Connective Tissue Remodeling Induced by Carbon Dioxide Laser Resurfacing of Photodamaged Human Skin

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Objective: To quantitatively examine the dynamics of molecular alterations involved in dermal remodeling after carbon dioxide (CO₂) laser resurfacing of photodamaged human skin.

Design: Serial in vivo biochemical analyses after laser therapy.

Setting: Academic referral center, Department of Dermatology, University of Michigan, Ann Arbor.

Subjects: Volunteer sample of 28 adults, 48 to 76 years old, with clinically evident photodamage of the forearms.

Intervention: Focal CO₂ laser resurfacing of photodamaged forearms and serial biopsies at baseline and various times after treatment.

Main Outcome Measures: Reverse transcriptase real-time polymerase chain reaction technology and immunohistochemistry were used to assess levels of type I and type III procollagens; matrix metalloproteinases (MMPs) 1, 3, 9, and 13; tropoelastin; fibrillin; primary cytokines interleukin 1β and tumor necrosis factor α, and profibrotic cytokine transforming growth factor β1.

Results: Production of type I procollagen and type III procollagen messenger RNA peaked at 7.5 and 8.9 times baseline levels, respectively, 21 days after treatment and remained elevated for at least 6 months. Increases in messenger RNA levels of several cytokines (interleukin 1β, tumor necrosis factor α, and transforming growth factor β1) preceded and/or accompanied changes in collagen levels. Marked increases in messenger RNA levels of MMP-1 (39 130-fold), MMP-3 (1041-fold), MMP-9 (75-fold), and MMP-13 (767-fold) were noted. Levels of fibrillin and tropoelastin rose in a delayed fashion several weeks after treatment.

Conclusions: The biochemical changes seen after CO₂ laser resurfacing proceed through a well-organized and highly reproducible wound healing response that results in marked alterations in dermal structure. These quantitative changes may serve as a means for comparison as other therapeutic modalities meant to improve the appearance of photodamaged skin are evaluated.

Arch Dermatol. 2004;140:1326-1332

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ARBON DIOXIDE (CO₂) LASER resurfacing remains one of the most effective treatments available for achieving facial rejuvenation. While several mechanisms may be involved in creating the cosmetic improvements seen after ablative laser resurfacing, up-regulated collagen production appears to be one of the more significant. Despite a number of histologic and ultrastructural studies of the effects of CO₂ laser therapy, relatively little is known about the resulting quantitative changes in collagen production and the time course over which these events occur. We thus sought to quantitatively examine the changes in collagen levels after CO₂ laser treatment of photodamaged human skin in vivo along with those of several key genes known to be involved in dermal remodeling.

We hypothesized that healing after CO₂ laser resurfacing would conform to fundamental wound healing mechanisms. However, we believed that the resulting quantitative changes in collagen production might be substantially greater than those seen after wounding related to other causes, thus at least partially accounting for the significant clinical improvements associated with this treatment modality. Despite the recent advent of multiple nonablative lasers and other devices aimed at creating improvements in the wrinkles associated with photoaging, ablative laser resurfacing remains the gold standard in the field in terms of efficacy. It is, therefore, an additional goal of this project to pro-
provide a quantitative picture of the biochemical changes associated with CO2 laser therapy against which the effects of other newer nonablative treatment modalities may ultimately be compared. While biochemical changes do not always translate directly into clinical alterations, such comparative information may eventually provide physicians and patients with information that is useful in therapeutic decision making.

