Stimulation of Cell Division by Argon and Nd:YAG Laser Trabeculoplasty in Cynomolgus Monkeys

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Although laser treatment of the trabecular meshwork is the most common form of surgery for glaucoma, the tissue response to this therapy is still incompletely understood. We applied argon or Nd:YAG laser to the trabecular meshwork of six monkeys. Cell division was identified by injecting tritiated thymidine into the anterior chamber 24 hr after laser application. Autoradiography of tissue sections revealed significantly more labeled cells in eyes treated with laser than in the untreated controls. In addition, cells in neighboring tissues such as iris, ciliary body and sclera showed labeling in association with laser application. Furthermore, comparison of argon-induced lesions with those caused by pulsed Nd:YAG suggests that there are quantitative and qualitative differences in the response of trabecular meshwork and surrounding tissues to these two forms of laser energy. Invest Ophthalmol Vis Sci 31:115-124, 1990

The trabecular meshwork (TM) constitutes the main pathway for conventional aqueous outflow. Some intrinsic or imposed alteration in the function of this tissue is generally considered to be the cause for the open-angle types of glaucoma. Many of the open-angle glaucomas can be successfully treated by applying laser energy to the trabecular meshwork, a procedure known as “trabeculoplasty,” although it is not known that a physical reshaping of the tissue is responsible for the beneficial effects of the treatment.

Wise and Witter1 proposed that laser treatment tightens an abnormally lax meshwork and thereby improves the facility of aqueous outflow. The most likely basis for such an effect would be heat-induced collagen shrinkage; the trabecular meshwork, like other collagen containing tissues, has been found to shrink with heating.2 But Van Buskirk et al3 found no improvement in outflow facility in enucleated human eyes treated with laser trabeculoplasty, even though other methods for physically increasing tension in the meshwork do improve the outflow facility of enucleated eyes.4 Possibly the laser treatment would have been effective if the eyes had been glaucomatous5; alternatively, it is possible that the laser produces its effect through a more complex process in which living tissues respond to laser wounds by altering their function.3

The current study was designed to extend our knowledge of the response of trabecular tissue to laser therapy through a study of acute effects on trabecular cell division in vivo. Two clinical forms of laser therapy—argon laser trabeculoplasty (ALT) and Nd:YAG trabeculoplasty—were studied in cynomolgus monkeys.

Materials and Methods

Animals

Six adult male or female cynomolgus (Macaca fascicularis) monkeys were used in this study. All procedures involving the animals were in accordance with the ARVO Resolution on the Use of Animals in Research.

The animals were anesthetized with intramuscular ketamine (Ketaset, Bristol Lab., Syracuse, NY) 10 mg/kg and xylazine (Rompun, Mobay Corp., Shawnee, KS) 0.5 mg/kg for preoperative evaluation 24 hr prior to laser therapy. A complete examination of the eye was carried out including direct ophthalmoscopy, slit-lamp biomicroscopy, applanation tonometry and gonioscopy to ensure there were no preexisting abnormalities.

Laser Treatments

Laser treatments were administered using either an argon blue-green laser (Synemed Inc., Berkeley, CA) or a Nd:YAG pulsed laser (AMO, Irvine, CA) with the animals anesthetized in the same manner. A Kaufman/Wallow gonioscopy lens (Ocular Instru-
ments, Inc., Bellevue, WA) was used to deliver the applications in all cases. One eye of each animal was randomly selected for treatment with either argon laser (three animals) or Nd:YAG laser (three animals). The opposite eye was an untreated control and was subjected only to gonioscopy.

The protocol for argon laser trabeculoplasty (ALT) included the following specifications: spot size, 50 μm; exposure, 0.1 sec; applications, 50 spots evenly distributed over 360° of the trabecular circumference. The power was adjusted to produce visible blanching of the tissue with or without formation of a single small gas bubble. Applications were made in one of two locations. Following the description of Melamed et al.,6,7 some spots were centered on the pigmented band. But our histologic studies on other similar animals showed that the pigmented band in these eyes is somewhat posterior to its relative position in human eyes. We therefore also placed some burns more anteriorly, in meshwork more directly adjacent to Schlemm's canal.

