Fluorescence Spectrum Analysis of Atherosclerotic Plaque Using Doxycycline

Manabu Miyagi, MD; Hitoshi Nakajima, MD; Tomitsugu Katoh, MD; Mikio Usui, MD; Tadashi Amemiya, MD; Yoshikazu Nagai, MD; Chiharu Ibukiyama, MD

Using doxycycline (DOXY), fluorescence spectrum analysis was performed on arteriosclerotic lesions, and the efficacy of this method was examined in basic and clinical studies. In the basic study, DOXY 50 mg was administered intravenously to arteriosclerotic rabbits, and the thoracoabdominal aorta removed. Fluorescence spectral analysis was performed on each specimen, and the fluorescence spectral pattern, peak intensity and degree of intimal hypertrophy were studied. In the clinical study, DOXY 200 mg was administered intravenously to 6 human subjects with stable angina and coronary arterial stenosis of greater than 90%, and coronary angiography, coronary angioscopy and fluorescence spectral analysis were performed. DOXY accumulation in the arteriosclerotic intima of rabbit aortae was confirmed. The fluorescence spectrum was monomodal, peaking at around 532 nm. In the noncalcification group, significant correlation was observed between peak intensity and arteriosclerotic intimal thickness. Using DOXY as a fluorescent marker, it was possible to assess the level of arteriosclerotic intimal hypertrophy. Clinically, it was possible to obtain the DOXY spectrum of the coronary arteries.

Key Words: Arteriosclerosis; Doxycycline; Fluorescence spectrum analysis

Various interventional therapies are presently used in the treatment of ischemic heart disease. In clinical trials in the US, percutaneous transluminal laser coronary angioplasty (PTLCA) was performed on 3,000 patients and 3,592 lesions using excimer laser, with a reported success rate of 84%. When this method is compared with percutaneous transluminal coronary angioplasty (PTCA) using a balloon, PTLCA can be performed on diffuse lesions in which PTCA is difficult, and it may be possible to use the laser to recanalize a chronic total occlusion, a lesion in which the passage of a guide wire would be very difficult. In addition, PTCA is contraindicated in ostial stenosis, and PTLCA is considered the first choice in closed chest treatment. In PTLCA, however, unexpected coronary perforation occurs in between 1.2 to 2.4% of cases; a rate which is obviously higher than for PTCA. As avoidance of coronary perforation is the paramount consideration in PTLCA, the requirement is for an appropriate guidance system to show the condition of the hypertrophic intima and the degree of ablation in real time, so that the atheroma alone is selectively ablated and the tunica media not irradiated.

At present, evaluation of coronary arteriosclerotic lesions is made using angioscopy and intravascular ultrasound, neither of which can be inserted into the coronary artery at the same time as the laser irradiation system and so cannot be the appropriate guidance system. Fluorescence spectrum analysis is a method by which the fluorescence induced by irradiation at specific wavelengths of an arteriosclerotic lesion is examined in detail to make the diagnosis of arteriosclerosis. A diagnostic system using autofluorescence generated from the constituents of arteriosclerotic lesions has been reported; but in each report the pattern of fluorescence is complicated, and various problems need to be solved before it can be of clinical use.

In our laboratory with a view to using photosensitive substances in fluorescence spectral analysis, we have studied the accumulation of HpD (hematoporphyrin derivatives) and NPe6 (mono-1-asparatylchlorine e6) in arteriosclerotic intima. However, because there are problems with the side effects of the light hypersensitivity of these substances, they are not used clinically at present except for photodynamic therapy of cancer.

Since the report in 1966 of their accumulation in arteriosclerotic lesions,13 the tetracycline group of antibiotics have been regarded as photosensitive substances with few side effects. Among the tetracyclines, doxycycline (DOXY) exhibits particularly intense accumulation in arteriosclerotic lesions. In the present study, we performed fluorescence spectral analysis on arteriosclerotic lesions using DOXY, to examine the possibility of its use in the detailed diagnosis of such lesions. Next, to examine the effectiveness of this method, we performed DOXY fluorescence spectral analysis on human coronary arteriosclerotic lesions in the clinical situation. In addition, in patients in whom directional coronary atherectomy (DCA) was performed, we examined the pattern of DOXY accumulation in the removed tissue.

