Journal of Fluorescence, Vol. 1, No. 2, 1991

Short Communication

Aortic Elastin Fluorescence After *in Vivo* Labeling with Congo Red

Oon Tian Tan,[1,3] Barbara Faris,[2,4] and Carl Franzblau[2]

Received February 4, 1991; revised May 9, 1991; accepted May 24, 1991

Congo red (CR) is an azo dye which not only preferentially binds to elastin, an extracellular matrix protein found in the media of arterial vessel walls, but also fluoresces when it binds to this protein. Protein solubilization data following laser irradiation of elastin:CR suspensions determined that the amount of elastin solubilized by laser irradiation increased with the increase in CR. The saturation point of CR to elastin was attained when 400 μg CR was added to 20 mg elastin suspension. When 20 ml of a 5% CR solution in 5% dextrose was administered intravenously, the CR was absorbed in levels sufficient to produce fluorescence of the main arteries in rabbits. Layers of tissue both in the media of the vessels and at the endothelial/intimal interface were clearly differentiated. Therefore, the elastin:CR complex appears to be an ideal system in which the elastin fluorescence could aid in distinguishing between normal and diseased tissue in certain pathological conditions, such as atherosclerosis and some types of breast tumors.

KEY WORDS: Aortic elastin; Congo red; fluorescence; laser irradiation.

INTRODUCTION

It has been known for several years that Congo red (CR) has a strong affinity for both amyloid and elastin fibers [1–4]. While a solution of CR alone does not exhibit fluorescence when monitored spectrofluorometrically, fluorescence is observed when the dye is bound to a protein moiety. We have previously shown that CR binds preferentially to elastin *in vitro* [5]. These earlier studies were done in neonatal rat smooth muscle cell cultures in which insoluble elastin was shown to account for as much as 50% of the total extracellular matrix proteins. It was demonstrated that the elastin in these cultures was preferentially stained with CR with no apparent adverse effects to the cells, even when the cells were grown in the presence of the dye for relatively long periods of time. A fibrous fluorescent network which resulted after the addition of CR to the cultures was shown to be identical to the elastin network in the extracellular matrix. Furthermore, the determination of the molar ratio of CR to elastin made it possible to estimate the amount of elastin solubilized after elastase injury of the cultures.

In the present study, blood vessel walls of rabbits were examined for fluorescence after the administration of CR to the animals. Also, the effect of laser irradiation at the appropriate wavelength of the elastin:CR complex was examined.

METHODS

Exposure of Elastin:CR Complex to Laser Irradiation

Twenty milligrams of powdered bovine ligamentum nuchae elastin per milliliter of Puck's saline was ho-

[1] The Laser Center at Boston University Medical Center, Boston, Massachusetts 02118.
[2] Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118.
[3] Present address: Department of Pathology, Boston City Hospital, 81 Harrison Avenue, Boston, Massachusetts 02118.
[4] To whom correspondence should be addressed at Department of Biochemistry K-225, Boston University School of Medicine, 80 East Concord Street, Boston, Massachusetts 02118.

1053-0509/91/0600-0147$06.50/0 © 1991 Plenum Publishing Corporation

mogenized and various amounts of CR (0–1600 μg) were added to each homogenate. These mixtures were allowed to stand overnight at 4°C, then centrifuged, and each pellet was washed thoroughly with Puck's saline. The pellet was reconstituted in the original volume of Puck's and 0.40 ml of each mixture was placed in one chamber of a four-chamber tissue culture slide. In a separate experiment, 0.20 ml of each mixture was diluted with 0.20 ml of Puck's saline (so that the final volume was 0.40 ml) before placing on the slide. After a few minutes, the elastin:CR particles adhered to the slide surface and each chamber was exposed to 100 single, 300-ns pulses of 504-nm irradiation (the wavelength which matches the absorption spectral peak of CR-labeled elastin) at 2.0 J/cm^2 using a 3-mm-diameter spotsize. The amino acid composition released into the supernatant was analyzed, with particular attention focused upon concentrations of glycine and alanine, since these two amino acids comprise 50% of the total amino acid composition of the elastin.

