Intimal Deposition of Functional von Willebrand Factor in Atherogenesis

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Abstract—During the formation of intimal thickening in normocholesterolemic rabbits, von Willebrand factor (vWF) is increased in the endothelial cells (ECs) and deposited in the intima. We investigated whether this also occurs during cholesterol-induced plaque formation, whether the synthesis of vWF increases, and whether this influences platelet adhesion. Rabbits were fed a cholesterol-rich (0.3%) diet for 26 weeks. Thereafter, half of the animals received a normal diet for another 26 weeks (cholesterol withdrawal). To induce intimal thickening in normocholesterolemic rabbits, collars were positioned around the carotid artery. Arterial segments were studied using immunohistochemistry, reverse transcription–polymerase chain reaction, electron microscopy, and platelet adhesion tests. Cholesterol treatment induced plaque formation in the aorta. The ECs had a cuboidal aspect, showed a dense immunoreactivity for vWF, a pronounced rough endoplasmic reticulum, and numerous Weibel-Palade bodies. There were subendothelial vWF deposits in the plaques and vWF mRNA was significantly increased as compared with controls. Similar changes were seen after collar-induced intimal thickening. After cholesterol withdrawal, both vWF mRNA and the ultrastructural morphology of the ECs normalized, and the vWF deposits disappeared from the plaque. Perfusion studies with anticoagulated rabbit blood over cross-sections of atherosclerotic aortas revealed increased vWF-mediated platelet adhesion in the subendothelial plaque region. Whereas rabbit platelets perfused through the lumen adhered to the same extent to de-endothelialized aortas of normocholesterolemic and atherosclerotic rabbits, vWF mediated platelet adhesion to endothelium was observed in atherosclerotic but not in normal aortas. Our results show an increased synthesis and (sub)endothelial presence of vWF after vascular injury, with functional consequences for platelet deposition on the vessel wall. (Arterioscler Thromb Vasc Biol. 1999;19:2524-2534.)

Key Words: atherosclerosis ■ intima ■ reverse transcription–polymerase chain reaction ■ hyperlipidemia ■ von Willebrand factor

Intimal thickening is a site of predilection for the development of atherosclerotic plaques. By positioning a flexible silicone collar around the rabbit carotid artery, a localized intimal thickening is induced within 14 days,1–3 which is probably the consequence of both media damage due to collar placement and the retention of cytokines and/or growth factors by the collar.1 The intimal thickening develops via migration of smooth muscle cells (SMCs) from the media to the intima and further SMC proliferation, similar to other models of intimal thickening and to the human situation.

We previously showed that during intimal thickening, the immunoreactivity for von Willebrand factor (vWF) increases in the endothelial cells (ECs) and that vWF is deposited in quantities in the extracellular space of the intimal thickening.2 The glycoprotein vWF is synthesized by only 2 cell types: ECs and megakaryocytes. One can discriminate the following 3 pools of vWF in the body: soluble plasma vWF, basement membrane (extracellular matrix) vWF, and cellular vWF found in storage granules of ECs and platelets (Weibel-Palade bodies and α-granules).4 The 3 pools contribute to adhesion of platelets and formation of a platelet plug during blood vessel injury. The basement membrane vWF may also promote EC adhesion in the intact vessel.4 Plasma vWF has an additional role in hemostasis because it carries factor VIII and protects it against proteolysis. Secretion of vWF from ECs can occur through 1 of the following 2 pathways: a constitutive pathway directly linked to synthesis (major pathway in cultured ECs) and a regulated pathway involving storage in the Weibel-Palade bodies and release after stimulation by secretagogues.5 In megakaryocytes and platelets, only the regulated pathway may be effectively operative.6 Accumulation of vWF in intimal thickening may be the result of increased synthesis and release by ECs, an influx from the plasma and/or platelets, or a decreased rate of removal of vWF from the extracellular matrix.7

The aim of the present study was to investigate whether the intimal deposition of vWF is due to an increased synthesis of...
the protein and whether subendothelial deposition of vWF also occurs after cholesterol-induced plaque formation and whether this has functional consequences for platelet adhesion.

