Detection of chymase-digested C-terminally truncated apolipoprotein A-I in normal human serum

Journal of immunological methods

Volume 369

Number 1-2

Page range 51-58

Year 2011-06

(C) 2011 Elsevier B.V.

NOTICE: this is the author’s version of a work that was accepted for publication in Journal of immunological methods. Changes resulting from the publishing process, such as peer review, editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version was subsequently published in PUBLICATION, Volume 369, Issues 1-2, 2011 DOI:10.1016/j.jim.2011.04.002

URL http://hdl.handle.net/2241/113676
Detection of chymase-digested C-terminally truncated apolipoprotein A-I in normal human serum

Yoko Usami¹, Kazuyuki Matsuda², Mitsutoshi Sugano², Nau Ishimine², Yuriko Kurihara¹, Tamaki Sumida¹, Kazuyoshi Yamauchi³, and Minoru Tozuka¹

¹Analytical Laboratory Chemistry, Graduate School of Health Care Sciences, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
²Department of Laboratory Medicine, Shinshu University Hospital, 3-1-1 Asahi, Matsumoto 390-8621, Japan
³Clinical Pathology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8575, Japan

Address correspondence to: Professor Minoru Tozuka, Analytical Laboratory Chemistry, Graduate School of Health Care Sciences, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan, Tel. +81-3-5803-5374; Fax. +81-3-5803-0166; E-Mail: mtozuka.alc@tmd.ac.jp

Abbreviations: apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; apoE, apolipoprotein E; LDL, low-density lipoprotein; HDL, high-density lipoprotein; RCT, reverse cholesterol transport; ABCA1, ATP-binding cassette transporter A1; LCAT, lecithin cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; MCP-1, monocyte chemotactic protein-1; KLH, keyhole limpet hemocyanin; OVA, ovalbumin; ELISA, enzyme-linked immunosorbent assay; WB, Western blotting; 2-DE, two-dimensional electrophoresis.
ABSTRACT

In atherosclerotic artery walls, mast cells, an inflammatory cell, are activated and secrete some proteases including chymase. Chymase, a chymotrypsin-like protease, cleaves the C-terminus of apolipoprotein A-I (apoA-I) at Phe225. This cleavage reduces the ability of apoA-I to promote the efflux of cellular cholesterol. The aim of this study is to detect C-terminally truncated apoA-I in normal human serum. For this purpose, we generated a monoclonal antibody that specifically recognizes C-terminally truncated apoA-I by immunizing mice with a peptide that corresponds to human apoA-I amino acid residues 216–225. The monoclonal antibody, termed 16-4 mAb, selectively reacted with recombinant C-terminally truncated apoA-I, but not recombinant full-length apoA-I. A two-dimensional electrophoresis analysis also indicated that only two out of six spots that contained apoA-I fragments and had a molecular mass of 26 kDa after chymase digestion reacted with the 16-4 mAb. We detected an extremely small amount of C-terminally truncated apoA-I in normal human serum by concentrating the serum through affinity chromatography using a 16-4 mAb-conjugated resin, and then performing Western blot analysis. The 16-4 mAb could be useful to examine whether C-terminally truncated apoA-I is associated with the progression of atherosclerosis.

Keywords: atherosclerosis, mast cell, proteolysis, biomarker, monoclonal antibody, reverse cholesterol transport
1. Introduction

