TUNEL Assay for Histopathologic Evaluation of Irreversible Chromosomal Damage following Nonablative Fractional Photothermolysis

Jordan P. Farkas, M.D.
James A. Richardson, D.V.M., Ph.D.
John E. Hoopman, C.M.L.S.O.
Spencer A. Brown, Ph.D.
Jeffrey M. Kenkel, M.D.
Dallas, Texas

Background: Fractional photothermolysis is extremely popular in skin rejuvenation and remodeling procedures. However, the extent of thermal cellular injury beyond the borders of the coagulated microcolumns produced with fractional phototherapy is undefined.

Methods: Six abdominoplasty patients were pretreated with the Lux1540 Fractional Erbium device (Palomar, Inc., Burlington, Mass.) at various clinical laser settings. After tissue excision, the panni were immediately biopsied. Biopsy specimens were fixed in formalin, embedded in paraffin, sectioned, and evaluated with the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL) procedure for cellular necrosis/apoptosis. Tissue was sectioned horizontally and longitudinally to help define the depth and distribution of the microcolumns of injury in a three-dimensional plane.

Results: The extent of cellular necrosis/apoptosis at variable depths within the epidermis and dermis was demonstrated successfully with the TUNEL technique. After the Lux1540 treatment, TUNEL-positive nuclei were identified in a vertically oriented fashion that extended from the epidermis into the papillary and reticular dermis, highlighting the areas of injury. The TUNEL-positive nuclei defined lesions that were approximately 175 to 225 μm in diameter and penetrated to variable depths (200 to 900 μm), depending on the fluence used for treatment (18 to 100 mJ).

Conclusions: TUNEL immunofluorescent labeling provided an accurate assessment of cellular damage within and surrounding the microthermal zones of coagulated collagen with respect to column depth and width. Because of its specificity, the TUNEL assay can be a useful adjunct to other histologic stains used to characterize cellular damage and matrix denaturation in skin treated with any fractional ablative or nonablative laser device. (Plast. Reconstr. Surg. 122: 1660, 2008.)

Fractional photothermolysis and fractional laser have revolutionized the development and practice of laser resurfacing and rejuvenation. Since the introduction of the Fraxel 1550 device by Reliant Technologies, Inc. (Mountain View, Calif.), fractional ablative and nonablative technologies have become increasingly more popular. Fractional devices have shown great potential for treating melasma, rhytides, acne scars, hyperpigmentation, photodamage, and various vascular lesions.1–5 The goal of fractional photothermolysis, as described by Manstein et al.,5 is to produce multiple microscopic zones of injury that are surrounded by a large surface area of unaffected tissue. The uninjured tissue surrounding the zones of injury is theorized to accelerate wound healing and lead to improved skin rejuvenation and remodeling.6 Unfortunately, little substantial histologic evidence validating its efficacy or benefit exists in the literature.
The 1540-nm fractional erbium glass laser (Fr1540) (Palomar Medical Technologies, Inc., Burlington, Mass.) is a nonablative laser that is capable of deep tissue penetration.7–11 The Fr1540 uses fractional methodology and distributes microarrays of energy at distinct foci throughout the treated region. The near-infrared wavelength, which is preferentially absorbed by water, allows for the option of treating superficial photo-induced wrinkles or focusing on deeper rhytides extending up to 1 to 2 mm from the skin surface. Minimal absorption by melanin at this wavelength allows deep penetration of photons and thermal energy to a depth between 400 μm and 2 mm.2,3,9–11

There are clinical studies and case reports regarding fractional photothermolysis with some of the newer fractional laser systems such as the Fraxel 1550 device. Coagulated microcolumns after initial injury have been well characterized using hematoxylin and eosin and Masson’s trichrome stains.2–5,12 However, a thorough understanding of the extent of thermal tissue damage beyond the coagulated microcolumns after fractional photothermolysis has yet to be elucidated.

Apoptosis is physiologic cell death caused by an energy-dependent suicide program.13–18 Apoptosis involves specific distinct morphologic, biochemical, and molecular alterations, including chromatin condensation, DNA fragmentation, and cellular collapse.15,16,19 Necrosis indicates morphologic changes related to cell death and spillage of cellular contents into the extracellular space. In contrast to necrosis, apoptotic cells maintain the integrity of their cell membranes, preventing the extravasation of their intracellular contents and thereby avoiding the induction of the cytokine-mediated inflammatory response seen with necrosis.15–17,19–25 Although apoptosis and necrosis have specific distinct morphologic features, the identification of the irreversibly damaged nuclei can be very helpful in determining the extent of tissue injury after ablative and nonablative laser treatments.

