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Nitric oxide produced by iNOS is associated with collagen synthesis in keloid scar formation

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Abstract

Nitric oxide (NO) has emerged as an important mediator of many physiological functions. Recent reports have shown that NO participates in the wound healing process, however, its role in keloid formation remains unclear. This study aimed to investigate the effect of NO on keloid fibroblasts (KF) and to determine the levels of inducible nitric oxide synthase (iNOS) expression in clinical specimens of keloid. Scar tissue from seven keloid patients with matched perilesion skin tissue controls was studied for inducible nitric oxide synthase expression and location. In addition, primary keloid and normal scar skin fibroblast cultures were set up to investigate the effects of NO in inducing collagen type I expression. Inducible nitric oxide synthase expression, and NO production were elevated in keloid scar tissues but not in matched perilesion skin tissues. Furthermore, exposure of KF to exogenous NO resulted in increased expression of collagen type I in a dose-dependent manner. NO exposure also induced time-course dependent collagen I expression that peaked at 24 h in KF. Taken together, these results indicate that excess collagen formations in keloid lesion may be attributed to iNOS overexpression.
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Wound healing is an essential process for injured tissue to recover its naturally intact structure. The healing process involves several steps including proliferation, migration, differentiation, and tissue collagen remodeling [1]. Keloid is characterized by excess collagen deposition. It is a lately spreading skin overgrowth and may be considered a plastic surgeon's nightmare. It recurs tenaciously when removed and is tumorigenic when transplanted [2]. Keloid fibroblasts (KF) have reduced growth factor requirements [3], higher growth rates, and reduced contact inhibitions [4]. Keloids exhibit a high rate of collagen and proteoglycan accumulation by fibroblasts [5], typical in the early phase of a wound but not in normal skin or scars [6]. Collagen synthesis is very important for healing wounds, being synthesized and secreted by fibroblast in soluble form and deposited extracellularly. All these steps have to progress in a coordinated manner and require the presence of various cytokines, growth factors, and other bioactive molecules [7].

The biological molecular nitric oxide (NO) is synthesized in rodent wounds [8]. NO is generated endogenously from L-arginine by nitric oxide synthase (NOS) [9]. Three forms of NOS are expressed in the human tissue, the two constitutive forms namely, the endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible form NOS (iNOS) [10]. The iNOS is calcium independent and provides NO production, iNOS mediates cytotoxic and cystostatic effects of the immune system, as NO possesses antibacterial,

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antiparasitic, and antitumor properties [6]. Recently, NO was found to regulate collagen catabolism by playing a role in wound collagen matrix deposition [11]. NO synthesis occurs for prolonged periods after injury. Human dermal fibroblasts have also been shown to express both eNOS and iNOS proteins which could be of importance during inflammatory and/or later stages of proliferation and tissue remodeling after skin injury in humans [12–14]. The amounts of NO synthesized by cNOS isoform are roughly a thousand times lower than those synthesized by the inducible isoform [15]. iNOS activity is the primary source of NO in healing wounds [16]. Although there are many studies indicating inhibition of collagen synthesis by NO in various cell types like arterioles, vascular smooth muscle cells, and pleural mesothelial cells [17–19], NO was also found to induce collagen synthesis in small intestinal lamina propria fibroblasts [20], and normal skin wound healing reepithelialization [21]. These studies indicate that iNOS and NO participate in the wound healing process.

In this report, we investigated whether NO produced by iNOS contributed to the overexpression of collagen in KF. We also determined iNOS and NO production levels in keloid patient skin lesions and analyzed the effects of exogenous NO on collagen synthesis in keloid fibroblasts.

Materials and methods

A total of seven keloid and perilesion skin tissue samples were obtained from seven Taiwanese patients (four women and three men, mean age 45.0 years; range 21–76 years). Skin tissues were excised from the chest in three patients, the ear lobe in three patients, and the shoulder region in one patient (duration of disease 1–6 months). Only typical, clinically clear-cut cases that extended beyond the original boundary of the wound were included in this study. None of the patients had received previous treatment other than pressure therapy. Seven site-matched normal scar skin specimens (two women and five men, mean age 43.9 years; range 26–66 years) were obtained at the time of other unrelated operations. Prior written informed consent was obtained from the patients and all procedures received the approval of the ethics board at Kaohsiung Veterans General Hospital and Taipei Medical University in adherence to the Helsinki Principles.

