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Published in:
Optical Methods for Tumor Treatment and Early Diagnosis: Mechanisms and Techniques

DOI:
10.1117/12.44046

1991

FLUORESCENCE CHARACTERISTICS OF ATHEROSCLEROTIC PLAQUE AND MALIGNANT TUMORS

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ABSTRACT

Two series of investigations utilizing laser-induced fluorescence (LIF) in characterizing diseased tissue are presented. In one *in vitro* investigation we studied the fluorescence from normal and atherosclerotically diseased arteries. In another, clinical, study we investigated the fluorescence *in vivo* from superficial urinary bladder malignancies in patients who had received a low-dose injection of Hematoporphyrin Derivative (HpD). Additionally, the fluorescence properties of L-tryptophan, collagen-I powder, elastin powder, nicotinamide adenine dinucleotide and β-carothene were investigated and compared with the spectra from the tissue samples. A nitrogen laser (337 nm) alone or in connection with a dye laser (405 nm) was used together with an optical multichannel analyzer (OMA) to study the fluorescence spectra. The fluorescence decay characteristics of atherosclerotic plaque were examined utilizing a mode locked argon ion laser, synchronously pumping a picosecond dye laser. A fast detection system based on photon counting was employed. The fluorescence decay curves were evaluated on a PC computer allowing up to three lifetime components to be determined. A fluorescence peak at 390 nm in fibrotic plaque was identified as due to collagen fibers, while a fluorescence peak at 520 nm was connected to β-carothene. The *in vivo* measurements of urinary bladder malignancies were performed with the optical fiber of the OMA system inserted through the biopsy channel of a cystoscope during the diagnostical procedure. The spectral recordings from urinary bladders, obtained at 337 nm and 405 nm excitation, revealed fluorescence features which can be used to demarcate tumor areas from normal mucosa. The fluorescence emission might also be useful to characterize different degrees of dysplasia.

0-8194-0516-7/91/$4.00

1. INTRODUCTION

Laser-induced fluorescence (LIF) as a method for probing human tissue has developed rapidly during recent years.[1-3] Sensitive techniques for detecting and localizing different diseases are very important. In many cases early diagnosis is of great importance for the prognosis, such as early discovery of endobronchial malignancies[4] and superficial urinary bladder malignant tumors.[5] For some types of human malignancies, known to be present because of the appearance of malignant cells in cytological tests, but not localized, no alternative diagnostic tool is available until the tumor has grown large enough to be visualized. Laser-induced fluorescence (LIF) as an additional diagnostic tool may improve the probability of earlier discovery. The method relies on the fact that different tissue types possess different fluorescence properties, thus enabling diseased areas to be discriminated from normal surrounding tissue.

When designing a diagnostical laser-based system intended for use in a clinical situation, there are several parameters that have to be considered. First of all a proper excitation wavelength has to be chosen depending on what chromophores in the tissue are of interest. For such a decision, a detailed knowledge of the fluorescence properties of the different chromophores is necessary. The absorption and emission profiles of molecules in tissue are rather broad and structureless. When excited at e.g. 337 nm the fluorescence maximum is at about 390 nm for tryptophan, 390 nm for collagen, 410 nm for elastin, 470 nm for nicotinamide adenine dinucleotide (NADH), 520 nm for β-carothene and 540 nm for melanin. In the fluorescence spectrum the contribution from various chromophores can be recognized, thus characterizing different tissue types. The fluorescence intensity at a few chosen wavelengths can also be used to characterize different tissue types.[6,7] Another aspect of the fluorescence is the excited state lifetime, that may vary for different chromophores.[8-12] By the use of a laser with a short pulse length, this feature can also be used for tissue diagnostics. Also the decay time of the polarization of the fluorescence shows some potential for tissue diagnostics.[13]

