Altered phospholipid-apoB-100 interactions and generation of extra membrane material in proteolysis-induced fusion of LDL particles

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Abstract **Lipid droplets and membrane material are produced in the extracellular matrix of the arterial intima during atherogenesis. Both in vitro and in vivo experimentation suggests that fusion of modified LDL particles leads to formation of such lipid droplets. Here we applied proton NMR spectroscopy to probe surface phospholipids phosphatidylcholine (PC) and sphingomyelin (SM) of LDL particles during proteolytic degradation of apolipoprotein B-100 (apoB-100). Initiation of apoB-100 degradation was accompanied by the abruptly increased intensity of the choline -N($C\underline{H}_3$)$_3$ resonance of PC molecules, indicating disruption of their interactions with apoB-100. However, subsequent particle fusion was accompanied by a steady decrease in the intensity of the choline resonances of both PC and SM. Electron microscopy of the proteolyzed LDL revealed irregularly shaped multilamellar membranes attached to aggregates of fused particles. This suggests formation of membrane material with low hydration, in which some of the atomic motions are hindered. Characterization of the behavior of the surface lipids of LDL particles during apoB-100 degradation and other types of LDL modification will aid in understanding molecular mechanisms leading to fusion and generation of multilamellar membrane material in the arterial intima during atherogenesis.**—Pentikäinen, M. O., M. T. Hyvönen, K. Öörni, T. Hevonoja, A. Korhonen, E. M. P. Lehtonen-Smeds, M. Ala-Korpela, and P. T. Kovanen. **Altered phospholipid-apoB-100 interactions and generation of extra membrane material in proteolysis-induced fusion of LDL particles.** ***J. Lipid Res.*** **2001.** 42: **916–922.**

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The initial stages of atherosclerosis are characterized by retention and accumulation of extracellular lipid in the form of lipid droplets (~100–400 nm in diameter), vesicles, and bilayer and multilamellar membrane structures in the arterial intima, the inner layer of the arterial wall (1–6). Work from several laboratories has indicated that fusion of native LDL particles (~22 nm in diameter) would be a modification that would give rise to cholesteryl ester-rich particles closely resembling the lipidic particles observed in atherosclerotic lesions (7–10). Fusion has also been observed to enhance the retention of LDL particles in the arterial intima by increasing the strength of their binding to aortic proteoglycans (11–13).

Particle fusion is induced in vitro by a variety of modifications of the LDL surface. These include proteolysis of the apolipoprotein B-100 (apoB-100) (14, 15) and hydrolysis of the surface phospholipids with sphingomyelinase (8, 11), phospholipase A_2 (16), or phospholipase C (17, 18). Modifications of the LDL surface are likely to occur in vivo, because a variety of extracellularly located proteases and lipolytic enzymes are present in the intima (19, 20). In our laboratory, we have previously characterized proteolytic fusion of LDL particles as an in vitro model for studying the processes in the arterial intima thought to be responsible for the formation of extracellular lipid droplets (10–12, 14, 15, 21). As part of our efforts to understand lipoprotein particle interactions, we developed a fusion assay based on ^{1}H NMR spectroscopy (22). In addition to monitoring particle size, ^{1}H NMR spectroscopy can provide physicochemical information about lipoprotein lipids and enable noninvasive follow-up of lipoprotein fusion (23–25).

An important finding has been that high field ^{1}H NMR spectroscopy permits individual detection of phosphatidylcholine (PC) and sphingomyelin (SM) molecules at the surface of LDL particles via the resonance of the choline head group -N($C\underline{H}_3$)$_3$ at about 3.2 ppm (25). Using this methodology, Murphy et al. (25) found that 19% of the total surface phospholipids of LDL are NMR invisible, ap-

Abbreviations: apoB-100, apolipoprotein B; EM, electron microscopy; PC, phosphatidylcholine; SM, sphingomyelin; TSP, sodium 3-trimethylsilyl[2,2,3,3-D_4] propionate.

