Induction of Rapid Atherogenesis by Perivascular Carotid Collar Placement in Apolipoprotein E–Deficient and Low-Density Lipoprotein Receptor–Deficient Mice

Jan H. von der Thüsen, MD; Theo J.C. van Berkel, PhD; Erik A.L. Biessen, PhD

Background—Perivascular collar placement has been used as a means for localized atherosclerosis induction in a variety of experimental animal species. In mice, however, atherosclerosis-like lesions have thus far not been obtained by this method. The aim of this study was the development of a mouse model of rapid, site-controlled atherogenesis.

Methods and Results—Silastic collars were placed around the carotid arteries of apolipoprotein E–deficient (apoE

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−/−) and LDL receptor–deficient (LDLr

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−/−) mice. The development of collar-induced lesions was found to occur predominantly in the area proximal to the collar and to be dependent on a high-cholesterol diet. Lesions were evident in apoE

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−/− mice after 3 weeks and in LDLr

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−/− mice after 6 weeks and were overtly atherosclerotic in appearance. Lumen stenosis reached 85% in apoE

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−/− mice and 61% in LDLr

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−/− mice 6 weeks after collar insertion. Expression levels of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 were increased both proximal and distal to the collar, whereas endothelial nitric oxide synthase expression was downregulated at the proximal site.

Conclusions—We propose that this model of collar-induced acceleration of carotid atherogenesis is of hemodynamic cause. It may serve as a substrate for sequential mechanistic studies concerned with the underlying cause and pathogenesis of atherosclerosis. The rapidity of lesion development will also aid the efficient screening of new potentially antiatherogenic chemical entities and the evaluation of therapies with limited duration of effectiveness, such as adenoviral gene therapy. (Circulation. 2001;103:1164-1170.)

Key Words: atherosclerosis ■ carotid arteries ■ cell adhesion molecules ■ hemodynamics

Atherosclerosis is a common and complex pathological process characterized by intimal foam cell accumulation and extracellular matrix deposition in medium and large-sized arteries.1 In the pursuit of a representative, reproducible, and practical model for this disease, several animal species and atherogenic stimuli have been used. Systemic approaches have included diet-induced hypercholesterolemia2 and genetically modified mouse models, including LDL receptor knockout (LDLr

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−/−),3 apolipoprotein E knockout (apoE

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−/−),4 and apolipoprotein E3-Leiden transgenic5 strains. Local lesion induction has been achieved by transluminal (eg, wire denudation)6 or extravascular (eg, perivascular collar placement)7 arterial manipulation. Perivascular collar placement offers the advantage of maintaining the structural integrity of the endothelium while inducing rapid, site-controlled lesion formation. The neointima in perivascular collar models tends to occur within the collar and is generally fibroproliferative in appearance, with limited foam cell formation and extracellular lipid deposition.8,9 Cholesterol feeding7 or local (oxidized) LDL application11 in conjunction with the placement of a perivascular Silastic collar have been shown to promote the development of more atherosclerosis-like lesions in the rabbit carotid artery. The underlying mechanism, however, appears to remain in essence fibroproliferative, and it could, therefore, be valuable to study the effect of perivascular collar placement in animals that are more susceptible to spontaneous and humanlike atherosclerosis. The aforementioned transgenic mouse models are eminently suited to this purpose because they are predisposed to developing severe atherosclerotic lesions, particularly when fed a high-fat diet.

