Macrophage metalloproteinases degrade high-density-lipoprotein-associated apolipoprotein A-I at both the N- and C-termini

Ivano EBERINI*, Laura CALABRESI†, Robin WAIT‡, Gabriella TEDESCHI§, Angela PIRILLO*, Lina PUGLISI*, Cesare R. SIRTORI† and Elisabetta GIANAZZA*†

*Dipartimento di Scienze Farmacologiche, Università degli Studi di Milano, via G. Balzaretti, 9, I-20133 Milano, Italy, †Centro E. Grossi Paoletti per lo Studio delle Dislipidemie, Dipartimento di Scienze Farmacologiche, Università degli Studi di Milano, via G. Balzaretti, 9, I-20133 Milano, Italy, ‡Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College of Science, Technology and Medicine, 1 Aspenlea Road, Hammersmith, London W6 8LH, U.K., and §Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Sezione di Biochimica, Università degli Studi di Milano, via G. Celoria, 12, I-20133 Milano, Italy

Atheromatous plaques contain various cell types, including macrophages, endothelial cells and smooth-muscle cells. To investigate the possible interactions between secreted matrix metalloproteinases and high-density lipoprotein (HDL) components, we tested the above cell types by culturing them for 24 h. HDL$_{3_4}$ (HDL subfractions with average sizes of between 8.44 nm for HDL$_{3_4}$ and 7.62 nm for HDL$_{2_3}$) were then incubated in their cell-free conditioned media. Proteolytic degradation of apolipoprotein A-I was observed with macrophages, but not with endothelial-cell- or muscle-cell-conditioned supernatant. Absence of calcium or addition of EDTA to incubation media prevented all proteolytic processes. The identified apolipoprotein A-I fragments had sizes of 26, 22, 14 and 9 kDa. Two-dimensional electrophoresis and MS resolved the 26 and the 22 kDa components and identified peptides resulting from both N- and C-terminal cleavage of apolipoprotein A-I. The higher abundance of C- than N-terminally cleaved peptides agrees with data in the literature for a fully structured α-helix around Tyr$^{18}$ compared with an unstructured region around Gly$^{185}$ and Gly$^{196}$. The flexibility in the latter region of apolipoprotein A-I may explain its susceptibility to proteolysis. In our experimental set-up, HDL$_{3_4}$ was more extensively degraded than the other HDL$_{3}$ subclasses (HDL$_{3_4}$ and HDL$_{2_3}$). Proteolytic fragments produced by metalloproteinase action were shown by gel filtration and electrophoresis to be neither associated with lipids nor self-associated.

Key words: atherosclerosis, mass spectrometry, matrix metalloproteinase, proteolysis, two-dimensional electrophoresis.

INTRODUCTION

Matrix metalloproteinases (MMPs), a family of calcium- and zinc-dependent endopeptidases with catalytic activity against extracellular matrix components [1], have been implicated in the progression of atherosclerotic plaques. In their active form they may contribute to vascular remodelling [migration and proliferation of smooth-muscle cells (SMCs) with neointima formation] and plaque disruption [2–5]. Ex-vivo, MMP-2 can be detected histochemically in normal human arteries together with its inhibitors, tissue inhibitor of metalloproteinases (TIMP)-1 and TIMP-2. In atheromas, on the contrary, MMP-9 and MMP-12 [11], and fibroblasts produce MMP-2 [12]. After stimulation with lipopolysaccharide (LPS), mouse macrophages express TNF-α and MMPs 2 and 9 [13].

