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The NH_2-terminal Region of Apolipoprotein B Is Sufficient for Lipoprotein Association with Glycosaminoglycans*

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Ira J. Goldberg‡§, William D. Wagner¶, Ling Pang‡, Latha Paka‡, Linda K. Curtiss‖, Jeanine A. DeLozier¶, Gregory S. Shelness¶, C. S. H. Young, and Sivaram Pillarisetti‡**

*From the Departments of ‡Medicine and **Microbiology, Columbia University College of Physicians & Surgeons, New York, New York 10032, the ¶Department of Pathology, Wake Forest University School of Medicine, Winston Salem, North Carolina 27157, and the ‖Scripps Research Institute, La Jolla, California 92037*

An initial event in atherosclerosis is the retention of lipoproteins within the intima of the vessel wall. The co-localization of apolipoprotein (apo) B and proteoglycans within lesions has suggested that retention is due to lipoprotein interaction with these highly electronegative glycoconjugates. Both apoB100- and apoB48-containing lipoproteins, *i.e.* low density lipoproteins (LDLs) and chylomicron remnants, are atherogenic. This suggests that retention is due to determinants in the initial 48% of apoB. To test this, the interaction of an apoB fragment (apoB17), and apoB48- and apoB100- containing lipoproteins with heparin, subendothelial matrix, and artery wall purified proteoglycans was studied. ApoB100-containing LDL from humans and human apoB transgenic mice and apoB48-containing LDLs from apoE knockout mice were used. Despite the lack of the carboxyl-terminal 52% of apoB, the apoB48-LDL bound to heparin-affinity gel as well as did apoB100-LDL. An NH_2-terminal fragment containing 17% of full-length apoB was made using a recombinant adenovirus; apoB17 bound to heparin as well as did LDL. Monoclonal antibodies against the NH_2-terminal region of apoB decreased apoB100 LDL binding to heparin, whereas antibodies against the LDL receptor-binding region did not alter LDL-heparin interaction. The role of the NH_2-terminal region of apoB in LDL interaction with matrix molecules was also assessed. Media containing apoB17 decreased LDL binding to subendothelial matrix by 42%. Moreover, removal of the apoB17 by immunoprecipitation abrogated the inhibitory effect of these media. Antibodies to the NH_2-terminal region decreased LDL binding to matrix and dermatan sulfate proteoglycans. Purified apoB17 effectively competed for binding of LDL to artery derived decorin and to subendothelial matrix. Thus, despite the presence of multiple basic amino acids near the LDL receptor-binding domain of LDL, the NH_2-terminal region of apoB is sufficient for the interaction of lipoproteins with glycoconjugates produced by endothelial and smooth muscle cells. The presence of a proteoglycan-binding site in the NH_2-terminal region of apoB may explain why apoB48- and apoB100-containing lipoproteins are equally atherogenic.

The hallmark of the atherosclerotic process, and the characteristic that distinguishes it from other inflammatory processes, is the presence of both intra- and extracellular lipid deposits (1). Despite a large body of data on interactions between lipoproteins and isolated proteins and cultured cells, the processes that lead to the accumulation of these lipoproteins and lipids in the artery wall are not well defined. Two types of lipoproteins have been clearly established as atherogenic. They are the apolipoprotein (apo)[1] B100-containing LDL and the apoB48-containing chylomicron remnants. Human genetic disorders resulting in increased circulating levels of either of these lipoproteins cause premature atherosclerosis (2, 3). Moreover, mice have been produced that have elevated blood concentrations of apoB100- or apoB48-lipoproteins, and increased plasma levels of either of these particles leads to atherosclerosis development (4, 5). Thus, a common and perhaps necessary requirement for atherogenesis is elevated plasma levels of lipoproteins containing at least the NH_2-terminal 48% of apoB.

The observation that elevated levels of apoB are the primary cause of atherosclerosis is supported by pathological data that also provide insights into how these lipoproteins accumulate within the artery. ApoB-containing lipoproteins are major components of the atherosclerotic plaque. Immunohistological studies of blood vessels have demonstrated the presence of LDL-like particles within the intima (6, 7). At least two processes can lead to increased LDL in atherosclerosis-prone regions. These regions could be more permeable to lipoproteins, or components of the artery wall could prevent egress of LDL after crossing the endothelial barrier. *In vivo* studies of the accumulation of LDL in cholesterol-fed rabbits have suggested that this latter process, termed lipoprotein retention, occurs in atherosclerosis-prone areas of the aorta (8).

If LDLs become associated with components of the artery, their egress would be prevented, and the LDLs would be retained in the artery. LDLs in atherosclerotic lesions are found in regions that are enriched in proteoglycans (9, 10), molecules that contain highly electronegative glycosaminoglycans (GAGs). Complexes of apoB-containing lipoproteins and proteoglycans have been purified from atherosclerotic portions of blood vessels (11). In addition, *in vitro* studies have shown that LDLs will associate with proteoglycans (12, 13) and that LDL-proteoglycan complexes can be produced under experimental conditions (14). Using heparin binding as a model for how LDL interacts with vessel wall proteoglycans, the prevailing view has been that heparin-binding regions of LDL that are predom-

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§ To whom correspondence should be addressed: Dept. of Medicine, Columbia University College of Physicians & Surgeons, 630 West 168th St., New York, NY 10032. Tel.: 212-305-5961; Fax: 212-305-5384; E-mail: IJG3@columbia.edu.