The purpose of this study was to investigate the degree of irreversible cellular injury following treatment with a nonablative fractional laser system (Fr1540) in vivo using the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate biotin nick end labeling (TUNEL) method. Apoptotic and necrotic cells were identified with a fluorescent histochemical staining technique that localizes DNA breaks through TUNEL as originally described by Gavrieli et al.26

PATIENTS AND METHODS
Six abdominoplasty patients of the senior author (J.M.K.) were treated with the Lux1540 nonablative fractional laser system (Palomar). The laser treatment was administered to multiple sites over the abdomen, immediately before the surgical procedure. The study was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center. Appropriate informed consent regarding all potential risks, objectives, and technical details were obtained for each participant.

The Fr1540 laser system contained an erbium glass laser with a microlens array that created a periodic assortment of individual microbeams. The microbeams were delivered to the skin through a simultaneously cooled sapphire glass window (17°C). The handpiece contained a 10-mm-diameter window with a 1-mm pitch array (microbeam density = 100 mB/cm²). The laser emitted a 1540-nm wavelength pulse with a well-defined grid of microbeams separated by 1 mm from the center of each individual beam. Energy fluences (18 to 100 mJ) at various pulse widths (10 to 30 msec) were examined following single- and multiple-pass treatments. Multiple passes consisted of re-treating the previously treated spots in a sequential “stamping” fashion at equivalent energy two to three times.

Punch biopsy specimens (8 mm) were obtained from the treated sites following surgical excision of the pannus, approximately 3 to 5 hours after laser treatment. Sections were placed in 10% neutral buffered formalin and placed on a shaker for 24 hours to ensure adequate fixation. After rinsing in 70% ethanol solution, the biopsy specimens were processed, embedded in paraffin, cut in serial horizontal or longitudinal sections (4 to 6 μm), and mounted on poly-L-lysine slides.

Histopathologic Evaluation
TUNEL
The assay was performed using the TUNEL kit from Promega Corp. (Madison, Wis.). Slides were incubated at 56°C for 15 minutes and deparaffinized in xylene, hydrated in graded ethanol solutions, and equilibrated in normal saline for 5 minutes and then in phosphate-buffered saline for an additional 5 minutes. Sections were fixed in 4% paraformaldehyde for 15 minutes and washed in phosphate-buffered saline. Sections were then permeabilized with 20 μg/ml of proteinase K (Promega) for 8 minutes at room temperature and prepared with 1:500 dilution of 10 mg/ml stock from the kit.

Sections were then washed in phosphate-buffered saline and postfixed in 4% paraformalde-
hyde, washed in phosphate-buffered saline, and equilibrated in 100 μl of equilibration buffer provided in the kit. Slides were then incubated flat in a humid chamber for 5 to 10 minutes. The terminal deoxynucleotidyl transferase reaction mix (45 μl of equilibration buffer, 5 μl of nucleotide mix, and 1 μl of terminal deoxynucleotidyl transferase enzyme) was prepared during the equilibration step and protected from light. Fifty microliters of the terminal deoxynucleotidyl transferase reaction mix was applied to each slide. Plastic coverslips were applied to each slide before incubation in a humid chamber protected from light for 1 hour at 37ºC. Slides were washed in 2× sodium saline citrate (Promega), rinsed and washed in phosphate-buffered saline, and counterstained with propidium iodide (Invitrogen Molecular Probes, Eugene, Ore.). Slides were then washed in double distilled water and coverslipped with Vectorshield (Vector Laboratories, Burlingame, Calif.).

**Fluorescent Microscopy**

Slides were evaluated using fluorescence excitation microscopy. TUNEL-positive nuclei demonstrated a bright green fluorescence using the approximately 470-nm (fluorescein isothiocyanate) fluorescence filter. Mouse thymus was used as the positive control. Review and photography of all histologic preparations were carried out on a Leica DM2000 photomicroscope (Leica Microsystems, Inc., Bannockburn, Ill.) equipped with bright field, epifluorescence, and incident angle darkfield illumination. All sections were reviewed with a board-certified pathologist.

The depths of the microcolumns of injury for the sections stained with hematoxylin and eosin and TUNEL were measured with a standardized ocular reticule micrometer by two blinded observers. Means and standard deviations were recorded and plotted using a standard software program (Excel, 2003; Microsoft Corp., Redmond, Wash.). A greater number of data points were collected for clinically relevant energy settings (50 to 65 mJ).