Cell culture

Primary fibroblast cultures were established from the keloids and normal scar skin specimens as described above. Dermis from the keloids and normal scar skin tissues was minced and incubated in a solution of collagenase type I (0.5 mg/mL) and trypsin (0.2 mg/mL) at 37 °C for 6 h. Cells were pelleted and grown in tissue culture flasks. Cells were grown at 37 °C in Dulbecco's modified Eagle's medium (Gibco/BRL, Gaithersburg, MD) supplemented with and 10% (vol/vol) fetal bovine serum (HyClone, USA) for primary culture and subsequent cultures, respectively, and 5 mg/mL L-glutamine in an atmosphere of 5% CO_2. Fibroblasts in the primary cultures were trypsinized with 0.05% trypsin/0.53 mM EDTA (Life Technologies, Carlsbad, CA) and passaged. All experiments were performed with third passage cells.

RT-PCR

Total RNA was isolated from the keloids, perilesion skin tissues, cultured KF, and NSSF with Tripure mRNA isolation reagent (Roche, Germany), as suggested by the supplier's instructions. The cDNA was reverse transcribed from total RNA by MMLV reverse transcriptase (USB, USA) with oligo(dT). Sequence of the PCR primers was as described below: iNOS sense 5′-CGGTGCTGTATT TCCTTACGAGGCGAAGAA-3′, iNOS anti-sense 5′-G GTGCTACTTGTTAGGAGGGTCAAGTAAAG-3′ [6]; collagen type I sense 5′-AATTCCTGGTCTGGGGCA CC-3′, collagen type I antisense 5′-GGCGGCCAGGGC TCCGAC-3′ [22]; GAPDH sense 5′-GTCCACTGGC GTCT TCACCACC-3′, GAPDH anti-sense 5′-AGGCA TTGCTGATGATCTTGAGGC-3′; and β-actin sense 5′-GTGGGGCGCCCCAGGCACCA-3′, β-actin antisense 5′-CTCCTTAATGTCACGCACGATTTC-3′. RT-PCR was performed with 2 μl cDNA samples containing 10 pmol of each primer, 200 μM dNTP, 2 mM $MgCl_2$, and 1 U *Taq* polymerase (Gibco BRL, MD) in a total volume of 25 μl with the following program: 5 min at 94 °C, 35 cycles of 1 min 94 °C, 1 min at 61 °C (iNOS), 62 °C (collagen type I), 54 °C (GAPDH), 60 °C (β-actin), 1 min at 72 °C, and 10 min at 72 °C. The amplified products were analyzed on a 2% agarose gel, stained with ethidium bromide, and photographed under UV light.

Western blotting

Skin tissue samples were excised and frozen immediately at −70 °C. The samples were homogenized in containing homogenizing buffer: 20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1.5 mg leupeptin, and protease inhibitor cocktail. The tissue extract was cleared by centrifugation at 13,000 rpm for 15 min. The amount of protein in the lysate was determined using Bradford method (Bio-Rad, USA). Total proteins were separated using 6% or 12% SDS–polyacrylamide gel, transferred onto nitrocellular membranes (Schleicher & Schnell, Keene, NH) in 25 mM Tris, 0.192 M glycine, pH 8.3, 20% methanol at 30 V overnight. The membranes were then blocked with 5% non-fat milk in 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20 overnight, and incubated with anti-β-actin (1:1000, Sigma, MO), anti-iNOS (1:1000, R&D, USA), or anti-human collagen type I (1:1000, Research Diagnostics, USA) for 2 h. The blots were further washed with Tris–HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20 for three times of 10 min each, and incubated with secondary antibody (1:5000, anti-rabbit or anti-mouse IgG, Santa Cruz,

CA) for 1 h. Antigens were visualized using chemiluminescence kit (ECL, Amersham, USA) followed by autoradiography (Hyperfilm, Amersham, USA).

Nitrate/nitrite concentration determination

Skin tissue was homogenized in PBS (pH 7.4) and centrifuged at 10,000*g* for 20 min at 4 °C. The supernatant was filtrated through 0.45 μm filter (Millipore, USA). The amounts of total proteins in the supernatant were determined using Bradford method. Equal amounts of total proteins were applied to the assay. The final products of NO in vivo are nitrite (NO_2^-) and nitrate (NO_3^-). The nitrate/nitrite (NO_x) concentrations were determined by Nitrate/nitrite Colorimetric Assay kit (Cayman, USA).

