Intraepidermal erbium:YAG laser resurfacing
Impact on the dermal matrix

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Background: Various minimally invasive treatments enhance the skin’s appearance. Little is known about the molecular mechanisms whereby treatments working at the epidermal level might alter the dermis.

Objective: We sought to quantify the molecular changes that result from erbium:yttrium-aluminium-garnet (Er:YAG) laser microablative resurfacing.

Methods: We performed biochemical analyses after intraepidermal Er:YAG laser resurfacing of 10 patients. Immunohistochemical analysis and polymerase chain reaction technology were utilized to measure key biomarkers.

Results: The basement membrane remained intact after intraepidermal microablation, as demonstrated by laminin γ2 immunostaining. Epidermal injury was demonstrated with acute up-regulation of keratin 16. An inflammatory response ensued as indicated by increases in cytokines interleukin 1 beta (IL-1β) and IL-8 as well as a substantial neutrophil infiltrate. Levels of cJun and JunB proteins, components of the transcription factor AP-1 complex, were also elevated. Up-regulation of extracellular matrix degrading proteinases matrix metalloproteinase 1 (MMP-1), MMP-3, and MMP-9 was noted. A transient increase in keratinocyte proliferation, as indicated by staining for Ki67, was observed. Increased expression of type I and type III procollagen was demonstrated.

Limitations: The data presented are those that resulted from a single treatment session.

Conclusions: Although microablation was confined to the uppermost epidermis, marked changes in epidermal and dermal structure and function were demonstrated after Er:YAG laser microablative resurfacing. We demonstrated substantial dermal matrix remodeling, including a degree of collagen production that compares favorably with some more invasive interventions. Dermal remodeling and stimulation of collagen production are associated with wrinkle reduction. Thus these results suggest that the skin’s appearance may be enhanced by creating dermal changes through the use of superficially acting treatments. (J Am Acad Dermatol 2011;64:119-28.)

INTRODUCTION

In recent years, there has been intense interest in developing increasingly less invasive techniques to improve the clinical signs of photoaging. Many nonablative laser systems have been utilized with these goals in mind.1-3 Although nonablative lasers have an impressive safety profile because the

Abbreviations used:
AP-1: activator protein 1
Er:Yag: erbium-yttrium-aluminium-garnet (laser)
IL: interleukin
KRT: keratin
MMP: matrix metalloproteinase
epidermis of treated skin generally remains intact, clinical results have been modest in terms of textural improvement. Alternatively, while traditional ablative laser resurfacing is, in many respects, the gold standard for facial rejuvenation, the procedure is fraught with the risk of significant complications and results in substantial down time.\textsuperscript{4-6}

To mitigate against the potential drawbacks of procedures that result in complete epidermal ablation, lower powered ablative erbium:yttrium-aluminium-garnet (Er:YAG) lasers have recently been used in an attempt to improve the appearance of human skin while largely preserving epidermal integrity. Superficial Er:YAG laser resurfacing employs technology with a long track record of efficacy now modified to produce a minimally invasive treatment. With respect to improving the uniformity of skin texture, this strategy theoretically relies on the ability of an intervention that acts primarily through intraepidermal injury to drive dermal extracellular matrix changes. To date, no study has produced detailed quantification of the dermal alterations that may result from such intraepidermal damage. Demonstration that controlled, superficial cutaneous injury results in significant dermal changes could result in the development of a paradigm shift in treatment strategies for photoaging. We thus sought to assess and quantify whether minimally invasive Er:YAG laser resurfacing has the ability to stimulate dermal matrix remodeling.

**METHODS**

**Human subject description, treatment, and tissue procurement**

This study was approved by the Institutional Review Board of the University of Michigan Medical School, and written informed consent was obtained from all study subjects prior to entry into the study. Patient recruitment occurred between Sept 1, 2007 and Nov 12, 2007. Patients included in the study were those of either gender of any racial/ethnic group who were at least 18 years of age with clinically evident photodamage of the forearm skin globally rated by investigators as at least moderate in severity. All included subjects were in generally good health and were judged to be willing and able to understand and comply with the requirements of the study protocol. Exclusion criteria included the use of oral retinoids within 1 year or topical retinoids to the forearms within 6 months of study entry, a history of abnormal scarring, the use of topical 5-fluorouracil to the forearm skin within 3 months of study entry, and a medical history or concurrent illness which investigators deemed unsafe for participation. Pregnant or nursing patients were also excluded.