Atherosclerosis partially results from an imbalance between the accumulation of low-density lipoprotein (LDL)-derived cholesterol in the intima and the cellular efflux of cholesterol by reverse cholesterol transport (RCT) (Jessup et al., 2006). Increased LDL particles in the plasma pass through the endothelial barrier and undergo modifications including oxidation (Navab et al., 1996), proteolysis (Paananen and Kovanen, 1994) and aggregation (Tabas, 1999). Modified LDLs are recognized by the macrophage scavenger receptor and taken up without feedback regulation (Greaves and Gordon, 2005). The subendothelial accumulation of cholesterol-filled macrophages, called foam cells, is a hallmark of atherosclerosis (Lusis, 2000). RCT is one of the principal antiatherosclerotic mechanisms that is induced by high-density lipoprotein (HDL) and its major protein, apolipoprotein A-I (apoA-I). ApoA-I promotes the efflux of excess cholesterol from macrophages via the ATP-binding cassette transporter A1 (ABCA1) and leads to nascent HDL (Wang et al., 2001). The plasma enzyme lecithin cholesterol acyltransferase (LCAT) promotes the esterification of cholesterol in nascent HDL to form the mature spherical HDL, which transports cholesterol back to the liver (Vedhachalam et al., 2007). Many inflammatory cells such as monocytes and lymphocytes migrate into the atherosclerotic artery wall, and HDL and LDL are modified by products secreted from inflammatory cells. Modified HDL has an impaired ability to promote cholesterol efflux and bind lipids, which consequently results in the progression of atherosclerosis (Shao et al., 2006a; Shao et al., 2006b).
Mast cells, a type of inflammatory cell, are filled with cytoplasmic secretory granules that contain two species of neutral serine proteases, tryptase and chymase (Kelley et al., 2000). Mast cells are activated by the complement proteins C3a and C5a, monocyte chemotactic protein-1 (MCP-1) and oxidized LDL, and secrete these proteases (Krishnaswamy et al., 2001). Interestingly, mast cells are found in atherosclerotic lesions throughout the progression of these lesions, and the proportion of activated mast cells correlatively increases with the progression of atherosclerosis (Kaartinen et al., 1994).

An in vitro experiment showed that HDL was degraded by chymase and that this degradation was caused by the proteolysis of apoA-I within HDL particles. Chymase cleaves apoA-I at either the N-terminus (Tyr18 and Phe33) or C-terminus (Phe225) (Lee et al., 2003). PreβHDL, the smallest particles that contains only one molecule of apoA-I and a few phospholipids, is strikingly sensitive to chymase-mediated proteolytic cleavage and rapidly depleted (Lee et al., 2000). In contrast, αHDL remains unchanged, although apoA-I in αHDL is cleaved and the generated fragments remain bound to αHDL particles. Treating HDL with chymase reduces its ability to promote cholesterol efflux from cells (Favari et al., 2004). Previous studies demonstrated that apolipoprotein A-II (apoA-II) and E (apoE) (Lee et al., 2002a) and cholesteryl ester transfer protein (CETP) (Lee et al., 2008) are also degraded and inactivated by chymase digestion. The C-terminal region of apoA-I is essential for both cholesterol efflux and lipid binding, suggesting that C-terminally truncated apoA-I is a potential novel biomarker for atherosclerosis.

In mice, the endogenous apoA-I levels in the intraperitoneal fluid were
significantly reduced when mast cell lysates were injected into the peritoneal cavity \textit{in vivo} (Judström et al., 2010). Similar to when mast cell deguranulation is induced, this treatment resulted in the degradation of both apoA-I and apoE in serum HDL particles. ApoE fragments but not apoA-I fragments were detected in the circulation, indicating that the apoE fragments remained HDL-associated while apoA-I fragments were released from HDL and rapidly eliminated from the circulation (Judström et al., 2010). To date, normal human serum has not been definitely shown to contain C-terminally truncated apoA-I. The major goal of this study is to develop a specific monoclonal antibody that recognizes C-terminally truncated apoA-I and to use this antibody to identify this fragment in normal human serum.
2. Materials and methods

2.1 Materials and reagents

Unless stated otherwise, all reagents were purchased from Wako Pure Chemicals (Tokyo, Japan). Blood samples were taken from apparently healthy 7 volunteers (the mean±SD of age and BMI are 28.1±11.9 and 20.9±1.9, respectively) who had provided informed consent. The study was approved by our institutional research ethics committee.

2.2 Purification of human apoA-I

HDL (d 1.063–1.21 g/mL) was isolated from pooled serum by ultracentrifugation (Havel et al., 1955). The isolated HDL was dialyzed against 20 mmol/L Tris-HCl (pH 7.4) containing 1 mmol/L EDTA overnight and then delipidated using ethanol : ether (3:2, v/v) at −20°C for 3 days. ApoHDL was washed twice with ether, dried, dissolved in 20 mmol/L Tris-HCl (pH 7.4) containing 1 mmol/L EDTA, 6.8 mol/L urea, and 0.1% 2-mercaptoethanol and applied to a gel-filtration column using Sephacryl S-200 (GE Healthcare Japan, Tokyo) (1.5 × 95 cm) equilibrated with the same buffer. The apoA-I–rich fractions were confirmed by Western blotting (WB), dialyzed against 20 mmol/L Tris-HCl (pH 7.4) containing 1 mmol/L EDTA, and then stored at −80°C until further use.