The aim of this study was to evaluate the intimal response of the carotid artery to Silastic collar placement in apoE

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−/− and LDLr

2

−/− mice, which are known to spontaneously develop extensive and complex atherosclerotic lesions. On an atherogenic diet, LDLr

2

−/− mice have relatively fibroproliferative intimal lesions at sites of hemodynamic predilection,3 whereas lesions in apoE

2

−/− mice are more heterogeneous and lipid-rich, even when a regular chow diet is given.4 We hypothesized that the flow disturbance caused by the placement of a mildly constrictive perivascular collar could result in site-controlled atherogenesis in these mouse strains; therefore, we applied such collars to the midsection of the common carotid artery, an easily accessible site of low natural occur-

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Figure 1. Schematic representation of carotid collar positioning and assessment sites.

herence of atherosclerosis. The extent as well as the composition of the lesions thus obtained was assessed to identify any potential differences in lesion development between the strains, thereby validating their relative value as a substrate for focal induction of atherogenesis. In addition, we used immunohistochemistry to determine endothelial integrity (von Willebrand factor [vWF]) and the expression levels of endothelial cell nitric oxide synthase (eNOS) and two endothelial adhesion molecules that are known to be involved in the development of atherosclerosis in humans (intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 [ICAM-1 and VCAM-1]) to further elucidate the underlying mechanisms of lesion formation in this model.

Methods

Animals

All animal work was approved by the regulatory authority of Leiden University and carried out in compliance with Dutch government guidelines. Male LDLr−/− and apoE−/− mice were acquired from TNO-PG and were 10 to 12 weeks old at the time of entry into the study. Unless otherwise stated, the animals received a Western-type diet (0.25% cholesterol and 15% cocoa butter, Hope Farms) 2 weeks before surgery. Diet and water were provided ad libitum.

Carotid Collar Placement

Collars were prepared from Silastic tubing (Dow Corning) and stored in 70% ethanol until further use. An inside diameter of 0.5 mm was chosen because an inside diameter of 0.3 mm resulted in a stenosis of ~30% because the average outside diameter of the common carotid at 80 mm Hg perfusion pressure was 0.36 mm. Mice were anesthetized by subcutaneous injection of ketamine (75 mg/kg, Eurovet), droperidol (1 mg/kg), fluanisone (0.75 mg/kg), and fentanyl (0.04 mg/kg) (all from Janssen-Cilag). Access to the anterior cervical triangles was gained through a sagittal anterior neck incision. Both carotid sheaths were opened, and the common carotid arteries were dissected free from the surrounding connective tissue, avoiding damage to the vagus nerves and the carotid bodies. The experimental animals, the neck wound was closed in one layer with interrupted silk sutures at this point (6-0, Braun). In the remaining animals, the wound was placed bilaterally around the common carotid arteries, and their axial edges were approximated by placement of 3 circumferential silk ties (Figure 1). Subsequently, the entry wound was closed and the animals were returned to their cage for recovery from anesthesia.

Histology and Immunohistochemistry

Cryosections were routinely stained with hematoxylin (Sigma Diagnostics) and eosin (Merck Diagnostica) and with oil red O (Sigma Diagnostics) for lipid visualization. Corresponding sections on separate slides were stained immunohistochemically with antibodies against a macrophage-specific antigen (MOMA-2, polyclonal rat IgG2a, diluted 1:10; Research Diagnostics Inc), α-smooth muscle cell actin (monoclonal mouse IgG2a (clone 1A4), dilution 1:500; Sigma), ICAM-1 (monoclonal rat IgG2a (clone BSA2), dilution 1:200; R&D Systems), VCAM-1 (monoclonal rat IgG2a (clone 429), dilution 1:100; Pharmingen), eNOS (monoclonal rat IgG1 (clone 3), dilution 1:20; Transduction Laboratories), and vWF (peroxidase-conjugated polyclonal rabbit Ig, dilution 1:100; Dako). The slides were incubated with primary antibody for 2 hours at room temperature, except for anti–VCAM-1 (+4°C overnight). Goat anti-mouse IgG peroxidase conjugate (dilute 1:100 and 1:500; Nordic) and goat anti-rat IgG alkaline phosphatase conjugate (dilution 1:100; Sigma Diagnostics) were used as secondary antibodies (1-hour incubation at room temperature), with 3,3′-diaminobenzidine (Sigma Diagnostics), nitro blue tetrazolium (Sigma Diagnostics), and 5-bromo-4-chloro-3-indolyl phosphate (Sigma Diagnostics) as enzyme substrates.

