Incubation of MDCO-216 (ApoA-IMilano/POPC) with Human Serum Potentiates ABCA1-Mediated Cholesterol Efflux Capacity, Generates New Prebeta-1 HDL, and Causes an Increase in HDL Size

Herman J. Kempen, Dorota B. Schranz, Bela F. Asztalos, James Otvos, Elias Jeyarajah, Denise Drazul-Schrader, Heidi L. Collins, Steven J. Adelman, and Peter L. J. Wijngaard

1 The Medicines Company (Schweiz) GmbH, Talstrasse 59, 8002 Zürich, Switzerland
2 Pacific Biomarkers LLC, 645 Elliott Avenue West, Suite 300, Seattle, WA 98119, USA
3 Lipid Metabolism Laboratory, Tufts University, 711 Washington Street, Boston, MA 02111, USA
4 Liposcience LLC, 2500 Summer Boulevard, Raleigh, NC 27616, USA
5 Vascular Strategies LLC, 5110 Campus Drive, Suite 150, Plymouth Meeting, PA 19462, USA

Correspondence should be addressed to Herman J. Kempen; herman.kempen@themedco.com

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1. Introduction

Repeated intravenous administration of recombinant ApoA-1 Milano complexed with POPC (previously known as ETC-216) was previously shown to reduce atherosclerotic plaque burden in experimental animals [1, 2] and in man [3]. After improvement of the manufacturing process development of this product (now named MDCO-216) has been resumed by The Medicines Company. We recently reported that infusion of MDCO-216 to cynomolgus monkeys led to rapid and...
drastic changes in lipoprotein levels and brisk stimulation of global and ABCA1-mediated serum cholesterol efflux capacity after 21 administrations every second day [4].

In the present work we demonstrate that the effect on serum cholesterol efflux capacity could already be obtained when MDCO-216 was incubated with human serum in vitro. We have also investigated the effects of the incubation of MDCO-216 with plasma on the HDL subpopulation profile and HDL prebeta-1 concentration, using a specific ELISA as well as nonnondenaturing 2D electrophoresis, and on HDL size and particle concentrations by $^1$H-NMR.

2. Materials and Methods

MDCO-216 is a complex of highly purified dimeric recombinant ApoA-I Milano and palmitoyl-oleoyl-phosphatidylcholine (POPC). Production of the recombinant protein in E. coli and its purification have been described [5]. Complexation with POPC was performed using a high-pressure homogenization procedure. The final product (stock solution) contained 13 mg/mL protein, 14 mg/mL POPC, 1.3 mg/mL Di-Na. hydrogen phosphate heptahydrate, 0.178 mg/mL Na. dihydrogen phosphate dehydrate, 0.8 mg/mL sucrose, and 62 mg/mL mannitol, pH 7.5.

2.1. Cholesterol Efflux Capacity. MDCO-216 was preincubated with human serum or 4% human serum albumin in concentrations between 100 and 1000 µg/mL (based on ApoA-I Milano protein) at 0 or 37°C. Global and ABCA1-mediated efflux capacity from j774 cells was assessed as described before [4] using 2% of these preincubation mixtures.

2.2. Prebeta-1 HDL Quantitation by ELISA. Prebeta-1 HDL in human plasma or in precubated plasma/MDCO-216 mixtures (see below) was measured at Pacific Biomarkers, using a commercial prebeta-1 HDL ELISA kit from Sekisui Medical Co. LTD. Samples taken from the preincubations were diluted 50-fold with 50% sucrose and kept at −70°C until analysis. The prebeta-1 HDL assay has been described in detail [6].

Preincubation of MDCO-216 with human plasma was done as follows.

Four solutions of drug were prepared:

(i) Vehicle: 6% sucrose and 1% mannitol in phosphate buffer, pH 7.4;
(ii) Solution High (10 mg/mL): 200 µL MDCO-216 stock + 60 µL Vehicle;
(iii) Solution Medium (3 mg/mL): 100 µL MDCO-216 stock + 330 µL Vehicle;
(iv) Solution Low (1 mg/mL): 50 µL Solution High + 450 µL Vehicle.

All four solutions were spiked into plasma samples of 3 separate donors (50 µL spike + 450 µL plasma) or into Vehicle to obtain final concentration of MDCO-216 at 0, 100, 300, and 1000 µg/mL. 250 µL of each mixture was placed on ice and 250 µL was incubated at 37°C in a water bath.

At the end of the incubation period, all samples, with and without added MDCO-216, either at 0°C or 37°C, were stabilized by diluting 1:21 with 50% sucrose (50 µL sample + 1 mL sucrose) and stored at −70°C until analysis.