METHODS

This study was approved by the institutional review board of the University of Michigan Medical School, Ann Arbor, and written informed consent was obtained from all study subjects. Twenty-eight subjects, aged 48 to 76 years, with clinically evident photodamage involving the forearms were recruited. For each subject, a focal area of one forearm was injected with 1% lidocaine with epinephrine to achieve anesthesia and then treated with a CO2 laser (Ultrapulse; Coherent, Inc, Santa Clara, Calif) for 2 passes at 300 mJ and 60 W, and with computer pattern generator settings of 3/5/6. Punch biopsies (3 mm) were obtained from the treated area at baseline and at up to 5 additional time points after the treatment. A minimum of 5 mm of laser-treated skin not taken for biopsy was left between each biopsy site. Treatment resulted in complete removal of the interfollicular epidermis. Total RNA was extracted from full-thickness skin samples, and messenger RNA (mRNA) levels of specific genes were quantified by real-time reverse transcriptase polymerase chain reaction technology.6 Tissue samples were analyzed for levels of matrix metalloproteinases (MMPs) 1, 3, 9, and 13; type I and type III procollagen; interleukin 1β (IL-1β); tumor necrosis factor α (TNF-α); transforming growth factor β1 (TGF-β1); tropoelastin; and fibrillin. Specific proteins were localized by immunohistochemistry using frozen sections. Sections (7 µm) were stained for MMP-1 with monoclonal antibody MAB 1346, MMP-3 with monoclonal antibody MAB 13412, MMP-9 with monoclonal antibody MAB 1346, MMP-3 with monoclonal antibody MAB 13412, MMP-9 with monoclonal antibody MAB 13415 (all MMP antibodies were obtained from Chemicon International Inc, Temecula, Calif), and type I procollagen (SP1.D8, developed by Heinz Furthmayr, MD, and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences, Iowa City). Presence of tissue-bound primary antibody was visualized with a secondary antibody-peroxidase complex.7

Biopsy specimens were obtained from 4 subjects at baseline and 6 months after treatment. These subjects participated in a study that included daily application of a moisturizer (Neutrogena Body Moisturizer Norwegian Formula; Johnson & Johnson, New Brunswick, NJ) for 3 weeks before CO2 laser treatment. All other aspects of the study for these subjects were similar to those described earlier. For biochemical analysis of skin samples obtained 6 months after treatment and the corresponding pretreatment control skin, epidermis and dermis were separated by laser capture microscopy (Leica LMD Laser Microdissection System; Leica Microsystems Inc, Bannockburn, Ill). Procollagen and MMP mRNA levels in the epidermis and dermis were quantified as described earlier.

Changes in biochemical end points during the course of the study were statistically evaluated by means of repeated-measures analysis of variance. Individual pairwise comparisons of values at each subsequent time with baseline levels were made with the Dunnett test. The type I error rate was set at 0.05. When necessary, logarithmic transformations of the data were made before analysis to achieve normality, and, when appropriate, the data are depicted on figures with logarithmic scaled ordinate axes. Summary statistics include means and standard errors. The data were analyzed with SAS statistical software (SAS Institute Inc, Cary, NC).

RESULTS

Primary cytokines IL-1β and TNF-α are rapidly induced in response to physical stress, and marked changes in the levels of these cytokines were noted in the acute phase of healing after CO2 laser resurfacing. The IL-1β mRNA levels were elevated (185-fold) 1 day after treatment and rose sharply between days 2 and 3 as levels increased to 694 times baseline (Figure 1A). The MMP-1 mRNA levels then declined rapidly between days 1 and 2 (Figure 1A). The MMP-3 mRNA levels remained highly elevated for 6 days after resurfacing, peaking at an 839-fold increase on day 6 and then rapidly declined between days 6 and 8 (Figure 1A). Increased TNF-α mRNA levels were initially observed during the first 3 days after treatment and were maximal (5.7-fold) on day 6 (Figure 1B). The TNF-α mRNA levels, like those of IL-1β, then fell sharply between days 6 and 8 after treatment (Figure 1B). These primary cytokines are known to induce MMPs, and we, therefore, next sought to examine levels of several MMPs.

