XeCl laser-induced fluorescence detection of peroxidized lipoproteins in lipid-rich atherosclerotic lesions

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ABSTRACT

Laser-induced fluorescence spectroscopy of arterial surfaces provides information about the composition of atherosclerotic plaques. The aim of the study was to determine whether accumulation of peroxidized lipoproteins in arterial walls, a process postulated to play a role in initiating atherosclerotic changes, can be demonstrated by fluorescence spectroscopy.

XeCl excimer laser (λ = 308 nm) induced fluorescence of human aortas containing early lipid-rich, non-collagenous lesions exhibited marked red shifts and broadening of the fluorescence spectra compared with spectra from non-atherosclerotic aortas. Similar profiles were observed in spectra obtained from oxidatively modified LDL, but not native LDL. In hypercholesterolemic rabbits with early foam cell lesions, spectral shifts resembled those of oxidized β-VLDL, the major lipoprotein accumulating in arteries of rabbits fed cholesterol.

XeCl laser-fluorescence spectroscopy of arterial surfaces may be useful for the identification of arterial plaques indicative of atherosclerosis in its early and probably reversible stages.

1. INTRODUCTION

Arteries with atherosclerotic changes exhibit altered autofluorescence responses that provide information about the fluorophore-chromophore composition of the underlying lesions. Characterization of arterial tissue by fluorescence spectroscopy has been performed with various lasers including the Ar-ion (476.5 nm)[1,3-7], XeCl (308 nm)[8,11], XeF (351 nm)[12], He-Cd (325 nm)[2,9], and N_2 (337 nm)[13] lasers. Advanced human atherosclerotic lesions as seen in unselected autopsy specimens are often highly collagenous and may contain surprisingly little lipid[14]. The fluorescence properties of such lesions are strongly influenced by the matrix proteins forming the fibrous caps that cover advanced plaques[9]. In contrast, during early atherogenesis, the subendothelial space is occupied by lipid-laden macrophages or foam cells, the major component of fatty streaks[15,16]. According to current concepts, lipoproteins accumulating in foam cell lesions undergo peroxidative and hydrolytic modifications[17-22]. It has been suggested that these lipoprotein modifications play an important role in attracting circulating monocytes and transforming them into lipid-laden foam cells[17].

In this paper we have tested the hypothesis that lipoprotein peroxidation products in early atherosclerotic lesions can be detected by fluorescence spectroscopy [23]. Experiments were performed using a XeCl laser because its wavelength (λ=308 nm) permits excitation of majority of tissue fluorophores and because near UV light can be delivered through flexible optical fibers adaptable to cardiovascular catheterization techniques.

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2. MATERIALS AND METHODS

2.1. Laser Fluorimeter

A XeCl excimer laser (model LPX-600, Lambda-Physik, Germany) producing 60-ns pulses of 308-nm light was used to elicit fluorescence spectra from arterial surfaces exposed to air or from lipoprotein solutions contained in high purity quartz cells (Fig. 1). XeCl laser fluence did not exceed 10^{-2} J/cm^2 to avoid changes in tissue autofluorescence due to laser irradiation.

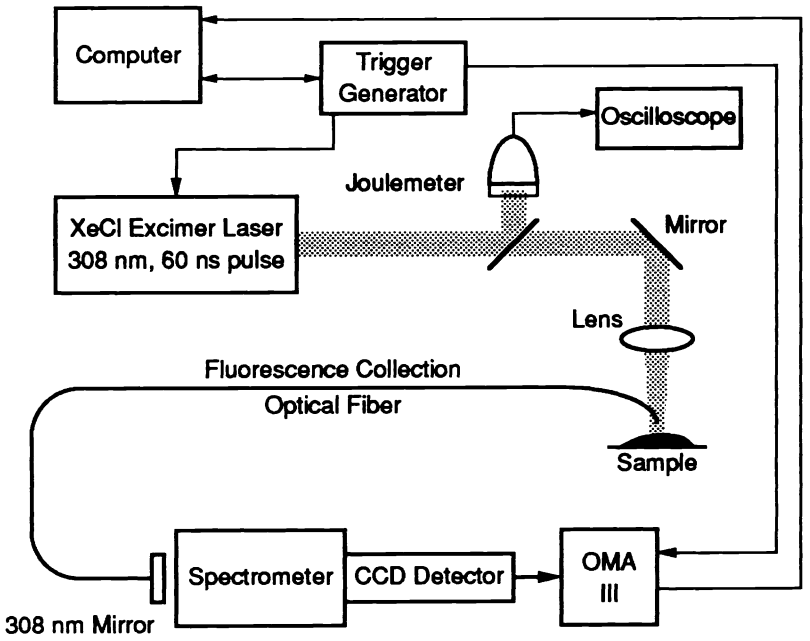

FIGURE 1.

