Characterization of a Human High Density Lipoprotein–Associated Protein, NA1/NA2 Identity With SP-40,40, an Inhibitor of Complement-Mediated Cytolysis

R.W. James, A.-C. Hochstrasser, I. Borghini, B. Martin, D. Pometta, and D. Hochstrasser

Two peptides, NA1 and NA2, which we previously suggested to be associated with high density lipoproteins (HDLs), have been purified. Polyclonal antibodies against each peptide and a monoclonal antibody against NA2 have been used to further characterize them and their association with HDL. Immunoblotting studies revealed that the peptides form a complex of molecular mass of approximately 80 kd. Agarose gel filtration showed coelution of NA1/NA2 and apolipoprotein (apo) A-I, the structural protein of HDL. This was confirmed by fast protein liquid chromatography, which further indicated that up to 60% of NA1/NA2 was located within the lower density range of the HDL spectrum. Complementary studies with anti-apo A-I immunoaffinity columns provided evidence that at least 40% of NA1/NA2 was associated with HDL, an association easily disrupted by ultracentrifugal manipulation. Finally, partial amino acid sequences showed virtually complete homology with a recently identified protein, SP-40,40, or cytolysis inhibitor. The protein is suggested to have a powerful inhibitory effect on complement-mediated cell lysis. Our results could thus furnish an explanation for the previously observed modulating influence of HDL on complement activity. (Arteriosclerosis and Thrombosis 1991;11:645–652)

Atherosclerosis is a complex process involving a multifaceted attack on the structural and functional integrity of the blood vessel wall. Cell damage figures prominently in our current understanding of the process, being implicated in the initiating (endothelial cell injury) and, more overtly, the concluding (necrotic debris in fibrous plaques) phases. In this context, the ability of the complement system, and, in particular, the terminal complement complex C5b9 to provoke cell lysis is an obvious consideration. The role of complement has remained peripheral to the central debate on the atherosclerotic process. There are, however, several studies suggesting the potential involvement of complement in the pathogenesis of the atherosclerotic plaque. Thus, factors that regulate complement activation may influence some aspects of the atherosclerotic process. Although the idea has received relatively little attention, it is interesting to note that less severe vascular lesions have been observed in animal models with an experimentally depleted or congenitally defective complement system.

High density lipoproteins (HDLs) exert a protective effect against the development of atherosclerotic disease. This is principally attributed to their ability to influence lipid metabolism, either by promoting transfer of cellular cholesterol to the liver for biliary excretion or by facilitating catabolism of triglyceride-rich lipoproteins, thus enhancing the removal of potentially atherogenic remnants. In a recent study, we demonstrated that the protein heterogeneity of HDL was more complex than previously shown. Several new proteins were described and partially characterized: in particular, two that were provisionally named NA1 and NA2. In a subsequent study, amino terminal sequence analyses indicated that they were previously unidentified plasma peptides. Based on values of molecular weight and isoelectric point determined by two-dimensional gel electrophoresis (2DGE), we also proposed that NA1 and NA2 were present in the cerebrospinal fluid (CSF). Concurrent studies by Murphy et al identified a novel protein in the C5b9 complex of immune
deposits. Reporting independently, Jenne and Tschopp have shown the protein (termed SP-40,40 or complement cytolytic inhibitor) to be a potent inhibitor of terminal complement complexes. Thus, it is a distinct possibility that the protein has an anti-cytolytic, protective function.

In this report, we have extended our studies of NA1/NA2 by purifying and preparing monospecific antibodies against the peptides. These have been used to examine their plasma distribution, together with that of apolipoprotein A-I (apo A-I), the structural protein of HDL. We demonstrate that NA1/NA2 corresponds to the recently identified complement cytolytic inhibitor, SP-40,40, and provide immunochromatographic confirmation that it is, in part, associated with HDL. As such, it raises the intriguing question as to whether anticytolytic activity could also be a feature of the beneficial influence of HDLs on the atherosclerotic process.

**Methods**

**Isolation of High Density Lipoprotein**

Blood samples were collected from normolipemic fasting subjects into tubes containing EDTA (8.8 mM). Plasma was subsequently fractionated by ultracentrifugation or immunoaffinity chromatography. Ultracentrifugal separation was effected either by sequential flotation or by means of density gradients. HDL was subsequently dialyzed against phosphate-buffered saline (PBS: NaCl 0.14 M, KCl 2.7 mM, phosphate buffer 10 mM; pH 7.4) containing EDTA (1 mM) and stored at 4°C.

