of 3% thioglycolate as described previously, and the number of macrophages was determined. More than 90% of peritoneal cells were positive for F4/80 and Gr1 by FACS analysis. Intravital microscopy of the interaction of leukocytes with the endothelium of the left carotid artery was performed after injection of 0.04 mg/kg acridine orange, as described. The details of the experimental procedure are provided in the online-only Data Supplement.

Statistical Analysis

Data are presented as mean±SEM. Statistical significance of cumulative concentration-response curves was analyzed by 2-way repeated-measures ANOVA. Plaque score, quantitative image analysis, and hyaluronan secretion in endothelial cells were compared by 1-way ANOVA followed by Bonferroni posttest. The comparison between 2 groups was performed with unpaired t tests. A value of P<0.05 was considered significant. Data analysis was performed with the use of GraphPad Prism 5.0 software.

Detailed descriptions of methods are provided as the online-only Data Supplement.

Results

Increased Atherosclerosis in Response to 4-MU

ApoE-deficient mice were fed a Western diet plus or minus 4-MU beginning at 4 weeks of age and were euthanized at 8, 15, and 25 weeks to quantify the extent of atherosclerosis. Surprisingly, treatment of mice with 4-MU resulted in increased aortic plaque burden after 21 weeks of treatment (Figure 1B). A trend toward increased plaque burden was already observed at 15 weeks. Increased atherosclerosis was also evident at the aortic root (Figure 1C). Hyaluronan accumulation in the atherosclerotic plaques and hyaluronan plasma concentration as analyzed by hyaluronan affinity histochemistry and hyaluronan enzyme-linked immunosorbent assay, respectively, were reduced (Figure 1C through 1E). Both parameters partially recovered during the experimental period. 4-MU had the same effect when applied to apoE-deficient mice with advanced atherosclerosis from 15 to 25 weeks (Figure IB and IC in the online-only Data Supplement). Analysis of hyaluronan accumulation at the aortic root revealed inhibition of hyaluronan deposition to an extent similar to that described above (Figure 1) for the 21-week treatment period. Plaque morphology and composition at the aortic root were analyzed in apoE-deficient mice treated from 4 to 25 weeks of age. The amount of plaque collagen and the fibrillar arrangement as judged by Sirius red staining and birefringence analysis were not affected by 4-MU (Figure II in the online-only Data Supplement). The immunostaining for SM-α actin showed a trend toward increased amounts of VSMC or more differentiated VSMC in the luminal part of the atherosclerotic plaque in response to long-term 4-MU treatment.

Endothelial Function Is Impaired in ApoE−/− Mice Treated With 4-MU

To analyze whether the proatherosclerotic effect of 4-MU was associated with the development of endothelial dysfunction, the acetycholine-dependent relaxation of aortic rings was analyzed. Acetycholine-dependent relaxation was impaired after treatment with 4-MU, as indicated by a right shift of the concentration-response curve and increased EC50 concentrations (Figure 2A). In contrast, the response to the nitric oxide donor 5-nitroso-N-acetyl-D,L-penicillamine was not affected by 4-MU treatment, suggesting that the sensitivity of VSMC to nitric oxide was normal (Figure 2B). Another apparent effect of the 4-MU treatment was the increased maximal contractile response to phenylephrine (Figure 2C). Taken together, these data suggest impaired endothelial function and increased contractility of aortic rings in response to long-term inhibition of hyaluronan synthesis. In turn, measurement of arterial blood pressure revealed an increase from 110.5±3.8 mm Hg in controls to 122.9±2.2 mm Hg (n=6; P<0.05) in apoE-deficient mice treated with 4-MU. To analyze whether the hypertensive effect of 4-MU is causally involved in the proatherosclerotic effect, the vasodilator dihydralazine was used to blunt the increase in systolic blood pressure (Figure 3A). Importantly, 4-MU increased the plaque score independently of effects on blood pressure (Figure 3B).