2.3. 2D Electrophoresis. Similar incubations of human serum at 0°C or 37°C were done with or without MDCO-216 and submitted to 2D electrophoresis as described in [7]. Gels were electrotransferred to nitrocellulose membranes and then reacted with either polyclonal goat antihuman apoA-I antibody recognizing both wild-type apoA-I and ApoA-I Milano or with a monoclonal antibody (17F3) specific for ApoA-I Milano, obtained from Mabtech, Nacka, Sweden.

2.4. $^1$H NMR. Changes in lipoprotein particle concentrations as a function of time after mixing MDCO-216 with serum were determined by NMR spectroscopy at Lipo-Science, Inc. (Raleigh, NC) using the modification of the published procedure for analyzing serum [8, 9]. After rapid mixing of a serum specimen with MDCO-216 (430 µg/mL final concentration) and transferring to the flow cell of a 400 MHz NMR analyzer (Bruker Biospin) maintained at 47°C, NMR spectra (130 sec acquisition time) were obtained every 4 minutes over a 1-hour time period. The midpoint of the first spectrum was 2.8 min after mixing, 6.8 min for the second, 10.8 for the third, and so forth. Separate spectra of MDCO-216 and serum at the same concentrations as in the mixture were also acquired. Digital addition of these 2 spectra created an artificial “time zero” (0 min) mixture spectrum to enable assessment of the lipoprotein composition prior to any particle remodeling induced by MDCO-216.

Lipoprotein subclass particle concentrations were calculated from the amplitudes of the spectroscopically distinct lipid methyl group signals emitted by each subclass, derived by deconvolution of the plasma methyl signal envelope at −0.8 ppm [8, 9]. This computational approach assumes that “the whole is the sum of its parts,” with the parts in the case of human serum comprising reference signals from all of the different-size spherical lipoprotein particles, ranging from the largest chylomicon to the smallest HDL subclass [8]. To successfully analyze mixtures of serum and MDCO-216, we expanded the deconvolution model to include an additional reference signal from MDCO-216 to account for its spectral contribution to the methyl signal envelope of the mixture. Since MDCO-216 is believed to be discoidal rather than spherical in structure, its NMR signal frequency cannot be used to infer its particle diameter nor can its deconvolution-derived signal amplitude be used to calculate its absolute concentration. To monitor and display (in Figure 4) the time-dependent changes in MDCO-216 relative concentration following its addition to serum, we arbitrarily set its “time zero” concentration to 10 µmol/L. Diameter ranges of the HDL particle subclasses quantified by NMR are as follows: large HDL-P 9.4–14 nm, medium HDL-P 8.2–9.4 nm, and small HDL-P 7.3–8.2 nm.
3. Results

3.1. Preincubation of MDCO-216 with Human Serum Potentiates ABCA1-Mediated Efflux but Not with Basal Efflux from J774 Cells. MDCO-216-HSA and MDCO-216-serum mixtures were compared for their capacity to efflux cholesterol from J774 cells. As shown in Figures 1(a) and 1(c), MDCO-216 preincubated with 4% HSA stimulated basal efflux and ABCA1-mediated cholesterol efflux in a concentration-dependent manner. At a concentration of 20.8 μg/mL (based on ApoA-I Milano protein) of MDCO-216 in the efflux medium, ABCA1-mediated efflux was about half of that obtained by 20 μg/mL free (uncomplexed) human wild-type apoA-I (Figure 1(c)).

Preincubation of serum with MDCO-216 at 37°C led to a concentration-dependent synergistic increase in ABCA1-mediated efflux (compare Figure 1(a) with Figure 1(b)), which means that the measured efflux of the serum-MDCO-216 combination exceeded the sum of the effluxes measured for the individual components. The synergistic effect was also clearly time-dependent, with about half of the increase seen after 10 minutes of preincubation at 37°C (not shown). After 60 min preincubation with serum, maximal stimulation of ABCA1 efflux was reached at 0.5 mg/mL MDCO-216, whereas MDCO-216 preincubated with HSA did not reach maximum at 1 mg/mL. The synergistic effect was seen in similar experiments with other sera having higher ABCA1-mediated efflux capacities (not shown).

Preincubation of serum with MDCO-216 also increased basal (cAMP independent) efflux, but the combination did not reach the sum of the individual components.

3.2. Preincubation of Human Plasma with MDCO-216 Protects against Loss of Endogenous Prebeta-1 HDL and Generates New Prebeta-1 HDL as Measured by ELISA and 2D Electrophoresis. As shown in Table 1(a), MDCO-216 itself was detected to some degree by the prebeta-1 HDL ELISA. After incubation
Table 1: Prebeta-1 HDL as measured by ELISA in MDCO-216 solutions mixed with buffer or plasma after 1 h incubation at 0 or 37 °C. MDCO-216 stock solution contained 13 mg/mL ApoA-I Milano dimer, 6% sucrose, 1% mannitol, and phosphate buffer pH 7.4. Vehicle solution contained 6% sucrose, and 1% mannitol in phosphate buffer, pH 7.4. Solution High (10 mg/mL protein) was prepared by mixing 200 μL MDCO-216 stock with 60 μL Vehicle. Solution Medium (3 mg/mL protein) was prepared by mixing 100 μL MDCO-216 stock with 330 μL Vehicle. Solution Low (1 mg/mL protein) was prepared by mixing 50 μL Solution High with 450 μL Vehicle. 50 μL of these Solutions was spiked into 450 μL Vehicle (Table 1(a)) or into 450 μL plasma (Table 1(b)) and incubated 60 minutes at 0 °C or 37 °C.