The specific focal forearm skin site to be treated was outlined and marked by an investigator. Measurements from anatomic landmarks (such as the wrist crease) were recorded to ensure that subsequent biopsy specimens were obtained from laser-treated areas. A baseline punch biopsy (4 mm in diameter) from untreated forearm skin was obtained under sterile conditions after anesthesia was achieved with locally injected 1% lidocaine. A localized area of photodamaged forearm skin was then treated with an Er:YAG laser (SmoothPeel, Candela Corp, Wayland, MA) using the following settings: 2940 nm wavelength, spot size 5 mm, and one pass at energy setting of approximately 750 mJ. Patients were instructed to liberally apply Vaseline or Aquaphor Healing Ointment to the treated skin several times per day until healed. Patients returned for follow up assessments and to provide additional skin samples on 4 additional visits at 1 day, 3 days, 7 days, and 14 days after the treatment. Biopsy sites were spaced a minimum of 0.5 cm apart to mitigate against the possibility that molecular changes due to wound healing at other biopsy sites would impact our measurements. Immediately after biopsy, skin specimens were embedded in optimal cutting temperature embedding medium (Tissue-Tek OCT compound; Miles, Naperville, IL), frozen in liquid nitrogen, and stored at \textdegree{}C until processing.

**RNA extraction, reverse transcription, and quantitative real-time polymerase chain reaction**

RNA extraction from whole skin biopsy specimens, reverse-transcription, and messenger RNA extracted.
quantification by real-time quantitative polymerase chain reaction was performed as previously described. Custom primers and probes were used for collagen I; collagen III; matrix metalloproteinases (MMP)-1, -3, -9; and 36B4 (sequences available upon request). All other primer-probe sets were validated gene expression assays (TaqMan; Applied Biosystems, Foster City, CA). Results are presented as fold change in treated versus untreated skin samples, normalized to transcript levels of house-keeping gene 36B4.

**cDNA preparation of microdissected skin samples**

Laser capture microdissection was performed as previously described to isolate interfollicular epidermis (without hair follicle infundibulum) and dermis from 14 μm-thick frozen sections. Tissue was collected in RLT buffer (Qiagen, Valencia, CA), and total RNA were isolated in the presence of carrier RNA by using an RNeasy Micro kit (Qiagen). Reverse-transcription was performed with random primers using a cDNA archive kit (Applied Biosystems). Complementary DNA was pre-amplified using a Taqman Pre-Amp Master Mix kit (Applied Biosystems) in a 14-cycle reaction and following the manufacturer’s instructions.

**Immunohistochemistry**

Skin biopsy specimens were cut into 7-μm frozen sections and immunohistochemical staining was performed by using the following primary antibodies: laminin γ2 (kaliretin: AbCam, Cambridge, MA), KRT16 (Novocastra/Vision Biosystems, Norwell, MA), Ki-67 (Biogenex, San Ramone, CA), neutrophil elastase (Dako, Carpinteria, CA), cJun (TransLab/BD Biosciences, San Jose, CA), JunB, MMP 1 (both from Santa Cruz Biotechnology, Santa Cruz, CA), MMP-3, MMP-9, or procollagen I (all from Chemicon/Millipore, Billerica, MA). Tissue-bound primary antibody was visualized with a secondary antibody-peroxidase-AEC system (Biogenex) as previously described.

**Protein extraction and procollagen I enzyme-linked immunosorbent assay**

Frozen sections (50-μm thick) were cut from OCT-embedded skin samples and placed onto polyethylene naphthalate foil-coated slides (Leica Microsystems, Bannockburn, IL). For each sample, dermal area was measured by means of the image analysis software component of a laser capture microscope (Leica AS LMD). Skin sections were next isolated from surrounding OCT by microdissection and collected into ice-cold protein extraction buffer (50 mM Tris hydrochloride, pH 7.4; 0.15 M sodium chloride; 1% Triton X-100; protease inhibitors [Complete Mini; Roche Diagnostics, Indianapolis, IN]) and homogenized by vortex mixing. Samples were centrifuged at 10,000g and 4°C for 5 minutes and supernatants were assayed for procollagen I by using an enzyme-linked immunoassay kit (Panvera, Madison, WI). Procollagen I protein concentrations were normalized to dermal volume.

