STUDY

In Vivo Stimulation of De Novo Collagen Production Caused by Cross-linked Hyaluronic Acid Dermal Filler Injections in Photodamaged Human Skin

Frank Wang, MD; Luis A. Garza, MD, PhD; Sewon Kang, MD; James Varani, PhD; Jeffrey S. Orringer, MD; Gary J. Fisher, PhD; John J. Voorhees, MD

Objective: To determine whether endogenous synthesis of new extracellular matrix may contribute to the degree and duration of clinical benefits derived from cross-linked hyaluronic acid dermal filler injections.

Design: In vivo biochemical analyses after filler injections.

Setting: Academic referral center.

Participants: Eleven healthy volunteers (mean age, 74 years) with photodamaged forearm skin.

Interventions: Filler and vehicle (isotonic sodium chloride) injected into forearm skin and skin biopsy specimens taken 4 and 13 weeks later.

Main Outcome Measures: De novo synthesis of collagen, the major structural protein of dermal extracellular matrix, was assessed using immunohistochemical analysis, quantitative polymerase chain reaction, and electron microscopy.

Results: Compared with controls, immunostaining in skin receiving cross-linked hyaluronic acid injections revealed increased collagen deposition around the filler. Staining for prolyl-4-hydroxylase and the C-terminal and N-terminal epitopes of type I procollagen was enhanced at 4 and 13 weeks after treatment ($P<.05$). Gene expression for types I and III procollagen as well as several profibrotic growth factors was also up-regulated at 4 and 13 weeks compared with controls ($P<.05$). Fibroblasts in filler-injected skin demonstrated a mechanically stretched appearance and a biosynthetic phenotype. In vitro, fibroblasts did not bind the filler, suggesting that cross-linked hyaluronic acid is not directly stimulatory.

Conclusions: Injection of cross-linked hyaluronic acid stimulates collagen synthesis, partially restoring dermal matrix components that are lost in photodamaged skin. We hypothesize that this stimulatory effect may be induced by mechanical stretching of the dermis, which in turn leads to stretching and activation of dermal fibroblasts. These findings imply that cross-linked hyaluronic acid may be useful for stimulating collagen production therapeutically, particularly in the setting of atrophic skin conditions.

Arch Dermatol. 2007;143:155-163

Author Affiliations: Departments of Dermatology (Drs Wang, Garza, Kang, Orringer, Fisher, and Voorhees) and Pathology (Dr Varani), University of Michigan Medical School, Ann Arbor. Dr Garza is now with the Department of Dermatology, University of Pennsylvania, Philadelphia.

INJECTABLE DERMAL FILLERS ARE becoming increasingly popular for improving skin contour defects related to aging (wrinkles and lines), depressed acne scars, and other traumatic or congenital conditions. Among the most widely used temporary fillers in the United States is cross-linked hyaluronic acid. The first filler of this type was approved by the Food and Drug Administration in December 2003, and now, several cross-linked hyaluronic acid dermal fillers are commercially available.[1] Of the 12 million cosmetic procedures performed annually in the United States, approximately 1 million currently involve this class of injectable fillers.[1]

CME course available at www.archdermatol.com

Hyaluronic acid is an endogenous polysaccharide found in highest concentrations in the skin and connective tissue.[2,3] In the skin, polymers of hyaluronic acid can bind water, forming a viscous substance that assists in hydration and turgor.[3,4] Accordingly, loss of hyaluronic acid with aging is associated with increased dehydration and wrinkling of the skin.[5]

Since hyaluronic acid is rapidly degraded in the skin,[3-5] chemical cross-linking of individual polymers provides increased stability.[5,6] In the United States, one

form of cross-linked hyaluronic acid is available under the trade name Restylane (Medicis Aesthetics Inc, Scottsdale, Ariz), also known as nonanimal stabilized hyaluronic acid (NASHA). Nonanimal stabilized hyaluronic acid is one of the most widely used dermal fillers in the United States. It is purportedly long lasting because, in addition to the stabilizing effect of cross-linking, it absorbs increasing amounts of water while being slowly degraded.[1,6,7] As such, it has been suggested that hydration helps treated skin maintain volume, even while the implanted material is broken down. Clinically, the benefits of NASHA have been reported to last 4 to 12 months.[1,8-11] This is at least twice as long as injectable collagen, the gold standard of dermal fillers.[8]

Because NASHA is relatively new, its biochemical effects in the skin are unknown. We surmised that changes in skin biology might occur in response to this filler. Normal skin is composed of an outer epidermis supported by an underlying dermis. The dermis consists mostly of secreted proteins, which collectively provide structural support for the skin and are known as the extracellular matrix. The major structural protein in the dermis is type I collagen, which comprises 90% of the dry weight of skin and diminishes with normal aging.[12-14] We found that NASHA injections stimulate de novo production of type I collagen. Additionally, we found that this induction may be mediated by mechanical stretching and consequent activation of collagen-producing cells (fibroblasts) in the dermis.

METHODS

TISSUE SAMPLES

All subjects signed informed consents, and the University of Michigan institutional review board approved this study. All subjects were volunteers and in good general health. The study was performed in the University of Michigan Clinical Research Program in the Department of Dermatology. Exclusion criteria included use of immunosuppressive medications, active infection of the skin, history of herpes simplex infection or connective tissue disease, and pregnancy. In an initial study (June 2004 through November 2004), a single NASHA injection in photodamaged forearm skin increased type I procollagen messenger RNA (mRNA) in 4 of 6 subjects after 1 week and in 6 of 6 subjects after 4 weeks. Based on these data, we enrolled additional subjects (6 men and 5 women, aged 64-84 years; mean age, 74 years), who received 3 injections of NASHA (0.7 mL each, 2-5 cm apart) in photodamaged dorsal skin of one randomly assigned forearm and 3 injections of vehicle (identical volume and spacing as NASHA) in the other forearm (conducted March 2005 through June 2005).

According to the package information accompanying NASHA syringes, the vehicle was identified as physiologic saline. Therefore, for our control injections we used 0.9% sodium chloride in sterile water (hereinafter "saline") obtained from our hospital pharmacy. For each subject, a transparent template was initially used to mark the locations of all injection sites relative to 3 or more specific skin landmarks (eg, moles, seborrheic keratoses, scars, hyperpigmented and/or hypopigmented spots, and hemangiomas). Photographs of the injection sites were also taken.

At 4 and 13 weeks, each injected site was pinpointed in a reliable manner by placing the transparent template directly over the skin of subjects' arms. For each injection site, skin samples (4 mm in diameter) were obtained under local anesthesia (lidocaine) at 4 and 13 weeks. Specimens were fixed in glutaraldehyde or frozen in optimal cutting temperature (OCT) medium at −70°C for later analyses.