One investigation focused on a possible role for MMPs in atherogenesis, when cholesterol accumulates in the cells of arterial intima [14]. After incubation with MMPs 3, 7 or 12, the ability of high-density lipoprotein (HDL$_{3}$) (HDL subfractions with average sizes of between 8.44 nm for HDL$_{3_4}$ and 7.62 nm for HDL$_{2_3}$) to induce the high-affinity component of cholesterol efflux from macrophage foam cells is markedly decreased, whereas preincubation with MMP-1 reduces cholesterol efflux only slightly and MMP-9 is ineffective [14]. The effects of the various MMPs appear to be reflected in their differential abilities to degrade small preβ-migrating particles present in the HDL$_{3}$ fraction: MMPs that strongly reduce cholesterol efflux cleave the C-terminal region of apolipoprotein A-I (apoA-I) and produce a major fragment of about 22 kDa [14].

The above results were obtained in an in vitro system, with the use of recombinant MMPs, often after short incubation times and in the presence of high protease-to-substrate ratios [14]. The present investigation had, therefore, the following aims: (i) to test the ability of MMPs, produced by different cell lines, to

Abbreviations used: apoA-I, apolipoprotein A-I; GGE, gradient gel electrophoresis; HDL, high-density lipoprotein; HUVEC, human umbilical vein endothelial cell; IPG, immobilized pH gradient; MMP, matrix metalloproteinase; 2-DE, two-dimensional electrophoresis; LPS, lipopolysaccharide; PAA, polyacrylamide; RPM, rat peritoneal macrophage; SMC, smooth-muscle cell; TIMP, tissue inhibitor of metalloproteinases; TNF-α, tumour necrosis factor α.

1 To whom correspondence should be addressed (e-mail Elisabetta.Gianazza@unimi.it).
Lipoproteins and their fragments were then fractionated by endothelial cells, SMCs and macrophages. Lipoproteins, apo-
common in vascular walls and atherosclerotic plaques, namely supernatants from the cultures of the three cell types most between our findings and published structural data and models.

In our experiments, HDL$_2$ was incubated for up to 72 h in supernatants from the cultures of the three cell types most common in vascular walls and atherosclerotic plaques, namely endothelial cells, SMCs and macrophages. Lipoproteins, apolipoproteins and their fragments were then fractionated by electrophoretic methods and characterized by biochemical, immunological and MS techniques.

**EXPERIMENTAL**

**Lipoproteins**

The HDL$_2$ subfraction was separated from plasma of healthy donors by sequential flotation on KBr [15] and dialysed against PBS containing 0.01 % (w/v) sodium EDTA.

**Cell culture and MMP activity testing**

Rat peritoneal macrophages (RPMs) were collected from control Sprague–Dawley rats by lavage with cold PBS and plated at a density of $1 \times 10^6$ cells/ml, or in one case $2 \times 10^6$ cells/ml. Human umbilical vein endothelial cells (HUVECs) and SMCs from rat aorta were grown until subconfluence. Medium 199 with or without 10 µg/ml LPS or, in some cases, 40–500 units/ml TNF-$\alpha$, was conditioned for 24 h with each cell line. In order to avoid interference from serum proteases and protease inhibitors, the medium did not contain fetal calf serum, except for HUVEC cultures, which contained residues of fetal calf serum from previous culture steps that were not rinsed away. The culture supernatants collected after centrifugation at 4°C for 20 min at 1500 g were tested for gelatinolytic and caseinolytic activities.

**Lipoprotein treatment**

The culture supernatants were made to 10 mM CaCl$_2$, then 400 µg/ml HDL$_2$ was added and incubation was allowed to proceed for up to 72 h at 37 °C. Residual HDL$_2$ was characterized using a combination of electrophoretic techniques.