**Statistical analysis**

Fold induction of biomarkers over time was compared to baseline levels with the repeated measures analysis of variance. Specific time-point comparisons were made with Dunnett’s test. The type I error rate was set at 0.05 for a two-tailed hypothesis. Summary data were described by using means and standard errors. The data were analyzed using SAS statistical software (SAS Institute, Inc, Cary, NC).

**RESULTS**

A total of 10 patients 44 to 75 years of age, with clinically significant photodamage of the forearm skin, were enrolled in the study and provided skin samples. Three patients were female and 7 were male. Patients tolerated the treatment well with no need for anesthesia. The procedure resulted in initial “frosting” of the treated skin, as expected. At initial follow-up visits, all patients were found to have erythema at the treatment sites that gradually resolved over the first 1 to 2 weeks after the laser procedure.

Histologically, superficial Er:YAG laser treatment resulted in minimal epidermal damage. Histological observations, 24 hours after treatment, indicated loosening of stratum corneum, and epidermal cell cohesion. These changes were associated with a thickening of the epidermis. On rare occasions, microvesicles were observable in the granular layer of the epidermis. In all cases, the epidermal basal layer was histologically unaffected and immunostaining for laminin γ2 confirmed the integrity of the basement membrane (Fig 1). When present, epidermal damage (presence of microvesicles) was seen 24 hours after treatment and was resolved 3 to 7 days after treatment.

Consistent with epidermal damage, we observed that Er:YAG laser treatment induced epidermal expression of keratin 16 (KRT16), a marker of epidermal injury (Fig 2). KRT16 was substantially elevated for 2 weeks after Er:YAG laser treatment. Interestingly, levels of heat shock protein 70, a sensitive indicator of the skin’s acute response to heat, were not found to be significantly altered by Er:YAG laser treatment (data not shown). Epidermal
injury was followed by a rapid and transient increase in keratinocyte proliferation, as assessed by positive staining for Ki67, a proliferation marker, which was induced by 5-fold 3 days after Er:YAG laser treatment (n = 6, P < .05), and returned to baseline at day 7 (Fig 3).

Epidermal damage triggered by Er:YAG laser treatment activated a wound healing response. Within the first day following Er:YAG treatment, we observed a marked increase in the inflammatory cytokines interleukin (IL)-1β (16-fold vs baseline, N = 10, P < .05) and IL-8, a potent chemoattractant for polymorphonuclear leukocytes (200 fold vs baseline, N = 10, P < .05). Tumor necrosis factor alfa (TNF-α) was not significantly induced by Er:YAG treatment (data not shown). A substantial cellular infiltrate, consisting primarily of polymorphonuclear cells, as assessed by positive staining for neutrophil elastase, was seen 24 hours after treatment and was noted to decline between day 3 and day 7 after treatment. Neutrophil infiltration was found to extend from the dermis into the epidermis.

The activator protein 1 (AP-1) transcription factor complex is known to regulate transcription of several important cytokines and MMPs in the skin.9 The latter are the enzymes primarily responsible for dermal matrix remodeling. AP-1 is a heterodimeric protein consisting of members of the Jun/Fos family of proto-oncogenes, which are known to be involved in stress-related transcriptional control. Levels of cJun protein were found to be significantly elevated in the epidermis, 13.5-fold (N = 10, P < .05) above baseline values 24 hours after the treatment, whereas JunB protein levels were also significantly increased in the epidermis, by 52-fold (N = 10, P < .05) as compared to baseline. Levels of cJun and JunB decreased toward baseline values over the subsequent 2 weeks (Fig 4).