Inhibition of Hyaluronan Synthesis Causes a Prothrombotic State

Because atherosclerosis and endothelial dysfunction increase the thromboembolic risk and because activation of platelets can itself promote progression of atherosclerosis, the thrombotic response was determined by photochemically induced injury to the carotid artery. Subsequently, the decrease of blood flow as a consequence of the formation of a platelet thrombus was measured. The time to stable occlusion of the
carotid artery was significantly shortened in mice treated with 4-MU, indicating accelerated thrombus formation (Figure 4A). In addition, the frequency of flow recovery representing the detachment of nearly occlusive thrombi was reduced after 4-MU treatment (Figure 4A). Thus, 4-MU caused a prothrombotic state in apoE-deficient mice. This prothrombotic response to 4-MU might be caused by increased platelet activation or by increased thrombin activation. A third cause might be facilitated or stronger interactions of platelets with the luminal surface because of the inhibition of hyaluronan synthesis. The basal platelet activation as evidenced by CD62P expression was measured by FACS and was not affected by 4-MU (Figure 4B). Furthermore, the CD62P expression by platelets activated with convulxin was decreased in apoE-deficient mice that received 4-MU (Figure 4C). Taken together, the results on platelets strongly suggest that systemic platelet activation is not involved in the prothrombotic response. Next, the endogenous thrombin potential was measured in the plasma of 4-MU-treated apoE-deficient mice as readout of total thrombin activity. No effect of 4-MU on thrombin activity was evident (Figure 4D). Because neither platelet activation nor thrombin activity was increased in response to inhibition of hyaluronan synthesis, it is concluded that inhibition of hyaluronan synthesis may have increased the interactions of platelets with the vascular wall. Therefore, the underlying mechanism for the vascular effects of 4-MU comprising (1) increased atherosclerosis, (2) increased blood pressure and impaired endothelial-dependent relaxation, and (3) a prothrombotic state may be loss or damage of the glycocalyx, which is critically dependent on the presence of hyaluronan.

4-MU Reduces the Glycocalyx

The hypothesis that disturbance of the glycocalyx was the underlying cause of the proatherosclerotic effects of 4-MU was addressed in further detail. For this purpose, the ultrastructure of the glycocalyx was analyzed after long-term treatment with 4-MU in myocardial capillaries, which are an established vascular bed to detect changes in the glycocalyx. The glycocalyx on the apical surface of EC was clearly detectable in controls (Figure 5A) and was indeed thinner and reduced after treatment with 4-MU (Figure 5B, 5D, and 5E). As a control, hyaluronidase-perfused hearts were analyzed and found to be nearly devoid of the glycocalyx (Figure 5C through 5E). If damage of the glycocalyx was indeed the underlying cause of the atherothrombotic effects of 4-MU, it must be postulated that the disturbance of the glycocalyx precedes the development of atherosclerosis and that large arteries prone to develop atherosclerosis were affected as well. Indeed, after 2 weeks of treatment with 4-MU, the glycocalyx of the innominate artery of apoE-
deficient mice was attenuated (Figure 6A). Furthermore, the capillary glycocalyx of the myocardium was also reduced after 2 weeks of treatment with 4-MU (Figure 6B). In addition, fluorescent hyaluronan staining of myocardial capillaries clearly demonstrated the loss of hyaluronan from the apical surface of the endothelium after 2 weeks of oral 4-MU application (Figure 6C).

4-MU Induces an Increased Inflammatory Response

Evidence is presented above that 4-MU interferes with the structure of the glycocalyx, which in turn might be responsible for the acceleration of atherosclerosis. To further define the pathways that may directly cause the progression of atherosclerosis as a consequence of the damaged glycocalyx, (1) endothelial permeability and lipid retention and (2) the inflammatory response were characterized. After long-term treatment with 4-MU, vascular permeability, as evidenced by the interstitial water content of the lungs, was not affected (Figure IIIA in the online-only Data Supplement). Furthermore, lipid retention in atherosclerotic plaques, as evidenced by Oil Red O and apoB-48, was not altered by long-term 4-MU treatment (Figure IIIB and IIIC in the online-only Data Supplement). Body weight, plasma cholesterol, and triglycerides showed a trend toward reduced values (Table). Therefore, neither enhanced lipid retention nor increased plasma lipids are causally involved in the proatherosclerotic effect of 4-MU. However, immunostaining of mac2 as indication for macrophage retention revealed significantly increased amounts of macrophages in plaques of the aortic root both after long-term treatment (Figure 7A) and after 2 weeks (Figure 7B) of treatment with 4-MU. Furthermore, intravital microscopy showed that 2 weeks of 4-MU treatment, which was effective in damaging the glycocalyx (Figure 6), increased adhesion of leukocytes at the endothelium of the carotid artery (Figure 7C). To investigate whether the augmented macrophage response is restricted to atherosclerosis or also occurs in an acute model of aseptic inflammation, macrophage recruitment in response to intraperitoneal injection of thioglycolate was quantified and found to be increased as well (Figure 7D). Thus, the present data show that perturbation of the glycocalyx and recruitment of leukocytes to vascular lesions occurred after 2 weeks of treatment and therefore clearly preceded the development of atherosclerosis.
Inhibition of Hyaluronan Synthesis in EC

If the conclusion is correct that 4-MU inhibits EC glycocalyx in vivo to trigger platelet adhesion and, importantly, inflammation as underlying causes of atheroprogession, hyaluronan synthesis by EC should be inhibited by the achieved plasma concentration of 4-MU. Therefore, after 2 weeks of treatment 4-MU plasma concentration was determined to be 640.3 ± 17.2 nmol/L (n = 12) by liquid chromatography–tandem mass spectrometry, and, subsequently, the effect of this concentration was determined on human coronary EC in vitro. Interestingly, 4-MU (600 nmol/L) inhibited hyaluronan secretion (77.7 ± 4.1% of untreated controls; Figure IV in the online-only Data Supplement). As a positive control, a high dose of 4-MU (300 μmol/L), which is commonly used in vitro, reduced endothelial hyaluronan secretion by half. Visualisation of the glycocalyx of the EC in culture was not performed because it has been shown in vitro under static and flow conditions that the endothelial glycocalyx is minute and not of hemodynamic relevance. Therefore, it is concluded that hyaluronan synthesis of EC is sensitive to the concentration of 4-MU achieved by oral application of 4-MU in mice.

