Fluorescence spectroscopy for identification of atherosclerotic tissue

F Bosshart, U Utzinger, O M Hess, J Wyser, A Mueller, J Schneider, P Niederer, M Anliker, and H P Krayenbuehl

Objective: Vessel perforation and limited steerability of the laser light are the major limitations of laser angioplasty. To improve steerability fluoresence spectroscopy has been proposed for identification of atherosclerotic plaques. The aim was to investigate this. **Methods:** Fluorescence spectroscopy with three different excitation wavelengths (325 nm, 380 nm, 450 nm) was tested in an emission range of 400 nm to 600 nm. Intensity ratios at 480/420 nm were determined in different types of blood vessels. Necropsy material from 40 patients (punch biopsies of 4 mm diameter from the coronary and carotid artery as well as from the ascending and descending aorta) was studied spectroscopically. Histological alterations of the vessel wall were assessed by a semiquantitative score (0 to 10 points): (a) normal tissue, 0 to 2 points (mean=0.25; n=38); (b) mild atherosclerotic lesions, 3 to 5 points (mean=3.35; n=39); (c) severe atherosclerotic lesions, $\geq$ 6 points (mean=6.75; n=43). **Results:** Best spectroscopic results were obtained with an excitation wavelength of 325 nm. In samples with severe atherosclerotic lesions the fluoresence spectra showed a significant reduction of the emitted wavelength intensities when compared to normal tissue. There was a clear separation of the fluorescence spectra between normal and mild as well as between normal and severe atherosclerotic lesions; normal tissue showed an increased intensity in the range from 420 nm to 540 nm, whereas atherosclerotic lesions had no or only a small peak at 480 nm. There was a significant correlation between the semiquantitative score (n=120) and the fluorescence ratio at 480/420 nm (excitation wavelength 325 nm) with a correlation coefficient of 0.87. The spectroscopic results showed no differences between the samples taken from different types of vessels. **Conclusions:** Fluorescence spectroscopy allows a reliable identification of normal and atherosclerotic lesions. The close correlation between the emitted light intensity ratio at 480/420 nm and the histological alterations of the vessel wall suggests a relationship between vessel wall fluorescence and the atherosclerotic alterations of the wall.

The basic problem of laser angioplasty remains vessel perforation due to limited steerability of the laser light. Several approaches have been used to reduce the risk of vessel perforation such as the hot tip laser probe with a metallic cap at the distal end of the laser and a sapphire tipped laser probe to focus the laser beam at a certain distance. Vessel perforation still occurred with the hot tip and sapphire tip although the risk was greatly reduced. More recently coronary angioscopy and fluorescence spectroscopy have been proposed for identification of atherosclerotic plaques and stenotic lesions.[1-7] However, angioscopy is limited by the fact that (1) the instrument can be used only for visualisation and not for treatment, and (2) the blood has to be replaced for short intervals by saline solution. Fluorescence spectroscopy has the advantages that the laser system can be used for detection and treatment, and that specific ablation of atherosclerotic plaques can potentially be performed without injury to the normal vessel. Previous studies have shown that fluorescence is reduced in the presence of atherosclerotic tissue.[2,3] Gaffney *et al*[4] and Deckelbaum *et al*[5] have found a direct relationship between plaque thickness and laser induced fluorescence.

Thus the purpose of the present study was (1) to assess the characteristics of fluorescence spectroscopy for identification of normal and atherosclerotic human tissue and (2) to make a direct comparison of three different excitation wavelengths; (3) to compare tissue taken from different anatomical sites; and (4) to relate the severity of atheromatous change semiquantitatively to the degree of alteration in the fluorescence spectrogram.

Methods

Necropsy material from 40 patients (mean age 57.8 years, range 16-87; fig 1) was obtained from the left anterior descending coronary artery, the ascending and descending aorta, and the carotid artery. Tissue samples of 5 × 10 mm were obtained from the coronary and the carotid artery and samples of 20 × 40 mm from the ascending and descending aorta. The specimens were removed 6 to 8 h after death and were stored at −24°C until spectroscopy was performed. One to four punch biopsies of 4 mm diameter were taken from each tissue sample and the punch biopsies were spectroscopically analysed. Following spectroscopic

Department of Internal Medicine, Medical Policlinic, Cardiology, University Hospital, 8091 Zurich, Switzerland: O M Hess, H P Krayenbuehl; Institute of Biomedical Engineering and Medical Informatics, Swiss Federal Institute of Technology, Zurich, Switzerland: F Bosshart, U Utzinger, J Wyser, P Niederer, M Anliker; Institute of Pathology: A Mueller, J Schneider. Correspondence to Professor Hess.