2.3 Cymase-mediated proteolysis of apoA-I

ApoA-I (1 mg/mL) was incubated with or without 0.1 BTEE unit/mL of chymase
(Elastin Products Company, Owensville, MO) in 20 mmol/L Tris-HCl (pH 7.4) containing 150 mmol/L NaCl and 1 mmol/L EDTA at 37°C for different time periods. The reaction was stopped by adding soybean trypsin inhibitor (Sigma-Aldrich Japan, Tokyo, Japan) at a final concentration of 100 µg/mL.

2.4 Generation of the monoclonal antibody

Synthetic peptides corresponding to sequences 216–225 of human apoA-I conjugated to keyhole limpet hemocyanin (KLH) or ovalbumin (OVA) were prepared for immunization and an enzyme-linked immunosorbent assay (ELISA), respectively. Mice (BALB/c) were immunized 7–11 times at approximately one week intervals by intraperitoneally injecting the KLH-conjugated peptide (100 µg/each time) in Freund’s adjuvant. The mouse with the highest titer against the peptide was identified using an ELISA with the OVA-conjugated peptide as the capture antigen, and then directly injected into the spleen with a final peptide boost (100 µg) one day before the fusion. Splenocytes were harvested from the mouse and fused with mouse myeloma cells (SP2). The hybridomas were cloned by three rounds of limiting dilutions. Only one cell line was selected based on its ability to recognize chymase-treated human apoA-I but not intact apoA-I, and then injected into the peritoneal cavity of mice. The mouse ascites was harvested and filtered through a 0.22-µm filter. The monoclonal antibody was determined to have an IgM isotype using IsoStrip (Roche Diagnostics, Indianapolis, IN), a mouse monoclonal antibody typing kit. The ascites was treated with ammonium sulfate (50%
saturated) to remove albumin and subsequently subjected to Sepharose CL-6B (GE Healthcare Japan, Tokyo, Japan) column chromatography (1.5 × 95 cm). NaN$_3$ (final concentration of 0.02%) was added to the partially purified IgM antibody (termed 16-4 mAb), and the antibody preparation was stored at 4°C.

2.5 Competitive ELISA

A 96-well plate (Polysorp, Nunc, Roskilde, Denmark) was coated with 100 µL/well of the OVA-conjugated synthetic peptide (50 µg/mL) in carbonate buffer (pH 9.6) at room temperature for 90 min. After washing three times with PBS containing 0.05% Tween 20 (Tween/PBS), 300 µL/well of 0.5% sodium casein in Tween/PBS was added to block the nonspecific binding sites and the plate was incubated at room temperature for 90 min. The plate was washed and incubated with 100 µL/well of the samples, including the OVA-conjugated synthetic peptide (0.04–0.4 µg/mL), purified human apoAI (10–70 µg/mL), and chymase-treated human apoA-I (10–70 µg/mL), containing the 16-4 mAb (final 1:1000 dilution) in PBS at room temperature for 90 min. After washing, 100 µL/well of a horseradish peroxidase-conjugated anti-mouse µ-chain antibody (1:1000 dilution, SouthernBiotech, Birmingham, AL) was added and the plate was incubated at room temperature for 90 min. After a series of final washes, 100 µL/well of SureBlue Reserve (Kierkegaard & Perry Laboratories, Gaithersburg, MD) was added for 1–5 min. The reaction was stopped by adding 100 µL/well of 0.3 mol/L H$_2$SO$_4$, and the absorbance at 450 nm was measured using SUNRISE RAINBOW-RC.
(Wako Pure Chemicals, Tokyo, Japan).

2.6 Preparation of recombinant apoA-I

Full-length apoA-I cDNA was PCR amplified using gene-specific primers (sense, 5’-ATGAAAGCTGCGTGCTG-3’ and antisense, 5’-TCACTGGGTGTTGAGCTTC-3’). To synthesize the cDNA encoding the C-terminally truncated apoA-I (Lys226→stop), an antisense mutagenic primer in which codon AAG was replaced with TAG (5’-CTAGAAGCTCTCCAGCAC-3’) was used with the sense primer described above. The amplified cDNA was cloned into the pcDNA4/HisMax TOPO TA expression vector (Invitogen, Carlsbad, CA). The purified plasmid was transfected into COS-1 cells using Lipofectamin™ 2000 (Invitogen, Carlsbad, CA) according to the manufacturer’s instructions. The transfected cells were cultured in 5% FBS/DMEM for 24 h, and then cultured in serum-free DMEM for the next 24 h. The culture supernatant was applied to a HisTrap HP column (1mL, GE Healthcare Japan, Tokyo, Japan), and the bound and unbound fractions were obtained as described in the manufacturer’s instructions. Both fractions were concentrated using an Amicon (Millipore, Bedford, MA).