There was a marked elevation in levels of MMP-1 mRNA beginning within 3 days after treatment (6335-fold) and peaking at 39 130 times basal levels on day 7 (Figure 2A). The MMP-1 mRNA levels then declined precipitously during the second week after treatment (Figure 2A). A similar temporal pattern was seen in the...
rise in MMP-3 mRNA levels, which rose rather quickly between days 2 and 7 after laser resurfacing, peaked at 1041 times baseline levels 7 days after treatment, and then sharply declined during the subsequent week (Figure 2B). A marked elevation in MMP-9 mRNA levels was also observed beginning within 3 days of resurfacing (27-fold) and peaking at 75 times baseline levels 6 days after exposure to the CO2 laser (Figure 2C). Although MMP-9 mRNA levels peaked at day 6 after treatment, in contrast to MMP-1 and MMP-3 levels, they remained elevated near peak levels for at least 28 days after resurfacing (42-fold) (Figure 2C). Interestingly, levels of MMP-13 mRNA rose in a comparatively delayed fashion starting about 1 week after treatment but not rising rapidly until after day 10 (Figure 2D). The MMP-13 mRNA levels peaked at 767 times basal levels on day 14 after treatment and subsequently declined during the following 2-week period, remaining somewhat elevated at 141 times baseline levels 28 days after resurfacing (Figure 2D). Levels of MMP-1, MMP-3, and MMP-9 mRNA were evaluated and found to have decreased to baseline values 6 months after treatment (data not shown).

Protein levels of MMP-1, MMP-3, and MMP-9 were also evaluated immunohistochemically. Enhanced protein staining at day 7, coinciding with the elevated mRNA levels described, was clearly evident for each of these 3 MMPs (Figure 3). The MMP-1 staining was noted to be extracellular and, to a lesser degree, intracellular. The MMP-3 staining was found to be primarily extracellular and highly associated with collagen fibers, while enhanced MMP-9 staining was found to be primarily intracellular with a much lesser degree of extracellular staining noted. While fibroblasts make MMP-1 and MMP-3 and thus account for the enhanced staining of these enzymes, these cells do not produce MMP-9. We believe that inflammatory cells, likely neutrophils and/or macrophages, are responsible for the MMP-9 staining described above. Matrix metalloproteinases are associated with both breakdown and remodeling of collagen, and we next examined levels of a cytokine, TGF-β1, known to be powerfully profibrotic, along with levels of type I and type III procollagen.

The TGF-β1 mRNA levels rose acutely within 3 days of laser resurfacing to 9.7 times baseline levels on day 2 after treatment and remained elevated at approximately 3.5 to 4 times baseline levels for at least 28 days after treatment (data not shown). Although TGF-β is a multifunctional cytokine, one of its major roles in skin is induction of procollagen biosynthesis, as described in the following paragraph.8

Coinciding with the decline in MMP-1 mRNA levels during the second week after resurfacing, type I and type III procollagen mRNA levels rose rather sharply starting after posttreatment day 10 (Figure 4A and B). By day 14, type I and type III procollagen mRNA levels had increased to 7.2- and 5.7-fold higher than at baseline, respectively. Levels of type I and type III procollagen mRNA remained elevated, and actually continued to rise somewhat during the subsequent week, ultimately peaking at 7.5 and 8.9 times basal levels, respectively, on day 21 after the treatment (Figure 4A and B). These levels then began to decline between days 21 and 28 after treat-
ment. Changes in type I procollagen protein levels were also clearly demonstrated immunohistochemically (Figure 5). At baseline, weak type I procollagen staining was primarily localized to the dermal-epidermal junction, and a lack of positive staining was seen below this in our photodamaged subjects. By 21 days after laser resurfacing, however, type I procollagen was found to be induced in the zone of repair between the dermal-epidermal junction and an underlying band of displaced elastotic material in the deep dermis (Figure 5). Six months after treatment, positive staining for type I procollagen persisted, although the intensity of the staining was lower than at day 21 (Figure 5). Skin samples obtained 6 months after laser resurfacing were also analyzed by means of laser capture microscopy so that dermal contributions to procollagen formation were assessed separately. With this technique, type I procollagen mRNA levels in the dermis were found to be elevated at 12.8 times baseline levels, and those of type III procollagen mRNA were noted to be 10.4-fold higher than at baseline.