Morphometry

Hematoxylin and eosin–stained sections were used for morphometric analysis. Each vessel was assessed in cross section at 3 levels: 0.5 mm proximal, in the mid-section, and 0.5 mm distal to the collar (Figure 1). The images were analyzed with a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems). The intimal surface area was calculated by subtracting the patent lumen area from the area circumscribed by the internal elastic lamina, whereas the medial surface area was defined as the area between the internal elastic lamina and the external elastic lamina. The intima/media ratio and the intima/lumen ratio were determined by dividing the intimal area by the medial area and the total area confined by the internal elastic lamina, respectively.

Cholesterol Assay

Nonfasting serum samples were obtained by femoral artery transection. Total serum cholesterol levels were quantified colorimetrically by enzymatic procedures (Roche), with Precipath used as internal standard.

Statistical Analysis

All groups consisted of 3 animals. Values are expressed as mean±SD. Data variance was analyzed by 1-way and 2-way ANOVA. A 2-tailed Student’s t test was used in the comparison of individual groups, which was used in a nonpaired form when comparing different animals and paired when comparing contralateral values in the same animal. A level of P<0.05 was considered significant.

Results

After recovery from anesthesia, the presence of perivascular collars did not affect normal functioning of the animals. This was corroborated by the absence of a difference in body weight or lipid levels between collar-treated animals and sham-operated control animals. There was no evidence of
collar-induced thrombosis in the analyzed common carotid arteries at any time point of analysis (from 1 day to 18 weeks). The data discussed below were derived from the right carotid arteries; these were not found to differ significantly from the contralateral side.

**Lesion Size**

One week after bilateral insertion of a 0.3-mm diameter collar, intimal thickening was not detectable in either strain (Figure 2 and Table 1). By 3 weeks, however, a neointima had appeared in the area proximal to the collar in apoE \(2/2\) mice. Six weeks after collar insertion, a significant increase in intimal surface area had occurred in both strains, although this was more extensive in the apoE \(2/2\) group (1.46±0.50×

\[10^5 \mu m^2\] in apoE \(2/2\), 0.59±0.23×10^5 \mu m^2 in LDLr \(2/2\); \(P<0.05\) compared with sham-operated in both strains). The increase with time in neointimal surface proximal to the collar was found to be significant in both apoE \(2/2\) and LDLr \(2/2\) mice (\(P<0.01\), 1-way ANOVA). The intimal area within and distal to the collar remained virtually unaltered at all time points examined; these values were confirmed to differ significantly from the values obtained at the proximal site (\(P<0.0001\), 2-way ANOVA). The medial surface area did not rise significantly with time at any site in either strain, and medial SMC loss was not found to have occurred at the time points examined (data not shown). No plaques were found in the corresponding sites of the common carotid arteries of sham-operated animals of either strain (Figure 3). The intima-media ratios of the proximal site are in agreement with the absolute data, being highest in both strains 6 weeks after insertion (apoE \(2/2\), 2.96±1.72; LDLr \(2/2\), 1.42±0.88). The degree of proximal lumen stenosis (as ex-

**Figure 2.** Cross-sectional intimal surface area (A and B), intima-media ratio (C and D), and degree of lumen stenosis (E and F) in collar-treated carotid arteries of apoE \(2/2\) and LDLr \(2/2\) mice. Animals were fed Western-type diet; lesion formation was assessed at 1, 3, and 6 weeks (\(n=3\) at each time point). Error bars represent SD. *Significant difference from sham-operated animals (assessed at 6 weeks, \(t\) test, \(P<0.05\)).

**Figure 3.** Cross-sectional intimal surface area in apoE \(2/2\) (A) and LDLr \(2/2\) (B) mice. Carotid arteries were analyzed at 6 weeks, after treatment with collar of 0.3 or 0.5 mm diameter, or after sham surgery. Animals were maintained on chow or Western-type diet. Error bars represent SD.