[1] The abbreviations used are: apo, apolipoprotein; NTAB, NH_2-terminal region of apoB; MB, monoclonal anti-apoB antibody; GAG, glycosaminoglycan; LDL, low density lipoprotein; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline; CMV, cytomegalovirus.

inantly found near the carboxyl-terminal region of apoB are responsible for LDL retention within arteries (15). This is because small peptides from this region contain multiple basic amino acids and bind more tightly to GAG than peptides from other regions of LDL (15–18). However, apoB48 lipoproteins that do not have this region are atherogenic (5). Therefore, if proteoglycan interaction is the initial step in atherosclerosis development, there must be additional proteoglycan-binding regions on these apoB48-containing lipoproteins or they must initiate atherosclerosis via an entirely different mechanism, an unlikely possibility.

The NH_2-terminal region of apoB (NTAB) is a relatively hydrophilic portion of the molecule that contains seven disulfide bonds and is thought to extend away from the surface of the LDL molecule (19). NTAB encompasses approximately 17% of the apoB and is at least 80 kDa. This region of apoB has been shown to bind to lipoprotein lipase (20), interact with the scavenger receptor (21), and bind to a triglyceride-rich lipoprotein receptor on macrophages (22). Moreover, NTAB is required for the initiation of assembly of apoB-containing lipoproteins in the endoplasmic reticulum (23, 24).

In this report, experiments are presented to demonstrate that NTAB binds to heparin affinity gels with an affinity equal to or greater than apoB100-containing LDL. In addition, apoB48-containing lipoproteins were observed to bind to heparin as well as LDL. Moreover, much of the binding of LDL to subendothelial matrix and purified arterial wall proteoglycans was inhibited by a truncated apoB protein containing the NH_2-terminal 17% of apoB. Based on these findings, we propose that NTAB contributes to the atherogenicity of LDL and remnant lipoproteins.

MATERIALS AND METHODS

Lipoprotein Isolation and ApoB Detection—Human and mouse LDL were isolated by sequential ultracentrifugation (25). Plasma was subjected to ultracentrifugation at $d < 1.019$ for 24 h at 40,000 rpm in a Beckman centrifuge at 10 °C as described previously (26). The floating lipoproteins containing very low and intermediate density lipoproteins were removed, $d = 1.012$ buffer was added to the infranatant, and the $d = 1.063$ solution was used for ultracentrifugation as above. For the mouse samples, the floating lipoproteins were recentrifuged to concentrate and dissociate any remaining apoE. LDL were dialyzed and the protein assessed by the method of Lowry *et al.* (27). LDL was radioiodinated using iodine monochloride as described previously (28), leading to 100–150 cpm/ng.

To confirm the presence of apoB and to detect any residual apoE, the mouse lipoproteins were subjected to Western blot analysis. SDS-PAGE and Western blotting were carried out as described previously (20). Briefly, nitrocellulose strips were incubated with the antibodies (1:500 dilution) for 1 h at room temperature, washed five times with phosphate-buffered saline containing 0.1% Tween 20, and then incubated with the corresponding secondary antibody coupled to horseradish peroxidase. The antibodies used included sheep anti-human polyclonal anti-apoB (Boehringer Mannheim), monoclonal anti-apoB antibodies (MBs), and polyclonal anti-apoE antiserum. The peroxidase reaction was developed with ECL Chemiluminescence Kit according to the manufacturer's directions (Amersham Life Sciences Ltd., Buckinghamshire, United Kingdom).

Monoclonal Anti-apoB Antibodies—Several monoclonal antibodies that interact with different regions of apoB were used. These antibodies have been characterized previously (29, 30). MB47 blocks a region of apoB required for LDL interaction with the LDL receptor. The epitope for MB47 has been localized to amino acids 3429–3453 and 3507–3523. MB19 interacts with an epitope within the first 100 amino acids of apoB (30) and has been used to inhibit lipoprotein lipase interaction with apoB (20). In addition, four commercially available antibodies were used (Ottawa Heart Institute Research Corporation, Ottawa, Canada). These were as follows: 1D1 epitope, amino acids 474–539; 2D8 epitope, amino acids 1438–1481; 4G3 epitope, amino acids 2980–3084; and 5E11 epitope, amino acids 3441–3569 (29). For experiments in which the effects of antibodies on LDL interaction with heparin-affinity gel were studied, an equimolar amount of the antibodies and LDL were incubated for 1 h at 4 °C prior to chromatography.

Adenovirus Production of Recombinant ApoB17—The recombinant, replication-defective adenovirus Ad.apoB17 was created in two steps. First the apoB17 cDNA fragment was cloned into the *Eco*RV and *Sal*I cloning sites of plasmid vector pACE (31). This contains, in order, the first 355 base pairs from the left end of the adenovirus genome, the cytomegalovirus (CMV) immediate early promoter, DNA that encodes splice donor and acceptor sites, cloning sites for the desired gene (in this case, *apoB17*), DNA encoding a poly(A) signal sequence from the mouse β globin gene, and approximately 6 kilobase pairs of adenovirus sequence extending from nucleotide 2966 through nucleotide 9197. The recombinant virus was created *in vivo* in 293 cells by homologous recombination between the *apoB17*-containing vector and plasmid JM17, as described (32). The recombinant virus is replication-defective in human cells other than 293 cells, which express adenovirus E1A and E1B. Following transfection of the two plasmids, infectious virus was recovered, the genomes were analyzed to confirm the recombinant structure, and then virus was plaque-purified, all by standard procedures (31). The control virus Ad-CMV was created in similar fashion from pACE lacking the ApoB17 insert.

Confluent 293 cells were infected with pACE-B17 and Ad-CMV at a multiplicity of infection of 100 plaque-forming units/cell. After 36 h, the media were removed and new, nonserum containing media (Dulbecco's modified Eagle's medium-1.5% BSA) were added to the cells. These media were collected after 8 h. 0.5 ml of each condition medium was used for immunoprecipitation and secretion of apoB17 into the medium was confirmed by Western blotting.