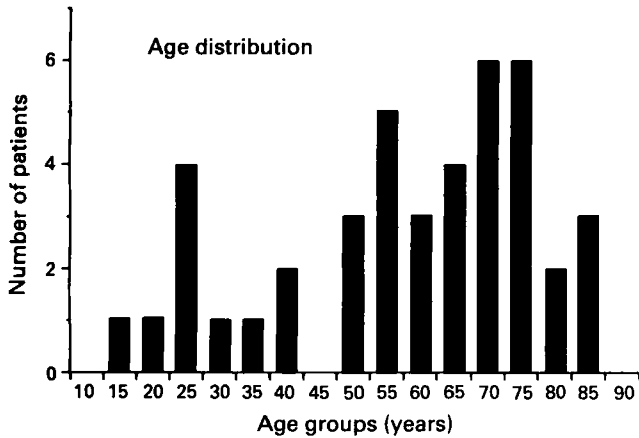

Figure 1 *Age distribution of the 40 patients who are included in the present study.*

evaluation, punch biopsies were fixed in buffered formaldehyde (4%) at room temperature for histological examination (fig 2). The effect of freezing on the emission spectrum was tested in 10 biopsy samples; spectroscopic evaluation was performed before and after freezing and identical wavelength spectra were obtained.

Spectroscopic analysis

Excitation wavelengths were selected in the ultraviolet (325 nm), the lower visible (380 nm) to the blue/green range (450 nm). A computer controlled monochromator was used to generate the wavelengths needed. The fluorescence inducing light beam was power controlled between 0 and 1 mW. A beamsplitter enabled transmission of part of the exciting light to a reference channel for regulation of the optical output. The incident light beam was focused and was projected at a constant angle of 45° on the intimal surface of the tissue sample which was suspended in saline solution at room temperature. The effect of the projection angle of the incident light beam was evaluated and no variations were found in the range from +20° to −20° except that a proportional decrease in the emitted light intensity was observed over the whole spectral range. The emitted light was focused by an achromatic lens and projected onto a slit of a spectrograph. No optical fibres were used for light transmission. A shutter was positioned in the optical path to limit irradiation of the tissue sample to the time period of fluorescence sampling. The emitted light was spectrally dispersed (grating 1200 groves·mm^{-1}, blaze 450 nm) and imaged on a photomultiplier system. The fluorescence

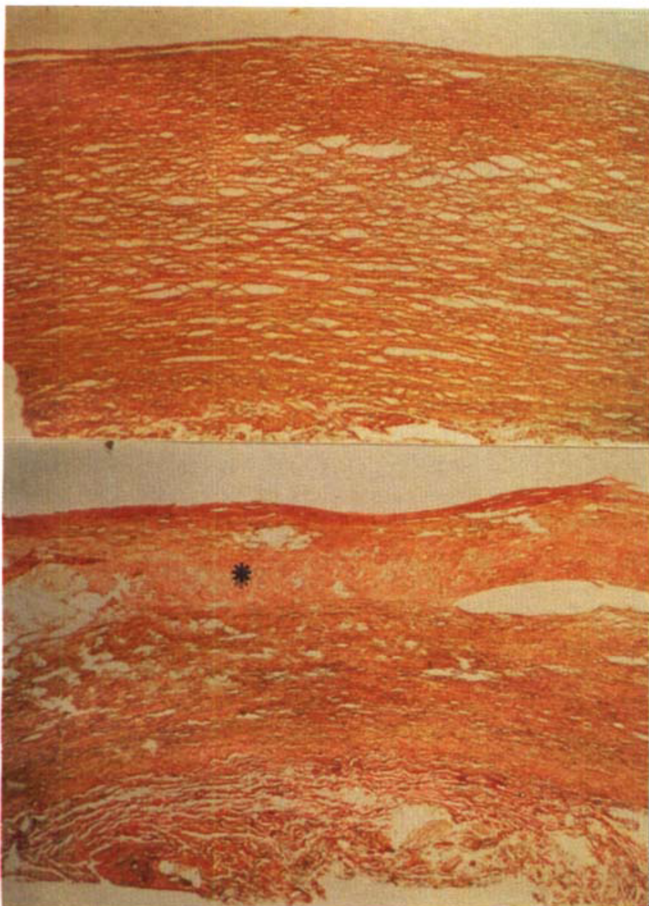

Figure 2 ***Photomicrographs of a punch biopsy from a normal (upper panel) and atherosclerotic vessel (lower panel). The normal vessel shows mild intimal thickening, whereas the atherosclerotic tissue sample shows a thickened intima (asterisk) with an increased amount of collagen and deposition of cholesterol crystals.***