2.7 Affinity chromatography

The partially purified monoclonal antibody (approx. 10 mg) was coupled to CNBr-activated Sepharose 4B (1 ml, GE Healthcare Japan, Tokyo, Japan) as described in
the manufacturer’s protocol. Serum (3 mL) obtained from the healthy volunteer (age; 55 yo, BMI; 24.3, serum HDL-cholesterol; 60 mg/dL, serum LDL-cholesterol; 126 mg/dL, serum apoAI; 126 mg/dL) was diluted 5-fold with Tween/PBS and applied to the monoclonal antibody-coupled-Sepharose 4B column. After washing the column with Tween/PBS, the bound proteins were eluted with 50 mmol/L glycine-HCl (pH 2.3) and immediately neutralized with 1 mol/L Tris. After dialyzing against PBS, the bound proteins were concentrated using an Amicon.

2.8 Western blotting (WB)

Western blotting was performed as previously described (Ishimine at al., 2010). Briefly, SDS-PAGE was performed using 12.5% polyacrylamide gel under reducing conditions. Isoelectric focusing was performed using 4% polyacrylamide gels containing 8 mol/L urea and 5% Pharmalyte (pH 4.0 – 6.5, GE Healthcare Japan, Tokyo). For two-dimensional electrophoresis (2-DE), the proteins separated by IEF were reduced, alkylated, and then subjected to SDS-PAGE. To detect apoA-I and truncated apoA-I, the separated proteins were electrophoretically transferred to PVDF membranes (Millipore, Bedford, MA). After blocking in 5% skim milk, the membranes were incubated with a goat anti-apoA-I polyclonal antibody (Academy Bio-Medical Company, Houston, TX) or the 16-4 mAb. Subsequently, the membranes were incubated with a POD-conjugated rabbit anti-goat IgG antibody (Medical & Biological Laboratories, Nagoya, Japan) or POD-conjugated rabbit anti-mouse µ-chain antibody (SouthernBiotech, Birmingham,
AL), respectively. The bands containing apoA-I and truncated apoA-I were visualized with 3,3’-diaminobentizin-4HCl and H₂O₂ (DAB system) or ECL Plus Western Blotting Detection Reagents (ECL system, GE Healthcare Japan, Tokyo, Japan).

3. Results

3.1 Proteolysis of human apoA-I by chymase

Purified human apoA-I was incubated with 0.1 BTEE unit/mL of chymase at 37°C. The cleavage kinetics were analyzed by SDS-PAGE followed by WB using an anti-apoA-I polyclonal antibody and developed with the DAB system. The intensity of the intact apoA-I band was attenuated over time and completely disappeared after a 120-min incubation (Fig. 1). In contrast, two major bands with apparent molecular masses of 26 and 24 kDa were newly detected, and the intensities of these bands peaked after approximately 30 and 60 min of digestion, respectively, and then gradually decreased. In contrast, incubating HDL₃ with the same chymase did not generate any apoA-I fragments (data not shown).

3.2 Preparation of an anti-C-terminally truncated apoA-I monoclonal antibody

To generate a monoclonal antibody specific for C-terminally truncated apoA-I, a 10-amino acid peptide corresponding to amino acid residues 216–225 of human apoA-I was synthesized and used as the antigen. A candidate hybridoma cell-line was selected based on the preferential recognition of chymase-treated apoA-I but not intact apoA-I. The monoclonal antibody produced by this hybridoma had an IgM isotype and was
named 16-4 mAb.
3.3 Competitive ELISA

To confirm the epitope recognized by the 16-4 mAb, a competitive ELISA was performed. When the synthesized peptide was used as both the coating material and the competing antigen, antigen concentration-dependent competition was observed (Fig. 2A). Similarly, concentration-dependence competition was observed when chymase-treated apoA-I was used as the competing antigen. However, intact apoA-I did not compete with the synthesized peptide (Fig. 2B, solid squares and solid circles, respectively). These findings indicated that the 16-4 mAb recognized an epitope in the synthesized peptide and within chymase-treated apoA-I but not intact apoA-I.