For some experiments, the apoB17 was removed from the 293 medium by immunoprecipitation as follows. ApoB17-containing medium was mixed with a 1⁄500 volume of polyclonal anti-apoB IgG and incubated at 4 °C overnight. The IgG and associated apoB17 were then removed by adding 0.05 volume of 10% protein A-Sepharose beads (Amersham Pharmacia Biotech) and centrifugation at 7000 rpm for 5 min in a microcentrifuge (Eppendorf model 5415C). This step was repeated a second time to remove any residual IgG. ApoB17 and IgG removal was confirmed by loss of the immunoreactive band by Western blot.

Purification of ApoB17—ApoB17 was isolated from medium obtained from adenovirus infected cells. The medium was incubated with heparin-affinity gel (Affi-Gel-Heparin, Bio-Rad) for 1 h at 4 °C. The gel was washed with Hepes buffer (pH 7.3) containing 0.15 M NaCl, and apoB17 was eluted with 0.5 M NaCl in Hepes buffer. The eluted apoB17 was detected by Western blot, and the fractions with the highest apoB17 content were pooled. ApoB17 was further purified by DEAE-cellulose chromatography. Heparin-eluted material was diluted to 0.15 M NaCl and loaded onto a DEAE column. The column was washed and apoB17 was eluted with 0.26 M NaCl in Hepes buffer. ApoB17 was detected by Western blotting, and the fractions containing apoB17 were pooled.

Heparin Affinity Chromatography—To assess lipoprotein and apoB binding to heparin, apoB17-containing 293 cell medium or isolated lipoproteins in low salt buffer (0.075 M NaCl, 10 mM Hepes, pH 7.4) were incubated with heparin affinity gel. The gel was first washed with 10 mM Hepes, pH 7.4, containing 0.075 M NaCl and 1.5% BSA. 3 ml of gel was then incubated for 2 h at 4 °C with either conditioned medium from 293 cells infected with pACE-B17, human LDL, mouse LDL from human-apoB100 transgenic mice, or mouse apoB48-containing lipoproteins from apoE null mouse. After binding, the heparin gel was packed into Bio-Rad poly-prep columns (0.8 × 4cm), and unbound media were collected. The gel was washed and either eluted sequentially with 2.5 volumes (8 ml) of 10 mM Tris-HCl, pH 7.4, containing 0.15 M, 0.4 M, and then 1 M NaCl or with a gradient (12.5 volumes) of 0.075–0.9 M NaCl. Fractions were either directly analyzed by SDS-PAGE or immunoprecipitated and analyzed by Western blotting.

For Western blot analysis, immunoprecipitation was carried out as described previously (33). Briefly, fractions collected from the unbound, 0.15, 0.4, and 1 M NaCl eluates were incubated overnight at 4 °C with 0.05 volume of 1:50 dilution of anti-apoB polyclonal antibody, followed by incubation with 0.05 volume of 10% protein A-Sepharose CL-4B beads for 3 h. The beads were washed, and bound proteins were eluted by boiling in 100 μl of sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol) for 5 min. Released proteins were analyzed by 5% SDS-PAGE, followed by Western blotting with anti-apoB polyclonal antibody as described above.

In some experiments, ^{125}I-LDL radioiodinated using iodine monochloride was used. To ensure that the labeled LDL interacted with proteoglycans in a manner similar to native LDL, the ^{125}I-LDL preparations were chromatographed using Affi-Gel-heparin. Only LDL that eluted in the same position as unlabeled LDL, and was therefore not damaged or oxidized during the labeling procedure, was used for sub-

sequent experiments.

Matrix Molecules—Subendothelial matrix was produced from bovine aortic endothelial cells as described previously (34). In brief, confluent monolayers of endothelial cells were washed three times with phosphate-buffered saline (PBS) and incubated for 5 min in a solution containing 20 mM NH_4OH and 0.1% Triton X-100 at room temperature. Detached cells were removed by washing three times with PBS followed by three times with minimum essential medium containing 3% bovine serum albumin (minimum essential medium-BSA). This procedure has been shown to leave the intact subendothelial matrix attached to the surface of the well.

To assess lipoprotein binding to isolated proteoglycans, 24 well plates (Falcon) were incubated overnight in borate buffer (pH 10) containing 10 μg/ml dermatan sulfate proteoglycans (Collaborative Biomedical Products, Bedford, MA). The unreacted regions of the plates were then blocked by incubating the plates in borate buffer containing 1.5% BSA for 2 h at room temperature. ^{125}I-LDL (5 μg/well) in PBS-1.5% BSA (PBS-BSA) was allowed to bind at 37 °C for 1 h. The plates were then washed three times with PBS-BSA. Bound apoB was extracted with 0.5 N NaOH for 1 h at room temperature, and the radioactivity was assessed (35).

Competition between LDL and ApoB17 for Binding to Dermatan Sulfate Proteoglycans—The role of the NH_2-terminal region of apoB in LDL association with purified arterial wall proteoglycans was assessed. Methods for these experiments are similar to those used in previous studies comparing the binding of different LDL species to proteoglycans (36). Decorin was isolated from human arteries as described (37). For these experiments, apoB17 containing a C-terminal FLAG epitope (DYKDDDDK) (apoB17F) was used. ApoB17F was produced by infection of Sf9 cells with recombinant baculovirus, followed by anti-FLAG immunoaffinity purification of the Sf9 culture medium. Purified apoB17 was quantified by the Lowry method (27).