Neointimal Hyperplasia

Next, the ligation of the carotid artery was used to rapidly induce a VSMC-rich vascular lesion, as described previously. Importantly, in this model 4-MU had no accelerating effect on neointimal hyperplasia as determined as neointimal area and intimal/media ratio 4 weeks (Figure 8) and 2 weeks after ligation (data not shown). Therefore, the aggravating effect of 4-MU is specific for atherosclerosis and does not apply to vascular lesions driven mainly by VSMC proliferation and migration.

Discussion

Hyaluronan synthesis and hyaluronan-degrading enzymes are thought to have potential as drug targets for treatment of cancer and possibly for cardiovascular disease as well. The present study is the first to investigate the manner in which inhibition of hyaluronan synthesis will affect the adult cardiovascular system with the use of a murine model of atherosclerosis. As a result, it is clearly shown that systemic inhibition of hyaluronan synthesis during the course of atherosclerosis increases plaque burden, likely because of disturbance of the endothelial glycocalyx and subsequently increased recruitment of macrophages to atherosclerotic lesions.

Application of the inhibitor of hyaluronan synthesis, 4-MU, caused 4 major effects on the vascular biology and pathology: (1) endothelial dysfunction and increased blood pressure, (2) increased thrombotic response, (3) increased inflammatory responses, and (4) increased atherosclerosis. In the investigation of the underlying mechanism, it was first addressed whether the hypertensive response to 4-MU was involved in the proatherosclerotic effect. However, because hydralazine effectively blunted the hypertensive effect of 4-MU but did not abolish the increase of plaque burden, the increase of blood pressure is unlikely to contribute significantly. Second, the interaction of activated platelets with the endothelium promotes atherosclerosis because of release of growth factors and thrombin and the incorporation of platelet thrombi into the lesions. This was also shown before in
apoE-deficient mice by inhibition of platelet adhesion (eg, by knockout of glycoprotein IIb integrin and subsequent inhibition of atheroprogression). Furthermore, increased platelet adhesion is also critical for the increased leukocyte recruitment to vascular lesions in apoE-deficient mice. Therefore, the prothrombotic response was considered as a potential mechanism contributing to the proatherogenic effect of 4-MU and was analyzed in further detail. However, the possibility that 4-MU itself activated platelets or the coagulation system was excluded on the basis of CD62P expression and endogenous thrombin potential. Therefore, it is likely that instead the interaction of platelets with the vascular wall was facilitated because of treatment with 4-MU. The endothelial glycocalyx is a network composed of hyaluronan, membrane glycoproteins, heparan sulfate proteoglycans such as syndecan-1, and bound plasma proteins. Both hyaluronan and heparan sulfate are essential for the integrity and function of the glycocalyx, as evidenced by enzymatic removal of these glycosaminoglycans. The dimension of the glycocalyx is variable depending on the type and location of the blood vessels (200 to 500 nm) but clearly exceeds the length of endothelial membrane proteins extending into the lumen. Therefore, the glycocalyx is thought to sterically impair the interaction of leukocytes and platelets to adhesion receptors and was analyzed in further detail. However, the possibility that 4-MU itself activated platelets or the coagulation system was excluded on the basis of CD62P expression and endogenous thrombin potential. Therefore, it is likely that instead the interaction of platelets with the vascular wall was facilitated because of treatment with 4-MU. The endothelial glycocalyx is a network composed of hyaluronan, membrane glycoproteins, heparan sulfate proteoglycans such as syndecan-1, and bound plasma proteins. Both hyaluronan and heparan sulfate are essential for the integrity and function of the glycocalyx, as evidenced by enzymatic removal of these glycosaminoglycans. The dimension of the glycocalyx is variable depending on the type and location of the blood vessels (200 to 500 nm) but clearly exceeds the length of endothelial membrane proteins extending into the lumen. Therefore, the glycocalyx is thought to sterically impair the interaction of leukocytes and platelets to adhesion receptors

Table. Body Weight and Plasma Analysis

<table>
<thead>
<tr>
<th></th>
<th>Weight, g</th>
<th>Cholesterol, mg/dL</th>
<th>Triglycerides, mg/dL</th>
<th>hsCRP, ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=5)</td>
<td>28.4±1.1</td>
<td>541.3±24.21</td>
<td>210.7±16.6</td>
<td>25.9±1.8</td>
</tr>
<tr>
<td>4-MU (n=5)</td>
<td>26.4±0.8</td>
<td>490.6±55.9</td>
<td>143.0±29.9</td>
<td>25.5±3.3</td>
</tr>
</tbody>
</table>