In some cases the optical properties of the endogenous chromophores are not sufficient to characterize different tissue types. In order to enhance the optical demarcation of malignant tumors it is possible to inject a tumor seeking drug. A clinically used drug is Hematoporphyrin Derivative (HpD)[14-19] with its characteristic double peaked fluorescence structure in the red region. As the drug is retained in malignant tissue the fluorescence is characterized by a dual peaked fluorescence in the red wavelength region, which is not seen in normal tissue. The intrinsic fluorescence from the tissue, the autofluorescence, on the other hand, has been shown to decrease in malignant tumors compared to normal tissue.[20] In the demarcation of malignant tumors it is advantageous to use both the HpD related fluorescence and the autofluorescence to enhance the tumor demarcation. The drug specific fluorescence and the autofluorescence can also be used to form a ratio which has the advantage to be dimensionless. Such a ratio will be independent of the distance to and the topography of the object and also to laser intensity variations, parameters that might be difficult to control. Furthermore, in

many cases the shape of the autofluorescence might contain information that can be used to increase the demarcation between tumor and the surrounding tissue.[20-23] This has been observed for many different tumor types using a nitrogen laser (337 nm) as an excitation source.

Apart from the diagnosis of malignant tumors, LIF may be incorporated for guiding the procedure of laser angioplasty,[24-28] a field in which a remarkable development has occurred during the last few years. Percutaneously performed laser angioplasty has opened up possibilities for easy and convenient treatment of narrowed or obstructed vessels also for patients in a poor general condition excluding them from major surgical procedures. Laser angioplasty utilizing the thermal effects of a Nd:YAG laser has successfully been used in large, peripheral arteries. Also, non-thermal excimer laser ablation has been used in peripheral arteries. Recently, the first reports on excimer laser ablation of coronary arteries have been published.[29-32] Reported complications utilizing laser angioplasty are dissection and perforation of the vessels. In cases of peripheral artery procedures this complication may not be severely damaging. However, in cases of coronary angioplasty such complications must be avoided. A guiding system for the laser action would improve the possibility to monitor the procedure inside the vessels and offer a better controlled laser ablation. The fluorescence characteristics from diseased and non-diseased areas of the vessel wall show remarkable spectral differences.

In this paper we report on two series of investigations in which we used LIF for tissue demarcation. In the first investigation we studied the *in vitro* fluorescence from human artery samples and showed that the autofluorescence is sufficient to demarcate normal vessel wall from atherosclerotically diseased tissue, and also that the *in vitro* fluorescence can be used to distingguish different tissue layers of atherosclerotic plaques and normal vessel wall. Furthermore, we have shown, that the *in vitro* fluorescence can be used to distingguish different stages of atherosclerosis. Thus, early fibrotic lesions can be distinguished from plaque regions with higher fat content and also from calcified plaque regions. The results are compared with the results from fluorescence recordings of a few pure substances and the origin of the different fluorescence bands are discussed. In the second trial we performed a clinical *in vivo* investigation on patients with different kinds of superficial bladder lesions including various degree of dysplasia and cancer *in situ*. The patients had been injected with a low dose of HpD.

2. MATERIAL AND METHODS

2.1. Fluorescence spectral recordings. As an excitation source a nitrogen laser (Laser Science VSL 337) was used alone or in conjunction with a dye laser, producing 3 ns pulses at 337 nm and 405 nm, respectively. The laser light was directed into a 600 μm fluorescence free quartz fiber, which was placed in contact with the object. The fluorescence light was collected by the same fiber and directed into an optical multichannel analyzer (EG&G PARC OMA III model 1460) equipped with an image-intensified 1024 element diode array detector. The overall spectral resolution of the system was about 10 nm. The system is described in detail in Ref. 23.

2.2. Fluorescence decay time recordings. A mode-locked Ar ion laser (Coherent Radiation CR 12) was used for synchronous pumping of a dye laser (Coherent Radiation CR 599) to obtain 6 ps pulses at 674 nm. The pulse train was frequency doubled to obtain a wavelength of 337 nm. The fluorescence was spectrally filtered with a 0.5 m monochromator (Bausch & Lomb) and detected with a microchannel plate photomultiplier (Hamamatsu R 1564 U). The trigger signal was obtained with a fast photo diode. The set-up includes a time-to-amplitude converter, fast amplifiers, constant fraction discriminators and a multichannel analyzer. The fluorescence decay curves were evaluated on a PC computer as composed of up to three lifetime components. A more detailed description is given in Ref. 15.