**Electrophoresis**

All experiments were performed under non-reducing conditions. Lipoprotein size was assessed by migration on 4–30 % T (where % T is the total monomer concentration in g/100 ml) polyacrylamide (PAA) gradients in Tris/borate/EDTA [16]. The pI values of apolipoproteins were evaluated by focusing on a non-linear 4–10 immobilized pH gradient (IPG) [17] in the presence of 8 M urea, and their $M_r$ values estimated by SDS/PAGE on 10–22 % T PAA in Tris/HCl/Tris/tricine buffer [18]. Two-dimensional electrophoresis (2-DE) was performed through a sequence of the above steps [19]. For qualitative evaluation and MS analysis the protein patterns were stained with either Coomassie Brilliant Blue or silver nitrate [20]; for immunological detection and N-terminal sequencing the proteins were electroblotted to either nitrocellulose in Tris/glycine buffer or to PVDF in 3-cyclohexylaminopropanesulphonic acid (Caps)/NaOH. Spot volumes in 2-DE patterns were quantified using PDQUEST (Bio-Rad, Hercules, CA, U.S.A.). Immunological detection was with a polyclonal anti-apoA-I antiserum followed by horseradish peroxidase-conjugated anti-rabbit IgG and ECL zymography. The assortment of apolipoproteins and their fragments in the various HDL$_2$ subclasses was investigated by a non-conventional two-dimensional electrophoretic technique, including migration on a 4–25 % T PAA gradient in Tris/borate/EDTA, for either 75 min or 24 h, and SDS/PAGE on 10–22 % T PAA as above.

**Matrix-assisted laser-desorption ionization MS**

In-gel digestion with trypsin was performed according to published methods [21–23]. Matrix-assisted laser-desorption ionization mass spectra were recorded with a ToFSpec 2E spectrometer (Micromass, Manchester, U.K.) equipped with a 337 nm nitrogen laser. The instrument was operated in the positive-ion reflectron mode at 20 kV accelerating voltage with time-lag focusing enabled. The matrix was a mixture of a-cyano-4-hydroxy-cinnamic acid and nitrocellulose [24]. For electrospray ionization experiments, desalted samples were loaded into palladium-coated borosilicate nanoelectrospray needles (Prota, Odense, Denmark) and mounted in the source of a
Q-Tof hybrid quadrupole/orthogonal acceleration time-of-flight spectrometer (Micromass).

N-terminal sequencing

Automated sequence analysis was performed on a pulsed-liquid sequencer (model 477; Applied Biosystems, Foster City, CA, U.S.A.) equipped with a 120A Applied Biosystems PTH analyser.

Gel filtration

A 4 ml sample of HDL$_3$, after a 3 day incubation in RPM supernatant, was fractionated by size on three serial Superose 6B columns (1.6 cm x 100 cm; Amersham Bioscience, Uppsala, Sweden), equilibrated in 10 mM Tris/HCl, pH 7.4, with 150 mM NaCl and 0.01% EDTA. The flow rate was 0.18 ml/min; the first 150 ml was discarded and fractions of 2 ml were collected. Aliquots of the eluate were analysed by SDS/PAGE, as above.

Phospholipid quantification

Phospholipid levels were determined by the enzymic method [25].

RESULTS

Figure 1 shows the zymograms for proteolytic activity (gelatinolytic, left-hand panel; caseinolytic, right-hand panel) on the supernatants after 24 h culture of HUVECs, SMCs and RPMs, without stimulation and after treatment with 10 μg/ml LPS. In all cases the amounts of secreted proteases and the ratios between proforms and mature forms were affected only to a minimal extent by LPS treatment; the same lack of effect on the above parameters was also observed with TNF-α treatment (50–400 units/ml [26]; results not shown).

Most proteases expressed by the three cell types have gelatin as their preferential substrate. SMCs and HUVECs both release MMP-2, while the latter cell line is the main producer of a caseinolytic enzyme identified as MMP-1. The major proteolytic enzymes secreted by RPMs are MMP-9 and MMP-12, with some MMP-3 and MMP-7 also being expressed.

