Permeation of human plasma lipoproteins in human carotid endarterectomy tissues: measurement by optical coherence tomography

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Abstract Atherosclerosis is an inflammatory process occurring in arterial tissue, involving the subintimal accumulation of LDL. Measurement of the rate at which LDL and other lipoproteins, such as HDL and VLDL, enter and exit the tissue can provide insight into the mechanisms involved in the development of atherosclerotic lesions. Permeation of VLDL, LDL, HDL, and glucose was measured for both normal and atherosclerotic human carotid endarterectomy tissues (CEA) at 20°C and 37°C using optical coherence tomography (OCT). The rates for LDL permeation through normal CEA tissue were \((3.16 \pm 0.37) \times 10^{-5} \text{ cm/s at } 20^\circ C\) and \((4.77 \pm 0.48) \times 10^{-5} \text{ cm/s at } 37^\circ C\), significantly greater \((P < 0.05)\) than the rates for atherosclerotic CEA tissue at these temperatures \((1.97 \pm 0.34) \times 10^{-5} \text{ cm/s at } 20^\circ C\) and \((2.01 \pm 0.23) \times 10^{-5} \text{ cm/s at } 37^\circ C\). This study effectively used OCT to measure the rates at which naturally occurring lipoproteins enter both normal and diseased carotid intimal tissue.

Atherosclerosis is the most common underlying cause of vascular disease, occurring in multiple arterial beds, including the carotid, coronary, and femoral arteries. Major efforts have concentrated on understanding the steps involved in the formation of atherosclerotic plaques. Considerable evidence now indicates that initiation of the inflammatory response involves accumulation of lipids and lipoprotein particles, particularly low-density lipoproteins (LDL), beneath the intimal layer of the arterial wall. This accumulation may be attributed to increased influx into and/or decreased efflux out of the intimal layer. Atherosclerosis also involves subintimal accumulation of inflammatory agents, such as tissue macrophages, which express scavenger receptors that bind modified lipoprotein particles. However, little is known about the rates at which substances permeate the arterial wall and how these rates may be related to the rate of atherosclerosis progression or regression. These processes may be closely linked to the rates at which lipoproteins permeate the individual layers of arterial tissues.

Several imaging techniques have been used to study the permeation of analytes in biological tissues, including ultrasound, magnetic resonance imaging (MRI), optical projection tomography (OPT), and optical coherence tomography (OCT). OCT is particularly attractive because it is a nondestructive, noninvasive technique that provides high-resolution, real-time images of biological tissues. OCT has been used extensively in tissue studies to measure permeation of different molecules and materials through various epithelial tissues, such as rabbit sclera, monkey skin, rabbit cornea, and porcine aorta.

In the present novel study, we used OCT to measure perfusion through yet another biological system, the intimal layer of the carotid arterial wall. This layer was obtained from human carotid endarterectomy (CEA) specimens.
which contained normal healthy segments (PC, EC) and diseased atherosclerotic segments (BC, IC) (Fig. 1). OCT has the potential for enabling investigation of the atherosclerotic process. Progression of this process is thought to involve the subintimal accumulation of modified LDL (15) in the subintimal space, while regression is believed to involve removal of cholesterol by HDL (16, 17) (reverse cholesterol transport), among other mechanisms. Our study was directed toward measuring and comparing the rates of VLDL, LDL, and HDL permeation into normal and atherosclerotic CEA tissue.

MATERIALS AND METHODS

Tissue preparation, characterization, and imaging

CEA specimens were obtained by surgical procedures performed at Methodist Hospital (Houston, TX) and stored at 4°C in PBS containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The study protocol was approved by the Institutional Review Board of the Baylor College of Medicine. The tissues contained relatively normal areas in the proximal common (PC) and external (EC) segments, and atherosclerotic areas in the bifurcation (BC) and internal (IC) segments (Fig. 1). The desired areas were cut from the tissue specimen with a 6 mm biopsy punch (Sargent, Inc.), and the resulting disks were transferred to a 96-well microtiter plate with the intimal surface facing upward. The discs were then classified as normal tissues or diseased tissues, the latter containing mostly lipidic, calcific, hemorrhagic, and/or fibrotic components. Each experiment was performed within 24 h after receiving the specimen. During OCT measurements, tissues were maintained at either 20°C or 37°C. Tissue viability was quantitatively measured with the LIVE/DEAD cell assay described by Allison et al. (19). About 300 µm sections were stained in 4 µM calcine AM and 2 µM ethidium homodimer, and then viewed by confocal microscopy. Live cells bind calcine AM at their surface and emit light at 515 (green) when excited at 495 nm. Dead cells allow entrance of ethidium and fluoresce at 635 nm (red) when excited at 495 nm. Unstained tissue (negative control) was used to determine tissue autofluorescence. Tissue treated with 70% ethanol for 30 min served as a positive control for dead cells. Positive control for live cells was a 3D culture of aortic smooth muscle cells.