IMMUNOHISTOCHEMICAL ANALYSIS

As described elsewhere,[15,16] OCT-embedded sections (7 µm) were stained for type I procollagen C-terminus (PIC5-5; Invitrogen, Carlsbad, Calif) and N-terminus (SP1.D8, obtained as described previously[15]); prolyl-4-hydroxylase α subunit (Acris, Hiddenhausen, Germany); and gelatinase B (Chemicon, Temecula, Calif). A subset of sections was fixed and stained for hyaluronic acid according to a modified protocol described previously.[17,18] Prior to staining, several sections were treated for 2 hours with 100 U/mL bovine testicular hyaluronidase (Sigma, St Louis, Mo) at 37°C. Subsequently, sections were incubated overnight with a biotinylated hyaluronic acid–binding protein (1:50) at 4°C (Calbiochem, San Diego, Calif) and developed as described previously.[15,18] All sections were counterstained with hematoxylin and examined under light microscopy. Image-Pro Plus software (version 4.1; Media Cybernetics, Silver Spring, Md) was used to quantify the area stained.

GENE EXPRESSION

After mRNA extraction from OCT-embedded samples, specific genes were quantified by real-time reverse transcriptase polymerase chain reaction, as described elsewhere.[15,16] Results were normalized to the mRNA level of the housekeeping gene *36B4*.

TRANSMISSION ELECTRON MICROSCOPY

Six ultrathin (0.1 µm), glutaraldehyde-fixed sections (plastic-embedded) were prepared as previously described.[19] Briefly, sections were stained with lead citrate–uranyl acetate (from EM Chemicals, Gibbstown, NJ) and observed with a Philips 400 Transmission Electron Microscope (Eindhoven, the Netherlands).

IN VITRO EXPERIMENTS WITH NASHA

Culture plates (24 wells) were coated overnight with type I collagen (5 µg/cm^2), and NASHA (200 µg/cm^2) was then added to a subset of wells. Fibroblasts were isolated from normal human skin as described previously[19] and seeded at 4×10^4 cells per well with KGM culture medium (Cambrex, Walkersville, Md) supplemented with calcium (Ca^{2+}) at a concentration of 1.4 mmol/L. Two days later, cells were visualized with light microscopy.

STATISTICAL ANALYSIS

The paired *t* test was used with 2-tailed *P* values and significance accepted as $P<.05$. Data are presented as average fold change ± 1 SE.

RESULTS

SUBJECTS

All 11 subjects had lightly pigmented skin, and on a 4-point clinical severity scale (none, mild, moderate, or severe), 8 had moderate photodamage on their forearms (eg, brown spots, lax skin, wrinkles, and/or uneven pigmentation),

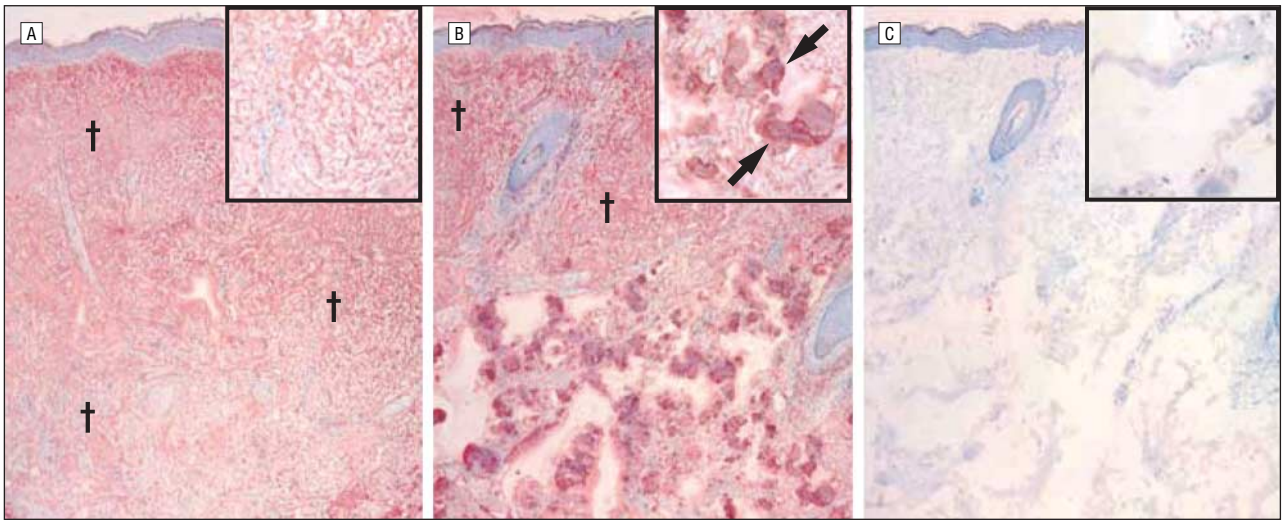

Figure 1. Visualization of injected cross-linked hyaluronic acid dermal filler in human skin in vivo. Photodamaged forearm skin was injected with isotonic sodium chloride vehicle (A) or nonanimal stabilized hyaluronic acid (NASHA) dermal filler (B). Shown here are representative tissue sections at 4 weeks after injection. Hyaluronic acid, both endogenous and injected, was detected using a biotinylated hyaluronic acid–binding protein. Positive staining appears red, while counterstaining with hematoxylin appears blue. Injected NASHA filler is denoted by arrows, while dermal areas containing endogenous hyaluronic acid are marked with daggers. C, To confirm staining specificity, a serial section from the same NASHA-injected sample was treated with hyaluronidase prior to the staining procedure. Original magnification for all main panels ×5; for insets ×40.

while 3 had mild-moderate photodamage. Following injections of NASHA and the saline vehicle into forearm skin, subjects experienced no adverse events except mild injection site tenderness for 24 to 48 hours.

Immediately following injections, intradermal saline and NASHA were visualized on each patient's arms as small bumps approximately 1.0 to 1.5 cm in diameter. After 4 and 13 weeks, we reliably located all saline and NASHA injection sites. Some NASHA-injected sites were still visible as small bumps (<1 cm), but the remaining NASHA- and saline-injected sites appeared flat.

INCREASED COLLAGEN SYNTHESIS IN NASHA-INJECTED SKIN

To begin assessing NASHA's biological effects, we stained tissue samples for hyaluronic acid. Saline-injected samples appeared normal and stained positively for endogenous hyaluronic acid (**Figure 1**A). In contrast, NASHA-injected skin at 4 and 13 weeks demonstrated well-circumscribed spaces in the middle and lower dermis. These spaces contained residual filler material that stained positively for hyaluronic acid (Figure 1B). This staining was abolished when samples were pretreated with a hyaluronic acid–degrading enzyme (hyaluronidase), thus verifying the specificity of our staining procedure (Figure 1C).