Methods

Experimental Animal Model The subjects were Japanese white rabbits (males, mean weight 3 kg). Each animal was anesthetized with intravenous pentobarbital at 25 mg/kg, and an incision made in the right inguinal region to expose the femoral artery. An incision was made in the...
artery, the distal end was ligated with 2.0 silk, and a 4Fr Fogarty balloon catheter was passed retrogradely up the artery from the proximal end. Under X-ray guidance, the balloon was passed to the thoracic aorta, then inflated with 1.5 ml of air, and pulled out to the bifurcation of the common iliac artery. This procedure was repeated 3 times. Subsequently, animals were kept on a 1.0% cholesterol diet for a period according to the protocol and used as the arteriosclerotic model for the following examinations.

All experimental animals were handled according to the Guidelines for Animal Experiments in Tokyo Medical College (J Tokyo Med Coll 1993; 51(3)).

Spectral Analysis
For fluorescence spectral analysis, an angioscope (Medical Science Co, Tokyo, Japan) was used. The angioscope is 3,000 mm in length, 1,500 mm in effective length and 0.75 mm in diameter, including the endoscope image fiber (3,000 imaging fibers), which doubles as the fluorescence detecting fiber, and the light source fibers (50 μm, 45 fibers). The endoscope tip was kept 1 mm from and perpendicular to the site chosen for measurement of fluorescence. The surface of the arteriosclerotic intima was irradiated by 405 nm from Hg-Xe light using a cut-off filter, and the generated fluorescence was picked up by the fluorescence detecting fiber. The generated fluorescence was amplified about 100 times using a spectroscopic analyzer, an image intensifier and a highly sensitive CCD camera, and the fluorescence spectrum displayed on a monitor. The spectral wave form thus depicted was computerized and analyzed using an optical multichannel analyzer (Multichannel Detector Data Acquisition System, ICONIX Co, Tokyo, Japan) to determine the wavelength and intensity of fluorescence (Fig 1).

Examination of Rabbit Aortae
Experiment 1 Six arteriosclerotic rabbits were kept on a 1.0% cholesterol diet for 2 weeks. The rabbits were killed with pentobarbital, the aorta was removed and incised, and fluorescence spectra were measured from the intima of the abdominal aorta at 4 sites at intervals of 1 cm. The aorta was incubated in a 2% DOXY solution in physiological saline for 30 min and then washed with physiological saline, and the fluorescence spectra were measured again from the same sites.

In addition, fresh-frozen sections of 5 μm in thickness were prepared before and after incubation from each specimen to measure the DOXY accumulation in the arteriosclerotic lesions using a fluorescent microscope (Newvanox, Olympus Co, Tokyo, Japan: excitation wave length 395–415 nm).

Experiment 2 Nine of the rabbits were kept on a 1.0% cholesterol diet for 2 weeks (3 rabbits), 4 weeks (3) and 8 weeks (3) to induce arteriosclerosis. Each rabbit was administered DOXY 50 mg intravenously, then killed 4 h later using pentobarbital, and the thoracoabdominal aorta exposed and incised. Fluorescence spectral analysis was performed on the intima of the thoracic and abdominal aortae at 4 sites each at intervals of 1 cm.

Each site to be analyzed was then fixed in 10% formaldehyde, embedded in paraffin, and sliced to prepare cross sections of 5 μm in thickness. The sections were stained with hematoxylin-eosin (HE) and elastica van Gieson (EVG), and divided histologically into either a
Clinical Study

Detection of Fluorescence Spectra From Human Coronary Arteries  Six patients with stable angina (age: 54.3±11.6 years; sex: male; target lesion: left anterior descending artery (LAD) in 4 patients and right coronary artery (RCA) in 2 patients), with 90% or more stenosis in the target coronary artery were studied. DOXY (200mg) was administered intravenously, and 4 h later coronary angiography (CAG) was performed. Coronary angioscopy and fluorescence spectral analysis by the double guiding catheter method were then performed, as follows.

