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Photosensitization of Experimental Atheromas by Porphyrins

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Arteriosclerotic arteries have been shown to fluoresce when treated with hematoporphyrin derivative. This study investigates the incorporation and distribution of a partially purified form of hematoporphyrin derivative (Photofrin II) in normal and arteriosclerotic rabbit aortas. A thoracoabdominal exploration was performed in 15 rabbits. Group I comprised normal rabbits, Group II normal rabbits given 5 mg/kg Photofrin II 48 hours before surgery, Group III arteriosclerotic rabbits and Group IV arteriosclerotic rabbits given 5 mg/kg Photofrin II 48 hours before surgery. Multiple aortic biopsy specimens for frozen section were taken from all rabbits. In addition, open laser endarterectomy (with an argon ion laser) was performed on Group III and Group IV rabbits. Frozen sections were studied by digital video fluorescence microscopy to determine the distribution of Photofrin II within the layers of the aortic wall.

The fluorescence of the intima of Group IV rabbits was found to be significantly greater than that of the intima, internal elastic lamina, media or adventitia of the other groups ($p < 0.01$) and significantly greater than that of the internal elastic lamina, media or adventitia of Group IV rabbits ($p < 0.01$). When open laser endarterectomy was performed, Group III rabbits required 103 ± 14 J/cm^2 and Group IV required 33 ± 3 J/cm^2 ($p < 0.01$). It is concluded that porphyrins are selectively localized within the intima of arteriosclerotic arteries. This localization sensitizes atheromas to argon ion laser light and facilitates laser endarterectomy.

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Three modes of laser-induced thermal destruction of atheromatous plaque have been suggested: 1) direct laser light absorption by plaque (1–3); 2) laser heating of a metal cap that is in direct contact with the plaque (4,5); and 3) a laser-heated fiberoptic tip that is in direct contact with the plaque (6,7). An early study (3) of laser application to arterial segments demonstrated blood-enhanced surface absorption of neodymium yttrium aluminum garnet (Nd-YAG) laser energy. Pigments released from irradiated erythrocytes were thought to adhere to the luminal surface of the vessel and acted as chromophores to potentiate the thermal reaction. In a similar manner, intermediary dyes such as Sudan Black have been used to stain the plaque and enhance surface absorption of Nd-YAG laser energy (1).

A derivative of the heme extract, hematoporphyrin derivative, has been shown to be selectively retained by a number of neoplastic and nonneoplastic tissues (8,9). Although the mechanism of this increased affinity is still unknown, fluorescence has been described in the atherosclerotic lesions of rabbits (10–12), a Patas monkey (10) and human cadaver aortas (13) when they are exposed to hematoporphyrin derivative. If accretion of hematoporphyrin derivative by atherosclerotic plaque is preferential relative to the remaining vessel wall, selective sensitization of the plaque to thermal destruction by an appropriate wavelength of laser light may be possible.

This study examines the incorporation and distribution of porphyrins within the wall of atherosclerotic rabbit aortas using digital video fluorescence microscopy (14–16) and the photosensitizing properties of certain porphyrins to argon ion laser radiation by open laser endarterectomy (17).

Methods

Animal preparation. Fifteen New Zealand white rabbits were chosen for study. They received humane care in compliance with the Animal Care Committee of the University of California, Irvine and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences (NIH publication 80-23, revised 1978). Eight

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rabbits underwent balloon catheter trauma to the thoracoabdominal aorta under general anesthesia (intramuscular acepromazine, 0.5 mg/kg body weight; xylazine, 3.0 mg/kg; ketamine, 50 mg/kg). To develop arteriosclerosis, these rabbits were fed a 2% cholesterol diet for 5 months. The remaining seven rabbits were fed a standard diet for 5 months.

The rabbits were divided into four groups. Group I comprised three normal rabbits. Group II comprised four normal rabbits that were given Photofrin II (5 mg/kg) (Photofrin Medical, Inc.) intravenously 48 hours before surgery. (Photofrin II is the trade name of a group of porphyrins primarily composed of di-hematoporphyrin ether but also contains small amounts of other porphyrins.) Group III comprised four arteriosclerotic rabbits and Group IV comprised four arteriosclerotic rabbits given Photofrin II (5 mg/kg) intravenously 48 hours before surgery. All animals were kept in the dark for 48 hours before study.