The Nd:YAG laser trabeculoplasty protocol was as follows: spot size, 20 μm; exposure, 14 nanoseconds; applications, eight to ten spots spaced approximately 200 μm apart in the superior trabecular meshwork; power, 4 mJ. We used a power setting somewhat lower than that used for Nd:YAG trabeculoplasty in human beings because preliminary work had demonstrated that these power levels consistently produced an open channel through trabecular meshwork connecting anterior chamber with Schlemm's canal. The applications were placed just anterior to the dark pigment band so that they would impact directly over Schlemm's canal. The superior chamber angle was treated so that any reflux bleeding would not settle on the lesions.

3H-Thymidine Administration

Twenty-four hours after the first thymidine infusion, the animal was anesthetized with ketamine and then given a lethal intracardiac dose of pentobarbital (Fort Dodge, Fort Dodge, IA). The eyes were rapidly enucleated and wrapped loosely in moist gauze. The eyes were then perfused with fixative at 15 mm Hg via a 23-gauge needle in the anterior chamber. The fixative was 2% glutaraldehyde, 1% formaldehyde, buffered with 0.1 M sodium cacodylate to 7.2 pH. After 30 min, the entire globe, still continuously perfused at 15 mm Hg, was immersed in the fixative solution. After 20–24 hr, the perfusion needle was withdrawn and the eye was placed in 0.1 M cacodylate buffer.

Dissection and Embedment

Each eye was positioned under an operating microscope, and the 12:00 limbus was marked for reference. Next a 5 to 6 mm central corneal button was dissected free and removed. The anterior chamber angle of laser treated eyes was examined under high magnification (×40) to locate the laser-treated sites. A 1 mm wedge of chamber angle containing an identifiable burn was then dissected free from the eye. This wedge of tissue also contained peripheral cornea, sclera overlying the burn, peripheral iris and ciliary body.

At least one wedge was removed from each control eye; for two control eyes a wedge was removed from each quadrant. This resulted in 12 tissue blocks from the control eyes. Seven blocks containing acute Nd:YAG laser lesions were prepared: four from one eye, two from another, and one from the third. Ten blocks containing argon laser lesions were prepared: four from each of two eyes, and two from the third. Prior to embedment, the blocks containing laser lesions were trimmed so that the edge destined for initial sectioning was already one-third to one-half way into a visible burn.

After final trimming of the tissue, the blocks were post-fixed in 2% buffered osmium tetroxide, dehydrated in graded ethanols, and embedded in EPOX 812 epoxy resin (E. Fullam, Inc., Schenectady, NY). Serial 1 μm sections were cut from each block and mounted sequentially in groups of three to six sections on three or four glass slides. Our standard sections were all made with the plane of section carefully oriented perpendicular to the canal of Schlemm. In three instances the plane of section was oriented parallel to Schlemm's canal and the plane of the iris. The slides with attached sections were coated with Ilford K.5 Nuclear Track Emulsion under safelight conditions, placed in a light-tight container and stored under refrigeration for 1 week. The exposed slides
were developed in Kodak D-19, fixed, rinsed and dried, and then stained with 1% toluidine blue buffered with 1% boric acid for 60 sec. The stained slides were rinsed, dried and coverslipped.

The identifying markers on the slides were covered, so that tabulations were made without knowledge of the treatment history. Independent readings confirmed the reproducibility of the counts. The only source of potential confusion was melanin. But these granules, being of different size and color, located in the cytoplasm, and in a different focal plane from the silver grains, were readily distinguishable from silver grains on top of the section over the nucleus. A formal counting rule adopted for the initial study required a minimum grain count of ten over the cell nucleus to consider a cell labelled. Later a smaller sample was reassessed using a counting rule of 20 grains.