Hydrolysis and Amino Acid Analysis

The supernatants after laser irradiation of the elastin:CR complex were brought to 6 *N* HCl by the addition of an equal volume of concentrated HCl. All samples were hydrolyzed, *in vacuo,* at 110°C for 20 h and then analyzed on a Beckman Model 6300 amino acid analyzer.

In Vivo Labeling of Rabbit Vessels with CR

Blood vessel walls of adult New Zealand rabbits were examined for fluorescence following administration of CR. The dye was given as a single intravenous administration of 20 ml of a 5% CR solution in 5% dextrose. Levels of CR in the serum of animals after intravenous administration of the dye were monitored spectrophotometrically. Fluorescence of histologically prepared sections of aortic tissue was observed on an inverted microscope (Nikon Diophot) equipped with epifluorescent optics and a 75-W mercury vapor light source. A 535- to 550-nm excitation filter was used in conjunction with a dichroic mirror (580 nm) and an emission barrier filter of 590 nm.

RESULTS

If various amounts of CR were added to 20 mg of powdered ligamentum nuchae elastin per ml of Puck's saline, the saturation point was attained when 400 μg of CR was added as judged by CR color not bound to the elastin. Up to the addition of 400 μg CR, all the color was absorbed to the elastin, while those tubes with the higher CR concentrations had color remaining in the supernatant. This observation was further supported by the protein solubilization results obtained after laser irradiation. The amino acid composition of the material released into the supernatant was analyzed, and as shown in Table I, the amount of glycine and alanine solubilized (the predominant amino acids in elastin) reached a maximum when 400 μg of CR was added to 20 mg of purified elastin/ml. Prior to that amount, there was an increase in elastin solubilization with increasing CR. The two sets of values refer to two separate experiments in which different volumes of the elastin:CR suspension was placed in each chamber of the microscope slide. Values not in parentheses were obtained when 0.40 ml suspension was used and those in parentheses were obtained when 0.20 ml suspension diluted with 0.20 ml Puck's saline was placed in each chamber. Although absolute values may vary between experiments, the trend was consistent in all experiments. However, it should be pointed out that this represented less than 1% of the total elastin.

In a series of animal experiments, it was determined that CR can be given by several routes of administration, all of which have resulted in the fluorescence of the elastin:CR complexes *in vivo.* When given systemically as a single intravenous administration, or administered orally once a week for 3 weeks (data not shown) to the rabbit, the CR was absorbed in levels sufficient to produce fluorescence of the main arteries in these animals. Serum levels of animals to which CR was intravenously

Table I. Elastin Solubilization by Laser Irradiation of Elastin–Congo Red Complex

CR[a]	Glycine (nmol)[b]	Alanine (nmol)[c]
0	1.4	n.d.[d]
12	6.7	2.9
50	11.3 (12.9)[e]	5.7 (6.7)
100	25.1 (23.4)	16.7 (14.2)
200	(42.6)	(23.2)
400	139.7 (122.5)	93.1 (80.5)
800	82.4	53.0
1600	123.0	80.7

[a]Micrograms of CR added to 20 mg of elastin suspension/ml.
[b]Nanomoles glycine per milliliter solubilized by laser irradiation.
[c]Nanomoles alanine per milliliter solubilized by laser irradiation.
[d]Not detected.
[e]Numbers in parentheses refer to values from a separate experiment in which one-half the volume of elastin:CR complex was placed in each chamber prior to laser irradiation. (Please refer to text.)