2.3. Pure substances. The fluorescence recordings of solutions and powders were performed in a quartz cuvette or an aluminum cup. The substances studied were: L-tryptophan (Sigma Lot 38F-0259), β-NADH (Sigma Lot 639 F6), β-carothene (Sigma type III Lot 78F-0317), bovine collagen-I powder (Sigma Lot 81451) and bovine elastin powder (Sigma Lot 68F-8180). Several other pure substances such as NAD^+, cholesterol and lecithin were also examined but found to exhibit low fluorescence. The excitation wavelength was 337 nm.

2.4. Atherosclerotic plaque. Fluorescence data were recorded from aortic samples collected from human autopsies within 24 h *post mortem*. The samples were rinsed from intraluminal blood and the inner vessel wall was exposed to the laser light (337 nm). The samples were cut at 45 degrees to the normal of the vessel wall to expose the different layers of the plaque. The samples were examined histopathologically for a precise description of the tissue composition.

2.5. Urinary bladder tumors. The patients were injected with Photofrin, a purified form of HpD (QLT, Vancouver, Canada) 48 hours prior to the investigations. Two drug doses, 0.35 and 0.5 mg/kg b.w., were used. Fluorescence spectra from various kinds of superficial lesions in the bladder wall were recorded, such as different kinds of dysplasia, carcinoma *in situ* and superficial bladder tumors. The investigations were performed in connection with cystoscopy with the optical fiber through the biopsy channel of the endoscope. Biopsy specimens of the investigated areas were taken for histopathological examination. Excitation at 337 nm or 405 nm were used.

3. RESULTS AND DISCUSSION

3.1. Atherosclerotic plaque. Fluorescence spectra were recorded in scans for various depths of the artery wall, starting from the endothelial layer of the vessel wall through the intimal layer, into the media and finally from the adventitia in the vessel wall. In Fig. 1 fluorescence spectra from a superficial fibrotic layer (a), a necrotic lipid-rich layer (b), from the non-diseased media (c) and from adventitia (d) underneath the plaque are shown. In the lower middle the spectrum of removed material from the lipidic part of the same plaque (e) and to the lower right the spectrum from the surface of a non-diseased artery wall (f) are presented. The fibrotic plaque differs from the normal wall through a much stronger fluorescence intensity at 390 nm. This is in agreement with previous studies by us and others, where the same difference was found between the surface of a plaque region and normal