Immunohistochemical

Skin tissue specimens were fixed in 10% buffered formaldehyde solution overnight. Dehydrated tissues were embedded in paraffin wax and cut at 5 μm thickness. The sections were stained by hematoxylin and eosin (H&E) or with Van Gieson's picrofuchsin stain. Serial sections were stained for iNOS using antibody against iNOS (1:100, Chemicon, CA) for 2 h at room temperature. The antigens were visualized using ABC kit with DAB as substrate (Santa Cruz, CA). The sections were counterstained with hematoxylin.

Collagen type I synthesis assay

Cultured KF and normal scar skin fibroblasts (NSSF) were grown in culture dishes and treated with different concentrations of NO donor DETA NONOate (diethylenetriamine NONOate; δ-NONOate, Cayman, USA) at 0, 125, 250, and 500 μM for 24 h in dose-dependent experiment. In addition, a time course experiment was performed. The cells were treated with 500 μM DETA, NONOate and harvested at different times of 0, 6, 12, 24, or 48 h. Protein lysates and cDNA were generated from the cells and analyzed by Western blot and RT-PCR to determine the effects of exogenous NO on collagen type I expression in these cells.

Statistical analysis

All data were reporter as means + SEM of at least three separate experiments. A paired Student's *t* test was employed for statistical analysis, with significant differences determined as $P < 0.05$.

Results

The iNOS expression and NO production were elevated in keloid scar tissue

To identify the roles of iNOS in Keloid formation, RT-PCR analysis was used to determine the levels of iNOS mRNA expression in seven keloid tissues and matched perilesion skin tissues. The results showed a significant increase of iNOS mRNA in all seven keloid tissues compared to perilesion skin tissue controls (Fig. 1A). Semi-quantitative analysis revealed 32.3 ± 8.6 fold increase in iNOS mRNA of keloid tissues compared with perilesion skin tissue controls ($P < 0.01$, Fig. 1B). In addition, Western blot analysis also showed iNOS expressions in all keloid tissues but not in perilesion skin tissue controls (Fig. 1C). Densitometry analysis showed 18.9 ± 6.5 fold overexpression of iNOS protein in seven keloid tissues compared to perilesion skin tissue controls ($P < 0.01$, Fig. 1D). nNOS and eNOS protein levels were also determined in keloid tissues and perilesion skin tissue controls. No significant differences in nNOS and eNOS protein expressions were found (data not shown).

To determine whether iNOS was functional in keloid tissues, NO_x (nitrate and nitrite) concentrations were quantitatively measured by using Nitrate/nitrite Colorimetric Assay kit in tissue extracts. Fig. 1E shows NO_x production in keloid tissues were 4.9 ± 0.9 fold higher than perilesion skin tissue controls ($P < 0.01$). These data showed increased production of NO were found only in keloid tissues. The increased NO concentrations may be associated with the elevated iNOS expression in keloid tissues.

To further determine the extent and location of iNOS protein expression in keloid tissue in situ, immunohistochemistry was performed using a monoclonal antibody specific for iNOS protein. To Fig. 2 reveals significant iNOS protein overexpression in the basal layer of seven keloid tissues (Figs. 2C and D). iNOS protein was also expressed in normal skin scar tissues but in lower levels as in keloid tissue (data not shown). These results showed the iNOS protein was expressed in the basal layer of keloid tissues and might play an important role in keloid collagen accumulation.

Collagen synthesis are activated by exogenous NO in keloid fibroblast

Seven primary KF cultures and seven site-matched NSSF cultures were set up to determine whether NO played a role in collagen synthesis. NO donor DETA NONOate was added to NSSF and KF at third passages in dose-dependent (0, 125, 250, and 500 μM) and time-course dependent (0, 6, 12, 24, and 48 h) experiments. Fig. 3A shows exogenous NO induced collagen type I mRNA expression in KF but not in NSSF in a dose-dependent manner. Quantitative analysis revealed collagen type I mRNA expression was significantly elevated in all seven KF after the addition of DETA NONOate (Fig. 3B, 5.7- to 22.7-fold increase, $P < 0.01$). Similar findings were also found in collagen protein expression in KF and not in NSSF after adding DETA NONOate. Fig. 3C shows that collagen type I protein expression in KF was enhanced by exogenous NO stimulation. Quantitative analysis observe that NO did not affect the collagen type I protein