Surgical protocol. The rabbits were anesthetized (intramuscular acepromazine, 0.5 mg/kg; rompun, 3.0 mg/kg; ketamine, 50 mg/kg), intubated and ventilated with a small animal respirator. A thoracoabdominal exploration was performed, the aorta was isolated, intravenous heparin (3.0 mg/kg) was administered and proximal and distal vascular control was obtained. In Groups I and II, multiple biopsy tissue samples (circumferential wall sections) of the aorta were obtained. In Groups III and IV, a longitudinal aortotomy was performed to expose the atheromatous plaques. Multiple circumferential biopsy specimens of atheromatous aorta were obtained and laser endarterectomy was performed on areas of the aorta not previously biopsied (usually separate endarterectomies in the abdominal aorta and in the thoracic aorta).

Laser radiation was delivered with a Coherent INNOVA 20 argon ion laser (mixed output of 488 and 514.5 nm) directed through a flexible 400 μm quartz fiber. A Coherent Power Meter, model 210, monitored laser radiation emanating from the fiber before and after each endarterectomy, and power was held constant at 1.0 W. The quartz fiber was hand-held and directed along the luminal surface of the aorta from a working distance of 2 to 4 mm. Energy density was estimated at 4 J/cm^2. The total incident energy was calculated using the exposure time and the endarterectomy surface area. Open laser endarterectomy was performed by creating a line of laser craters with 1.0 second exposures at the proximal and distal ends of an atheroma. The craters were eccentric corresponding to the angulation of the incident beam. Crater diameters were measured in all cases. Continuous wave radiation connected the end point craters and developed the cleavage plane loosening the atheroma from endarterectomy surface. Laser light was used to dissect the plaque from the media and to thermally fuse the end points once the plaque had been removed. At no time did the quartz fiber contact the artery. The aortas were removed for histologic and fluorescence study. On completion of aortic biopsies or endarterectomy, or both, the rabbits were killed by barbiturate injection.

Histologic preparation. The aortic biopsy sections were embedded in O.C.T. compound (Tissue Tek II, 458 embedding medium) and quick-frozen on dry ice. Serial 4 μm coronal sections were prepared on a cryostat (A.O. Cryocut) at -28°C. Adjacent alternate sections were taken, one stained with hematoxylin-eosin and one examined under the fluorescence microscope for porphyrin fluorescence. The endarterectomy surface and end points were fixed in 3% glutaraldehyde in phosphate buffer, dehydrated and embedded in paraffin. The samples were serially sectioned at 6 μm and stained with hematoxylin-eosin for light microscopy.

Digital video fluorescence microscopy. The fluorescence intensity correlates with the amount of porphyrins within the tissues (16). Coronal sections of arterial wall were viewed through a Zeiss RA epifluorescence/phase contrast microscope (excitation filter BP 390-440 nm; barrier filter LP 590) at 16 × magnification. Light from a 50 W mercury lamp was directed to the tissue section by a chromatic beam splitter. Fluorescent images were detected by a low light level silicon intensified video camera (Venus model TV 2M, Zeiss Instruments, Inc.) that was mounted on the microscope trinocular viewing port. Using a GYYR II MK videorecorder, the images were recorded on ½ inch (1.27 cm) reel to reel videotape. There were 22 sections taped in Group I, 25 taped in Group II, 28 in Group III and 40 in Group IV. The number of sections selected in each group varied according to the pairs of fluorescence sections and hematoxylin-eosin sections that matched exactly and contained all three layers of the aortic wall. All histologic layers of the aortic wall were scanned in succession and recorded on the videotape.

The image processing system used to display the videotape images for analysis (14) and the system for densitometry and the modifications necessary to adapt the software for fluorescence (15,16) have been described. Selected images from each videotaped specimen were displayed on the image processing monitor, averaged by the system and stored in the computer. The relative fluorescence of the various histologic regions was then measured on the stored images. Each stored image was composed of a 512 × 512 pixel display, each pixel having an eight bit resolution. This corresponded to a gray scale where black = 0 and bright white (saturation) = 225. On each analyzed image several 30 × 30 pixel areas were measured to give the average gray value for each histologic region.

Statistical analysis. Comparison of the relative fluorescence intensity for each histologic layer of the aorta within each group and among the four groups was made with Hotelling's T^2 test (18). Comparison of the energy required to perform each step of the argon ion laser endarterectomy was made with Student's t test. Differences amounting to a value of $p < 0.05$ were considered significant.