Fluorescence spectroscopic setup. XeCl excimer laser pulse at 308 nm was used to excite arterial surfaces or isolated lipoproteins in solution. The emitted fluorescence was collected with an optical fiber and transmitted to a spectrometer coupled to an optical multichannel analyzer. An interference mirror reflecting 99 % of radiation at 308 nm was placed in front of the spectrometer entrance slit to attenuate back-scattered laser light. Emitted fluorescence was evaluated in the spectral region between 330 and 600 nm

The irradiated region of the sample was approximately 1mm in diameter, a compromise between spatial selectivity and detectability of fluorescence emission. The emitted fluorescence was collected with fused silica optical fibers (Diaguide, Fort Lee, New Jersey) with a core diameter not exceeding 1/10 that of the irradiated area in order to avoid shadowing of the target under irradiation. The end of the optical fiber was positioned perpendicular to the target surface and its tip was precisely centered above the target at a distance equal to the length of the excitation beam diameter. These precautions minimize fluorescence distortion due to scattering and reabsorption within the tissue[24].

Collected fluorescence was transmitted through a fiber optical system to a spectrometer coupled to a gated multichannel spectral analyzer (model OMA III, EG&G Princeton Applied Research, Princeton, New Jersey). Fluorescence was induced by a single laser pulse and recorded during a 20-ns gate positioned at the maximum amplitude of the fluorescence pulse. Before each experiment spectral calibration of spectrometer was performed.

A high reflectance (99 %) mirror (308 FR-1D-FL, ARC Corp., Acton, Massachusetts) was placed in front of the spectrometer entrance slit to suppress 308 nm back scatter from the sample. To improve the signal-to-noise ratio, at least four (average 7) fluorescence spectra were obtained from each target site. Profiles of the spectra were numerically smoothed using a narrow interval procedure. Smoothed spectra from different arteries and different arterial locations were compared by superimposing them. To facilitate comparison of the spectra, they were normalized with respect to integrated fluorescence intensity.

2.2. Studies with human lipoproteins and arteries

Human lipoproteins were fractionated by ultracentrifugation of pooled plasma collected in EDTA (1mg/ml) as previously described [25]. The lipoproteins were dialyzed against phosphate-buffered saline containing 0.01% EDTA. Protein in the samples was determined by the Lowry method using serum bovine albumin as a standard [26]. Oxidative modification of the low density lipoprotein fraction (LDL, density 1.019 - 1.063 g/ml) was performed by exposing native LDL for 24 h to cultured rabbit endothelial cells equilibrated with serum-free F10 medium [25]. Samples of native LDL serving as control were subjected to incubations in F10 medium containing no cells. All LDL samples were re-isolated by ultracentrifugation. The extent of lipoprotein modification was assessed by measurements of thiobarbituric acid - reactive substances (TBARS) and electrophoretic mobility as previously reported [25].

Human aorta samples were obtained at *post mortem* examination from 19 patients within 6 hours after death. The wide age range of the donors (10-73 years) insured the collection of specimens with or without atherosclerosis of varying severity [15]. All samples were examined rapidly after excision to avoid artifacts resulting from prolonged storage. Throughout the experiments, the specimens were superfused with saline (21 ^{o}C) to avoid tissue desiccation. Six to ten samples, approximately 1cm^2 in size, were cut out from each aorta at sites thought to contain no atherosclerotic changes, fatty lesions (fatty streaks), or advanced fibrous plaques. Areas selected for spectroscopic study were marked with India ink and subsequently excised for histologic examination[6]. The photomicrographs of histologic sections were evaluated by two blinded independent observers and categorized according to the Stary classification of atherosclerotic plaques [15].

Sections exhibiting no or minimal changes including varying degrees of intimal thickening were called nonatherosclerotic (normal zones, Stary type I). Fatty streaks (early lesions) corresponded to lesions containing layers of macrophages (foam cells) and extracellular lipid particles (Stary lesion type II). These lesions contained by definition no stainable collagen deposits. Samples exhibiting such lesions were obtained from the aorta of one child and four adults. Advanced atheromas represented lesions collected from middle-aged patients (56-73 years old) with either fibro-lipid (Stary lesion type IV) or purely fibrous (collagenous) plaques (Stary lesion type V). In our context, the term fibrous plaque (Stary lesion type V) was used, when irradiation sites of the superficial *intima* (1mm thick subendothelial layer) contained a collagenous cap free of stainable lipid. In contrast, when irradiation sites contained in addition to collagen any stainable lipid, the lesions were called fibro-fatty (Stary type IV).