Lipoproteins were isolated by sequential ultracentrifugation (20), dialyzed against PBS, and concentrated by ultrafiltration (XM-100 Amicon). Tissues were imaged in the presence of VLDL (4.5 × 10^6 Da, >80 nm diameter, 4.20 × 10^7 mole particles/l), LDL (2.1 × 10^6 Da, ~21 nm diameter, 1.80 × 10^6 mole particles/l), HDL (0.25 × 10^6 Da, ~8 nm diameter, 8.50 × 10^6 mole particles/l), and glucose (180 Da, ~1 nm diameter, 4.44 × 10^4 mole particles/l). Solutions were stored at 4°C and equilibrated to the requisite temperature (20°C or 37°C) 30 min before each experiment.

Each tissue sample was immersed in PBS and imaged for 5 min to acquire baseline data. Subsequently, a particular analyte (VLDL, LDL, HDL, or glucose) was added to the well containing the tissue sample, and image acquisition continued for another 40 min. A schematic representation of the methods used for these experiments is shown in Fig. 2.

Imaging system

The OCT technique is based on the principle of interferometry. A beamsplitter divides the light coming from the laser source and projects it into a sample arm and a reference arm. Light scattered and back reflected from the tissue is combined with light returned from the reference arm, and a photodiode detects the resulting interferometric signal. Interference data is collected across the surface of the sample, resulting in 2D and 3D images with micrometer-scale resolution and several millimeters penetration depth, depending on tissue type. The 2D OCT images are averaged into a single 1D distribution of light in depth on a logarithmic scale. Optical properties of tissue as a function of permeating molecule concentration can be dynamically monitored and assessed by analyzing the in-depth light attenuation profile (21).

A time-domain OCT system (Imalux Corp., Cleveland, OH) was utilized in this study. The optical source of the OCT system was a low-coherence near-infrared broadband laser diode, now commercially available, (Superlum, Inc., Moscow, Russia) with a wavelength of 1510 ± 15 nm and an output power of 3 mW. The in-depth resolution was 0.2 pix/µm. The system’s scanning functionality was conducted with an endoscopic probe using a single-mode optical fiber for scanning along the x-axis. Piezoelectric modulation of the fiber length enabled in-depth scanning along the z-axis. A 2D image with dimensions of 2.2 (z-axis depth) × 2.4 mm was obtained every 4 s continuously, corresponding to each complete scan both laterally and vertically. These 2D images were then laterally averaged (over ~1 mm) for speckle noise suppression, producing a single 1D curve on a logarithmic scale.

Data analysis

The OCT signal slope (OCTSS) method, previously described by us in detail (12), was used to calculate the permeability rate of lipoprotein particles and glucose molecules moving through CEA tissues. As they traveled through the carotid tissue, they caused changes in the optical properties of the sample. These changes were reflected in the slope of the selected region (slab) within the OCT signal, as seen in the OCTSS graphs (Fig. 3). The selected slabs occurred at approximately the same depth (100 µm from the intimal layer) and varied in thickness from 100 to 150 µm. A more elaborate explanation of these effects of the changes within the tissue samples is included in the supplementary online data. The time required for the permeating species to move through the selected region (t_{spp}) was estimated to begin at the point of OCTSS decrease, reflecting a drop in the attenuation of the tissue due to refractive index matching caused by the permeation of the analyte, and to end at the point of OCTSS increase, where a reverse process starts. The average permeability rate in cm/sec (Ps) was calculated by dividing the thickness of the selected region (t_{spp}) by the time (Ps = t_{spp}/t_{spp}). Representative OCTSS graphs for LDL and HDL permeation into normal and diseased CEA tissues are shown in Fig. 3. In Fig. 3B-D, the monitored regions were around 100 µm away from the intimal layer. In Fig. 3A, however, the region at a lower depth (around 300 µm)
was chosen to demonstrate a lag time between the addition of lipoproteins and the point at which the slope changes due to arriving particles. Representative OCT signals from glucose and VLDL permeation experiments are included in the supplementary online data.

