Differential Effects of Apolipoprotein A-I–Mimetic Peptide on Evolving and Established Atherosclerosis in Apolipoprotein E-Null Mice

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Background—Apolipoprotein (apo) A-I and apoA-I–mimetic peptides showed promise to prevent atherosclerosis development. Using a bypassed vein graft model in apoE-null mice, we evaluated the effects of oral or intraperitoneal administration of an apoA-I–mimetic peptide on evolving atherosclerotic lesions in the vein graft and compared such effects on the established atherosclerotic lesions in aortic sinus in the same mice.

Methods and Results—We used apoE-null mice in which a segment of inferior vena cava was grafted into the right carotid artery at 16 weeks of age. Native aortic atherosclerotic lesions (established atherosclerosis) and vein-graft atherosclerotic lesions (evolving atherosclerosis) were assessed 4 weeks after daily oral (0.3 mg/mL) or intraperitoneal (50 μg in 200 μL saline) administration of an apoA-I–mimetic peptide, D4F. Mice receiving saline or water without D4F served as controls. Both oral and intraperitoneal administration of D4F reduced vein-graft atherosclerotic (evolving lesions) plaque size by 43% and 42%, plaque lipid by 70% and 49%, and macrophage immunoreactivity by 63% and 62%, respectively, compared with controls. In contrast, D4F had no effect on the native aortic sinus atherosclerotic lesions (established lesions).

Conclusions—Oral and intraperitoneal administration of the apoA-I–mimetic peptide D4F significantly reduced rapidly evolving atherosclerotic lesions in vein grafts but not established atherosclerotic lesions in aortic sinus. These observations suggest that the type of atherosclerotic lesions and the time of initiation during the course of lesion evolution modulate the beneficial effects of apoA-I–mimetic peptides on atherosclerosis. (Circulation. 2004;110:1701-1705.)

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Methods

Mice and Vein Graft Procedure
ApoE-deficient mice (Jackson Laboratory, Bar Harbor, Me) were fed a high-fat, high-cholesterol diet containing 21% (wt/wt) fat and 0.15% cholesterol from 6 weeks of age throughout the duration of the experiment. At 16 weeks of age, the vein graft surgery was performed as described previously. Briefly, mice were anesthetized with intraperitoneal (IP) injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). The right common carotid artery was isolated and dissected free of surrounding tissues. The artery was transected and a plastic cuff placed at both cut ends. Both ends of the artery were everted over the cuffs and secured with a 9-0 silk ligature. A segment of inferior vena cava harvested from donor mouse was then interposed between the 2 ends of the carotid artery by sleeving of the segment of inferior vena cava harvested from donor mouse was then used to graft the carotid artery. The graft was clamped with a vascular clamp, and a 25-gauge needle was inserted into the graft to allow for injection of heparin. The graft was then flushed with heparin (30 U) and saline (0.9%) to clear the lumen of the graft. The graft was then anastomosed end-to-end to the common carotid artery, and the neck was closed. The mice were then given daily IP injection of 30 U heparin for 3 days to prevent in situ thrombosis within the graft.

D4F Treatment
Apolipoprotein-mimetic peptide (D4F, DWFKAFYDKVAEK-FKEAF) was administered for 4 weeks after vein graft surgery by the oral route with drinking water (0.3 mg/mL) or by daily IP injection (50 μg in 200 μL saline) (n=10). Mice receiving 0.9% saline IP (n=7) or oral water (n=11) served as controls for the IP and oral D4F groups, respectively. The D4F doses chosen had been shown to be effective in reducing atherosclerotic lesions. The D4F peptide was prepared and changed every other day. The mice were euthanized at 4 weeks after vein graft surgery.

Plasma Collection and Analytical Methods
Blood was collected via retro-orbital venous plexus puncture before euthanasia. In some mice in the D4F IP group, the blood was collected 1 hour after D4F injection to determine D4F concentration by reverse-phase high-performance liquid chromatography as described previously. Serum was stored at -80°C until analysis. Total cholesterol levels were measured with a commercially available kit (Sigma).