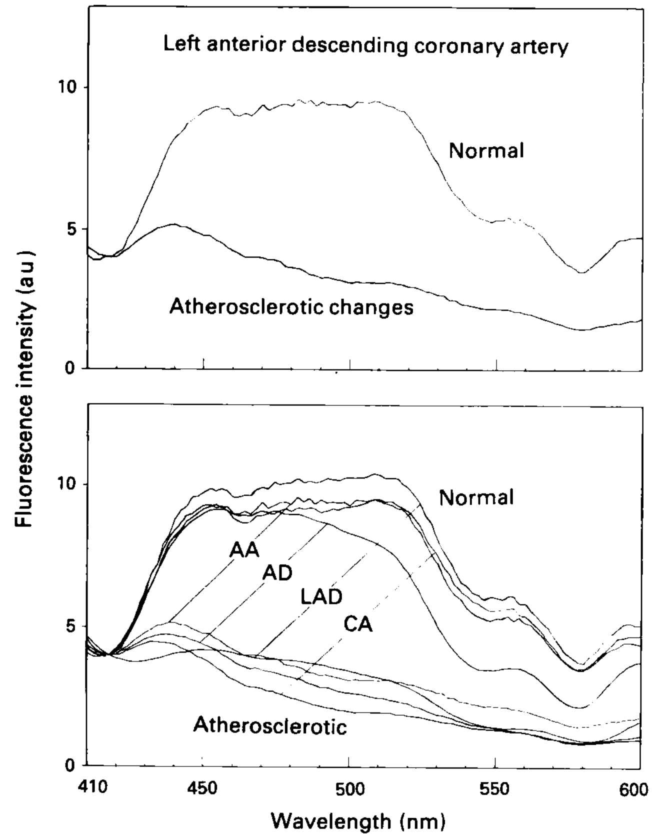

Figure 3 *Typical fluorescence spectra (upper panel) of a normal and atherosclerotic left anterior descending coronary artery (excitation wavelength 325 nm). There is a flattening of the fluorescence spectrum of the atherosclerotic tissue sample in the range of 420 to 550 nm. Fluorescence spectra (lower panel) of the ascending aorta (AA), descending aorta (AD), left anterior descending coronary artery (LAD), and carotid artery (CA) from a normal (upper four tracings) and an atherosclerotic vessel (lower four tracings). The four different vessel types show no major differences in the fluorescence spectrum in the presence of normal or pathological vessel structure. There are some differences in the spectrum between 490 and 600 nm of the normal descending aorta when compared to the other vessels but these differences were within the normal range (see fig 4). A reduced fluorescence intensity is observed between 420 and 550 nm for the four atherosclerotic vessels. The necropsy specimens were obtained from different patients. au=arbitrary units.*