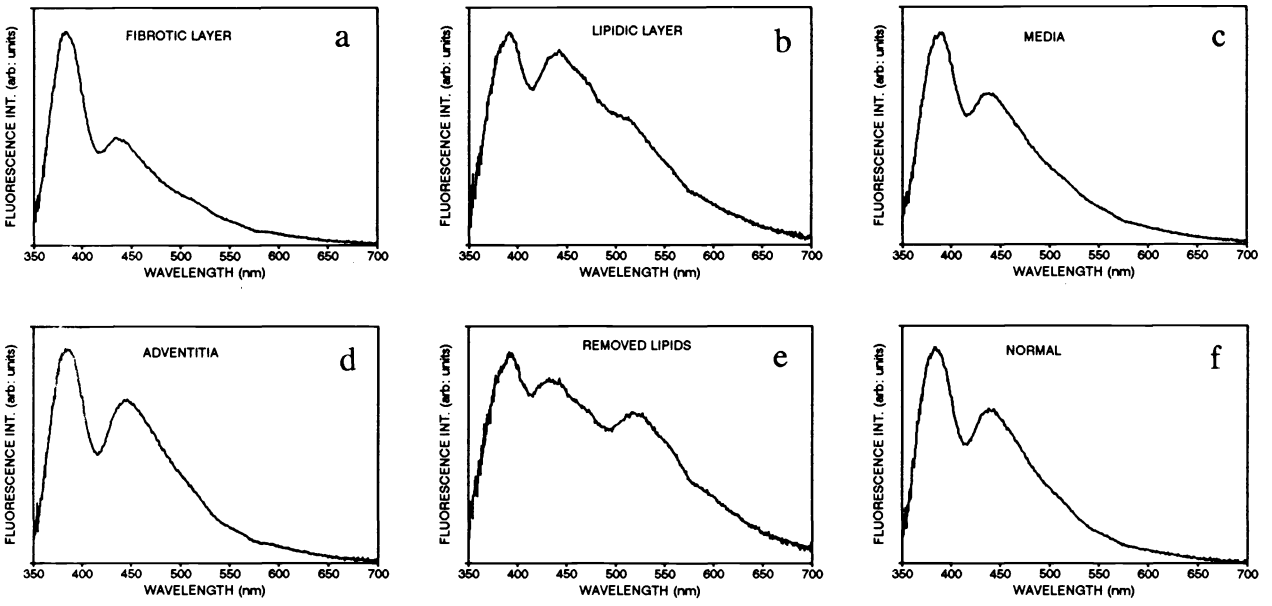

Fig. 1. Fluorescence spectra from the superficial fibrotic layer (a), lipid rich layer (b), the media (c) and the adventitia (d) of a plaque region. Also a spectrum from the lipid contents of the same fatty plaque is shown (e). Finally, a spectrum from the surface of a non-diseased artery wall in given (f). The excitation wavelength was 337 nm.

vessel wall.[27,33] The two spectra from lipid rich material (b and e), especially the one from removed lipid content (e), also exhibit special features. A fluorescence band is peaking at about 520 nm. This characteristic feature can be used to recognize lipidic regions of plaque. The spectral dip at 420 nm is due to the reabsorption by hemoglobin[34,35].

The origin of the different fluorescence bands is of importance for determination of a suitable plaque demarcation criterion to be used in a guiding system for percutaneous laser ablation. Hence, the fluorescence from some selected pure substances was studied. The results are presented in Fig. 2, where the fluorescence spectra from L-tryptophan, collagen-I powder, elastin powder, NADH and β-carothene are presented. The spectra are not plotted on the same scale and should be compared only regarding the spectral shape. As already pointed out above, there is a major difference between plaque and normal vessel wall at about 390 nm. In Fig. 2 we can find three possible candidates contributing to this peak namely tryptophan, collagen or elastin. Collagen is a very brightly fluorescing substance with its fluorescence maximum at 390 nm. Elastin has a very broad spectral width of the fluorescence with the peak at about 410 nm. It is likely that the fluorescence at 390 nm is a mixture of the spectral contribution from collage and elastin with higher collagen signal in the fibrotic plaque.[33] Also tryptophan may contribute to the signal at 390 nm.

