Focal accumulation of an apolipoprotein B-based synthetic oligopeptide in the healing rabbit arterial wall

(apolipoprotein E/synthetic peptides/arterial imaging)

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ABSTRACT The functions of surface-accessible domains of apolipoprotein (apo) B, the protein moiety of low density lipoprotein (LDL), are unknown, aside from its role in the LDL receptor-binding domain, which lies toward the carboxyl-terminal end of apoB. Since LDL accumulation in arterial lesions does not depend on recognition of LDLs by a cell-surface receptor, we synthesized an oligopeptide with the sequence of the trypsin-accessible domain of apoB that lies closest to the aminoterminal end of the protein and compared its biological activity to that of another synthetic oligopeptide with the sequence of the heparin- and apoB/apoE receptor-binding domains of apoE. (Tyrosine was added at the amino-terminal end of each peptide to facilitate radiolabeling.) The 18-amino acid apoB-based peptide included residues 1000–1016 of apoB, for which no function has been previously described. In radioautographs, the 125I-labeled peptide accumulated focally at the healing edges of regenerating endothelial islands in the balloon catheter deendothelialized rabbit aorta. In contrast, the 21-residue apoE-based peptide, which included residues 129–148 of apoE, accumulated diffusely and uniformly throughout the deendothelialized areas of the aorta. The data show that focal binding of the apoB-based peptide delineates arterial lesions and suggest that this arterial wall-binding domain of apoB mediates accumulation of LDLs in arterial lesions.

Atherosclerosis is undetectable by angiography or by ultrasound imaging and flow studies until late in its course, when atherosclerotic plaques narrow, and often occlude, the arterial lumen. Earlier detection would be facilitated by the ability to locate lesions through characteristics other than their space-occupying properties. Since pathological arterial wall accumulation of cholesterol carried by low density lipoproteins (LDLs) is characteristic of atherosclerosis, we tested radiolabeled LDL for its ability to accumulate in arterial lesions rapidly enough to be useful diagnostically. We have recently shown (1) that human atherosclerotic lesions can be imaged with the γ scintillation camera through their focal intramural accumulation of 99mTc-labeled LDL. However, the effectiveness of 99mTc-LDL as an imaging agent was sometimes decreased by the relatively long half-life of LDL in human plasma (2–3 days) compared with the short physical half-life of 99mTc (6 hr), as indicated by the fact that in some patients, radioactivity in the blood obscured atherosclerotic lesions in the vessel wall (1).

By using the balloon-catheter deendothelialized rabbit aorta (2), we had previously demonstrated by radioautography that 125I-labeled LDL accumulated primarily at the leading edges of regenerating endothelial islands and to a lesser extent in deendothelialized areas (3). The results led to the development of a technique for labeling LDL with 99mTc (4), an isotope more suitable than 125I for external imaging, and subsequently led to adrenal imaging in rabbits (5) as well as imaging of human atherosclerotic lesions (1), both of which also depended on focal accumulation of radiolabeled LDL. The hypothesis underlying the present study was that the specificity of LDL accumulation in arterial lesions is mediated by one or more domains of apolipoprotein (apo) B that have the specific properties necessary to direct LDL to the lesions. The goal of the present study was to determine whether a short synthetic peptide based on the primary amino acid sequence of the protein moiety of LDL, apoB (6–9), would accumulate focally in healing rabbit arterial wall; because of its low molecular weight, it was hoped that the peptide would also be cleared rapidly from plasma. From our past ability to extrapolate successfully from the rabbit model (3–5) to human imaging (1, 10), we anticipated that a peptide that produces focal radioautographic images in the rabbit should also image human atherosclerosis. A trypsin-accessible domain from apoB (11), which was furthest from the region of the LDL receptor-binding site, was chosen for synthesis and study because we had shown previously that methylated LDL, which is not recognized by any cell-surface receptor, accumulated to a greater extent than native LDL at the leading edge of regenerating endothelium in the “ballooned” rabbit aorta (12). The apoB-based synthetic peptide (SP) was designated apoE SP-4 and contained amino acid residues 1000–1016 of apoB-100: (Tyr)-Arg-Ala-Leu-Val-Asp-Thr-Leu-Lys-Phe-Val-Thr-Gly-Ala-Lys-CONH2 (an amino-terminal tyrosine not present in the native sequence was added to facilitate radioiodination).