Figure 2 compares the SDS/PAGE patterns of HDL$_3$ after a 3 day incubation in medium conditioned for 24 h by HUVECs, SMCs or RPMs, with and without the addition of 10 μg/ml LPS, with that of normal HDL$_3$. The major lipoprotein components (apoA-I and dimeric apoA-II) are marked, together with contaminating albumin in HUVEC supernatants. No change in apoA-II concentration was detected in any sample. The black arrow points to a proteolytic fragment with a mass of approx. 22 kDa, most probably derived from apoA-I and found in RPM samples. Figure 4 (see below) demonstrates in RPM-conditioned medium the presence of further proteolytic fragments, about 26 kDa in size, which failed to be resolved by one-dimensional electrophoresis at high sample loads (Figure 2, grey arrow).

HDL$_3$ incubation in RPM-conditioned media without CaCl$_2$ or with CaCl$_2$ and EDTA failed to produce any protein digestion (results not shown). The MMPs that were newly secreted by

© 2002 Biochemical Society
I. Eberini and others

Figure 4 Structural characterization of proteolytic fragments after HDL₃ incubation in RPM-conditioned medium

After 3 days at 37 °C in medium 199 conditioned for 24 h with 2 x 10⁶ cells/ml, lipoproteins were desalted and concentrated by trichloroacetic acid/acetone precipitation and subjected to 2-DE: isoelectric focusing (IEF) on a 4–10 IPG in the presence of 8 M urea followed by SDS/PAGE on a 10–22% T PAA gradient in Tris/HCl/Tris/tricine buffer. Proteins were then stained directly for spot excision and MS processing, or electroblotted on to PVDF for N-terminal sequencing.

RPMs after LPS treatment (Figure 1) did not modify the pattern of HDL digestion. The interpretation was that these MMPs were expressed minimally and were inactive on this specific substrate.

Figure 3 shows the changes in the electrophoretic pattern for HDL₃ and constituent apolipoproteins as a function of time of incubation in RPM-conditioned medium. Figure 3 (top panel) shows the results of a gradient gel electrophoresis (GGE) which allows the size of the lipoproteins to be evaluated. The smallest particles (HDL₃– arrow) appeared to be selectively degraded with time. The small decrease in overall concentration between the 0 and 3 treatments is due to non-specific protein adsorption to a salt precipitate that forms upon addition of calcium to the cell-culture supernatant. The middle panel of Figure 3 shows an SDS/PAGE for assessing apolipoprotein size. The 22 kDa fragment (Figure 3, middle panel, black arrow) is the only resolved newly formed peptide, which accumulated with time. Figure 3 (bottom panel) shows isoelectric focusing of apolipoproteins, dissociated and denatured by the presence of 8 M urea. Incubation for 3 days in non-conditioned medium brought about a shift in the balance (Figure 3, bottom panel, lane 3 compared with lane 0) between the 0 and −1 isoforms of apoA-I, which differ by an Asn → Asp deamidation event [27]. Incubation in conditioned medium results in the appearance (Figure 3, bottom panel, lanes C3 and LPS3 compared with lane 0) of bands at a pI of approx. 5.15 (arrow).

Figure 4 shows the 2-DE map of a HDL₃ sample incubated for 3 days in medium 199 conditioned for 24 h with 2 x 10⁶ RPMs/ml. The harsher digestion conditions, the higher protein load and the greater resolution of the analytical procedure allow for the identification of a large number of peptides. Some have a molecular mass of 26 kDa, which could not be resolved from the major apoA-I band in one-dimensional runs (see above). The peptides with mass values of approx. 22 kDa appear to be very heterogeneous in charge, whereas the densest spots are resolved at pI < 5.25, several minor components migrate at pI > 5.25. By densitometric evaluation of the 2-DE pattern (Table 1), the volume of apoA-I-derived peptides equals that of the intact parent protein, i.e. under these incubation conditions apoA-I has been degraded proteolytically by about 50%. A parallel analysis of an HDL₃ sample incubated for 3 days in medium 199 conditioned for 24 h by 1 x 10⁶ RPMs/ml demonstrates a 20% proteolytic degradation of apoA-I (results not shown).