![Figure 2](http://circ.ahajournals.org/)

![Figure 3](http://circ.ahajournals.org/)

<table>
<thead>
<tr>
<th>Collar Size/Diet</th>
<th>0.3 mm, Western Type</th>
<th>0.5 mm, Western Type</th>
<th>0.3 mm, Sham, Chow</th>
<th>0.5 mm, Sham, Western Type</th>
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</thead>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 1</td>
<td>0.07±0.04</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>Week 3</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Week 6</td>
<td>1.46±0.46</td>
<td>0.08±0.03</td>
<td>0.4±0.53</td>
<td>0.03±0.01</td>
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<tr>
<td>LDLr (2/2)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 1</td>
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<td>ND</td>
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<tr>
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<tr>
<td>Week 6</td>
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<td>0.01±0.003</td>
<td>0.006±0.004</td>
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<tr>
<td>ApoE3-Leiden</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 26</td>
<td>0.005±0.0001</td>
<td>ND</td>
<td>ND</td>
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</tr>
</tbody>
</table>

ND indicates not done. Values are expressed as \(10^5 \mu m^2\±SD.

TABLE 1. Cross-Sectional Intima Surface Area Proximal to Collar in ApoE \(2/2\), LDLr \(2/2\), and ApoE3-Leiden Transgenic Mice
pressed by the intima-lumen ratio) was highest in the apoE−/− mice, being 36±26% at 3 weeks and approaching occlusion (85±5%) by 6 weeks. The corresponding values for LDLr−/− mice were 9±7% and 61±23%. Distal and midsection sites did not display a significant increase in intima-media or intima-lumen ratio. A neointimal response was not observed in animals of either strain 6 weeks after insertion of a nonconstrictive 0.5-mm diameter collar (Figure 3; 0.08±0.03×10⁵ μm² in apoE−/−, 0.02±0.0005×10⁵ μm² in LDLr−/−; P<0.05). LDLr−/− mice on chow diet had not developed collar-induced lesions by 6 weeks (P<0.05; serum total cholesterol [TC] 261.6±39.4 mg/dL as compared with 1281.2±298.4 mg/dL on Western-type diet). Lesions in chow-fed apoE−/− mice (serum TC, 731.0±85.4 mg/dL) were found to be considerably but not significantly smaller than in their Western-type diet–fed counterparts (serum TC, 1126.6±29.5 mg/dL), as depicted in Figure 3 (0.40±0.53×10⁵ μm² versus 1.46±0.50×10⁵ μm²). ApoE3-Leiden transgenic mice did not develop collar-induced plaques for up to 6 months after collar placement despite having been on a Western-type diet (serum TC, 229.6±48.6 mg/dL; 0.005±0.0001×10⁵ μm²).

Lesion Composition
The composition of the observed plaques was determined by a variety of histological and immunohistochemical staining techniques. Routine hematoxylin and eosin staining revealed that collar-induced plaques in LDLr−/− mice remained homogeneous and relatively cellular with advancing maturity, whereas plaques in apoE−/− mice were seen to become increasingly heterogeneous, consisting of an acellular necrotic core and a well-defined fibrous cap by 6 weeks (Figure 4). Significant extracellular matrix deposition was demonstrated in mature plaques by Weigert’s staining method (data not shown). Oil red O staining revealed extensive intracellular and extracellular lipid deposits in the plaques of both strains, which included abundant cholesterol crystal clefts. Early plaques contained a particularly high number of foam cells; these were more numerous and appeared to be more lipid-rich in apoE−/− mice than in LDLr−/− mice (Figure 5). On immunohistochemical staining, the majority of these cells were positive for MOMA-2 (Figure 5), thus confirming their monocytic origin. Staining for smooth muscle actin was restricted to the media in early lesions, but differentiated smooth muscle cells (SMCs) appeared in the intima at 6 weeks, when positive cells were found to be primarily located in the fibrous cap (Figure 5).