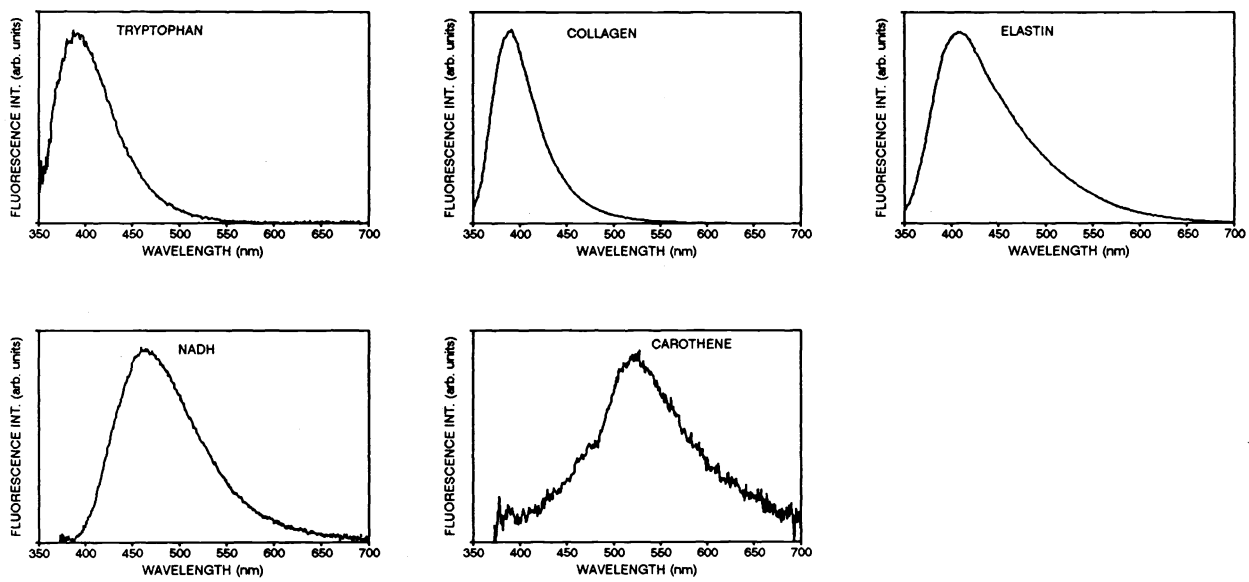

Fig. 2. Fluorescence spectra from L-tryptophan, collagen I powder, elastin powder, NADH and β-carothene. The excitation wavelength was 337 nm.

In the spectra shown in Fig. 1, a fluorescence peak at about 520 nm is seen in the lipidic part of the plaques, and even more prominently in the spectrum of the removed lipidic content. Comparing with Fig. 2, we find, that β-carothene has its fluorescence peak at 520 nm. Most probably β-carothene, which is known to be present in lipid-rich plaques, is responsible for the fluorescence peak at 520 nm. In Fig. 2 the fluorescence spectrum of NADH is also presented. NADH has been reported to be the major fluorophore in, e.g., muscle tissue under UV excitation.[36-39] Such a fluorescence band cannot be observed in the plaque spectra in Fig. 2.

In order to get more information from the fluorescence the decay time of the fluorescence was studied for the plaque samples as well as for the pure substances. Including this information in the fluorescence analysis, more reliable conclusions can be drawn. The results from the fluorescence decay time measurements are listed in Table I for various pure substances as well as for different plaque layers and normal artery wall. First of all, we find a major similarity in the data recorded at 380 nm for tissue samples of media and adventitia underneath plaque regions, superficial normal vessel wall and from pure elastin. This is in agreement with the results from the spectral investigation of the samples. Thus, it is very likely that the major fluorophore in the non-diseased vessel wall is elastin. It is very important for a plaque detection system to be used during laser angioplasty to be able to distinguish between plaque and normal vessel wall, but it is of equal importance that the laser ablation stop before entering the media and adventitia. In this context, it is interesting to note the similarity between

Substance/Sample	λ (nm)	A_1 (%)	A_2 (%)	A_3 (%)	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)
Tryptophan	380	27.7	72.3		2.78	1.47	
Collagen I	380	29.7	45.5	24.8	9.94	4.99	0.78
Elastin	380	9.2	36.1	54.8	7.83	2.65	0.50
Fibrotic layer	380	18.1	38.4	43.5	7.12	2.33	0.42
Lipidic layer	380	7.7	32.7	60.2	5.41	1.28	0.22
Media layer	380	11.0	36.9	52.1	5.91	1.77	0.35
Adventitia layer	380	12.0	37.9	50.0	5.81	1.62	0.28
Normal vessel wall	380	9.9	31.8	58.3	6.03	1.74	0.22
Elastin	520	41.5	58.5		6.73	1.43	
NADH	520	22.8	77.2		0.58	0.24	
β-Carothene	520	6.6	27.8	65.5	9.65	1.96	0.35
Fibrotic layer	520	12.9	37.2	49.9	9.15	2.87	0.51
Lipidic layer	520	7.3	31.1	61.7	9.72	2.21	0.37
Media layer	520	13.8	40.4	45.8	8.74	2.76	0.48
Adventitia layer	520	14.2	38.6	47.2	8.08	2.33	0.35

