Overexpression of Hyaluronan in the Tunica Media Promotes the Development of Atherosclerosis

Song Chai, Qing Chai, Carl C. Danielsen, Peter Hjorth, Jene R. Nyengaard, Thomas Ledet, Yu Yamaguchi, Lars M. Rasmussen, Lise Wogensen

Abstract—The arterial content of hyaluronan (HA) undergoes diffuse changes as part of the diabetic macroangiopathy. Because HA influences the phenotype of vascular cells in vitro such as proliferation, migration, and secretion, it is tempting to speculate that diabetes-induced hastened cardiovascular disease may be linked to the increased amount of HA. To explore the pathophysiological role of altered HA content in the arterial wall in vivo, we created transgenic (Tg) mice with HA overexpression in smooth muscle cells (SMCs) in large and small vessels, targeted by the α smooth-muscle-cell-actin (αSMA) promoter fused to the human hyaluronan synthase 2 (hHAS2) cDNA. RT-PCR demonstrated hHAS2 mRNA expression in the tunica media of large and small vessels. In situ hybridization confirmed that hHAS2 mRNA was targeted to the SMCs. The aortic HA content was elevated in the Tg mice, and by immunohistochemistry, it was seen that HA accumulated in the tunica media. The secretory profile of high- and low-molecular HA was similar in wild-type and Tg animals. Overproduction of HA in the aorta resulted in thinning of the elastic lamellae in Tg mice. Our data suggest that this may lead to increased mechanical stiffness and strength, as determined by controlled stretching until failure. Finally, overproduction of HA on the genetic background of the ApoE-deficient mouse strain promoted atherosclerosis development in the aorta. These results indicate that a single component of the diabetic macroangiopathy, diffuse accumulation of HA, accelerates the progression of atherosclerosis. (Circ Res. 2005;96:583-591.)

Key Words: hyaluronan | biomechanics | atherosclerosis | diabetes | transgenic

Hyaluronan (HA) is a large, nonsulfated glycosaminoglycan (GAG) produced in the vasculature by smooth muscle cells (SMCs) and endothelial cells (ECs). It is synthesized at the inner face of the cell membrane by hyaluronan synthases 1 to 3 (HAS 1 to 3), followed by translocation to the outer surface and the intercellular space.1 Recent in vitro investigations have shown that high (HMW) as well as low molecular (LMW) forms of HA influence important cellular functions, such as proliferation, migration, and secretory capabilities.1–4 There seems to exist a delicately regulated balance between production, sizing, and removal of HA that is central to its biological functions during normal conditions. If this balance is disturbed, it has been hypothesized that disease may develop.5 The notion that HA is likely to take part in the development of vascular pathologies has been nourished by a number of observations, which show altered HA amounts in several arterial disease entities such as diabetic angiopathy, atherosclerosis, and restenosis.6–8 During the build-up of the atherosclerotic plaques in humans, some areas with local accumulation of HA appear.9–11 This is in contrast to the situation in patients with diabetes in whom the HA accumulation is disseminated in tunica media and is regarded as one important element in a series of diffuse matrix changes in the vessels.6,12 The diabetic angiopathy deserves some specific comments. First, a very high and equal frequency of cardiovascular disease (CVD) exists among men and women with diabetes for as yet unexplained reasons.13–15 In line with this, it is interesting that in subjects with long-term diabetes the increasing mortality from CVD is independent of any well-known risk factors such as hypertension, dyslipidemia, and microalbuminuria.16,17 Second, the vessel wall thickness is increased in juvenile patients with a diabetes duration of 5 years.18,19 Third, histochemical and biochemical records demonstrate that atheromatosis free arterial wall segments from patients with diabetes contain excessive amounts of HA, type IV collagen, and fibronectin in the tunica media.6,12 Finally, in vitro studies reveal that factors from the diabetic metabolism affect the release of HA from SMCs.20 In concert these studies indicate that matrix alterations in the arterial wall in diabetes may be part of a
generalized angiopathy. We hypothesize that these changes make the vessel wall more susceptible to atherothrombotic stimuli, thus contributing to the increased prevalence of CVD in diabetic patients. To address the biological effects of increased HA content in the arteries in vivo, we created transgenic mice with disseminated overexpression of HA in the tunica media of large and small vessels. We investigated the ultrastructural changes in the arterial wall, the stiffness and strength of aortic rings, and the influence on the development of atherosclerosis in ApoE-deficient mice.

Materials and Methods

Production of hHAS2 Transgenic Mice

A restriction fragment containing the mouse αSMC-actin 5′ flanking region SMP8 (−1074 bp, 63 bp of 5′UT and 2.5 kb of intron 1), the human hyaluronan synthase 2 (hHAS2) cDNA, and a SV40 polyA terminator sequence21−22 was gel-purified using GeneCleanII (BIO101) and microinjected into pronuclei of fertilized eggs from F1 (C57BL/6J × DBA/2J) females crossed with F1 (C57BL/6J × DBA/2J) males (Taconic Europe, Rey, Denmark). The progeny from backcrosses to C57BL/6J were identified as transgenic (Tg) or nontransgenic (WT) by polymerase chain reaction (PCR) with primers complementary to sequences in the SV40 fragment. hHAS2 transgenic animals (>3–generation C57BL/6J) were crossed with ApoE-deficient (ApoE−/−) mice (>10-generation C57BL/6J) (Bomholtgaard Breeding and Research Center) to generate animals that were hHAS2−/− or hHAS2 ApoE−/− (>3 to 4 generation). Unless otherwise stated, mice were euthanized by cervical dislocation. For details, including legal approvals, see the expanded Material and Methods in the online data supplement available at http://circres.ahajournals.org.