A 8Fr guiding catheter was fixed in the coronary arterial ostium, a 0.014-inch guide wire inserted into the distal coronary artery, and a 5Fr inner guiding catheter inserted to just proximal to the stenotic lesion using the over-the-wire method. The angioscope was advanced to the tip of the

noncalcification or calcification group.

Fig 2. Fluorescence microscopic photograph (A) and fluorescence spectrum (B) of rabbit aorta before treatment with DOXY.

Fig 3. Fluorescence microscopic photograph and fluorescence spectrum of rabbit aorta after incubation in DOXY solution. (Upper) Microscopic photograph (A) and fluorescence spectrum (B) of type II (AHA) atherosclerotic lesions. (Lower) Microscopic photograph (A) and fluorescence spectrum (B) of type III (AHA) atherosclerotic lesions. Yellow-green fluorescence of DOXY was observed in the hypertrophied atherosclerotic intima, and its accumulation was localized to the intima. The fluorescence intensity was greater for the type III atheroma.
inner guiding catheter, and a bloodless view prepared using warmed physiological saline (containing 5,000 units of heparin in 1,000 ml) at a flushing rate of 0.3–3.0 ml/s to obtain an angioscopic image. Next, under angioscopic control the tip of the angioscope was allowed to make contact with the vascular wall, and then pulled out 1 mm, keeping the distance between the angioscope tip and the vascular wall constant. The angioscopic image fiber was connected to the spectrum analyzer, and the light source was changed from light to an Hg-Xe lamp filter at a wavelength of 405 nm to record the DOXY fluorescence spectrum.

During the procedure, isosorbide dinitrate was continuously infused into the coronary artery at a rate of 1–2 ml/h to prevent coronary spasm. Subsequently, the inner guiding catheter and angioscope were withdrawn to a normal section of the same coronary artery for angioscopic observation and to record the fluorescence spectrum. A comparison was made of the peak intensity of the spectra obtained between the stenotic lesion and the normal area.

The clinical study was conducted after approval by the ethical committee of Tokyo Medical University Hospital.

**Examination of the Site of DOXY Accumulation in Tissue Removed by DCA** In the cases in which DCA was performed on stenotic lesions, fresh frozen tissue sections were prepared and examined under the fluorescence microscope. The same specimens were also immunohistochemically stained using HE with using antibodies for proliferating cell nuclear antigen (PCNA), smooth muscle cell actin, fibroblasts and macrophages.

**Statistical Analysis**

The various indices in each group were expressed as the mean ± SD. The correlation between 2 variables, such as the peak intensity and the thickness of intima, in the basic...
Results

Basic Study

Examination of Rabbit Aortae: Experiment 1 The fluorescence spectra and microscopic findings before incubation with DOXY are shown in Fig 2. The intima appeared pale green, but fluorescence was minimal. In the spectral analysis, the wave form of the received fluorescence spectrum was almost flat. Fig 3 shows the fluorescence microscopic findings after incubation with DOXY, which revealed atheromas corresponding to the histological classification of both type II and type III. In both these types, the typical DOXY yellow-green fluorescence can be seen localised to the intima, and gradually fading toward the medial side. The fluorescent spectographs both show a monomodal pattern with a peak position at about 532 nm. The intensity was greater for the type III atheroma, with greater intimal hypertrophy, but no change was seen in the spectral pattern.

Examination of Rabbit Aortae: Experiment 2 The peak position and the peak intensity were compared between the noncalcification and calcification groups (Fig 4). No significant difference was found between the 2 groups in the wavelength of the peak position (533±1.24 nm vs 532±0.71 nm), and the overall mean peak position for all subjects was 532±0.62 nm. The noncalcification group exhibited a significantly higher peak intensity, however (0.964±0.114 vs 0.338±0.005 p<0.0001) (Fig 5). When the relationship between the thickness of the intima and the peak intensity obtained at that site was examined in the noncalcification group, a significant positive correlation was found. In the calcification group, no correlation was found (Fig 6).