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parently because apoB-100 immobilized 27% of the PC molecules. This and a fluorescence spectroscopic study (26) provide evidence of the preferential interaction of apoB-100 with PC and suggest that SM resides in a bulk lipid phase at the surface of the LDL particle. A model of the interaction between apoB-100 and PC was illustrated in a review of LDL structure (27).

Here, we modified LDL particles with proteolytic enzymes, α-chymotrypsin or trypsin, and studied the molecular interactions at the surface of the proteolyzed particles by ^{1}H NMR, together with electron microscopy (EM) and biochemical experimentation. The experiments revealed that, in addition to triggering fusion of LDL particles, proteolysis induced time-dependent changes in the PC and SM resonances, apparently by disrupting the apoB-100-PC interaction at the onset of proteolysis, and later, during particle fusion, by generating low-hydrated membrane material. This process mimicks the extracellular formation of lipid droplets and membrane material in the arterial intima during atherogenesis.

MATERIALS AND METHODS

Materials

[1,2-^{3}H]cholesteryl linoleate, [^{14}C]PC, and PD-10 and Superose 6 HR columns were from Amersham Pharmacia Biotech (Piscataway, NJ); α-chymotrypsin (from bovine pancreas), deuterium oxide (D_2O), deuterated chloroform ($CDCl_3$) and methanol (CD_3OD), and trypsin (from bovine pancreas) were from Sigma (St. Louis, MO). Purified phospholipid transfer protein was a kind gift from P. Somerharju (University of Helsinki, Helsinki, Finland).

Isolation and modifications of LDL

Human LDL (d = 1.019–1.050 g/ml) were isolated from plasma of healthy volunteers by sequential ultracentrifugation (28). The isolated LDL was dialyzed extensively against buffer A (150 mM NaCl, 1 mM EDTA, pH 7.4), filtered through a 0.22 μm pore size filter, fractionated, and stored at 4°C. In some experiments, the LDL was labeled with [^{3}H]cholesteryl linoleate as described previously (15). LDL was labeled with [^{14}C]PC by incubation of sonicated [^{14}C]dipalmitoylphosphatidylcholine vesicles with LDL in the presence of purified phospholipid transfer protein for 18 h at 37°C followed by reisolation of the labeled LDL by size-exclusion chromatography over two Superose 6 HR columns connected in series.

The NMR measurements were carried out in buffer B (137 mM NaCl, 2.7 mM KCl, 10 μM Na_2EDTA, 10 mM phosphate, pD 7.0 in D_2O) (25). To change the buffer, LDL was gel filtered twice through PD-10 columns equilibrated and eluted with buffer B. The amounts and concentrations of LDL are expressed in terms of protein, as determined by the procedure of Lowry et al. (29) with bovine serum albumin as standard. LDL (1 mg/ml) was proteolyzed by incubation with α-chymotrypsin (0.1 or 0.025 mg/ml) or trypsin (0.1 mg/ml) at 37°C in buffer B for the indicated times (15, 21, 22). In some experiments, lipids of native and modified LDL were extracted into deuterated chloroform–methanol 2:1 (v/v) according to Bligh and Dyer (30) and analyzed by proton NMR.

The association between surface and core lipids in proteolyzed particles was analyzed with LDL particles labeled with both [^{3}H]cholesteryl linoleate and [^{14}C]PC in density-gradient ultracentrifugation and in size-exclusion chromatography over Superose 6 HR columns. Samples were processed for thin-section EM exactly as described previously (21), and viewed in a JEOL (Tokyo, Japan) JEM-1200EX transmission electron microscope at the Electron Microscopy Core Facility (Institute of Biotechnology, University of Helsinki).