Immunofinity fractionation of plasma was performed as described. Briefly, plasma (0.25 ml) was passed three times through an affinity column containing polyclonal antibodies against apo A-I. After washing the column with PBS containing 0.5 M NaCl, bound lipoproteins were eluted with thiocyanate (3 M in 20 mM phosphate buffer, pH 7.0) and dialyzed as described above.

**Purification of NA1/NA2**

NA1 and NA2 were purified by a combination of liquid-phase isoelectrofocusing (IEF) and high-resolution 2DGE (R.W. James et al., unpublished observations). Briefly, plasma (20 ml) was fractionated by liquid-phase IEF (Rotofor, Bio-Rad, Richmond, Calif.) into 20 fractions representing pH increments from 3.0 to 10.0. The fractions containing NA1/NA2 were then subjected to preparative 2DGE (see below). After electrophoresis, the protein pattern was visualized by CuCl2 staining, and spots representing NA1 and NA2 were excised from the gel. Protein was recovered by electroelution (Bio-Rad elution chamber).

**Polyacrylamide Gel Electrophoresis**

Single-dimensional gel electrophoresis and 2DGE were performed exactly as described. For analytical 2DGE, less than 20 μg protein was used; preparative 2DGE employed 1.5 mg protein. Protein patterns obtained after single- or two-dimensional electrophoresis were electrotransferred to nitrocellulose sheets using the Bio-Rad Transblot cell. Subsequent processing of the sheets was performed as described.

Analytical 2DGE gels were also silver stained and scanned using the MELANIE system. This computerized system allows the relative staining intensities of different protein spots to be compared.

**Production of Antibodies**

Polyclonal antibodies were obtained by conventional immunization procedures by injecting rabbits separately with NA1 and NA2. Monoclonal antibodies were prepared as described, and monoclonality was assured by limited dilution cloning.

**Gel Filtration**

Plasma (2.0 ml) was fractionated (4°C) on an agarose column (Biogel A5m, Bio-Rad; 100×2.0 cm) by elution with NaCl (0.9% wt/vol) containing EDTA (1 mM). Fractions of 2.0 ml were collected and analyzed for lipid and protein components. Fractionation of plasma (0.1 ml) was also performed using the Fast Protein Liquid Chromatography system (Superose 12-HR 10/30; Pharmacia, Uppsala, Sweden). The column was developed (room temperature) with 10 mM tris(hydroxymethyl) aminomethane (pH 8.0) and 0.15 M NaCl at a flow rate of 0.5 ml/min, with a pressure of 8 atm. Fractions of 0.25 ml were collected.

**Amino Acid Analyses**

NA2, purified as described above, was subjected to reverse-phase chromatography on a Vydac C-4 (Vydac, Hesperia, Calif.; 250×4.6 mm) using a CH3CN gradient (solvent A, 0.12% trifluoroacetic acid in water; solvent B, 0.1% trifluoroacetic acid in CH3CN) with a linear gradient from 5% solvent B to 60% solvent B over 55 minutes. The protein fraction was monitored at 225 and 280 nm absorbance and collected manually. NA2 was then digested with endoproteinase Asp-N (Boehringer Mannheim, Mannheim, F.R.G.) following the manufacturer's instructions. After 16 hours of digestion, peptides were recovered by high-performance liquid chromatography as described above and sequenced.

**Other Analyses**

Enzyme-linked immunosorbent assays (ELISAs) were performed as described previously. Briefly, microtiter plates were coated overnight with antigen (100 μl; 10 μg/ml), then incubated with diluted antiserum or culture medium containing monoclonal antibodies. Bound antibodies were revealed with the appropriate immunoglobulin G-specific second antibody coupled to alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.).

Total protein was measured by the procedure of Lowry et al. Apo A-I was quantified by electroim-
Results

Purification of NA1/NA2 and Production of Monospecific Antibodies

The combined liquid-phase IEF and preparative 2DGE gave rise to highly purified preparations of NA1 and NA2. The results are shown in Figure 1, together with Western blots using monospecific, polyclonal antibodies against NA1 and NA2. Similar immunoblotting studies confirmed that NA1 and NA2 are also present in CSF (not shown).

During the course of these studies, it became apparent that the behavior of NA1/NA2 was sensitive to the presence of reducing agents. Under nonreducing conditions, in plasma (and CSF; not shown) the apparent $M_r$ doubled to 80 kd, as shown in Figure 2. Monospecific, polyclonal antibodies against both NA1 and NA2 (Figure 2A) and the monoclonal antibody against NA2 (Figure 2B) recognized the higher-molecular-weight protein, suggesting that the peptides form a complex linked by a disulfide bond.