Endothelial Expression of Adhesion Molecules and eNOS
Endothelial integrity was confirmed by staining for vWF in all 3 sites examined (Figure 6). Endothelial ICAM-1 expression was seen circumferentially throughout the common carotid artery in both collar-treated and sham-operated animals. Expression at the proximal and distal sites, however, was strongly upregulated by placement of a 0.3-mm-diameter collar, whereas expression within the collar was reduced (Figure 6). ICAM-1 expression by endothelial cells overlying collar-induced lesions decreased with increasing lesion maturity. Furthermore, its expression did not appear to be dependent on the administration of a high-cholesterol diet (data not shown). VCAM-1 expression was detected in a limited number of endothelial cells in the proximal and distal sites and found to be downregulated in the intracollar area (Figure 7). eNOS expression was found to be limited to several contiguous areas of the endothelium. Attenuation of eNOS staining was observed proximal to the collar 1 week after collar insertion (Figure 7).
Discussion

Since the initial studies by Booth et al., the rabbit has been the favored animal model for the investigation of collar-inflicted neointima formation. However, placement of a pericarotid or perifemoral collar has also been shown to induce intimal accumulation of SMCs in rats and mice, respectively. In all of these models, the neointima develops primarily in the collared section, with little involvement of the adjacent proximal or distal uncuffed artery. A range of causative mechanisms has been suggested for the source of these lesions. Thus, direct mechanical or chemical injury of the media by the collar may trigger the migration and proliferation of SMCs within the intima. Alternatively, indirect activation of the media might result from the disruption of its innervation and blood supply caused by damage to the perivascular nerve plexus and/or adventitial vasa vasorum. Furthermore, the presence of a collar has been found to attenuate endothelial NO synthesis and to reduce reactivity to NO. Finally, it may also elicit a localized inflammatory response, resulting in diapedesis and intimal accumulation of leukocytes.

In contrast, the collar-related plaques formed in our model are located primarily in the area proximal to the collar. Minor effects are observed in the distal area, whereas the area inside the collar remains unaffected (Figure 2). These plaques are therefore presumed to differ in pathogenesis from those seen in the above-mentioned models. The location of the lesions, with maximal stenosis occurring at some distance from the collar, implies that induction of atherosclerosis by the collar through a direct mechanical or chemical injury is unlikely. The absence of

Figure 5. High-power (×1000) view of proximal plaque at 6 weeks after collar insertion in apoE−/− (A, B, and C) and LDLr−/− (D, E, and F) stained for lipid (with oil red O; A and D), MOMA-2 (B and E), and actin (C and F).

