# Histological Changes and Unscheduled DNA Synthesis in the Rabbit Cornea Following 213-nm, 193-nm, and 266-nm Irradiation

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## ABSTRACT

**PURPOSE:** To examine the acute outcome of corneal irradiation in adult rabbits for 193-, 213-, and 266-nm laser wavelengths.

**METHODS:** Animals were randomly allocated to one of three groups and were treated with 213-nm quintupled Nd:YAG laser, a 193-nm excimer laser, or a 266-nm quadrupled Nd:YAG laser (n=6 per group, two exposure durations). Thermal damage was assessed histologically and the extent of DNA damage estimated by measuring unscheduled DNA synthesis in corneal epithelial and stromal cells using $^{3}H$ thymidine autoradiography.

**RESULTS:** For the 193- and 213-nm groups, irradiation did not induce thermal damage. Moreover, cells displaying unscheduled DNA synthesis represented <4% of the total cell numbers with no difference between groups ($P>.05$). By contrast, the 266-nm laser led to stromal melting and vacuolation; unscheduled DNA synthesis levels were elevated over the other groups ($P<.05$).

**CONCLUSIONS:** Corneal laser ablation with the 213-nm Nd:YAG laser resulted in similarly low levels of thermal and DNA damage to those produced using the clinically accepted 193-nm excimer laser. [*J Refract Surg.* 2007;23:477-481.]


The argon-fluoride 193-nm excimer laser is a widely used and innovative tool in therapeutic and refractive corneal surgery. It achieves precise photoablation of corneal tissue with minimal thermal damage to adjacent tissue structures.[1-3] However, the requirement for fluorine gas to generate the beam gives rise to health and safety concerns. The laser itself is also bulky and requires frequent maintenance checks.[4,5]

To circumvent many of these disadvantages, there has been a growing interest in the development of Nd:YAG 213-nm (5th Harmonic) solid-state laser technologies. Preliminary corneal ablation studies indicate minimal tissue damage and smooth surfaces, outcomes comparable to those obtained with the 193-nm ArF-excimer laser.[4,6] Moreover, the homogenous beam with a stable energy distribution and small spot size enables high precision corneal sculpting.[7] Faster pulse repetitions (300 to 400 Hz) reduce ablation times, which, in combination with a small beam size, may reduce long-term consequences of thermal damage, including haze. The laser has been successfully used for human corneal surgery[8,9] and outcomes have been good with no complications observed in follow-up studies for up to 12 months,[10] with longer-term studies currently underway.

However, for the 213-nm Nd:YAG laser to be clinically acceptable, its ultraviolet-induced carcinogenic and mutagenic potential must be assessed. A significant consequence of laser

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TABLE 1

**Pulse Count Conditions for Each Wavelength**

| Wavelength | Fluence ($mJ/cm^2$) | Pulse Count | |
|---|---|---|---|
| | | Exposure 1 | Exposure 2 |
| 193 nm | 180 | 400 | 800 |
| 213 nm | 150 | 300 | 450 |
| 266 nm | 150 | 300 | 600 |

irradiation with wavelengths between 220 and 320 nm is DNA damage,[3,11] including cyclobutyl pyrimidine dimers, single-strand DNA chain breaks, and DNA-protein cross-links.[12,13] Outcomes include mutagenesis, carcinogenesis, and cell death.[5,14,15] Damage occurs when DNA absorbs light wavelengths and is maximal at the peak absorbances of 190 and 260 nm.[15] In vitro and in vivo studies suggest a lower rate of DNA damage for the 193-nm wavelengths compared to the deleterious 243- or 254-nm wavelengths.[1,3] However, the only study of DNA damage following 213-nm solid-state laser irradiation reported in vitro levels intermediate between those produced in vivo by gas excimer 193-nm and 248-nm laser ablation.[16] However, the experiments were compromised by the use of an early version of the solid-state laser that likely produced a range of wavelengths. In addition, results are difficult to interpret in the context of corneal surgery because of the use of in vitro models.

This study reports the results of corneal ablations in vivo in rabbits. A well-characterized 213-nm wavelength solid-state laser free of contaminating wavelengths was used; 193-nm excimer and 266-nm wavelength lasers served as controls. Thermal damage was assessed by light microscopy. To provide optimal assessment of DNA damage, $^3$H thymidine autoradiography was used to assess the extent (unscheduled DNA synthesis) per cell, reflecting DNA repair.[3] The studies showed no difference between 213- and 193-nm wavelengths for any of the parameters tested.