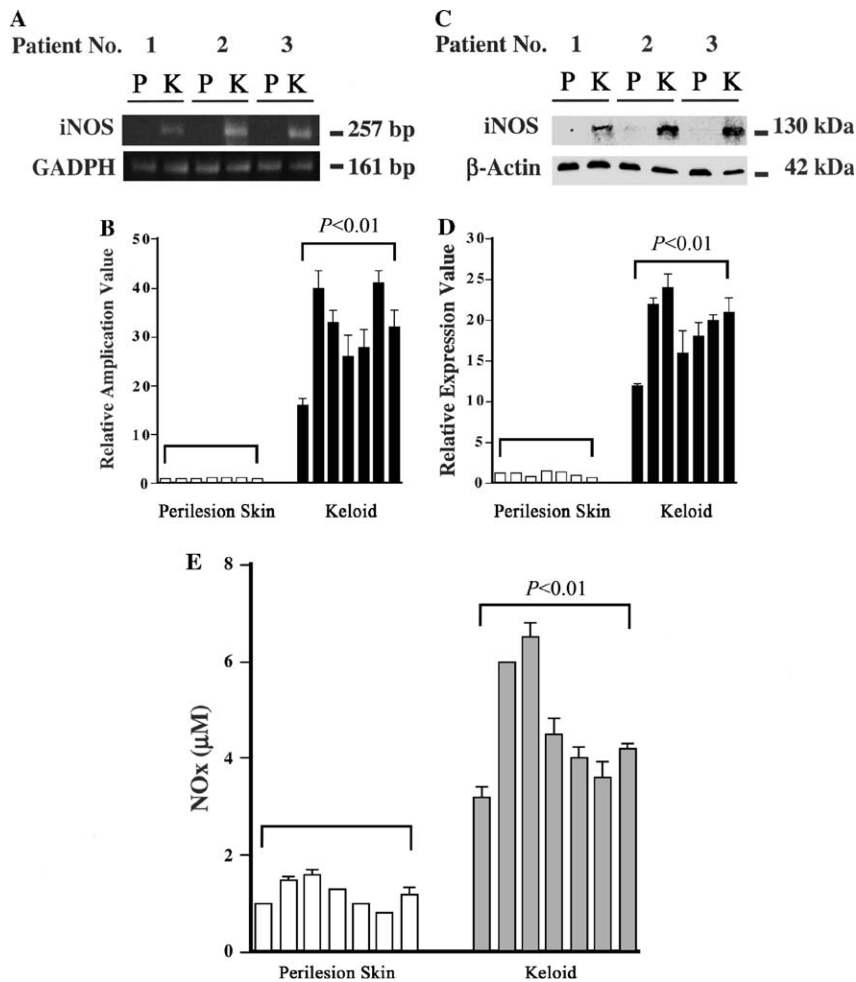

Fig. 1. Expression of iNOS gene and NO_x production in keloid lesion and normal perilesion tissues. (A) RT-PCR shows the iNOS gene expression in three representative keloid patients (K) and matched perilesion skin (P) tissues. Note the expression of iNOS mRNA was enhanced in all seven representative keloid tissue samples compared to perilesion controls. (B) Relative amplification values of iNOS RT-PCR calibrated by the iNOS mRNA expression versus GAPDH mRNA expression. (C) Western blot analysis of iNOS protein expression in three representative keloid patients and matched perilesion skin tissues. (D) Relative expression values of iNOS protein calibrated by the iNOS versus β-actin protein expression. Note iNOS protein expression in keloid tissues was significantly higher than perilesion skin tissues. (E) NOS activity in keloid and matched perilesion skin tissues. The NOS activity was determined by measuring NO products.

basal expression in NSSF, but exogenous NO stimulated the collagen type I expression in KF in a dose-dependent manner. The collagen type I protein expression was elevated from a 6.2- to 13.1-fold increase in seven KF compared to NSSF cultures when the DETA NONOate concentrations was increased from 125 to 500 μM ($P < 0.01$, Fig. 3D).

The effects of NO on collagen expression were also investigated in time course experiments. The results showed exogenous NO induced highest collagen type I mRNA (Fig. 4A) and protein (Fig. 4C) expressions in KF at 24 h. Fig. 4B reveals a 2.5- to 3.3-fold increase in collagen type I RNA expression after adding DETA NONOate for 6–24 h compared to time 0 control ($P < 0.05$). Collagen type I protein expressions were elevated from a 1.6- to 4.4-fold increase compared to time 0 control (Fig. 4D). These results showed that exogenous NO induced collagen type I expression in KF in a dose-dependent manner, with the highest expression at 24 h after DETA NONOate treatment.