As a control, another lipoprotein-based synthetic peptide based on the amino acid sequence of apoE (13) was tested. It was designated apoE SP-2; in addition to an amino-terminal tyrosine to facilitate iodination, it contained amino acids 129–148 of apoE: (Tyr)-Ser-Thr-Glu-Glu-Leu-Arg-Arg-Leu-Ala-Ser-His-Leu-Arg-Lys-Arg-Leu-CONH2. This domain contained the overlapping apoB/apoE receptor-binding site and heparin-binding site of apoE (14, 15). It is also reported to be 67% homologous with a known heparin-binding site (residues 3359–3367) on apoB that was also coincident with the apoB domain suspected of being responsible for LDL binding to the receptor (9). The apoE-based peptide known to be implicated in LDL receptor binding was tested, rather than its apoB counterpart, because at the time our study began, the identity of the receptor-binding domain(s) of apoB was less certain. A peptide implicated in binding to the LDL receptor was tested as a control because the earlier results with methylated LDL (12) suggested that the receptor-binding domain of apoB was

Abbreviations: apo, apolipoprotein; LDL, low density lipoprotein; SP, synthetic peptide; apoE SP-4 or apoE SP-2, synthetic peptide based on primary amino acid sequence from apoB or apoE.

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1436
either not necessary for, or not involved in, focal accumulation of the lipoprotein in arterial lesions.

MATERIALS AND METHODS

Peptide Synthesis and Purification. ApoB SP-4 was synthesized by the Merrifield solid-phase method (16) with a DuPont 250 peptide synthesizer on a benzhydrylamine resin (Pierce, 0.4 mmol of NH₂ per g of resin). The tert-butoxycarbonyl group was used for amino-terminal protection and was removed at each step of the synthesis with trifluoroacetic acid/dichloromethane, 1:1 (vol/vol). Side chains were protected with the following groups: benzyl for threonine and tyrosine, β-benzyl ester and γ-benzyl ester for aspartic and glutamic acids, 2-chlorobenzoxycarbonyl for lysine, and p-tosyl for arginine. Glutamine was coupled as the p-nitrophenyl ester without side chain protection. An equimolar ratio of tert-butoxycarbonyl amino acid and diisopropyl carbodiimide was used for each coupling; these reagents were added in a 2.5-fold molar excess and coupled for 1.5 hr. Addition of amino acids was monitored by the Kaiser test (17). Incomplete couplings were repeated until a negative Kaiser test was obtained. With amino acids that showed a positive Kaiser test after three repeats, the peptide was acetylated with acetic anhydride to block the small amount of underivatized peptide chains before proceeding to the next coupling.

The peptide was cleaved from the resin with 10 ml of hydrogen fluoride and 2 ml of anisole per g of resin at 0°C for 45 min. The crude peptide was extracted with diethyl ether to remove anisole, dissolved in 1% aqueous acetic acid, separated from the resin by filtration, and lyophilized. It was purified to homogeneity by high-performance liquid chromatography (Beckman model 110B solvent delivery system and Beckman model 165 UV detector) on a reverse-phase Vydac C₈ column. Thin-layer chromatography of apoB SP-4 showed a single band with an Rf of 0.21 in a solvent system consisting of the upper phase of a mixture of glacial acetic acid/1-butanol/water, 1:4:5. Fast atom bombardment-mass spectrometry gave a protonated molecular ion peak (M + H)⁺ at m/z = 2010.0, in good agreement with the calculated value of 2010.2. On amino acid analysis, experimental values for the relative abundance of each amino acid in the peptide were in good agreement with theoretical values. The lyophilized peptide was stored at −20°C.

Peptide apoE SP-2 was a gift from William F. DeGrado (DuPont). 

Iodination of Peptides. Iodination of peptides was carried out by Hunter and Greenwood’s chloramine-T method (18). In general, 400 μg of peptide in phosphate buffer (2 mg/ml, pH 7.4) was added to 1 mCi of Na¹²⁵I (New England Nuclear; 1 Ci = 37 GBq); this was followed by addition of 30 μl (8 mg/ml) of chloramine T in phosphate buffer. After 30 sec, the reaction was quenched by addition of 60 μl of Na₂S₂O₄ (8 mg/ml) in phosphate buffer. The labeled peptide was separated from unbound reactants by chromatography on a Sephadex G-25 column (1 × 50 cm), eluted with 0.1% acetic acid containing 1% human serum albumin. Column fractions containing the iodinated peptide were pooled, neutralized with 0.1 M NaHCO₃, and used for injection into rabbits. Labeling efficiency for both peptides was about 90%.