## MATERIALS AND METHODS

### Animals and Laser Irradiation

Pigmented half-lop rabbits (n=18) were maintained in the Animal Care Unit (UWA) and provided with food and water ad libitum. Procedures conformed to the National Health and Medical Research Council (Australia) guidelines and were approved by the institutional Animal Ethics Committee. Animals were randomly allocated to one of three groups and were treated with a 213-nm quintupled Nd:YAG laser, a 193-nm excimer laser, or a 266-nm quadrupled Nd:YAG laser source (n=6 per group). One animal from each group was used to establish ablation conditions for each laser: number of pulses required to perforate the cornea and optimal ablation rate (fluence). Two pulse conditions were used: high and low and were selected so as to produce a similar ablation depth (Table 1). The five remaining rabbits in each group received 300-µm slit ablations in each eye and a low (left eye) and high (right eye) pulse count at each wavelength (Table 1). Fluence of the 193- and 213-nm lasers was chosen based on clinical parameters.[8,17] For the 266-nm laser where no clinical equivalent exists, ablations were performed at 150 $mJ/cm^2$ so as to be comparable to the 213-nm laser. In addition, the wavelength was inefficient at ablating tissue at any attempted fluence.

### Tissue Preparation and $^3$H Incorporation

Animals were anesthetized by intravenous inoculation of 3 mL of 0.4 mL Rompin/mL Ketamine (Ilium Veterinary Products, Smithfield, Australia), and then euthanized by lethal injection of 3 mL Lethobarb (Virbac, Peakhurst, Australia). Immediately after ablation, eyes were enucleated and the cornea dissected and temporarily floated in Dulbecco's modified eagle's medium (Invitrogen, Grand Island, NY) at 37°C. The cornea was then incubated in Dulbecco's modified eagle's medium in 10% fetal calf serum (Invitrogen, Mt Waverly, Australia) with $^3$H-thymidine (final activity 20 µCi/mL) at 37°C and 5% $CO_2$ for 2 hours. The mean time interval between laser ablation and the start of incubation was 1.25 hours.

Following incubation, corneas were thoroughly rinsed in phosphate buffered saline, fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), and stored at 4°C for 3 days. The slit zone was cut down to 3 $mm^2$ pieces, dehydrated through a series of ethanols, and embedded in Epon 812 resin (ProSciTech, Queensland, Australia). Thirty non-serial 2-µm sections were cut transverse to the lesion and placed on clean, gelatin subbed glass slides.

### Autoradiography

Slides were dip-coated in NTB-2 emulsion (Kodak, Collingwood, Australia) at 43°C. Excess emulsion was drained off and wiped from the back of the slide. Slides were placed on a cooled stainless steel tray for 20 minutes, laid flat at room temperature for 1 hour, and stored overnight in lightproof boxes containing silica gel (Ajax, Chemicals, Bibra Lakes, Australia). On the following day, a fresh batch of silica gel was placed in the boxes and stored at 4°C for 2 weeks. After 2 weeks,

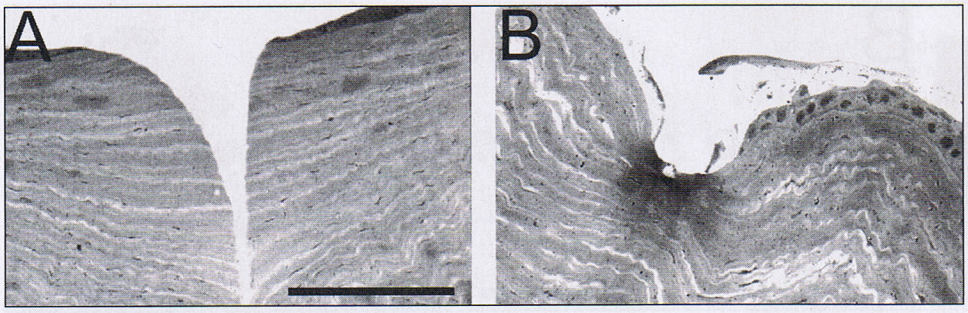



**Figure 1.** Photomicrograph of toluidine blue stained corneal section through the ablation zone for **A)** 213-nm and **B)** 266-nm wavelengths. No evidence of thermal damage is visible in the 213-nm ablated tissue, whereas significant tissue undulation and vacuolation is apparent in the 266-nm ablated tissue. Scale bar: 300 μm.

sections were developed in D-19 developer (Kodak), washed in distilled water, fixed in rapid fixer (Kodak), washed in running water, stained with toluidine blue, and viewed under a light microscope.

