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DIFFERENTIAL EFFECTS OF ND-YAG LASER ON COLLAGEN AND ELASTIN PRODUCTION BY CHICK EMBRYO AORTAE IN VITRO

Relevance to Laser Angioplasty for Removal of Atherosclerotic Plaques*

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ABSTRACT: Aortae from 17-day old chick embryos were subjected to irradiation with a Nd:YAG laser at energy densities varying from 1.2 - 4.7 x 10^3 J/cm^2. The aortae were pulse-labeled in vitro with [^{3}H]proline or [^{14}C]valine, and the synthesis of collagenous polypeptides and soluble elastin was examined by SDS-polyacrylamide gel electrophoresis, followed by fluorography and quantitative scanning densitometry. Irradiation of the aortae with Nd:YAG laser resulted in inhibition of the synthesis of the extracellular matrix proteins. The production of collagen was inhibited to a considerably larger degree than the production of elastin. Thus, the biosynthetic pathway for collagen production appears to be more susceptible to laser inhibition than the corresponding pathway for elastin production. These observations may have relevance to laser angioplasty which has been proposed to be applicable for removal of atherosclerotic plaques in human vessels. Specifically, the results suggest that inhibition of the extracellular matrix production may result in weakening of the vessel wall with subsequent aneurysm formation and rupture.

Recent observations in the literature suggest that lasers might be useful for removal of atherosclerotic plaques in aorta and other blood vessels (2-4). These studies, largely morphologic, have demonstrated that laser energy can be delivered precisely to remove atherosclerotic, both calcified and non-calcified lesions. Although several studies have suggested that the removal of the lesions can be performed without substantial damage to the surrounding blood vessel tissue (5-8), the consequences of laser irradiation in terms of metabolic alterations and connective tissue response have not been evaluated.

Since lasers, such as those used for angioplasty, have been shown to affect cell biology and connective tissue metabolism (9), the potential hazards of laser irradiation to the aorta could include aneurysms and perforation of the vessel wall, distal embolization and obstruction of the lumen, and altered viability and connective tissue response of the resident fibroblast populations (10-12).

*A preliminary report of this study was given at the Annual Meeting of the Western Region, Society for Investigative Dermatology, February 9, 1984, Carmel, CA (1).

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Several reports have demonstrated that lasers can specifically influence collagen in tissues and fibroblast cultures. For example, Nd:YAG laser has been shown to inhibit the synthesis of collagen both in human skin fibroblast cultures in vitro (13-15), as well as in pig skin in vivo (16). Low energy lasers, such as He-Ne and Ga-As lasers, have been shown to enhance the synthesis of procollagen by cultured human skin fibroblasts (17,18). Thus, connective tissue modulation may take place also in humans subjected to laser angioplasty for the removal of atherosclerotic lesions.

To examine the potential biological consequences of laser angioplasty to the functional and structural integrity of blood vessels, in this study we examined the effects of a Nd:YAG laser on the synthesis of collagen and elastin, the two major fibrillar components of the blood vessels. The results indicate that Nd:YAG laser modulates collagen and elastin synthesis in a differential manner.

MATERIALS AND METHODS: Aortae and associated large blood vessels were dissected from 17-day old chick embryos, rinsed with modified Krebs medium and blotted dry (19). Aortae, four per well, were placed in 96-well microtiter plates and subjected to laser irradiation. The laser used was a Nd:YAG laser (Endolase, Inc.), a continuous wave laser at 1064 nm with a maximum power output of 60 W. The aortae were irradiated at the distance of 10 cm from the tip of the fiberoptic for 3, 6, 9 and 12 seconds, producing energies in the range of 1.2 - 4.7 x 10^3 J/cm^2 (13).