3.4 Specificity of the 16-4 mAb for C-terminally truncated apoA-I

To confirm the specificity of the 16-4 mAb, the recombinant intact apoA-I (ri-apoA-I) and recombinant C-terminally truncated apoA-I (rt-apoA-I) in the unbound fractions of the HisTrap HP column were analyzed by WB using the 16-4 mAb and developed with the ECL system (Fig. 3). Although ri-apoA-I was detected by WB using a polyclonal anti-apoA-I antibody and had the same molecular weight as purified serum apoA-I (Fig. 3A, lanes 1 and 2), the 16-4mAb did not detect any bands (Fig. 3B, lanes 1
and 2). In contrast, the rt-apoA-I was detected by both the polyclonal antibody and the 16-4 mAb and had a smaller molecular mass (approx. 26 kDa) than intact apoA-I (Fig. 3A and 3B, lanes 3 and 4). Similar results were obtained when the culture supernatant was directly analyzed. The polyclonal anti-apoA-I antibody reacted with both ri-apoA-I and rt-apoA-I regardless of a conjugated His-tag. However, the 16-4 mAb reacted only with the untagged and His-tagged rt-apoA-I (data not shown). These results clearly indicated that the 16-4mAb selectively recognized the C-terminally truncated apoA-I, which lacks residues 226–243.

3.5 Production of C-terminally truncated apoA-I by chymase digestion of apoA-I

ApoA-I was digested with chymase for various time periods (similarly treated samples shown in Fig. 1) and then analyzed by WB using the 16-4 mAb and the DAB system (Fig. 4). The digested fragment had an apparent molecular mass of 26 kDa and was detected by the polyclonal anti-apoA-I antibody after a 10-min incubation. The band intensity slightly increased after a 30-min incubation and was maintained up to 120 min (Fig. 4A). In contrast, a band with an apparent molecular mass of 26 kDa was detected by the 16-4 mAb after a 30-min incubation and its intensity clearly continued to increase up to 120 min (Fig. 4B). These increasing intensities were not obviously
proportional to that of the 26 kDa digested apoA-I fragments that were detected by the polyclonal antibody.

3.6 2-DE of chymase-digested apoA-I

Chymase-digested apoA-I was also analyzed by 2-DE followed by WB. After blotting, the proteins on the PVDF membranes were sequentially probed using the two antibodies, and the DAB system. First, the membrane was probed with the 16-4 mAb to detect the C-terminally truncated apoA-I, and then with the anti-apoA-I polyclonal antibody to detect intact apoA-I and its fragments (Fig. 5). The intact apoA-I was separated into two major and three minor bands by IEF (Fig. 5B, 0 min). All five bands had almost the same molecular weight by SDS-PAGE (2D) (Fig. 5C, 0 min). After apoA-I was treated with chymase, some of the novel bands appeared by IEF in more basic and acidic directions (Fig. 5B, 20 min and 60 min). The 2-DE patterns showed many additional spots with lower molecular weights than the intact apoA-I (Fig. 5C, 20 min and 60 min). There were at least six spots with an apparent molecular mass of 26 kDa; however, only two of these spots were detected by the 16-4 mAb (white arrows and inset D).
3.7 Detection of C-terminal truncated apoA-I in human serum

When normal human serum was directly analyzed by WB using the 16-4 mAb and the DAB system, C-terminally truncated apoA-I was not detected (data not shown). To verify whether C-terminally truncated apoA-I is present in normal human serum, the serum was concentrated by 16-4 mAb-coupled affinity chromatography in order to bind any C-terminally truncated apoA-I. The bound fraction (Fig. 6C) as well as intact (Fig. 6A) and chymase-treated (Fig. 6B) apoA-I were analyzed by SDS-PAGE followed by WB. The 16-4 mAb detected a band in the bound fraction that migrated at the same molecular mass (26 kDa) as the chymase-digested apoA-I fragment. In the bound fraction, a relatively large amount of intact apoA-I was also detected using the anti-apoA-I polyclonal antibody, likely indicating that a large amount of intact apoA-I in the serum non-specifically bound to the column compared to C-terminally truncated apoA-I.
4. Discussion