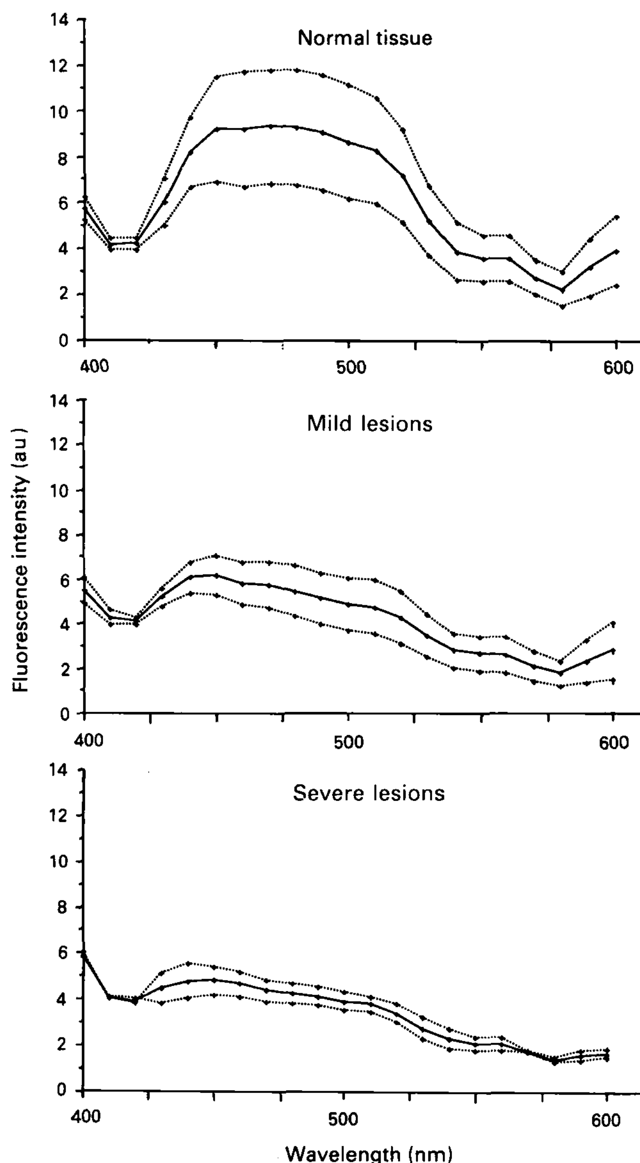

Figure 4 Averaged fluorescence spectra (excitation wavelength 325 nm) for 38 normal (upper panel; mean ± 1 SD), 39 vessels with mild lesions (middle panel), and 43 vessels with severe atherosclerotic lesions (lower panel). There is a progressive loss of the fluorescence intensity between 420 and 550 nm from the normal to the atherosclerotic tissue sample. For further explanations see text.

spectrum was analysed over a wavelength range from 300 to 600 nm (fig 3). The background noise was subtracted from each spectrum to correct for non-zero background and then digitised. Fluorescence intensity was measured in increments of 1 nm, the intensity in arbitrary units. For comparison purposes and to decrease variability all spectra were normalised by the intensity at 418 nm. This reference point was selected because all fluorescence spectra showed a common deflection point at 418 nm (fig 3). The collected data were displayed and stored on an IBM PC-AT for further computations.

Histological evaluation

After spectroscopic analysis of the tissue samples the punch biopsies were fixed and cut in 4 micron thick sections and were stained with haematoxylin-eosin, van Gieson elastin (fig 2), alcian blue, and periodic acid Schiff solution. Only

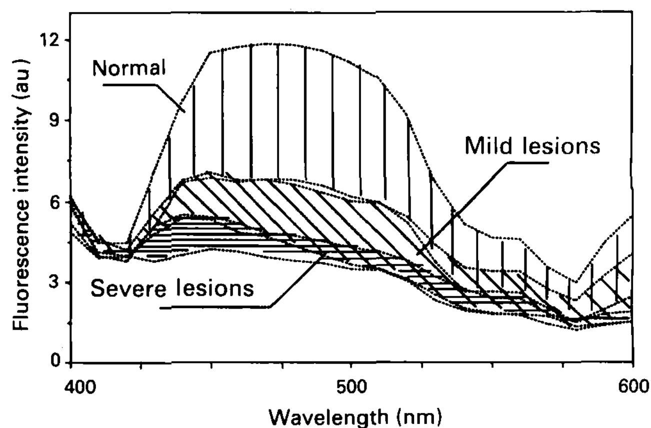

Figure 5 Overlay plot of the three averaged fluorescence spectra (excitation wavelength 325 nm) for normal tissue, mild lesions, and severely atherosclerotic lesions. Values are means and upper and lower limits (± 1 SD) of the fluorescence intensity. There is a clear separation between the normal tissue and mild lesions but some overlap exists for the mild lesions and the severely atherosclerotic lesions in the wavelength range ≥480 nm. au=arbitrary units.

sections from the centre of the biopsy were histologically evaluated. The following histological criteria were used for semiquantitative evaluation:

1 Intimal thickening (with collagen formation)
2 Foam cells
3 Cholesterol crystals
4 Calcification
5 Mucoid substances (intima and/or media).

These five criteria were used to assess the severity of the histological changes of each biopsy sample. The topographical extent of the five histological criteria was used as a measure of the severity of atherosclerotic alterations. For each criterion no changes were graded as 0 points, mild alterations as 1 point, and severe alterations as 2 points. Mild to severe alterations were graded as 1.5 points. All points of the five evaluated criteria were added (= semiquantitative score) such that a maximum of 10 points (5 × 2) could be achieved per biopsy. An average score of zero to 2 points was considered to be *normal*, a score of 3 to 5 points as mild (= *mild atherosclerotic lesions*), and a score of 6 to 10 points as severe histological alterations (= *severe atherosclerotic lesions*). The evaluation was carried out blinded by the same pathologist who was unaware of the spectroscopic result. Intraobserver variability was tested in 10 biopsy samples of 10 different patients; the mean difference of the semiquantitative score between the two measurements was 0.9 and the standard deviation of the mean difference 1.3 (mean score: 4.3 points).