Next, we stained tissue for markers of newly synthesized type I collagen. Since collagen is initially produced as a soluble precursor (called *procollagen*), we first used an antibody against the C-terminal domain of type I procollagen. This antibody stains intracellular and extracellular epitopes, and accordingly, we found increased intracellular and extracellular dermal staining in NASHA-treated samples compared with controls (**Figure 2**A). At 4 and 13 weeks, staining occurred particularly in areas surrounding the filler.

Similar to the C-terminal marker, we found increased staining in NASHA-injected samples at 4 and 13 weeks using an antibody against the N-terminal domain of type I procollagen. This antibody binds an intracellular epitope, and correspondingly, we observed increased intracellular staining, especially in cells embedded within connective tissue fibers surrounding the filler (Figure 2B). Intracellular staining also occurred in cells more proximal (adjacent) to the filler, but no staining occurred inside spaces containing the filler.

Finally, we stained for prolyl-4-hydroxylase, an enzyme that modifies proline residues in procollagen to allow stable assembly of mature type I collagen. This enzyme level is elevated within fibroblasts that are actively producing procollagen.[20] In NASHA-treated skin at 4 and 13 weeks, staining for this marker was increased compared with controls and appeared similar to staining for the N-terminal antibody (Figure 2C).

To further evaluate these findings, we used real-time reverse-transcriptase polymerase chain reaction technology to quantify gene expression (mRNA) for types I and III procollagen. Type III procollagen associates with type I procollagen, and both are coordinately regulated in the skin.[21,22] At 4 and 13 weeks, respectively, expression was increased a mean ± SE of 10.5 ± 2.4-fold ($P = .003$) and 17.6 ± 6.9-fold ($P = .04$) for type I procollagen, and 7.9 ± 1.8-fold ($P = .004$) and 11.6 ± 4.6-fold ($P = .045$) for type III procollagen (data not shown).

EXPRESSION OF GROWTH FACTORS AND MATRIX METALLOPROTEINASES WITH NASHA TREATMENT

To investigate potential mechanisms underlying collagen induction following NASHA injections, we measured gene expression of growth factors known to stimulate cutaneous collagen deposition.[16,23] Compared with controls, we found that expression for connective tissue growth factor and all 3 isoforms of transforming growth factor β was increased with NASHA injections

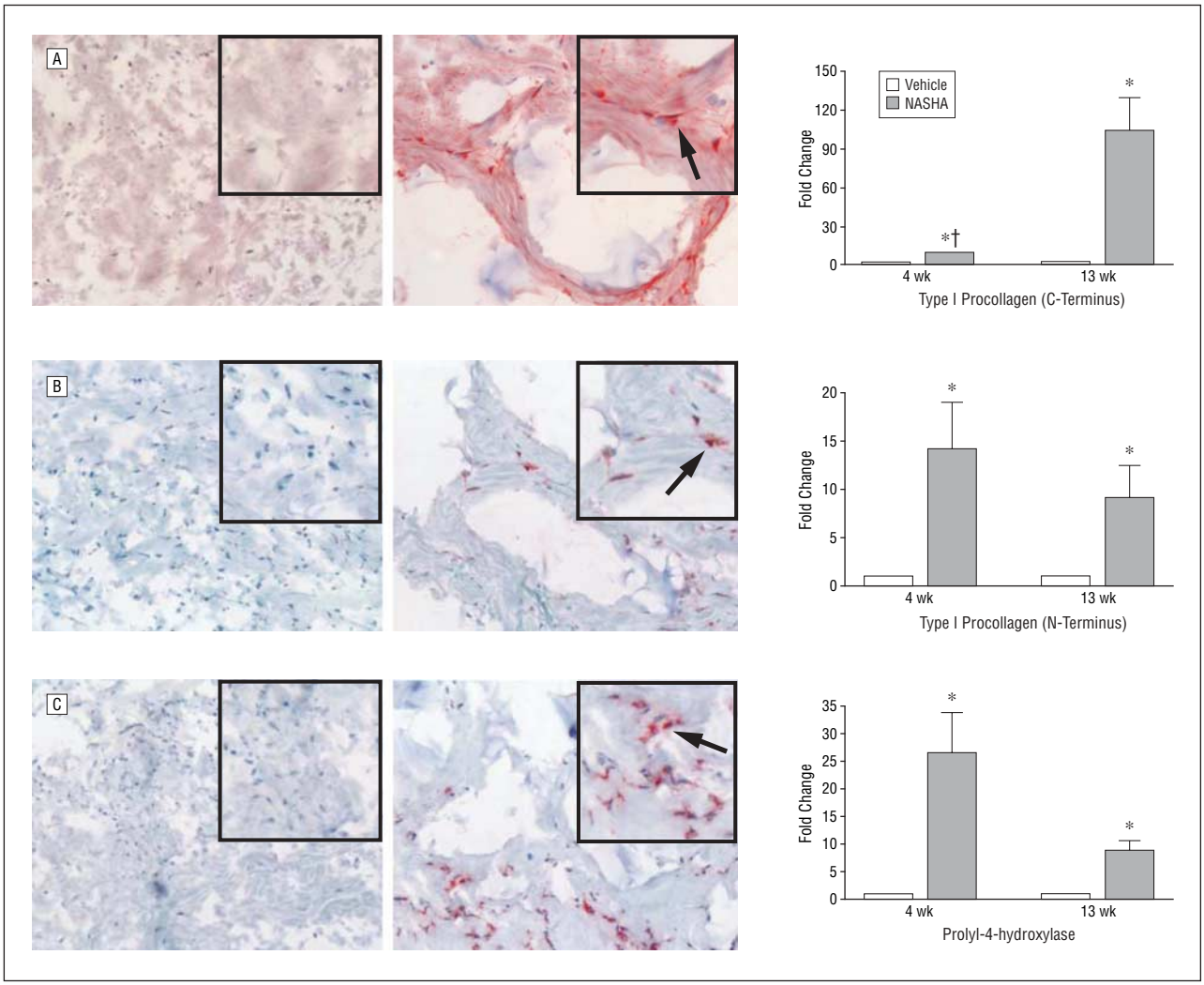

Figure 2. Cross-linked hyaluronic acid injections induce de novo collagen synthesis in photodamaged human skin in vivo. Following injection with isotonic sodium chloride vehicle (left panels) or nonanimal stabilized hyaluronic acid (NASHA) (middle panels), skin samples were stained for markers of type I collagen production, including the C-terminus of type I procollagen (A), the N-terminus of type I procollagen (B), and prolyl-4-hydroxylase (C). Shown here are representative sections at 4 weeks after injection. Positive staining appears red, while counterstaining with hematoxylin appears blue. Examples of positively stained fibroblasts are denoted by arrows. Open spaces in NASHA images (middle panels) correspond to areas that contained filler material (see Figure 1). Original magnification for all main panels ×20; insets ×32. Bar graphs depict quantification of dermal staining for each antibody in skin treated with vehicle or NASHA at 4 and 13 weeks (n=11). Control data are normalized to 1. Data for NASHA-injected samples are presented as mean + SE–fold change. *P<.05. †1.5 SE.