Values are mean±SEM. ApoE-deficient mice were treated for 21 weeks with 4-MU according to the experimental design in Figure 1. Subsequently, body weight, plasma cholesterol, plasma triglycerides, and high-sensitivity C-reactive protein (hsCRP) were determined.
such as intercellular adhesion molecule, vascular cell adhesion molecule, or von Willebrand factor. Thus, it was considered in the present study that 4-MU interferes with the structural and functional integrity of the glycocalyx. After ruthenium red fixation, the glycocalyx was analyzed by electron microscopy and was indeed found to be reduced after 2 weeks and 21 weeks of 4-MU treatment in both capillaries and the innominate artery. Thus, a defect of the glycocalyx was already established early after application of the drug and therefore preceded the development of the prothrombotic phenotype and atherosclerosis. Furthermore, hyaluronan synthesis in cultured EC was effectively inhibited by the plasma concentration of 4-MU that was reached by oral 4-MU application in the present study. This finding supports the current working hypothesis that oral administration of 4-MU has adverse vascular effects based on the inhibition of the endothelial glycocalyx.

Another major finding was increased macrophage content in atherosclerotic lesions after 4-MU application, which was considered a potentially important mechanism causing the proatherosclerotic effect of 4-MU. The assumption of steric hindrance of interactions between leukocyte and EC through the glycocalyx is supported by the fact that adhesion of leukocytes to the endothelium increases on the enzymatic removal of the glycocalyx. This finding was complemented in the present study by increased macrophage retention in vascular lesions after short-term and long-term treatment with 4-MU. Furthermore, 2-week treatment with 4-MU also increased the number of macrophages that were recruited in an acute model of inflammation. In support of this, increased interactions between leukocytes and the endothelium were detected in 4-MU-treated apoE-deficient mice by intravital microscopy in the present study. The data therefore suggest that adhesion and subsequent transmigration of macrophages at sites of inflammation are increased because of the perturbed glycocalyx in response to 4-MU and that this effect is not restricted to atherosclerosis, as evidenced by the peritonitis model. Recent evidence supports the conclusion that disturbance of the glycocalyx accelerates atherosclerosis. In apoE*3-Leiden mice, the glycocalyx was found to be decreased both in response to an atherogenic diet and at lesion-prone locations in the vascular tree. Furthermore, the thickness of the glycocalyx was negatively correlated with intimal-medial thickening. In human type 1 diabetes mellitus, it was observed that the degree of disturbance of the glycocalyx and the release of hyaluronan into the plasma were increased compared with in nondiabetic controls and were positively correlated with carotid artery intima-media thickness.

As an alternative mechanism, increased endothelial permeability was considered. However, no evidence for tissue edema and increased lipid retention was detected. It is likely that residual amounts of hyaluronan and the heparan sulfate proteoglycans maintain endothelial function sufficient to prevent endothelial leakage.

On the basis of the present results, the inhibition of endothelial hyaluronan synthesis and subsequent perturbation of the endothelial glycocalyx likely are the underlying mechanisms of the prothrombotic and proinflammatory state in apoE-deficient mice receiving 4-MU. Importantly, this hypothesis was strongly supported by the fact that attenuated glycocalyx was detected in microvessels and macrovessels after 2 weeks of treatment with 4-MU and that hyaluronan secretion of cultured EC is decreased in response to the actual
plasma concentrations of 4-MU. In turn, the strong proinflammatory effect may represent the major effector mechanism that aggravates atheroprogession in this model. Apparently, disturbance of the glycocalyx overrides the inhibition of the synthetic VSMC phenotype, which could have been expected as a result of inhibition of neointimal hyaluronan synthesis. This conclusion is further supported by the present finding that in a model of VSMC-driven neointimal hyperplasia, 4-MU had no accelerating effect.