Methods

Collar-Induced Intimal Thickening

Male New Zealand white rabbits (2.5 to 3 kg) (Rijksstation voor Kleinveeteelt, Merelbeke, Belgium) were anesthetized with sodium pentobarbital (30 mg/kg IV). Both carotid arteries were surgically exposed and dissected from the surrounding tissues. A soft silicone collar was placed around the left carotid artery. The right carotid artery was sham operated; i.e., it was isolated from the surrounding connective tissue and the vagus nerve and was exposed to a similar stretch as the contralateral collared artery. All the rabbits of the collar group were fed a normal diet. Animals were killed by an overdose of pentobarbital at 7 days (transmission electron microscopy experiment) or 14 days. A segment of the sham-operated and collared carotid artery was fixed in 4% formalin. The remaining part was frozen as fast as possible in liquid nitrogen for RNA isolation.

Cholesterol-Induced Atherosclerotic Plaques

Male New Zealand white rabbits (2.8 to 3.5 kg) were fed a diet supplemented with a low-dose cholesterol (0.3%) for 26 weeks (n=20). Ten randomly selected animals were killed after this period (chol 26 wk group); the other animals were fed a normal diet for another 26 weeks (chol cholesterol withdrawal (chol wd) group). At this period, these animals were also killed for study. A control group was included. Serum was stored at -20°C and total cholesterol, LDL and VLDL cholesterol, and triglycerides were determined. For histological examination, a segment of the thoracic aorta was fixed in 4% formalin. The remaining part of the thoracic aorta of 4 rabbits of the control group, 4 rabbits of the cholesterol group, and 4 rabbits of the cholesterol withdrawal group was frozen as fast as possible in liquid nitrogen for RNA isolation. In a second experiment, platelet adhesion studies were performed (cf. infra).

In addition, 8 rabbits were given an even lower cholesterol diet (0.125%) for 9 weeks, and the evolution of platelet numbers was followed with time.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction Analysis

Total RNA was isolated using the guanidinium isothiocyanate and phenol/chloroform extraction method. Reverse transcription-polymerase chain reaction (RT-PCR) was performed with an automatic thermal cycler (MultiCycler PTC-200, MJ Research Inc) using a 1-step RT-PCR system (Titan, Boehringer Mannheim). The avian myoblastiosis virus–reverse transcriptase was applied for first strand synthesis and an enzyme blend, which consisted of Taq DNA polymerase and Pwo DNA polymerase for the PCR part. The following specific primers were used: rabbit vWF mRNA sense (5’ CAG CAA ACG GAG CCT GTC CT 3’ [BankGen accession No. S64544, positions 102 to 121]), rabbit vWF mRNA antisense (5’ AGC CAT GTG TTG CAG TCC AT 3’ [BankGen accession No. S64544, positions 352 to 371]). Rabbit β-actin mRNA sense (5’ GCC GCA CCA GGG CGT 3’ [BankGen accession No. X60733, positions 189 to 203]) and rabbit β-actin mRNA antisense (5’ ATG GCC GGC GTG TTG AAC 3’ [BankGen accession No. X60733, positions 453 to 470]) primers were used as controls. The final concentrations in the RT-PCR mixture (50 µL) were sense and antisense primer, 0.4 µmol/L each; dNTP, 0.2 mmol/L each; dithiothreitol, 5 mmol/L; MgCl₂, 1.5 mmol/L; RNA inhibitor, 8 U; total RNA, 1 µg; and enzyme mix, 1 µL. Reverse transcription was performed at 50°C for 30 minutes. The thermocycling parameters were denaturation of the template at 94°C for 2 minutes; 10 cycles consisting of incubations at 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 45 seconds; 25 cycles consisting of incubations at 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 45 seconds plus cycle elongation of 5 seconds for each cycle; followed by a prolonged elongation time of 7 minutes at 68°C. Products were analyzed by agarose gel electrophoresis (2.5%, Gibco BRL Life Technologies) and visualized by Sybr Green I nucleic acid gel stain (1:10 000 dilution of the commercially available stock solution, FMC BioProducts) under ultraviolet light. Quantification of the bands (optical density×mm²) was done using the Diversity One 1.1 software (pdi).

Immunohistochemistry

The immunohistochemical reactions were carried out by an indirect peroxidase antibody conjugate method using a polyclonal antibody raised in sheep against human factor VIII–related antigen (vWF), dilution 1:250 (Binding Site). To enhance antigenicity the sections were pretreated with 1 mg trypsin/mL tris(hydroxymethyl)aminomethane buffer, pH 7.8, at 37°C for 10 minutes (Fluka). The specificity of the primary antibody against vWF has been reported previously. The polyclonal sheep anti-vWF antibody was visualized by a pig anti-sheep peroxidase as a secondary antibody (Binding Site,) by using aminoethy lacarbazol as a chromogen. For negative controls, primary antibody was omitted.