Discussion

The regulation and function of iNOS in the skin appears to mainly be associated with inflammatory and immune responses and is implicated into the pathogenesis of a wide range of human skin diseases [23]. Our findings demonstrate that iNOS mRNA had elevated expression in the skin lesions of seven patients with keloid but not in perilesion skin tissue controls. The iNOS protein expression in three patients was also conspicuously increased in keloid lesions when compared to the perilesion skin tissue controls. Both mRNA and protein of iNOS were clearly raised in keloid lesions. Experimental evidences also suggested that increased expression of iNOS, which is calcium independent, is related to various pathological processes, and

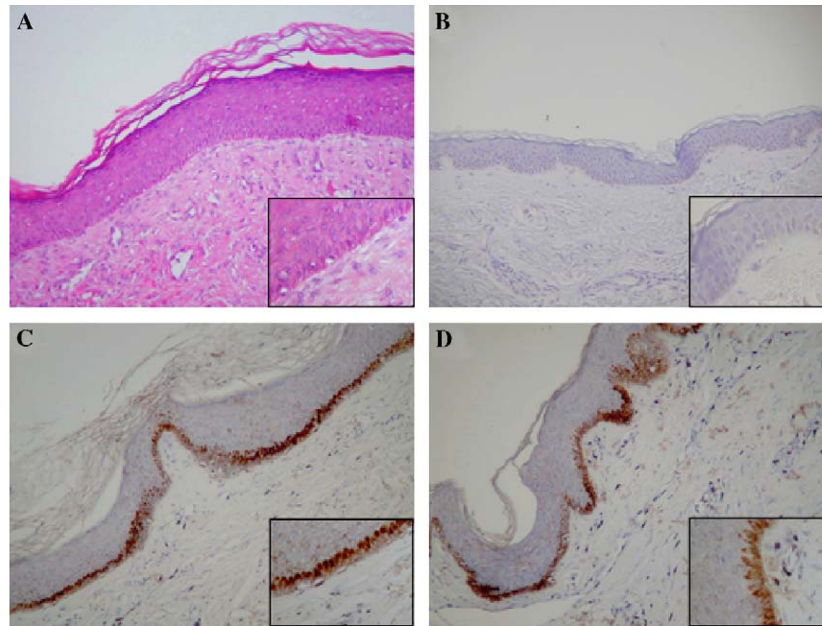

Fig. 2. Immunohistochemical analysis of keloid and matched normal skin tissues with iNOS antibody. (A) H&E staining of a representative keloid tissue (100×). Inset represents a 400× power-field view. (B) iNOS immunohistochemical staining analysis in a representative perilesion skin tissue. Note no expression of iNOS (100×). (C–D) Two representative keloid tissues stained positive with iNOS antibody (C patient 2, D patient 3). Note the brown colored DAB deposits revealed positive iNOS protein only in the basal layer of keloid lesions.