All resolved spots were processed using MS fingerprinting to assess their derivation from either apoA-I or apoA-II. For the most abundant components, N-terminal sequencing was performed after blotting on to a PVDF membrane. The results of such analytical procedures are summarized in Table 1. Peptides cleaved at both the N- and C-termini were detected; the former with a higher pI, the latter with a lower pI than apoA-I. The identification of peptides with masses of approx. 14 and 6 kDa (by SDS/PAGE) as well as bands with pI values both lower and higher than the reference apoA-I (in isoelectric focusing) as apoA-I fragments was assessed further by immunoblotting procedures (results not shown).

The question of how cleaved apoA-I associates with lipoproteins was addressed in two ways: a specifically designed 2-DE set-up, with GGE followed by SDS/PAGE (Figure 5), and gel filtration followed by analytical SDS/PAGE (Figure 6).

A control sample and an HDL₃ sample incubated for 3 days in medium 199 conditioned for 24 h by 1 x 10⁶ RPMs/ml were
Table 1  Relative abundance and peptide MS coverage of apoA-I proteolytic fragments as resolved by 2-DE

Spots refer to those shown in Figure 4. Under N-terminus (sequencing), the numbers indicate the first amino acid at the N-terminus of each fragment according to the sequence of intact apoA-I; this was amino acid no. 1 for two fragments (that are confirmed as only C-terminally cleaved) and amino acid no. 19 for another fragment (confirmed as N-terminally cleaved). Total spot volume (%) refers to the percentage abundance of individual fragments with respect to total apoA-I (intact + fragments) loaded in the gel.

<table>
<thead>
<tr>
<th>Spot</th>
<th>N-terminus (sequencing)</th>
<th>Peptide coverage (MS)</th>
<th>Total spot volume (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact apoA-I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>18.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>30.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>48.5</td>
</tr>
<tr>
<td>pI &lt; 5.27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>11–215</td>
<td>13.5</td>
<td></td>
</tr>
<tr>
<td>d</td>
<td>11–215</td>
<td>6.1</td>
<td></td>
</tr>
<tr>
<td>e</td>
<td>11–188</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>f</td>
<td>11–188</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>g</td>
<td>11–173</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>h</td>
<td>11–188</td>
<td>15.9</td>
<td></td>
</tr>
<tr>
<td>i</td>
<td>119–188</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>j</td>
<td>11–97</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>45.3</td>
</tr>
<tr>
<td>pI &gt; 5.27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>k</td>
<td>24–226</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>l</td>
<td>24–215</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>m</td>
<td>24–215</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>97–206</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>o</td>
<td>97–226</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>196–239</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>6.2</td>
</tr>
</tbody>
</table>

Figure 5  Assessment of the distribution of apoA-I, apoA-II and of their proteolytic fragments among the various HDL₃ subclasses

Control (0) and RPM-digested HDL₃ (C3) were separated by 4–25%T GGE either until the tracking dye migrated the entire length of the gel (75 min at 120 V in a 6 cm slab; left-hand panel) or until proteins reached their pore limit PAA concentration (24 h; right-hand panel); the Coomassie Brilliant Blue-stained patterns after this step are shown at the top. Gel strips from the GGE run (1 mm thick, cast on GelBond fail) were equilibrated in Tris/tricine/SDS buffer and embedded at right angles on 10–22%T PAA slabs for SDS/PAGE. The 2-DE protein pattern was stained with silver nitrate. Arrows point to approx. 26 and 22 kDa components, detectable in C3 compared with 0 after 75 min but lost in the anodic compartment after 24 h.

DISCUSSION

Limited proteolysis has been applied to the study of apoA-I in different types of HDL and reconstituted lipoprotein, for structural, topological and functional purposes. Maximal suscep-
the onset and progression of atherosclerosis. Contrary to the situation in healthy arterial walls, in which the constitutive expression of MMP-2 is balanced by the presence of TIMPs, atheromas contain a variety of MMPs in their active form (MMPs 1, 2, 3, 9 and 11). Since lipoproteins and other serum components do enter the atheromatous lesion there is scope for enzyme–substrate interaction.