Statistics

The fluorescence spectra of the normal tissue (n=38) and of the mild (n=39) and severe atherosclerotic lesions (n=43) were averaged over the whole emitted wavelength range and a mean curve ± 1 standard deviation (SD) was calculated for the three groups (figs 4 and 5). The above calculations were performed for the data from excitation wavelengths 325 nm and 380 nm. The data from excitation wavelength 450 nm are not included due to large scatter. The intensity of the fluorescence spectrum was measured at 480 nm and 420 nm (excitation wavelength 325 nm and 380 nm, respectively) and the intensity ratio of 480/420 nm was calculated for each

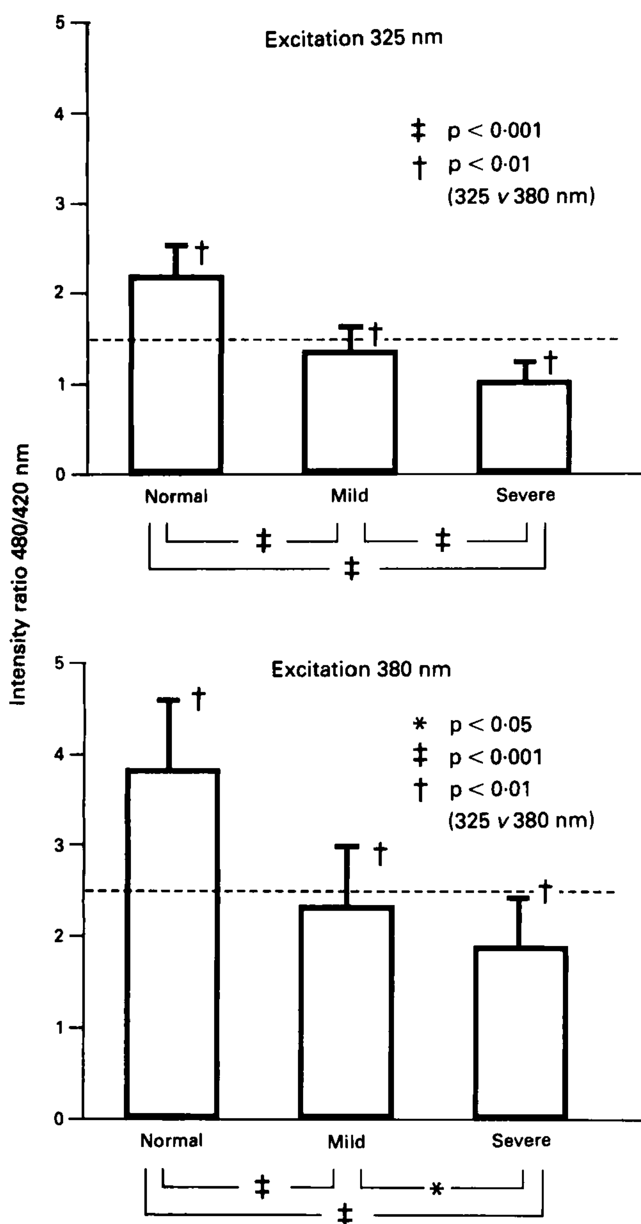

Figure 6 *Intensity ratios at 480/420 nm for normal tissue and for tissue with mild and severe atherosclerotic lesions, with an excitation wavelength of 325 nm (upper panel) and 380 nm (lower panel). Columns are means, bars=SD. The highest ratios were observed for normal tissue and the lowest ones for severely atherosclerotic tissue. The ratios were significantly higher for all tissues using an excitation wavelength of 380 nm rather than 325 nm but standard deviation, and thus scatter, was larger for the higher excitation wavelength. Dashed lines=lower limit of normality.*

biopsy sample. These ratios were plotted against the semiquantitative histological score using a semilogarithmic regression analysis. The coefficient of variation (standard deviation divided by mean value) was calculated for the intensity ratio at 480 nm divided by 420 nm using the excitation wavelength at 325 and 380 nm. The intensity ratios for normal tissue and for mildly and severely atherosclerotic tissue (excitation wavelengths 325 and 380 nm) were compared by a one way analysis of variance; when the analysis was significant, the Scheffé procedure was applied. A p value less than 0.05 was considered to be significant.