Using the monospecific antibodies described above, a series of studies was undertaken to examine the plasma distribution of apo A-I and NA1/NA2.

Gel Filtration

Plasma from fasting human subjects was fractionated by gel filtration using either agarose columns (separation time of $\approx$2 days) or the much more rapid fast protein liquid chromatography system (separation time of $\approx$1 hour). The elution profile for apo A-I from the agarose column (Figures 3A and 3B) coincided with that for NA1/NA2, the peak tube for apo A-I (pooled fraction 6, tubes 151–153) corresponding to that for NA1/NA2 as shown in Figure 3B. Virtually no immunoreactive NA1/NA2 was detected in fractions corresponding to very low density (VLDL) and low density (LDL) lipoproteins (lanes V and L of the immunoblot, Figure 3B).

Recent studies have suggested that apolipoproteins loosely associated with lipoproteins could dissociate during the relatively long time necessary for fractionation on agarose columns. This was the reason we elected to use the Superose column, with its accelerated separation time. An example of the elution profile obtained from the Superose column is given in Figure 4. Three peaks of material immunoreactive with NA1/NA2 antibodies were discernible. Peak 1 (tubes 24 and 25) occurred at the leading edge of the HDL peak, that is, in the lower-density region of the HDL density spectrum. Peaks 2 (tubes 30 and 31) and 3 (tubes 34 and 35) correspond in molecular mass to the dimeric and monomeric forms of NA1/NA2. When assessed by scanning the immunoblots, approximately 60% of the total immunoreactive material was in the region of peak 1. The elution profile also confirmed that very little NA1/NA2 was associated with VLDL/LDL.

Immunoaffinity Chromatography on Anti-Apolipoprotein A-I Columns

Three sets of studies were performed using plasma from fasting human subjects and an affinity column containing polyclonal antibodies against apo A-I.
FIGURE 2. Photograph of Western blots of human plasma separated by single-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (panel A) or two-dimensional gel electrophoresis (2DGE) (panel B) and immunoblotted with anti-NA1/NA2 antibodies. Panel A shows autoradiograms of reduced (lanes 1–3) and nonreduced (lanes 4–6) human plasma incubated with polyclonal antibodies to NA1 (lanes 2,5), NA2 (lanes 3,6), and a mixture of both antibodies (lanes 1,4). Bound antibodies were revealed with iodine-125–labeled goat anti-rabbit immunoglobulin G (IgG). Panel B shows 2DGE performed on nonreduced (left) and reduced (right) human plasma by isoelectrofocusing in two separate rod gels, then laying half of each rod gel on top of the same second-dimension SDS-PAGE slab gel. Arrow indicates direction of decreasing molecular weight. Western blots of resulting profiles were performed using monoclonal antibody F4OM1H3:C7 against NA2 and were revealed with peroxidase-labeled rabbit anti-mouse IgG.

In the first series of studies, whole plasma and the fraction retained by the immunoaffinity column were analyzed by analytical high-resolution 2DGE. (Representative gels of such fractions are given in Reference 15, Figure 1A, and Reference 14, Figures 6A and 6B.) Silver-stained gels, scanned and analyzed by the MELANIE program, were used to examine the staining intensities of NA1/NA2 and fibrinogen, relative to that of apo A-I. As shown in Table 1, the ratio of NA1/NA2 to apo A-I was considerably higher in the retained fraction as compared with that of whole plasma. This was not the case for the quantitatively more important non-HDL–associated plasma proteins, of which fibrinogen is given as an example.

A second set of ELISA studies examined various preparations of HDL using antibodies against NA1/NA2. HDL isolated by immunoaffinity chromatography showed a significantly greater degree (p<0.001)

FIGURE 3. Profiles of fractionation of human plasma (2.0 ml) by agarose gel filtration. Elution was monitored by absorbance at 280 nm, and fractions (2.0 ml) were analyzed for lipids and protein. Panel A: Elution profile. Panel B: Fractions corresponding to very low density lipoprotein (V, tubes 98–108) and low density lipoprotein (L, tubes 109–126) were pooled; fractions corresponding to high density lipoprotein were pooled in threes (fraction 1, tubes 136–138; up to fraction 13, tubes 172–174). Equivalent plasma volumes of each pool were analyzed by single-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotted with a mixture of anti-NA1/NA2 antibodies. Immunoblot is superimposed on the region of Figure 3A corresponding to tubes 137–174. Albumin (of similar M, to NA1/NA2) eluted in tube 12. ••••, Cholesterol (μg/ml); ×•×•, protein optical density at 280 nm; ••••, triglycerides (μg/ml); •—•, apolipoprotein A-I (apo A-I) (μg/ml).
Profiles of fractionation of human plasma by Superose fast protein liquid chromatography. Elution was monitored by absorbance at 280 nm, and fractions (0.25 ml) were analyzed for lipids and protein. Panel A: Elution profile. Panel B: Fractions were pooled in twos, starting at tube 12 of Figure 4A (pooled tube 1, tubes 12,13; pooled tube 15, tubes 40,41). Equivalent plasma volumes of each pool were analyzed by single-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotted with a mixture of anti-NA1/NA2 antibodies. Immunoblot is superimposed on the region of Figure 4A corresponding to tubes 12–41. (Albumin eluted in pooled tube 13.) ○—○, Cholesterol (µg/ml); ×—×, protein optical density at 280 nm; ■—■, apolipoprotein B (apo B) (µg/ml); □—□, apo A-I (µg/ml).