Table 1. Average Gray Value (fluorescence analysis) for Each Histologic Region of Arteriosclerotic Rabbit Aortas

Group	Intima	Internal Elastic Lamina	Media	Adventitia
I, Normal	62.32 ± 0.19	62.23 ± 0.16	64.56 ± 0.18	58.42 ± 0.14
II, Normal + Photofrin II	63.35 ± 1.35	66.86 ± 0.78	67.13 ± 0.66	63.38 ± 0.77
III, Arteriosclerotic	68.60 ± 0.72	70.53 ± 0.81	68.54 ± 0.70	58.97 ± 0.65
IV, Arteriosclerotic + Photofrin II	75.42 ± 1.04*	69.27 ± 0.68	66.94 ± 0.90	61.90 ± 1.02

*$p < 0.01$. Each number represents the mean of the average gray values (fluorescence intensity) measured in the respective area of an artery.

Results

Fluorescence analysis. Fluorescence was initially visualized by gross observation of the luminal surface under ultraviolet light (Wood's lamp). In Group I (normal), Group II (normal plus Photofrin II) and Group III (arteriosclerotic), no fluorescence was observed. In Group IV (arteriosclerotic plus Photofrin II), salmon-pink fluorescence of the intima was seen. No fluorescence was observed in the adventitia or surrounding tissues. After laser endarterectomy in Group IV rabbits, fluorescence was observed only in areas of residual plaque. There was no fluorescence from the endarterectomy surface.

Histologic sections of defined layers of the aortic wall (Table 1) were assessed for relative fluorescence intensity over the range of 590 to 630 nm. By using image enhancement in combination with the low light level video camera, we were able to detect and quantify fluorescence not detectable by routine visual methods. This technique was sensitive enough to detect autofluorescence in the normal rabbit aortas (Group I) that served as the control group (Fig. 1). There was an increase over the fluorescence seen in the control group in the internal elastic lamina, media and adventitia when normal rabbits were given Photofrin II (Group II) (Fig. 2). When arteriosclerotic rabbit aortas (Group III) were examined, autofluorescence was noted in the intima, internal elastic lamina and media (Fig. 3) and there was a significant ($p < 0.01$ from control) increase in fluorescence detected in the intima of arteriosclerotic rabbits pretreated with Photofrin II (Group IV) (Fig. 4).

Photosensitization. There were 10 laser endarterectomies performed in Group II rabbits and 10 in Group IV. Total incident energy per unit area was 103 ± 14 J/cm^2 (mean ± SE) for argon ion laser endarterectomy in arteriosclerotic rabbits (Group III). This was reduced to 33 ± 3 J/cm^2 by pretreatment with Photofrin II ($p < 0.01$) (Group IV). This significant difference was evident for each step of endarterectomy except for fusion of end points (Table 2). The reduction of energy requirements was most striking for the dissection of the cleavage plane within the media. Photofrin II-treated rabbits (Group IV) required 11 ± 1 J/cm^2 for the dissection and nontreated rabbits required 48 ± 8 J/cm^2 ($p < 0.01$). Craters were established with 1.0 second exposures at 1.0 W along the transverse diameter