RNA Isolation and RT-PCR

Total RNA was reversed transcribed to gain cDNA. Amplification of cDNA was performed using the following primers: hHAS2, 5′-GCTACACCTCATCTACCC-3′ and 5′-GACTTCTTCTTT-TTTCACCCC-3′. Murine β-actin (mβ-actin), 5′-CTACAACT-GAGCTCGGTGTGC-3′ and 5′-GTCCAGACGAGGTGAGG-CATG-3′ and accomplished as described online. The PCR products were separated by electrophoresis in a 2% agarose gel with ethidium bromide. The intensity of the visualized bands was measured in arbitrary absorbance units with a BIO-RAD UV-gel camera. Results are presented as the ratio between the density of hHAS2 and mβ-actin. For details, see expanded Material and Methods.

In Situ Hybridization

Hybridization of deparaffinized sections from selected organs was performed with 35S-labeled sense or antisense RNA probes (6 × 104 cpm/section) according to standard methods (see expanded Material and Methods).

HA Immunoassay

Tissues were excised, and the aorta was dissected into intimamedia. The tissues were weighed, homogenized in 2 mL of 0.01 mmol/L Tris-HCl and 0.2 mmol/L EDTA pursued by digestion with protease from Streptomyces Griseus (1 mg/mL, Sigma) overnight at 37°C and followed by heat inactivation (100°C for 30 minutes). After centrifugation (30 000g for 10 minutes), the HA content in the supernatants was measured by an HA ELISA (Chugai Diagnostics Science Co). The detection limit of the assay was 10 ng/mL and the intra-assay CV% was 3.6% to 4.7%. The plasma HA concentration was measured using the same ELISA.

Histochemistry

We stained paraffin or cryosections with standard methods with orcin, biotinylated HABP (1:250, Seikagaku Co), and antibodies against αSMC-actin (1:150, NeoMarkers) and CD11b/CD18 (Mac1) (1:150, Chemicon) (see expanded Material and Methods).

Determination of HA Molecular Weight by High-Performance Liquid Chromatography

The aorta (Tg: n = 6, WT: n = 4) was dissected and chopped into small pieces that were distributed in two wells (90-well plate) and incubated at 37°C for 24 hours in MEM containing 40 μCi D-[6-3H]-glucosamine-hydrochloride (Amersham Biosciences, UK). Tissue from two mice were pooled, homogenized, and extracted with guanidinium-chloride. Diluted supernatants were passed through an anion exchange cellulose column (DEAE-52, Whitman). The HA-containing elute underwent dialyze and freeze-drying. The samples were resuspended in 0.1 mol/L CH3COONH4, pH 7.2. One part was treated with a Hyaluronidase (Streptomyces hyaluronidicus) (15 U/μL, Calbiochem, VWR Int. GmbH), the other sample was left untreated. All samples (Tg, n = 3; WT, n = 2), were separated by high-performance liquid chromatography (HPLC) (Superox 6 HR 10/30 column) (PharmaciaBiotech). Fractions were collected and counted. For details, see expanded Material and Methods.

Electron Microscopy

Aortic ring profiles were embedded in Epon for electron microscopy and 50-nm sections were examined ultrastructurally with a digitalized Philips CM10 electron microscope (SIS 3.0). All vascular profiles were scored according to the amount of the intercellular ground substance (clear areas) and the degree of elastin fragmentation (0 = absent/low, 1 = moderate, 2 = severe). The width of 4 to 7 elastic lamellae, including membrana elastica interna and externa, and the distance between the lamellae, were measured at two or four positions chosen at random. Mean values of the parameters were obtained for each mouse before group means were calculated. All measurements were performed in a blinded fashion. For details, see expanded Material and Methods.

Mechanical Testing and Hydroxyproline Content

Freshly excised aortas were stored at −20°C until analysis. The lower thoracic aorta was cleaned of adhering fat and loose connective tissue. Aortic ring specimens (1-mm high; in average 3.5 mm thick and 3 to 4 generation). Unless otherwise stated, mice were euthanized by cervical dislocation. For details, including legal approvals, see the expanded Material and Methods in the online data supplement available at http://circres.ahajournals.org.