RESULTS

Comparison of ApoB100 and ApoB48 LDL Association with Heparin-Gel—To test whether lipoproteins containing apoB100 have higher affinity for heparin than do apoB48-containing lipoproteins, LDL were isolated from human apoB100-expressing and apoE knockout mice. The apoE knockout particles were used to prevent any confounding effects of apoE in the apoB48 particles on the association with heparin. Human LDL contained almost entirely apoB100, and the particles from the E knockout mice were almost entirely apoB48 particles. Neither lipoprotein had detectable apoE by Western blot.

Both lipoproteins were used for heparin-affinity chromatography and eluted either stepwise with increasing concentrations (Fig. 1*A*) or with a gradient of NaCl (Fig. 1*B*). Only trace amounts of the apoB100 lipoproteins did not bind to heparin and were present in the 0.075 M NaCl buffer, run through. The Western blots of the recovered protein in each eluate are shown in Fig. 1*A*. Some of the associated apoB100-LDL was eluted with 0.15 M NaCl (physiologic ionic strength). Additional apB100-LDL remained bound and eluted with 0.4 M NaCl; no further apoB100 LDL was eluted with 1 M NaCl. Most apoB48 LDL also associated with heparin in the 0.075 M salt buffer. The elution pattern of this LDL was similar to that of the B100 particles: most of the LDL eluted using 0.15 M NaCl and 0.4 M NaCl. Therefore, absence of the C-terminal region of apoB did not result in decreased heparin binding.

To further compare the elution of these two LDLs, 100 μg of both lipoproteins were allowed to bind to heparin-affinity gel at the same time and were eluted with a salt gradient. As shown in Fig. 1*B,* both apoB100- and apoB48-LDL bound with similar affinities and eluted at a salt concentration of about 0.25–0.3 M NaCl.

ApoB17 Binding to Heparin-containing Gel—We next tested whether an NH_2-terminal fragment of apoB would directly bind to heparin. ApoB17-containing medium (6 ml) was mixed with an equal volume of 20 mM Tris (pH 7.4) to reduce its ionic strength to 0.075 M NaCl and then was applied to heparin-affinity gel (3 ml). The gel was sequentially washed with increasing ionic strength, and the apoB was concentrated by immunoprecipitation. Densitometric scans of the Western blots were used to estimate the amount of apoB100 and apoB17 eluted with each NaCl concentration. As shown in Fig. 2, human LDL, eluted in the identical position to the LDL obtained from the human apoB100 expressing transgenic mice; *i.e.* production of the lipoproteins in the mouse or human led to similar heparin binding. ApoB17 also associated with heparin. Some apoB17 eluted with 0.15 M NaCl, but the majority of apoB17 was eluted with 0.4 M NaCl. A small amount of the apoB17, less than 20%, remained and was dissociated with the 1 M NaCl buffer. When eluted with a salt gradient, most apoB17 eluted from the heparin between 0.25 and 0.3 M NaCl (not shown). Therefore, apoB17 bound to heparin with an affinity similar to that of apoB100 and apoB48 LDL. This suggested that the amino-terminal 17% of apoB could mediate apoB100-LDL association with heparin.

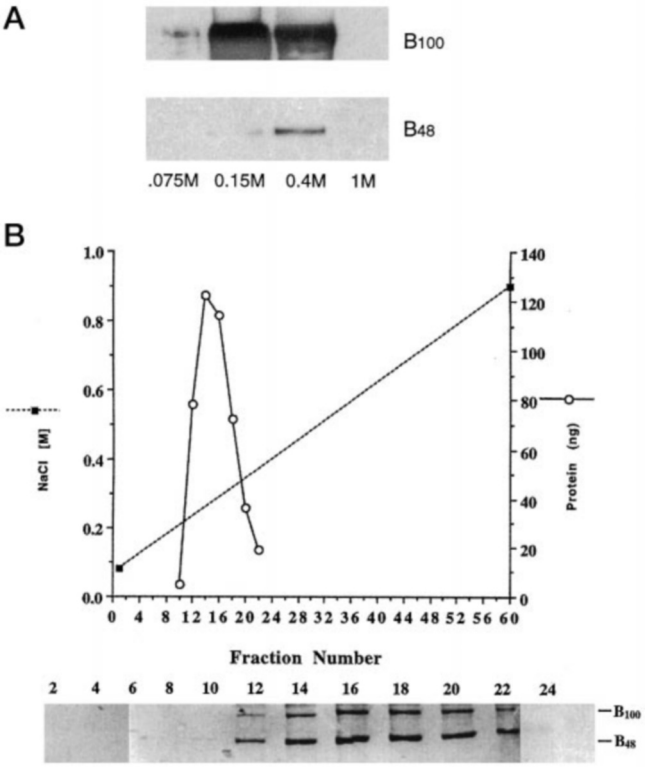

FIG. 1. **Comparison of heparin affinity of apoB100- and apoB48-containing lipoproteins.** Lipoproteins of density 1.019–1.063 g/ml were isolated from plasma of human apoB100-expressing transgenic mice and apoE knockout mice. The lipoproteins were concentrated by a second ultracentrifugation, dialyzed, and allowed to bind to 3 ml of heparin-affinity gel in buffer containing 0.075 M NaCl. Lipoproteins were eluted with increasing concentrations of NaCl, either step-wise (8 ml of each buffer) (*A*) or with a gradient (0.075–0.9 M NaCl) (*B*) in the same buffer. The eluted apoB was analyzed by SDS-PAGE and identified by Western blotting (*A*) or Coomassie Blue staining (*B*). Both apoB48 and B100 eluted at similar salt concentration (~0.26 M NaCl).

