Pre-Beta High Density Lipoprotein
Unique Disposition of Apolipoprotein A-I Increases Susceptibility to Proteolysis

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Apolipoprotein A-I-containing lipoproteins (high density lipoproteins, HDL) can be separated into two subfractions, which have pre-beta and alpha electrophoretic mobilities, respectively. These fractions differ in both composition and structure. Some preparations of pre-beta-migrating HDL, but not alpha-migrating HDL, were found to contain two polypeptides with Mr of approximately 26 and 14 kDa, which are scission products of apolipoprotein (apo) A-I. They are recognized by monospecific antibodies to apo A-I and have N-terminal sequences identical to those of mature apo A-I. This proteolytic scission of apo A-I occurs primarily after venipuncture. Immediate addition of protease inhibitors minimized the appearance of the fragments in plasma. To study the relative susceptibilities of pre-beta and alpha HDL to proteolysis, the lipoproteins were incubated in vitro with plasmin. The apo A-I in pre-beta HDL was extensively degraded, but that in alpha-migrating HDL was degraded to a much lesser extent, indicating that the appearance of apo A-I fragments in pre-beta HDL was due to enhanced sensitivity to proteolysis. To varying degrees, thrombin, kallikrein, elastase, arginine C endopeptidase, and chymotrypsin also appear to cleave pre-beta HDL faster than alpha HDL. Most of the proteases generated a 12 to 14 kDa peptide fragment under conditions of limited cleavage. These results suggest that the conformational state of apo A-I in pre-beta-migrating HDL or its spatial relationship to lipids is significantly different from that of apo A-I in alpha-migrating HDL. Furthermore, this conformation of apo A-I appears to expose a protease-sensitive region near the midpoint of the sequence. Finally, when studies of pre-beta HDL are undertaken, care should be taken to prevent proteolytic degradation of this particle. (Arteriosclerosis 10:25–30, January/February 1990)

High density lipoproteins (HDL) are a collection of subpopulations with different compositions and properties. One such HDL subpopulation has pre-beta electrophoretic mobility (pre-beta HDL). This group of lipoproteins has a composition that is distinctly different from the bulk of alpha-migrating HDL. Pre-beta HDL also appear to possess structural properties that distinguish them from the bulk of HDL. The in vitro proteolytic degradation of apolipoprotein (apo) A-I has been demonstrated for the enzymes plasmin, thrombin, trypsin, and chymotrypsin. The degradation of apo A-I on intact HDL has also been studied, allowing the identification of exposed and protected regions. But no apparent differences in the proteolytic degradation of HDL fractions HDL₁ and HDL₂ have been determined. We describe here the enhanced sensitivity of the apo A-I in pre-beta HDL to in vitro proteolytic cleavage when compared to alpha HDL.

Methods

Isolation of Plasma

Plasma was isolated from freshly drawn venous blood by centrifugation at 10,000 g for 30 minutes at 4°C in the presence of preservatives and inhibitors, 0.04% ethylenediaminetetraacetic acid (EDTA), 0.05% NaN₃, 1 μg/ml gentamycin, 0.3 mg/ml benzamidine, 1 mM PMSF, 0.13% e-amino caproic acid, and 10 μg/ml alpha₂ macroglobulin, final concentrations.

Isolation of Pre-Beta- and Alpha-migrating High Density Lipoproteins

Immunosorbed HDL were isolated by selected-affinity immunosorption, as described previously. Briefly, apo A-I-containing lipoproteins (immunosorbed HDL) were recovered from plasma by retention on an anti-apo A-I-Sepharose column and were eluted by 0.2 M acetic acid, pH 3.0. The pH of the eluate was adjusted immediately to 7.0 with 2 M Tris, pH 10.0. The antibodies used to make the selected affinity column were isolated through the use of an apo A-I-Sepharose column and the same elution buffer. After concentration and dialysis, the immunosorbed HDL...
Figure 1. Electrophoretic separation of apoproteins (apo) from pre-beta high density lipoprotein (HDL). A. Sodium dodecyl sulfate 5% to 25% polyacrylamide gel electrophoresis of two different pre-beta HDL preparations compared to a set of molecular weight standards and stained with Coomassie blue. B. Autoradiograph of an immunoblot of the transferred proteins from a similar gel. A monospecific anti-apo A-I antisera was used for the identification of protein bands.