^{1}H NMR spectroscopy

Experimental. The ^{1}H NMR data were obtained at 37°C with a Bruker (Leipzig, Germany) DRX 500 MHz spectrometer at the NMR Laboratory (University of Oulu, Oulu, Finland). A sealed coaxial insert (outer diameter of 2 mm) containing an external standard, sodium 3-trimethylsilyl[2,2,3,3-D_4] propionate (TSP, 8 mM) and $MnSO_4$ (0.6 mM) in 99.8% D_2O, was placed in a 5-mm NMR tube containing 0.465 ml of sample (22). In each experiment 64 flame ionization detection (FID) signals of 64K data points were accumulated, using a pulse repetition time of 6.6 s and 90° pulses. The spectral width was 6.25 kHz. LDL and LDL plus α-chymotrypsin or trypsin samples were incubated in the magnet for up to 48 h and data were recorded every 10 to 60 min. Four different experiments with α-chymotrypsin and two with trypsin-treated LDL particles were carried out, leading to follow-up data sets, each consisting of 48 to 286 individual spectra. The measured FID signals were Fourier transformed to the frequency domain spectra, using exponential apodization of either 0.0 or 0.3 Hz. At the concentrations and under the experimental conditions used, over the frequency range studied, α-chymotrypsin or trypsin gave a hardly detectable broad signal and thus did not disturb the information about the LDL lipids (data not shown). Spectra of lipid extracts of native and proteolyzed LDL in deuterated choloroform–methanol 2:1 (v/v) were acquired as described above, except that a 20-s pulse repetition time was applied and no external standard was used.

Spectral analysis. The terminal fatty acid methyl -C$\underline{H}_3$ and the PC and SM choline head group -N(C$\underline{H}_3$)$_3$ resonances were subjected to line-shape fitting analysis to resolve the areas and chemical shifts of the resonances. In the spectra of native and proteolyzed LDL the -C$\underline{H}_3$ resonance (at about ~0.8 ppm) was fitted with four Lorentzian components. The -N(C$\underline{H}_3$)$_3$ resonance was fitted with two components, corresponding to the signals from SM (~3.24 ppm) and PC (~3.25 ppm). In the spectra of the lipid extracts, nine resonance peaks from ~0.73 to 0.62 ppm were used to describe the total -C$\underline{H}_3$ area. A linear baseline and a phase parameter were also estimated for the resonance regions analyzed. A total line shape-fitting algorithm of the PEak reseaRCH (PERCH) software (University of Kuopio, Kuopio, Finland) was applied (31).

Particle size-dependent chemical shifts. A physical model has been developed for lipoprotein particle structure to explain the size-dependent chemical shifts in the ^{1}H NMR resonances of lipoprotein lipids (32). This phenomenon is attributed to the anisotropy of the magnetic susceptibility due to orientation of the surface monolayer of the lipoprotein particles. Solution of the magnetic field equations for a spherical particle with an isotropic core and a radially oriented surface gave a simple equation linking the frequency of the ith NMR line, ν_i, and the lipoprotein particle radius, R (in SI units):

$$\nu_i(R) = \nu_i^0 + \frac{2}{3}\nu_0\Delta\chi\ln\frac{R}{R-\Delta} \qquad \textit{Eq. 1)}$$

Here ν_i^0 is the asymptotic value of ν_i at limit $R \to \infty$, ν_0 is the operating frequency of the spectrometer, $\Delta\chi$ is the anisotropy of the magnetic volume susceptibility of the particle surface, and Δ is the thickness of the surface. As the frequency ν_i is an explicit function of the radius R, this model explains in a natural way the

systematic dependence of the frequency observable with NMR on the size of the lipoprotein particles. This theoretical formulation has been shown to provide a means for dynamic follow-up of lipoprotein particle fusion and also for distinguishing fusion from aggregation of the particles (22). Using the chemical shifts of the fatty acid -C$\underline{H}_3$ resonances of lipoprotein lipids, Equation 1 was applied here to probe the fusion of LDL particles in the proteolytically treated samples and to calculate and follow up the average size of the LDL particles in the samples. The thickness, Δ, and the anisotropy of the magnetic susceptibility, $\Delta\chi$, of the surface monolayer were kept constant for particles of different sizes, and values of 2.0 nm for Δ and of -0.223 for $\Delta\chi$ (in SI units) were used according to previous work (22, 32).