Effects of Monoclonal Antibodies on LDL Binding to Heparin—Monoclonal antibodies were used to block regions of apoB and prevent their accessibility to heparin. MB47 and 5E11 are antibodies that block LDL interaction with the LDL receptor-binding region of apoB. In the experiment shown in Fig. 3*A,* MB47 was added to apoB100-LDL at a concentration sufficient to inhibit LDL uptake by LDL receptor-up-regulated cells (38). In separate experiments, both MB47 and 5E11 antibodies blocked LDL degradation by fibroblasts over 80% (data not shown). Most of the LDL eluted from heparin at 0.15 M NaCl.

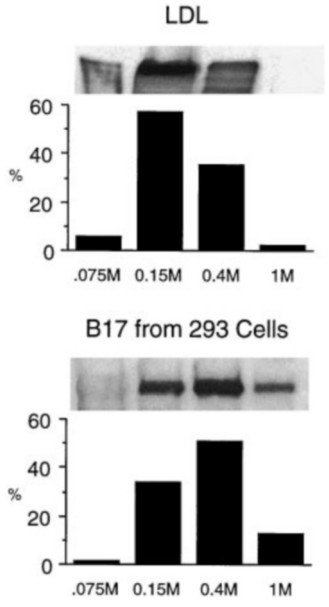

FIG. 2. **Human LDL and apoB17 binding to heparin.** Human LDLs were isolated by ultracentrifugation, and apoB17 was produced in the medium of 293 cells after infection by an apoB17-producing adenovirus. The medium and human LDL were applied to a heparin-affinity column in 0.075 M NaCl and the column was eluted with buffer containing increasing concentrations of NaCl, as described in Fig. 1. The eluted apoB was assessed by Western blot analysis as described under "Materials and Methods." Densitometric estimates of the recovery of apoB100-lipoproteins and apoB17 in each fraction after a step elution are shown.

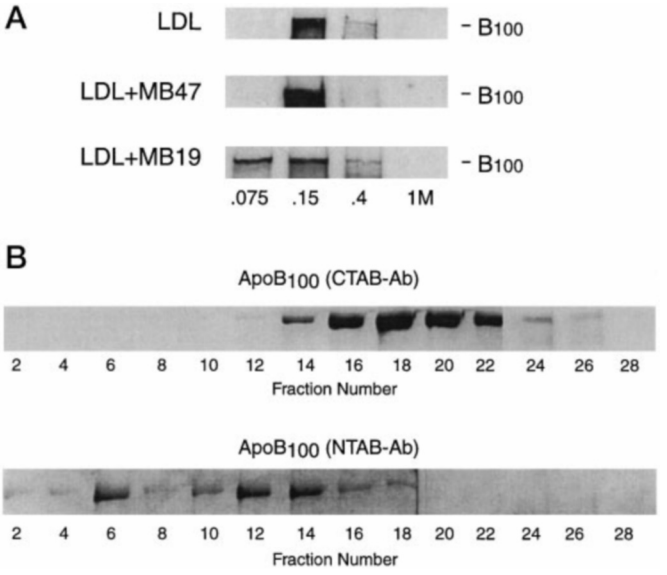

FIG. 3. **Antibodies to the NH_2-terminal region but not the carboxyl-terminal region of apoB inhibit LDL binding to heparin-agarose.** *A,* human LDL was incubated for 1 h with an equimolar amount of monoclonal antibodies to apoB (*MB*). Heparin-affinity chromatography of human LDL was performed in the presence of MB19, an antibody directed to the NH_2-terminal region, or MB47, an antibody that inhibits LDL binding to the LDL receptor. The gel was eluted stepwise with increasing concentrations of NaCl, and apoB was assessed by SDS-PAGE and Coomassie Blue staining. *B,* human LDLs (200 μg) were incubated with equimolar concentrations of MBs either to the carboxyl-terminal region (4G3 epitope, amino acids 2980–3084, and 5E11 epitope, amino acids 3441–3569 (*CTAB*)) or to the NH_2-terminal region (1D1 epitope, amino acids 474–539; 2D8 epitope, amino acids 1438–1481 (*NTAB*)), both at equimolar concentrations for 1 h at 4 °C. Heparin-agarose chromatography was then performed as described in Fig. 1. Fractions were analyzed by SDS-PAGE and stained with Coomassie Blue.

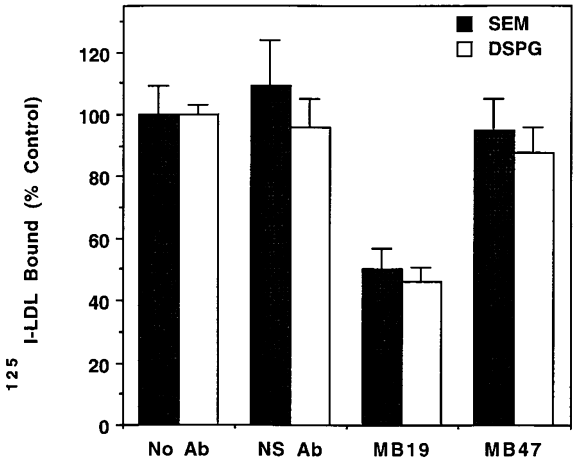

FIG. 4. **Effect of monoclonal anti-apoB antibodies on LDL binding to subendothelial matrix and dermatan sulfate proteoglycans.** Subendothelial matrix (*SEM*) was prepared from confluent endothelial cells as described under "Materials and Methods." SEM-containing (*A*) or dermatan sulfate proteoglycan-containing (*B*) wells were incubated with ^{125}I-LDL (5 μg/ml) for 1 h in Dulbecco's modified Eagle's medium-1.5% BSA in the presence or absence of MBs. Unbound LDL was removed, and bound LDL was eluted with 0.5 N NaOH and counted.