Immune Response to D4F
To determine whether daily injection or oral drinking of D4F peptide elicited any immune response in mice, an ELISA was used to determine antibody titers to D4F. Briefly, the plates were coated with D4F peptide (20 μg/mL) or nLDL/Cuox LDL (20 μg/mL) overnight. After thorough washing with PBS containing 0.05% Tween-20 (PBS-T) and blocking with Superblock (Pierce, Catalog No. 37535) for 5 minutes at room temperature, 100 μL of diluted mouse serum (1:100) samples were added to the wells and incubated at room temperature for 2 hours, followed by anti-mouse IgG-horseradish peroxidase (1:5000) or IgM-horseradish peroxidase (1:10000) incubation at room temperature for 2 hours. The color was developed with ABTS [2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), diammonium salt] and hydrogen peroxide as substrate, and absorbance was read at 405 nm.

Serum Amyloid A Levels
As a marker of systemic inflammation, serum amyloid A (SAA) levels in mouse plasma were determined with a commercially available ELISA kit (BioSource International) in accordance with the manufacturer’s protocol.

Tissue Preparation and Histomorphometry of Vein-Graft and Aortic Sinus Lesions
At euthanasia, the grafted inferior vena cava and the base of the heart containing the aortic root were harvested and embedded in OCT compound (Tissue-Tek), frozen on dry ice, and stored at -80°C. Serial 6-μm cross sections of vein grafts were collected on slides for histological analysis after being stained with hematoxylin-eosin or oil red O to visualize lipid deposition according to a standard protocol. Smooth muscle cell or macrophage content was visualized by smooth muscle α-actin immunoreactivity (Sigma, 1:100) or macrophage immu-

| TABLE 1. Effects of 4-Week Treatment of IP or Oral D4F on Body Weight, Plasma Cholesterol Level, and Accelerated Atherosclerotic Vein Graft Lesions |
|-----------------|-----------------|-----------------|-----------------|
|                 | Control         | D4F             | P               |
| IP group, n     | 7               | 10              |                 |
| BW at death, g  | 28.2±3.3        | 27.4±2.1        | NS              |
| Cholesterol, mg/dL | 1424±467       | 1272±294        | NS              |
| Plaque size, mm² | 0.96±0.33       | 0.55±0.37       | 0.03            |
| MOMA stain, %   | 17.5±4.6        | 6.6±0.3*        | <0.01           |
| Lipid content, % | 12.4±6.5        | 6.4±3.8         | 0.027           |
| Oral group, n   | 11              | 12              |                 |
| BW body weight at death, g | 29.8±4.6 | 28.7±2.1 | NS              |
| Cholesterol, mg/dL | 1692±454       | 1011±326        | <0.01           |
| Plaque size, mm² | 0.93±0.42       | 0.53±0.28       | 0.01            |
| MOMA stain, %   | 13.8±4.7        | 5.2±2.5         | <0.01           |
| Lipid content, % | 19.1±7.0        | 5.8±6.0         | <0.01           |

*P<0.05 was considered significant.

Statistical Analysis
Data are presented as mean±SD. Groups were compared by use of t tests. A value of P≤0.05 was considered significant.

Results

Body Weight, Plasma Cholesterol, and D4F Levels
At euthanasia, there was no difference in body weight between control and D4F-treated mice in either the IP or oral groups (Table 1). Plasma cholesterol levels in each group exceeded 1000 mg/dL, and there was no difference in cholesterol levels between control and D4F-treated mice in the IP group. However, plasma cholesterol of the oral D4F group was lower than that of the oral control group (Table 1). High levels of serum D4F concentration were detected in the circulation after IP D4F injection (3767±1183 pmol/mL, n=5).

Development of Vein Graft Lesions
The phenotypic composition of accelerated atherosclerosis in the vein graft lesions 4 weeks after grafting were assessed by use of various staining techniques. Smooth muscle cells were distributed primarily in the region near the lumen, ie, the cap region of the plaque, whereas lipid content and macrophage immunoreactivities were found under the cap region in the body of the vein graft lesions (Figure 1).

Thus, the accelerated atherosclerotic lesions in these vein grafts are remarkably similar to advanced atherosclerotic lesions in native arteries.

Effects of D4F on Vein-Graft Lesions (Evolving Lesions)
Oral as well as IP D4F significantly reduced the size of atherosclerotic plaque, by 43% and 42%, respectively, compared with controls (Table 1, Figure 2). This reduction in plaque size was associated with a significant reduction of

norreactivity (Serotec, 1:25), respectively. To detect interstitial collagen, Masson’s trichrome staining was performed. Sections containing aortic sinus were stained to assess plaque size and lipid deposition. Three equally spaced cross sections were used in each mouse to qualify the neointimal lesion using image analysis software (Image-Pro Plus).
intraplaque lipid, by 70% and 49%, and macrophage immunoreactivity, by 63% and 62%, respectively (Table 1, Figures 3 and 4). However, there was no significant difference in percent collagen content or percent smooth muscle cell immunoreactivity between the control and D4F groups in either the IP or oral groups (data not shown).