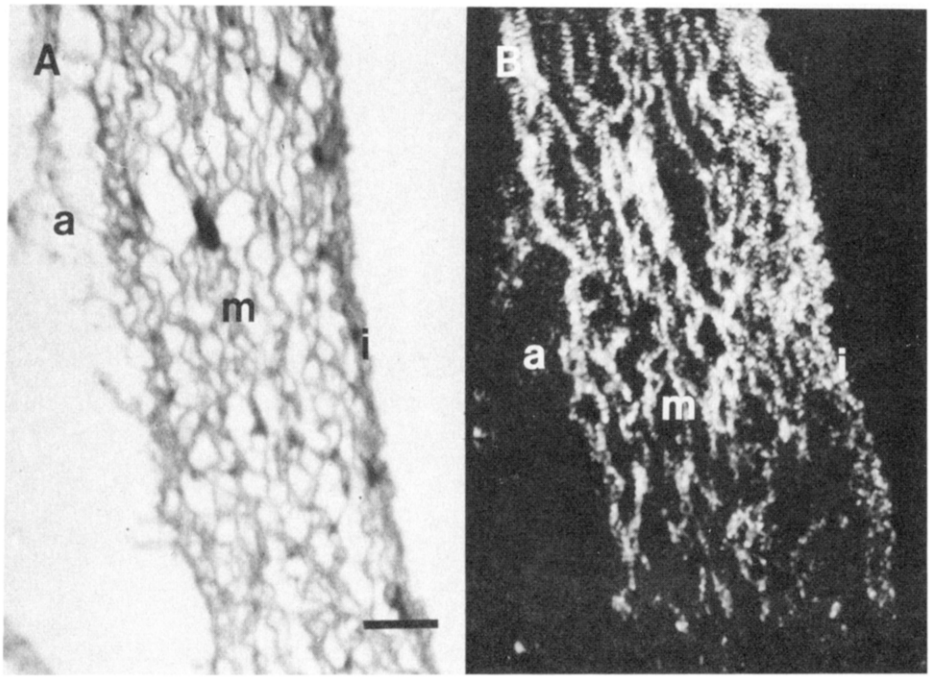

Figure 1. Group I. Coronal sections of a normal rabbit aorta. **A,** Frozen section shows normal architecture with the elastic fibers of the media (**m**) constituting the thickest portion of the artery (hematoxylin-eosin stain) (calibration bar = 100 μm). **B,** Adjacent section examined by digital videofluorescence microscopy shows homogeneous fluorescent activity in the media. a = adventitia; i = intima.

JACC Vol. 9, No. 3
March 1987:639–46

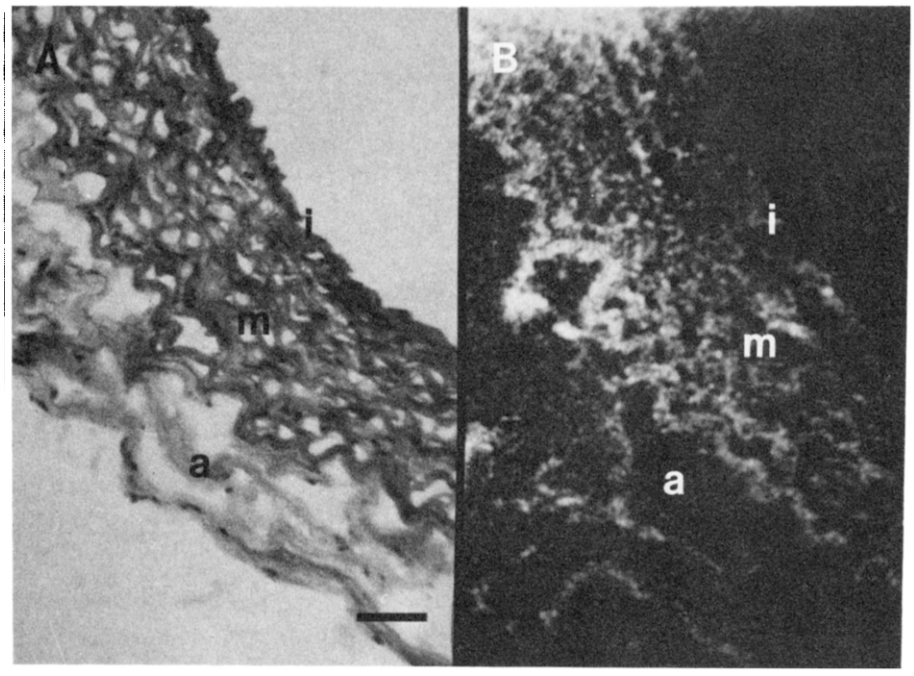

Figure 2. Group II. Coronal sections of a normal rabbit aorta 48 hours after injection of Photofrin II (5 mg/kg). **A,** Hematoxylin-eosin stain shows normal architecture of the aorta. **B,** Adjacent section examined by digital video fluorescence microscopy shows homogeneous fluorescence within the media (**m**). There is no increase in fluorescence compared with Figure 1. a = adventitia; i = intima; calibration bar = 100 μm.

of the opened aorta. Mean crater diameter (widest point) measured 0.5 ± 0.1 mm for arteriosclerotic aortas and 0.7 ± 0.1 mm for arteriosclerotic aortas pretreated with Photofrin II (p = NS). No discernible difference was noted in the depth of the crater at light microscopic resolution (430 ×).

Light microscopic study of the endarterectomy surfaces and end points showed a characteristic narrow band of coagulation necrosis consisting of nuclear pyknosis surrounded by a diffuse region of damage. These changes occurred to a greater extent subjacent to the transition between the end point and endarterectomy surface (Fig. 5). The endarterectomy surface was smooth and the elastic fibers of the media were undisturbed in all experiments (Fig. 6). Aortas from rabbits treated with Photofrin II were indistinguishable histologically from nontreated aortas. In all cases, there was no evidence of increased nontarget tissue damage in rabbits given Photofrin II.