2.3. Studies with lipoproteins and arteries from rabbits with and without hypercholesterolemia

Relationships between spectroscopic characteristics of plasma lipoproteins and alteration in arterial autofluorescence were further evaluated in rabbits. Male albino New Zealand rabbits were placed on standard chow or on 1% rabbit cholesterol pellets as previously described[28]. After a 10 week dietary period, plasma total cholesterol measured by the cholesterol oxidase method averaged 1.2 ± 0.2 (SE) and 31.1 ± 9.0 mM in six control and six cholesterol-fed rabbits, respectively. Plasma was fractionated as described for human samples. The major cholesterol-carrying lipoprotein fraction in cholesterol-fed rabbits is recovered at a density of < 1.006 g/ml (beta very low density lipoprotein, β-VLDL)[20]. Samples of β-VLDL were oxidized by incubation with endothelial cells[25]. Spectroscopic evaluation of rabbit aortas and native or oxidized rabbit lipoproteins (β-VLDL) were performed as described above.

2.4. Analysis of Spectra and Statistics.

Sample groups selected according to their histologic categories yielded in general similar fluorescence spectra (except Stary group IV) as assessed by gross fluorescence band-shapes. To assess

variance of measured spectra in each histologic category, two parameters were determined for each spectrum: (1) the position of the fluorescence maximum and (2) the fluorescence band-width at half maximal amplitude (FWHM). Standard deviations (SD) of the parameters (1) and (2) from the mean values for Stary categories I (no or minimal changes) and V (lipid free fibrous caps) were very small (< 4 nm). Slightly larger values (< 10 nm) were obtained for the Stary category II (fatty streaks). In contrast, for Stary category IV (mixed fibro-lipid plaques), standard deviations were large (>100 nm), reflecting the compositional variance of the lesions. Therefore, measured fluorescence spectra belonging to Stary categories I, II, and V were averaged, but for fibro-fatty lesions we report here only selected examples of fluorescence spectra.

The statistical significance of differences (p-values) between mean values of fluorescence maximum position for Stary groups I, II, and V was evaluated by the analysis-of-variance procedure using the SAS/STAT statistics program. The generalized linear model (GLM) employing a multiple comparison test yielded p-values for each pair of Stary categories I, II, and V. P -values of < 0.01 were considered to represent significant intergroup differences. Fluorescence spectra for rabbit aorta samples and different lipoprotein preparations were analyzed by the same statistical procedure.

3. RESULTS

3.1. Human samples

Averaged fluorescence spectra from 6 human LDL pools before and after their oxidative modifications are shown in Figure 2. The native LDL profile exhibits a single fluorescence peak at approximately 340 nm (Fig. 2, continuous line). Oxidative modification of LDL produces a prominent spectral shift toward the red with a maximum at about 430 nm. Standard deviations of the position of fluorescence maxima from the mean value do not exceed 4 nm for native and 6 nm for oxidized LDL. In addition, there is a significant broadening of the spectrum consistent with an overlap of several fluorescence bands (Fig. 2, dashed line). The fluorescence band-width for native and oxidized LDL were 55±4 nm and 145±6 nm, respectively.

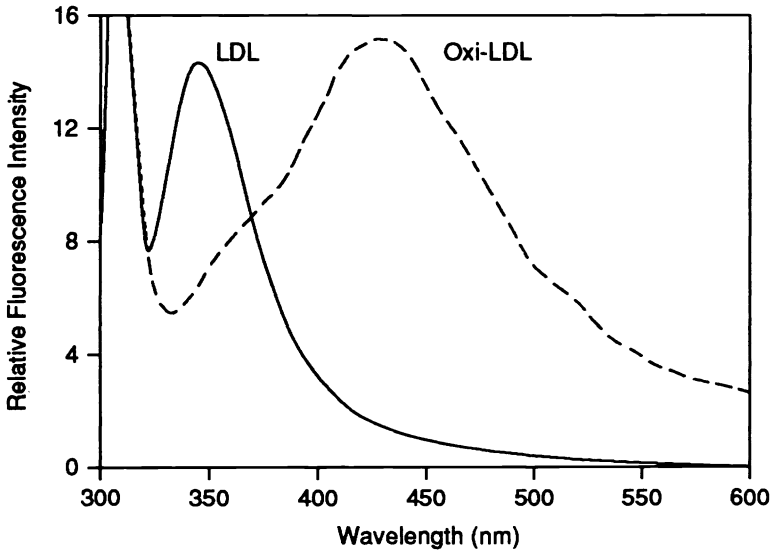

FIGURE 2.