**Effects of D4F on Native Atherosclerosis (Established Lesions in Aortic Sinus)**

The effects of D4F on native atherosclerosis after IP or oral administrations for 4 weeks after surgery are summarized in Table 2. There was no difference in plaque size or lipid content in the aortic sinus sections. Macrophage immunoreactivity was slightly reduced with IP D4F, but oral D4F had no effect (Table 2).

**Immune Response and SAA Levels**

There was no significant difference in IgM or IgG titers against peptide D4F or IgG titers against nLDL/Cu-oxLDL between D4F-treated mice and controls (Table 3).

SAA is an acute-phase reactant that can be induced by interleukin (IL)-6. It has been shown previously that apoA-I–mimetic peptide reduced IL-6 expression after an inflammatory insult. Therefore, we hypothesized that apoA-I–mimetic peptide may modulate SAA level. However, in our study, we did not find an effect of D4F on SAA levels in either the IP or oral drinking groups (IP group: control, 103±129 µg/mL; D4F, 72±138 µg/mL; P=0.64; oral group: control, 49±57 µg/mL; D4F, 113±136 µg/mL; P=0.2).

**Discussion**

In this study, we used a vein-graft model in apoE-null mice fed a high-cholesterol diet to evaluate the effects of D4F, an apoA-I–mimetic peptide made from D-amino acids, on vein-graft and native aortic atherosclerosis. This model provides a unique opportunity to evaluate the effect of an intervention on rapidly evolving atherosclerotic lesions (vein-graft lesions) and well-established lesions (native aortic sinuses and aortic lesions) in the same animal. The morphological pattern and distribution of various lesion components (lipids, smooth muscle cells, macrophages) in our model were similar to those previously reported.

Several clinical studies examining the pathology of vein-graft atherosclerosis, risk factors for vein graft atherosclerosis, and therapeutic interventions effective against vein-graft atherosclerosis have generally demonstrated similarities between native and vein-graft atherosclerosis. Similarly, the histomorphometric features of vein-graft atherosclerosis show remarkable similarities to those of native arterial atherosclerosis, and factors such as hyperlipidemia, hypertension, cigarette smoking, and inflammation play critical roles in both disease processes, even though the time course of plaque evolution is accelerated in vein grafts. This accelerated atherogenesis presumably is a result of early...
endothelial damage and hemodynamic stresses to which vein grafts are exposed when placed in the arterial circulation.

This study demonstrates that 4 weeks of daily oral or IP administration of D4F (1) significantly reduces evolving atherosclerotic lesions in the vein graft, with concomitant decrease in plaque lipid content and inflammation, and (2) has no significant effect on established native aortic sinus atherosclerotic lesions. These findings suggest that D4F may differentially affect early and evolving atherosclerosis compared with established advanced atherosclerosis, emphasizing the importance of time of initiation of therapy in reference to the stage of atherosclerosis evolution. Our findings seem to differ from those reported by Navab et al., who demonstrated a significant reduction in native aortic atherosclerosis in hyperlipidemic mice with oral D4F. However, Navab et al initiated D4F therapy at 4 weeks of age, when aortic lesions have not yet formed, and continued treatment for 6 weeks, whereas in our study, therapy was begun at 16 weeks of age, when aortic lesions are already established, and continued treatment for 4 weeks. Thus, the significant reduction of native aortic atherosclerosis observed by Navab et al may be attributable to early initiation of therapy and/or longer duration of therapy. Additional studies involving initiation of therapy after establishment of atherosclerotic lesions with longer duration of treatment will be necessary to determine whether even established or advanced lesions could be favorably modulated by prolonged D4F therapy.