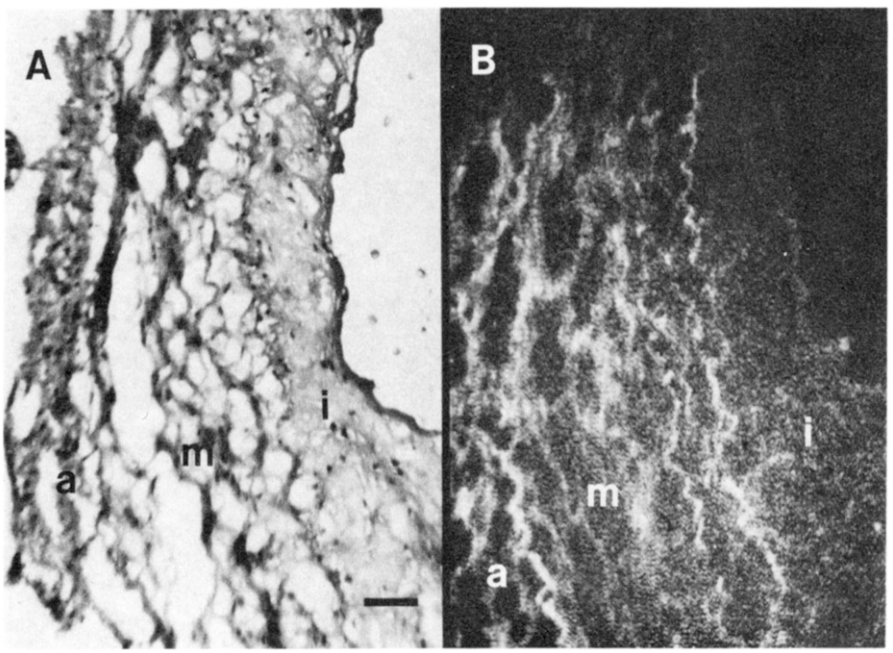

Figure 3. Group III. Coronal sections of an arteriosclerotic rabbit aorta. **A,** Hematoxylin-eosin stain of a frozen section shows a thickened arteriosclerotic intima (**i**) and spaces between the elastic fibers due to fatty infiltration. **B,** Adjacent frozen section examined for fluorescence shows no significant difference in fluorescence from that in Figures 1 and 2. a = adventitia; m = media; calibration bar = 100 μm.

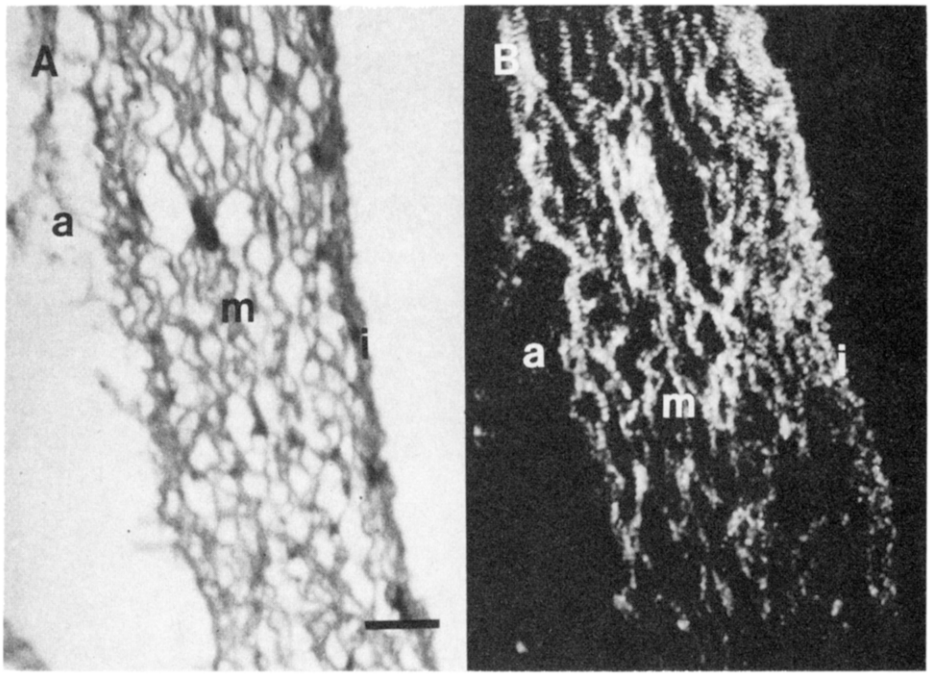

Figure 4. Group IV. Coronal section of an arteriosclerotic rabbit aorta 48 hours after injection of Photofrin II (5 mg/kg). **A,** Hematoxylin-eosin stain shows markedly thickened atheromatous intima (**i**). There is attenuation of the elastic fibers of the media (**m**). **B,** Adjacent section examined by digital video fluorescence microscopy shows significantly increased fluorescence in the intima (atheroma). This shows that Photofrin II is localized in the atheroma. a = adventitia; calibration bar = 100 μm.