(**Figure 3**A). At 4 weeks, each gene was significantly induced (P<.05), while at 13 weeks transforming growth factors β2 and β3 remained significantly upregulated.

We then measured gene expression of matrix metalloproteinases. These enzymes play an important role in balancing collagen production vs breakdown.[24] For instance, collagenase 1 and collagenase 3 cleave mature collagen, while stromelysin 1 and gelatinase B further cleave collagen fragments.[24,25] Altogether, these 4 enzymes are capable of degrading all components of dermal extracellular matrix. Compared with vehicle, we observed no statistically significant changes (P>.05) in mRNA levels for collagenase 1, collagenase 3, and stromelysin 1 at 4 or 13 weeks after NASHA treatment (data not shown). Gelatinase B mRNA expression was not significantly changed at 4 weeks (P=.07) but exhibited a statistically significant elevation of mean±SE 8.9±3.3-fold at 13 weeks (P=.04). However, immunostaining did not demonstrate significant changes (P=.09) in gelatinase B protein expression at 13 weeks (data not shown).

We also examined gene expression of tissue inhibitors of matrix metalloproteinases, which are endogenous proteins that prevent excessive extracellular matrix breakdown.[24] In NASHA-treated skin, we observed increased expression of tissue inhibitor of matrix metalloproteinases 1, 2, and 3 at 4 weeks (P<.05 for each) (Figure 3B).

FIBROBLASTS DEMONSTRATE A BIOSYNTHETIC PHENOTYPE WITH NASHA INJECTIONS

To further investigate mechanisms for collagen induction by NASHA treatment, we examined fibroblast morphologic characteristics for stretching, a finding that correlates with increased collagen synthesis.[19,26,27] To identify dermal fibroblasts, tissue sections were stained for the C-terminus of type I procollagen (as first depicted in

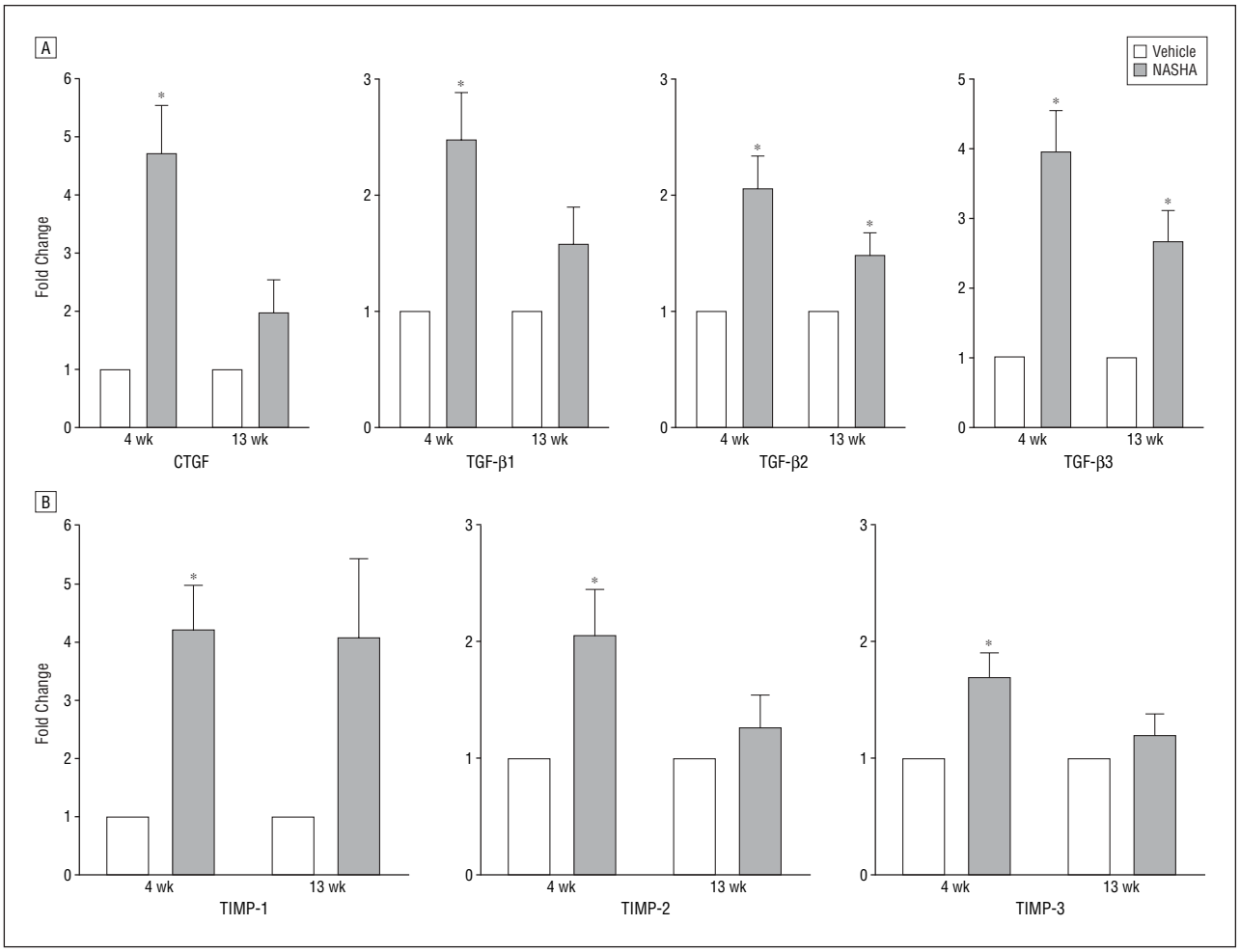

Figure 3. Cross-linked hyaluronic acid injections induce gene expression of profibrotic growth factors and tissue inhibitors of matrix metalloproteinases in human skin in vivo. Total messenger RNA was extracted from skin injected with nonanimal stabilized hyaluronic acid (NASHA) or vehicle, and transcripts for the indicated genes were quantified using real-time reverse-transcriptase polymerase chain reaction. A, Expression for profibrotic growth factors, including connective tissue growth factor (CTGF) and the 3 isoforms of transforming growth factor β (TGF-β1, TGF-β2, and TGF-β3). B, Expression for 3 tissue inhibitors of matrix metalloproteinases (TIMP-1, TIMP-2, and TIMP-3). Data at 4 and 13 weeks are presented. Data for controls (skin treated with isotonic sodium chloride vehicle) are normalized to 1, and data for NASHA-injected skin are depicted as mean + SE–fold. *$P<.05$.