Assay of Collagen and Elastin Production. Immediately following the laser irradiation, the aortae were placed in 1 ml of modified Krebs medium supplemented with 5% dialyzed fetal calf serum, 50 µg/ml of ascorbic acid and 50 µg/ml of β-aminopropionitrile (19). Following a 60 min preincubation, radioactive amino acids, either [^{3}H-2,3]-L-proline or [^{14}C]valine, were added, and the aortae were incubated for an additional 180 min at 37°C. At the end of the incubation, 100 µl of 20% SDS containing 8 M urea was added, the samples were boiled for 5 min and homogenized with a Polytron tissue homogenizer. The samples were then centrifuged at 18,000 x g for 30 min at 25°C. Aliquots of the supernatant were subjected to SDS-polyacrylamide slab gel electrophoresis using 6% gels (20). In some experiments, the extracted proteins were subjected to digestion with bacterial collagenase (21) prior to SDS-polyacrylamide gel electrophoresis. Aliquots of the supernatants were also dialyzed against 1 mM Tris-HCl containing 0.15 M NaCl, pH 7.5, and taken for assay of DNA (22) and total protein (23). Aliquots were hydrolyzed in 6 M HCl for 18 hrs at 120°C, and assayed for total incorporation of radioactivity into nondialyzable macromolecules, as well as for synthesis of [^{3}H]hydroxyproline (24).

In separate experiments, the lanes containing radioactive polypeptides were cut out from the 6% SDS-polyacrylamide slab gels, immersed in 70% formic acid equilibrated in N_2, and incubated with cyanogen bromide, 2 mg/ml for 3 hrs at 25°C (25). The strips of gel were then placed on top of a 10% SDS-polyacrylamide gel and re-electrophoresed in a second dimension.

Following SDS-polyacrylamide gel electrophoresis, the radioactive bands were visualized by fluorography (26). The relative distribution of bands, representing collagenous polypeptides and tropoelastin, were quantitated by scanning densitometry using an automatic computing densitometer (ACD 18, Gelman Sciences, Inc.). The relative distribution of collagen and elastin was calculated from the sum of the polypeptides representing these two proteins.

RESULTS: For measurement of the biosynthesis of connective tissue macromolecules, aortae were first incubated with radioactive proline, and the newly-synthesized proteins were extracted and examined by SDS-polyacrylamide gel

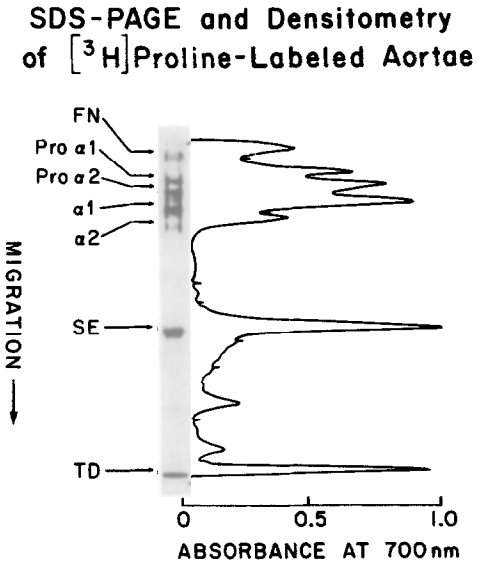


Figure 1. Synthesis of extracellular matrix proteins by chick embryo aortae in vitro. Aortae were incubated with [^{3}H]proline, the ^{3}H-labeled proteins extracted with SDS at 100°C and examined by SDS-polyacrylamide slab gel electrophoresis, followed by fluorography (left lane). The radioactive peptides in the fluorograms were identified as representatives of fibronectin (FN), proα and α chains of procollagen and collagen, and soluble elastin (SE) by the criteria indicated in the text. The relative quantities of the newly-synthesized proteins were evaluated by scanning densitometry at 700 nm, as shown by the tracing on the right. TD indicates the migration position of bromphenol blue tracking dye.