RESULTS

LDL was proteolyzed extensively with α-chymotrypsin so that after 6 h no intact apoB-100 was present in SDS-PAGE and at 24 h 60% of the apoB-100 had been cleaved to trichloroacetic acid (TCA)-soluble fragments (<5,000 kDa) (15, 22). Proteolysis was accomplished within the NMR spectrometer and thus we were able to follow the kinetics of the proteolysis-induced fusion and the changes in the mobilities of the surface lipids on the LDL particles. **Figure 1** shows the choline head group -N(C$\underline{H}_3$)$_3$ resonances of PC and SM in the ^{1}H NMR spectrum of native LDL and in its lipid extract, illustrating how the behavior of PC and SM choline head groups can be studied separately. Figure 1 also shows the time-dependent behavior of the -C$\underline{H}_3$ resonance, which shifted toward higher frequencies, indicating particle fusion during α-chymotrypsin digestion (Equation 1) (22, 32).

Analysis of the areas of the choline head group resonances in the ^{1}H NMR spectra showed an increase in PC resonance, which accords with disruption of the tight apoB-100-PC interactions (**Fig. 2**). Unexpectedly, however, after 4 h of proteolysis, the area of the PC resonance started to decrease, whereas SM resonance started to decrease immediately. Similar behavior was observed for both PC and SM, but at a lower rate, when trypsin or a lower concentration of α-chymotrypsin was used (not shown). Interestingly, detailed analysis of the -C$\underline{H}_3$ resonance revealed a slight shift toward lower frequencies before the major shift toward higher frequencies was observed (Fig. 2). The area of -C$\underline{H}_3$ groups also decreased slightly (0–7%) during the incubations (not shown). Absence of a peak corresponding to free choline groups (at 3.17 ppm) suggested that the decreases in PC and SM resonances were not due to hydrolysis of the PC and SM molecules by, for instance, phospholipases (not shown). Moreover, in our previous studies we have found that during proteolytic modification the lipids of LDL are not hydrolyzed (7). Thus, in contrast to oxidative modification of LDL (33), the endogenous phospholipase A_2 of LDL, that is, the platelet-activating factor acylhydrolase, is not activated during proteolytic treatment of LDL. When lipids were extracted from the modified particles in solvent and analyzed by ^{1}H NMR, the areas of PC and SM (-C$\underline{H}_3$ as a reference) were found to be similar in native LDL (0.20 for PC and 0.074 for SM) and in LDL proteolyzed for 4 h (0.20 and 0.079), 12 h (0.19 and 0.071), 24 h (0.24 and 0.086), and 48 h (0.17 and 0.067). This further demonstrates that the PC and SM molecules were intact, but that, in the modified particles, the mobilities of their head groups were inhibited. Taken together, it appeared that proteolytic degradation of apoB-100 rapidly increased the mobility of the head groups of PC molecules and later induced particle fusion, during which both PC and SM molecules formed structures in which their atomic mobilities were restricted, especially in the head group region.

To find out whether there had been any changes in particle morphology that could explain the observed behavior of PC and SM, we studied the modified particles by EM. When samples were cast in agarose, stained, and thin sec-