Figure 2A). In NASHA-treated skin at 4 and 13 weeks, we observed many stretched fibroblasts embedded in connective tissue fibers surrounding spaces that contained NASHA (**Figure 4**A). Some cells were directly adjacent to these spaces, but none were inside. Many stretched fibroblasts contained abundant rough endoplasmic reticulum, indicating a heightened state of protein synthesis (Figure 4B).[28] Compared with controls, these cells also had more surface area contacting nearby collagen fibers. In saline-treated skin, dermal fibroblasts did not possess stretched shapes or conspicuous rough endoplasmic reticulum (Figure 4).

Given these observations, we cultured fibroblasts with and without NASHA added to collagen films that mimic dermal extracellular matrix (data not shown).[26] In the presence of collagen alone, fibroblasts were distributed evenly. In cultures containing NASHA, the filler material was localized to scattered pockets (as seen in vivo), and fibroblasts were found surrounding the filler. No cells were identified within the filler, suggesting that fibroblasts do not preferentially populate or bind the filler.

COMMENT

It is generally assumed that many dermal fillers improve the appearance of skin by occupying physical volume. Indeed, we visualized the presence of injected NASHA in skin, suggesting that this filler's effects are derived in part from space filling. To further investigate potential mechanisms for this filler's long-lasting cosmetic benefits, we assessed the biological response of skin to NASHA. We found that NASHA injections induce type I collagen production in photodamaged forearm skin. Currently, there is no evidence to suggest that biological responses fundamentally differ in skin from various anatomic sites.[13,29] As such, it is likely that induction of type I collagen enhances NASHA's clinical duration in facial skin, where the filler is most commonly injected.

We observed increased intracellular and extracellular immunostaining for type I procollagen synthesis in NASHA-treated skin. This pattern is consistent with the production of type I collagen, a protein that is synthesized inside cells and secreted into the surrounding

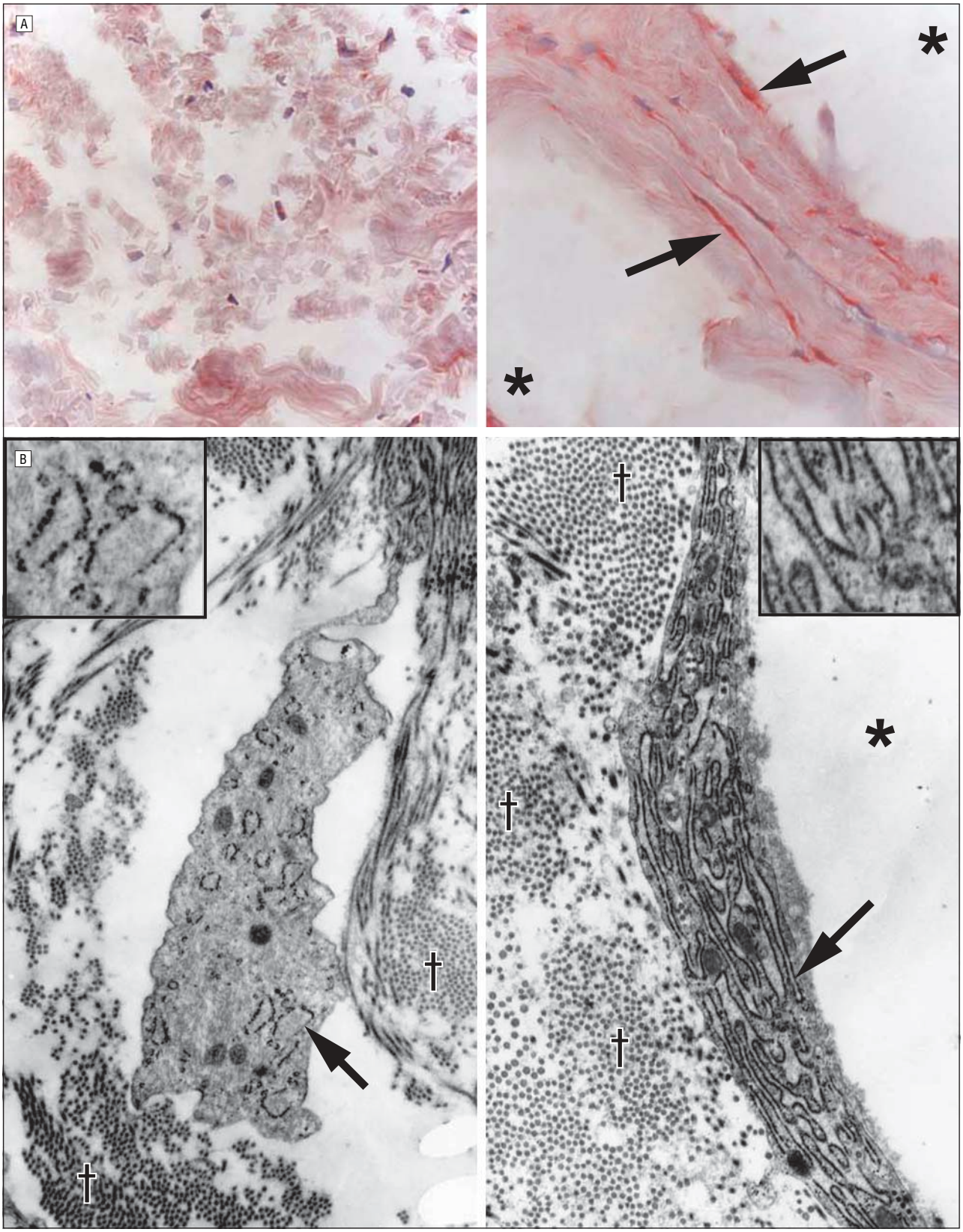

Figure 4. Fibroblasts exhibit a stretched morphologic shape and synthetically active phenotype in skin treated with nonanimal stabilized hyaluronic acid (NASHA). Fibroblasts were visualized in skin injected with isotonic sodium chloride vehicle (left images) or NASHA (right images). Shown are representative tissue sections at 4 weeks after treatment. Open spaces (asterisks) in the NASHA images are consistent with areas that contained filler material. A, Tissue sections were stained for the C-terminus of type I procollagen (as first depicted in Figure 2A), and positively stained fibroblasts (arrows) appear dark red. Cell nuclei were counterstained with hematoxylin (blue) (original magnification ×40). B, Fibroblasts were examined with transmission electron microscopy. Daggers denote collagen fibers/fibrils in the extracellular matrix. Arrows denote rough endoplasmic reticulum, which are intracellular structures involved in synthesizing proteins, including collagen. These are shown at higher power in the insets (main panels original magnification ×2200; insets ×5300).

