Relationship of Lipoprotein-Associated Phospholipase A_2 and Oxidized Low-Density Lipoprotein in Carotid Atherosclerosis

Supplemental Materials/Methods

I. Tissue Collection and Processing

Human CEA tissues were collected immediately in RNALater upon excision from subjects. A 50mL tube with ~30mL of RNALater was provided to the surgical team. The tissues were submerged in ice cold RNALater until processed (~2 hours). The CEA tissues were rinsed in fresh RNALater and incubated for 48 hrs at 4^0C, with a change of RNALater at 24 hrs. The RNALater was removed and the tissue was stored at -80^{o}C (days) until thawed on ice for mounting, sectioning, or homogenization. We have determined that RNALater preserves not only the quality of RNA but proteins as well. Additionally, RNALater contains 10mM EDTA, which is sufficient to prevent storage oxidation. Blood samples were drawn from 20 of the patients who had undergone CEA, thereby providing 20 tissue/blood matched pairs. Fasting bloods were drawn into Vacutainer tubes containing EDTA. After cells were sedimented by centrifugation (3000x g,15min) the plasma was decanted and stored at -20 C^0 for analysis an average of 4.5 months after tissue acquisition.

II. Total Protein Extraction

STE buffer (1.2mL, 100mmol/L NaCl, 25mmol/L Na_2EDTA, pH 8.0, 10mmol/L Tris-HCl, pH 8.0) was added to the 5mm segments of CEA tissues for 15min incubation

on ice, followed by tissue mincing on a glass plate cooled to 4°C. The minced tissue was homogenized (30-45sec., level 7 speed, Brinkmann Polytron, Switzerland), and treated with an equal volume (~2mL) of 2X homogenization buffer; 2mmol/L Na Citrate, 2mmol/L cysteine, and 1X PBS with Complete Protease Inhibitor tablets (Roche). This extraction buffer is designed to prevent protein degradation and further LDL oxidation during processing and storage. Samples were incubated on ice for 10min, followed by centrifugation at 3,750 rpm (30min). Supernatant was stored at –80° C and protein concentration determined by Bio-Rad D_C Protein Assay using BSA as a standard. No detergent was introduced into the protein extraction buffers (STE and 2X homogenization) to avoid the possibility of detergent-induced alteration of the oxLDL structure and the water solubility of Lp-PLA_2 and oxLDL.

III. Antibody Conjugation

The following protocol was used to prepare each antibody-conjugate (4E6- Alexa 488 or Quantum Dot525, 2C10-Alexa546, 4B4-Alexa 546, apoB-100-Quantum Dot625): 0.2mg of lyophilized antibody (with no protein carrier or amine containing buffer) was dissolved in 100µl of 0.1mol/L sodium bicarbonate; 0.3mg of Alexa fluorescent succinimidyl ester (amine-reactive) dye was dissolved in 30µl DMSO, and 10µl of the unstable dye solution was immediately added to the antibody solution. The mixture was agitated gently in the dark for 30 min. Another 10µl aliquot of the dye solution was added to the reaction mixture and stirred gently for 30min in the dark. This reaction mixture was then transferred to a 3,500 MWCO dialysis cassette (Pierce, Slide-A-Lyzer) and dialyzed against one liter of 1X PBS for 7 days, with buffer changes every two days.

The antibody-fluorophore conjugate was then chromatographed on a gel filtration column (G-25 Sephadex) to remove any traces of unconjugated fluorophore. Eluted protein was diluted to 20ng/mL then stored (4^{o} C) in dark.

IV. Immunohistochemistry

CEA tissue segments (5mm) were sectioned (10μm) transversely to the long axis of the CEA tissue. Frozen sectioning was performed with a Leica CM-130 cryotome and sections were mounted on positively charged glass slides to enhance adherence (SuperFrost Plus, Fisher). Immunohistochemical experiments used the following reagents: antibody mAb-4EB directed against human oxLDL (Mercodia); antibody mAb-2C10 directed against Lp-PLA $_2$ (DiaDexus, Inc); and antibody pAb against apoB100. These antibodies were directly conjugated to Alexa Fluor dyes or Quantum Dots (Molecular Probes) by succinimidyl ester coupling or by quantum dot antibody labeling, per manufacturer's instructions.