Discussion

Intraluminal laser angioplasty requires a parallel orientation of laser light to the vessel lumen and close approximation between the laser fiber and the treated lesion (1–3). Vessel perforation is common (3,19,20). For this reason, a number of investigators (4,5) have begun work with laser-heated metal caps instead of the bare-ended laser fiber, but the metal cap can deliver only thermal energy, not laser light.

Our approach has been to use the laser fiber in an open procedure, laser endarterectomy, to ensure precise dissection of the atheroma without the danger of arterial perforation (3,17,21,22). Selective photosensitization may offer another way to obviate the complications of intraluminal laser use. This approach could result in the destruction of plaque with less chance of perforation because of greatly reduced laser energy requirements. The photosensitizing properties of porphyrins to laser light are well known from tumor studies (9,23,24).

Previous studies. Spears et al. (10) have shown that hematoporphyrin derivative causes fluorescence in arteriosclerotic arteries. This finding has been confirmed by others (11–13). Yet there is no proof that hematoporphyrin derivative localizes only in the target tissue, that is, the atheroma, and there is no proof that this localization sensitizes plaques to laser light. If, for example, the entire artery takes up hematoporphyrin derivative, then there would be no selective photosensitization of the atheroma and the entire vessel would still be vulnerable to perforation (although at reduced laser energy).

Spears et al. (10) observed fluorescence in the aortas of arteriosclerotic rabbits and an arteriosclerotic monkey by examining the specimens under ultraviolet light 48 hours after intravenous infusion of hematoporphyrin derivative (5 mg/kg). Cortis et al. (11) reported fluorescence in the aortas of arteriosclerotic rabbits by examining the specimens under ultraviolet light 24 hours after intravenous infusion of hematoporphyrin derivative (15 mg/kg). They also observed these specimens through an angioscope to demonstrate that fluorescence could be thus identified. Litvack et al. (12) observed fluorescence in the aortas of arteriosclerotic rabbits by examining the specimens under ultraviolet light 48 hours to 2 weeks after intravenous infusion of hematoporphyrin derivative (10 mg/kg). They also observed histologic sections of these specimens under ultraviolet light and reported

Table 2. Energy Requirements for Argon Ion Laser Endarterectomy

Group	Crater (J)	End Point (J)	Cleavage Plane (J/cm^2)	Fusion (J)	Total (J/cm^2)
III, Arteriosclerotic	25 ± 3	49 ± 11	48 ± 8	20 ± 4	103 ± 14
IV, Arteriosclerotic + Photofrin II	21 ± 3*	36 ± 7*	11 ± 1†	20 ± 4	33 ± 3*

*$p < 0.01$; †$p < 0.001$.