In this study, we generated a monoclonal antibody (16-4 mAb) that specifically recognizes C-terminally truncated apoA-I, which can be produced by chymase-mediated digestion in the atherosclerotic lesions. To generate the 16-4 mAb, mice were immunized with a 10-amino acid peptide that corresponds to human apoA-I amino acid residues 216–225, which corresponds to the newly generated C-terminus after chymase digestion. The specificity of the 16-4 mAb was demonstrated by a competitive ELISA and WB using chymase-digested apoA-I and recombinant C-terminally truncated apoA-I, respectively (Fig. 2–4). Although this peptide sequence is present in intact apoA-I, the 16-4 mAb did not recognize intact apoA-I, which raises two possibilities. First, the tertiary structure of the new C-terminus of apoA-I after chymase digestion could be altered. The C-terminus of intact apoA-I, which is encoded by amino acids 228–243, forms one of ten α-helical segments in the apoA-I molecule (Brouillette et al., 2005). This α-helix is abolished when chymase cleaves the molecule at residue 225, and the new C-terminus may have an altered conformation that is recognized by the 16-4 mAb. Second, the region containing residues 216–225 of apoA-I, which is the same sequence that was used for immunization, might be masked by intramolecular interactions or lipids. In the lipid-free form of intact apoA-I, the C-terminal α-helix
interacts with the N-terminal helix bundle, resulting in a C-terminal domain that is relatively unexposed to solvents. In the first step in which apoA-I binds to phospholipids, the interaction between the C-terminal $\alpha$-helix and N-terminal domain is lost and the C-terminal $\alpha$-helix binds to lipids (Koyama, et al., 2009). Thus, the C-terminal $\alpha$-helix of intact apoA-I would mask the region recognized by the 16-4 mAb, and therefore intact apoA-I in both the lipid-free and lipid-bound forms would not react with the 16-4 mAb.

A previous report showed that treating HDL with chymase (40 BTEE units/mL) resulted in the disappearance of pre$\beta$-HDL, a minor containing fraction, and 10- and 26-kDa apoA-I fragments remained in $\alpha$HDL (Lee et al., 2000). It was also shown that chymase degraded approximately 35% of apoA-I contained in discoidal pre$\beta$-migrating reconstituted HDL particles (9.6-nm diameter), and generated two major bands of 26 and 24 kDa after a 6-h incubation (Lee et al., 2003). The 26- and 24-kDa bands contained apoA-I fragments that lacked either the N-terminal 18 (Tyr18) or C-terminal 18 (Phe225) residues or the N-terminal 33 (Phe33) residues, respectively. The 24-kDa band did not contain any fragments that were simultaneously cleaved at both ends (Tyr18 and Phe225) (Lee et al., 2003). In the present study, similar 26- and 24-kDa bands were generated; however, lipid-free apoA-I was completely degraded within a 2-h
incubation with 0.1 BETT unit/mL chymase (400-fold lower concentration than the HDL degradation experiment described above) (Fig. 1). The 26-kDa fragment was detected by WB using the 16-4 mAb, suggesting that apoA-I in HDL particles as well as lipid-free apoA-I, at least in part, was cleaved at residue Phe225.

The specificity of the 16-4 mAb for the C-terminally truncated apoA-I was confirmed by WB using a recombinant C-terminally truncated apoA-I as the antigen (Fig. 3B). We used COS-1 cells to produce apoA-I with or without the C-terminal 18 amino acids in intact apoA-I. A relatively small amount of the His-tag–free fraction, corresponding to intact or C-terminally truncated apoA-I, was observed in the culture supernatant, indicating that some of those proteins were secreted after the signal peptides were digested, according to the innate ability of COS-1 cells. We concluded that the His-tag–free fractions contained intact and C-terminally truncated apoA-I but not proapoA-I and C-terminally truncated proapoA-I, because the molecular masses of both proteins were the same as the purified human apoA-I and chymase-treated 26-kDa fragment that reacted with the 16-4 mAb (Fig. 3). Consequently, the 16-4 mAb specifically reacted with the recombinant C-terminally truncated apoA-I regardless of the presence of a His-tag and/or signal peptide, but not with any form of intact apoA-I.