Analysis of Atherosclerosis in hHAS2 ApoE−/− and hHAS2 ApoE−/− Transgenic Mice

The heart and entire aorta were removed from hHAS2 ApoE−/− and hHAS2 ApoE−/− littermates after 16 to 20 weeks on normal diet. The processing of the root is described in the expanded Material and Methods. The average atherosclerotic lesion area in three levels was determined using Olympus BX50 microscope and analysis software. The extent of atherosclerosis in the aortic arch and thoracic aorta was measured en face by visualizing cholesterol deposits with Oil Red O. The surface area of the lesion within the arch was expressed as percentage of the arch covered by lesion calculated by means of analysis software including multiple-image alignment. The area with positive staining in the thoracic aorta was calculated by using a frame (175 349.1 μm2/44 crosses; CAST, Olympus) that was moved stepwise, from side to side all along the aorta. All analyses were made in a coded fashion by the same investigator.

Statistics

Statistical analysis between two groups of data were performed by Student t test after exclusion of variance in-homogeneity (tested by
Increased Aortic HA Content

The HA content (ELISA) in selected organs from 8- to 14-month-old mice are given in the Table. The HA amount in aorta was higher in Tg mice of line 5986 (n=14-month-old mice are given in the Table. The HA amount in selected organs from 8- to 12-month-old mice are given in the Table. The HA amount in the selected organs was similar when comparing the two lines of transgenic mice (Table). A similar pattern of HA production in the kidney and bladder was seen in younger, 4- to 5-month-old Tg mice (line 5986) (Bladder, 44.9±11.2 ng/mg; Kidney, 1.31±0.64 ng/mg; n=6) versus WT mice (Bladder, 46±11.2 ng/mg; Kidney, 0.35±0.05 ng/mg; n=5) (P<0.05 and P=0.01, respectively). Immuno-histochemistry (IHC) demonstrated that HA was localized in the tunica media of the aorta (n=4 for both line 5986 and 5990), and the smaller vessels in the kidney (n=4 for both line 5986 and 5990) and retina (n=9, line 5986) of Tg mice (Figure 2B). On the other hand, WT mice exhibited weak HA expression (for each organ n=4 to 7). In the bladder, HA was present in the connective tissue layer of the mucosa and the muscularis in all tested mice; however, it was difficult to detect any differences in the staining pattern in Tg (n=4 for both line 5986 and 5990) versus WT mice (n=4) (data not shown). The mean plasma HA concentration was similar among Tg mice of line 5986 (705±156 ng/mL; n=8), line 5990 (621±98 ng/mL; n=8), and WT mice (650±80 ng/mL; n=9) (P<0.05).

Molecular Weight Characterization

The distribution of HMW and LMW HA forms produced in vitro from cultured slices of aorta exhibited a similar pattern in Tg (n=3) versus WT mice (n=2) (Figure 3). In all incidents, the HA isolated and purified from overnight incubated aorta slices were eluted as two major molecular size species G1 (fractions 8 to 10) [Tg, 55±5.2% (n=3); WT, 53±6% (n=2); G2 (fractions 16 to 20) Tg, 40±5% (n=3); WT, 44±6% (n=2)]. The ratio between the peaks was 1.5±0.29 in Tg (n=3) versus 1.3±0.31 in WT (n=2) mice. The first peak corresponds to the void volume equivalent to a molecular size of >1×10^6 Da. The second peak corresponds to molecular size species a little larger than 1.1×10^5 Da. Both peaks represent HA, because they disappeared after treatment of parallel handled samples with Streptomyces hyaluronlyticus before HPLC (data not shown).

Thinning of the Elastic Membranes in the Aorta of Transgenic Mice

Orcein staining did not reveal any obvious differences in the vascular morphology between WT and Tg mice (Figure 4). By electron microscopy we found, however, that the average width of the elastic lamellae in Tg (n=8) and WT (n=7) mice was 0.763±0.097 and 0.926±0.083 μm, respectively.

Figure 1. hHAS2 RNA expression in 3- to 6-month-old Tg and WT mice. Results are mean±SEM (n=4 to 6).
(P<0.001). The distance between the elastic lamellae (Tg, 3.831±1.015 μm; WT, 3.861±1.142 μm) and the total media thickness (Tg, 26.7±4.26 μm; WT, 25.4±7.9 μm) were similar in the two groups. More ground substance was visible in the space between the elastic lamellae and the SMCs in Tg versus WT mice (P<0.01). Debris from the elastic fibers was more often seen in Tg mice than in normal animals, and fragmentation of the elastic membranes was clearly more evident in Tg mice versus WT controls (P<0.01) (Figure 4).