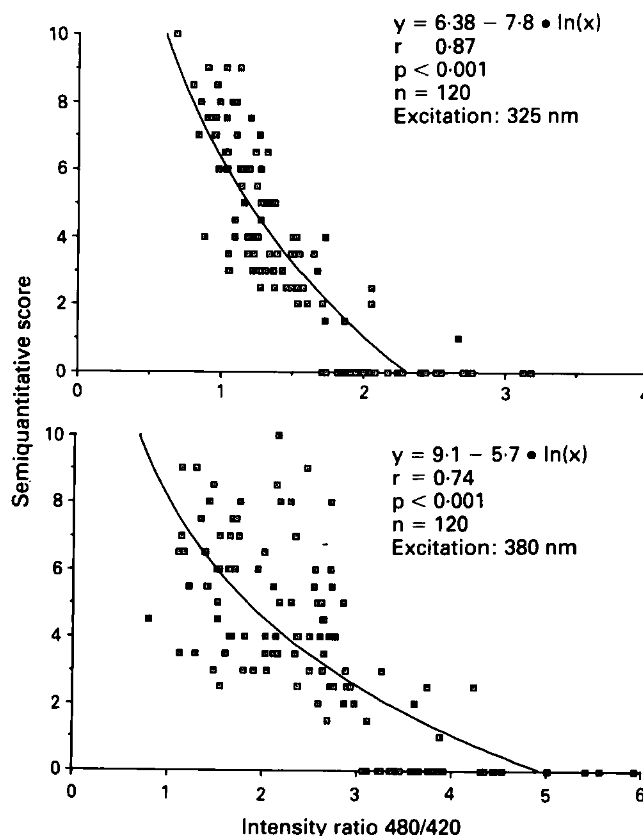

Figure 7 *Semilogarithmic plots of the semiquantitative histological score (ordinate) and the fluorescence intensity ratio at 480/420 nm for an excitation with 325 nm (upper panel) and 380 nm (lower panel). Normal tissue (semiquantitative score ≤2) showed an intensity ratio at 480/420 nm which is equal or larger than 1.5 for an excitation wavelength of 325 nm and equal or larger than 2.5 for an excitation wavelength of 380 nm. r=correlation coefficient, p=probability, n=number of samples.*

Results

A typical biopsy sample of the ascending aorta in a patient with normal arteries and in another with atherosclerotic alterations is shown in fig 2. A characteristic fluorescence spectrum of the left anterior descending coronary artery from a normal and a pathological vessel (top panel) and from the ascending and descending aorta, the left anterior descending coronary artery, and the carotid artery of patients with normal arteries and patients with atherosclerotic lesions (bottom panel) are presented in fig 3.

Fluorescence spectroscopy

Fluorescence spectra from excitation wavelengths of 325 nm, 380 nm, and 450 nm were obtained from all samples. Since fluorescence spectra from 450 nm excitation showed much scatter with no clear separation between normal and atherosclerotic tissue, these spectra were not quantitatively evaluated and thus these data are not included in the present analysis. Individual and mean data (with standard deviations) for excitation wavelengths of 325 nm and 380 nm are shown in fig 6. The ratio of the fluorescence intensity at 480 nm divided by that at 420 nm showed higher values for the longer excitation wavelength but also more scatter with a larger coefficient of variation (CV=0.25 for 380 nm; CV=0.18 for 325 nm, $p<0.01$). Therefore the averaged fluorescence spectra from the excitation wavelength of 325 nm are depicted for normal tissue, tissue with mild lesions,

and tissue with severely atherosclerotic lesions (mean values with standard deviations) in figs 4 and 5. There is a separation between normal and atherosclerotic tissue although there is some overlap between the spectra of the mildly and severely atherosclerotic lesions in the range of 480 nm to 600 nm, whereas the two spectra are separated between 420 nm and 480 nm (fig 5).

Intensity ratios for excitation wavelength of 325 and 380 nm – Using 325 nm excitation wavelength, the fluorescence intensity of normal tissue (fig 3) was high at 480 nm and low at 420 nm, the ratio (480 nm fluorescence intensity divided by 420 nm fluorescence intensity) having a mean value of 2.16 with a lower limit of 1.5. Using 380 nm excitation wavelength on normal tissue, the 480/420 nm fluorescence intensity ratio had a mean value of 3.81 with a lower limit of 2.5. Mildly and severly atheromatous tissue had lower 480/420 nm fluorescence intensity ratios than normal tissue, their mean values being 1.36 and 1.09 respectively using 325 nm excitation wavelength, and 2.31 and 1.88 respectively using 380 nm excitation wavelength. The mean values of normal, mildly and severely atheromatous tissue differed significantly (fig 6).

Semiquantitative histological score – The mean score amounted to 0.25(SD 0.5) for nomal vessels, 3.35(0.6) for mild atherosclerotic lesions, and 6.75(1.5) for severely atherosclerotic lesions.