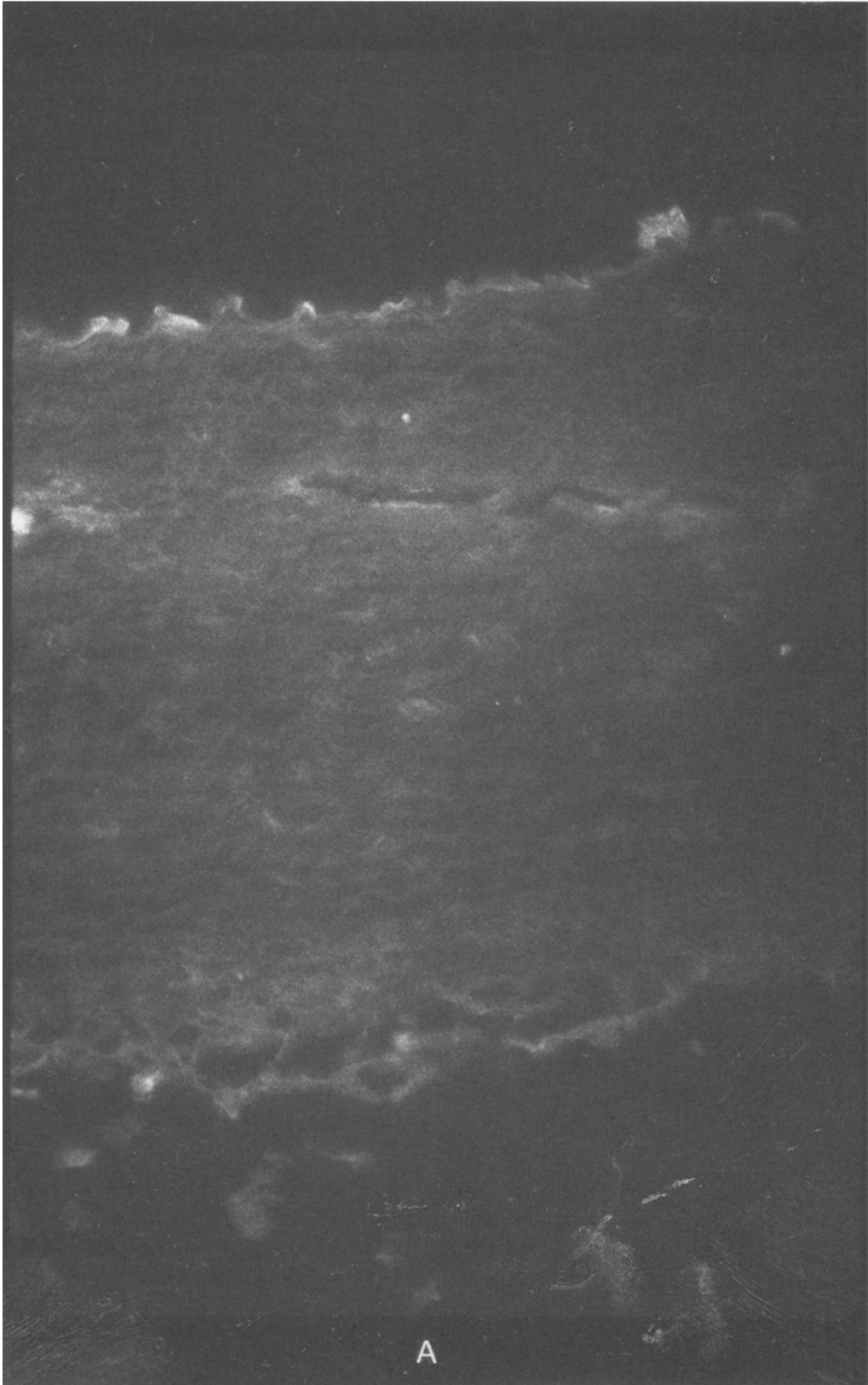

Fig. 1A. Fluorescence in aorta of rabbits treated with Congo red. Autofluorescence of control rabbit aorta (rabbit not exposed to Congo red).