Incubation with MB47 led to no appreciably change in LDL elution from heparin-gel. In contrast, MB19 increased the amount of LDL not associating with heparin and found in the 0.075 M NaCl fraction. Therefore, antibodies that block the NH_2-terminal but not the LDL receptor-binding region decreased LDL binding to heparin. These data support the hypothesis that regions other than the LDL-receptor binding region of apoB can interact with heparin.

A similar experiment was performed with a second set of monoclonal antibodies to the NH_2-terminal and the LDL receptor-binding regions of apoB. In this experiment, shown in Fig. 3*B,* the antibodies (equimolar concentrations) were allowed to associate with the LDL for 1 h at 4 °C, the LDL-antibody complexes were mixed with the heparin-affinity gel, and the gel eluted with a continuous gradient from 0.075 to 0.9 M NaCl. As in Fig 3*A,* the antibody directed against the NH_2-terminal, but not the antibody that blocks LDL interaction with the LDL receptor, decreased LDL binding to heparin (Fig. 3*B*). Addition of C-terminal antibodies (4G3 and E11 (CTAB)) did not alter the elution pattern of LDL from that of Fig. 1B. In contrast, in the presence of NTAB antibodies (1D1 and 2D8), LDL was eluted in two peaks in fractions both at a lower salt concentration than that of untreated LDL (Fig. 1*B*). Therefore, two different combinations of monoclonal antibodies gave similar results, implicating the NH_2-terminal region of apoB in LDL-heparin interaction.

Effects of MB19 and MB47 on LDL Interaction with Matrix Proteins—Because the promotion of atherosclerosis is thought to require LDL interaction with matrix proteoglycans, the effects of MBs on LDL association with subendothelial matrix and with isolated proteoglycans were assessed. As shown in Fig. 4*A*, MB19 decreased LDL binding to subendothelial matrix by approximately 50%, whereas MB47 had little effect on LDL association with matrix.

Because subendothelial matrix contains a number of proteins that may interact with LDL, immunoinhibition was assessed using plates that were coated with dermatan sulfate proteoglycans. Shown in Fig. 4*B* are the effects of MB19 and MB47 on LDL association with dermatan sulfate proteoglycans. MB47 had little effect on LDL binding, but MB19 inhibited LDL interaction with the dermatan sulfate approximately 50%.

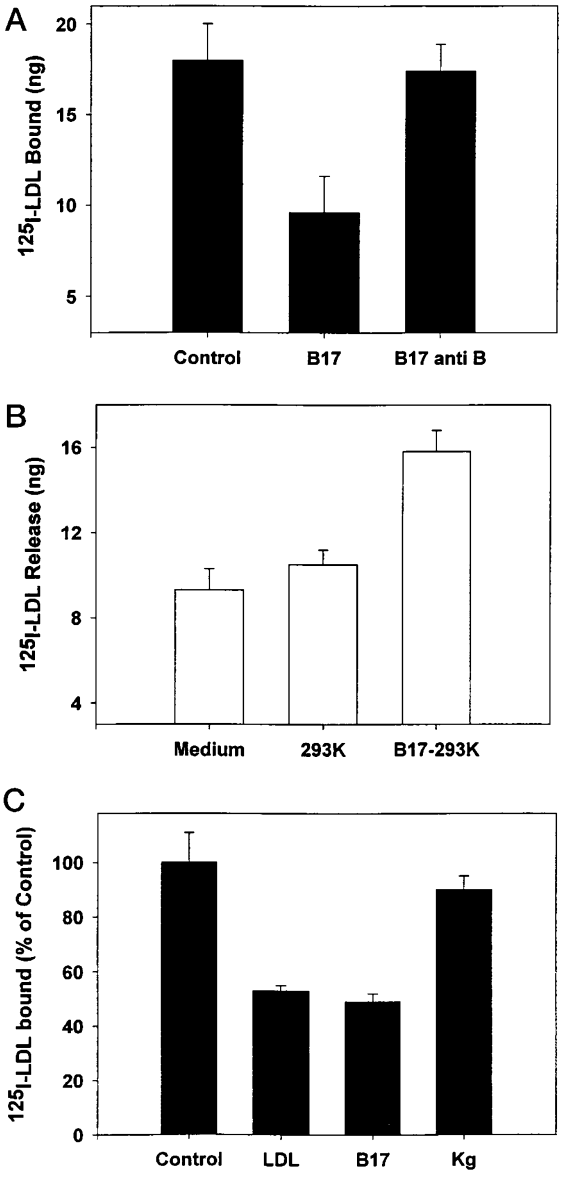

FIG. 5. **LDL association with subendothelial matrix in the presence of apoB17.** *A,* LDL association with subendothelial matrix. This was assessed as described in Fig. 4. The LDL was added in medium from 293 cells, medium from 293 cells expressing apoB17, and the apoB17-containing medium after immunoprecipitation of the apoB17 (denoted apoB17 anti B). *B,* release of bound LDL by apoB17. In this experiment, ^{125}I-LDL was first allowed to bind to subendothelial matrix for 1 h at 37 °C. Unbound LDL was removed, and matrix containing bound LDL was incubated for 1 h at 37 °C with plain medium or medium obtained from control adenovirus infected 293K cells (*293K*) or medium from 293K cells infected with apoB17-producing adenovirus (*B17–293K*). Released LDL was counted. *C,* competition with purified proteins. LDL association with subendothelial matrix was assessed as described in Fig. 4. ^{125}I-LDL was mixed with 20 μg unlabeled LDL (*LDL*), 1 μg of purified apoB17, or 10 μg of high molecular weight kininogen (*Kg*) and added to wells containing subendothelial matrix. Binding was carried out at 37 °C for 90 min. Unbound radioactivity was removed, and bound radioactivity was extracted with 1 N NaOH.