Although changes in collagen are often the primary focus when the effects of CO₂ laser resurfacing are consi-
sidered, we also sought to examine alterations in elastic tissue. Tropoelastin mRNA levels rose gradually after laser resurfacing beginning during the second week after the procedure and were still rising 28 days after treatment, when these levels were 2.4 times those found at baseline (Figure 6A). Tropoelastin mRNA levels remained elevated for at least 6 months in 3 of 4 subjects (data not shown). Similarly, mRNA levels of another major component of elastin fibers, fibrillin 1, were also noted to gradually increase after resurfacing, rising to 3.2 times basal levels 28 days after treatment (Figure 6B). In addition, as already noted, the disorganized elastotic material commonly associated with photodamaged skin was found to be displaced deeper into the dermis after the treatment, and this was clearly seen immunohistochemically (data not shown).

**COMMENT**

Carbon dioxide laser resurfacing is clearly efficacious in producing cosmetic improvements in patients' skin.9-12
However, relatively little is known about the details regarding the molecular mechanisms involved. Hypothesized mechanisms have included collagen contraction, physical ablation of photodamaged tissue, and neocollagenesis. Although clinical cutaneous rejuvenation may actually be based on a combination of these factors, we sought to examine in detail the latter aspect of laser resurfacing. Several studies have examined new collagen formation after laser resurfacing from a histologic standpoint, but to our knowledge, there has been no prior detailed quantitative examination of the changes in collagen and several key genes involved in dermal remodeling after CO2 laser treatment.

Much of the data presented are based on real-time reverse transcriptase polymerase chain reaction technology, which quantifies tissue mRNA levels. The advantages of this technique are at least 2-fold. First, it allows for the production of truly quantitative data. Second, it is an extremely sensitive technique that enables detection of even very subtle molecular changes in very small tissue samples. While the measurements made are at the mRNA level, previous work regarding collagen biochemistry in human skin indicates a high degree of correlation between mRNA levels and those of associated proteins when dealing with the key molecules measured in this study.

The biochemical changes noted after CO2 laser resurfacing proceed through a well-organized and highly reproducible wound healing response. Some of the earliest changes include elevations in the levels of both IL-1β and TNF-α. These proinflammatory cytokines are involved in fundamental mechanisms associated with the tissue repair process, and each is known to induce MMPs. It thus makes sense that shortly after IL-1β and TNF-α levels rise, those of several important MMPs are found to be markedly elevated. Specifically, we see significant elevations to nearly 40 000 times baseline levels in collagenase 1 (MMP-1), an enzyme that catalyzes the first step of collagen degradation. Here, we believe that MMP-1 is induced by the laser procedure to degrade patients' photodamaged collagen. A similar temporal pattern, with peak enzyme levels occurring 1 week after resurfacing, is seen for stromelysin (MMP-3), an enzyme that degrades both partially degraded collagen and other matrix proteins such as proteoglycans and elastin.

To cleave a collagen molecule, at least 2 types of MMPs, both a collagenase and a gelatinase, are required. Levels of gelatinase B (MMP-9) are also found to be maximally elevated approximately 1 week after the treatment. However, unlike MMP-1 and MMP-3, whose levels decline rather sharply 2 weeks after laser resurfacing, levels of MMP-9 taper much more slowly. In fact, MMP-9 levels remained elevated at near peak levels (42-fold higher than at baseline) 28 days after resurfacing. The action of a gelatinase follows that of a collagenase in the process of breaking down a collagen molecule, and the comparatively delayed decline in MMP-9 levels fits well with this concept. The long-lived elevation in MMP-9 suggests residual degradation of MMP-1-generated collagen fragments by MMP-9.

We hypothesize that clearance of fragmented, photodamaged collagen by increased MMP levels facilitates the formation and deposition of new collagen. Quantitatively we see a marked elevation in both type I and type III procollagen levels, which peak between 2 and 3 weeks after CO2 laser resurfacing after a decline in MMP-1 and MMP-3 levels. The fact that MMP-1 levels fall just before the bulk of new collagen formation protects this new collagen from MMP activity, and thus permits a net gain in collagen in the dermis. Type I and type III procollagen levels remain elevated for at least 6 months after the laser therapy. This is in keeping with the clinical observation that patients' skin continues to improve in appearance for many months after laser resurfacing.