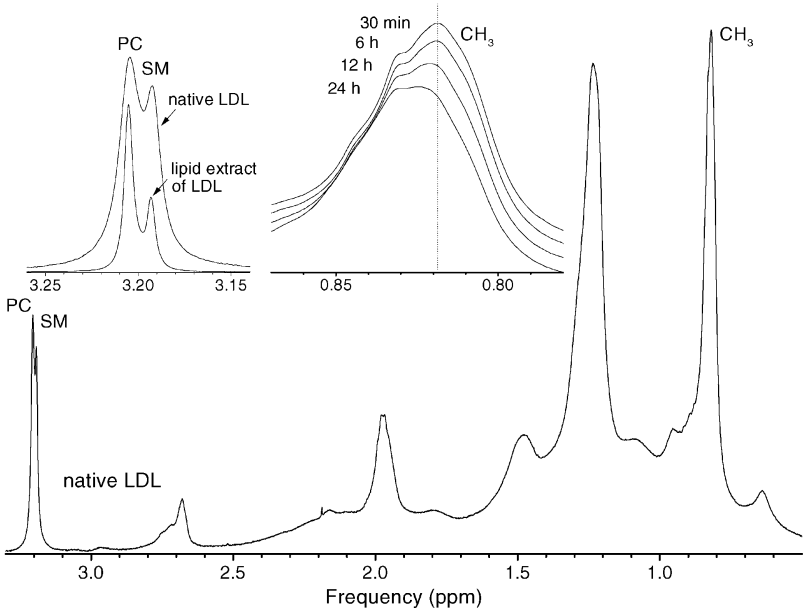

Fig. 1. The ^{1}H NMR lipid resonances in the aliphatic region of native LDL spectrum. Left inset: PC and SM resonances in the native LDL spectrum and in the spectrum of organic lipid extract of the native LDL sample. Right inset: -C$\underline{H}_3$ resonance and its shift, which is due to particle fusion during proteolysis of LDL by α-chymotrypsin. Note that the chemical shift of the extract spectrum has been adjusted to match the PC and SM resonances observed in the LDL spectrum, whereas the other spectra are referenced to the external TSP reference at 0.0 ppm.

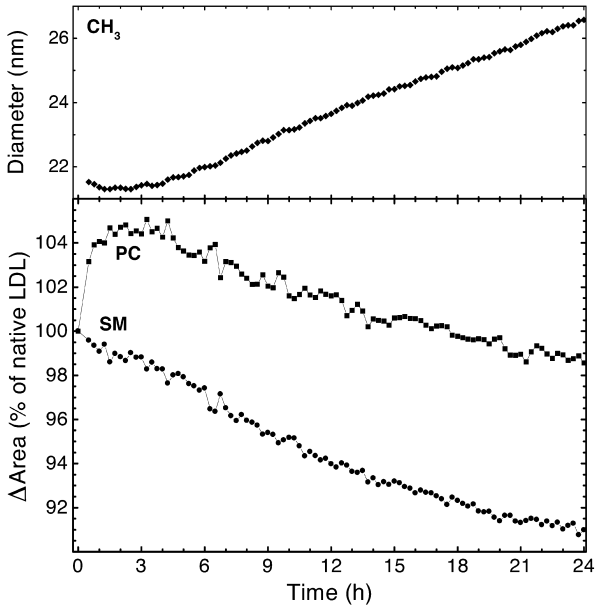

Fig. 2. The change in LDL diameter calculated from the shift of the -C$\underline{H}_3$ resonance by Equation 1 (top) and the change in the area of PC and SM peaks in the ^{1}H NMR spectra as a function of time for LDL incubated at 37°C in the NMR spectrometer in the presence of α-chymotrypsin (bottom).

tions were analyzed, we observed not only fused particles, but also large amounts of membranous material (**Fig. 3**). Most of this material was associated with the fused particles, which were bound to each other (i.e., formed an aggregate), and many of the membranes were found to be multilamellar. To ascertain whether the excess of membrane material had formed vesicles, phospholipid and cholesteryl ester double-labeled proteolyzed LDL was analyzed by size-exclusion chromatography and by density gradient ultracentrifugation. The two methods showed that the distribution of phospholipids and cholesteryl esters was similar (not shown), indicating that no separate vesicles had been formed from the excess of membrane material.

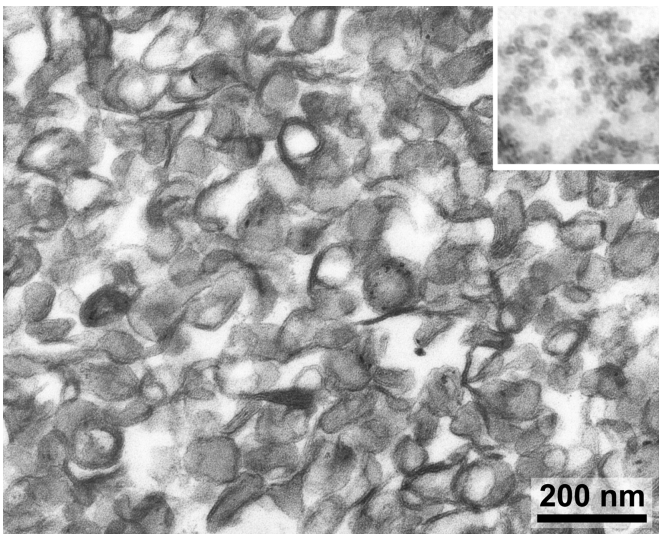

Fig. 3. Transmission electron micrograph of native LDL and LDL proteolyzed for 48 h at 37°C by α-chymotrypsin. Note the extensive aggregation of the proteolyzed particles and the multilamellar membranous material protruding from the fused particles. Inset: Native-sized LDL particles.