FIGURE 4. Profiles of fractionation of human plasma by Superose fast protein liquid chromatography. Elution was monitored by absorbance at 280 nm, and fractions (0.25 ml) were analyzed for lipids and protein. Panel A: Elution profile. Panel B: Fractions were pooled in twos, starting at tube 12 of Figure 4A (pooled tube 1, tubes 12,13; pooled tube 15, tubes 40,41). Equivalent plasma volumes of each pool were analyzed by single-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotted with a mixture of anti-NA1/NA2 antibodies. Immunoblot is superimposed on the region of Figure 4A corresponding to tubes 12–41. (Albumin eluted in pooled tube 13.) ○—○, Cholesterol (µg/ml); ×—×, protein optical density at 280 nm; ■—■, apolipoprotein B (apo B) (µg/ml); □—□, apo A-I (µg/ml).

FIGURE 5. Bar graph of immunoreactivity of human high density lipoprotein (HDL) preparations with anti-NA1/NA2 antibody mixture. HDLs (10 µg/ml) were coated onto microtiter wells and incubated with saturating concentrations of a mixture of antisera against NA1/NA2. Bound antibody was revealed with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G. A, B, and C, total HDL (d=1.063–1.21 g/ml), HDL2 (d=1.063–1.125 g/ml), and HDL3 (d=1.125–1.21 g/ml) isolated by sequential ultracentrifugation18; D and E, HDL2 and HDL3 isolated by density gradient ultracentrifugation19; F, fraction retained by anti-apolipoprotein A-I immunoaffinity chromatography.14 Results are expressed as optical density (OD)±SD, n=8, developed over 30 minutes (background OD of 0.08). *p<0.001 compared with all other preparations; **p<0.001 compared with preparations A, B, and C.

Amino Acid Analyses

Partial amino acid analysis was performed on NA2, purified as described above. The amino terminal sequence previously established13 was confirmed and extended. In addition, two internal amino acid sequences were obtained. These are given in Figure 7, together with the amino terminal sequence previously obtained for NA1.14 Comparable amino acid sequences translated from cDNA sequences for SP-40,4031 and cytolysis inhibitor17 are also given. There is virtually complete homology between the three sequence sources.

Table 1. Ratios of Relative Staining Intensities of NA1/NA2, Apolipoprotein A-I, and Fibrinogen

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Plasma</th>
<th>HDL</th>
</tr>
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<tbody>
<tr>
<td>NA1/NA2</td>
<td>0.14±0.01</td>
<td>0.49±0.09</td>
</tr>
<tr>
<td>Apo A-I</td>
<td>0.95±0.28</td>
<td>0.14±0.07</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>0.16±0.06</td>
<td>2.81±0.96</td>
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Silver-stained, high-resolution two-dimensional gel electrophoresis gels of whole plasma and the fraction (from the same plasma) retained by an anti–apo A-I affinity column were scanned and analyzed by the MELANIE system.24–26 Values represent relative staining intensities±SD of indicated proteins in samples from three normolipemic subjects. HDL, high density lipoprotein; apo, apolipoprotein.
FIGURE 6. Photograph of Western blots of fractions bound and not bound by anti-apolipoprotein A-I immunoaffinity column. Equivalent plasma volumes of bound (1,3) and unbound (2,4) fractions were analyzed by single-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotted with a mixture of anti-NA1/NA2 antibodies (1,2, reduced; 3,4, nonreduced). Bound antibodies were revealed with iodine-125-labeled goat anti-rabbit immunoglobulin G, and autoradiograms were scanned with the MELANIE system to allow comparison of relative intensities.