Alterations of the Biomechanical Properties of the Aorta in Transgenic Mice

The maximal load and the maximal stiffness were significantly different among the groups of both the 3-month-old (P=0.029 and P=0.028, respectively) and 6-month-old mice (P=0.009 and P=0.061, respectively) (Figure 5). At 3 months of age, both the maximal load and the maximal stiffness were higher in Tg mice of line 5986 (n=6) versus age-matched WT mice (n=10) (both P<0.05), whereas the

| HA Content (ng/mg tissue) in 8- to 14-Month-Old hHAS2 Mice on C57 Background and in 4- to 5-Month-Old hHAS2 Mice on ApoE−/− Background |
|---|---|---|---|---|---|
| | Kidney | Bladder | Aorta | Arcus Aorta | Aorta Thoracicus |
| **C57 background** | | | | | |
| Tg 5986 | 3.48±0.30* (n=8) | 128.58±7.15 (n=8) | 166.48±24.61* (n=12) |  |
| Tg 5990 | 2.70±0.36 (n=4) | 128.54±9.48 (n=4) | 268.42±70.57* (n=4) |  |
| WT | 2.06±0.27 (n=7) | 122.13±15.0 (n=7) | 53.88±4.74 (n=9) |  |
| **ApoE−/− background** | | | | | |
| hHAS2−/− ApoE−/− | 1.94±0.70 (n=5) | 62.64±21.68* (n=5) | 227.31±31.70* (n=5) | 157.41±13.20* (n=5) |
| hHAS2−/− ApoE−/− | 1.49±0.54 (n=5) | 33.68±9.69 (n=5) | 56.11±25.0 (n=5) | 76.17±26.37 (n=5) |

Mean±SD. *P<0.05 Tg vs WT.
values of line 5990 (n=6) were in between. At 6 months of age, the maximal load was higher in Tg mice of line 5986 (n=6) versus age-matched WT mice (n=8) and mice of line 5990 (n=6) (both \( P < 0.05 \)), whereas the maximal stiffness, with the highest value in mice of line 5986, tended to be different among the groups (\( P = 0.061 \)) (Figure 5). In all tested groups of mice, both the aortic extensibility and the total collagen content expressed as mg per mm of aortic circumference were similar (data not shown).

**Promotion of Atherosclerosis in hHAS2\(^+\)ApoE\(^{-/-}\) Transgenic Mice**

We backcrossed hHAS2 transgenic mice (line 5986) with ApoE\(^{-/-}\) animals to obtain hHAS2\(^+\)ApoE\(^{-/-}\) and hHAS2\(^+\)ApoE\(^{-/-}\) animals. The HA staining was profound in the tunica media of the aortic root (n=9), the aortic arch (n=6), and the thoracic aorta (n=1) in hHAS2\(^+\)ApoE\(^{-/-}\), whereas it was weak in hHAS2\(^+\)ApoE\(^{-/-}\) animals (root, n=9, aortic arch, n=7, and thoracic aorta, n=1) (Figure 6A). The HA content was higher in the aortic arch, thoracic aorta, and the bladder in hHAS2\(^+\)ApoE\(^{-/-}\) versus hHAS2\(^+\)ApoE\(^{-/-}\) mice (all \( P < 0.05 \)) (Table). hHAS2\(^+\)ApoE\(^{-/-}\) mice exhibited only marginally elevated HA levels in the kidney (\( P = 0.28 \)) (Table). At 16 to 20 weeks of age, the extent of the atherosclerotic lesions in the aortic root were higher 80 and 160 \( \mu \)m from the valve cusps in hHAS2\(^+\)ApoE\(^{-/-}\) (n=15) versus hHAS2\(^+\)ApoE\(^{-/-}\) (n=9) (both \( P < 0.05 \)) (Figure 7A). The area (%) of lipid deposits in the aortic arch was increased in hHAS2\(^+\)ApoE\(^{-/-}\) (n=17) versus hHAS2\(^+\)ApoE\(^{-/-}\) (n=10) (\( P < 0.001 \)) (Figure 7B). This difference

**Figure 3.** Gel filtration chromatography (Superose 6.0 HR) of isolated HA produced by aorta slices from Tg (solid line) and WT mice (dotted line) cultured in the presence of [\( ^{1}H \)]-glucosamine-hydrochloride-labeled HA. Molecular weight (MW) standards: (1) maltoheptose (1153.02 Da) and (2) maltohexose (990.86 Da). \( V_0 \) indicates void volume. Representative elution pattern is shown.

**Figure 4.** Morphology of the aortic tunica media from WT (A through C) and Tg (D through F) mice. Orcein staining shows the elastic component (black) (A and D). Electron microscopy demonstrates that the SMCs fill out the space between the elastic lamellae in WT mice (→) (B) and a normal arrangement of collagen bundles along the elastic structures (→) (C). In Tg animals, the SMCs seem to withdraw from the elastic lamellae leaving an increased amount of intercellular ground substance (→) and collagen bundles have a loose arrangement and are absent in some areas (▶) (E and F). The SMC appears fusiforme (→) (F). Deposits of fragmented elastic material (▶). Original magnification: A and D, \( \times 100 \); B, C, E, and F, Bar=1 \( \mu \)m.

**Figure 5.** Maximal load (A) and maximal stiffness (B) were estimated of aortas isolated from Tg and WT mice at 3- or 6-months of age. N indicates Newton. Mean±SEM. \( * P < 0.05 \).