As noted, AP-1 transcription factor is a major regulator of a subset of MMPs involved in matrix remodeling.9 MMP-1 can initiate cleavage of collagen fibrils. Once cleaved, collagen is further degraded by MMP-3 and MMP-9. Thus, we quantified...
transcription levels of AP-1-regulated MMPs: MMP-1, MMP-3, and MMP-9. We observed that levels of MMP-1 and MMP-3 significantly rose to 230-fold (N = 10, P < .05) and 140-fold (N = 10, P < .05), respectively, above baseline levels 24 hours after treatment (Fig 5, A and B). MMP-9 levels were also substantially elevated to 10.4-fold (N = 10, P < .05) above pretreatment values within 24 hours of laser treatment (Fig 5, C). Laser capture-microdissection revealed that Er:YAG laser treatment induced MMP-1 and MMP-3 gene expression in both epidermis and dermis (Fig 6, A and B). Immunohistochemistry confirmed that MMP-1 and MMP-3 proteins were localized in the epidermis and dermis, whereas MMP-9 protein was localized to sites of primary inflammation (Fig 6, C-E).

During wound healing, the initial inflammatory phase is followed by remodeling of the dermal extracellular matrix, which in turn is followed by elevated production of type I and type III collagen. Following Er:YAG laser treatment, levels of procollagen I mRNA were found to be significantly elevated by 2.1- and 2.8-fold versus baseline, respectively, 7 and 14 days after treatment (N = 10, P < .05). Similarly, there was a 2.4- and 3.8-fold increase in procollagen III mRNA 1 and 2 weeks, respectively, following the procedure (N = 10, P < .05) (Fig 7, A). Consistent with increased RNA levels, procollagen I protein levels were elevated, by 4.3- and 5.5-fold as compared to baseline, 7 and 14 days, respectively, following the laser procedure (N = 10, P < .05) (Fig 7, B). Immunohistochemistry revealed that Er:YAG laser treatment stimulated procollagen I synthesis in fibroblasts throughout the mid to upper dermis (Fig 7, C).

**DISCUSSION**

Treatments such as dermabrasion, medium depth and deep chemical peels, and traditional fully ablative carbon dioxide and erbium laser resurfacing have been utilized to physically or chemically remove the epidermis, wound the dermis, and thereby stimulate collagen production and dermal remodeling. Alternatively, nonablative laser and light-based therapy is intended to heat the dermis while sparing the epidermis, thus creating changes in the dermal matrix with less risk and minimal downtime.10-13 The effects of some nonablative laser and light-based treatments may be enhanced with the use of topical photosensitizers; recently, photodynamic therapy has evolved to include cosmetic applications.14-16 While clinical results have sometimes been promising, issues related to photosensitivity are a substantial drawback of these treatment modalities. Another relatively recent approach to the treatment of photoaging is the use of fractionated nonablative and ablative technologies.17-20 Here the goal is to enhance safety and minimize downtime by treating a fraction of the skin’s surface area while sparing intervening islands of skin.

The use of superficially acting, low-powered Er:YAG laser therapy represents a modified treatment strategy by utilizing a well-studied, “tried and true” technology that has historically mainly been used in fully ablative laser therapy. The potential efficacy of such an approach depends on the ability of a laser acting on the epidermis to stimulate meaningful changes in the dermal matrix. Indeed,
the current study provides significant evidence for the validity of this tactic. The fact that the basement membrane in treated skin remained intact strongly suggests that the treatment’s initial impact was confined to the epidermis. Furthermore, levels of heat shock protein 70 were not significantly altered by the laser therapy. This finding suggests that thermal effects of the treatment were minimal and that the dermal changes noted are unlikely to have been caused by direct dermal heating, given the very superficially acting treatment settings used in our study protocol. The fact that no histologic evidence of thermal changes in the upper dermis could be demonstrated further substantiates this conclusion.

We have previously demonstrated the correlation between epidermal KRT16 levels and eventual collagen production, and this finding held true with superficial Er:YAG laser resurfacing as well. Elevated KRT16 levels are indicative of epidermal activation, and we believe that the controlled injury caused by the laser therapy in turn led to the production of collagen in the dermis. The relative importance of the resulting inflammatory response remains to be assessed.