electrophoresis (Fig. 1). Fluorograms of the gels containing the radioactive polypeptides revealed several bands which could be identified to represent distinct connective tissue proteins. First, fibronectin with an apparent molecular weight of 220 - 240 kd was identified by its relative mobility in the polyacrylamide gel (27). Secondly, collagenous polypeptides, proα1, proα2, α1 and α2, were identified on the basis of their mobility in relation to known polypeptide standards of proα and α chains of type I collagen (28,29). In addition, these polypeptides were susceptible to digestion with bacterial collagenase (not shown). Thirdly, a radioactive polypeptide migrating with an apparent molecular weight of 72 kd was identified as soluble elastin by three criteria. First, these polypeptides migrated in the same position as purified tropoelastin standards with a known molecular weight of 72 kd (30). Secondly, when the proteins were labeled with radioactive valine instead of radioactive proline, the relative intensity of this band was considerably enhanced in comparison to collagen and fibronectin (19) (Fig. 2). Thirdly, two-dimensional electrophoresis of the [^{14}C]valine-labeled aorta proteins indicated that the polypeptide with a molecular weight of 72 kd was not

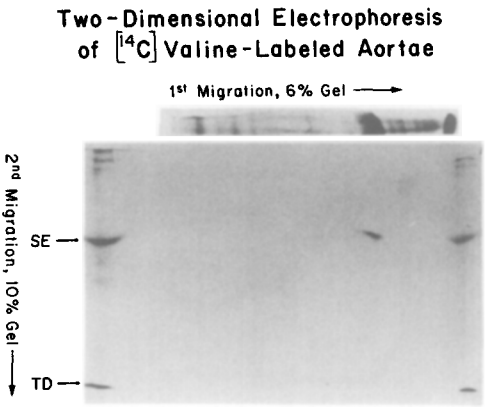
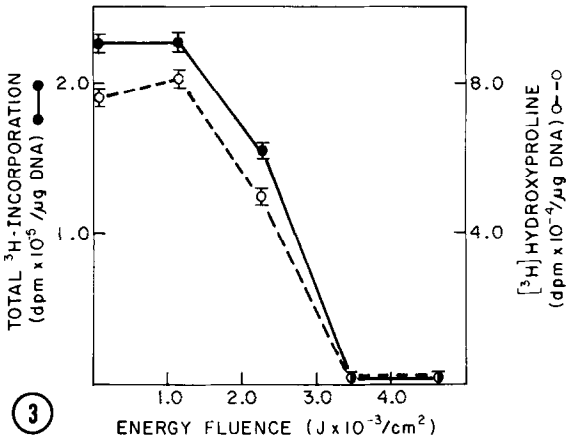


Figure 2. Demonstration that the 72 kd polypeptide, representing soluble elastin (SE), resists digestion by cyanogen bromide. Aortae were incubated with [^{14}C]valine and the ^{14}C-labeled proteins were examined by SDS-polyacrylamide slab gel electrophoresis (1st dimension), using 6% polyacrylamide gel, as in Fig. 1. One lane, visualized by fluorography, was subjected to cyanogen bromide digestion, placed on top of a 10% polyacrylamide gel and electrophoresed in 2nd dimension. The results demonstrate that the 72 kd polypeptide migrates with SE in undigested samples, which were electrophoresed on the second gel at the far end lanes.

susceptible to cyanogen bromide digestion (Fig. 2). Since elastin is known to be devoid of methionine (31), and on the other hand, cyanogen bromide digestion cleaves polypeptides at methionyl residues (25), these observations strongly support the identity of the 72 kd polypeptide as soluble elastin.

Treatment of aortae with Nd:YAG laser at different energy fluences demonstrated that at 2.3 J/cm^2, this laser significantly affected the total incorporation of radioactive proline into non-dialyzable macromolecules, and energies of 3.5 and 4.7 J/cm^2 further inhibited the protein synthesis (Fig. 3). The synthesis of [^{3}H]hydroxyproline paralleled the inhibition of total incorporation of ^{3}H-radioactivity in the same specimens (Fig. 3). Examination of the newly-synthesized radioactive proteins by SDS-polyacrylamide gel electrophoresis indicated, however, that the synthesis of collagen was reduced to a larger degree than elastin synthesis (Fig. 4). Thus, the biosynthetic pathway of collagen appeared to be more susceptible to laser inhibition than the corresponding pathway for soluble elastin.