JACC Vol. 9, No. 3
March 1987:639–46

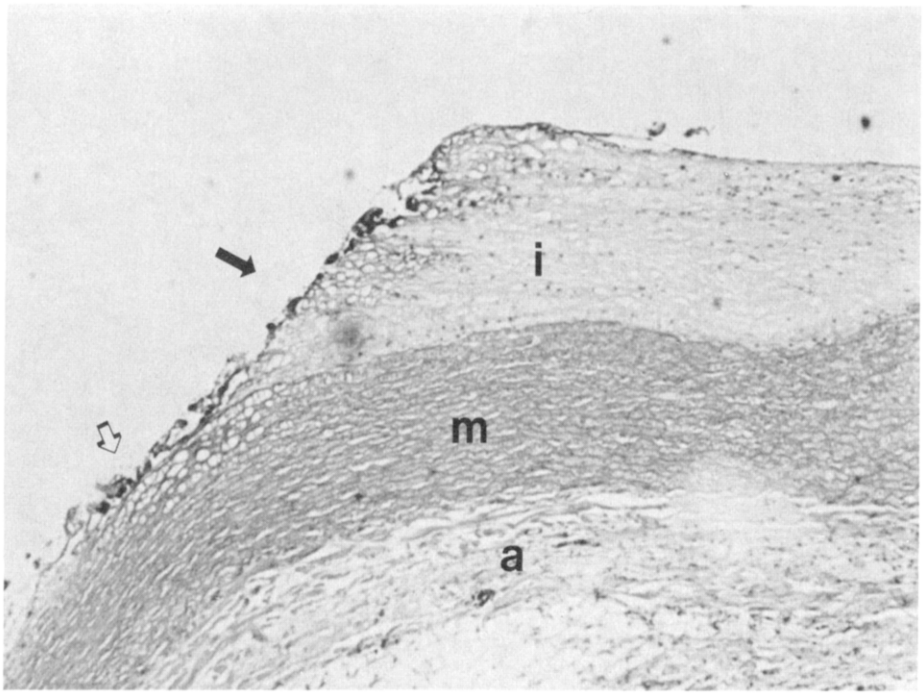

Figure 5. Longitudinal section of a distal end point of an arteriosclerotic rabbit aorta after photosensitized argon ion laser endarterectomy. Low power magnification shows a tapered transition from media (**m**) (**open arrow**) to intima (**i**) (**closed arrow**). There is surface carbonization where the layers are fused with vacuolization of cells immediately beneath the fusion line. a = adventitia. Hematoxylin-eosin stain; original magnification × 10, reduced by 26%.

that most of the fluorescent activity was localized in the atheromatous plaque. These three studies depended on visual identification of fluorescence. The naked eye is a poor instrument for the detection of fluorescence, however, and it cannot quantify fluorescence (25). Hence, the conclusion reached by these investigators that hematoporphyrin derivative localizes only in atheromas is made on the basis of subjective interpretations of fluorescence.

Only Kessel and Sykes (13) used instrumentation to quantify porphyrin accumulation in arteriosclerotic arteries. They used a fluorometer to quantify the fluorescent signal of sections of human cadaver aortas incubated in different

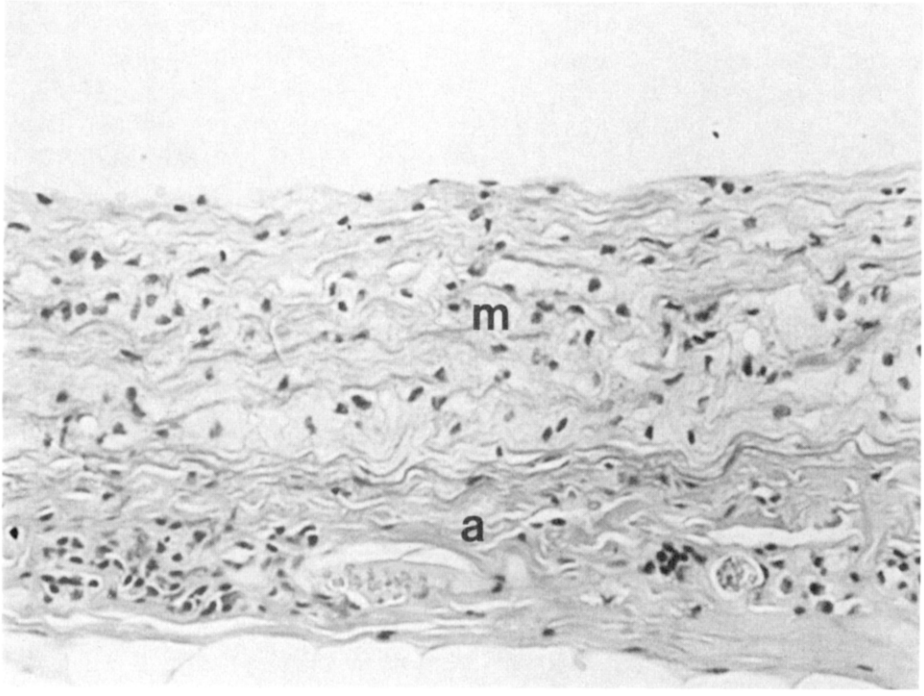

Figure 6. Longitudinal section of an arteriosclerotic rabbit aorta after photosensitized argon ion laser endarterectomy. High power magnification shows a smooth endarterectomy surface within the media (**m**). The internal elastic lamina has been removed and there is no charring along the surface. The remaining media and adventitia (**a**) are undisturbed. Hematoxylin-eosin stain; original magnification × 40, reduced by 25%.

porphyrins. They showed accumulation of hematoporphyrin derivative and they documented that the major components that accumulated were hematoporphyrin, coproporphyrin and uroporphyrin. These are all hydrophilic compounds that are not associated with tumor localization. These data are contrary to the theories of Spears et al. (10) and Litvack et al. (12) that the lipophilic components of hematoporphyrin derivative are responsible for localization in arteriosclerotic arteries. The study of Kessel and Sykes (13), however, involved in vitro accumulation of porphyrins and their claim that the plaques were the site of porphyrin localization is not made on the basis of a histologic determination of fluorescence, but rather on the measurement of the entire arterial section.