The present study provides novel information about the effect of systemic inhibition of hyaluronan synthesis on the progression of atherosclerosis and vascular homeostasis. The results suggest that systemic inhibition of hyaluronan synthesis is unfavorable and supports the conclusion that inhibition of hyaluronan synthesis, if attempted, should be targeted locally (eg, during exploration as antineoplastic treatment strategies). Importantly, cardiovascular side effects should be considered during the future development of hyaluronan synthesis inhibitors for therapeutic interventions.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Hyaluronan is an integral extracellular matrix component that plays crucial roles in, for example, development and homeostasis of cartilage and skin. However, increased hyaluronan production is associated with tumor progression and vascular disease. Hyaluronan accumulates during neointimal thickening in atherosclerotic plaques and restenotic lesions. In the neointima, it contributes to volume expansion and supports the proliferative and secretory phenotype of vascular smooth muscle cells. Therefore, inhibition of hyaluronan synthesis has been considered as a strategy to limit neointimal thickening and atheroprediction. On the other hand, recent research has established hyaluronan on the luminal surface of vascular endothelial cells to be a critical constituent of the endothelial glycocalyx, which has strong vasoprotective functions. In the present study, it is shown in a murine model of atherosclerosis that inhibition of hyaluronan synthesis by an oral hyaluronan synthesis inhibitor surprisingly enhances inflammatory and thrombotic responses and in the long term increases atherosclerosis. This adverse effect was attributed to a partial loss of the endothelial glycocalyx. Of note, hyaluronan synthesis inhibitors are effective in inhibiting tumor progression in mouse models and may be tested clinically to enhance the response to antitumor strategies. In light of the present results, it may be crucial to avoid adverse effects on the endothelial glycocalyx because damage of the glycocalyx may lead to increased atherothrombotic risk and enhance inflammatory cell recruitment.
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SUPPLEMENTAL MATERIAL
ONLINE MATERIAL & METHODS

Analysis of atherosclerosis and plaque composition

Tissue processing and fixation

The heart and entire aorta were fixed in 4 % neutral buffered paraformaldehyde for 24 hours and subsequently transferred into 20 % sucrose in PBS solution. Hearts were frozen in tissue freezing medium (Leica Nussloch, Bensheim, Germany) in liquid isopentane at -40°C and 14 μm cryosections of the aortic root were prepared for immunohistochemical staining.

Plaque burden of the aorta and the aortic root

Atherosclerotic plaques were visualized by Oil-red-O staining of lipid deposits. Subsequently images of en face preparations of the whole aorta and the aortic root were taken and the percentage of plaques in relation to the entire aortic surface accordingly to the aortic root calculated as plaque score in percent of total area using ImageJ 1.37v software (NIH).

Immunohistochemistry

Smooth muscle cells (SMC) were stained with a monoclonal mouse anti-α-SM-actin antibody (clone 1A4, 1:1000, Sigma, Deisenhofen, Germany). Secondary antibody to detect anti-α-SM-actin was obtained from Santa Cruz (Santa Cruz, USA). Affinityhistochemistry of hyaluronan was performed with HA binding protein, detected with FITC-labeled streptavidine (2 μg/ml, Calbiochem, Bad Soden, Germany). Detection was performed using diaminobenzidine (Zytomed, Berlin, Germany) as a chromogen. Macrophages were detected by an antibody against mac2 (1:400, Cedarlane, Burlington, Canada) and a Rhodamine Red-X conjugated goat anti-rat IgG (preabsorbed to rodent, 1:400, Jackson ImmunoResearch, Suffolk, UK) was used as a secondary antibody. Apo B48 was detected using K23300R (BioDesign, Saco, ME, 1:50) recognizing mouse apo B48 and human apo B100.
Histochemistry

Cryosections of the aortic root were stained for collagen accumulation, which was detected by picrosirius red staining. Qualitative analysis of collagen deposition was performed using polarized light microscopy and birefringence analysis. In addition, lipid staining of cryosections was performed using Oil-red-O.

Blood pressure measurement and endothelial function

Systolic blood pressure (SBP) was measured by a non-invasive computerised tail cuff system (Visitech System BP-2000, Apex, USA) as described previously. Endothelial function was determined by acetylcholine dependent relaxation of aortic rings in organ bath experiments as described previously. Briefly, 5 mm segments of the thoracic aorta were repeatedly depolarised with 80 mM KCl. Submaximal pre-contraction of aortic rings was carried out with 200 nM phenylephrine. Endothelium-dependent relaxation was assessed by cumulative administration of acetylcholine from $10^{-9}$ to $10^{-5}$ M. Endothelium-independent relaxation to increasing concentrations of the NO-Donor S-Nitroso-N-acetyl-D,L-penicillamin (SNAP) ($10^{-10}$ – $10^{-5}$ M) was examined after pre-contraction with 10 µM phenylephrine.

Plasma analysis

Blood was collected by heart puncture and anti-coagulated with 100 mM EDTA in isotonic sodium chloride solution. Plasma was prepared via centrifugation at 850 x g for 15 min at 4ºC and stored at −20ºC for later analysis. Total cholesterol and triglycerides were subsequently quantified using the enzymatic in vitro tests Fluitest® TG and Fluitest® CHOL (Analyticon Biotecnologies, Lichtenfels, Germany). High sensitive C-reactive protein (hsCRP) was quantitatively determined by a highly sensitive two-site enzyme-linked immunoassay (mouse hsCRP ELISA, K-Assay®, Kamiya Biomedical Company, Seattle, USA). Hyaluronan was
determined by a sandwich protein binding assay from Corgenix (HA-Test Kit, Corgenix, Colorado, USA).