DISCUSSION: The results of this study indicate that Nd:YAG laser inhibits the production of extracellular macromolecules by chick embryo aortae *in vitro*. The magnitude of the effect was, however, different in case of collagen and elastin production: Collagen synthesis was reduced to a considerably larger degree than



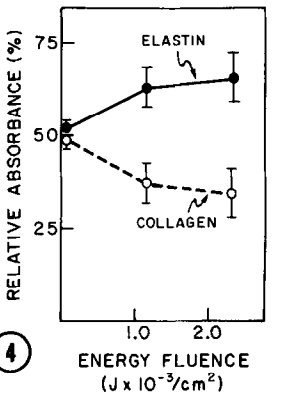


Figure 3. Inhibition of the incorporation of [^{3}H]proline into non-dialysable macromolecules and the synthesis of [^{3}H]hydroxyproline by Nd-YAG laser. Chick embryo aortae were first subjected to laser irradiation at energy fluences indicated and then incubated with [^{3}H]proline. The total ^{3}H-radioactivity and [^{3}H]hydroxyproline, expressed as dpm per µg DNA in the tissue specimens, were determined, as indicated in the text.

Figure 4. The relative synthesis of collagen and elastin in chick embryo aortae subjected to laser irradiation. The aortae were treated with Nd-YAG laser at energy fluences indicated, and then labeled with [^{3}H]proline, as in Fig. 3. The radioactive polypeptides were separated by SDS-polyacrylamide slab gel electrophoresis, and the relative synthesis of collagenous polypeptides (represented by proα and α chains) and soluble elastin was determined by scanning densitometry, as in Fig. 1.

the production of elastin. Collagen and elastin are two major fibrillar components of vessel wall in aorta and associated blood vessels (19). Both collagen and elastin apparently play an important role in providing tensile strength and elasticity to the vessels. In support of this conclusion are several experimental situations where either the production of collagen and elastin is prevented (32) or their fiber formation and subsequent stabilization by covalent inter-molecular crosslinks are perturbed (33). In many of these situations, fragility of blood vessels leading to rupture can occur. Also, several heritable disorders of connective tissue, such as the Marfan syndrome, the Ehlers-Danlos syndrome types IV and IX and the Menkes' syndrome, which involve abnormalities in the structure and metabolism of collagen and/or elastin, are characterized by fragility of the blood vessels (34). In fact, rupture of the major arteries is a leading cause of demise for patients with the Marfan syndrome and the Ehlers-Danlos syndrome type IV (34). These observations then attest to the critical role that collagen and elastic fiber networks play in providing structural and mechanical integrity to the blood vessels.

Several recent studies have addressed the utilization of lasers for angioplastic removal of atherosclerotic plaques. This concept was orginally proposed in 1963 by McGuff et al. (see 35) who used a pulsed ruby laser to destroy plaques in cadaver vessels. With the advent of laser technology, several additional lasers, including argon, CO_2 and Nd:YAG lasers, have been utilized for similar purposes (2-8, 10-12). Furthermore, development of delivery systems has made these lasers potentially applicable for treatment of living human subjects through catheterization. Most of the studies thus far have examined the effects of lasers on blood vessel morphology. The results indicate that the atheromatous lesions can be removed with precision, and the amount of tissue removed is dependent on the power, pulse-frequency, wave-length and the mode of deposition of laser energy.

Some previous studies have addressed the potential hazards of laser angioplasty: These include weakening of the vessel wall, resulting in aneurysms and rupture (10). Weakening of the blood vessel wall, could easily result from inhibition of the synthesis of collagen and elastin, as demonstrated in the present study. The inhibition of the collagen and elastin production may result from the thermal effects of the laser on the surrounding tissue (14), containing cells, such as fibroblasts and smooth muscle cells (19), producing collagen and elastin. Thus, further development of lasers with minimal thermal effects, such as krypton-fluoride excimer laser at UV range (36,37), might potentially avoid some of the consequences of the laser irradiation which depend on thermal damage to the surrounding tissue.

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