Fluorescence profile of native (nonoxidized) human LDL (continuous line). There is a fluorescence maximum at approximately 340 nm with no appreciable additional features. Fluorescence profile of LDL oxidized by incubation with endothelial cells (dashed line). Compared with native LDL, the spectrum exhibits a marked red shift. Additionally, there is a broadening of the spectrum, consistent with multiple fluorophores.

The increased fluorescence of oxidized LDL at visible wavelengths is associated with a change in the absorption spectrum. Differences between the average absorption spectra from 6 native and 6 oxidized LDL samples in the wavelength-range 240 nm - 800 nm are illustrated in Figure 3. The absorption bands present in native samples at 450, 480, and 520 nm are absent after oxidative modification. Qualitative and quantitative differences between spectra from native and oxidized LDL very similar to those depicted in Figures 2 and 3 were observed in a total of six preparations from six different plasma pools.

Figure 4a (continuous line) shows an average fluorescence profile elicited from 3 samples of a 12 year old child aorta. These samples had no structural signs of atherosclerosis, including an absence of foam cells and stainable extracellular lipid. The non-atherosclerotic intima exhibited an abundance of finely reticulated material known to represent proteoglycan ground substance (Stary type I lesion[15]). The spectrum from this sample reveals a broad emission band with a fluorescence maximum at about 370 nm, smaller peaks at 382 nm, 390 nm, and 430 nm, and a broad shoulder extending into the yellow range. The FWHM value is about 105 nm. Virtually identical spectra were obtained from the aorta of a 10 year old child and two young adults. The average spectrum from the juvenile aortas with minimal changes (Stary type I, 10 samples from 4 aortas) was very similar to that depicted by the continuous line in Figure 4a except that its major maximum was shifted 4 nm toward longer wavelengths.

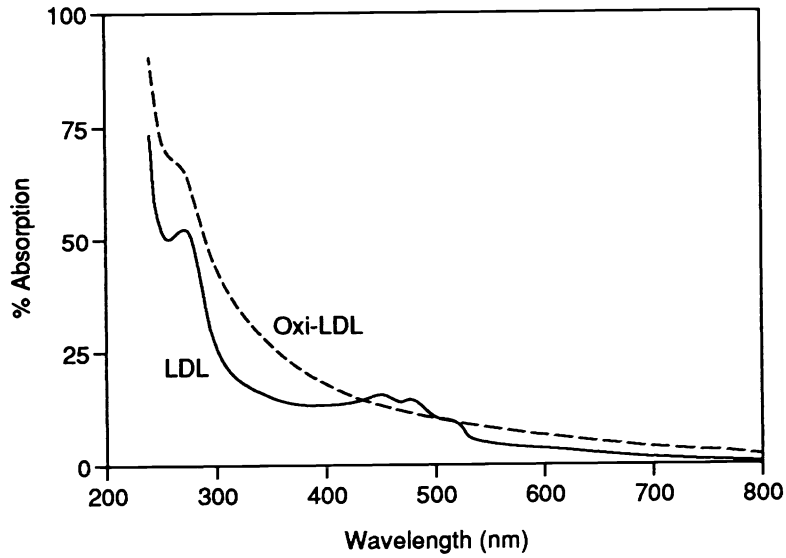

FIGURE 3.

Absorption spectra of native (continuous line) and oxidized (dashed line) LDL in solution. Oxidative modification results in a loss of absorption bands in the 430 - 540 nm wavelength region.

Figure 4a (dashed line) depicts an average fluorescence spectrum taken from an adult aorta containing abundant foam cells and numerous extracellular Oil Red O - positive particles and pronounced thickening of the aortic wall, but no collagenous deposits (Stary lesion type II, 9 lesions from 7 aortas). Compared with the non-atherosclerotic aorta (continuous line), there is a broadening of the spectrum with a marked shift towards the red and a maximum occurring at about 430±9 nm. Lipid-rich lesions (Stary type II) collected from a total of five aortas yielded similar spectra with a FWHM value of 170±10 nm. The p-value for the significance of the difference in position of the fluorescence maximum between lipid-rich plaques versus non-atherosclerotic (normal) human aorta walls was 0.0007.