After oral administration, biologically active intact D4F can be detected in the circulation, and in feeding concentrations of 0.05 to 2.0 mg/mL, D4F shows a flat dose-response in terms of atherosclerosis inhibition. The precise mechanism of the atheroprotective effect of D4F remains to be defined. Previous studies have shown that apoA-I-mimetic peptides, including D4F, have no significant effect on circulating lipoprotein profile but reduce the susceptibility of LDL to oxidation, increase the antiinflammatory activities of HDL, and attenuate hyperlipidemia-induced endothelial dysfunction, in part by restoring coupled endothelial nitric oxide synthase activity through enhanced heat-shock protein 90 interactions with endothelial nitric oxide synthase. Recently, the mechanism of action of oral D4F in apoE-null mice was shown to involve rapid formation of cholesterol containing 7- to 9-nm particles, with pre-β mobility enriched in apoA-I and paraoxonase activity. As a result, lipoprotein lipid hydroperoxides are reduced, endogenous HDL becomes antiinflammatory, and HDL-mediated cholesterol efflux and reverse cholesterol transport from macrophages are stimulated.

Because D4F was previously shown to reduce IL-6 expression, we evaluated the effect of D4F on levels of SAA, an inflammatory molecule that is upregulated by IL-6 in mouse. However, we did not observe a significant effect of D4F on SAA levels. It is possible that we assessed the effect of D4F on SAA level late in the course, because IL-6 levels peak 7 days after inflammatory insult and tend to decline afterward. Navab and colleagues have recently reported that oral D4F induces the rapid formation and clearance of small 8-nm HDL-like particles containing apoA-I, D4F, and paraoxonase 1, which convert mature HDL particles from proinflammatory to antiinflammatory in apoE-null mice.

**TABLE 2. Effects of D4F on Native Aortic Atherosclerosis**

<table>
<thead>
<tr>
<th>Group</th>
<th>Plaque Size in Aortic Sinus, mm²</th>
<th>Lipid Content in Aortic Sinus Plaque, %</th>
<th>Macrophage Immunoreactivity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP Control</td>
<td>0.42±0.12</td>
<td>18.1±6.8</td>
<td>20.3±4.2</td>
</tr>
<tr>
<td>D4F (n=10)</td>
<td>0.50±0.09</td>
<td>18.0±6.7</td>
<td>15.2±3.7†</td>
</tr>
<tr>
<td>Oral Control</td>
<td>0.38±0.12</td>
<td>14.4±2.9</td>
<td>14.9±4.8</td>
</tr>
<tr>
<td>D4F (n=12)</td>
<td>0.38±0.17</td>
<td>13.9±2.3</td>
<td>15.7±6.0</td>
</tr>
</tbody>
</table>

*No statistical difference between control and D4F-treated mice in either IP or oral group.
†P=0.02 vs IP control group.

**TABLE 3. Antibody Response Against D4F or LDL in the IP Group**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Control (n=7)</th>
<th>D4F (n=10)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM against D4F</td>
<td>0.102±0.064</td>
<td>0.071±0.027</td>
<td>0.18</td>
</tr>
<tr>
<td>IgG against D4F</td>
<td>0.059±0.022</td>
<td>0.062±0.024</td>
<td>0.81</td>
</tr>
<tr>
<td>IgG against nLDL</td>
<td>0.065±0.019</td>
<td>0.078±0.034</td>
<td>0.36</td>
</tr>
<tr>
<td>IgG against CuoxLDL</td>
<td>0.212±0.050</td>
<td>0.286±0.177</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Antibody titers are expressed in optical density units.
In summary, D4F treatment for 4 weeks significantly reduced evolving atherosclerosis in the bypassed vein grafts, with a concomitant decrease of intraplaque lipid and macrophage content without a clear effect on the size or composition of established native aortic atherosclerosis. Our data, together with the observations by Navah et al., suggest that initiation of this novel therapy in the early stage of atherogenesis is likely to be important. Thus, our data have potentially important implications for the clinical testing of apoA-1–mimetic peptides, because in humans, most eligible patients for such therapy are likely to already have preexisting atherosclerosis.

Potential Limitations of the Study

Although many histomorphological similarities exist between native arterial and vein-graft atherosclerosis in both experimental animals and humans, some cautions need to be exercised when interpreting the differential effects on native aortic versus vein-graft atherosclerosis reported in our study. We cannot dismiss the possibility that the differential effects of D4F on vein-graft versus native atherosclerosis could be because of the differences in the underlying pathogenesis (such as different time courses of atherogenesis, differences in hemodynamic stresses, and origin of cellular components in the plaques) between native aortic atherosclerosis and vein-graft atherosclerosis. Additional studies may be necessary to determine the relative influences of time of initiation/duration of intervention and type of atherosclerosis as a determinant of response to D4F.

References

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