Present findings. In our study, the incorporation and distribution of a purified porphyrin mixture from hematoporphyrin derivative (called Photofrin II) was studied throughout the histologic layers of the arterial wall by digital video fluorescence microscopy at the wavelengths known to represent porphyrin fluorescence (590 to 630 nm). The results clearly show native fluorescence in normal and arteriosclerotic arteries. There was no change in the fluorescence of normal arteries treated with Photofrin II, but the fluorescence of arteriosclerotic arteries was significantly enhanced by Photofrin II and was specifically concentrated in the atheromatous intima. In fact, there was a significant gradient of activity from the atheromatous intima to the internal elastic lamina to the media to the adventitia (Table 1). The greatest concentration of Photofrin II occurred in the intima and internal elastic lamina of arteriosclerotic arteries that are the histologic layers above the cleavage plane of an open endarterectomy. If porphyrins sensitize atheromas to laser light, then laser endarterectomy of Photofrin II-treated atheromas should require less energy than that of nontreated atheromas, as shown in our study (Table 2). Open laser endarterectomy required significantly less energy when Photofrin II was given, particularly in the critical step of dissection of the cleavage plane beneath the internal elastic lamina.

Laser endarterectomy versus laser angioplasty. Laser endarterectomy is the ideal method for the evaluation of photosensitization of atheromas because this procedure requires several well defined steps to remove the atheroma from the artery and leave a smooth surface. Litvack et al. (12) used laser angioplasty in an attempt to demonstrate photosensitization of atheromas by hematoporphyrin derivative. They had no control laser angioplasty group, however, and they did not localize the site of laser exposure. They used a Rhodamine B dye laser (636 nm) pumped by an excimer laser. This is an experimental laser system that is not clinically relevant at this time. We used an argon ion laser (488 and 514.5 nm) that is a standard clinical laser with wavelengths in the range of one of the best absorption peaks of porphyrins (26). Because the argon ion laser has a shorter wavelength than does the dye laser, there is less penetration in tissue, thus allowing for better control of laser energy for endarterectomy. By using a surgical procedure, the target site was well localized and precise evaluations of the energy requirements could be made. We serially sectioned the arteries to study the quality of the endarterectomies. There was no histologic difference between the photosensitized and nonphotosensitized laser endarterectomy specimens. All of the specimens showed a smooth endarterectomy surface with normal architecture of the media and adventitia and an even transition at the end points. The only sites of thermal injury were the end points, which were welded by direct laser exposure. The smooth surface without thermal injury is the result of laser energy directed tangentially at a natural cleavage plane. We have previously shown (3,17,21,22) similar results for argon ion laser endarterectomy. Although the energy requirements between the two groups were significantly different, photosensitization could not improve on a technique that already causes little tissue damage and has a reported low incidence of perforations.

Application of porphyrin administration. In certain solid tumors, differential concentrations of porphyrins by the diseased tissue relative to surrounding normal tissue may be achieved by waiting a given amount of time from porphyrin administration (9,24). At 48 hours after Photofrin II infusion, we achieved a therapeutic ratio of 1.13 between the structures above and those below the cleavage plane. The phenomenon of preferential incorporation or selective retention of Photofrin II, or both, by atheromatous plaque relative to the remaining vessel wall may be further exploited by adjusting the interval between Photofrin II administration and laser therapy to improve this therapeutic ratio. Litvack et al. (12) noted fluorescence in rabbit atheromas 72 hours after hematoporphyrin derivative infusion and "faint fluorescence" for up to 2 weeks afterward. Their dose was 10 mg/kg, however, whereas in clinical use the standard dose is 2.5 mg/kg. Also, their qualitative observation of fluorescence does not prove that the atheromas were photosensitized 72 hours or 2 weeks after infusion, and without quantitative evaluations of the porphyrin activity in the histologic layers of the arterial wall, there may be no differential concentration (therapeutic ratio) of porphyrins beyond 48 hours. Indeed, cancer patients who receive hematoporphyrin derivative usually achieve their optimal therapeutic ratio within 72 hours, but retain traces for up to 6 weeks after treatment.

Conclusions. We have verified the concept originally proposed by Spears et al. (10) that porphyrins are incorporated in atheromas by providing objective measurements of porphyrin fluorescence. By demonstrating reduced laser energy requirements for open laser endarterectomy, we have shown that this incorporation results in photosensitization

JACC Vol. 9, No. 3
March 1987:639–46

of atheromas to argon ion laser light. If porphyrins can be used clinically in association with laser therapy, we would expect the outcome to be selective removal of plaque with reduced risk of perforation or other laser-induced side effects.

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