In the paper of Lindstedt et al. [14] recombinant MMPs were at high concentrations. In the present investigation, we tried to reproduce the assortment and relative concentration of MMPs in an ‘atheromatous’ environment, in the presence of MMP levels typical of cell-culture supernatants (around 1 μg/ml) [34].

Atheroma cells have morphological and functional properties different from their resting counterparts: SMCs display a secretory phenotype, epithelial cells a prothrombotic phenotype, whereas macrophages may turn into lipid-laden foam cells [35]. Different stimuli (TNF-α and LPS, in increasing concentrations) were applied to the cells in culture during the 24 h conditioning period. These treatments had only minimal effects on MMP production by the cell lines. This unresponsiveness is most likely linked to cell-culture conditions: RPMs as recruited in the peritoneal cavity were in an activated state, and unlike their behaviour in healthy vessels, SMCs and HUVECs were assayed when in a proliferating state.

It is remarkable that the MMPs with the greatest effect on apoA-I were secreted by macrophages, which are not typical components of the arterial wall but abundant in atheromatous lesions [35]. In contrast, neither smooth muscle nor endothelial cells released proteolytic enzymes able to interact with HDL₃. In vivo, apoA-I proteolysis by MMPs can thus be associated with atherosclerotic plaques from their onset onwards. After entering atheromatous lesions, apoA-I may compete with matrix components as a substrate for MMPs, and reduce the ability of arterial wall enzymes to effect remodelling and eventual plaque rupture. This could be part of the mechanism by which HDLs act as anti-atherogenic factors. However, MMP proteolytic action eventually inactivates HDL₃, particularly HDL₃₉₄, the most efficient particles in removing cellular cholesterol [36]. Several authors (e.g. [14,37,38]) have already reported that proteolytic removal or mutation of the C-terminal region of apoA-I (amino acids 222–243) can reduce intracellular cholesterol efflux. Therefore lipid deposition in the early lesions cannot be limited further by this physiological protection mechanism.

Our data show clearly that, among HDL₃ subclasses, HDL₃₉₄ is degraded preferentially while, among HDL₃ components, apoA-I is a much more susceptible substrate than apoA-II. The reason for HDL₃₉₄ selective disruption could be ascribed to the relatively low lipid content in comparison with other HDL₃ subclasses (HDL₃₃ and HDL₃₄₅). Indeed lipid-free apoA-I is degraded easily by proteolytic enzymes, with minimal selectivity in sequence targeting [33]. The extensive degradation of HDL₃₉₄ corresponds to the accumulation of massive amounts of proteolytic fragments: as resolved by 2-DE after 3 days of incubation, these amount to 20 % of total apoA-I-related material with samples from 1 × 10⁶ RPMs/ml, and to 50 %, with 2 × 10⁶ RPMs/ml (Figure 4).

Two alternative theoretical models, based on amphipathic α-helices, have been proposed for the tertiary structure of lipid-bound apoA-I (residues 44–243). In the first, the picket-fence model of Phillips et al. [39], the helices run anti-parallel to one another (intramolecular protein–protein interactions) and parallel to the lipid acyl chains. In the second, the belt model of Segrest et al. [40], the helices run perpendicularly to phospholipids (intermolecular protein–protein interactions). A novel hairpin folding of each apoA-I monomer has been proposed, with
most helices perpendicular to the phospholipid acyl chains and a random head-to-tail and head-to-head arrangement of the two apoA-I molecules [41].

