Ultrastructure of Collagen Thermally Denatured by Microsecond Domain Pulsed Carbon Dioxide Laser

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Background: Clinical improvement in photodamaged skin after carbon dioxide (CO₂) laser resurfacing is thought to result in part from thermal collagen shrinkage. The presence of such collagen has not been unequivocally demonstrated. To identify and characterize the morphological features of collagen after CO₂ laser exposure, we irradiated ex vivo human facial skin and bovine calcaneus tendon with microsecond domain pulsed CO₂ laser energy and examined specimens for histopathological and ultrastructural changes in collagen.

Observations: In dermis and tendon, 3 zones of collagen structure were apparent on electron microscopy. The first, most superficial zone demonstrated loss of collagen structure. The second zone consisted of admixed normal collagen fibers and thickened collagen fibers. Zone 3 consisted of normal-appearing collagen fibers.

Conclusions: Ultrastructural examination of irradiated collagen revealed distinct morphological zones of denatured collagen fibers. Partially denatured fibers had an increased diameter consistent with lineal shrinkage. Zonal distinction was undetectable by light microscopy. Ultrastructurally, the zones of denatured collagen located above the normal fibers correlated with the zone of altered material seen on light microscopy. These findings suggest that collagen fiber shrinkage does occur after pulsed CO₂ laser irradiation and that this phenomenon contributed, at least in part, to the immediate tissue contraction observed clinically.

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**MATERIALS AND METHODS**

Fresh (<3 hours old) ex vivo facial human skin removed as redundant tissue during reconstruction provided a clinically relevant in vitro model for laser resurfacing and its effects on dermal type I collagen; fresh (<12 hours old) bovine calcaneus tendon served as a concentrated array of essentially pure type I collagen for this study. Fresh specimens were wrapped in saline-moistened gauze and stored at 4°C before irradiation. Room temperature specimens were individually exposed to 1 (tendon and skin) or 2 (skin only) passes each with 3 different CO2 laser systems (UltraPulse 5000C, Coherent Medical, Palo Alto, Calif; TruPulse, Palomar Medical Technologies, Beverly, Mass; and SilkTouch, Sharplan, Allentown, NJ). The laser parameters that we used in this study are shown in the Table. Skin specimens were irradiated perpendicular to the skin surface and cylindrical tendon specimens, once sectioned longitudinally to reveal a flat internal surface, were irradiated perpendicular to the open face so created. Two specimens each were irradiated with 1 or 2 passes using 0% (TruPulse and SilkTouch) or 10% (UltraPulse) pulse overlap and with interpass debridement using a saline-dampened cotton-tipped applicator. Each area was approximately 1.0 cm². Specimens were fixed for LM in 10% buffered formalin, processed in paraffin, and stained with hematoxylin-eosin. Specimens were fixed for transmission electron microscopy (EM) in glutaraldehyde and prepared as previously described.

Briefly, ultrathin (1-µm) sections were cut with a microtome (MT-7, RMC, Tucson, Ariz.), placed on copper grids, and stained with uranyl acetate-lead citrate. The tissue was then examined with a transmission EM (EMU-4, RCA, Camden, NJ).

Tissue welding depends on heat-induced collagen changes. In a laser-welded rat carotid artery, loss of collagen periodicity and increased fibril caliber where fibrils interdigitate at the weld were cited as the structural basis for the laser-induced welding effect. Also, welded rat-tail tendon demonstrates gross tissue contraction. Tang et al. examined collagen structure transitions created by diode laser (830-nm) welds of rat aorta using multiple 8-second laser pulses. Within the welds, collagen fibrils demonstrated a variety of morphological changes. Adjacent collagen fibers might show normal ultrastructural features (diameter, birefringence, and periodicity), intermediate changes (increased diameter with normal periodicity but reduced birefringence), or complete denaturation (loss of recognizable fibrillar structure, periodicity, and birefringence). Moreover, all these changes could occur within single fibers, and when periodicity was observed, fibers always retained a 55-nm regular banding pattern. Tang and colleagues attributed the presence of adjacent normal and denatured fibers to differential thermal stability of different collagen types within the vessel wall and to protection by overlying elastic tissue.