Transforming growth factor β1 is powerfully profibrotic, and the elevated levels of this cytokine in CO2 laser–treated skin, peaking 2 days after treatment but remaining high for at least 28 days after resurfacing, act to support ongoing new collagen formation. The increased TGF-β1 levels reported herein are in contrast to those noted by Nowak et al in a previous study regarding levels of this cytokine after CO2 laser treatment in an in vitro model that used keloidal and normal dermal fibroblast cell lines. In that study, there was a trend toward suppression of TGF-β in both normal and keloidal fibroblast-derived cell lines after CO2 laser exposure. Whether this difference is due to the fact that the current study specifically examined photodamaged skin rather than normal and keloidal tissue or perhaps is based on inherent differences between our in vivo study design and the in vitro nature of the previous work is not clear.

Levels of collagenase 3 (MMP-13) were noted to rise later than those of MMPs 1, 3, and 9, peaking 2 weeks after CO2 laser treatment. Evidence suggests that MMP-13 is involved in remodeling of collagen, which occurs in the later stages of wound healing, rather than in initial collagen degradation. Our data support the concept that MMP-13 functions to remodel newly produced collagen in CO2 laser–treated skin, rather than acts to break down older, photodamaged collagen molecules. Further research regarding the role of MMP-13 in dermal remodeling is needed to clarify this point.

Myofibroblasts are specialized, differentiated fibroblasts that are distinguished from dermal fibroblasts by expression of α–smooth muscle actin, increased capacity to contract collagen fibrils, and elevated production of type I collagen. Myofibroblasts are found in healing full-thickness wounds such as those due to burns or surgical incisions and are associated with wound contracture and scarring. We were unable to detect myofibroblasts (α–smooth muscle actin–positive fibroblasts) at any time after CO2 laser resurfacing. Thus, healing after CO2 laser resurfacing differs qualitatively from that of other types of wounds. This difference may stem from the comparatively superficial nature of CO2 laser–induced wounds. Since myofibroblasts are associated with scarring, one might speculate that the dearth of these cells in normally healing CO2 laser–induced wounds may be of cosmetic benefit.

While we focused mainly on collagen when considering the cosmetic improvements brought about by CO2 laser therapy, we also found comparatively modest but clear changes in the levels of tropoelastin and fibrillin 1, 2 major components of elastic fibers. Levels of these molecules were still rising 28 days after treatment, and tropoelastin mRNA levels remained elevated 6 months...
of CO2 lasers, but with a more favorable safety profile. The challenge now is to create a device that produces biochemical changes approaching those of CO2 laser resurfacing. The hallmark of photodamaged skin, deeper into the dermis after laser resurfacing may be of clinical benefit. A similar displacement of elastic material has been demonstrated in the past after dermabrasion. It should be noted that we attempted to mimic clinical practice in terms of our laser settings and the in vivo use of photodamaged skin as our source for tissue samples. In our study, we used the forearms as our treatment site because of practical limitations in the number of biopsy specimens that could be obtained from the face. While there may exist subtle differences in responses of photodamaged forearm and facial skin to CO2 laser resurfacing, there is no evidence that fundamental wound healing mechanisms should differ between these sites. Still, we cannot rule out the possibility that the molecular alterations seen after resurfacing of facial skin may be somehow different from the changes demonstrated in this study.

We hope that the quantitative data presented will serve as a “measuring stick” against which the biochemical effects of other lasers—including nonablative lasers—may be compared. Such comparison may ultimately contribute to the design of more efficacious devices aimed at achieving cutaneous rejuvenation. Data presented in this article delineate specific alterations in key enzymes and cytokines that are associated with the production of the desirable quantity of new collagen formation brought about by CO2 laser resurfacing. The challenge now is to create a device that produces biochemical changes approaching those of CO2 lasers, but with a more favorable safety profile.

Accepted for Publication: January 23, 2004.

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Funding/Support: This study was supported by a research grant from the Dermatology Foundation, Evanston, Ill.

REFERENCES