Table I. Fluorescence decay data for L-tryptophan, collagen I powder, elastin powder, β-carothene and NADH. The excitation wavelength was 337 nm. The data listed are wavelength (ns), fluorescence decay amplitudes (%) and fluorescence decay times (ns) according to $I(t) = \sum_k A_k \exp(-t/\tau_k)$

the normal artery and, on the other hand, media and adventitia. The fluorescence decay times for fibrotic plaque at 380 nm are longer than those for non-diseased tissue. Furthermore, the amplitude of the slow decay component is stronger than for e.g. normal vessel wall, resulting in a slower overall fluorescence decay for the fibrotic plaque layer. The amplitude of the slow decay component is also very high for pure collagen, higher than that for elastin. The fluorescence characteristics with a prominent fluorescence band at 390 nm and a slow fluorescence decay, are in agreement with the biological composition of fibrotic plaque tissue with a high content of collagen fibers.

As already mentioned above, the fluorescence spectrum of tryptophan is quite similar to that of collagen for 337 nm excitation. However, it is clear from Table I that the fluorescence decay time is much shorter for tryptophan than for collagen and elastin. Thus, tryptophan may not contribute to the fluorescence for the excitation wavelength used. Table I also reveals a

slightly lower amplitude for the slow decay component for lipidic plaque compared with the other tissue samples. The same can be observed for β-carothene solution at 520 nm. It is possible that β-carothene is partly responsible for the faster fluorescence decay of lipidic plaque. Of interest is also the very fast fluorescence decay of NADH, which supports our conclusion that NADH is not of importance for the plaque fluorescence.

3.2. Urinary bladder tumor lesions. The evaluated data from a scan across three flat bladder tumors with normal surrounding mucosa in between are shown in Fig. 3. The patient was injected with 0.5 mg/kg b.w. Photofrin. The