DISCUSSION

The detection of PC and of SM molecules via the choline head group -N(C$\underline{H}_3$)$_3$ resonance allowed probing of the behavior of the surface lipids of LDL during digestion of an important component of the LDL surface, apoB-100. Similar to the original findings by Murphy et al. (25), we found that during LDL proteolysis, the mobility of the PC head groups increases initially, probably because of diminishing immobilization of the PC head groups by apoB-100. This is in good agreement with the proposed pentapartite NH_3-α_1-β_1-α_2-β_2-α_3-COOH structure for apoB-100 at the LDL surface (34), where the β_1 and β_2 clusters have been suggested to associate irreversibly with lipids (35, 36). Molecular graphics of a model β sheet and PC in our review of LDL structure (27) illustrated how, at the molecular level, the mobility of the PC head groups could be inhibited by apoB-100. The slow decrease rather than increase in the mobility of SM during apoB-100 proteolysis demonstrates the difference in the behavior of PC and SM in the LDL surface, and suggests that SM resides in domains that are not tightly associated with apoB-100.

Interestingly, at the onset of LDL proteolysis, which was associated with the increase of PC mobility, the -C$\underline{H}_3$ resonance shifted toward lower frequencies. The reason for this

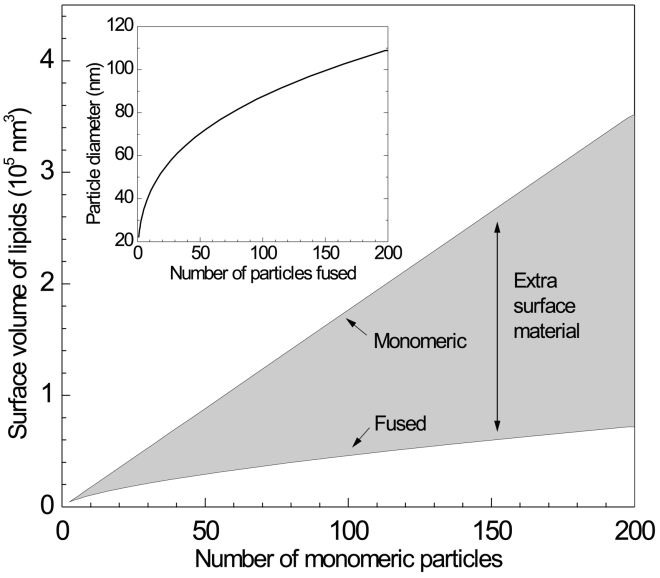

Fig. 4. Formation of extra surface material during proteolytic fusion of LDL particles. Shown is the surface volume of the lipids present in proteolyzed LDL [assuming that 70% of the surface volume is lipids (37)] and the surface volume needed to cover a fused particle. Inset: The diameters calculated for spherical fused LDL particles. In the calculations the diameter of native LDL was taken as 22 nm and the thickness of the surface layer was taken as 2 nm.

phenomenon is not clear, but could be due to condensation of the particles on loss of surface protein before sufficient surface defects were formed in the particles to allow their fusion. At a later stage of proteolysis, the kinetics of the decrease in the mobilities of PC and SM were found to follow the kinetics of LDL fusion. We have previously found that fusion only takes place after extensive proteolysis of apoB-100 (10, 11, 14, 15). This can be achieved with proteases having broad specificity, such as α-chymotrypsin, trypsin, and pronase, which yield small (TCA-soluble) fragments that are released from the particle surface (15). In contrast, specific proteases that, although they cleave apoB-100, do not release any fragments have been found not to trigger LDL particle fusion (15). These findings accord with the above-described hypothesis, suggesting that the shift of -C$\underline{H}_3$ resonance toward lower frequencies was due to loss of surface protein before the actual fusion process started.