Clinical Study

Fig 7 shows the CAG findings in a male patient (35 year old) with effort angina, in which there was 90% stenosis of Segment 6 of the LAD and a normal section proximal to the stenotic lesion. On angioscopy, a white vessel wall with a smooth surface was seen, corresponding to stable atheroma in the classification of angioscopic findings according to Mizuno et al. Fig 8 shows the fluorescence spectrum obtained from the stenotic lesion at the stenotic and normal sites shown in Fig 7. The spectrum of DOXY had a peak at 560 nm at both sites, but the stenotic lesion gave a high value for the peak intensity. When the peak intensities at the normal sections (6 sites) and the stenotic lesions (6 sites) obtained from the 6 patients were compared, the stenotic lesions gave significantly higher values (0.848±0.139 vs 0.473±0.058 p<0.05).

Fig 9 shows the findings of fluorescence microscopy of the tissue specimens dissected by DCA. Yellow-green DOXY-specific fluorescence was observed, indicating that DOXY accumulates in human coronary arteriosclerotic lesions. Sites positive for anti-PCNA antibodies, anti-smooth muscle cell actin antibodies, anti-fibroblast antibodies and anti-macrophage antibodies did not correspond to the sites of DOXY accumulation.
Discussion

Fluorescence Spectral Analysis of Arteriosclerotic Lesions Using DOXY

Basic Study  A few studies have used tissue autofluorescence for fluorescence spectral diagnosis of arteriosclerotic lesions. However, because collagen, elastin and carotenoids, all of which exhibit autofluorescence, are nonspecific constituents of the various layers of vessel walls\(^{17,18}\), it is difficult to identify whether the origin of fluorescence is in the arteriosclerotic intima or in the media. We therefore investigated the use of photosensitive substances that are specifically incorporated into the arteriosclerotic intima. In the basic study, DOXY administered intravenously accumulated only in the arteriosclerotic intima. Even when the vascular wall was incubated in a DOXY solution at a higher concentration, there was no accumulation in the tunica media. From these results, because DOXY was selectively accumulated in the arteriosclerotic intima, it may be assumed that the intensity of fluorescence reflects the degree of intimal hypertrophy. Furthermore, when the peak intensity of the DOXY spectrum and the thickness of the intima of rabbit aorta, determined histologically, were compared, a good correlation was obtained between the 2 variables. On the other hand, accumulation of DOXY varies with changes in the tissue properties of the arteriosclerotic intima. In particular, DOXY accumulation was minimal in calcified lesions, the fluorescence intensity was low, and there was no correlation with the degree of intimal hypertrophy. From the preceding, it can be assumed that (1) the cellular component is decreased in intensely calcified lesions and DOXY accumulation is therefore decreased, (2) lesion calcification prevents transmission of the excited light, and (3) calcification inhibits permeation of DOXY into the intima.

In the present study, the source of the excited light used was 405 nm Hg-Xe lamp filter light. We considered that the degree of penetration of light into the intima and the intensity of the fluorescence would change with the intensity of excited light. In the present study, it was assumed that the correlation between the degree of intimal hypertrophy and the intensity of fluorescence observed in the noncalcification group was because the degree of intimal hypertrophy was mild, and the excited light penetrated sufficiently. Further study is needed into more intense light sources, such as lasers.