The vWF deposits were quantified by using a color image analysis system (PC_image) as described elsewhere. For this purpose, a rectangle with a length of 100 µm along the luminal margin was drawn. The parallel segment of the internal elastic lamina formed the opposite outer border of this rectangle. Within each intimal rectangle, the area of vWF immunoreactivity (brown color) was measured in a standardized way. The cutoff value for the vWF immunoreactivity was defined by the RGB (red-green-blue) levels of the vWF staining in the cytoplasm of the ECs. Measurements were repeated within several intimal rectangles until the vWF deposits were quantified along the whole circumference of the artery. For each artery, summation of the data of all intimal rectangles was expressed as immunoreactive area (µm² per 100 µm ECs).

Double immuno stains were performed for vWF and α-SMC actin and for vWF and macrophages (RAM-11 antibody). The vWF antibody was visualized using a peroxidase technique and aminoethylcarbazol as a chromogen. The α-SMC actin or RAM-11 antibody was visualized using an alkaline phosphatase technique and Fast Blue BB salt (Sigma) as a chromogen.

The presence of adhering platelets in aorta segments from atherosclerotic rabbits was verified immunohistochemically using a monoclonal anti-GPIb antibody (G28E5) raised in our laboratory against human GPIbα-chain but cross-reactive with rabbit GPIbα. Bound G28E5 was detected via secondary peroxidase conjugated goat antiovine-1g antibodies (Dako) and staining essentially as reported by van Zanten et al[19] using diaminobenzidine (0.5 mg/mL) supplemented with CoCl₂ (0.25 mg/mL) and NiNH₄SO₄ (0.2 mg/mL) in 50 mmol/L Tris-HCl buffer, pH 7.6, to increase the sensitivity of detection.

Transmission Electron Microscopy

Segments of the sham-operated and collared carotid artery (collar experiment) and the thoracic aorta (cholesterol and cholesterol withdrawal group) were cut in small arterial rings. The rings were rinsed in a cacodylate buffer and were postfixed in glutaraldehyde 0.5% (vol/vol) in 0.1 mol/L sodium cacodylate buffer (pH 7.4) for 24 hours. After fixation, the specimens were washed with sucrose phosphate buffer and postfixed for 30 minutes in 1% (vol/vol) osmium tetroxide in 0.1 mol/L sodium cacodylate buffer (pH 7.4). After dehydration in ethanol and propylene oxide, they were embedded in Epon. Selection of the zones most characteristic for the lesions was done on semithin sections stained with toluidine blue. Thin sections were cut with an LKB ultratome, stained with 2% uranyl acetate, and examined with a Jeol 1200 EX electron microscope at 80 kV.

In Vitro Platelet-Vessel Wall Interactions Under Flow Conditions

Rabbit aorta cross-sections (7 µm) were mounted on Dünhardt coated coverslips as described elsewhere[8-11] and were perfused in a parallel-plate flow chamber at 1300 s⁻¹ with normal citrate anticoagulated rabbit blood for 5 minutes. After perfusion, the sections were fixed with 0.5% glutaraldehyde and bound platelets were detected with the anti-GPIbα antibody G28E5, as described above.
In addition, the thoracic aorta from normocholesterolemic and atherosclerotic rabbits was harvested, divided in segments, and opened longitudinally. After careful de-endothelialization of some segments with cotton-wool sticks, both nondamaged and damaged rabbit aorta segments were mounted in a circular perfusion chamber. The aorta segment (0.5 cm² exposed to the perfusate) was perfused for 5 minutes at 1100 s⁻¹ with normal anticoagulated rabbit blood supplemented with autologous 111In-labeled rabbit platelets (1 to 2×10⁶ cpm/mL blood). As a consequence of the labeled platelet addition, total platelet numbers increased by 15% to 20%. After perfusion, segments were carefully rinsed, and the number of bound platelets (radioactivity measurement) was calculated as a function of the total platelet number in the perfusate. The role of vWF in platelet deposition was investigated by performing perfusions in the presence of increasing concentrations of the antihuman vWF antibody Ajv-W-2 (0 to 10 μg/mL) (gift of Ajinomoto Co, Inc, Yokohama, Japan, code Ajv-W-2), cross-reactive with the A1 domain of rabbit vWF and neutralizing vWF binding to its receptor on the platelet, the GPIb/VIIa complex. The specificity of platelet binding was further investigated in the presence of inhibitors of platelet activation such as iloprost (1 μg/mL, Iomédine, Schering-Plough), the NO-donor sodium nitroprusside (100 μmol/L, Sigma), and prostaglandin E₁ (2 μmol/L, Sigma). In addition, perfusions were performed with the NO synthase inhibitor Nω-nitro-L-arginine methyl ester (400 μmol/L, Sigma).