### Cell Counts

Epithelial cells and keratocytes were counted within 100 μm from the ablated edge, and 100 μm deep from the base of the ablation. Grossly damaged cellular fragments bordering the lesion edge were not included. Background $^3$H thymidine labeling was estimated as the average number of intranuclear grains within cells encompassing a 50 μm$^2$ area located >500 μm outside of the lesion. Non-labeled cells were defined as containing a number of grains equal to or less than background levels. Sparsely labeled cells represent cells undergoing unscheduled DNA synthesis and were defined as containing intranuclear grains above background levels and up to 15 intranuclear grains per cell. Heavily labeled cells are not indicators of DNA damage but are thought to be spontaneously dividing cells and were defined as those containing >15 intranuclear grains per cell.[3] To normalize for variation between animals, sparsely labeled cells were expressed as a percentage of the total number of cells within the counting region (sum of sparsely labeled cells + heavily labeled cells + non-labeled cells). The mean percentage and standard deviation (SD) for epithelial cells and keratocytes was computed for each group. Analysis of variance (Dunnett's post-hoc test) was performed to evaluate differences in the mean percent of sparsely labeled cells for each group.

## RESULTS

### Thermal Effects

Light microscopic examination of all samples revealed a V-shaped ablation extending through the corneal epithelium and deep into the stroma (Fig 1A). The edges of the ablation zone in corneas ablated with the 193- and 213-nm lasers appeared uniform with no evidence of stromal melting, although the base of the ablation consistently showed undulations. In comparison, in corneas ablated by the 266-nm laser, stromal collagen appeared irregular and vacuolated, suggesting thermal damage to the tissue at the edges and base of the ablation zone (Fig 1B).

### Quantification of Unscheduled DNA Synthesis

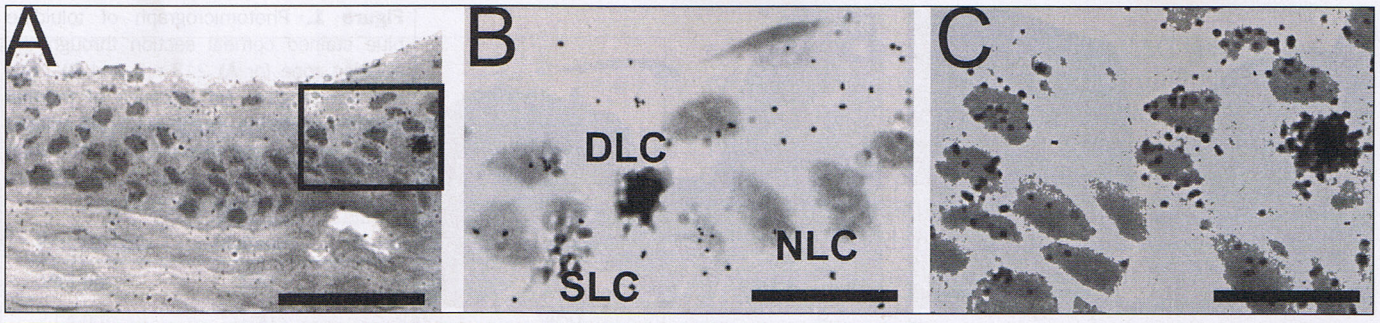
Background numbers of intranuclear $^3$H thymidine grains were between 4 and 5 for all samples. Grains were randomly distributed between nuclear and non-nuclear elements, supporting a nonspecific origin. In all sections, the region of interest surrounding the ablation site contained non-labeled cells (<5 grains/cell), sparsely labeled cells (6 to 15 grains/cell), and heavily labeled cells (>15 grains/cell). No significant differences were seen in the percentage of sparsely labeled cells in the high and low pulse count groups (Student *t* test; $P>.05$), and values were therefore pooled within laser groups (Fig 2).