**RESULTS**

After treatment with the Fr1540 device on the abdominal skin, an obvious pattern of histopathologic injury was observed. The single-pass laser treatments demonstrated distinct microcolumns of thermal damage that were clearly defined by TUNEL-positive nuclei. Each lesion measured approximately 150 to 225 μm in diameter at the epidermis and tapered in a conical fashion into the papillary or reticular dermis. The individual microcolumns were distributed uniformly over the treated surface with 800 to 1000 μm of unaffected tissue between lesions (Fig. 1). TUNEL-positive nuclei were observed following all Fr1540 treatments. Energy settings of 25 to 30 mJ affected the nuclei of fibroblasts that were identified 400 μm from the skin surface, whereas fluences of 90 to 100 mJ damaged deeper nuclei up to 1000 μm deep. Treatment energies were related directly to the depth of the TUNEL-positive nuclei identified within the dermis (Fig. 2). The microcolumn depths for tissue stained with both TUNEL and hematoxylin and eosin tissue were measured and

![Fig. 1. Sections of skin at low and higher magnification (10× and 20×) demonstrating the TUNEL-positive nuclei, highlighting the microcolumns of injury. Each lesion measured approximately 150 to 225 μm in diameter at the epidermis and tapered in a conical fashion into the papillary or reticular dermis. The individual microcolumns were distributed uniformly over the treated surface and separated by 800 to 1000 μm of unaffected tissue between lesions (left, 68 mJ/15 msec; right, 90 mJ/15 msec).](image-url)
evaluated. Linear relationships between energy and depth of injury were observed with both stains but were not significantly different (Fig. 3).

The TUNEL-positive cells highlighted the vertical microcolumns of injury passing from the epidermis through the dermal-epidermal junction and into the underlying dermis to various depths. When compared with the epidermis, fewer TUNEL-positive nuclei were identified in the papillary and reticular dermis on account of its hypocellular nature. However, the TUNEL-positive nuclei of fibroblasts in the dermis delineated the affected areas from the surrounding uninjured cells. The microcolumns of injury were separated from one another by regions of unaltered TUNEL-negative cells in the epidermis and dermis. However, some of the affected nuclei within the microcolumns of injury were TUNEL negative.

At higher magnification, morphology of the TUNEL-positive nuclei was characterized by fragmented, pyknotic, or streamed bright green fluorescent nuclei. The nuclei of the stratum lucidum and granulosum within the affected microcolumns did not demonstrate a positive TUNEL signal, in contrast to the cells of the stratum spinosum and stratum basale (Fig. 4).

Horizontal sections were examined to evaluate the spatial relationship and diameter of the microcolumns of injury. However, identification of the pattern of injury was challenging because of the hypocellularity of the dermis. Areas of injury were difficult to localize and indeterminate. Thermal injury was clear when the laser energy beam affected a resident sebaceous gland, hair follicle, or blood vessel within treated dermis (Fig. 5).

With multiple-pass treatments, a random non-uniform pattern of damage separated by variable distances of unaffected skin was observed. As opposed to the relatively narrow, well-delineated columns of injury seen with a single pass, multiple...
passes resulted in large areas of TUNEL-positive nuclei and overlapping of the microcolumns of injury (Fig. 6).

The TUNEL method provided details regarding the acute cellular damage profile that was not possible with hematoxylin and eosin and Masson’s trichrome. Although these stains provided information regarding collagen denaturation and epidermal disruption, the TUNEL-labeled nuclei defined the distinct borders of the microcolumns and the depth of the cellular injury into the dermis (Fig. 7). However, as mentioned previously, the penetration of the coagulated columns was not always consistent with the TUNEL-positive nuclei. Some areas of treated tissue with obvious coagulation did not elicit a TUNEL-positive signal in the surrounding nuclei. It should be noted that the hypocellularity of the dermis sometimes makes it difficult to definitively conclude the true depth of injury with the TUNEL method.
DISCUSSION

When assessing thermal injury in human skin, standard histologic staining methods, such as hematoxylin and eosin and Masson’s trichrome, leave room for subjective interpretation with regard to extent of tissue injury and identification of borders of the regions of tissue damage. Subtleties in tincture of dyes and morphologic alterations of the tissue make it difficult to draw confident conclusions regarding the extent of laser-induced injury. Using an in vivo human model, the TUNEL method is helpful in defining the acute damage profile following laser treatments. Tangible evidence of cellular injury helped to decrease some of the ambiguity of defining the laser-induced skin injury.

With the constant influx of new laser treatment devices into the marketplace, a histologic verification of laser-induced injury is essential. Optimizing clinical settings for a given laser device is difficult if the acute damage profile for a given laser system in vivo at various parameters has not been defined. The TUNEL method provides insight into tissue response to the optical energy at the cellular level and a more detailed understanding of the total affected surface area and damage profile after laser treatment than is possible with common histologic stains.