Animals and Surgery. New Zealand White rabbits (about 3 kg each) were obtained from Hazleton Research Animals (Denver, PA). Balloon-catheter deendothelialization of the aorta was performed as described (3). The animals were allowed to heal for 4 weeks before intravenous injection of 200–400 μCi of labeled peptide. Four rabbits received apoE ¹²⁵I-labeled SP-2 (¹²⁵I-SP-2) and nine rabbits received apoB ¹²⁵I-labeled SP-4 (¹²⁵I-SP-4). Two of the former and six of the latter received peptide that had been mixed with a cholesterol ester:phospholipid microemulsion (molar ratio, 1:1) (gift of Mary Walsh; Boston University) (19). The microemulsion was used in early experiments but not later ones because it gave no greater, and often less marked, accumulation than peptide alone.

Blood samples were drawn immediately after injection of labeled peptide and at intervals thereafter to measure plasma radioactivity.

Aortic Specimens. In most experiments labeled peptide was allowed to circulate for 4–5 hr; in some, peptide circulated for 24 hr. Twenty minutes before sacrifice, each rabbit was injected intravenously with 5 ml of a 0.5% solution of Evans blue dye (Allied Chemical, National Aniline Division, New York), which stains areas of deendothelialized aorta blue (20). The animal was then sacrificed and the aorta was removed. The adventitia was stripped off and the remaining aorta was washed with normal saline. The aorta was opened along the ventral surface, pinned out, and washed again with saline. After being photographed, the aorta was fixed for 2 hr in 10% trichloroacetic acid.

En Face Radioautography. Fixed vessels were washed with saline; excess moisture was removed, and the vessels were covered with a single layer of plastic wrap (SaranWrap). They were then placed between two sheets of high-speed x-ray film (Kodak orthofilm OM-1) and stored in a Kodak X-Omatic cassette containing two DuPont Cronex Lightning Plus intensifier screens at −70°C for 1–5 weeks. Times of exposure were 4–5 weeks for apoB SP-4 and 3 days to 1–2 weeks for apoE SP-2.

RESULTS

Removal of apoE ¹²⁵I-SP-2 and apoB ¹²⁵I-SP-4 from Plasma. The disappearance of radioactivity from plasma was identical for both peptides and was very rapid (Fig. 1). After 2–5 min, 85% of the radioactivity had disappeared from plasma. By 30 min, the amount of radiolabel in plasma was <10% of the injected dose. Results for peptide with microemulsion were similar to those for peptide alone. The disappearance of the peptides from plasma was very rapid compared to that of the removal of human LDL, which has a half-life of about 21 hr in the rabbit (21).

Accumulation of apoE ¹²⁵I-SP-2 and apoB ¹²⁵I-SP-4 in Rabbit Aorta. In Fig. 2, an en face radioautograph of a healing

![Graph](image-url)
active because of their small size in relation to the limit of resolution of en face radioautography. Other reendothelialized islands, where healing had progressed more rapidly, demonstrated no radioactivity in their centers. This is evident in Fig. 3, which shows a segment of an aorta from a rabbit injected with SP-4 mixed with a cholesterol ester/phospholipid microemulsion. Here the radioactive edges of healing islands are clearly distinct from the nonradioactive centers of the islands. In this rabbit, apoB SP-4 was allowed to circulate for 24 hr. By 24 hr <1% of the injected dose remained in plasma, but the intensity of radioactivity at the healing edges was still high. Focal accumulation of apoB SP-4 was seen consistently in rabbits injected with that peptide.

In Fig. 4, a radioautograph and photograph of an aorta of a rabbit injected with apoE $^{125}$I-SP-2 are compared. With this peptide the pattern of accumulation was very different from that of apoB SP-4. The regenerating endothelial islands had very little radioactivity, and there was no evidence of focal accumulation at the edges of the islands (Fig. 4). The still deendothelialized abdominal aorta showed a moderately high, uniformly diffuse level of radioactivity, which was clearly demarcated from the minimal level of radioactivity in the unballooned thoracic aorta. The pattern of diffuse accumulation in deendothelialized areas was consistently seen in animals injected with apoE SP-2.