After apoA-I was cleaved by chymase (0.1 BTEE unit/mL), the 26-kDa fragment
recognized by the 16-4 mAb appeared by WB after a 30-min digestion, and the band intensity increased with the digestion time. These apparent intensities were not relative to the intensities of the 26-kDa fragments detected by the anti-apoA-I polyclonal antibody (Fig. 4). These findings suggest that some fragments in the 26-kDa band were not recognized by the 16-4 mAb and that a portion of the 26-kDa fragments could be N-terminally (Tyr18) truncated apoA-I. Chymase digestion of apoA-I produced many spots on 2-DE (Fig. 5). Two out of 6 spots with the same molecular mass (26 kDa) were recognized by the 16-4 mAb. The major and minor spots could correspond to the C-terminally truncated apoA-I and its deamidated form, respectively. Deamidation commonly occurs during apoA-I purification, especially when using a buffer containing a high concentration of urea (Ghiselli et al., 1985). Some of the other spots could correspond to the N-terminally (Tyr18) truncated apoA-I, including its deamidated forms, as the isoelectric points of these spots were higher than that of intact apoA-I, which is consistent with a theoretical change in the isoelectric points based on the N-terminal amino acid sequence that was removed from intact apoA-I upon chymase digestion. In addition, several lower molecular weight spots including N-terminally (Phe33) truncated apoA-I were detected with the anti-apoA-I polyclonal antibody. These findings suggest that lipid-free apoA-I has more chymase cleavage sites than
lipid-bound apoA-I.

Previous *in vitro* studies demonstrated that various proteases, including tryptase (Lee et al., 2002b), transthyretin (Liz et al., 2007), matrix metalloprotease (Lindstedt et al., 1999), plasmin and kallikrein (Lindstedt and Kovanen, 2000), degrade apoA-I in HDL particles. Similarly, studies have shown that apoA-I is degraded *in vivo* by activated mast cells (Judström et al., 2010). Many types of proteases within atherosclerotic lesions degrade apoA-I and effect cellular cholesterol efflux, consequently contributing to the progression of atherosclerosis. Although a few reports have detected protease-digested apoA-I fragments in human serum, one report detected 26- and 22-kDa fragments in the serum of patients with acute myocardial infarction (Eberini et al., 2007). In the present study, an apoA-I fragment that was truncated at Phe225 was identified in the serum of healthy subjects. However, the concentration of C-terminally truncated apoA-I in normal serum is extremely low because this fragment was not detected by WB without partially purifying the serum samples. Approximately 200 ng of bound proteins were obtained from 3 mL of serum by 16-4 mAb-coupled affinity column chromatography. The results indicate that most of the proteins were non-specifically bound proteins because a relatively large amount of non-specific adsorption of intact apoA-I compared to C-terminally truncated apoA-I was detected by
WB using the anti-apoA-I polyclonal antibody (Fig. 6C).

A biomarker that sensitively reflects a personal risk of myocardial and cerebral infarction has not been established. However, several recent studies reported new biomarkers that predict the progression of atherosclerosis (Koenig, 2007; Packard and Libby, 2007; Pineda et al., 2009). Mast cell migration and activation are known to increase proportionately with the progression of atherosclerosis (Kaartinen et al., 1994). Therefore, the C-terminally truncated apoA-I that is produced by mast cell chymase and activated in atherosclerotic lesions could directly reflect the progression of atherosclerosis. The presence of this truncated apoA-I also indicates a reduction in the most suitable acceptor for ABCA1-mediated cholesterol efflux. In healthy subjects, mast cells are not activated, and consequently there are extremely low levels of C-terminally truncated apoA-I in the serum. On the other hand, patients with progressive atherosclerosis have many activated mast cells in atherosclerotic lesions, possibly indicating that C-terminally truncated apoA-I is dramatically increased.

To evaluate the usefulness of C-terminally truncated apoA-I as a biomarker for the progression of atherosclerosis, it will be necessary to develop a convenient assay such as an ELISA. Measuring the levels of C-terminally truncated apoA-I may provide predictive information for both atherosclerosis patients and individuals with traditional
risk factors for coronary heart disease.

5. Conclusion

We have produced the monoclonal antibody (16-4 mAb) specifically reacted with C-terminally truncate apoA-I, which is known to be induced by chymase derived from mast cells in atherosclerotic lesions, using a peptide corresponded to human apoA-I amino acid residues 216–225 as an antigen. No cross reaction was observed between 16-4 mAb and full-length apoA-I. C-terminally truncated apoA-I was detected by 16-4 mAb in normal human serum, hopefully the 16-4 mAb could be useful to examine whether C-terminally truncated apoA-I is associated with the progression of atherosclerosis.
Acknowledgements

We thank Professor Takayuki Honda (Department of Laboratory Medicine, Shinshu University) for generously providing assistance to our collaborators and coauthors, Dr Kazuyuki Matsuda, Dr Mitsutoshi Sugano, and Nau Ishimine. We also thank Innovative Antibody Engineering Laboratory, Advance Inc. for advice on generating the monoclonal antibody. The study was approved by the Ethics Committee of TMD University (No. 614). This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 21590611).
References


scavenger receptors. J. Lipid Res. 46, 11-20.