Statistical Analysis
In the collar experiment, the sham-operated and collared arteries were compared using the 2-tailed paired Student’s t test. In the cholesterol experiment control group, cholesterol group and cholesterol withdrawal groups were compared using ANOVA followed by the Student-Newman-Keuls test. The serum lipid values at the start of the experiment and after 26 weeks of cholesterol supplementation or 26 weeks cholesterol followed by 26 weeks cholesterol withdrawal were compared using the paired Student’s t test. Comparison of the degree of platelet adhesion with rabbit (sub-)endothelium was done via the Kruskal-Wallis test. The number of circulating platelets of the degree of platelet adhesion with rabbit (sub-)endothelium was done via the Kruskal-Wallis test. The number of circulating platelets was applied for these purposes. Differences were considered significant at P<0.05.

Results
Serum Lipid Values
After feeding rabbits a cholesterol-rich diet (0.3%) for 26 weeks the total serum cholesterol levels increased from 43±6 to 1245±148 mg/dL (P<0.001), the LDL cholesterol levels from 7±1 to 396±140 mg/dL (P<0.05), and the VLDL cholesterol levels from 20±8 to 412±46 mg/dL (P<0.001). The triglyceride levels did not significantly change (from 91±12 to 105±45 mg/dL). After 26 weeks of cholesterol withdrawal, the total cholesterol normalized to 28±2 mg/dL, the LDL cholesterol to 6±1 mg/dL, and VLDL to 8±2 mg/dL. The level of the triglycerides amounted to 65±9 mg/dL.

vWF mRNA
Intimal Thickening
vWF mRNA was detected both in sham-operated and collared arteries as the expected 270-bp amplified product. After collaring the RT-PCR signal for vWF mRNA was significantly increased (P<0.05, n=5). Omitting either the RNA or the RT step did not result in a band on the gel. The signal for β-actin mRNA, which was used as internal control, did not significantly differ between the sham-operated and collared arteries (Figures 1A and 2A). The ratio of vWF mRNA to β-actin mRNA was significantly increased (0.81±0.07 versus 0.48±0.05 in sham, P<0.05, n=5).

vWF Deposition
Intimal Thickening
The sham-operated carotid arteries were lined by ECs showing a granular staining for vWF. Deposition of vWF in the subendothelium or the media was not found (Figure 3A). After 14 days of collar placement, a thickening of the intima composed of α-SMC actin-positive SMC had formed. The ECs showed a dense flocculent immunoreactivity for vWF. vWF was deposited in the matrix of the intimal thickening (Figure 3B). Macrophages were not present in the intimal...
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mRNA, which was used as a control, was not significantly differ-
ent. The localization of the vWF deposits was further docu-
ted using double immunostaining for vWF and α-SMC actin and for vWF and macrophages (RAM-11 stain). In the thoracic aorta of rabbits fed a diet supplemented with 0.3% cholesterol for 26 weeks, vWF-immunoreactive granules were present in the ECs, and vWF was deposited in the spaces between the SMCs of the plaque (Figure 3I). There was an accumulation of macrophages in the plaque, but vWF was not present within these macrophages (Figure 3J). After 26 weeks of cholesterol withdrawal, the vWF deposits had disappeared from the plaque (Figures 3K and 3L). Rare macrophages remained in the plaque (Figure 3L), and vWF immunoreac-
tivity was present in the ECs.

Transmission Electron Microscopy

Intimal Thickening

Transmission electron microscopy showed that after collar-
ing, the ECs had lost their normal flattened shape, became cuboidal, and contained a pronounced rough endoplasmic reticulum. Weibel-Palade bodies, which are single-
membrane-bound cylindrical rod-like bodies that contain a number of microtubules set in an electron-dense matrix, were more abundant in comparison with sham-operated carotid arteries (Figures 5A and 5B).