Assuming that the thickness of particle surface is constant, in a large particle the ratio of the surface to core volume is much less than in a small particle. Accordingly, as illustrated in **Fig. 4**, although hydrolysis of apoB-100 is expected to decrease the surface volume of LDL up to ~30% (37), the amount of surface lipids required to cover a fused particle is much less than is present in the proteolyzed particles. For example, when a lipid droplet with a diameter of 100 nm is generated from fusion of ~150 LDL particles, only ~22% of the surface lipids present in the proteolyzed LDL particles is needed to cover the surface of the fused particle. The result of the calculation indicating the formation of extra surface material from LDL is consistent with the experiments in which changes in the PC and SM mobilities were observed and with the observations in EM of the membranes extending from the fused LDL particles. Although pro-

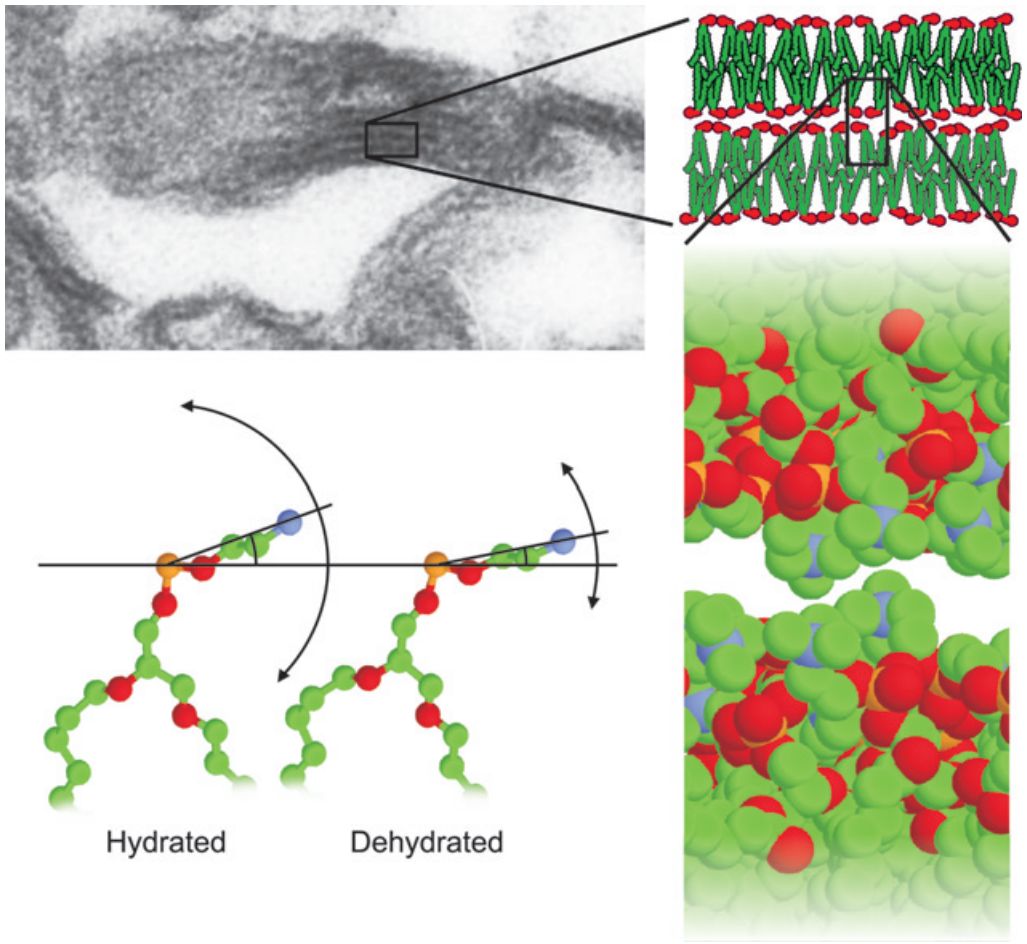

Fig. 5. Electron micrograph of multilamellar membranous material extending from a fused LDL particle (top left) together with the visualization of a hypothetical multilamellar structure at the molecular level (top right) and the head group regions of opposing layers at the atomic level (bottom right). The illustration at the atomic level demonstrates the close contacts between the head groups that are likely to occur with low hydration of opposing layers. The molecular graphics were made with RasMol software (RasWin Molecular Graphics, Windows version 2.6) and the model was constructed by reducing the spacing between the opposing layers, which were taken as snapshots of a molecular dynamics simulation of a palmitoyl-linoleoyl-PC bilayer (53). All the atoms are represented by spheres with 1.2-Å radius. The hydrogen atoms and water molecules were omitted for clarity. The possible changes in the head group orientation and mobility due to dehydration are illustrated by the phosphorus-nitrogen vectors (bottom left). The two phosphorus-bound oxygen atoms, the three nitrogen-bound carbon atoms, and all the hydrogen atoms in the PC molecules were omitted for clarity. The color coding for the bottom figures is as follows: blue for nitrogen, yellow for phosphorus, red for oxygen, and green for carbon.

teolytic LDL fusion can initially be explained by hydrophobic interactions between particles having surface defects due to apoB-100 degradation, we do not know what forces drive LDL fusion even though there is an excess of surface components.

The multilamellar membrane structure seen in EM will explain the decrease in the mobilities of the head groups of PC and SM, and also the slight effect in the methyl groups of the fatty acid chains. These observations are potentially relevant to human atherosclerosis, because multilamellar membranes have been observed in human atherosclerotic plaques (2), and moreover, early experiments with ^{13}C NMR spectroscopy have shown broadening of resonances of