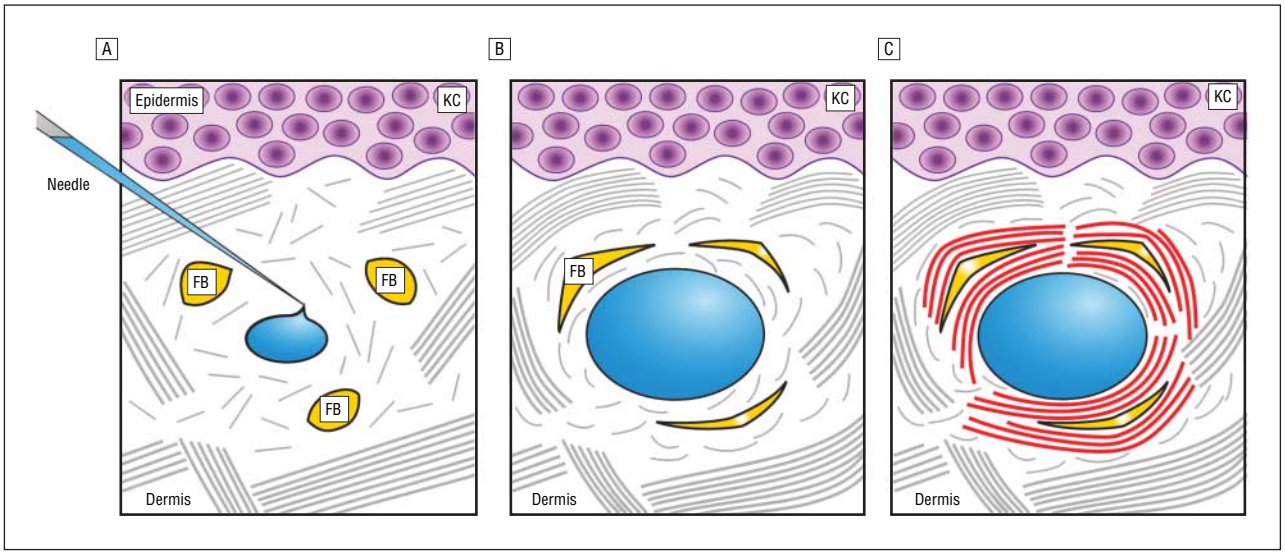

Figure 5. Working model of mechanical tension (stretching) induced by nonanimal stabilized hyaluronic acid (NASHA) injections as a mechanism for collagen induction in human skin. Normal skin consists of an outer epidermis composed mostly of keratinocytes (KCs), and an underlying dermis consisting mostly of extracellular matrix proteins, which are synthesized by fibroblasts (FBs). Types I and III collagen fibers are the major structural components of the extracellular matrix. In contrast to younger skin, which contains intact collagen fibers, photodamaged older skin (depicted here) contains areas of fragmented collagen fibers. A, NASHA is shown as preferentially localizing in areas containing more highly fragmented collagen fibers, since these regions may be more accommodating. B, This results in stretching of existing collagen fibers (curved lines), which is sensed by nearby fibroblasts through cell surface receptors such as integrins. In response, fibroblasts become morphologically stretched (B) and activated to produce extracellular matrix components (C), including new, intact collagen fibers (red lines).

extracellular matrix. The shape and localization of positively stained cells were consistent with fibroblasts, the major producer of collagen in skin. We also found that gene and protein expression for procollagen remained elevated at least 13 weeks, suggesting that NASHA continually activated collagen synthesis pathways in the skin.

We considered that receptor-mediated interaction with injected NASHA may be able to stimulate fibroblasts. Fibroblasts can bind endogenous hyaluronic acid through various receptors, including CD44, the receptor for hyaluronan-mediated motility, intercellular adhesion molecule, and others.[3,18,30] Binding to hyaluronic acid has been implicated in cellular activation, migration, inflammatory activity, and other processes.[3,18] In vivo and in vitro, we observed that fibroblasts preferentially adhered to collagen matrix rather than to NASHA. While these observations do not completely rule out a direct stimulatory role for injected NASHA, they suggest that cellular binding to NASHA does not occur to an appreciable degree and, therefore, is not likely involved in inducing collagen production.

Our findings instead suggest that mechanical tension may play a critical role in stimulating collagen synthesis. We found mechanically stretched fibroblasts in extracellular matrix areas surrounding spaces occupied by NASHA. In vivo, these stretched cells were localized near the filler, but not always directly contacting the material (Figure 2 and Figure 4). This observation, together with our in vitro findings, suggests that NASHA injections alter cellular production of collagen by stretching existing collagen fibers. This stretching, in turn, may impose mechanical tension on nearby fibroblasts, which interact with collagen fibers through cell surface integrin receptors.[27]

Indeed, increased mechanical tension in extracellular matrix has been shown to induce morphologic stretching of fibroblasts and initiation of collagen synthesis.[19,21,25-27,31-36] In contrast to cells in relaxed substrates, fibroblasts cultured in mechanically tense collagen lattices assume a stretched, spindlelike shape rather than appearing short and round. These fibroblasts reorganize their actin cytoskeleton so as to align themselves along lines of tension. Furthermore, stretched fibroblasts increase the production of connective tissue components such as types I and III collagen. It is thought that this reflects a dynamic cellular response for counteracting extrinsically applied tension.[26,27]

Similarly, mechanical tension is maintained in younger skin because intact collagen fibers provide a stable scaffold to which fibroblasts can bind. In contrast, photodamaged older skin contains a greater proportion of fragmented collagen.[24,25] This fragmentation reduces the structural integrity of the extracellular matrix, resulting in less mechanical tension on fibroblasts and, hence, the appearance of "collapsed" (spherical) fibroblasts and reduction of collagen production.[19,25] Since NASHA was localized to discrete areas within photodamaged skin, we believe that NASHA may preferentially flow into areas containing fragmented fibers, since these regions may be more accommodating. The introduction of NASHA may "push" on existing fibers and thereby create a more rigid scaffold that approximates younger dermis. This provides increased mechanical tension, stimulating fibroblasts to produce new, intact collagen (**Figure 5**).

In addition, we found that NASHA injections may promote collagen synthesis by inducing connective tissue growth factor and transforming growth factor β. These profibrotic growth factors stimulate collagen production in fibroblasts and play a key role in connective tissue production during wound repair.[16,37,38] Although the magnitude of induction was relatively modest, small amounts of growth factors often produce biologically significant effects.[38,39] As such, production of growth fac-

tors likely contributes to new extracellular matrix synthesis in NASHA-treated skin.