Fig 4. Er:YAG laser treatment induces transcription factor AP-1 in human skin in vivo. Photodamaged forearm skin was treated with a single pass of Er:YAG laser as described in the Methods section. Skin samples were obtained from untreated (No Tx) and treated skin at indicated times posttreatment. Skin sections were immunostained for transcription factor AP-1 components cjun (A) and JunB (C). A, cjun-positive staining is red; counter staining is blue. Images are representative of 6 subjects. (Original magnification: ×40.) B, Quantification by image analysis of cjun staining at indicated times after Er:YAG laser treatment, in human skin in vivo. N = 10; Asterisk, P < .05. C, JunB positive staining is red; counterstaining is blue. Images are representative of 6 subjects. (Original magnification: ×40.) D, Quantification by image analysis of JunB staining at indicated times after Er:YAG laser treatment, in human skin in vivo. N = 10. Asterisk, P < .05.
Mechanistically, the laser therapy resulted in a highly organized cascade of molecular events that are known to be associated with dermal matrix remodeling. The resulting induction of the AP-1 transcription factor led to up-regulation of both proinflammatory cytokines known to be important in tissue healing and MMPs, which are vital to remodeling of dermal structural proteins.

It is noteworthy that the degree of collagen production induced by the minimally invasive Er:YAG laser procedure compares favorably to that produced by a number of other interventions. This finding is consistent with the relatively small body of primarily clinical literature exploring the effects of superficial, minimally ablative Er:YAG laser resurfacing. For example, Tay and Kwok have recently demonstrated substantial clinical efficacy of such an approach in the treatment of mild to moderate atrophic acne scarring. In addition, Drnovsek-Olup, Beltram, and Pizem demonstrated histologic evidence of dermal changes induced by minimally ablative Er:YAG laser therapy.

Additional validity for the concept of epidermal injury leading to dermal changes is provided by recent reports of the ability of microdermabrasion to invoke a wound-healing response in human skin in vivo. These studies demonstrate that superficial abrasion of the epidermis stimulates MMP and collagen production. These findings suggest that interventions acting purely on epidermal keratinocytes may lead to the production of soluble factors that drive dermal changes. This concept of epidermal injury being sufficient to drive dermal changes is also in keeping with current thinking about the pathogenesis of photoaging. In the current study, a treatment targeting the epidermis only was again found to result in dermal remodeling.

Although the laser treatment examined in the current study resulted in substantial collagen production, it is possible that even greater collagen induction might have been demonstrated if dermal changes had been assessed at later time points. Collagen production remained elevated for at least 2 weeks after the treatment. Previous work examining dermal matrix changes after fully ablative carbon dioxide laser resurfacing demonstrated peak collagen production occurring 3 weeks after treatment. Therefore collagen induction by Er:YAG laser therapy might have continued to increase at later time points. Furthermore, the data presented herein are those that resulted from a single treatment session. If repeated exposure to the superficially acting Er:YAG laser results in additive collagen production, we might expect that a series of treatments might result in enhanced clinical effects.

**Fig 5.** Er:YAG laser treatment induces expression of matrix metalloproteinases in human skin in vivo. Photodamaged forearm skin was treated with a single pass of Er:YAG laser as described in the Methods section. Skin samples were obtained from untreated (No Tx) and treated skin at the indicated times posttreatment. Total RNA was prepared from the specimens and mRNA levels of MMP-1 (A), MMP-3 (B), and MMP-9 (C) were quantified by real-time reverse transcriptase polymerase chain reaction (RT-PCR). N = 10. Asterisk, P < .05.
The current study raises the possibility that enhanced appearance of human skin may be achieved by creating dermal changes through the use of superficially acting treatments. The impressive changes demonstrated herein indicate that such a treatment paradigm might be important in the design of future technology and treatment protocols. The fact that minimally ablative treatments are associated with little risk and modest downtime may make these therapeutic approaches all the more popular in the years to come. There is now the need for additional translational research to determine the correspondence of clinical and biochemical results with various aesthetic interventions. However, such work is dependent on the willingness of cosmetically oriented patients to provide multiple skin samples. In addition, this type of research will require substantial numbers of patients and will be very costly. These are among the challenges slowing progress in the development of more effective procedures to enhance human appearance.

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REFERENCES