choline groups in plaque phospholipids, indicating their motional restriction (38). The decreased mobility is likely to be due to low hydration of the multilamellar membranes, for which the molecular organization is illustrated in **Fig. 5**. Decreased motion due to dehydration has been observed in models of phospholipid membranes (39–41): with dehydration, the effective size of the polar head group decreases, the lateral packing of the fatty acid chains tightens, and thermal motion decreases. This tighter packing in the bilayer plane affects both the head groups and the chains of the lipid molecules. A decrease in the rate of head group motion as a function of dehydration has also been demonstrated by an increased deuterium spin-lattice relaxation rate (42). The enhanced effect on the head groups as compared with the effect on the chains could be attributed to the close contacts between the choline groups of opposing layers with low hydration, as demonstrated by molecular dynamics simulations (43, 44). The close contacts between opposing cholines may tend to make the head groups more rigid. In fact, deuterium order parameters have revealed that, with low hydration, the average orientation of the head group dipole changes toward the plane of the membrane (42, 45). Close contacts between opposing bilayers, as well as proposed changes in head group orientation and mobility, are illustrated in Fig. 5. Interestingly, the mobilities of PC and SM appear to decrease similarly during the course of proteolysis. This suggests that PC and SM enter the modified surface structures in roughly equal quantities, even though the surface components of native LDL particles are suggested to form different domains rather than to be homogeneously distributed (27). Unfortunately, whether the unesterified cholesterol, which is partially distributed on the LDL surface, enters into the modified surface structures could not be determined with the present methodology.

The use of ^{1}H NMR in studies of LDL and complex lipid systems is not simple, yet combining ^{1}H NMR with other methods can give novel molecular information about the particles that is not available by any other method. LDL fusion has been suggested to be an important process in the initiation of lipid accumulation in the arterial intima during atherogenesis (20, 46). Because apoB-100 has been shown to be hydrolyzed to a variable degree in LDL isolated from the arterial wall (47–52), proteolysis of LDL is a potential modification contributing to LDL fusion in the arterial intima. Characterization of the behavior of surface lipids during proteolytic fusion of LDL, as was done in the present study, should help us to understand the molecular organization of the LDL surface during apoB-100 degradation and also during other types of LDL modification, and ultimately should aid in designing molecules that attenuate fusogenic interactions of the modified LDL particles.

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