### (a)

| Solution spiked     | Vehicle | Low  | Medium | High |
|---------------------|---------|------|--------|------|
| Final MDCO-216 concentration (µg/mL protein): |         |      |        |      |
| Incubated at 0 °C  | 0       | 12   | 44     | 118  |
| Incubated at 37 °C | 0       | 5    | 13     | 52   |
| Prebeta-1 HDL in µg/mL (% of added) |         |      |        |      |

### (b)

| Incubation temp.  | 1 h 0 °C |         |        |      |
|-------------------|----------|---------|--------|------|
| Solution spiked   | Vehicle  | Low     | Medium | High |
| Final MDCO-216 concentration (µg/mL protein): |         |      |        |      |
| Spiked in plasma 1| 62       | 75     | 101    | 105  |
| Spiked in plasma 2| 126      | 126    | 131    | 121  |
| Spiked in plasma 3| 112      | 144    | 143    | 125  |
| Spiked in plasma average | 100    | 115    | 125    | 117  |
| % of plasma spiked with vehicle** | 115%      | 125%   | 117%   | |

### Incubation temp.  | 1 h 37 °C |         |        |      |
|---------------------|----------|---------|--------|------|
| Solution spiked     | Vehicle  | Low     | Medium | High |
| Final MDCO-216 concentration (µg/mL protein): |         |      |        |      |
| Spiked in plasma 1  | 5        | 77     | 162    | 379  |
| Spiked in plasma 2  | 12       | 89     | 153    | 375  |
| Spiked in plasma 3  | 23       | 127    | 173    | 401  |
| Spiked in plasma average | 13    | 98     | 163    | 385  |
| % of plasma spiked with vehicle** | 733%      | 1220%  | 2888%  | |

*Value for MDCO-216 spiked in plasma minus value for MDCO-216 spiked in Vehicle (Table 1(a)) in µg/mL. **(value for plasma plus MDCO-216 minus value for MDCO-216 in vehicle)/value for plasma spiked with vehicle.

of the Low, Medium, and High Solutions at 0 °C, the measured amount was about 12% of the real amount of MDCO-216 protein present, whereas after incubation at 37 °C this was reduced to about 5%.

Table 1(b) shows that preincubation of plasma alone at 37 °C caused loss of nearly 80% of the prebeta-1-HDL ELISA compared to plasma kept at 0 °C. After preincubation of plasma with 100, 300, and 1000 µg/mL MDCO-216 at 0 °C the amounts of prebeta-1-HDL ELISA ascribable to plasma were 115, 125, and 117%, respectively, of the amounts in plasma incubated with Vehicle. However, after incubation of these mixtures at 37 °C amounts of prebeta-1-HDL ELISA increased to 733%, 1220, and 2888%, respectively, of plasma incubated with Vehicle. This suggests that during preincubation at 37 °C the added MDCO-216 led to generation of new prebeta-1 HDL in a dose-dependent manner.

After similar incubations, samples were subjected to 2D electrophoresis. As shown in Figure 2(a), incubation of serum alone at 37 °C leads to a marked loss of prebeta-1 HDL and also some loss of small (alpha-4 and alpha-3) HDL (compare left with middle blot in left panel). MDCO-216 alone showed several alpha-mobility particles with sizes between 8 and 9.5 nm, but no particles with prebeta-1 mobility (right-hand blot in right panel). After incubation of serum spiked with increasing concentrations MDCO-216, there was a prominent increase of prebeta-1 concentration, accompanied with a decrease/disappearance of small alpha-3 and alpha-4 particles and a relative increase in large alpha-1 and alpha-2 particles. Cross immunoprobning the membranes with the ApoA-I Milano-specific antibody (17F3) (shown in Figure 2(b)) indicated that apoA-I Milano was present in alpha-2 and alpha-1 particles. At higher
Figure 2: Effect of preincubation of human plasma alone or with MDCO-216 at 37°C. (a) After 2D electrophoresis, the blots were reacted polyclonal Ab against human apoA-I (also reacting with ApoA-I Milano). Asterisk denotes position of albumin. (b) 2D electrophoresis of the same samples of (a), with the blots now reacted with MAb 17F3 specific for ApoA-I Milano.
concentrations ApoA-I Milano was also observed in particles similar to alpha-4. However, no ApoA-1 Milano was detected in prebeta-1 HDL.