Figure 2. Plasmin digestion of pre-beta- and alpha-migrating high density lipoprotein (HDL). Alpha and pre-beta HDL were incubated for 1 hour at 4°C with or without plasmin. The apoproteins were analyzed by sodium dodecyl sulfate 5% to 25% polyacrylamide gel electrophoresis. A. Alpha HDL without plasmin. B. Alpha HDL with plasmin. C. Pre-beta HDL without plasmin. D. Pre-beta HDL with plasmin. An amount of sample equivalent to 5 µg of undigested protein was applied to each lane.

Protease Incubation of High Density Lipoprotein

Aliquots of 25 µg of pre-beta or alpha HDL protein in 80 µl of reaction buffer (10 mM Tris, pH 8) were incubated at 4°C in the presence or absence of 14 mUnits purified plasmin (Boehringer-Mannheim, West Germany). After 1 or 12 hours, the incubation was terminated by the addition of decyl sulfate to a final concentration of 0.1%, and the samples were boiled for 1 minute. Protease digestions by 2 µg elastase (Boehringer-Mannheim, West Germany), 0.18 U kallikrein, 1.2 U thrombin (Sigma, St Louis, MO), 10 µg chymotrypsin (Sigma), or 1.0 U arginine-C endoprotease (Sigma) were performed as described for plasmin, except that the incubations were for 0.5 to 1 hours at room temperature.
**Analysis of Proteins**

The apoproteins were analyzed by electrophoresis in discontinuous 5% to 25% gradient gels in the presence of 0.1% sodium dodecyl sulfate (SDS), 0.025 M Tris, 0.192 M glycine (pH 8.9) buffer. The samples were prepared by addition of an equal volume of solubilizing buffer containing 4% SDS, 0.125 M Tris, and 20% glycerol (pH 6.8) and were boiled for 30 seconds. In all gels, each lane contained an amount of protein equivalent to 5 µg based on the protein content measured before incubation with proteases. After electrophoresis, the gels were either stained with Coomassie blue R 250 or electrophoretically transferred to nitrocellulose sheets. The transferred proteins were analyzed by reaction with anti-apo A-I antisera and 125I-labeled protein A. The apo A-I fragments were separated by high-performance liquid chromatography (HPLC) on a Mono Q ion-exchange column (Pharmacia, Uppsala, Sweden). The peptides were eluted with a 0.0 to 0.3 M NaCl gradient in the presence of 6 M urea and 0.005 M Tris (pH 7.4). The isolated proteins were then subjected to amino acid sequence analysis on an Applied Biosystems model 470 A protein microsequencer.

**Results**

The protein patterns observed in several preparations of pre-beta HDL obtained from several donors at different times fell into two groups. Some preparations contained predominantly intact apo A-I, while others also contained proteins of approximately 26 kDa and 14 kDa, along with traces of other bands (Figure 1A). These three bands were detected by immunoblotting with anti-apo A-I antisera (Figure 1B), indicating that the 26 kDa and 14 kDa bands were fragments of apo A-I. Indeed, after isolation of the 28, 26, and 14 kDa bands by HPLC, it was determined that the N-terminal sequence (4 amino acids) of each band was identical to that reported for mature apo A-I. At the same time, no 26 or 14 kDa fragments were found in concurrent preparations of alpha HDL.

The degradation of apo A-I in pre-beta HDL appeared to occur after blood drawing. We found that the appearance of the proteolytic fragments in pre-beta HDL could be minimized by the addition of protease inhibitors, especially alpha 2 macroglobulin, to blood immediately after phlebotomy. The degree of degradation did not appear to be dependent upon a subject's plasma lipid or lipoprotein contents. In fact, the degradation of pre-beta HDL from a single subject was variable, depending upon the length of time the sample was unprotected by protease inhibitors. The progression of degradation of a single sample was found to be a function of storage time without protection by protease inhibitors.

To investigate this enhanced sensitivity of pre-beta HDL to proteolytic degradation, we performed a series of in vitro incubations of the isolated HDL subfractions with plasmin.
After an incubation of 1 hour, plasmin had a modest effect on the apo A-I of alpha HDL, creating a light band at approximately 14 kDa. However, the apo A-I of pre-beta HDL was degraded, broadening the band at 28 kDa and forming a prominent band at approximately 14 kDa (Figure 2). Below the lower band, a faint zone of staining material appeared, probably representing the degradation products of the C-terminal portion of apo A-I. After 12 hours, the 28 kDa apo A-I of alpha HDL did not appear to undergo further degradation, whereas intact apo A-I had disappeared from pre-beta HDL, leaving only faint bands (Figure 3). Since equal amounts of protein were analyzed, the decrease in the intensity of the apo A-I band truly reflects degradation of this protein. Interestingly, prolonged incubation of alpha HDL with plasmin did cause the disappearance of apo A-I and the 14 kDa fragment of apo A-I, yet no further degradation of apo A-I was observed. This suggests that in alpha HDL, only a portion of the total apo A-I is susceptible to plasmin degradation under the conditions used. As demonstrated by these results, the apo A-I of pre-beta HDL appears to be more sensitive to proteolysis by plasmin when compared to the apo A-I of alpha HDL.