Lp-PLA_2 antibody, 2C10, is a mouse anti-human monoclonal antibody generated by immunizing mice with purified human Lp-$PLA_{2.}$ (3). OxLDL antibody, 4E6, is a mouse anti-human monoclonal antibody generated by immunizing mice with human LDL either oxidized with copper or modified with MDA. Hybridomas were screened to isolate specific antibodies with ELISA. 96-well plates were coated with MDA-LDL or OxLDL. Human anti-apoB-100 antibody was generated against human LDL. Purified LDL was isolated by ultracentrifugation at d 1.016-1.063 and delipidated by diethyl ether:ethanol (3:1) extraction. The moist protein precipitate was solubilized in 20 mM Tris, 10mM sodium decyl sulfate, pH 8.25 and subjected to size exclusion

chromatography on Sepharose S-200. The purified apoB100 was used for goat immunization. Antiserum was chromatographed over LDL-Sepharose to obtain affinity purified anti-apoB-100. The antibody reacted with apoB-100 and apoB-48 but not with other apolipoproteins.

Tissue Immunostaining: Slides were dehydrated (vacuum desiccator, 20min) and treated with UV light for 24hr and/or 0.1% sodium borohydride-PBS (30min) to remove background fluorescence. A hydrophobic boundary was drawn onto the slide around the tissue section with an Immunopen (Vector Labs., Burlingame, CA). The following abbreviated procedures were performed; i) Removal of OCT: 150µl of 70% ethanol treatment for 15 min, 150µl of water for 10 min, 150µl of 1X PBS for 10 min. ii) Blocking step: 150µl of blocking solution (4% mouse serum, 100µg/ml non-immune IgG_1 (isotype), and 1mg/ml bovine serum albumin in 1X PBS) for 1hr on orbital shaker. iii) Antibody incubation: 20µl of antibody (20ng/ml) with 50µl of 1X PBS-T (Tween-20, 0.05%) for 2hrs on an orbital shaker in the dark at ambient temperature. iv) Washing (ambient temperature, gentle orbital shaking, and dark): 150µl of 1X PBS-T for 15min, 150µl of 1X PBS-T with 4,6-diamidino-2-phenylindole (DAPI) nuclear stain (1:20,000, 5mg/mL stock) for 10min, 150µl of 1X PBS-T for 15 min. v) Mounting: one drop of 4% *n*-propyl gallate in glycerol was added to the tissue to preserve fluorescent signal, 1.5mm thick 24X40 coverslip (Fisherbrand), and sealed by clear finger nail polish. Samples were stored at -20^{o} C. Slides were imaged with the Delta Vision Spectris System with deconvolution fluorescence microscopy (Issaquah, WA). Lenses of 20X, 40X, 60X, and 100X were used in this study. Control slides were treated with unconjugated-antibody prior to incubation with conjugated-antibody.

OxLDL Controls: Purified oxLDL, 10µl of 28mU/L (Mercodia, Uppsala, Sweden), was transferred to positively charged glass slides (SuperFrost Plus, Fisher) for 30 min. Immunohistochemistry was performed as stated in the supplemental data (II). Human anti-oxLDL (4E6)-Alexa Fluor Dye 488 conjugate was used as probe.

Fluorescent Microbead Standards: Fluorescent microbeads with a diameter of 0.02µm (FluoSpheres® aldehyde-sulfate microspheres, yellow-green fluorescent (505/515), Invitrogen Molecular Probes, Eugene, OR) were placed on positively charged slides (SuperFrost Plus, Fisher). Slide mounts were treated with 4% *n*-propyl gallate in glycerol.