The average percentages of sparsely labeled cells were 3.39% (SD 2.8) for 213-nm, 3.4% (SD 2.9) for 193-nm, and 29.41% (SD 8.61) for 266-nm ablation (Table 2). No significant difference was noted in the percentage of sparsely labeled cells for 213- and 193-nm ablation ($P>.05$). However, the percentage of sparsely labeled cells in 266-nm ablations was significantly higher than in 213- and 193-nm ablations ($P<.05$).

## DISCUSSION

In an acute in vivo rabbit model of corneal laser surgery, the solid-state 213-nm (5th Harmonic) Nd:YAG laser has similar tissue ablation characteristics to those of the clinically accepted 193-nm wavelength excimer laser. Moreover, both wavelengths induce comparably low levels of DNA damage, resulting in <4% of corneal cells undergoing unscheduled DNA synthesis.

Our results, showing an absence of significant thermal damage using 193- or 213-nm lasers, match previous in vitro and in vivo animal studies showing excellent outcomes for both lasers, with minimal scarring/undesirable histological outcomes and good healing.[4,6,18,19] Our results also confirm previous study results showing significant tissue damage using the higher 266-nm wavelength.[5] Transmission electron microscopy and longitudinal studies of corneal haze



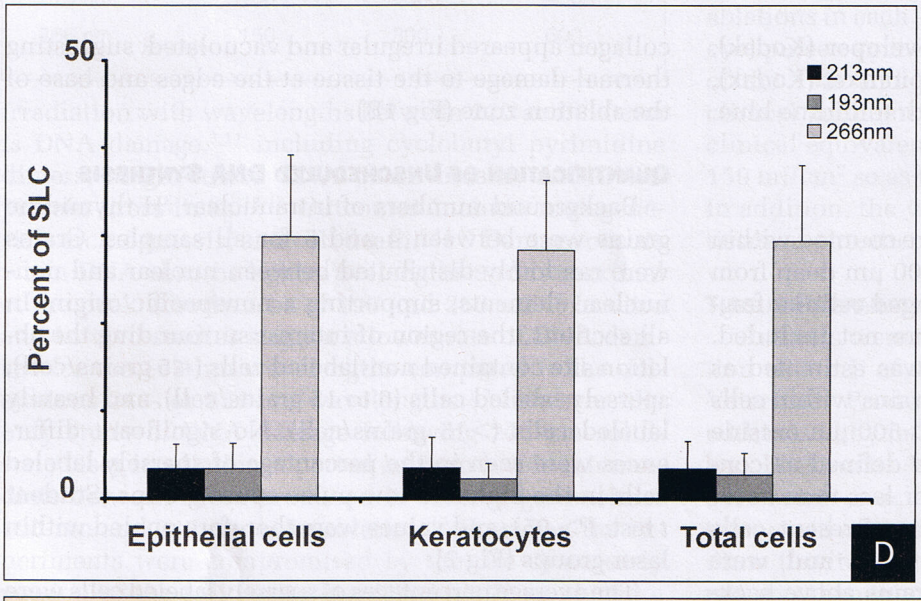




**Figure 2.** Photomicrographs of $^{3}H$ autoradiography on corneal tissue counterstained with toluidine blue. **A)** Low power panel is for orientation, boxed area is magnified in (C). High power panels show sections of **B)** 213-nm and **C)** 266-nm ablated corneas following autoradiography procedure. Non-labeled cells (NLC) (<5 grains), sparsely-labeled cells (SLC), and densely labeled cells (DLC) are indicated in (B). **D)** Histogram shows percent of sparsely labeled cells of the total number of epithelial cells, keratocytes, and total number of cells in the counting zone for 193-, 213-, and 266-nm wavelengths. Scale bars: A, 50 μm; B,C: 10 μm.