3.3. Remodeling of Serum HDL upon Incubation with MDCO-216 Assessed by $^1$H-NMR. The phospholipid in MDCO-216 gave rise to a methyl NMR signal centered at 0.79 ppm (Figure 3). A signal of this frequency would correspond to that from a medium-size spherical HDL particle [8], but because the structure of MDCO-216 may not be spherical, it is not currently possible to deduce its size or structure from its NMR characteristics. Also in Figure 3 the methyl signal envelopes of the serum sample (red), the artificial “0 min” mixture created by digital addition of the serum and MDCO-216 signals (black), and the signals resulting from incubation of MDCO-216 and serum for 2.8 min (blue) and 10.8 min (green) are shown. Qualitative inspection of these curves indicates a time-dependent reduction of signal amplitude in the region coming from by small HDL particles (ca. 0.77 ppm) and a corresponding increase in signal coming from medium- and large-size HDL subclasses (ca. 0.79–0.82 ppm). Prebeta-1 HDL particles do not appreciably contribute signal to this region of the NMR spectrum due to their very low lipid content.

The quantitative assessment via deconvolution analysis of changes in MDCO-216 and HDL particle subclass concentrations brought about by addition of MDCO-216 to serum is shown in Figure 4. Not shown are data for the VLDL and LDL subclasses which were unaffected by addition of MDCO-216, nor for the HDL subclasses at longer time points since further changes beyond 10.8 min were not observed. The signal from MDCO-216 decreased rapidly over time and was not detectable in the 10.8 min spectrum and beyond. Over the same short time interval, the concentration of small HDL particles fell >95% while there was a concomitant increase in medium-size HDL and a substantial 5-fold increase in the concentration of large HDL particles. These subclass changes led to a net decrease in total HDL particle concentration from 47 to 40 μmol/L.

4. Discussion

The data presented here show that incubation of plasma or serum with MDCO-216 in vitro causes a strong increase in ABCA1-mediated cholesterol efflux capacity, concomitant with increased average HDL size and generation of new prebeta-1 HDL containing only wild-type apoA-I. Recently, very similar changes were reported for incubation of human serum with CSL-112 [10]. CSL-112 consists of two molecules wild-type apoA-I complexed with 110 molecules soybean lecithin. A strong increase in ABCA1-mediated efflux was observed after incubation of CSL-112 with human serum at 37°C in vitro. Incubation of 1 mg/mL CSL-112 with serum at 37°C was described to strongly increase prebeta-1 HDL concentration, whereas CSL-112 was hardly detectable with the prebeta-1-HDL ELISA assay. The increase reported by these authors was very similar to that
reported here (Table 1) after incubating serum with 1 mg/mL MDCO-216. The authors report that upon one-dimensional nondenaturing gradient PAGE this incubation led to marked generation of particles with the same size (about 5.6 nm) as seen after incubation of serum with free apoA-I. In our case, native 2D electrophoresis demonstrated a marked increase in particles with prebeta-1 mobility and apparent size of 7.2 nm. Miyazaki et al. recently provided evidence that the particles detected as prebeta-1 HDL upon 2D electrophoresis and by the Sekisui ELISA actually consist of lipid-free apoA-I [11].

Our 1H-NMR measurements showed rapid conversion of MDCO-216 and small-sized HDL into larger-sized HDL particles, with a concomitant decrease in the total HDL particle concentration. There was no evidence from the deconvolution analysis that MDCO-216 persisted in its native structural state beyond about 10 minutes of incubation with serum, but we cannot rule out the possibility that it may have been transformed into a different structure(s) with unknown NMR properties. The validity of the NMR deconvolution approach to deduce HDL subclass changes, caused by addition of MDCO-216, is supported both by the observed agreement with the HDL changes seen by 2D electrophoresis and by numerous reports in the literature of agreement between NMR-assessed lipoprotein changes and those assessed independently using various separation techniques [12–16]. The latter also applies for the lack of effect on LDL and VLDL size and particle numbers inferred by NMR deconvolution.

Taken together, the data reported here support the hypothesis that upon incubation with plasma or serum, MDCO-216 fuses with small endogenous alpha-migrating HDL particles to produce larger HDL particles containing both apoA-I WT and ApoA-I Milano. As a side effect of this fusion, free apoA-I WT is liberated which is detected as prebeta-1 HDL. Similar results have been described for an apoA-I mimetic peptibody after intravenous administration in mice [17]. The question remains whether the increase in ABCA1-mediated efflux is brought about solely by these newly-formed prebeta-1 HDL particles or whether the altered (larger) HDL particles now containing ApoA-I Milano also contribute to the increased cholesterol efflux capacity of serum.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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