Correlations between intensity ratios and semiquantitative histological score – The correlation coefficient between the natural logarithm of the intensity ratio at 480/420 nm (excitation wavelength 325 nm) and the semiquantitative score was 0.87 (fig 7). A similar but slightly less close correlation (r=0.82) was found between the natural logarithm of the intensity ratio of 480/420 nm (excitation wavelength 380 nm) and the semiquantitative score (fig 7).

Discussion

Fluorescence spectroscopy has been used for characterisation of normal and atherosclerotic tissue under in vitro conditions by a number of researchers.[1,2,4,7-12] These investigators have used necropsy material and excitation wavelengths of 248 nm,[3] 308 nm,[6] 325 nm,[10] and 458 nm.[4] The intensity of fluorescence of wavelength 400 to 500 nm emitted by atheromatous arterial wall in response to laser excitation has been shown to be much less than that of emitted by normal arterial wall.[4,5,10] Sartori and coworkers[2] reported an increased light intensity from calcified but reduced intensity from fatty atherosclerotic tissue when excited with an argon laser (wavelength 458 nm). It was postulated that laser light induced fluorescence is due to fluorophores that originate in deep arterial wall structures and emit a broad unstructured spectrum between 500 and 600 nm. More recent studies have shown, however, that the emitted fluorescence is reduced in atherosclerotic tissue, probably due to an absorption of the emitted light from deeper wall structures by the overlying atherosclerotic tissue.[4-6,10,11] Furthermore, Gaffney and coworkers[4] have shown a close correlation between different intensity ratios of the emitted light and intimal wall thickness in human atherosclerotic coronary arteries. Deckelbaum and coworkers[5] also observed a close correlation between plaque thickness and laser induced fluorescence. Neither study further specified the severity of histological alterations or differences in spectral analysis from different vessels and tissues. Typically, human atherosclerotic plaques consist of multiple structures such as collagen, elastin, lipids, cholesterol crystals, foam cells, etc. Which structure is responsible for the fluorescence or absorption of the emitted fluorescence is not clear; recently, Laifer and coworkers[8] described a direct relation between the shape and intensities of the fluorescence spectra and different substances. According to these investigators[8] the emitted light from the tissue layers is thought to originate from the media and to be dependent on the collagen/elastin ratio. In the presence of atherosclerotic plaques the collagen/elastin ratio is increased and thus the fluorescence intensity is reduced.

The purpose of the present study was to compare the emitted fluorescence spectra from human necropsy specimens with a semiquantitative histological score, to evaluate the selectivity of different excitation wavelengths for detection of normal and atherosclerotic tissue, and to investigate the influence of the type of vessel (elastic or muscular) on the fluorescence spectra.

Fluorescence spectra from normal tissue and from mildly and severely atherosclerotic tissue

Spectroscopic evaluation of human vessel tissue showed a strong fluorescence in the range of 420 to 520 nm after an excitation with monochromatic light of 325 nm. The fluorescence spectrum is similar for normal tissue of the ascending and descending aorta as well as the coronary and carotid artery (fig 3). A decreased fluorescence was observed in tissue samples with mild or severe atherosclerotic lesions. There was a direct relationship between the extent of the atherosclerotic changes and the reduction in the emitted light intensity (fig 4). Both the absolute fluorescence intensities and the 480/420 nm fluorescence intensity ratios in atheromatous tissue are less than those in normal tissue, the intensity ratios of the majority of atheromatous samples falling below the lower limit of normality – 1.5 for 325 nm excitation, and 2.5 for 380 nm excitation (fig 6). At both excitation wavelengths there was a correlation between the fluorescence intensity ratio and the semiquantitative score (fig 7) although less scatter was observed for the excitation wavelength at 325 nm (correlation coefficient 0.87) than 380 nm (correlation coefficient 0.74). The intensity ratio approaches 1 in the presence of severe atherosclerotic alterations (fig 4) indicating that the greater the atherosclerotic change the flatter becomes the fluorescence spectrum, whereas the normal spectrum shows the highest intensity values between 420 and 540 nm (fig 4). Interestingly, the spectra of the four different vessels (ascending and descending aorta, carotid artery, and coronary artery) showed no qualitative differences, although the aorta and the carotid artery are of the elastic type and the coronary artery of the muscular type (fig 3). Age had no direct influence on the fluorescence spectra and no correlation was observed between age and the fluorescence intensities at 480/420 nm of normal and atherosclerotic tissue.