An average spectrum of 3 crude preparations of human aortic elastin is depicted in Figure 4b (continuous line). The broad spectral band extends from the ultraviolet to the yellow range (FWHM equals 105 ±4 nm) with a maximum at 407±3 nm. Similar spectra have been obtained from elastin with the use of a He-Cd laser[9]. Compared with elastin, the fluorescence spectrum of the non-atherosclerotic aorta appears to be shifted toward shorter wavelengths. This may reflect non-elastin matrix proteins such as glycosaminoglycans abundant in L-tryptophan (Figure 4b, dashed line), an amino acid not contained in elastin[28]. The XeCl laser induced fluorescence spectrum of tryptophan was characterized by a position of the maximum at 362±3 nm and a FWHM value of 65±3 nm.

Figure 5 shows an average fluorescence profile from fibrous caps and representative fluorescence spectra from mixed fibro-fatty plaques. The average fluorescence spectrum for well developed fibrous caps without stainable superficial lipid (Stary type V, 18 lesions from 12 aortas) is drawn with a continuous line. The spectrum is narrower than that of the non-atherosclerotic human aorta (Fig. 4a, continuous line), lacking the broad shoulder in the yellow range (FWHM is 85±5 nm). The position of the maximum for the fibrous plaque fluorescence is at 382±4 nm. The spectrum of fibrous plaques resembles

that of crude collagen[8,9]. The p-values of the significance of the difference in the position of fluorescence maxima between fibrous plaques versus normal human aorta, and between fibrous versus lipid-rich plaques were 0.0012 and 0.0016, respectively.

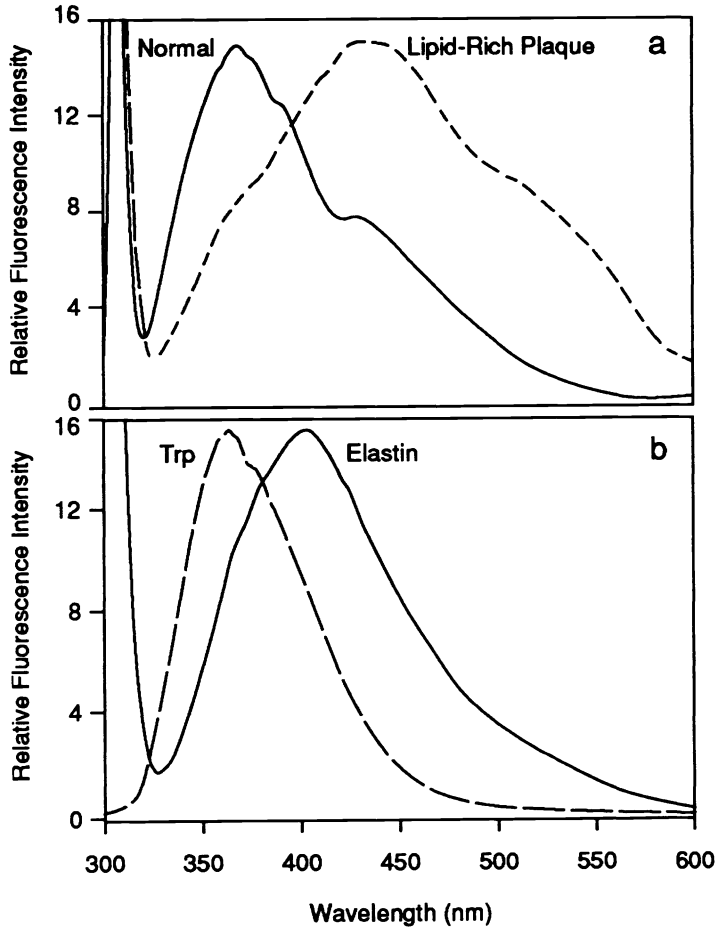

FIGURE 4.

(a) Fluorescence spectrum from human aortas: Continuous line - spectrum from the aortic surface of a 12 year old child (Stary lesion type I - slight intimal thickening with ground substance accumulation, but without microscopic signs of atherosclerosis). Dashed line - spectrum from the aorta of a young adult at a site containing a non-collagenous lipid-rich lesion (Stary type II), (b) Spectrum from a crude preparation of human aortic elastin (continuous line) and from L-tryptophan in aqueous solution (dashed line).

Figure 5 (dashed lines) depicts also spectra from irradiation sites containing both collagenous and abundant lipidic deposits (fibro-fatty plaques, Stary type IV lesions). Fibro-fatty plaques were the most frequent lesions in our autopsy material (79 Stary type IV lesions in 15 aortas). The spectra from such lesions appear to combine features of pure fatty lesions and lipid-free collagenous caps.