TABLE 2

**Percent of Sparsely Labeled Cells of Epithelial Cells, Keratocytes, and Total Cells Counted**

| | Wavelength | | | | | |
|---|---|---|---|---|---|---|
| | 193 nm | | 213 nm | | 266 nm | |
| | % SLC | SD | % SLC | SD | % SLC | SD |
| Epithelial cells | 3.401631 | 2.916549 | 3.394153 | 2.809972 | 29.88041 | 9.285443 |
| Keratocytes | 2.240129 | 1.75246 | 3.492312 | 3.499725 | 28.36727 | 7.924869 |
| Total cells | 2.759972 | 2.458026 | 3.436311 | 3.147517 | 29.40915 | 8.615575 |

*SLC = sparsely labeled cells, SD = standard deviation*

development are currently underway in our laboratory to further elucidate the long-term outcomes of corneal ablation using the 213-nm laser.

Regarding DNA damage, our results complement those of others showing that 213- and 193-nm lasers induce negligible levels of unscheduled DNA synthesis and do not cause significant mutagenic or oncogenic tissue changes.[1,2,15,20-22] The low impact at 193-nm wavelength is thought to be due to cytoplasmic membrane components shielding the nucleus at these wavelengths, thus limiting the photon dose reaching the cellular DNA.[1,2,20,22-24] Presumably this mechanism also accounts for the low impact of the 213-nm wavelength.

The present results contrast with an in vitro study of hamster lung and human corneal fibroblasts by Kaido et al,[16] suggesting that the 213-nm is more cytotoxic and mutagenic than the 193-nm laser. However, the cells were covered by a thin fluid layer during irradiation and the subablative fluences applied were too low to cause any evaporation. Wavelength transmission

through fluid is four orders of magnitude (10,000×) more efficient for the 213-nm compared to the 193-nm wavelength.[25] Therefore during the experiment, cells exposed to the 213-nm wavelength effectively received significantly higher doses of radiation. In the present study, in vivo ablation of rabbit corneas closely mimicked the clinical situation, suggesting that a clinically relevant dose of solely 213-nm wavelength does not induce DNA damage in vivo.

An additional concern with the study by Kaido et al[16] was that the delivery system for the 213-nm radiation may have contained contaminating 266-nm radiation. Along with others, we have shown significant DNA damage following irradiation with 266 nm, reflecting DNA's peak absorbance between 240 and 270 nm.[26] In contrast, the solid-state laser used in the present study incorporated a double prism mechanism with beam blocks, preventing any 266-nm wavelength from reaching the eye.

The 213-nm Nd:YAG laser and the clinically accepted 193-nm excimer laser result in equivalent acute cellular outcomes in vivo. However, additional advantages of the solid-state 213-nm lasers, including lower thermal effects[25] and higher precision,[8] suggest that the laser may produce a better chronic outcome.[8-10] A further benefit is the improved operator handling and safety. The 213-nm solid state laser has the potential to become a powerful therapeutic tool for refractive surgery.