5986 [ ]
5990 [ ]
WT [ ]
was also present in the descending aorta (hHAS2^{+/-}/ApoE^{+/-} \textit{n}=15) and hHAS2^{+/-}/ApoE^{+/-} \textit{n}=10 \textit{(all }P<0.05\text{)} (Figure 7C). The SMCs were seen throughout the tunica media (Figure 6B). The distribution of SMC-positive cells in the plaques in the aortic root was similar in hHAS2^{+/-}/ApoE^{+/-} \textit{n}=5 versus hHAS2^{+/-}/ApoE^{+/-} \textit{n}=8. They were primarily seen under the fibrous capsule and scattered around in the central area of the plaques (Figure 6B). The presence of HA was obvious in areas with SMCs in hHAS2^{+/-}/ApoE^{+/-} but not in hHAS2^{+/-}/ApoE^{+/-} (Figure 6B). Both genotypes exhibited HA staining in areas with cholesterol crystals. This may, however, be due to background staining (Figure 6B). Also the distribution of CD11b/CD18-positive cells in the plaques in the aortic root was similar in hHAS2^{+/-}/ApoE^{+/-} \textit{n}=8 versus hHAS2^{+/-}/ApoE^{+/-} \textit{n}=7. The macrophages were primarily seen central in the lesion and in the underlying tunica media and adventitia (Figure 6B).

**Discussion**

This study clearly indicates that raised HA levels in the aortic wall significantly transform the property of the vessel, leading to reduced thickness of the elastic lamellae, altered biomechanical properties, and most importantly, an increased susceptibility to atherogenic insults. This is in line with observations reported by Renard et al who suggest that GAG accumulation is one of the first steps in initiation of diabetes-induced atherosclerosis.

Our investigations used transgenic mice in which hHAS2 expression, driven by the murine \alpha SMC-actin promoter, was able to generate arterial hHAS2 RNA, leading to raised HA levels with a normal distribution of HMW versus LMW forms. We found a relatively high HA content in WT aorta, which at a first glance seems contradictory to the almost absent HA staining in the tunica media in the WT mice. It should be underlined, however, that the vessel extracts probably include some components from the tunica adventitia that normally contain HA. Clearly, our data show that accumulation of HA occurs especially in the tunica media. The circulating HA levels were similar in transgenic and WT mice. This suggests that HA in our model acts in an autocrine or paracrine fashion. It is generally believed that HA throughout the body is cleared by receptor-mediated uptake mediated...
by the hyaluronan receptor for endocytosis (HARE), which is found in lymph node, liver, and spleen sinusoidal endothelial cells. Tissue HA is mainly removed by lymph and passed to the lymph nodes in which it is degraded. Approximately 15% of HA exits the lymph nodes, enters the blood, and is removed by the liver sinusoidal endothelial cells, which has a rather high clearance capacity. Together, it is not surprising that the HA plasma levels are normal in Tg mice.

The higher HA content tend to increase the maximal load and the maximal stiffness of the vessel in hHAS2 transgenic mice. This observation was manifest in one of our transgenic lines (5986) at 3 and 6 months of age. Furthermore, a trend toward increased maximal load and maximal stiffness was seen in 3-month-old mice of line 5990. However, this trend disappeared with age. Our observations suggest that HA may influence the biomechanical properties of the aorta. Hyaluronan promotes the shrinkage of collagen gels by aortic SMCs in vitro. This observation is believed to be associated with changes in collagen reorganization and cell shape and partly mediated by activation of the CD44 signaling pathway. In line with this is the observation that blocking of RHAMM (receptor for HA-mediated motility) is linked to attenuated collagen gel contraction by SMCs isolated from the bladder exposed to stretch injury. Furthermore, in accordance with the minimal strength and stiffness of elastin compared with collagen, the thinning and fragmentation of the elastic membranes in the Tg mice were not found to be reflected in neither reduced mechanical strength, stiffness, and extensibility of the aorta or changes in the toe-part of the load-strain curves corresponding to the normal in vivo range of loading (data not shown). Therefore, the observed changes of the elastic membranes more likely contribute to collagen reorganization resulting in increased strength and stiffness of the aortic wall in Tg mice. This is in line with observations in a mouse model of Marfan syndrome and in experiments evaluating the impact of elastases on aortic elasticity, in which fragmentation of the elastic membranes is associated with increased stiffness of the vessel wall.