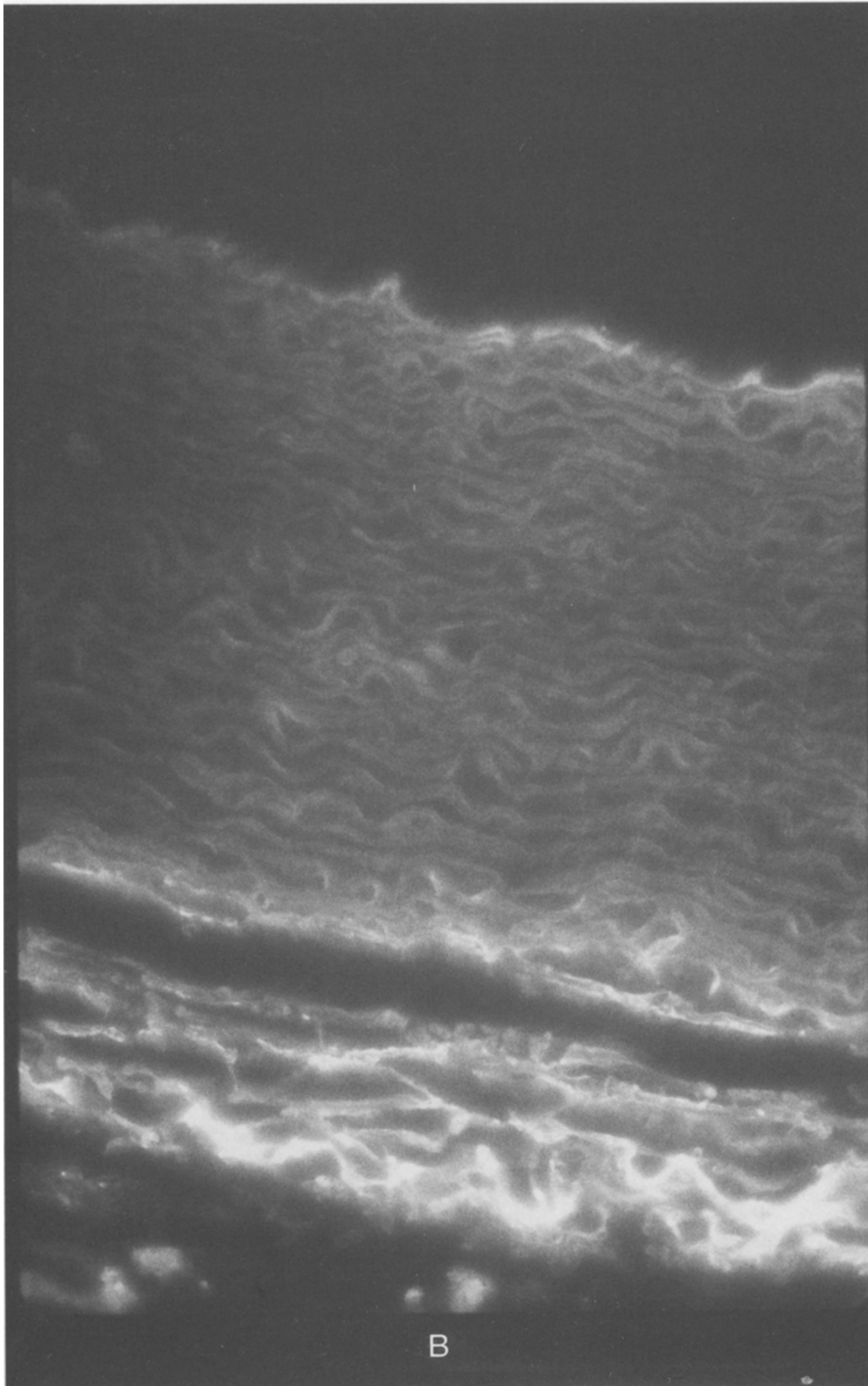

Fig. 1B. Fluorescence in aorta of Congo red-treated rabbit. Note that the fluorescence is localized to the elastic fibers of the vessel wall. Sections were monitored on a Nikon Diophot inverted microscope equipped with epifluorescent optics. A 535- to 550-nm excitation filter was used in conjunction with a dichroic mirror (580 nm) and an emission barrier filter of 590 nm.

administered reached a peak in 10 min. These levels gradually decreased in concentration to barely detectable levels by 3 h.

Histologically, some fluorescence is clearly evident in the elastic tissue of the blood vessels (Fig. 1A). However, the uptake of CR after intravenous administration greatly enhanced the autofluorescence already present in the media of rabbit arteries (Fig. 1B). These observations clearly demonstrate the ability of the elastin in the vessel wall to be labeled with CR, resulting in a fluorescent complex, and that the animal tolerates administration of the dye.