Future implications

The present analysis confirms a relationship between degree of atheroma and fluorescence spectroscopy. A clear difference in the emitted fluorescence spectrum was observed between normal and abnormal vessels (mild lesions and severely atherosclerotic lesions) indicating that reliable detection of atherosclerotic plaques by fluorescence spectroscopy is possible. An intensity ratio of ≤ 1.5 for an excitation with 325 nm monochromatic light and a ratio of ≤ 2.5 for an excitation with 380 nm is clearly abnormal and can serve as a threshold criterion for detection of atherosclerotic tissue by fluorescence spectroscopy. It has to

be realised, however, that in vivo measurements are more critical than in vitro measurements due to catheter movement (aiming) and influences of blood (absorption of light). Some of these problems might be overcome either by a direct contact of the laser probe and the vessel wall (no catheter movement and no absorption of light by blood) or by flushing the segment under observation with saline. The influence of saline has been evaluated in the present analysis and has been found to have no effect on the emitted fluorescence spectra.

Nevertheless, further in vivo studies are certainly needed before a clinical application of fluorescence spectroscopy for on line tissue recognition is feasible. Geschwind and coworkers[13] have reported preliminary data on the success of spectroscopy guided laser angioplasty in the peripheral arteries of 19 patients. They concluded that the safety of the procedure can be improved by the spectroscopic guidance but the unexpected high frequency of mechanical vessel damage by the relatively stiff catheter appeared to limit the applicability of this system.

Received 25 June 1990; accepted 15 January 1992

We are very grateful for the invaluable help of Dr U Zollinger from the Institute of Forensic Medicine of the University of Zürich for the supply of tissue samples from young patients. The study was supported by a grant from the Swiss National Science Foundation.

Key terms: laser; fluorescence spectroscopy; histological score; atherosclerosis; excitation wavelength; monochromatic light; coronary angioplasty

1 Clarke RH, Isner JM, Gauthier T, *et al.* Spectroscopic characterization of cardiovascular tissue. *Lasers Surg Med* 1988;**8**:45–59.
2 Sartori M, Weilbaecher D, Valderrama GL, *et al.* Laser-induced autofluorescence of human arteries. *Circ Res* 1988;**63**:1053–9.
3 Laufer G, Wollenek G, Hohla K, *et al.* Excimer laser-induced simultaneous ablation and spectral identification of normal and atherosclerotic arterial tissue layers. *Circulation* 1988;**78**:1031–9.
4 Gaffney EJ, Clarke R, Lucas A, Isner J. Correlation of fluorescence emission with the plaque content and intimal thickness of atherosclerotic coronary arteries. *Lasers Surg Med* 1989;**9**:215–28.
5 Deckelbaum L, Stetz M, O'Brien K, *et al.* Fluorescence spectroscopy guidance of laser ablation of atherosclerotic plaque. *Lasers Surg Med* 1989;**9**:205–14.
6 Laufer G, Wollenek G, Rüeckle B, *et al.* Characteristics of 308 nm excimer laser activated arterial tissue photoemission under ablative and non-ablative conditions. *Lasers Surg Med* 1989; **9**:556–71.
7 Sartori M, Sauerbrey R, Kubodera S, Tittel FK, Roberts R, Henry PD. Autofluorescence maps of atherosclerotic human arteries – a new technique in medical imaging. *IEEE J Quant Electron* 1987;**10**:1794–7.
8 Laifer LI, O'Brien KM, Stetz ML, Gindi GR, Garrand TJ, Deckelbaum LI. Biochemical basis for the difference between normal and atherosclerotic arterial fluorescence. *Circulation* 1989;**80**:1893–901.
9 Hoyt CC, Richards-Kortum RR, Costello B, *et al.* Remote biomedical spectroscopic imaging of human artery wall. *Lasers Surg Med* 1988;**8**:1–9.
10 Andersson PS, Montan S, Svanberg S. Multispectral system for medical fluorescence imaging. *IEEE J Quant Electron* 1987; **10**:1798–805.
11 Leon MB, Lu DY, Prevosti LG, *et al.* Human arterial surface fluorescence: atherosclerotic plaque identification and effects of laser atheroma ablation. *J Am Coll Cardiol* 1988;**12**:94–102.
12 Richards-Kortum R, Mehta A, Hayes G, *et al.* Spectral diagnosis of atherosclerosis using an optical fiber laser catheter. *Am Heart J* 1989;**118**:381–91.
13 Geschwind HJ, Dubois-Rande JL, Shafton E, Boussignac G, Wexman M. Percutaneous pulsed laser-assisted balloon angioplasty guided by spectroscopy. *Am Heart J* 1989;**117**: 1147–52.