RESULTS

The permeation rates for VLDL, LDL, HDL, and glucose at 20°C and 37°C through normal and diseased CEA tissues were quantified using OCT. The rates for VLDL in normal and diseased tissues were $(1.13 \pm 0.26) \times 10^{-5}$ cm/sec and $(1.50 \pm 0.21) \times 10^{-5}$ cm/sec at 20°C and $(1.20 \pm 0.25) \times 10^{-5}$ cm/sec and $(1.75 \pm 0.34) \times 10^{-5}$ cm/sec at 37°C. The rates for LDL at 20°C were $(3.16 \pm 0.37) \times 10^{-5}$ cm/sec in normal tissues and $(1.97 \pm 0.34) \times 10^{-5}$ cm/sec in diseased tissues. However, at 37°C the rates for LDL were $(4.77 \pm 0.48) \times 10^{-5}$ cm/sec in normal tissues and $(2.01 \pm 0.23) \times 10^{-5}$ cm/sec in the diseased tissues. The measured rates for HDL in CEA tissues were $(1.57 \pm 0.26) \times 10^{-5}$ cm/sec for normal tissues and $(2.01 \pm 0.32) \times 10^{-5}$ cm/sec for diseased tissues at 20°C and $(2.42 \pm 0.24) \times 10^{-5}$ cm/sec for normal tissues and $(2.43 \pm 0.31) \times 10^{-5}$ cm/sec for diseased tissues at 37°C. In addition to the above-mentioned lipoproteins, the rates of 20% glucose solution in CEA samples were quantified. The permeability rates in normal and diseased tissues were $(3.51 \pm 0.27) \times 10^{-5}$ cm/sec and $(6.31 \pm 0.61) \times 10^{-5}$ cm/sec at 20°C and $(3.70 \pm 0.44) \times 10^{-5}$ cm/sec and $(5.70 \pm 0.48) \times 10^{-5}$ cm/sec at 37°C. A comprehensive comparison of the permeability rates is summarized in Fig. 4.

The experimental variability in the measurements stems from the use of various tissue samples and inter- (5.1%; n = 4) and intra-operator (1.3%; n = 5) variability.

The viability of cells in the CEA tissues used for OCT measurements was evaluated with the LIVE/DEAD cell assay. This was chosen to demonstrate a lag time between the addition of lipoproteins and the point at which the slope changes due to arriving particles. Representative OCT signals from glucose and VLDL permeation experiments are included in the supplementary online data.

**Fig. 2.** Schematic representation of tissue preparation and experimental procedure. A: Tissues were cut using a 6 mm diameter biopsy punch. B: Tissues were stored in PBS in a microtiter plate. C: Functional imaging was performed using an OCT system. D: An analyte was added after acquiring 50 images for baseline data.

**Fig. 3.** Representative OCT signal slopes of LDL and HDL permeation in normal and diseased tissue. The time required for the permeating species to move through the selected region ($t_{region}$) is indicated by a green marker.
was 37.0%. The mean increase for these lipoproteins through diseased tissue was 13.2%. The most striking effect of temperature increase on permeability rate was observed with LDL in normal tissues with an increase of 50.9%.

Our second hypothesis was that the permeation rate would be inversely related to lipoprotein size; i.e., the largest lipoprotein would exhibit the smallest rate of permeation. This hypothesis proved valid for the comparison of VLDL (>80 nm) versus HDL (8-11 nm). However, this hypothesis did not hold for LDL. Its permeation rate through normal tissue at 37°C [(4.77 ± 0.48) × 10^{-5} cm/s] was 97.1% greater than the rate for HDL [(2.42 ± 0.24) × 10^{-5} cm/s] under the same conditions. This unexpected behavior of LDL prevailed only with normal tissues. With diseased tissue at 20°C, the rate increased (1.50 < 1.97 < 2.01) with decreasing particle size (VLDL > LDL > HDL). A similar trend was observed at 37°C (1.75 < 2.01 < 2.43) with diseased tissue.

**DISCUSSION**

Our initial hypothesis was that increasing temperature would increase permeation rates for each lipoprotein. This was, in fact, the case for all three lipoproteins permeating through normal and diseased tissues. The mean rate increase for VLDL, LDL, and HDL through normal tissue was 37.0%. The mean increase for these lipoproteins through diseased tissue was 13.2%. The most striking effect of temperature increase on permeability rate was observed with LDL in normal tissues with an increase of 50.9%.