Figure 6. ICAM staining (with alkaline phosphatase, using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrates) and vWF staining (with peroxidase – 3,3′-diamino-benzidine as substrate) of proximal (A), intracollar (B), and distal sites (C) 1 week after collar insertion in apoE−/− mice. Note that vWF staining is present throughout artery; ICAM-1 staining is increased in sites distal and particularly proximal to collar.
lesions at the level of the collar also indicates that diminished removal of waste products from the vessel wall or disruption of the perivascular nerve plexus are unlikely to play a major pathogenic role. Moreover, the limited thickness of the murine carotid media, which consists of 3 SMC layers, obviates the need for a transmural blood supply. The initial lesions seen in our model contain mainly foam cells of monocytic origin, in addition to extracellular lipid deposits. With progressive maturation, SMCs appear in the cap of the lesion and extracellular matrix is deposited. This suggests that the pathogenesis of these lesions depends on lipid accumulation as an initial stimulus rather than migration and proliferation of SMCs. This is corroborated by the dependency of lesion development on high-cholesterol feeding, which was found to be relative in apoE−/− mice and absolute in LDLr−/− mice. The lesional distribution pattern found in our model mirrors the distribution of lipid deposition in aortic coarctation models in cholesterol-fed rats and cynomolgus monkeys, in which lipid deposition was found to be highest proximal to the stenosis, with relative sparing and absence of lesion development distal from the stenotic segment. The deposition of lipids in these models is known to be rheologically determined in part, whereas it may also reflect a localized increase in endothelial permeability and/or retention of LDL. These lesions were found to correspond to areas of low shear stress and disturbed laminar flow. It is conceivable that equivalent hemorheological conditions exist in our model, in which a collar of 0.3-mm inside diameter was found to cause a stenosis of ~30% and attenuation of flow disturbance by placement of a nonconstrictive collar resulted in considerably delayed atherogenesis. Hemodynamic factors also play an important indirect role in human and experimental atherogenesis, through modulation of vascular adhesiveness for leukocytes. The endothelium assumes a pivotal role in these processes because the expression of various endothelial genes known to be upregulated in the endothelium of atherosclerotic plaques and lesion-prone sites in humans and experimental animals have been found to be shear-stress responsive, including ICAM-1, eNOS, and VCAM-1. Atherogenesis is known to occur predominantly at sites of disturbed flow, typified by low and oscillating shear stress. The application of oscillatory shear stress to endothelial cells in culture has been found to greatly enhance ICAM-1 expression and downregulate eNOS expression in comparison with steady laminar flow as well as inducing a transient rise in VCAM-1 levels. In our model, the expression of ICAM-1 and VCAM-1 is in agreement with these findings, being higher at sites of disturbed flow (proximal and distal to the collar) than in the high laminar shear stress environment within the collar (Table 2 and Figures 6 and 7). Conversely, downregulation of eNOS was observed at the proximal site (Table 2 and Figure 7). It should be noted that besides shear stress, circumferential deformation has also been found to augment ICAM-1 and VCAM-1 expression, potentially through the generation of reactive oxygen species. This type of deformation is more likely to occur in the “free” proximal and distal sites than within...
TABLE 2. Endothelial Cell Expression Levels of Proatherogenic Cell Adhesion Molecules, eNOS, and vWF in Collar-Treated and Sham-Operated Carotid Arteries of ApoE−/− Mice 1 Week After Surgery

<table>
<thead>
<tr>
<th></th>
<th>Proximal</th>
<th>Intracellular</th>
<th>Distal</th>
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<tbody>
<tr>
<td>ICAM-1</td>
<td>++</td>
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<td>VCAM-1</td>
<td>+</td>
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</tr>
<tr>
<td>eNOS</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>vWF</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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</tr>
</tbody>
</table>

+++ indicates strong, circumferential expression; ++, circumferential expression; +, expression in cell clusters or individual cells; and 0, no evident expression.

the collar, where the artery is prevented from full extension by the “splinting” effect of the collar.

Summary

Our model is characterized by site-controlled and accelerated atherogenesis, which mirrors the differences seen between apoE−/− and LDLr−/− mice in spontaneous atherosclerosis. The differences in lesion composition reflect the more fibrocellular plaques normally seen in LDLr−/− mice and the more lipid-rich and heterogeneous plaques typical of apoE−/− mice. In addition, the temporal pattern of lesion development is in agreement with the more delayed and diet-dependent atherogenesis in LDLr−/− mice in comparison with apoE−/− mice. It may offer several advantages over conventional animal models of mechanically induced atherosclerosis. First, closer resemblance to human plaque morphology, endothelial expression pattern, and plaque pathogenesis should ensure its relevance as a model of human atherosclerosis per se. This is of importance in the validation of mechanistic studies and may improve the relevance of in vivo assessment of potential therapeutic strategies. Second, rapid atherogenesis will allow efficient screening of potentially antiatherogenic new chemical entities and the evaluation of therapies with a limited duration of effectiveness. The latter category includes many adenoviral vectors, the expression of which often does not exceed a few weeks. Last, the possibility of controlled lesion induction in easily accessible sites is very much suited to further intraluminal instrumentation and application of therapeutic agents. This will also prove valuable in the development of a more representative murine model for restenosis because a double-injury restenosis model based on collar-inflicted atherosclerosis is likely to reflect the complex pathogenesis seen in clinical practice.

References

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