Clinical Study  Chutorian et al reported the accumulation of tetracycline, seen under ultraviolet light, in segments of human aorta obtained at autopsy, and in surgically removed carotid atheroma, bathed in tetracycline solution\(^{19}\). In the present study, because the yellow-green DOXY specific fluorescence was seen in specimens obtained from DCA, this is the first time that the accumulation of DOXY in coronary atheroma has been confirmed clinically. Watari et al reported that they measured HpD fluorescence in the aortae of arteriosclerotic rabbits in vitro and in vivo, and that it is possible to estimate from its intensity the degree of intimal hypertrophy\(^{10}\). When we compared the strength of DOXY fluorescence in the coronary arteries at the stenotic site and at the normal site, it was significantly greater at the stenosis. However, the strength of fluorescence decreases with the square of distance, and also changes with the angle of entry of excited light into the tissue. Accordingly, as outlined earlier, we attempted at the time of measuring fluorescence to maintain a constant distance between the endscope tip...
and the vessel wall, but with this system it was impossible to achieve a uniform angle of entry of excited light into the tissue for each point of measurement. We therefore consider that clinical studies using this system of making comparisons of fluorescent intensity will have limitations. However, to our knowledge this is the first report of clinical measurements, using transcutaneous cannulization, of fluorescence in coronary atheroma with a comparative study. Bartorelli et al20 and Morguet et al21 used coronary arterial autofluorescence in a feedback system in laser angioplasty, but because it has become possible to clearly differentiate the fluorescence of DOXY, a substance which is not naturally present in the body, from autofluorescence, this method was considered to be a simple and smart guidance system. With further improvements to this system, it should be useful in studies of coronary atherosclerosis.

In recent years, dispatch catheters22 and new needle injection catheters23 have been used clinically for local intracoronary drug administration. We previously reported that administration of HpD in a very small amount using a dispatch catheter allows the local accumulation of significant amounts of HpD in the arteriosclerotic intima when compared with systemic administration.23 Because HpD exhibits red-orange fluorescence with peaks at 630 nm and 690 nm in response to ultraviolet irradiation, it can be differentiated from autofluorescence more clearly, but intense light hypersensitivity occurs following systemic administration. Using these catheters, low-dose local administration of substances such as HpD, which shows excellent photosensitivity but cannot be administered systemically due to light hypersensitivity, may be possible and this method may have wider application.

Mechanism of Doxycycline Accumulation in Arteriosclerotic Lesions

On fluorescence microscopic examination of rabbit aortae 4 h after intravenous administration, DOXY was localized to the intima of the arteriosclerotic lesion and no accumulation was seen in the media or adventitia. In addition, the most intense fluorescence was observed on the luminal side of the intima, and gradually decreased toward the medial side. From those results it was considered that, 4 h post-administration, DOXY is not taken up by specific cells of the arteriosclerotic intima, but rather nonspecifically permeates into the intima from the vascular lumen. In special immunostaining of the DCA tissue specimens, no accumulation of the specific constituents of arteriosclerosis was observed. In studies of intracellular DOXY fluorescence, it has been reported that fluorescence is selectively intense in mitochondria25 and that fluorescence is emitted by chelation of intracellular Ca and Mg ions.26 However, concerning DOXY accumulation in arteriosclerotic lesions as well as intracellularly, there are a number of unclear areas that will require further study.

Chutorian et al reported that when they bathed aortae in tetracycline solution, uptake was complete within 10 min and was unchanged up to 2 h later.19 In the present study, DOXY was accumulated in coronary atheroma 4 h post-administration, but further study is needed to determine the optimum time after administration.

Conclusion

Using a photosensitive substance, doxycycline, analysis of the fluorescence spectrum of arteriosclerotic lesions was performed. (1) The accumulation of DOXY in the arteriosclerotic intima of rabbit aortae was confirmed. The fluorescence spectrum obtained was monomodal, with a peak at about 332 nm. (2) The peak intensity of fluorescence of DOXY for rabbit aortae was significantly higher in the noncalcification group than in the calcification group. In the noncalcification group, a significant correlation was observed between the peak intensity and the thickness of the arteriosclerotic intima. (3) From specimens obtained at DCA, the accumulation of DOXY in arteriosclerotic lesions in human coronary arteries was confirmed. (4) Clinically, it was possible to obtain a DOXY fluorescent spectrum of the coronary arteries; however, fluorescent spectral assessment of the degree of arteriosclerosis using the peak intensity will require further improvements to the system.

Acknowledgments

We would like to express our gratitude to Professor Hiroyuki Shimada and Lecturer Junko Nishikawa, of the First Department of Pathology, Tokyo Medical University, for their guidance in the preparation of the fresh frozen tissue sections and immunohistological staining.

References