Figure 5 (long dashed line) depicts a fluorescence spectrum with two major bands obtained from a lesion with a thick collagenous cap and abundant subjacent lipid. The intense band with a maximum at 384 nm and a FWHM value of about 83 nm resembles the fluorescence spectrum of pure fibrous caps, whereas the broad band with a maximum at about 427 nm and a FWHM value of about 175 nm is suggestive of a lipid-rich lesion. Figure 5 (short dashed line) shows another example of a mixed fibro-lipid lesion with a thin collagenous cap incompletely masking subjacent lipid. The structural and chemical variability of fibro-fatty lesions is reflected in marked spectral variability that precludes useful statistical analysis.

3.2. Rabbit samples

Compared with human LDL, oxidative modification of rabbit β-VLDL produced a noticeable but less prominent spectral change, a phenomenon that may reflect VLDLs decreased susceptibility to oxidation[19].

Figure 6 (continuous line) shows the average spectrum of the β-VLDL fraction (d<1.006 g/ml) from plasma of 6 normocholesterolemic rabbits. Figure 6 (long and short dashed lines) depicts average fluorescence profiles from 6 unoxidized and 6 oxidized β-VLDL samples prepared from pooled plasma of 6 hypercholesterolemic rabbits. The rabbit lipoprotein spectra are asymmetrical, suggesting that more than one fluorophore contributed to the total fluorescence pattern. The spectrum of normocholesterolemic rabbit plasma exhibits a major peak at 410±5 nm and minor peaks at about 450 nm and 468 nm (Figure 6, continuous line). These minor peaks become the major emission maxima in unoxidized β-VLDL prepared from hypercholesterolemic rabbit plasma (Figure 6, long-dashed line).

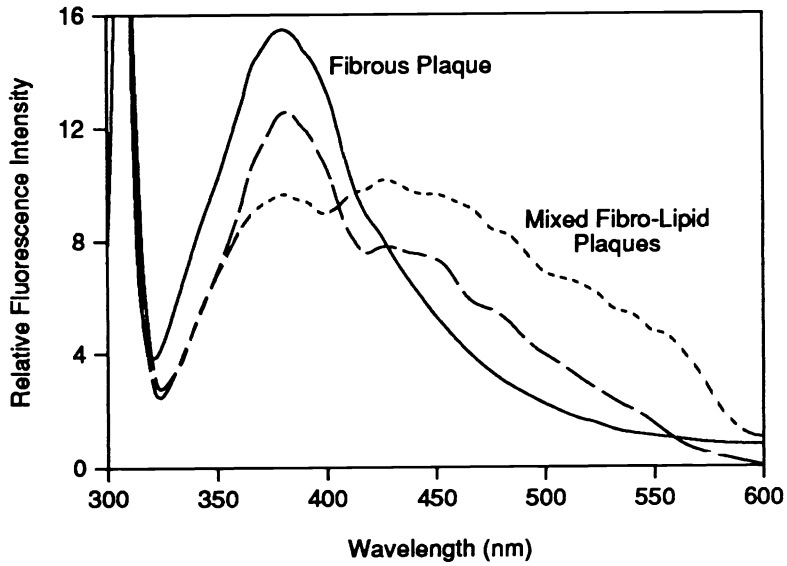

FIGURE 5.

Continuous line - Average fluorescence spectrum of of 18 thick fibrous caps containing no Oil-Red-O-positive material Dashed lines - Fluorescence spectra of advanced fibro-fatty plaques (Stary type IV). One lesion was abundant in extra- and intracellular lipid and had a thin collagenous cap (short-dashed line); another lesion had a thicker collagenous cap with deposits of extracellular Oil-Red-O-positive material (long-dashed line). Note that the spectra of mixed fibro-fatty plaques have features in common with spectra from histologically pure fatty and pure fibrous lesions.

Oxidation of β-VLDL produces a broadening of the spectrum, which is less marked than that observed with oxidation of human LDL. Oxidation changes the FWHM value from 86±4 nm to 100±8 nm that broadens the fluorescence band-width, but only 14 nm. In contrast to the oxidation of human LDL, oxidative modification of rabbit β-VLDL shifts the fluorescence spectrum toward the blue with the appearance of new maxima at approximately 382±3 and 394±3 nm (Figure 6, short-dashed line).