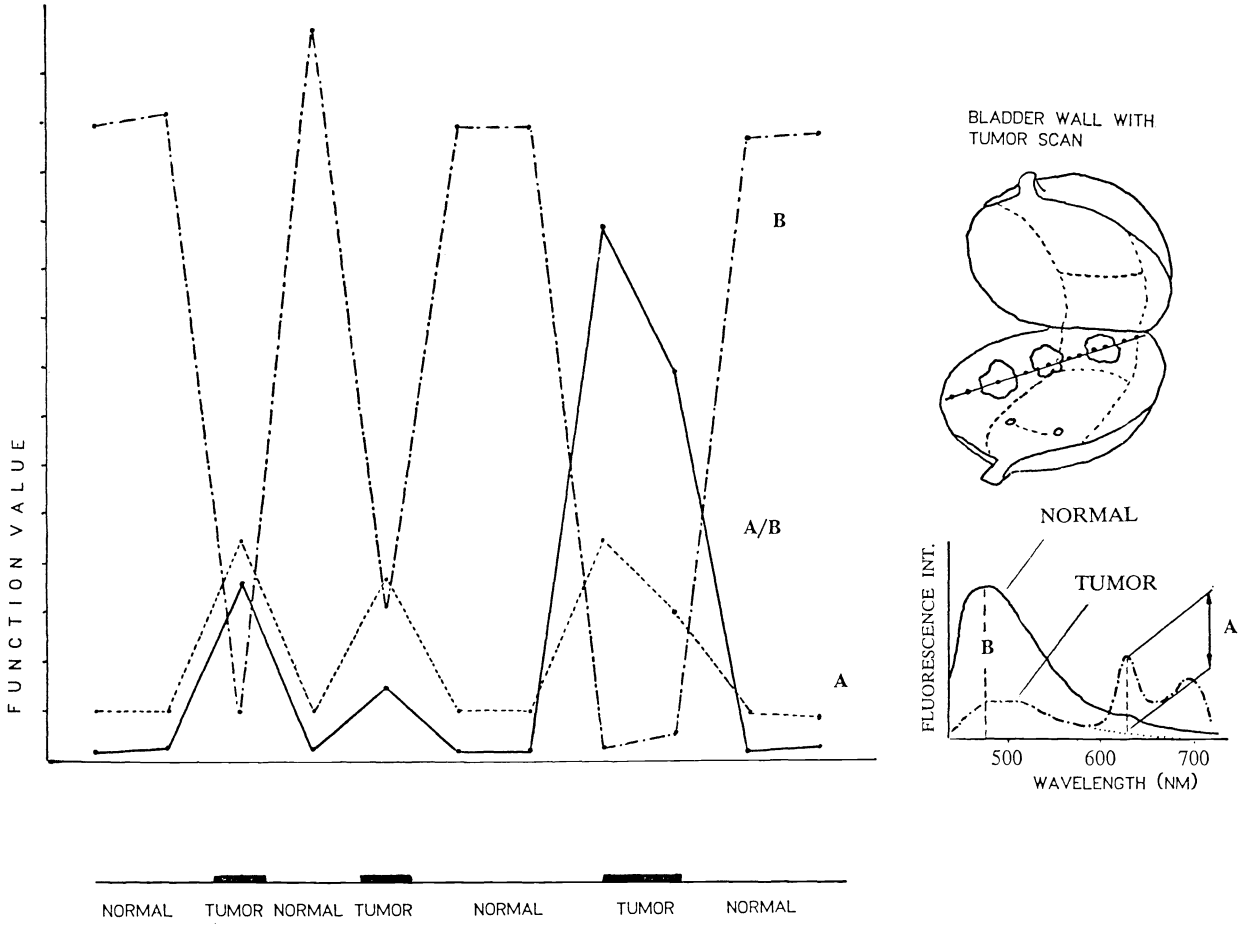

Fig. 3. Fluorescence data obtained in a scan across three malignant superficial bladder tumors with healthy mucosa surrounding the lesions. The fluorescence investigations were performed during cystoscopy in connection with diagnostical procedures. The fiber probe was placed through one of the biopsy channels of the endoscope. The patient had been injected with 0.35 mg/kg b.w. Photofrin 48 hours prior to the investigation. The location of the bladder tumors as well as two typical fluorescence spectra of tumor and normal mucosa are shown to the right. (From Ref. 38).

excitation wavelength was 405 nm. Two typical fluorescence spectra from tumor and normal mucosa are also shown. The autofluorescence signal at about 470 nm is denoted B, while the drug specific fluorescence at 630 nm is marked with an A. As seen in the figure there is a clear sign of the drug related fluorescence peaking at about 630 nm and 690 nm in the tumor spectrum, while no such porphyrin signature can be recognized in the spectrum from the normal mucosa. Another clear difference between the two spectra is the intensity of the autofluorescence (B), with a low intensity in the tumor area and a much higher in the normal mucosa. If the drug specific fluorescence at 630 nm is divided by the autofluorescence signal at 470 nm an enhanced demarcation of the tumor area is obtained. This is illustrated in Fig. 3 where the intensities of A and B are plotted together with the dimensionless ratio A/B.

In Fig. 4 fluorescence emission spectra from normal bladder mucosa and a superficial malignant tumor are shown for excitation at wavelengths 405 nm and 337 nm in a patient injected with 0.35 mg/kg b.w. Photofrin 48 hours prior to the investigation. As can be seen in the figure the tumor area is recognized by a very low autofluorescence intensity in the blue-green wavelength region for both excitation wavelengths. The drug specific signal at about 630 nm can be identified for both excitation wavelengths although better at 405 nm, which is close to the prominent absorption peak for porphyrin at 405 nm. The spectra excited at 337 nm show another difference in the autofluorescence with the maximum intensities occuring at wavelengths separated by 80 nm. The prominent fluorescence contribution at about 390 nm for the normal mucosa is not present for the malignant tumor. A possible chromophore candidate contributing to the fluorescence at 390 nm is elastin.