Competition between NTAB and LDL for Binding to Subendothelial Matrix—As a further test of the role of NTAB in LDL matrix interaction, radioactive LDL association with matrix was assessed in conditioned medium from 293 cells and from 293 cells that were infected with apoB17 producing adenovirus. As shown in Fig. 5*A,* the apoB17-containing medium reduced LDL binding by 42%. To determine whether this effect was specifically due to apoB17 rather than other components of the

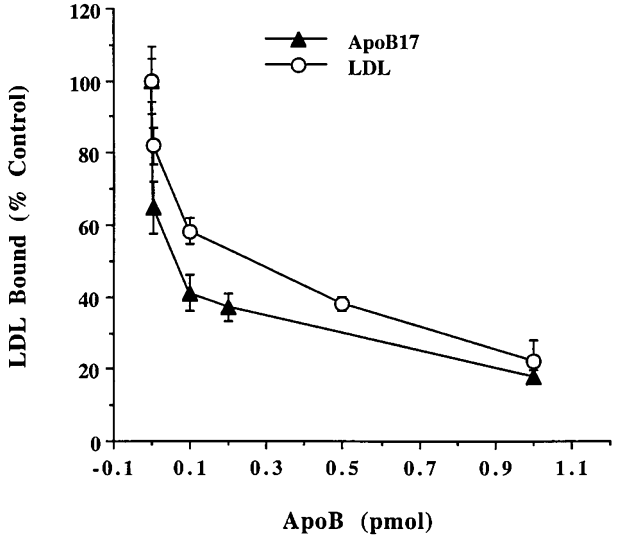

FIG. 6. **Competition of LDL association with artery-derived decorin by apoB17.** Purified decorin was radiolabeled, and its association with LDL coated microtiter plates in the presence of increasing concentrations of LDL or apoB17 was assessed.

apoB17-adenovirus infected medium, the same media were used after removing the apoB17 by immunoprecipitation (Fig. 5*A, B17 anti-B*). The apoB17-depleted medium did not block LDL binding to matrix.

In a different experiment ^{125}I-LDL was first allowed to bind to subendothelial matrix and then incubated for 1 h at 37 °C with plain medium, medium obtained from control adenovirus infected 293K cells, or medium from 293K cells infected with apoB17 adenovirus. As shown in Fig. 5*B*, apoB17 medium released greater amounts of LDL. Therefore, soluble NH_2-terminal fragments of NTAB compete with LDL and decrease its association with matrix.

Next, we compared the effects of LDL, purified apoB17, and kininogen (another heparin-binding protein (molecular mass, ~115,000 Da)) on LDL binding to subendothelial matrix. Both LDL (100 μg, a 20-fold excess) and apoB17 (1 μg, an approximately equimolar amount) inhibited LDL association approximately 50%. Kininogen (10 μg) had no effect. These data suggest that apoB17 was a better competitor than unlabeled LDL for interaction with matrix components including proteoglycans. In this same experiment, the LDL was incubated in medium containing 50 units/ml of heparin. Heparin decreased the amount of associated LDL by 54%, from 5.3 ± 0.6 to 2.46 ± 0.3 ng. This suggests that some LDL interaction with matrix is not to glycosaminoglycans.

ApoB17 Competition with LDL for Association with Decorin—To further confirm that NTAB was responsible for LDL binding to proteoglycans and to assess in a more quantitative manner the abilities of LDL and apoB17 to compete for LDL-proteoglycan association, additional studies were conducted using artery-derived decorin. Decorin is the major dermatan sulfate containing proteoglycan of artery. The system employed maximizes the LDL-proteoglycan binding by initially using an interaction binding buffer consisting of 50 mM NaCl, 5 mM calcium, and finally a physiologic buffer of 150 mM NaCl. For these studies microtiter plates were coated with LDL, and the association of radioiodinated artery derived decorin was assessed in the presence of increasing concentrations of either LDL or apoB17. As expected, LDL effectively and totally competed for binding of decorin to LDL (Fig. 6). When apoB17 was used to compete for decorin binding to LDL, essentially all binding to LDL was inhibited. For both LDL and apoB17, less than 1 pmol was necessary for maximum competition. Three

separate experiments were completed to assess binding affinities using molar levels of either apoB17 or LDL required to inhibit 50% of decorin binding to LDL. On average, 0.16 ± 0.02 pmol (mean ± S.E.) of apoB17 and 0.40 ± 0.03 pmol of LDL were required. For these experiments, six levels of the DYKDDDDK peptide used in purification of apoB17, ranging from 10 to 300 pmol, had no influence binding decorin to LDL (data not shown). These results indicate that apoB17 binds to artery derived decorin, and this binding affinity is significantly ($p < 0.05$) greater than the binding affinity of intact LDL particles.

DISCUSSION

A central paradigm for atherogenesis is that the process begins by the retention of lipoproteins on vessel wall proteoglycans. Pathological and biochemical information has supported this thesis. Moreover, studies by several investigators showed that regions of apoB would associate with heparin and other GAG (15–18). However, these experiments, using short peptides, implicated regions close to the LDL-receptor binding domain (amino acids 3359–3367) as the heparin binding, and by inference, atherogenic region of apoB. Thus, a widely held assumption is that this region of apoB causes cholesterol-carrying lipoproteins to become pathologic. Our data suggest that this is not the only portion of apoB that will increase lipoprotein association with GAG.