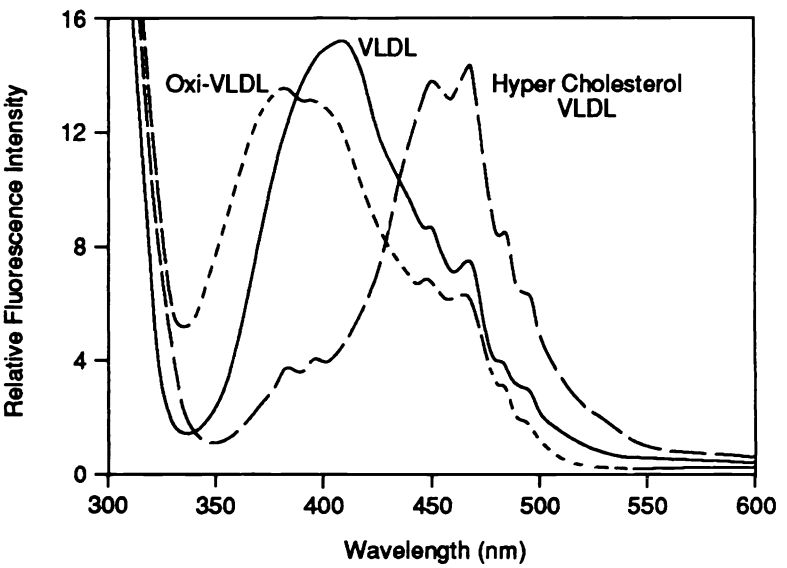

FIGURE 6.

Fluorescence profile of native (nonoxidized) VLDL isolated from a pool of normocholesterolemic rabbit plasma (continuous line). Fluorescence spectrum of β-VLDL isolated from a pool of hypercholesterolemic rabbit plasma (long-dashed line). Oxidative modification of β-VLDL produces a spectral shift towards the shorter wavelength range (short-dashed line).

Figure 7 (continuous line) depicts the average spectrum from the aortas of 6 rabbits without hypercholesterolemia. As opposed to the non-atherosclerotic human aorta with a well developed intimal layer abundant in glycosaminoglycans, the native rabbit intima is extremely thin with the internal elastic membrane in close apposition to the endothelial cell layer. The fluorescence spectrum has a band-width of 103±4 nm and shows a major fluorescence peak at 407±3 nm, a fluorescence signature characteristic of elastin (Fig. 4a)[9].

Figure 7 (dashed line) depicts the average spectrum from the thoracic aortas of 6 cholesterol-fed rabbits at the site of an advanced foam cell lesion. The spectrum exhibits a major peak at 382±2 nm and lesser peaks at 394±2 nm, 450±4 and 468±4 nm, features found in the spectrum of oxidized β-VLDL (Figure 6, short-dashed line). The general profile of fluorescence spectra and FWHM values for lipid rich plaques in rabbits strongly resemble that of the oxidized β-VLDL. Spectra from rabbit aortas occupied by foam cell lesions were very reproducible and were seen in all rabbits both at the level of the aortic arch and descending thoracic aorta. The p-value of the significance of the difference in position of fluorescence maxima between lipid plaques and normal rabbit aorta walls was 0.0011.

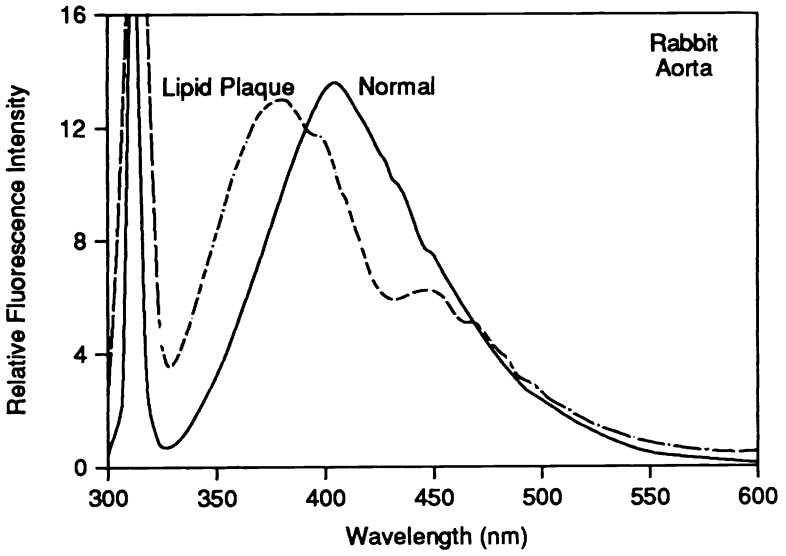

FIGURE 7.

Fluorescence spectra from the aortic surface of a normocholesterolemic rabbit (continuous line) and from that of a hypercholesterolemic rabbit at a site containing a multilayered foam cell lesion (short-dashed line). The spectrum from the diseased rabbit aorta has similarities with that from oxidized β-VLDL (Figure 6, short-dashed line).