Finally, we considered the possibility that NASHA injections may stimulate wound repair mechanisms in the skin. Several dermatologic cosmetic procedures, such as carbon dioxide laser resurfacing and microdermabrasion, stimulate collagen production by inducing wound healing.[29,40] The early stages of wound healing are characterized by inflammation and dermal collagen degradation by matrix metalloproteinases. In later stages, breakdown of collagen is replaced by deposition of new collagen. This remodeling is associated with improved appearance of aged skin. In the present study, our earliest skin samples were taken at 4 weeks. Since this would correspond to later stages of wound healing, we cannot completely eliminate wound repair responses as contributing to collagen production in NASHA-treated skin. However, based on expression of matrix metalloproteinases and their regulators (tissue inhibitors of matrix metalloproteinases), collagen deposition in NASHA-treated skin may be promoted in part by reduced collagen breakdown after 4 weeks.

Overall, our findings indicate that NASHA injections induce robust collagen production through several potential mechanisms, including the mechanical stretching of fibroblasts, stimulation of growth factors, and inhibition of collagen breakdown. Of these, mechanical stretching may be the most important. This is because mechanical stretching has been shown to stimulate fibroblasts to produce growth factors and tissue inhibitors of matrix metalloproteinases while also inhibiting expression of matrix metalloproteinases.[26,33] Thus, mechanical stretch may act as a proximal stimulus that accounts for many of our observations.

To our knowledge, NASHA is the first hyaluronic acid filler to clearly demonstrate substantial and sustained induction of collagen synthesis in the dermis. It remains to be seen whether other hyaluronic acid fillers, such as hylan B (sold as Hylaform and Hylaform Plus; Inamed Aesthetics, Santa Barbara, Calif), another form of NASHA (sold as Captique; Genzyme Corporation, Cambridge, Mass), or a recently approved version of cross-linked hyaluronic acid (sold as Juvéderm; Allergan Inc, Irvine, Calif), can also stimulate collagen deposition in skin. Decreased collagen levels likely contribute to wrinkle formation,[13] and as such, the stimulation of collagen production by NASHA may explain in part this filler's cosmetic benefits.

Furthermore, histologic studies indicate that injected NASHA lasts 6 to 9 months in skin.[5,8] This correlates well with NASHA's clinical duration, suggesting a predominantly volume-derived effect during this time. However, as more NASHA injections are received in a particular area of skin over time, we predict that collagen will accumulate at that location. This is because the half-life of dermal collagen is estimated to be 15 years.[41] Thus, although it is unclear whether a single NASHA injection stimulates enough collagen to create noticeable clinical changes, we hypothesize that collagen accumulation from multiple NASHA injections over time may yield durable cosmetic effects. Long-term studies correlating biochemical changes to observable clinical improvements may clarify this.

Finally, our findings suggest that cross-linked hyaluronic acid may have application as a stimulator of collagen in conditions of collagen deficiency, including atrophic skin disorders (eg, human immunodeficiency virus–related lipodystrophy or steroid-induced dermal atrophy). Additionally, in fibrotic conditions such as scleroderma or keloids, we suggest that mechanical stretch may be a potential pathologic mechanism worth investigating in future studies.

Accepted for Publication: August 9, 2006.
Correspondence: Frank Wang, MD, Department of Dermatology, University of Michigan Medical School, 1618 A. Alfred Taubman Healthcare Center, 1500 E Medical Center Dr, Ann Arbor, MI 48109 (frawang@umich.edu).
Author Contributions: Drs Wang and Garza contributed equally to this article. Drs Wang and Fisher had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design*: Garza, Kang, Varani, Orringer, Fisher, and Voorhees. *Acquisition of data*: Wang, Garza, Kang, Varani, and Fisher. *Analysis and interpretation of data*: Wang, Garza, Kang, Varani, Orringer, Fisher, and Voorhees. *Drafting of the manuscript*: Wang. *Critical revision of the manuscript for important intellectual content*: Wang, Garza, Kang, Varani, Orringer, Fisher, and Voorhees. *Statistical analysis*: Wang. *Obtaining funding*: Voorhees. *Administrative, technical, or material support*: Fisher, Varani, and Orringer. *Supervision*: Fisher, Kang, Orringer, and Voorhees.
Financial Disclosure: None reported.
Funding/Support: Medicis Aesthetics donated cross-linked hyaluronic acid–containing syringes for research purposes but had no involvement in the design or conduct of the study or in the collection, management, analysis, and interpretation of the data. Medicis was not involved in the preparation or review of the manuscript. This study was supported by the University of Michigan Department of Dermatology Cosmetic Research Fund; grants 5T32 AR007197 (F.W.) and 1K24 AR01259 (S.K.) from the National Institutes of Health, Bethesda, Md; and the Babcock Research Endowment, University of Michigan, Ann Arbor.
Acknowledgment: We thank Laura VanGoor, BFA, for assistance with graphic material; Ted Hamilton, MS, for assistance with statistical analysis; and Craig Hammerberg, PhD, for technical assistance.

REFERENCES

1. Matarasso SL, Carruthers JD, Jewell ML. Consensus recommendations for soft-tissue augmentation with nonanimal stabilized hyaluronic acid (Restylane). *Plast Reconstr Surg*. 2006;117:3S-34S.
2. Sakai S, Yasuda R, Sayo T, Ishikawa O, Inoue S. Hyaluronan exists in the normal stratum corneum. *J Invest Dermatol*. 2000;114:1184-1187.
3. Weindl G, Schaller M, Schafer-Korting M, Korting HC. Hyaluronic acid in the treatment and prevention of skin diseases: molecular biological, pharmaceutical and clinical aspects. *Skin Pharmacol Physiol*. 2004;17:207-213.
4. Tammi MI, Day AJ, Turley EA. Hyaluronan and homeostasis: a balancing act. *J Biol Chem*. 2002;277:4581-4584.
5. Duranti F, Salti G, Bovani B, Calandra M, Rosati ML. Injectable hyaluronic acid gel for soft tissue augmentation. A clinical and histological study. *Dermatol Surg*. 1998;24:1317-1325.