Labelled trabecular cells were recorded as being juxtacanalicular, or anterior (to Schlemm's canal), middle (adjacent to Schlemm’s canal) or posterior (to Schlemm's canal) corneoscleral TM. Cells were counted as corneoscleral TM cells if they were in a normal position on a trabecular beam, that is, flattened against the underlying beam and contiguous with neighboring TM cells. If a cell were rounded or if it were not in a typical TM cell relationship to a trabecular beam, it was not counted. Although juxtacanalicular cells do not invest trabecular beams, they were identified on the basis of their morphology, their relationship to surrounding tissues, and, in some instances, a typical functional characteristic—giant vacuoles. Inflammatory cells, which were found in both treated and control eyes, were distinguished from TM cells on the basis of their rounded shape, characteristic cell surface or nuclear morphology, and their limited physical contact with the meshwork, and were not counted.

A mitotic index was calculated for each section by dividing the total number of labelled trabecular cells by the total number of trabecular cells present. Since the total number of trabecular cells did not vary greatly in these sections, we generally determined the total cell count on one section per slide, and used that count for the determination of all sections on the slide. A mean mitotic index was calculated for each tissue block and for each eye in the study. Because the number of animals was small, and since we did not know whether the data were normally distributed, we used a nonparametric statistical test, the Wilcoxon Signed Rank Test.

The primary interest of this study was the behavior of trabecular meshwork cells after exposure to argon or Nd:YAG laser. The preparation of the specimens was therefore designed to give thorough and consistent samples of this tissue. Adjacent tissues were of course present in all sections, but the extent of their representation depended on the dissection. Cornea, sclera, ciliary body and iris, to the degree they were present, were examined in every section, and any labelled cells found in these cells were recorded.

**Results**

Control eyes showed very little trabecular cell labelling. Eight of the 12 control tissue specimens contained no labelled TM cells on any of the sections cut from these blocks. The remaining four control tissue blocks showed infrequent labelling: only 14 of 61 sections from these blocks had one or more labelled cells. And, when labelling was present in control sections, it was sparse. Generally only one cell was labelled; the exceptions were two sections, each with two labelled cells.

Cell labelling was increased substantially in association with both argon (Fig. 1) and Nd:YAG (Fig. 2) laser lesions. Every argon laser tissue block (n = 10) produced some sections with labelled cells (Fig. 3); altogether 62% of sections cut from these blocks had at least one labelled cell. With argon laser-treated tissue, when labelling was present in a cross-section of the TM, the average number of labelled cells was 7.95. Preferential labelling of the anterior TM was not evident in either group.

A mitotic index for each section was calculated, and these data are presented in Table 1 (A and B). A graphic display of the distribution of mitotic indices for each section of each eye is presented in Figure 6. For statistical comparison, the mean mitotic index for each laser-treated eye was compared with the mean index for the paired control. The mean mitotic index was significantly different (P = 0.036), laser greater than control.

Since individual specimens from a single eye are subject to a common environment, considering all specimens from an eye in aggregate (as above) seemed the most appropriate method of comparison. However, some observations also suggested that each laser lesion may represent an independent response. For example, if we continued to cut sections from blocks with laser lesions, we eventually reached tissue well away from the burn center. These adjacent tissues were less actively labelled (or even unlabelled) when compared to the sections taken from the center of the lesion. In the three blocks sectioned parallel to
Schlemm's canal, we found that labelled cells were closely associated with the burn center. Finally, there is a fairly wide variability in laser lesion labelling. While some dramatically high mitotic indices were observed (eg, Table 1B, Observation 9), three of the anterior argon laser burns (Table 1B, Observations 8, 10, 13) produced indices no higher than the most active control tissues—even though other lesions in the same eyes were actively labelled.

Since these observations suggested some autonomy of individual lesions within eyes, we also subjected the data to statistical analysis considering each specimen as an independent observation. The mean mitotic index from each of 12 control blocks was paired
with a laser block from the opposite eye. When there were more laser blocks than control blocks for a given eye pair, the least active laser block was used to be conservative. The laser and control treatment groups were significantly different ($P = 0.0025$). In addition, each type of laser burn was compared to tissues from its paired control eye in the same manner. This analysis showed that both lasers caused significantly greater labelling than controls (both $P = 0.036$). Finally, the two lasers were compared, again using the most conservative blocks, and the Nd:YAG showed significantly more labeling than the argon (Wilcoxon Rank Sum, $P = 0.0163$). A comparison of the two lasers based on randomly selected blocks (rather than
Fig. 5. Nd:YAG laser lesion. Labelled cells are seen on the outer wall of Schlemm's canal (arrows) as well as in the trabecular meshwork. SC = Schlemm's canal. Bar = 100 μm.

least active blocks) showed a less significant difference (P = 0.0782).