Discussion

These results demonstrate that a plasma component with a potent anticytolytic capacity is associated, in part, with HDL. The conclusions are based on gel filtration studies (Figures 3 and 4), which show coelution of NA1/NA2 and apo A-I, the structural protein of HDL, with specific association being confirmed by the experiments with the anti-apo A-I immunoaffinity column (Figures 5 and 6 and Table 1). Amino acid analyses (Figure 7) clearly show coidentiy of NA1/NA2 and SP-40,40, or cytolysis inhibitor.

Association of the protein with lipoproteins appears to be essentially limited to HDL, as little NA1/NA2 could be detected immunologically in lower-density lipoproteins (Figures 3 and 4). This observation parallels previous suggestions that the anticytolytic activity of lipoproteins was primarily restricted to HDL. In their investigations of the phenomenon, Rosenfeld et al concluded that apo A-I and apo A-II were the primary inhibitors, acting after addition of C9. This does not appear to have been confirmed. In contrast, SP-40,40, or cytolysis inhibitor, would appear to be most active at an earlier stage (C5b7) according to results from Jenne and Tschopp. Its mode of action appears to be similar to that of another fluid-phase inactivator, vitronectin, or S-protein. That is, it can compete with membrane lipids for lipophilic regions of C5b7 exposed during formation of the complex, preventing insertion of the latter into lipid bilayers. Interestingly, in their article, Rosenfeld et al reported that whole HDL was more active at the C5b7 level, as had been previously reported by other investigators. It suggests that at least part of the inhibitory activity documented by these groups could be attributed to NA1/NA2 (SP-40,40). Indeed, examination of the sodium dodecyl sulfate gels of Rosenfeld et al (Reference 34, Figure 3) showing the most potent anticytolytic activity reveals the presence of bands in the 40-kd region, which disappear or are diminished under nonreducing conditions.

Based on studies with the anti-apo A-I column (Figure 6), it can be estimated that some 40% of NA1/NA2 is associated with HDL. This compares favorably with the conclusions of Murphy et al, who proposed that as much as 40% of SP-40,40 (NA1/NA2) was present in a nonmonomeric form, perhaps complexed with other plasma proteins. Furthermore, rapid gel filtration of plasma (Figure 4) suggests that an even greater percentage of NA1/NA2, as much as 60%, is present in a higher-molecular-weight form. In this respect, recent studies suggesting that prolonged
gel fractionation procedures, which had been considered to minimally perturb lipoprotein structure, can cause apolipoprotein dissociation from HDL, and perhaps of relevance, the association between NA1/NA2 and HDL is labile, as indicated by the disruption occasioned by ultracentrifugation (Figure 5). This has also been observed for other HDL apolipoproteins. Evidently, an anticytolytic function for NA1/NA2 diverges from the concept of lipoprotein-associated proteins as being primarily involved in lipid transport. However, there is a growing list of HDL-associated proteins with no apparent relevance to lipid transport (see Reference 14). Perhaps this is indicative of a more extensive transport function for HDL. Neither can a direct role for NA1/NA2 in lipid transport be presently excluded, given the high affinity with which NA1/NA2 binds to apo A-I (R.W. James et al, unpublished observations).

As mentioned previously, there is mounting evidence of a role for complement in the pathogenesis of atherosclerosis, although the manner of its involvement is far from clear. Consequently, factors that modulate complement-mediated cytolytic activity could have a bearing on the development of the atherosclerotic plaque. In addition to the fluid-phase inhibitors S-protein and SP-40,40 (NA1/NA2), other membrane-bound inactivators acting at various points in the complement cascade have been described. A recent report suggests that several of these regulators are present in human atherosclerotic lesions. It remains to be established unambiguously that the HDL–NA1/NA2 complex is of physiological relevance to the modulation of complement activity, although earlier studies lend credence to such a possibility. Nevertheless, our demonstration that a protein reported to be a powerful inhibitor of complement-mediated cytolysis is associated, in part, with HDL is particularly intriguing. The latter is compounded by suggestions that the complement-activating capacity of a lipid complex recently isolated from atherosclerotic plaques may be linked to its high content of unesterified cholesterol. A primary function of HDL is thought to be elimination of cholesterol, initially in its unesterified form; this is facilitated by the small size of the HDL particle, permitting extensive irrigation of extravascular spaces. HDLs already have a well-defined antiatherosclerotic role linked to lipid metabolism.

The question now arises as to whether one feature of this antiatherosclerotic effect could be a potential capacity to modify complement-mediated cell lysis. This consideration merits further attention, as it links two apparently distinct antiatherosclerotic features. It also provides the basis for proposing an expanded beneficial influence of HDL on atherosclerotic disease.

Acknowledgments

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R W James, A C Hochstrasser, I Borghini, B Martin, D Pometta and D Hochstrasser

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