6. Biesman B. Soft tissue augmentation using Restylane. *Facial Plast Surg*. 2004;20:171-177.
7. Andre P. Evaluation of the safety of a non-animal stabilized hyaluronic acid (NASHA–Q-Medical, Sweden) in European countries: a retrospective study from 1997 to 2001. *J Eur Acad Dermatol Venereol*. 2004;18:422-425.
8. Lemperle G, Morhenn V, Charrier U. Human histology and persistence of various injectable filler substances for soft tissue augmentation. *Aesthetic Plast Surg*. 2003;27:354-366.
9. Narins RS, Brandt F, Leyden J, Lorenc ZP, Rubin M, Smith S. A randomized, double-blind, multicenter comparison of the efficacy and tolerability of Restylane versus Zyplast for the correction of nasolabial folds. *Dermatol Surg*. 2003;29:588-595.
10. Olenius M. The first clinical study using a new biodegradable implant for the treatment of lips, wrinkles, and folds. *Aesthetic Plast Surg*. 1998;22:97-101.
11. Yoon ES, Han SK, Kim WK. Advantages of the presence of living dermal fibroblasts within Restylane for soft tissue augmentation. *Ann Plast Surg*. 2003;51:587-592.
12. Fisher GJ, Datta SC, Talwar HS, et al. Molecular basis of sun-induced premature skin ageing and retinoid antagonism. *Nature*. 1996;379:335-339.
13. Griffiths CE, Russman AN, Majmudar G, Singer RS, Hamilton TA, Voorhees JJ. Restoration of collagen formation in photodamaged human skin by tretinoin (retinoic acid). *N Engl J Med*. 1993;329:530-535.
14. Talwar HS, Griffiths CE, Fisher GJ, Hamilton TA, Voorhees JJ. Reduced type I and type III procollagens in photodamaged adult human skin. *J Invest Dermatol*. 1995;105:285-290.
15. Kang S, Cho S, Chung JH, Hammerberg C, Fisher GJ, Voorhees JJ. Inflammation and extracellular matrix degradation mediated by activated transcription factors nuclear factor-kappaB and activator protein-1 in inflammatory acne lesions in vivo. *Am J Pathol*. 2005;166:1691-1699.
16. Quan T, He T, Kang S, Voorhees JJ, Fisher GJ. Ultraviolet irradiation alters transforming growth factor-β/smad pathway in human skin in vivo. *J Invest Dermatol*. 2002;119:499-506.
17. Lin W, Shuster S, Maibach HI, Stern R. Patterns of hyaluronan staining are modified by fixation techniques. *J Histochem Cytochem*. 1997;45:1157-1163.
18. Tammi R, MacCallum D, Hascall VC, Pienimaki JP. M. H, Tammi M. Hyaluronan bound to CD44 on keratinocytes is displaced by hyaluronan decasaccharides and not hexasaccharides. *J Biol Chem*. 1998;273:28878-28888.
19. Varani J, Schuger L, Dame MK, et al. Reduced fibroblast interaction with intact collagen as a mechanism for depressed collagen synthesis in photodamaged skin. *J Invest Dermatol*. 2004;122:1471-1479.
20. Olsen BR, Berg RA, Kishida Y, Prockop DJ. Collagen synthesis: localization of prolyl hydroxylase in tendon cells detected with ferritin-labeled antibodies. *Science*. 1973;182:825-827.
21. Lambert CA, Colige AC, Lapiere CM, Nusgens BV. Coordinated regulation of procollagens I and III and their post-translational enzymes by dissipation of mechanical tension in human dermal fibroblasts. *Eur J Cell Biol*. 2001;80:479-485.
22. Orringer JS, Voorhees JJ, Hamilton T, et al. Dermal matrix remodeling after nonablative laser therapy. *J Am Acad Dermatol*. 2005;53:775-782.
23. Ignotz RA, Massague J. Transforming growth factor β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J Biol Chem*. 1986;261:4337-4345.
24. Fisher GJ, Wang ZQ, Datta SC, Varani J, Kang S, Voorhees JJ. Pathophysiology of premature skin aging induced by ultraviolet light. *N Engl J Med*. 1997;337:1419-1428.
25. Varani J, Perone P, Fligiel SE, Fisher GJ, Voorhees JJ. Inhibition of type I procollagen production in photodamage: correlation between presence of high molecular weight collagen fragments and reduced procollagen synthesis. *J Invest Dermatol*. 2002;119:122-129.
26. Kessler D, Dethlefsen S, Haase I, et al. Fibroblasts in mechanically stressed collagen lattices assume a "synthetic" phenotype. *J Biol Chem*. 2001;276:36575-36585.
27. Silver FH, Siperko LM, Seehra GP. Mechanobiology of force transduction in dermal tissue. *Skin Res Technol*. 2003;9:3-23.
28. Nagata K. Expression and function of heat shock protein 47: a collagen-specific molecular chaperone in the endoplasmic reticulum. *Matrix Biol*. 1998;16:379-386.
29. Orringer JS, Kang S, Johnson TM, et al. Connective tissue remodeling induced by carbon dioxide laser resurfacing of photodamaged human skin. *Arch Dermatol*. 2004;140:1326-1332.
30. Day AJ, Prestwich GD. Hyaluronan-binding proteins: tying up the giant. *J Biol Chem*. 2002;277:4585-4588.
31. Delvoye P, Wiliquet P, Leveque JL, Nusgens BV, Lapiere CM. Measurement of mechanical forces generated by skin fibroblasts embedded in a three-dimensional collagen gel. *J Invest Dermatol*. 1991;97:898-902.
32. Fisher GJ, Choi HC, Bata-Csorgo Z, et al. Ultraviolet irradiation increases matrix metalloproteinase-8 protein in human skin in vivo. *J Invest Dermatol*. 2001;117:219-226.
33. Grinnell F. Fibroblast-collagen-matrix contraction: growth-factor signalling and mechanical loading. *Trends Cell Biol*. 2000;10:362-365.
34. Grinnell F. Fibroblast biology in three-dimensional collagen matrices. *Trends Cell Biol*. 2003;13:264-269.
35. Lambert CA, Colige AC, Munaut C, Lapiere CM, Nusgens BV. Distinct pathways in the over-expression of matrix metalloproteinases in human fibroblasts by relaxation of mechanical tension. *Matrix Biol*. 2001;20:397-408.
36. Lambert CA, Soudant EP, Nusgens BV, Lapiere CM. Pretranslational regulation of extracellular matrix macromolecules and collagenase expression in fibroblasts by mechanical forces. *Lab Invest*. 1992;66:444-451.
37. Quan T, He T, Kang S, Voorhees JJ, Fisher GJ. Connective tissue growth factor: expression in human skin in vivo and inhibition by ultraviolet irradiation. *J Invest Dermatol*. 2002;118:402-408.
38. Takehara K. Growth regulation of skin fibroblasts. *J Dermatol Sci*. 2000;24(suppl):S70-S77.
39. Massague J. How cells read TGF-β signals. *Nat Rev Mol Cell Biol*. 2000;1:169-178.
40. Karimipour DJ, Kang S, Johnson TM, et al. Microdermabrasion: a molecular analysis following a single treatment. *J Am Acad Dermatol*. 2005;52:215-223.
41. Verzijl N, DeGroot J, Thorpe SR, et al. Effect of collagen turnover on the accumulation of advanced glycation end products. *J Biol Chem*. 2000;275:39027-39031.