FIGURE LEGENDS

Figure 1. Proteolysis of human apoA-I by chymase

Purified human apoA-I (1 mg/mL) was incubated with (+) or without (−) 0.1 BETT unit/mL of chymase at 37°C for the indicated time periods. The cleaved products were analyzed by SDS-PAGE followed by WB using an anti-apoA-I polyclonal antibody and the DAB system. M indicates the molecular weight marker.

Figure 2. Competitive ELISA for chymase treated apoA-I

To confirm the epitope that is recognized by the 16-4 mAb, a competitive ELISA was performed. A 96-well plate was coated with a synthetic peptide corresponding to residues 216–225 of human apoA-I that was conjugated to ovalbumin (OVA). Mixtures of the 16-4 mAb (final 1:1000 dilution) and the indicated concentrations of the OVA-conjugated synthetic peptide (A), purified human apoA-I (B; ●), or chymase-treated human apoA-I (B; ■) in PBS were added to evaluate competitiveness. After the reaction, a horseradish peroxidase-conjugated anti-mouse µ-chain antibody was used to detect the amount (as absorbance at 450nm) of bound 16-4 mAb to the peptides. The data are shown as the means ± standard deviations (SD) of three replicates.
**Figure 3. Specificity of the 16-4 mAb**

To evaluate the specificity of the 16-4 mAb, recombinant C-terminally truncated apoA-I was examined by WB. Purified human apoA-I (lane 1), recombinant human apoA-I (lane 2), chymase-treated human apoA-I (lane 3), and recombinant C-terminally truncated human apoA-I (lane 4) were subjected to SDS-PAGE followed by WB with the polyclonal anti-apoA-I (A) and 16-4 mAb (B) antibodies. The bands were visualized with the ECL system.

**Figure 4. Recognition of chymase-digested C-terminally truncated apoA-I by the 16-4 mAb**

Purified human apoA-I (1 mg/mL) was incubated with (+) or without (−) 0.1 BTEE unit/mL of chymase at 37°C for the indicated time periods. The cleaved products were analyzed by SDS-PAGE followed by WB using the polyclonal anti-apoA-I (A) and 16-4 mAb (B) antibodies. The bands were visualized with the DAB system.

**Figure 5. Two-dimensional electrophoresis for chymase treated apoA-I**

ApoA-I (0 min) and chymase-treated apoA-I (20 and 60 min) were analyzed by two-dimensional electrophoresis (2-DE). After incubating with (20 and 60 min) or without (0 min) 0.1 BTEE unit/mL chymase, the cleaved products were analyzed by
isolectric focusing followed by WB using the 16-4 mAb (A) and polyclonal anti-apoA-I (B) antibodies. Similarly, after 2-DE (C), the C-terminally truncated apoA-I was visualized by WB using the 16-4 mAb antibody (inset D), and then the membranes were restained with the polyclonal anti-apoA-I antibody. The C-terminally truncated apoA-I was distinguished as dark black spots (white arrows). White arrowheads indicate intact apoA-I. All bands and spots were developed using the DAB system.

**Figure 6. Identification of C-terminally truncated apoA-I in normal human serum**

Serum (3 mL) diluted 5-fold with Tween/PBS was applied to Sepharose 4B that was conjugated to the partially purified 16-4 mAb. The bound fraction (C), as well as the purified apoA-I (A) and chymase-treated (120 min) apoA-I (B), were analyzed by SDS-PAGE followed by WB using the polyclonal anti-apoA-I (p) and 16-4 mAb (m) antibodies. The bands were visualized using the DAB system.
Fig. 1

<table>
<thead>
<tr>
<th>incubation time (min)</th>
<th>0</th>
<th>120</th>
<th>5</th>
<th>10</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>chymase</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Fig. 2

A

B

ABS (450nm)

peptide (mg/mL)

proteins (mg/mL)
Fig. 4

A and B: Western blot analysis showing the presence of chymase at 26kDa over time. The table below summarizes the results:

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>0</th>
<th>120</th>
<th>10</th>
<th>30</th>
<th>60</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>chymase</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Fig. 6

Protein bands at 28kDa and 26kDa are shown in the diagram.