The small differences in mobility of undegraded apo A-I observed in Figures 2, 3, and 4 appear to be due to variations in interlane migration. The N-terminal sequences of the apo A-I in both alpha HDL and pre-beta HDL were found to be identical. When the proteins of alpha and pre-beta HDL were mixed and subjected to electrophoresis, there was only one apo A-I band visible and no broadening of this band was detected.

Pre-beta and alpha HDL were also subjected to degradation by thrombin, kallikrein, elastase, arginine C endoprotease, and chymotrypsin (Figure 4). To varying degrees, the apo A-I in pre-beta HDL appears to be more sensitive to cleavage by most of these proteases than the apo A-I in alpha HDL, under conditions of limited digestion. Thrombin and kallikrein both generated a low molecular weight fragment from pre-beta HDL apo A-I, whereas no degradation of alpha HDL was apparent. Elastase and chymotrypsin extensively digested pre-beta HDL with lesser degradation of alpha HDL. In most cases, proteolytic degradation of pre-beta HDL led to the formation of a 12 to 14 kDa fragment, suggesting that a region of hypersensitivity to proteolysis exists when apo A-I assumes the conformation or exposure that it possesses in pre-beta HDL.

Discussion
We have demonstrated that the apo A-I of pre-beta HDL has a much greater sensitivity to proteolytic cleavage by plasmin and several other endoproteases than does the apo A-I of alpha HDL. This implies that the apo A-I exists in a different environment or conformation on pre-beta HDL than on alpha HDL. Pre-beta HDL contain 90% protein, which is predominantly apo A-I. The unique composition of pre-beta HDL apparently gives the particles a structure that is quite different from...
that of the bulk of HDL. Specifically, the helicity of apo A-I in pre-beta HDL is significantly less than that of apo A-I in alpha HDL.2 Also the lipid-poor environment found in pre-beta HDL may increase the exposure of a protease-sensitive region on apo A-I. Previous studies have shown that isolated apo A-I is degraded by plasmin,3,4 thrombin,4 trypsin,5,6 and chymotrypsin5 in vitro, consistent with this possibility.

The proteolytic cleavage of apo A-I in pre-beta HDL that we found appeared to occur after phlebotomy, because immediate addition of protease inhibitors minimized the formation of fragments. The original inhibition cocktail contained the antibiotics, azide and gentamycin, which suggests that the cleavage is not due to microbial proteases but is caused by protease activity found in blood. Such an event would be analogous to the cleavage of apo B-100 by kallikrein.14-1518 The exact protease responsible is unknown; however, possible candidate proteases include an elastase-like metalloprotease that has been described in association with ultracentrifuged HDL,17,18,19 as well as the serine proteases involved in the clotting cascade and in thrombolyis.

While the proteolysis we described occurred during in vitro incubations, the cleavage may have physiological importance. Bachorik et al.20 have observed that when HDL are exposed to cultured hepatocytes, the apo A-I is degraded into fragments similar to those found in pre-beta HDL in this study. Furthermore, Gregg and associates21 have recently shown that apo A-I partially degraded to approximately 26 kDa is removed from circulation more rapidly than nondegraded apo A-I. Since degradation of pre-beta HDL appears to yield such fragments, it is possible that pre-beta HDL might comprise a special kinetic compartment of apo A-I with increased turnover.

Initial cleavage of the apo A-I on pre-beta HDL, and to some extent alpha HDL, by plasmin as well as several other proteases generated apo A-I fragments of approximately 12 to 14 kDa. This region of cleavage spans amino acid residues 100 to 120 and may correspond to the so-called “hinged domain” that has been hypothesized to explain observed quantized changes in HDL diameter.22 The hypothesis suggests that this domain becomes excluded from the surface monolayer of an HDL particle as its diameter decreases. Such a conformational change might expose this region to proteolytic cleavage.

The finding that degradation of apo A-I in pre-beta HDL occurs and can be minimized by the immediate addition of protease inhibitors indicates that future studies directed at the quantitation or analysis of this lipoprotein should involve protection against proteolytic cleavage of apo A-I.

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