Examination of the tissues adjacent to the trabecular meshwork also revealed different patterns of thymidine labelling depending on the treatment. The corneal epithelium was a regular site for labelled cells in all specimens regardless of treatment. Control tissues showed a near total absence of labelling in adjacent tissues other than corneal epithelium. In 136 sections cut from 12 different blocks of untreated control tissue, only one labelled cell—a scleral fibroblast—was found in an adjacent tissue other than corneal epithelium.

By contrast, the sclera external to a Nd:YAG laser lesion commonly showed labelling of scleral fibroblasts (Figs. 4, 7); six of seven Nd:YAG blocks contained labelled cells with a rather uniform distribution of label—61 of 95 sections. Some sections were densely labelled, with as many as ten positive cells. Labelling of scleral fibroblasts in association with argon lesions was less frequent (four of ten blocks) and less dense.

Cell labelling in the iris and ciliary body adjacent to argon lesions was considerably more pronounced than in either controls or Nd:YAG lesions. No control tissues had labelling in the iris, and only one labelled iris cell (a vascular endothelial cell) was found in the Nd:YAG specimens. In contrast, all but one of the ten argon laser blocks showed labelling of iris vascular endothelial cells (Fig. 8); the labelling was quite uniform (89 of 134 sections) and often dense (several sections had more than ten labelled cells).

Labelling of cells in the ciliary body was almost exclusively associated with argon laser lesions. Control tissues had no ciliary body labelling, while three sections from one Nd:YAG block had one labeled cell each. However, five of the ten argon laser blocks (38 of 67 sections) did show some labelling in the ciliary body (Fig. 9), with as many as 14 cells labelled in a section. The labelled cells appeared to be stromal.

Table 1. Specimens examined

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Multiple sections (average 12) were cut from each block of tissue. A mitotic index was calculated for each section; the average of those indices for each block is presented in the righthand column of Table 1. (Table IA, Control specimens; Table IB, Laser-treated Specimens.) The more anterior argon burn placement is represented as ARG(A), the more posterior, ARG(P). Comparison of the mean mitotic index for control eyes vs. laser-treated eyes revealed a significant difference between groups (P = 0.036).
cells, and the most frequent location was somewhat posterior to the scleral spur.

Finally, thymidine labelling was reassessed using a counting rule of 20 instead of ten for reasons detailed in the discussion. The proportion of cells scored as labelled diminished in both the control (by 21%) and lasered (by 39%) groups, however the statistical difference between groups remained significant ($P = 0.036$).

Discussion

Laser trabeculoplasty using either argon or Nd:YAG laser stimulates uptake of tritiated thymidine and presumed cell replication. This method is widely used to mark dividing cells, though it is not absolutely specific, since DNA repair can occur in nondividing cells. We believe the dense labelling found in this study is most adequately explained by thymidine uptake in association with cell replication.

For our 1 μm thick sections we selected, initially, a counting rule of ten or more grains over a nucleus as evidence of S phase DNA synthesis. Bylsma et al. selected 20 grains or more for their 3 μm thick sections, which calculates to 13 grains for a 1 μm thick section. Nuss et al. in a study specifically designed to distinguish between scheduled and unscheduled DNA synthesis, considered 15 grains over nuclei in
2–3 μm thick sections to indicate S phase DNA synthesis; this correlates with 10 grains in 1 μm thick sections, and supports our use of this counting rule. Nonetheless, following the initial analyses of the data we wished to determine the effect produced by a counting rule of 20. Using a small sample of representative sections it was found that this 2-fold change in the minimum grain count did not change the $P$ value of the difference between the labeled nuclei of control and lasered tissue.