The most prominent observation in the present study is that ApoE-deficient animals with increased HA levels in the vessel wall develop accelerated atherosclerosis. It should be underlined that atherosclerosis is absent in our hHAS2 mice on the normal C57 genetic background. Thus, the presence of one element of the diabetic macroangiopathy in combination with an atherogenic milieu, but without hyperglycemia, accelerates the development of atherosclerotic lesions. Several observations have implicated HA in a variety of the biological processes involved in atherogenesis, but our data show for the first time, that disseminated accumulation of HA accelerates the atherogenic process in vivo. Several mechanisms are likely to play a role in the augmented atherogenesis. First, a recent report has described an attenuated development of atherosclerosis in mice genetically engineered to lack CD44. Because HA is the principal ligand for CD44, the observation is compatible with our results. Recruitment of macrophages was decreased and SMCs were in a quiescent state in the CD44-deficient mice. These are in line with in vitro data showing that HA accumulation may be important for macrophages/foam cell migration and activity through HA-CD44 receptor interactions. Likewise, SMC proliferation and migration is modulated by HA. Thus, a HA-rich environment is believed to promote SMCs as well as macrophage secretory capabilities, migration, and proliferation. Clearly, both alterations in the phenotypic stage of SMCs and macrophages may play a role in the increased atherogenesis. Presently, we can only say that there is no overwhelming difference in the pattern of macrophage infiltration of the atherosclerotic plaques in hHAS2/ApoE−/− versus hHAS2−/− mice. However, it is as yet unknown whether the functional status of the infiltrating macrophages and SMCs are altered. Secondly, augmented atherogenesis may relate to the observed biomechanical alterations, because it has been hypothesized that increased arterial stiffness may give rise to an atherosclerosis-prone vessel wall. This proposal is based both on observational data about relations between arterial stiffness and atherosclerosis and on experimental results, linking increased arterial stiffness with marked alterations in endothelial cell func-
tion, putatively leading to vascular damage. Finally, it is possible that lipoprotein transport may be altered due to modifications in the arterial wall, and because HA binds lipoproteins, this may accelerate atherogenesis in hHAS2 ApoE−/− mice. It is intriguing that our findings are in agreement with a recent publication by Sussmann et al. They report that upregulation of HAS2 expression via EP2 and IP receptors may contribute to HA accumulation during the development of the atherosclerotic plaques, thereby mediating proatherogenic functions of COX2. On the other hand, downregulation of HAS2 expression may mediate the antiatherogenic properties of COX2 inhibitors. The exact mechanisms behind the HA-induced atherogenesis, however, needs further exploration.

In summary, our in vivo observations suggest that accumulation of HA in the aortic tunica media is followed by an increased mechanical stiffness and strength of the aorta. Furthermore, the accumulation of HA stimulates the development of atherosclerosis in ApoE−/− mice. Based on the observations reported in this study, we propose that increased levels of HA in the vascular wall promote the progression of atherosclerotic lesions. Moreover, the obtained knowledge of disseminated HA accumulation as a promoter of atherogenesis may have implications for the understanding of diabetic angiopathy. In diabetes, the amount of HA, and other extracellular matrix components in the tunica media, is increased in atheromatosis-free areas. Our data are compatible with the notion that raised areas of HA in the tunica media, as part of the diabetic macroangiopathy, may facilitate the development of atherosclerosis among patients with diabetes, with HA being one missing factor linking diabetes and diabetes-induced atherosclerosis.

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References


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Production of hHAS2 transgenic mice

A restriction fragment containing the mouse αSMC-actin 5’flanking region SMP8 (-1074 bp, 63 bp of 5’UT and 2.5 kb of intron 1), the human hyaluronan synthase 2 (hHAS2) cDNA and a SV40 polyA terminator sequence 1,2 was gel-purified using GenecleanII (BIO101, La Jolla, CA) and microinjected into pronuclei of fertilized eggs from F1 (C57BL/6J x DBA/2J) females crossed with F1 (C57BL/6J x DBA/2J) males. When the pups reached three to four weeks of age, DNA was extracted from 3 mm long tail biopsies and the genotype was determined by the polymerase chain reaction (PCR) with primers complementary to sequences in the SV40 fragment (forward: GGGAGCAGTGGTGGAATGCC and backward: GGGGGAGGTGTGGGAGGTTT). The following generations of progeny from backcrosses to C57BL/6J were identified as transgenic (Tg) or non-transgenic (WT) by polymerase chain reaction (PCR) with primers complementary to sequences in the SV40 fragment. hHAS2 transgenic animals (>3-generation C57BL/6J) were crossed with ApoE-deficient (ApoE -/-) mice (>10–generation C57BL/6J) (Bomholtgaard Breeding and Research Center, Ry, Denmark) to generate animals that were hHAS2⁺ApoE -/- or hHAS2⁻ApoE -/- (>3-4 generation). At the second backcross homozygotic ApoE-deficient animals were identified by PCR with the following primers: Forward: AAAGCAGGCGACGACGATAG and backward: AGAGGAACACAGGAAGGAG. All mice were given free access to standard chow and water. Unless otherwise stated stated mice were sacrificed by cervical dislocation. The animal studies were conducted in accordance with the guidelines of and approved by The Animal Experiments Inspectorate, Denmark (#1999/561-218, #2003/561-682) and the Danish Working Environment Service (#20000004676/3, # 20010011479/8, #200300194040/3).