Similar thermal alterations have been observed in cutaneous collagen, but with a more distinctly zonal pattern. Zweig et al. examined the lateral thermal damage along erbium and CO2 laser incisions in murine skin. Both lasers delivered approximately 25 J/cm² within 250-microsecond pulses using an effective beam diameter of 0.36 mm, overlapped by 50%. Two zones of thermally altered collagen were described. Within the “coagulation zone,” collagen fibers were swollen and devoid of optical birefringence and periodicity. Within the “transition zone,” both denatured and normal collagen fibers were intermixed. Birefringence of fibers within the zone was reduced and their periodicity “partly recognizable.” These changes were correlated with those found in murine skin immersed for 160 seconds in a controlled temperature bath. Under these conditions, collagen fiber denaturation began at 57°C; the number and diameter of swollen fibers increased at 65°C (periodicity partly maintained); and fiber coagulation was nearly complete at 73°C. The width of the transition zone caused by the CO2 laser was approximately double that produced by the erbium laser (375 ± 52 µm vs 198 ± 70 µm, respectively).

Common speculation asserts that the zone of shrunken collagen seen immediately after resurfacing human skin acts as a tightened scaffold over which new collagen is laid down in the delayed phase of wound healing. Some believe that this taut scaffold contributes to the final improvement seen months after laser resurfacing. In human laser skin resurfacing, collagen fiber shrinkage has been assumed, but not unequivocally demonstrated. In this study we sought to identify, verify, and characterize by ultrastructural analysis a zone of collagen fiber shrinkage after microsecond domain pulsed CO2 laser exposure.

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**RESULTS**

No gross tissue shrinkage was observed after first passes of CO2 laser irradiation on skin and tendon specimens. With second passes of laser energy, immediate tissue...
shrinkage was evident. Light microscopy after laser resurfacing revealed superficial tissue ablation in the skin and tendon specimens. A layer of denatured collagen, characterized by tinctorial change and loss of birefringence on polarized LM, subtended each laser impact site. In tendon specimens cut in cross section, this layer assumed a scalloped or festooned configuration (Figure 1). In concert with this configuration, undulating variations in the thickness of altered collagen were observed in tendon specimens cut longitudinally, but presumably slightly tangentially to this cross-sectional pattern. The thickness of the layer of altered collagen measured 16 to 120 µm in the skin and 8 to 80 µm in the tendon specimens. While residual thermal collagen damage zone thickness correlated positively with pass number and pulse duration, the small number of specimens prohibited meaningful statistical analysis of this trend. Electron microscopic analysis in both skin and tendon revealed 3 distinct zones of collagen fiber morphological change; these zones were most clearly evident in the densely packed tendinous collagen (Figure 2 through Figure 4). In the most superficial tissue, just subtending the laser impact, zone 1 consisted of homogenous, completely denatured collagen, without discrete fibrillar components. Just below this, zone 2 demonstrated a significant population of less structurally defined collagen fibers of increased diameter interspersed among normal collagen fibers. Where seen in longitudinal section, these zone 1 and 2 fibers of increased diameter also manifested a loss of normal collagen fiber banding periodicity (Figure 4). Finally, ultrastructurally normal collagen fibers were found in zone 3.

The relative thicknesses of zones 1 and 2 were measured on ultrastructural samples of tendon alone. Zone 1 thickness measured approximately 60 and 50 µm, and zone 2 approximately 9 and 15 µm, for single- or double-laser passes, respectively (all lasers). It must be noted that the indistinct zonal margins created in the transition between zones 1 and 2 made measurement difficult. Also, these measurements represent a sampling of a very small area within each specimen. Thus, these measurements should be viewed as estimates.

Measurements of collagen fiber diameter were carried out on tendon specimens, since these morphologi-cal zones were largest and most distinct in these specimens. The total population of collagen fibers measured within zone 2 of the treated tendon had a mean ± SD fiber diameter of 0.24 ± 0.12 µm (n = 912) compared with 0.14 ± 0.07 µm (n = 898) of fibers from the untreated tendon. There was no significant difference in the mean fiber diameter in zone 2 between the 3 laser systems. Visual examination of the fiber structure in zone 2 of the treated tendon revealed 2 subpopulations. Structurally normal fibers, identical to those found in zone 3, had a fiber diameter of 0.15 ± 0.06 µm (n = 491), and thickened fibers had a fiber diameter of 0.35 ± 0.06 µm (n = 421) (Figure 5). Measurements of smaller numbers of fibers in skin specimens revealed similar-caliber fibers in similar subpopulations.