Cholesterol-Induced Atherosclerotic Plaques

After 26 weeks of cholesterol feeding, the plaques were covered by an uninterrupted layer of ECs with a cuboidal aspect and a pronounced rough endoplasmic reticulum. Weibel-Palade bodies were abundantly present (Figure 6A). However, after 26 weeks of cholesterol withdrawal, the plaques were covered by an uninterrupted layer of ECs, which had become flattened, similar to control arteries, and their ultrastructural morphology had normalized. Weibel-Palade bodies were scarcely present (Figure 6B).

Platelet-Vessel Wall Interactions

Mild cholesterol feeding (0.125%) was associated with a progressive reduction in circulating platelet numbers from 669 000 ± 54 000 platelets/μL platelet-rich plasma at day 0 to 551 000 ± 76 000 platelets/μL at the end of the 9th week (P = 0.0026). The immunohistochemical detection of vessel wall associated platelets via the platelet membrane protein GPIbα confirmed the presence of essentially single adhering platelets to the aorta endothelium of cholesterol-fed rabbits but not to normocholesterolemic rabbits. Therefore, perfusion experiments were performed with normal rabbit blood over cross-sections prepared from the aorta and the carotid artery of hypercholesterolemic and normocholesterolemic rabbits.

Platelet Adhesion to Aorta Cross-Sections

In agreement with similar perfusions performed over human lesions, platelet deposition on normal aorta occurred pri-
marily in the adventitia (Figures 7A and 7C). However, the weak adhesion observed in the media area was concentration-dependently inhibited by AJvW-2, confirming that platelet adhesion occurred in a vWF-dependent fashion to vessel wall collagens. Higher AJvW-2 concentrations were required to prevent adventitial platelet adhesion than medial adhesion (Figure 7E). Perfusion with normal rabbit blood over atherosclerotic cross-sections revealed a higher platelet deposition both in the medial and the intimal area (Figures 7B and 7D). Except for the adventitial platelet deposition, aggregate formation was concentration-dependently inhibited by AJvW-2 (Figure 7F). These experiments suggested that the higher platelet reactivity with the atherosclerotic rabbit vessel wall was related to vWF but did not enable distinction between platelet adhesion via vascular vWF and plasma vWF-mediated platelet binding to cross-section exposed collagens. Therefore, additional perfusions were performed over nonde-nuded rabbit endothelium of hypercholesterolemic and normocholesterolemic rabbits and over de-endothelialized subendothelium.

Figure 3. A, Sham-operated carotid artery at day 14. The ECs show a granular staining for vWF. Arrowhead indicates internal elastic lamina. Bar=10 μm. B, Carotid artery collared for 14 days. The ECs demonstrate a dense immunoreactivity for vWF. Deposition of vWF is present in the luminal part of the extracellular matrix of the intimal thickening. Arrowhead indicates internal elastic lamina. Bar=10 μm. C, Control rabbit thoracic aorta. The ECs show a granular staining for vWF. Arrowhead indicates internal elastic lamina. Bar=10 μm. D, Thoracic aorta of a rabbit fed a diet supplemented with 0.3% cholesterol for 26 weeks. vWF deposition was diffusely present in the plaque. Arrowhead indicates internal elastic lamina. Bar=10 μm. E, Thoracic aorta of a rabbit fed a diet supplemented with 0.3% cholesterol for 26 weeks. The ECs demonstrate a dense immunoreactivity for vWF. Deposits of vWF are prominently present in the luminal part of the plaque, just beneath the ECs. Bar=10 μm. F, Thoracic aorta of a rabbit fed a diet supplemented with 0.3% cholesterol for 26 weeks. vWF deposits are present in the deeper regions of the plaque. Arrowhead indicates internal elastic lamina. Bar=10 μm. G, Thoracic aorta after 26 weeks cholesterol withdrawal. The ECs demonstrate immunoreactivity for vWF. The vWF deposits in the plaque have disappeared. Arrowhead indicates internal elastic lamina. Bar=10 μm. H, Higher magnification of photomicrograph G. Bar =10 μm. I, Double immunostain for vWF (brown) and α-SMC actin (blue) of a thoracic aorta of a rabbit fed a diet supplemented with 0.3% cholesterol for 26 weeks. The vWF-immunoreactive granules are present in the ECs and deposited in the spaces between the SMCs of the plaque. Bar=10 μm. J, Double immunostain for vWF (brown) and macrophages (RAM-11 stain, blue) of a thoracic aorta of a rabbit fed a diet supplemented with 0.3% cholesterol for 26 weeks. In the plaque there is an accumulation of macrophages. vWF is not present within macrophages. Bar=10 μm. K, Double immunostain for vWF (brown) and α-SMC actin (blue) of a rabbit thoracic aorta after 26 weeks of cholesterol withdrawal. The vWF deposits have disappeared from the plaque. vWF immunoreactivity is only present in the ECs. Arrowhead indicates internal elastic lamina. Bar=10 μm. L, Double immunostain for vWF (brown) and macrophages (RAM-11 stain, blue) of a rabbit thoracic aorta after 26 weeks of cholesterol withdrawal. Rare macrophages remain in the plaque. The vWF deposits in the plaque have disappeared. vWF immunoreactivity is present in the ECs. Arrowhead indicates internal elastic lamina. Bar=10 μm.
Platelet Adhesion to Endothelium From Normocholesterolemic and Hypercholesterolemic Rabbits