Platelet activation and endogenous thrombin potential

Platelet-rich plasma (PRP) was prepared by centrifugation (3,000 rpm, 40 seconds) and percentage of platelets was determined by the PE-conjugated rat-anti-mouse anti CD41 antibody (BD Pharmigen) using a Cytomics FC 500 Cytometer (Beckmann Coulter). PRP contained 98-99 % CD41 positive particles. Subsequently, platelet activation was measured by expression of P-selectin (CD62P) as determined by FITC-conjugated rat-anti-mouse anti CD62P antibody (BD Pharmigen) and the respective FITC-conjugated isotypic control (mouse IgG1-FITC, Beckman Coulter) as previously described. PRP was pre-incubated with 5 µg/ml convulxin (Alexis) to determine changes in maximal platelet activation. Analysis was performed using CXP Analysis Software 2.2. Endogenous thrombin potential (ETP) was measured in platelet-poor plasma (PPP) using a modified thrombinoscope method. Briefly, PPP (15 µl), platelet membranes (10 µl), 1 pmol innovin as source of tissue factor, PBS (55 µl) and recalcification buffer (20 µl, 20 mM HEPES, pH 7.35, BSA 60 mg/ml and 100 mM Ca²⁺ (final concentration)) containing the fluorogenic substrate (ZGly-Gly-Arg-7-amino-4-methylcoumarin, Bachem) were mixed and thrombin generation was monitored for 60 minutes using a Thrombinoscope (Fluoroskan Ascent, Thermo Electron Corporation). Resulting curves were analyzed using Thrombinoscope Analysis 3.0 software. The measurement of the ETP represents the kinetics and the total amount of thrombin activation in a given sample.

Electron microscopy of the glycocalyx

To preserve the glycocalyx animals were perfused at 105 mmHg with a 0.3 % ruthenium red solution according to Luft for 30 min and post fixed with the same fixation solution (1h).
Subsequently pieces were washed in 0.1 M cacodylate buffer for 30 min and stored at 4° C in the same solution overnight. Post-fixation was performed at room temperature for 3 h using 4 % osmiumtetroxide, 0.5 M cacodylate buffer (CB) and 0.3 % ruthenium red. Subsequently, tissue pieces were washed with 0.1 M CB. After dehydration of tissues using increasing ethanol concentrations (30 %, 50 %, 70 %, 80 %, 90 % and 96 %, 60 min each) pure ethanol was applied (3 x 10 min) followed by propylenoxide (3 x 20 min). Subsequently tissues were embedded in EPON® (Polysciences) and EPON® blocs were cut on a Reichert-Jung Ultracut E® ultra microtome set to a thickness of 80 nm. Sections were then mounted on 200 mesh hexagonal copper grids and treated with 1 % uranyl acetate (5 min) and 0.4 % lead citrate (3 min). A Zeiss transmission electron microscope (EM 902A) was used for final investigation at 80 KV at magnifications from 3,000 to 140,000 x. Digital image acquisition was performed by a MegaViewII slow-scan-CCD camera connected to a PC running ITEM® 5.0 software (Soft-imaging-systems); images were stored as uncompressed TIFF files in 16 bits of gray. The glycocalyx was analyzed in 3 animals for each condition in case of the 25 week time point and in 5-6 animals per treatment for the 6 week time point. Subsequently the glycocalyx was quantified by measuring the electron-dense material on the apical surface of 7 myocardial capillaries per animal and on 7 sections of the *A. brachiocephalica*. Each capillary was photographed at 5 randomly selected sites (140000-fold magnification) and an area of 300 x 200 pixels was placed at the apical endothelial plasma membrane. The glycocalyx was then measured as area fraction in percent using ImageJ 1.37v software (NIH). Furthermore, the diameter of the glycocalyx was determined at 20 randomly selected sites per image. Results were averaged per blood vessel and animal. As a control hyaluronidase (*Streptomyces hyalurolyticus*, Sigma, 20 IU/ml) was infused prior to fixation revealing only minute remnants of the glycocalyx.
Detection of endothelial glycocalyx by fluorescent HA staining

Cryosections (20 µm) were fixed in acid–formalin/methanol (3.7 % formaldehyde/PBS, 70 % methanol and 5 % glacial acetic acid, all v/v) for 20 minutes. The slides were blocked with 5 % bovine serum albumin for 1 hour at room temperature and were subsequently incubated with biotinylated HAbP (2 µg/ml; Seikagaku, Tokyo, Japan) at 4°C overnight. After three washes with phosphate-buffered saline (PBS), the sections were incubated with streptavidin-FITC (Dako, Carpenteria, CA, USA) in PBS containing 1 % bovine serum albumin. Furthermore, endothelial cells were visualized by CD31 staining. The monoclonal anti-mouse antibody against CD31 (Abcam, Cambridge, UK) was detected by sheep anti-rat Rhodamine-Red™ conjugate (Jackson, Suffolk, UK). As controls sections were digested with Streptomyces hyaluronidase prior to staining, which abolished hyaluronan staining (data not shown). Sections were embedded in ProLong Gold antifade reagent containing DAPI (Invitrogen, Germany). Imaging of the sections was performed using a Zeiss Axio Observer Z1 microscope and a 63x objective. The glycocalyx was analyzed employing ImageJ 1.42. Resulting area fractions were normalized to the amount of endothelial cells as apparent by CD31 staining.