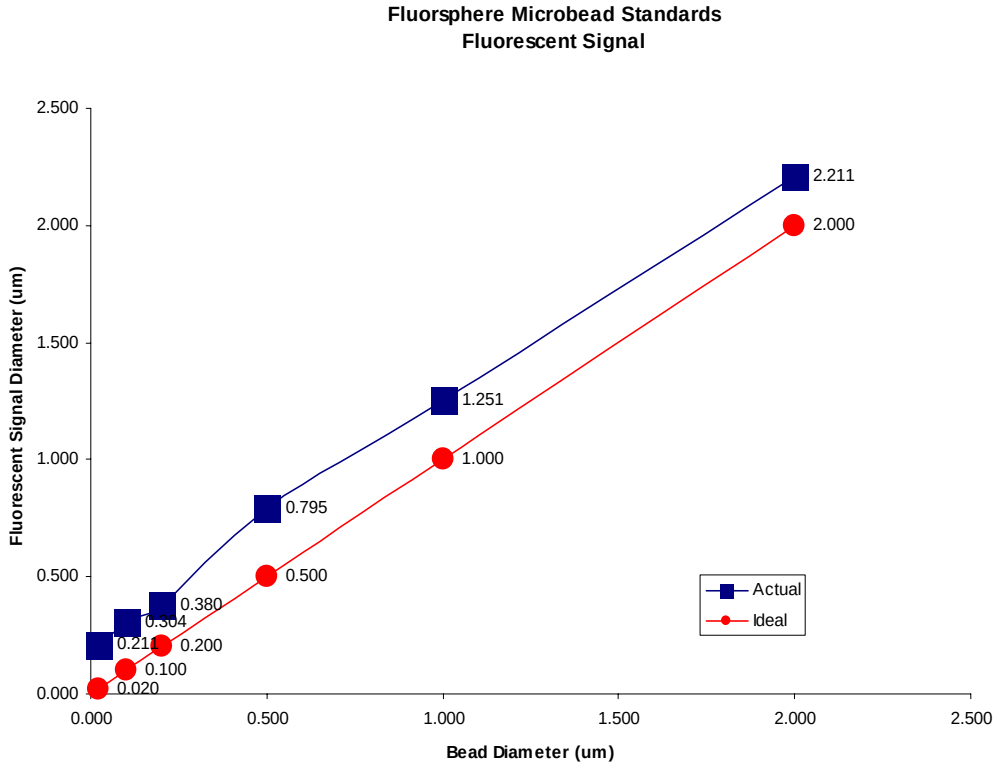
Immunohistochemistry of plasma oxLDL: Freshly isolated plasma was smeared onto polysilanated slides. Slides were treated with 0.01% glutaraldehyde, washed, then immuno-stained as previously described.

V. Identification of individual fluorescent oxLDL particles

Using immunohistochemistry to image individual oxLDL particles is novel, however this required operating at the limits of our microscopy system (fluorescence, deconvolution). We originally measured the fluorescence diameter of an average sized positive free-standing oxLDL signal in tissue to be 200nm using deconvolution microscopy (Figure 4F). Immunofluorescence measurements of purified oxLDL (with a known diameter of ~20nm) also resulted in a fluorescence diameter measurement of 200nm (Figure 4G). Similarly, fluorescent microbeads (20nm) appeared to have a diameter of 200nm as determined by the software distance measurement tools (Softworx,

Deconvolution Microscopy Software) (Figure 4H). However, the fluorescent signal diameter is an overestimation, and was determined to be an order of magnitude greater than the actual size of individual LDL particles. Fluorescence signals in the sub-micron range are often difficult but not impossible to measure accurately due to their limits of resolution (Supplemental References 3 and 4). The true size of the oxLDL-positive particle was established by comparison to known standards and was determined to be ~20nm. We performed fluorescence distance analysis for a range of beads with known diameters (Supplemental Data 1). In these experiments we used a range (20nm, 100nm, 200nm, 500nm, 1µm, and 2 µm) of fluorescent microsphere standards and imaged these beads at 100X+ enhanced, followed by distance measuring using the Softworx distance tool.

VI. Supplemental Data .



Supplemental References:

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