4. DISCUSSION

The major finding of this study is that arteries undergoing early atherosclerotic changes exhibit an altered autofluorescence that may reflect the fluorescent properties of the lipoproteins accumulating in the tissue.

In the case of human atherosclerosis, lipoprotein retained in the arterial intima belongs predominantly to the low density fraction (LDL)[21,22], possibly because of its high binding affinity to intimal glycosaminoglycans[30]. Owing to its abundance in polyunsaturated cholesterol esters, LDL is particularly susceptible to oxidation[19]. It is of interest that several of the features observed in the spectra of oxidatively modified LDL, such as position of maximum, fluorescence band-width, and nonsymmetrical complex profile are also seen in the spectra from pure fatty lesions in human aorta.

The opposite oxidation-shifts of the fluorescence spectra of human LDL and rabbit β-VLDL is an interesting finding that may be explained on the basis of the different compositions of these two lipoproteins. Compared with LDL, β-VLDL is much less abundant in polyunsaturated cholesterol esters and it contains apoproteins other than apo-B (apo-E and apo-C's). These differences may account for disparate oxidative modifications and may explain the opposite shifts.

The similarities of the fluorescence spectra for oxidized lipoproteins in solution and lipid-rich plaques in both rabbits and humans support the view that oxidized lipoproteins are important determinants of the fluorescence of lipid-rich atherosclerotic plaques [20].

The fluorescence properties of LDL have been ascribed to the tryptophan residues of apoprotein B_{100} [19,31-33]. Oxidative modification of LDL is known to alter amino acid residues in apo B_{100}. One important change involves lysine residues which undergo covalent linkage with lipid peroxidation products[21,32,33]. It has been suggested that peroxidation-related changes in LDL autofluorescence reflect 4-hydroxynonenal-amino acid condensation products[32]. It is important to note, however, that the abundance and state of oxidation of polyunsaturated fats might by themselves contribute to changes in lipoprotein fluorescence [32,34,35].

Whereas the autofluorescence of early lipid-rich lesions in humans and rabbits may reflect the fluorescence properties of the dominant lipoprotein accumulated in tissue, the autofluorescence of arteries containing no superficial lipid deposits may express mainly the fluorescence of matrix proteins directly underlying endothelial surfaces. In non-atherosclerotic arteries with a thin intima, the fluorescence may reflect the abundance of elastin, whereas in arteries with advanced lesions, collagen may become the dominant determinant [9].

The fluorescence spectrum of normal human aorta is not identical to that of elastin, although it displays some important features of elastin fluorescence such as the long broad shoulder in the range from 450 nm to 550 nm . Although elastin contributes to the fluorescence of normal aorta, other fluorophores in the well developed human aortic intima may be important. In contrast, in the rabbit aorta where the intima is very thin with the internal elastic membrane located directly under the endothelial layer, the fluorescence spectrum is similar to that of elastin (compare continuous line profiles at Fig. 4b and Fig.7).

In the normal human aorta, the position of the fluorescence maximum differs from that of pure elastin (370 nm vs 407 nm), which probably reflects other tryptophan-containing matrix proteins in the subendothelial space. The similarity between fluorescence spectra of normal aorta and elastin reported by Deckelbaum et al.[9] may have resulted from the use of a longer excitation wavelength (325 nm vs 308 nm in this study). The longer effective penetration depth of He-Cd laser radiation may lead to the induction of fluorescence from deeper structures including the inner elastic membrane.

Overall, our findings confirm earlier reports that fluorescence spectroscopy may be a useful tool to characterize the composition of atherosclerotic lesions [1-13]. Fluorescence profiles of normal aorta wall and various plaques excited by the laser pulse at 308 nm differ significantly. Because of its ability to effectively excite numerous tissue fluorophores, the XeCl laser radiation at 308 nm may be particularly useful for diagnostic applications. Angioscopic detection of arterial tissue containing peroxidized lipoproteins might prove important, since they may signal arteries at risk for the development of advanced lesions. Lesions with dense collagen may have a limited potential for true regression (absolute rarefaction of collagen) [36]. Therefore, identification of atherosclerosis before dense collagenization has occurred may be important for the prevention of advanced disease and its inevitable complications. According to current concepts, atherosclerotic plaques at risk for thrombotic complications may contain abundant foam cells [37]. Therefore, the occurrence in close proximity of fluorescence spectra indicative of fibrous and lipid-rich loci might help in the detection of advanced plaques with inflammatory cellular components, lesion characteristics thought to be risk-factors for rupture and trombosis.

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