Peritoneal macrophages

For the preparation of peritoneal macrophages a 3 % solution of thioglycolate (Sigma-Aldrich, Deisenhofen, Germany) was boiled and autoclaved. Before i.p. injection thioglycolate was warmed to 37°C. Four days after thioglycolate injection the mouse was anesthetized for the collection of peritoneal macrophages. Warm PBS (5mM EDTA) was injected into the mouse peritoneal (5 ml/mouse) which was gently massaged for a moment. Subsequently some air was injected into the peritoneal cavity and peritoneal fluid was collected. Peritoneal macrophages were kept on ice and the amount per animal was counted on a Hausser hemocytometer. Peritoneal macrophages were identified by flow cytometry.
using F4/80 and Gr1 as markers. More than 90 percent of the peritoneal cells were macrophages in untreated apoE-deficient mice and mice treated with 4-MU.

**Intravital microscopy**

To analyse leukocyte attachment to endothelium in the carotid arteries an intravital microscopy approach was chosen. Mice were anesthetized with 5 mg/kg Ketamin /Xylazin. Skin was removed beginning at the cheek stopping close to sternum. The salivary glands were replaced and the connective tissue was removed from the right jugular vein. The jugular vein becomes ligated and catheterized. The left carotid artery was dissected and micro surgically a black contrast plate was placed beneath. Then the mouse was transferred to the stage of the intravital microscope (DM2500MH, Leica Microsystems, Wetzlar, Germany). The carotid artery was covered with warm PBS, which prevented tissue drying, and provided a liquid bridge between the water immersion lens (20-fold magnification) and the tissue. Afterwards the fluorescent dye acridine orange (Sigma-Aldrich, Deisenhofen, Germany) was injected via the catheter at a concentration of 2 mg/kg body weight but not exceeding 100 µl total volume to reduce volume overload. Immediately, all nuclear cells in the blood flow appeared to be fluorescent. Acridine orange labeled leukocytes were video recorded for 15 minutes with a digital video camera (Kappa opto electronics, Gleichen, Germany). All animals were in the age of 6 weeks and were sacrificed by final overdose of anesthesia at the end of the measurement. The quantitative assessment of leukocytes-endothelium interaction within the vessel (i.e. adhesion) was performed by off-line analysis of recorded video images using the image-analysis-system Cap-Image, Firma Zeintl, Heidelberg. Video records (30 sec each) of the same area of the carotid vessel were analysed at 3, 4 and 5 minutes after injection of acridine orange. Adherent leukocytes were defined as cells that did not move or detach from the endothelium within the observation period of 30 seconds. Adherent leukocytes were quantitated by manually counting the total number of adherent leukocytes along a 100 µm
long and 150 µm broad defined segments, and the counts were expressed as the number of adherent cells/30 sec/mm² of the vessel surface. The surface area was calculated from diameter and length of the observed vessel segment (determined by Cap-Image; Firma Zeintl, Heidelberg), assuming cylindrical geometry of the artery.

**Tissue edema**
To address whether the disturbance of endothelial HA may have caused increased endothelial leakage, the interstitial water content of lungs was determined as the difference between wet weight and dry weight as described previously¹⁰. As a positive control the effect of *i.p.* injection of 100 µg histamine after 2 hours was used.

**Neointimal hyperplasia**
The animals were anesthetized and the left common carotid artery was dissected and ligated near the carotid bifurcation as described previously¹¹. All animals were sacrificed 28 days after ligation of the carotid artery. Mice were perfusion fixed for 3 minutes with 4 % *p*-formaldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.3) as described¹². After excision of the left and right carotid arteries, the vessels were fixed in 4 % *p*-formaldehyde for 6 hours. The carotid arteries were dehydrated and embedded in paraffin. Serial sections, 5 µm thick were cut, for morphometric analysis by hematoxylin-eosin staining sections every 50 µm were used. Morphometric analysis was carried out on digital images of these vessels with image analysis software Leica Analysis Software (Leica Microsystems, Wetzlar, Germany). The circumferences of the lumen, *Elastica lamina interna* und *Elastica lamina externa* were determined by tracing along the different vessel layers.
LC-MS/MS analysis of 4-MU plasma concentration