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![Fig. 5](image-url)  
**Fig. 5.** Tissue viability test. A: Smooth muscle cell (SMC) treated with ethanol (negative control). B: 3D culture of SMC (positive control). C: Normal CEA tissue. D: Calcified CEA tissue. Green indicates live cells; Red indicates dead cells.
Our third hypothesis was that lipoproteins would permeate more rapidly through diseased tissues than normal tissues. This hypothesis proved valid at 20°C for VLDL (mean = 38.5%) and HDL (mean = 14.2%). For LDL at 20°C, however, the normal tissues exhibited 47.7% greater mean permeability than diseased tissues. For normal carotid tissues at physiological temperature (37°C), the permeability rate of LDL was significantly higher than that of glucose. This result suggests the presence of a facilitated cellular transport mechanism specific to LDL. Previous reports have indicated that LDL permeates the endothelial layer of aortic tissue primarily by two pathways: vesicles (22, 23) and leaky junctions (24–26). Through the process of endocytosis, vesicle transporters enable endothelial cells to take up LDL, with little effect arising from concentration differences between the intracellular and extracellular matrix. Leaky junctions within the endothelium originate from apoptotic events and the formation of lesions due to an increase in the rate of cellular turnover. An in vitro study of LDL transport compared the significance of these two pathways and found that leaky junctions accounted for <90% of LDL flux, whereas vesicles accounted for <10% (27). Leaky junctions are temperature-dependent, being more abundant and greater in size at 37°C than at 20°C. These differences could account for the dramatic increase in the permeability rate of LDL when the temperature is raised to 37°C.

The glucose permeation measurements revealed interesting properties of the normal and diseased arterial wall. The rate of glucose permeation through normal tissues was not significantly increased by raising the temperature from 20°C to 37°C (3.51–3.70), suggesting that the normal internal permeability to small molecules is at or near its maximal value in this temperature range. Similarly, glucose permeation through diseased tissues was not considerably changed upon raising the temperature from 20°C to 37°C (6.31–6.57). Significantly, the rate of glucose permeation through diseased tissues at 20°C (37°C) was 79.9% (54.0%) greater than through normal tissues. The higher permeation rates for the diseased tissues suggest the presence of interstitial defects that permit influx of small molecules like glucose, but not large particles like lipoproteins.

This study benefited from several unique advantages: 1) OCT does not perturb the CEA tissue or permeating species; 2) both normal and diseased tissue could be obtained from the same tissue specimen; and 3) the amount of tissue and permeating species required was small, allowing adequate experiment replication. However, the study had significant limitations: 1) the permeating species did not compete with other components that would be present in normal blood; 2) measurements were performed using static, nonflowing solutions; and 3) the CEA tissue contained only the intimal layer and plaque, not the media or adventitial layers.

Our observation that LDL permeates CEA tissue faster than other lipoproteins is consistent with a much earlier report that 125I-LDL enters the normal arterial wall rapidly and interacts with it (28). This interaction includes both binding to the arterial wall and degradation of the radio-labeled LDL protein. These studies were extended later in the balloon de-endothelialized rabbit aorta, which was injected with 125I-LDL. Anterior scintigrams demonstrated sequestration of the labeled lipoprotein by the aorta (29). In a separate study, 125I-LDL was also shown to accumulate in the carotid arteries of patients previously injected with the labeled lipoprotein (29). The retention of LDL in the arterial wall may be controlled by atherin, a protein that avidly binds LDL and is found only in atherosclerotic lesions, not in normal intima (30).

CONCLUSIONS

The permeability rates of VLDL, LDL, HDL, and glucose in normal and diseased human CEA tissue at 20°C and 37°C was measured using OCT. A significant difference ($P < 0.05$) between 20°C and 37°C in normal tissue was found for the permeability rate of LDL, $(3.16 \pm 0.37) \times 10^{-5}$ cm/s and $(4.77 \pm 0.48) \times 10^{-5}$ cm/s, respectively, supporting previous suggestions for an arterial transport mechanism specific to LDL. OCT has proven to be a non-invasive and nondestructive method for determining the permeability rates of lipoproteins and other small analytes in CEA tissues.

Direct experimental measurement of depth-resolved lipoprotein influx rates for normal and diseased vasculature is essential for our understanding of the complexities of atherosclerotic plaque formation. The results presented in this study testify to the feasibility of using OCT to measure the rates at which a small molecule like glucose and a large particle like a lipoprotein permeate through the arterial tissue.

To calculate the rate of net accumulation of lipoproteins in the arterial wall, it is essential to determine their influx and efflux rates. OCT is well suited for both determinations. Quantification of the rates of flux into and out of vascular tissues may unveil specific mechanisms involved in the processes of reverse cholesterol transport and atherosclerosis regression.

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