In our study, argon laser treatment caused cell division to be maximal in the immediate vicinity of the laser lesion. In a similar study in organ culture, Acott et al.9 also found stimulation of cell division by ALT, but the distribution was more widespread—involving both treated and untreated areas equally. They note that such a widespread effect is consistent with a medium-born regulatory signal for cell replication. The focal activity we found in our study is not inconsistent with this hypothesis. If the lesions themselves produce a diffusible mitotic stimulant(s), their influence would be widely and evenly distributed in an organ culture system. In vivo, on the other hand, factors produced locally in the TM would be carried out of the eye by the normal flow of aqueous humor, allowing little impact on more distal tissues.

We noted no preferential labelling of the anterior TM cells. We found all TM cells capable of incorporating label, and the distribution was highly variable. In organ culture, the initial post-laser labelling was concentrated in the anterior TM.9 This difference may reflect cell migration, species variation or other dissimilarities in the two experiments.

One of the advantages of in vitro culture is the ability to control the environment and thereby eliminate some sources of variability, but clinical treatments such as ALT may trigger any of a wide range of responses operative in the living organism. And while several laboratories have demonstrated trabecular cell replication in culture,10,13 few have addressed the question in vivo.14 For this reason, a further careful comparison of TM response to laser in culture versus in vivo should help clarify which aspects of the response are controlled by neuronal or secondary phys-
The pulsed Nd:YAG laser also produced focal stimulation of trabecular cell division, at least as potently as the argon laser. Organ culture studies using Nd:YAG have not been reported, so comparison is not possible with our labelling results.

Qualitative comparisons of adjacent cornea, sclera, iris and ciliary body were possible, although only a variable amount of these tissues were present. The corneal epithelium was a regular site of labelled cells consistent with its rapid replication rate. Other tissues differed in their labelling, depending upon the treatment the eye received. A strong tendency for labelling of iris vascular endothelium in argon-treated tissues raises the question of endothelial proliferation contributing to the breakdown of the blood-aqueous barrier in ALT. A potential concern raised by this finding is the possibility of long lasting change in the iris vessels making the eye more prone to future blood-aqueous barrier breakdown and/or vasoproliferation. Labelling of ciliary body stromal cells was a prominent feature of some argon-treated eyes, and was rare or nonexistent in the other groups. The consequences of such changes are unclear, but their exclusive association with the argon lesion underscores the variable responses of these tissues to differing stimuli.

The pulsed Nd:YAG laser produced an impressive response in the trabecular meshwork cells. In addition, frequent labelling of scleral fibroblasts external to the impact was observed. Since very few scleral fibroblasts were labelled in the other groups, this response seems to be specific for the Nd:YAG lesion, and may relate to the greater physical disruption caused by this instrument. It seems that the pulsed laser stimulated division almost entirely in its direct path: the primary target, trabecular meshwork, and the sclera behind it. The thermal effect of the argon laser, on the other hand, stimulates the direct target, the trabecular meshwork, but also stimulates iris and ciliary body indirectly. The potential mechanisms for indirect stimulation include heat transfer, neuronal mechanisms, or local hormones or growth factors.

Our studies show that laser stimulation of trabecular cell replication occurs in vivo, and that the stimulus may be either an argon thermal lesion or a pulsed Nd:YAG lesion. Considering the decrease in trabecular cell density reported with age and glaucoma, this response to laser application may be an important factor in producing the benefits of laser trabeculoplasty. Laser stimulation may produce a net increase in trabecular cellularity, and it may even select a population which is inherently more healthy, as Bylsma et al have suggested. On the other hand, trabecular cell proliferation might take the form of a membranous covering of the meshwork with potentially adverse effects on aqueous outflow. Further studies of these replicating cells, especially regarding long-term functional and/or morphologic characteristics, should reveal what role such cellular alterations play in the mechanism of laser trabeculoplasty.

Key words: autoradiography, laser trabeculoplasty, trabecular meshwork, tritiated thymidine, wound healing

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