4-MU concentrations in plasma of apoE deficient mice were determined by high-performance liquid chromatography coupled with mass spectrometry (LC-MS/MS, Applied Biosystems/MDS Sciex API5000 LC/MS/MS. To apoE KO mice plasma samples (10 µL) acetonitrile (20 µL) containing the internal standard (20 ng/mL umbelliferone) was added. After thorough mixing, the samples were centrifuged for 5 minutes at 3600 rpm at approximately 4°C, and the supernatant was diluted 1:7 (v/v) with formic acid (0.05 %). Thirty µL of each sample were chromatographed on a reversed-phase column (YMC-Pack Cyano, 50x4.6 mm, 5µm) and eluted with a solvent system consisting of 0.05 % formic acid and methanol (50:50, v:v). The analytes were monitored by LC-MS/MS with a SRM method as follows: Precursor → product ion for 4-MU m/z 175 → m/z 133 and for the internal standard m/z 161 → m/z 133, all analyses were in negative mode. Under these conditions 4-MU and the internal standard were eluted after approximately 1 min and 0.9 min, respectively. The Analyst software (version 1.4.2, Applied Biosystems/MDS Sciex, Thornhill, Ontario, Canada) was used for evaluation of all chromatograms. The limit of quantification for apoE deficient mice plasma samples was 2.324 ng/mL. The response from calibration standards was linear from 2.324 to 363.2 ng/mL and the coefficient of correlation for all measured sequences was at least 0.998. The inter-day precision and analytical accuracy ranged from 3.0 to 7.6 % and from 96.0 to 100.1 %, respectively. The intra-day precision and analytical accuracy ranged from 3.4 to 10.0 % and from 91.8 to 102.8 %, respectively. The mean absolute recovery of 4-MU in apoE KO mice plasma over the whole concentration range was determined as 97.5 ± 3.3 % and for the internal standard at the working concentration as 89.7 ± 1.4 %.
**Endothelial cell culture**

Human coronary endothelial cells (EC) were obtained from PromoCell (Heidelberg, Germany) and cultured under routine conditions according to the instructions of the distributor using the recommended media and supplements. The experiments were performed to analyze whether the plasma concentration of 600 nM 4-MU (Sigma-Aldrich, Deisenhofen, Germany) as determined by LC-MS/MS was indeed effective to inhibit HA-synthesis of EC. For this purpose cells were treated acutely for 24 hours with 4-MU at 600 nM. 4-MU at 300 µM was used as positive control. At the end of the experimental period the concentration of HA was determined in the conditioned medium as described above for the plasma samples using the Corgenix® assay and normalized to total cellular protein.

**Literature**


online Fig. 1

A

Western-diet

0 4 15 25 weeks

B

plaque burden aorta [%]

control 4-MU

C

plaque area aortic root [mm²]

control 4-MU

D

HA

control 4-MU

aortic root [AF %]

control 4-MU
online Fig. 2

A

**collagen**

control

4-MU

B

**collagen**

control

4-MU

C

**α-actin**

control

4-MU
online Fig. 3

A

B

C
online Fig. 4

![Graph showing HA-secretion [% of control] for different concentrations of 4-MU. The graph compares control, 600 nM 4-MU, and 300 µM 4-MU treatments.]
Online Figure 1. Increased atherosclerosis in apoE<sup>−/−</sup> mice after 10 weeks of treatment with 4-MU. A, Experimental design. B, Aortic en face preparations stained with Oil red O and plaque burden of control and 4-MU treated mice. C, Morphometric quantification of plaque area at the aortic root. D, HA staining at the aortic root and area fraction (AF) of HA staining at the aortic root as determined by image analysis, bars = 100 µm; n = 6; *, p<0.05.

Online Figure 2. Plaque composition in response to 4-MU. Plaque composition was analyzed at the aortic root with respect to collagen and VSMC content. No significant differences were detected by image analysis displayed on the right side of the respective photographs; bars = 100 µm; n = 6. A, Collagen was stained by picrosirius red. B, Collagen fibril arrangement was assessed by birefringence analysis (polarized light) of picrosirius red stained sections. C, VSMC content as visualized by immunostaining of alpha-SM actin (red).

Online Figure 3. Interstitial water content and lipid retention. ApoE-deficient mice were treated with 4-MU for 2 weeks (A) to analyze interstitial water content as readout for endothelial permeability and for 21 weeks (B, C) to analyze lipid retention in the plaque matrix at the aortic root. A, wet weight and percentage of water loss of lungs derived from animals treated with 4-MU (2 weeks), n = 9; or as a positive control with histamine (100 µg, i.p., 2 hours), n = 5; *, p<0.05 versus control. B, Lipid accumulation as determined by Oil red O staining. C, Apo B48 accumulation as determined by immunostaining. Quantitative data were obtained by digital image analysis and displayed on the right side of the respective photographs, bar = 100 µm, n = 5.

Online Figure 4. 4-MU inhibits endothelial HA-synthesis in vitro. Human coronary EC were treated with 600 nmol/L 4-MU representing the plasma concentration of the in vivo
experiments or with a high dose of 4-MU (300 mmol/L) as positive control. The application of 4-MU was short term (24 h), mean ± SEM, n = 6-11; *, p<0.05.