RNA isolation and RT-PCR

Total RNA was isolated from selected organs with Trizol (5 ml per organ) (Invitrogen, Denmark) and quantified by spectrophotometry at 260 and 280 nm. One μg of RNA was treated for 15 min at

RNA isolation and RT-PCR

Total RNA was isolated from selected organs with Trizol (5 ml per organ) (Invitrogen, Denmark) and quantified by spectrophotometry at 260 and 280 nm. One μg of RNA was treated for 15 min at
room temperature with 1U DNAase I in 10 µl reaction buffer (Invitrogen, Denmark), followed by inactivation at 65°C for 10 min. Subsequently, cDNA was prepared by incubating with 200 U MMLV-reverse transcriptase (Invitrogen, Denmark) in 30 µl reaction mixture (50 mmol/L Tris, pH 8.3, 75 mmol/L KCl, 3 mmol/L MgCl₂, 0.5 mmol/L dATP, 0.5 mmol/L dTTP, 0.5 mmol/L dGTP, 0.5 mmol/L dCTP and 30 pmol of random hexamers) for 75 min at 37°C, followed by 15 min at 95°C. PCR was performed using 2 µl RT-solution in 20 µl reaction mixture (20 mmol/L Tris, pH 8.4, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.5 mmol/L dATP, 0.5 mmol/L dTTP, 0.5 mmol/L dGTP, 0.5 mmol/L dCTP, 2.5 U DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland)) and 500 pmol of each of the primers: hHAS2: 5’-GCATCACACCTCATCATCC-3’ and 5’-GACTTCTCTTTTTCCACCCC-3’. Murine β-actin (mβ-actin): 5’-CTACAATGAGCTGCAGTGCC-3’ and 5’-GTCCAGACGCAGGATGGCATG-3’. Amplification of cDNA was accomplished by two initial cycles of 95°C (60s), 54°C (60s) and 73°C (60s), followed by a number of rounds at 94°C (45s), 54°C (45s) and 73°C (45s). In the final cycle the samples were incubated for 2 min at 73°C. To ensure a dose-dependent PCR for the semi-quantitative PCR protocol we determined the optimal number of cycles for each primer set as described ³. In short, the number of cycles, which gave rise to a weak band was selected for evaluation of the samples, i.e. 35 and 38 cycles for mβ-actin and hHAS2, respectively. The PCR products were separated by electrophoresis in a 2% agarose gel with ethidium bromide. All PCR reactions gave rise to products of the expected molecular size. The intensity of the visualized bands was measured in arbitrary absorbance units with a BIO-RAD UV-gel camera. Results are presented as the ratio between the density of hHAS2 and mβ-actin. RNA samples without MMLV-RT and samples without RNA did not give rise to cDNA amplification.
**In situ hybridization:**

Selected organs were removed, fixed in 4% paraformaldehyde for 20 hours at 4°C and embedded in paraffin. Deparaffinized sections were digested with proteinase K (10 µg/ml) for 30 min at 20°C and prehybridized for 2-3 hours at 42°C in hybridization buffer (see online expanded Material and Methods). Hybridization with 35S-UTP-labeled sense or antisense RNA probes (6 x 10^5 cpm/section) was done for 16 hours at 50°C in a humidified chamber, followed by washing, dehydration and drying. Sections were dipped in KODAK NTB2 emulsion and developed after exposure for 3 and 6 weeks. Sense and anti-sense RNA probes were prepared by in vitro transcription of linearized plasmid containing the hHAS2 cDNA.

**Immunohistochemistry:**

Organs were dissected from the surrounding tissue and removed. One piece of tissue was placed in Tissue-Tek® OCT compound (Miles, USA) and snap frozen in liquid nitrogen. The other piece was fixed in 4% paraformaldehyde for 24 hours at 4°C. Eyes were fixed for one hour in 4% paraformaldehyde. After dehydration in 70% ethanol the lenses were removed and each eye was divided in two parts. Thereafter, dehydration continued in 96% and 100% ethanol and the tissue was embedded in paraffin. Paraffin- or cryo-sections were blocked and stained by standard methods. The biotinylated secondary antibodies were labelled with avidin-horseradish-peroxidase complexes using an ABC-kit (Vector Laboratories, CA, USA). The peroxidase was visualised with 3.3 diaminobenzidine (DAB) (Sigma, MI, USA) and the slides were contrasted using 1.5% methylthioninchloride. For staining with HABP deparaffinized sections were blocked with 0.02 mmol/L glycine for 10 min, followed by incubation with 2% normal goat serum (Vector Laboratories, CA, USA). Endogenous peroxidase activity was blocked by incubation with 1% H₂O₂ in methanol for 15 min at 20°C. Thereafter the sections were incubated for 18 hours at 4°C with
biotinylated hyaluronic acid binding protein (HABP) (1:250) (Seikagaku, Japan). The biotinylated HABP was labeled with ABC-kit (Vector Laboratories, CA, USA) and visualized with DAB as above. Finally, the slides were dehydrated and mounted. Some sections were pre-treated with 1 mg/ml (825 U/ml) hyaluronidase (Sigma, MI, USA) at 37°C for 30 min serving as negative control for the staining procedure. We used a mouse blocking system (Lab Vision Corporation, Fremont, CA, USA) when the primary antibodies were of murine origin (αSM-actin). For each staining we included a control in which the primary antibody was omitted. Paraffin sections were also processed for staining of the elastic membranes with orcein.