This study is not without limitations. First, the Fr1540 device is commonly used to treat photodamage, or pigmentation, of facial skin. Our in vivo histologic results were collected from the abdominal pannus of abdominoplasty patients that did not have any of these pathologic skin changes. Second, abdominal skin differs morphologically from human facial skin. Facial skin has a thinner epidermis and a greater density of hair follicles and sebaceous glands, and is considerably more vascular than abdominal skin. These different anatomical characteristics may account for a significantly different damage profile than that demonstrated with this in vivo abdominal skin model. Further study in vivo on human facial skin is currently underway.

It must be emphasized that this is not a clinical article. Clinically, most treatment regimens consist of multiple passes per treatment with anywhere from four to eight treatment sessions over a 4- to 6-month period. The results reported here follow a single treatment session on the abdomen with only one to three passes of the handpiece. The acute damage profile may be considerably different after each treatment session throughout the wound-healing period.

CONCLUSIONS

The increasing popularity of fractional technology in ablative and nonablative laser treat-
Fig. 6. Sections of skin demonstrating the wide area of TUNEL-positive nuclei after a multiple-pass treatment. (Left) Section treated with 58 mJ/15 msec with three passes and (right) section treated with equal energy with five passes of the handpiece. Significantly wider regions of TUNEL-positive nuclei were recognized within the multiple-pass–treated areas and fewer unaffected cells were identified between the microcolumns of injury, as opposed to the relatively narrow microcolumns and wider regions of unaffected cells seen with the single-pass treatments.

Fig. 7. Sections of skin stained with hematoxylin and eosin (left) and the corresponding TUNEL-labeled section (right). The borders of the coagulated columns of collagen are less distinct in the hematoxylin and eosin sections than in the corresponding TUNEL sections. The TUNEL method highlights the cellular injury surrounding the treated region and assists in the evaluation and delineation of the injured area when used in concert with the hematoxylin and eosin sections (50 mJ/15 msec; above, 10×; below, 20×).
ments has produced many unanswered questions. Fractional photothermolysis is dependent on the preservation of the unaffected regions of skin between thermally damaged areas to potentially accelerate the wound-healing process and lead to a shorter recovery period.\textsuperscript{5,6} A more extensive histopathologic evaluation of the wound-healing process following these clinical fractional treatments over time is needed. Once an accurate assessment of acute tissue injury following the laser treatment has been established, future studies evaluating the skin reaction to multiple treatments, wound healing, neocollagenesis, and skin remodeling can be pursued.

Because fractional technology is still in its early developmental phases, certain laser parameters such as dosing, timing, energy, pulse width, and microbeam density for optimal collagen remodeling and restructuring for the various fractionated laser systems have yet to be defined. The TUNEL method may be a useful adjunct when used with other commonly used histologic staining methods to evaluate acute tissue injury, providing detailed information with regard to the acute cellular damage profile within the skin after laser treatment that is not available with hematoxylin and eosin or Masson’s trichrome staining. An in-depth histopathologic evaluation of acute skin reaction and injury following fractional laser treatments will lead to appropriate tailoring of treatments, consistency, reproducibility, improved clinical outcomes, and higher overall patient satisfaction with fractional laser skin surgery. To improve the efficacy and reproducibility of fractional nonablative treatments, further investigation into laser tissue interaction and wound healing is needed.

\textbf{Jeffrey M. Kenkel, M.D.}

Department of Plastic Surgery
Clinical Center for Cosmetic Laser Treatment
University of Texas Southwestern Medical Center
at Dallas
1801 Inwood Road
Dallas, Texas 75390-9163
jeffrey.kenkel@utsouthwestern.edu

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**Instructions for Authors: Update**

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Beginning in July of 2007, *PRS* has required all articles reporting results of clinical trials to be registered in a public trials registry that is in conformity with the International Committee of Medical Journal Editors (ICMJE). All clinical trials, regardless of when they were completed, and secondary analyses of original clinical trials must be registered before submission of a manuscript based on the trial. Phase I trials designed to study pharmacokinetics or major toxicity are exempt.

Manuscripts reporting on clinical trials (as defined above) should indicate that the trial is registered and include the registry information on a separate page, immediately following the authors' financial disclosure information. Required registry information includes trial registry name, registration identification number, and the URL for the registry.

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- http://www.trialregister.nl/trialreg/index.asp (Netherlands Trial Register)
- http://www.umin.ac.jp/ctr (UMIN Clinical Trials Registry)