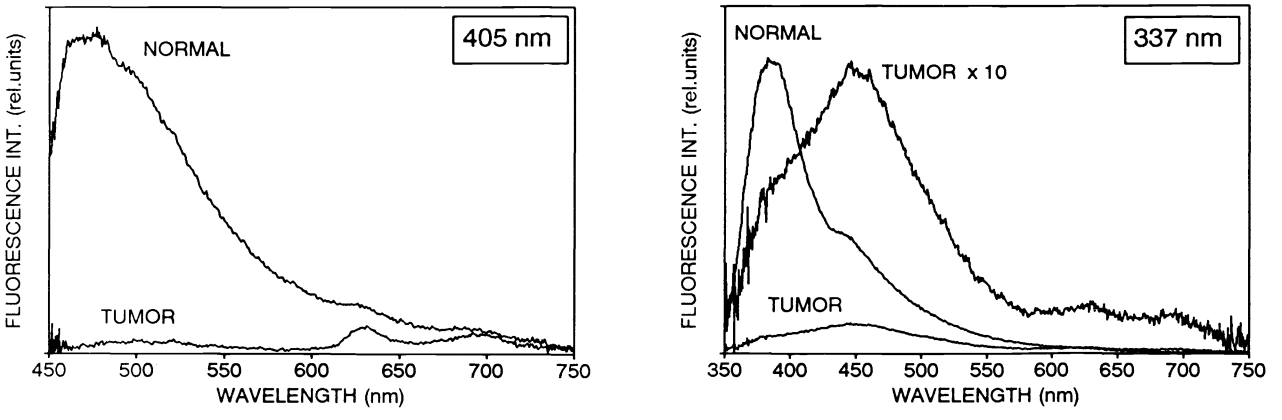

Fig. 4. Fluorescence spectra from a malignant superficial bladder tumor and normal mucosa. The excitation wavelength was 405 nm (left) and 337 nm (right). The tumor spectrum obtained at 337 nm excitation is also shown for 10 times intensity magnification. The patient had been injected with 0.35 mg/kg b.w. Photofrin 48 hours prior to the investigation.

In Fig. 5 we give some examples of the fluorescence signature of two different stages of dysplastic transformation, mild and severe dysplasia in the bladder wall together with the signature of normal mucosa. The autofluorescence peaking at about 470 nm shows a decreasing intensity in the regions of dysplasia. Beside the difference in the autofluorescence there is also a sign of porphyrin in the spectra from the dysplastic regions, which is not seen in the spectrum from the normal mucosa. A known chromophore fluorescing at about 470 nm is NADH. It has been suggested by us and others that the decreased autofluorescence in malignant tissue might be due to less content of NADH[36-39]. It is very likely that the decrease of the autofluorescence is due to a decreasing content of NADH as the dysplastic transformation of the bladder wall proceeds.

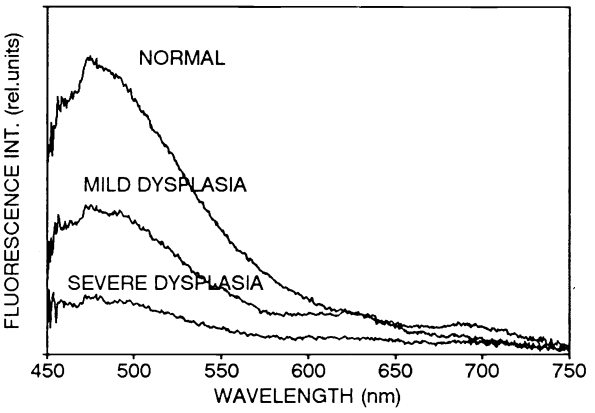

Fig. 5. Fluorescence spectra from normal urinary bladder wall and from different grades of dysplasia. The excitation wavelength was 405 nm. The patient had been injected with 0.35 mg/kg b.w. 48 hours prior to the investigation.

ACKNOWLEDGEMENTS

This work was supported by the Swedish Cancer Society, the Swedish Council for Medical Research, the Knut and Alice Wallenberg Foundation and the Swedish Board for Technical Development. The assistance work by Gertrud Wohlfart and the support by Anders Gustafson are gratefully acknowledged.

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