## REFERENCES


1. Green H, Boll J, Parrish JA, Kochevar IE, Oseroff AR. Cytotoxicity and mutagenicity of low intensity, 248 and 193 nm excimer laser radiation in mammalian cells. *Cancer Res.* 1987;47:410-413.
2. Lubatschowski H, Kermani O, Otten C, Haller A, Schmiedt KC, Ertmer W. ArF-excimer laser-induced secondary radiation in photoablation of biological tissue. *Lasers Surg Med.* 1994;14:168-177.
3. Nuss RC, Puliafito CA, Dehm E. Unscheduled DNA synthesis following excimer laser ablation of the cornea in vivo. *Invest Ophthalmol Vis Sci.* 1987;28:287-294.
4. Gailitis RP, Ren QS, Thompson KP, Lin JT, Waring GO III. Solid state ultraviolet laser (213 nm) ablation of the cornea and synthetic collagen lenticules. *Lasers Surg Med.* 1991;11:556-562.
5. Rasmussen RE, Hammer-Wilson M, Berns MW. Mutation and sister chromatid exchange induction in Chinese hamster ovary (CHO) cells by pulsed excimer laser radiation at 193 nm and 308 nm and continuous UV radiation at 254 nm. *Photochem Photobiol.* 1989;49:413-418.
6. Ren Q, Simon G, Legeais JM, Parel JM, Culbertson W, Shen J, Takesue Y, Savoldelli M. Ultraviolet solid-state laser (213-nm) photorefractive keratectomy: in vivo study. *Ophthalmology.* 1994;101:883-889.
7. Guirao A, Williams DR, MacRae SM. Effect of beam size on the expected benefit of customized laser refractive surgery. *J Refract Surg.* 2003;19:15-23.
8. Anderson I, Sanders DR, van Saarloos P, Ardrey WJ IV. Treatment of irregular astigmatism with a 213 nm solid-state, diode-pumped neodymium:YAG ablative laser. *J Cataract Refract Surg.* 2004;30:2145-2151.
9. Roszkowska AM, Korn G, Lenzner M, Kirsch M, Kittelmann O, Zatonski R, Ferreri P, Ferreri G. Experimental and clinical investigation of efficiency and ablation profiles of new solid-state deep-ultraviolet laser for vision correction. *J Cataract Refract Surg.* 2004;30:2536-2542.
10. Roszkowska AM, De Grazia L, Ferreri P, Ferreri G. One-year clinical results of photorefractive keratectomy with a solid-state laser for refractive surgery. *J Refract Surg.* 2006;22:611-613.
11. Jones CA, Huberman E, Cunningham ML, Peak MJ. Mutagenesis and cytotoxicity in human epithelial cells by far- and near-ultraviolet radiations: action spectra. *Radiat Res.* 1987;110:244-254.
12. Lerman S. Biological and chemical effects of ultra violet radiation. In: Lerman S, ed. *Radiant Energy of the Eye.* New York, NY: Macmillan Publishing Co; 1980:115-131.
13. Takakura K, Ishikawa M, Hieda K, Kobayashi K, Ito A, Ito T. Single-strand breaks in supercoiled DNA induced by vacuum-UV radiation in aqueous solution. *Photochem Photobiol.* 1986;44:397-400.
14. Feld MS, Kramer JR. Mutagenicity and the XeCl excimer laser: a relationship of consequence? *Am Heart J.* 1991;122:1803-1805.
15. Kochevar IE, Walsh AA, Green HA, Sherwood M, Shih AG, Sutherland BM. DNA damage induced by 193-nm radiation in mammalian cells. *Cancer Res.* 1991;51:288-293.
16. Kaido TJ, Kash RL, Sasnett MW, Twa M, Marcellino G, Schanzlin D. Cytotoxic and mutagenic action of 193-nm and 213-nm laser radiation. *J Refract Surg.* 2002;18:529-534.
17. Dougherty PJ, Wellish KL, Maloney RK. Excimer laser ablation rate and corneal hydration. *Am J Ophthalmol.* 1994;118:169-176.
18. Ren Q, Gailitis RP, Thompson KP, Lin JT. Ablation of cornea and synthetic polymers using a UV (213nm) solid-state laser. *IEEE Journal of Quantum Electronics.* 1990;26:2284-2288.
19. Ren Q, Simon G, Parel JM. Ultraviolet solid-state laser (213-nm) photorefractive keratectomy: in vitro study. *Ophthalmology.* 1993;100:1828-1834.
20. Gebhardt BM, Salmeron B, McDonald MB. Effect of excimer laser energy on the growth potential of corneal keratocytes. *Cornea.* 1990;9:205-210.
21. Hieda K, Hayakawa Y, Ito A, Kobayashi K, Ito T. Wavelength dependence of the formation of single-strand breaks and base changes in DNA by the ultraviolet radiation above 150 nm. *Photochem Photobiol.* 1986;44:379-383.
22. Kochevar IE. Cytotoxicity and mutagenicity of excimer laser radiation. *Lasers Surg Med.* 1989;9:440-445.
23. Kochevar IE, Walsh AA, Held KD, Gallo RL, Mirro J. Mechanism for 193-nm laser radiation-induced effects on mammalian cells. *Radiat Res.* 1990;122:142-148.
24. Munakata N, Hieda K, Kobayashi K, Ito A, Ito T. Action spectra in ultraviolet wavelengths (150-250 nm) for inactivation and mutagenesis of Bacillus subtilis spores obtained with synchrotron radiation. *Photochem Photobiol.* 1986;44:385-390.
25. Dair GT, Ashman RA, Eikelboom RH, Reinholz F, van Saarloos PP. Absorption of 193- and 213-nm laser wavelengths in sodium chloride solution and balanced salt solution. *Arch Ophthalmol.* 2001;119:533-537.
26. Kochevar IE. Photobiology: basic science. *Dermatol Clin.* 1986;4:171-179.