In the rabbit injected with apoB SP-4 (Fig. 2), the V-shaped line of demarcation between the deendothelialized abdominal and unballooned thoracic region seen with Evans blue had no corresponding V-shaped line in the radioautograph of that aorta. Although there should be regenerating endothelium at the boundary between ballooned and unballooned aorta, focal accumulation of apoB SP-4 at the boundary was not seen in any studies. Although the reason for this is unknown, the significant point here is that apoB SP-4 did not accumulate in undisturbed (normal thoracic) aorta.

**DISCUSSION**

Two lipoprotein-based short synthetic peptides, apoB SP-4 and apoE SP-2, were tested for their ability to accumulate focally in the healing deendothelialized rabbit aorta.
accumulation, a necessary prerequisite for external imaging of arterial lesions with the \( \gamma \) scintillation camera, occurred only with apoB SP-4, and it occurred as early as 4.5 hr after injection, rapidly enough to be useful for external imaging. Thus, in addition to the receptor-binding domain of LDL, another functional domain, an arterial wall-binding domain, represented by apoB SP-4, has been demonstrated. The intense focal accumulation of apoB SP-4 at the healing edges of regenerating islands was indistinguishable from that shown by LDL (3). In contrast, apoE SP-2 showed diffuse accumulation throughout still deendothelialized areas of the aorta.

Since the sequence of apoE SP-2 has considerable homology to the LDL receptor-binding domain of apoB (9), the results suggest, in accord with previous data (12), that the receptor-binding domain of apoB is not involved in focal accumulation of the lipoprotein in arterial lesions. Preliminary experiments in our laboratory have tested this hypothesis further with a recently synthesized apoB-based peptide containing an LDL receptor-binding domain.

Naturally occurring atherosclerotic lesions are rarely deendothelialized. Thus, an important point, vis-a-vis external imaging, is that in the ballooned rabbit artery model, apoB SP-4 accumulates in regions where regenerating endothelium is present and not in unballooned thoracic aorta with normal endothelium. In preliminary data from the Watanabe heritable hyperlipidemic rabbit, another model for human atherosclerosis, apoB SP-4 accumulated focally in atherosclerotic lesions, but apoE SP-2 did not. Apparently, the regenerating endothelium of healing islands and the endothelium overlying atherosclerotic lesions share the ability to mediate accumulation of apoB SP-4.

The difference in accumulation patterns of the lipoprotein-based peptides may be related to the clusters of positive charge on apoE SP-2, which are not present on apoB SP-4. Specific apoB and apoE binding to the LDL receptor and to connective tissue glycosaminoglycans is thought to result from ionic interaction between positively charged domains on the ligand and negatively charged regions at the binding site (22–27). ApoE SP-2 has a net positive charge of +5 and binds rapidly to deendothelialized artery, presumably by ionic interaction to exposed arterial wall glycosaminoglycans. ApoB SP-4 has no clusters of positive charge, a net charge of +2, and no obvious binding to deendothelialized arterial wall. Thus, we infer that the arterial wall-binding pattern of apoB SP-4 at the regenerating edges of endothelial islands [and the similar pattern of LDL itself (3)] is more complex than that of SP-2. What other mechanism might mediate such a distinctive binding pattern is as yet unknown.

Fig. 3 shows that focal accumulation of apoB SP-4 was still intense 24 hr after injection, at a time when there was <1% of the injected dose remaining in plasma. The prolonged persistence of the radioiodinated peptide in arterial lesions suggests strongly that it was protected from catabolism by cellular lysosomal enzymes and thus not intracellular, but bound to some component of extracellular matrix. Since apoE SP-2 also appeared to bind to extracellular matrix, but did not accumulate focally in association with regenerating endothelium at the edges of healing lesions, the implication is that extracellular matrix associated with regenerating endothelium differs from that found elsewhere in the arterial wall. The ability of apoB \( ^{125}\text{I}-\text{SP-4} \) to remain focally bound in healing lesions at a time when plasma levels of radioactivity were very low should enhance visualization of arterial lesions.

The results with apoB SP-4 indicate that this oligopeptide represents a domain of LDL apoB that may be as important for pathological LDL arterial wall binding as the LDL receptor-binding domain is for regulating intracellular cholesterol levels. Important questions that remain to be determined include whether apoB SP-4 is unique or one of several domains on apoB that accumulate selectively in arterial lesions; what the relationship is between the structure of these domains and arterial wall binding; and how charge and amphiphilicity are related to the interaction of apolipoproteins with the arterial wall. We believe that the use of oligopeptides to elucidate the mechanisms of LDL arterial wall binding, in particular, should help us to learn more about how LDL is implicated in atherogenesis.
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