DISCUSSION

Elastin, an extracellular matrix protein, is a major component in the media of arterial vessels. Previous studies from our laboratory [5] determined that CR preferentially stained elastin fibers in the extracellular matrix of neonatal rat aortic smooth muscle cell cultures. Small amounts of CR for as little as 10 min [unpublished data] were sufficient. Of particular interest is the observation that while a solution of the dye alone did not fluoresce, the elastin:CR complex exhibited a strong fluorescence. The dye was not toxic to the cells even when grown in the presence of CR for relatively long periods of time. The animal studies described here further demonstrated that CR could easily and efficiently be administered without any apparent adverse effects to the animals, while still "tagging" elastin. Therefore, the elastin:CR complex appears to be an ideal system in which the elastin fluorescence could be clearly observed in tissue.

Since atherosclerosis is a disease responsible for over 10 million cases of ischemic vascular disease in the United States and is becoming even more significant as the average age of the population increases, the safe removal of atherosclerotic lesions in blood vessels would be invaluable. Plaque, the characteristic lesion, is composed of proliferative smooth muscle cells, macrophages (invested with lipid to varying degrees), necrotic cells, and extracellular connective tissue. Although rapid progress has been made in the application of lasers in the clinical treatment of these lesions, certain problems remain. Complications of laser angioplasty, including the risk of perforation during plaque resection, the disruption of arterial wall media producing aneurysms, and the potential risk of accelerating atherosclerosis through nonspecific laser-induced intimal injury, are well documented. The "ideal" treatment would be one where only plaque is removed and adjacent healthy blood vessel wall structures are left intact.

A recent study published by Deckelbaum *et al.* [6] demonstrated differences in fluorescence among normal aorta, thin yellow fatty plaque, and thick white atheromatous plaque. The fluorescence spectrum of normal aorta was at 514 nm, while the fluorescence maxima for white and yellow plaque were 448 and 538 nm, respectively. Since the absorption maximum of CR-labeled elastin is 504 nm with a fluorescence (emission) maximum of 590 nm, this elastin:CR complex provides its own distinctive peak, which lies beyond the fluorescence maxima of normal aorta as well as white and yellow plaque. The ability to differentiate between these two types of tissue provides a system whereby the laser beam can be inactivated when it encounters the strong characteristic pattern of fluorescence in the intima and/or media underlying plaque in atheromatous blood vessels. Hopefully, such studies should ultimately aid in the designing of a "fail-safe" detection and ablating system for coronary laser angioplasty.

The studies with purified elastin powder demonstrated that elastin can be solubilized when complexed with CR by laser irradiation. While only small amounts of elastin were solubilized in these studies, the possibility that more of the fluorescent complex was "nicked" or damaged, but not solubilized, exists. However, these experiments strongly suggest that one could remove elastin in pathological conditions in which an abnormal amount of elastin is present, as in certain types of breast tumors [7]. Therefore, such a fluorescent protein–dye complex could prove to be a powerful tool in the diagnosis and/or treatment of certain diseases.

ACKNOWLEDGMENTS

This work was supported by NIH Grants AR385-04, HL13262, and HL19717.

REFERENCES

1. B. Highman (1946) *Arch. Pathol.* **41,** 559–562.
2. G. Romhanyi (1971) *Virchows Arch. (Pathol. Anat.)* **354,** 209–222.
3. D. A. Hall (1966) *Biochem. J.* **101,** 29–36.
4. H. M. Kagan, N. A. Hewitt, and C. Franzblau (1978) *Biochim. Biophys. Acta* **322,** 258–268.
5. A. C. Hospelhorn, B. Faris, P. T. Mogayzel, Jr., O. T. Tan, and C. Franzblau (1988) *J. Histochem. Cytochem.* **36,** 1353–1358.
6. L. I. Deckelbaum, J. K. Lam, H. S. Cabin, K. S. Clubb, and M. B. Long (1987) *Lasers Surg. Med.* **7,** 330–335.
7. A. Kadar (1984) in A. Ruggeri and P. M. Motta (Eds.), *Ultrastructure of the Connective Tissue Matrix,* Martinus Nijhoff, Boston.