Our data show that NTAB alone or associated with lipoproteins can interact with heparin and matrix proteoglycans. The following data support this. 1) ApoB48 lipoproteins, which do not contain the LDL-receptor binding region, bind to heparin as well as or better than apoB100-LDL does. 2) Antibodies to NTAB, but not the LDL-receptor binding region, decrease LDL association with heparin. 3) Soluble fragments of NTAB bind to heparin better than LDL does. 4) Blocking NTAB with MB or competition with soluble NTAB decreases LDL interaction with subendothelial matrix. 5) ApoB17 competes with LDL for binding to decorin, the major dermatan sulfate proteoglycan of the arteries. Together, these data suggest that the interaction of NTAB with proteoglycans is the basis for retention of at least some apoB-containing lipoproteins within the artery. This assumes that retention is dependent on LDL proteoglycan interactions.

Although apoB100 in LDL may contain a number of heparin-binding regions when peptides of apoB are produced, our data suggest that LDL interaction with heparin involves NTAB. The observation that MB19, the anti-NTAB antibody, inhibited LDL binding to heparin and dermatan sulfate should not be interpreted as evidence for the involvement of a specific epitope in this process. Rather the relatively large antibody, approximately 150 kDa, probably produced steric hindrance of the smaller, less than 100-kDa NTAB. In contrast, MB47, which inhibits LDL interaction with the LDL receptor, does not mask a region that is essential for LDL-heparin interaction. The competition experiments using soluble apoB17 demonstrate that this protein had heparin binding properties and are suggestive that apoB17 prevents LDL binding to matrix by competing with a similar region on apoB. The observation that NTAB binds more tightly to heparin than does LDL was not unexpected. We had previously observed that a fragment of apoB was found on the surface of cultured endothelial cells and that this protein was dissociated from the cells by treatment with heparin (20).

Prior theories implicating the LDL-receptor binding region of apoB in LDL retention within arteries were consistent with the biochemical data showing that small peptides near this region bound to heparin. The interpretation of these data required the assumption that this region of apoB was situated on the lipoprotein particles in a manner identical to that in the small soluble peptide fragments that were used in the biochemical studies. Other heparin-binding peptides that were identified in the NTAB had less positive charge and did not bind to heparin with as great an affinity. The extrapolation of these observations to that of LDL requires exposure of the peptide on the LDL surface and tertiary configuration changes that do not mask these regions or alter their charge density, *i.e.* by ionic interaction with negatively charged amino acids outside of these peptides. The configuration of NTAB when not associated with lipid or even when on apoB48 could differ from its structure in apoB100. Several groups of investigators using protease digestion have shown that NTAB exposure is increased as very low density lipoprotein is converted to LDL (40–42). Perhaps, for this reason, some larger very low density lipoproteins are less atherogenic because they bind more poorly to proteoglycans. Similarly, it may be that NTAB contribution to the atherogenicity of apoB48-lipoproteins may be relatively greater than its role in apoB100 LDL. It should be noted that LDL interactions with proteoglycans are a relatively low affinity process compared with that of other heparin-binding molecules (43). For this reason it has been postulated that "bridging" molecules might be required to mediate this process (44).

Recently, Boren *et al.* (45) created an apoB molecule in which lysine 3363 was mutated to glutamic acid. LDL containing this mutated apoB had a defect in binding to the dermatan sulfate-containing proteoglycan, biglycan; interaction with heparin and decorin was not reported. These data suggest that multiple sites may be important in LDL binding to different proteoglycans or that this mutation produces alterations in apoB structure outside the basic amino acid cluster that was being investigated. Such an effect would not be unprecedented as heparin binding often involves the tertiary structural arrangement of basic amino acids.

Studies of the interactions of other proteins with heparin have shown that mutation of individual amino acids does not necessarily lead to identification of heparin binding regions. For example, several studies to define the heparin-binding region of lipoprotein lipase showed that mutagenesis of basic residues reduced, but did not eliminate, lipoprotein lipase-heparin interaction (46–48). In addition, mutations of lipoprotein lipase outside of the putative heparin-binding regions sometimes also result in molecules with defective heparin binding, presumably because the conformation of the protein is altered. More recent studies using chimeric molecules in which large regions of lipoprotein lipase and hepatic lipase were interchanged have shown that a different region mediates heparin binding (49, 50). Therefore, protein interaction with heparin appears to be a complex molecular interaction that, at least in some cases, is not modulated by a single charged amino acid unless that amino acid is essential for the conformation of a larger region of the protein.

Demonstrating that NTAB mediates lipoprotein association with matrix proteins allows a more consistent understanding of why apoB-lipoproteins are atherogenic. Previous investigators have postulated that apoB48-lipoproteins required additional proteins for retention. Because most of these lipoproteins are remnants found in the postprandial period or in the plasma of patients with dysbetalipoproteinemia, one hypothesis was that apoE, a well known heparin-binding protein, mediates proteoglycan interaction. This hypothesis, however, is inconsistent with two recent observations. 1) ApoE knockout mice have severe atherosclerosis; thus, the apoB48 remnants are retained within the matrix in the absence of apoE. 2) Production of apoE by macrophages, including those within the arterial wall, is anti-atherogenic (39, 51). The anti-atherogenic actions of apoE

are exclusive of apoE actions to reduce plasma lipoproteins. Therefore, the hypothesis that apoE promotes atherosclerosis by causing retention of apoB48 remnants is not tenable.

What are the pathobiological implications of these primarily biochemical observations? 1) The data lead to an alteration in our view of lipoprotein atherogenicity and allow a more consistent mechanism that explains why apoB100- and apoB48-lipoproteins are equally atherogenic. 2) By beginning to define the atherogenic portion of lipoproteins, a molecular target for intervention at the level of apoB interaction with artery wall proteins becomes discernable. If the results presented in this manuscript can be extended to studies in animal models of atherosclerosis, the potential exists to seek agents that prevent apoB-matrix protein interactions as preventative and/or therapeutic agents for coronary artery disease.

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