**COMMENT**

Previous studies examining the LM sequelae of CO2 laser resurfacing have recognized changes in the cutaneous extracellular matrix, composed predominantly of type I collagen. While our findings confirm similar changes present by LM, we determined that EM is required to discern the finer morphological alterations in collagen fibers immediately after resurfacing.
Ultrastructural examination of human dermal and bovine tendon collagen subjected to microsecond CO₂ laser pulses did reveal distinct zones of completely and partially denatured collagen fibers, similar to those described by Zweig et al.¹⁷ This gradation of morphological alterations in collagen fibers mirrors that seen in tissue welding¹⁴,¹⁵ and collagen fiber dissolution experiments.⁵-⁹ The increased cross-sectional diameter of partially denatured collagen fibers observed in zone 2 was consistent with linear shrinkage of these fibers. Excessive heating, occurring closest to the laser energy impact, causes the complete fiber denaturation observed in zone 1. In zone 2, some fibers, or certain areas of fibers, absorbed sufficient heat energy to allow relaxation of hydrogen bonding and linear shrinkage of the fibers (partial denaturation). These findings suggest that collagen fiber shrinkage did occur after pulsed CO₂ laser irradiation and that this phenomenon contributed, at least in part, to the immediate tissue contraction seen clinically. Since tendon contains a single collagen type, it is unlikely that the admixture of normal and denatured collagen fibers in zone 2 results from differences in imino acid content. It is perhaps more likely that there are differences in local heat diffusion through the tendon and relative cooling of 1 fiber by heat uptake in an adjacent fiber.

An intact tertiary structure is also necessary for diffraction of plane polarized light (birefringence). Loss of tertiary structural integrity of the triple helix is associated with loss of birefringence, owing to disruption of collagen fibril arrangement in larger collagen fibers, and with tintorial change, owing to exposure of stain-binding sites within the unraveled collagen fiber.¹ Because of the irregular nature of the transition boundary and the inability to simultaneously assess exactly the same tissue by LM and EM, it is difficult to judge whether zones 1 and 2, which are seen distinctly on EM, are both found within the zone of altered dermal matrix that is seen on LM. One can distinguish 10- to 15-µm objects on LM at high power, yet no authors have reported a readily distinguishable zone of intermediate damage. Since disrupted zone 2 fibers would stain differently and manifest altered birefringence, our inability to discern a layer of intermediate degree alteration argues that the zone 2

![Image](image1.png)

Figure 3. Transmission electron micrograph of bovine tendon collagen after carbon dioxide laser irradiation (UltraPulse 5000C, Coherent Medical, Palo Alto, Calif; 1 pass), looking more closely at zones 2 (2) and 3 (3) (original magnification ×40,000; bar = 1 µm).

![Image](image2.png)

Figure 4. Transmission electron micrograph of ex vivo human skin after carbon dioxide laser irradiation (UltraPulse, 1 pass), demonstrating the zone 2 of collagen fibril features in the papillary dermis. Thermal denaturation and separation of basilar keratinocytes have occurred in what would have been zone 1 change. The arrows indicate enlarged zone 2 collagen fibrils. Loss of normal type I collagen banding periodicity is evident in denatured collagen fibers seen in longitudinal section (original magnification ×29,000; bar = 1 µm).

![Image](image3.png)

Figure 5. Collagen fibril diameter before and after treatment of bovine tendon with a high-energy carbon dioxide laser. The number at the top of each bar represents the number of fibrils counted.

![Image](image4.png)

Figure 6. Transmission electron micrograph of ex vivo human skin after carbon dioxide laser irradiation (UltraPulse, 1 pass), demonstrating the zone 2 of collagen fibril features in the papillary dermis. Thermal denaturation and separation of basilar keratinocytes have occurred in what would have been zone 1 change. The arrows indicate enlarged zone 2 collagen fibrils. Loss of normal type I collagen banding periodicity is evident in denatured collagen fibers seen in longitudinal section (original magnification ×29,000; bar = 1 µm).
The role of shrunken collagen fibers in long-term clinical improvement after laser resurfacing has not been delineated

differential tensile strength of completely and partially denatured collagen fibers is unknown. Previous studies have shown that partially denatured collagen does maintain at least 30% of its tensile strength. Our findings show that zone 2 in skin consists of a mixture of partially denatured and normal collagen fibers. Together, these findings suggest that this layer may have enough tensile strength to account for the maintenance of tissue contraction that is seen clinically. The fate of these 2 layers over time has not been established. If the layers are sloughed within days, much as chemically denatured collagen sloughs after chemical peeling, it is difficult to imagine that they could be the source of persistent tissue contraction. Gross tissue contraction seen months after resurfacing may instead be attributable to extracellular matrix remodeling or myofibroblast activity.