By scanning the primary sequence of apoA-I for the known target sites of MMPs and by computing the physico-chemical parameters of the resulting peptides with a module running on the ExPASy server (http://www.expasy.org/tools/pi_tool.html), we evaluated the pI for all fragments containing an intact N-terminus to be lower than 5.27, and for all fragments containing an intact C-terminus to be higher than 5.27, the pI of native apoA-I. Cuts near the N-terminus of the apoA-I molecule, despite being reported as occurring with other proteases [28,29], had not been described previously with MMPs [14]. The relative abundance of acidic compared with alkaline peptides, as listed in Table 1, demonstrates that, in a test medium closely resembling the situation in vivo, the C-terminal region of apoA-I is most susceptible to MMP digestion. Actually, alkaline peptides are found in appreciable amounts only under harsher experimental conditions, such as incubation in culture media conditioned with 2 × 10^5 RPMs/ml (comparison with incubation in different culture media not shown).

Reasons for greater susceptibility to proteolysis of the specified amino acid targets are probably related to topology. NMR data [42] on apoA-I(142–187), a peptide with a sequence identical to residues 142–187 of apoA-I, in either dodecylphosphocholine or SDS micelles, show the C-terminal region as unstructured due to the presence of Gly185 and Gly186. On the contrary, diffraction data on Δ(1–43), a mutant lacking the indicated residues, crystallized in lipid-free form under high-salt conditions were assigned a C-terminus of the region as unstructured [43]. More recently the same authors reported a different crystallization protocol for Δ(1–43), including detergents in a low-ionic-strength solution, which resulted in a different structure [44] that is not yet resolved completely [45]. The accessibility to proteasines of susceptible bonds in the apoA-I C-terminal region, shown by our experiments, strengthens the hypothesis that amino acids around Tyr189 are not in a structured conformation.

In a recent NMR investigation, the structure of a C-terminally truncated form, Δ(187–243), assigned Tyr189 to an α-helix [46]; in agreement with the above discussion, this provides a hypothetical basis for the higher resistance to proteolysis of the N- versus the C-terminus in apoA-I under our experimental conditions.

Our data demonstrate that proteolytic fragments of apoA-I produced under the specified experimental conditions dissociate from lipids upon cleavage. These findings were unexpected in view of the current models of HDL structure (picket-fence [39] and belt [40]; see above), which assume the amphipatic α-helix to be the main motif in the secondary structure of this apolipoprotein. The driving force in determining HDL structure should be the main motif in the secondary structure of this apolipoprotein. The amphipatic α-helix, spanning most of the protein sequence [47]. Assuming that a single cut in apoA-I would not cause very extensive α-helix unfolding, these data are difficult to account for, unless very strong co-operative binding is assumed. Our observations suggest a much higher affinity for lipids in the C-terminus: a cut in this region results in prompt dissociation of the 26 and 22 kDa fragments from HDL. Mutation of specific C-terminal hydrophobic residues have been reported to diminish the ability of apoA-I to bind to HDL [48].

The released fragments appear not to aggregate: in a gel chromatography set-up, the approx. 26 kDa component elutes before the approx. 22 kDa component (Figure 6), and their migration rate in a time course of non-equilibrium GGE versus dimeric apoA-I is suggestive of their being in monomeric form (results not shown). Indeed the C-terminal domain of apoA-I seems to be involved in the self-association of the protein [48].

We thank Dr F. Nardi for providing SMCs, Dr M. Camera and Ms F. Meloni for supplying HUVECs and Dr G. Chiesa, Dr E. Vegato, Dr A. Cignarella (all from Dipartimento di Scienze Farmacologiche, Milan, Italy), Professor D. Spady (Southwestern Medical Center, University of Dallas, Dallas, TX, U.S.A.) and Professor I. J. Goldberg (Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY, U.S.A.) for stimulating discussion. This work was supported in part by grants from Università degli Studi di Milano (FIRST) and MURST (COFIN 2000–2001: Structural studies on hydrophobic molecule-binding proteins) to E.G., and a grant in aid by Esperion Therapeutics, Ann Arbor, MI, U.S.A.

REFERENCES