During the perfusion in a cylindrical flow chamber of cotton-wool damaged subendothelium was inhibitory, as evident from radioactivity measurements in postperfusion plasma (not shown) and from control experiments in the presence of cytokines IL-1β, monocytes, and sodium nitroprusside, which substances had no effect on this background signal (not shown). Similarly, neither AJvW-2 nor NAD-dependent nitric oxide synthase had any effect on this low value (not shown).

In contrast to non-denuded controls, perfusions over non-denuded atherosclerotic aorta revealed specific platelet adhesion (P=0.001; Figure 8A). This specific platelet adhesion (ie, corrected for background signal) equaled 0.015±0.005% of circulating platelets, corresponding to an average specific deposition around 600 000 platelets/cm² endothelium. This adhesion was concentration-dependently inhibited by AJvW-2 with an IC₅₀ around 2.5 μg/mL (Figure 8B). Perfusions at 300 s⁻¹, revealed no specific platelet adhesion (Figure 8A), in agreement with the absence of vWF interactions with its receptor on platelets at these low shear rates.

Discussion

In normal arteries, the ECs secrete vWF both in the plasma and toward the subendothelial space, where a minimal deposition of this glycoadhesive protein is present in the basal lamina and plays a role in the adhesion of the ECs. However, ECs can also be stimulated to release additional amounts of vWF basolaterally under conditions of “stress” (eg, hemodynamic changes). In cultured ECs, approximately 5% of the newly synthesized vWF is directed to the Weibel-Palade bodies. The majority of the protein is secreted constitutively.

The present study showed that cholesterol-induced plaque formation was associated with an increased ratio of vWF mRNA to β-actin mRNA and deposition of vWF in the plaque. The plaques that covered the plaques formed an unruptured layer but showed cytoplasmic vWF accumulation, a pronounced rough endoplasmic reticulum, and abundant Weibel-Palade bodies, similar to advanced human atherosclerotic plaques. Therefore, our results point to an increased synthesis of vWF by the ECs after a cholesterol-rich diet. The reason for the vWF deposition is unclear. Hypercholesterolemia may be a factor that can regulate vWF gene transcription, as suggested by the present study. This could be explained by the presence of inflammatory mediators in atherosclerotic plaques or by a direct action of cholesterol on vWF synthesis. It has been shown that activated transcription factor nuclear factor-κB (NF-κB) is present in the ECs covering an atherosclerotic lesion. Activation of NF-κB by oxidized-LDL may lead to transcription and enhanced synthesis of adhesion molecules but possibly also of vWF. In addition, in cultured ECs, LDL enhances the concentration of cell-related vWF. In rabbits on a 0.3% cholesterol diet the increase of serum cholesterol levels is only partly due to the increase of LDL cholesterol levels; VLDL cholesterol levels are also increased. LDL cholesterol and βVLDL cholesterol may have different effects on endothelium. However, a dense immunoreactivity for vWF was only present in ECs covering an atherosclerotic plaque or intimal thickening. In those parts of the aorta or carotid artery that were lesion-free, the ECs showed a normal vWF expression despite that they had been in contact with the same amount of blood lipids.