**Determination of HA-molecular weight (MW)**

The aortas from six transgenic (Tg) and four wild-type (WT) mice were dissected and chopped into small pieces that were distributed in two wells (96-well-plate) and incubated at 37°C for 24 hours in MEM containing 40 μCi D-[6-3H]-glucosamine-hydrochloride (Amersham Biosciences, UK). Tissue and medium were separated and saved at -80°C. Tissue from two mice were thawed, pooled, homogenized and extracted with 4 mmol/L guanidinium-chloride, 50 mmol/L CH₃COOH, 5 mmol/L benzamidine HCl, 0.5 mmol/L N-Ethylmaleimide, 1 mmol/L PMSF, pH 5.8. Samples were centrifuged at 30.000 g at 4°C. The supernatants were diluted x 20 in urea buffer (6 mol/L CH₄N₂O, 50 mmol/L CH₃COOH, 10 mmol/L EDTA, 5 mmol/L N-Ethylmaleimide, 5 μg/ml albumin, pH 5.8) and passed through a DEAE-52 column. The HA-containing fractions were eluted with 6 mol/L CH₄N₂O, 0.6 mol/L CH₃COOH, 10 mmol/L EDTA, 5 mmol/L N-ethylmaleimide, and 5 μg/ml albumin, pH 5.8. The eluate was dialyzed (25 mmol/L NH₄HCO₃) and freeze-dried twice. The samples were resuspended in 0.1 mol/L CH₃COONH₄, pH 7.2, centrifuged and the supernatants were saved. One part was treated with Hyaluronidase (*Streptomyces hyalurolyticus*) (15 U/μl, Calbiochem, VWR Int. GmbH, Germany) at 37°C for 1 h, the other part was left
untreated. All samples were counted in a scintillation counter, 50 µl was injected and separated by MW using a Superose®FLPC® column (PhamaciaBiotech) at a flow rate of 0.5 ml/min. Fractions were collected and counted.

**Electron microscopy (EM)**

Anaesthetized mice (90 mg/kg BW Mebumal ip) were sacrificed by opening the chest and perfusion through the heart with cold 4% formaldehyde (100 mmHg). The aortic arch and the descending aorta to the level of arteria renalis were separated from the lower part of the aorta. The upper part was cut into cross-sectional rings A-E, A being close to the heart. The first ring (A) was stained with 1% osmium and 2% tannic acid to enhance the contrast of the elastic membranes. The tissue was embedded in Epon for electron microscopy and 50 nm sections were examined ultrastructurally with a digitalized Philips CM10 electron microscope (SIS 3.0). All measurements were performed in a blinded fashion. The width of 4-7 elastic lamellae, including membrana elastica interna and externa, and the distance between the lamellae, were measured at two or four positions chosen at random using the orthogonal intercept lengths in a plane section. Mean values of the parameters were obtained for each mouse before group means were calculated. All vascular profiles were scored 0-2 by a blinded observer according to the amount of the intercellular ground substance (clear areas) and the degree of elastin fragmentation (0=absent/low, 1=moderate, 2=severe).

**Mechanical testing and hydroxyproline content**

Freshly excised aortas were coded and stored at –20°C until analysis in a 50 mmol/L Tris-HCl (pH 7.4) buffer used throughout the testing procedure. Before testing the lower thoracic aorta was cleaned of adhering fat and loose connective tissue. One-mm-high aortic ring specimens (in average
3.5 specimens per aorta) were cut between intercostal arteries. For mechanical testing, each specimen was mounted in a materials testing machine (Alwetron TCT5, Lorentzen & Wettre, Stockholm, Sweden), i.e. with two parallel steel wires (d=0.35 mm) through the lumen. Then the specimen was stretched (10 mm/min) until failure by pulling the wires apart in a direction perpendicular to the wires while soaking in buffer at room temperature. Following failure the collagen content in the ring specimen was estimated by hydroxyproline determination. From the on-line recorded load-deformation values the load-strain curve was calculated and maximal load and maximal stiffness (i.e. maximal slope of the load strain curve) were derived. In calculation of strain, the original circumference of the ring specimen was defined as the circumference at which the specimen attained a minimal load (1% of maximum). Mean values of the parameters were obtained for each mouse before group means were calculated.

**Analysis of atherosclerosis in hHAS2⁺ApoE -/- and hHAS2⁻ApoE -/- transgenic mice**

After 16-20 weeks on normal diet, hHAS2⁺ApoE -/- and hHAS2⁻ApoE -/- littermates were perfused with PBS under anesthesia (Mebumal 90 mg/kg), and the heart and entire aorta were removed. With standardized interval (80 µm) sections of the aorta root were cut and stained for elastin. The beginning of the aortic root was recognized by the presence of circumferential elastic membranes and valve cusps. This section and two sections 80 and 160 µm downstream, were used to estimate the amount of plaque. The average atherosclerotic lesion area was determined using Olympus BX50 microscope and Analysis software. The extent of atherosclerosis in the aortic arch and thoracic aorta was measured en face. Cholesterol deposits were visualized by staining with Oil Red O. The surface area of the lesion within the arch was expressed as percentage of the arch covered by lesion calculated by means of Analysis software including multiple images alignment. The area with positive staining in the thoracic aorta was calculated by using a frame
(175,349.1 µm²/144 crosses (CAST, Olympus, Denmark)) that was moved stepwise, from side to side all along the aorta. All analyses were made in a coded fashion by the same investigator.

Reference List