Qualitative immunohistochemical and quantitative platelet adhesion studies showed that in atherosclerotic aortas there was an increased adhesion of platelets to the ECs, which was mediated by vWF, possibly explaining the drop of platelet numbers throughout atherogenesis. After mechanical removal of the ECs, the degree of platelet adhesion to the subendothelium was similar in atherosclerotic and control aortas. Perfusion studies over cross-sections of atherosclerotic aortas revealed increased vWF-mediated adhesion in the plaque area in comparison with normal sections. This shows that an increased platelet adhesion results both from a direct effect mediated via (sub)endothelial vWF and an indirect effect exerted via reactive plaque collagens, this binding, however, being mediated via plasma vWF. It is conceivable that the
increased production of vWF by ECs not only resulted in subendothelial vWF deposition but probably also in an increase in plasma vWF. High vWF levels may reflect endothelial dysfunction and may be an indicator of poor prognosis of cardiovascular events.23–27 However, we were unable to measure vWF levels in rabbit plasma.

Finally, we demonstrated that cholesterol withdrawal normalized vWF mRNA levels, the EC morphology, and their number of Weibel-Palade bodies, and it led to the disappearance of the vWF deposits from the plaques. Possibly, vWF is taken up by macrophages, which subsequently disappear from the plaques after cessation of cholesterol feeding.14 However, we did not find a colocalization of vWF and RAM-11.

Also in a collar-induced intimal thickening that almost completely consisted of smooth muscle cells, an increase in
Figure 6. Transmission electron photomicrographs. A, Thoracic aorta of a rabbit fed a cholesterol-rich (0.3%) diet for 26 weeks. The ECs are cuboidal and show a pronounced rough endoplasmic reticulum (RER) and many Weibel-Palade bodies (WP). Bar=1 μm. B, Thoracic aorta after 26 weeks of cholesterol withdrawal. The ECs have a normal flattened shape. The RER is less pronounced and WP-bodies are scarcely present. Bar=1 μm.
Figure 7. Platelet adhesion to aorta cross-sections. Immunocytochemical staining of GPIbα on platelets deposited on rabbit aorta cross-sections of normocholesterolemic (A, C, and E) and hypercholesterolemic (B, D, and F) rabbits after perfusion with rabbit anticoagulated blood in a parallel plate flow chamber at a shear rate of 1300 s⁻¹ for 5 minutes in the absence (A, B, C, and D) and presence (E and F) of the neutralizing anti-vWF antibody AJvW-2 (10 μg/mL). The position of the internal elastic lamina is indicated by arrowheads. Bar=500 μm (A and B) or 50 μm (C, D, E, and F).
vWF mRNA, deposition of vWF in the intima, and changes in the ultrastructural morphology of the ECs were seen, although the rabbits were normocholesterolemic. In this case, the deposition of vWF could be related to shear stress, an increased biosynthesis and/or release of vWF mediated by inflammatory stimuli.

The increased synthesis and intimal deposition of vWF in atherosclerotic plaques and intimal thickenings may have important implications for human vascular disease. The possible importance of vWF deposition is underscored by the fact that it is not limited to atherosclerotic plaques and intimal thickenings but that it occurs after balloon angioplasty, in an increased adhesion of platelets to the vessel wall, as compared with normal aortas. Also, the subendothelial vWF deposits were functional with regard to platelet adhesion in the plaque, as shown by both types of perfusion studies. This may have important clinical consequences, eg, during plaque rupture when vWF deposits in the plaque become exposed to blood. It has been shown that vWF is essential for the development of occlusive thrombosis and that it appears to support the progression of a platelet-fibrin microthrombus to an occlusive thrombus both after cholesterol-induced atherosclerosis and after stenosis and pinch-injury in the pig. Although severe von Willebrand disease did not protect completely against the development of atherosclerosis, autopsy findings in 3 patients with von Willebrand disease types IIB and III revealed the presence of atherosclerotic lesions without occlusive arterial thrombi. The organization and incorporation of mural thrombi appears to be an important component of atherosclerotic plaque growth.

In conclusion, our results show an enhanced synthesis and subendothelial deposition of vWF during atherogenesis in rabbits. The raised vWF production facilitated platelet adhesion to activated endothelium and the intraplaque vWF deposits remained functional and may promote platelet adhesion on plaque rupture. These findings may be relevant for our understanding of endothelial cell activation during atherogenesis.

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References


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