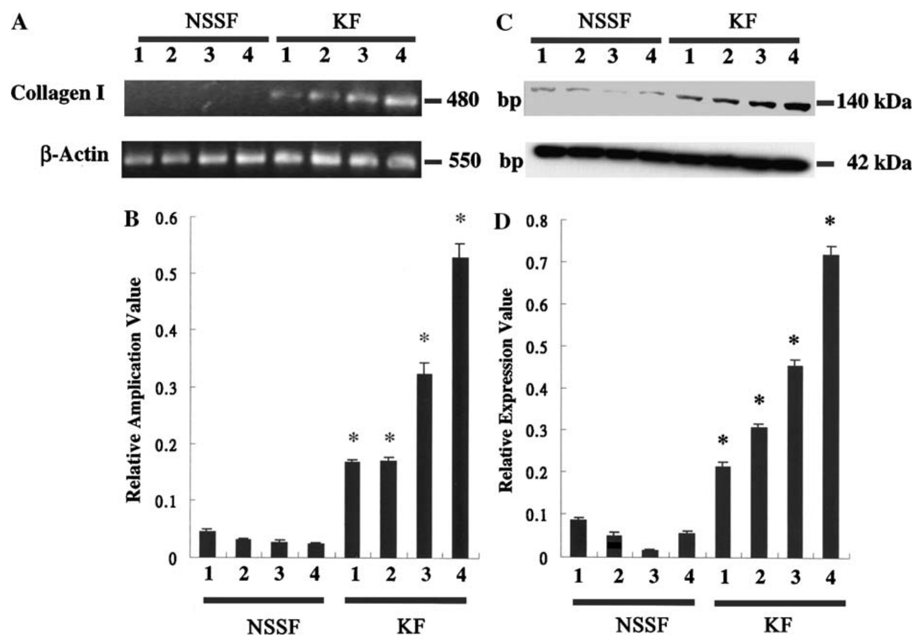

Fig. 3. Type I collagen expression in primary KF versus NSSF. Lane 1: control (0 μM DETA NONOate); lane 2: 125 μM DETA NONOate; lane 3: 250 μM DETA NONOate; lane 4: 500 μM DETA NONOate. (A) RT-PCR analysis of collagen type I mRNA expression in NSSF and KF. Note dose-dependent increase of collagen type I mRNA expression in KF but not in NSSF. (B) Calibration of the collagen type I mRNA expression versus β-actin mRNA expression in NSSF and KF. Note collagen type I mRNA expression in KF was significantly higher than NSSF ($^{*}P < 0.01$, $n = 3$). (C) Western blot analysis of collagen type I protein expression in NSSF and KF. (D) Calibration of the collagen type I protein expression versus β-actin protein expression in NSSF and KF. Note collagen type I protein expression in KF was significantly higher than in NSSF ($^{*}P < 0.01$, $n = 3$).

such as inflammation and cancer [24]. The iNOS was also reported to induce the healing of skin and the intestinal mucosa, the killing of certain bacteria, regulation of T cell proliferation and differentiation (Th1 vs Th2), and control of leukocyte recruitment [25].

Previous reports have shown increased iNOS expression in psoriasis [26], atropic skin [27], and contact dermatitis [28]. Our results showed significant expression of iNOS protein in the basal layer of keloid tissues. The cutaneous basement membrane zone (basal layer) composed of

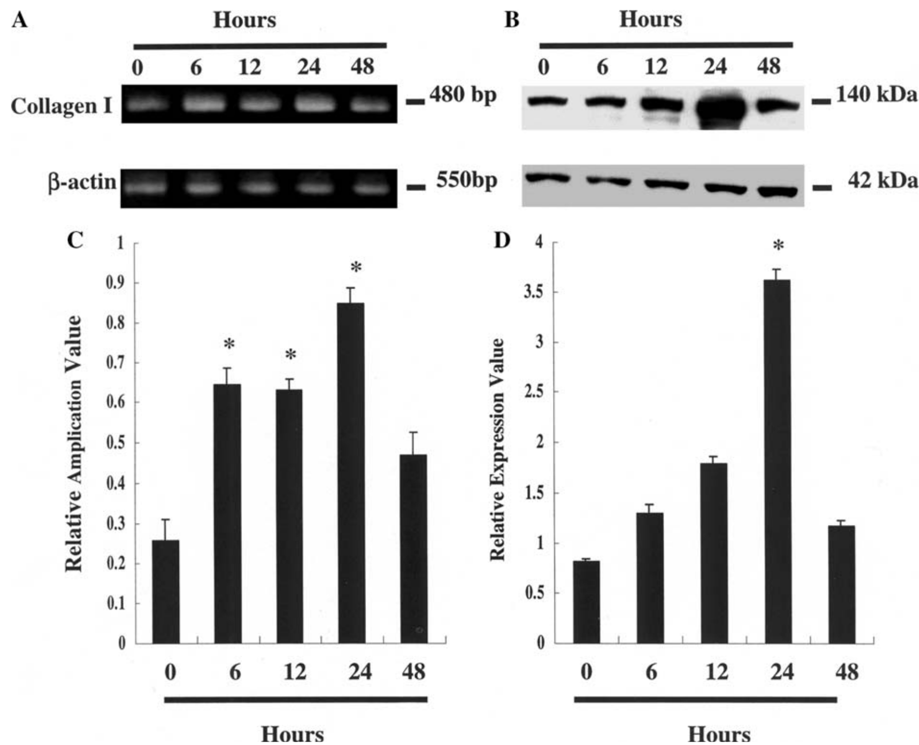

Fig. 4. Time-course collagen type I RNA and protein expression in KF after adding NO donor DETA NONOate. KF was treated with 500 μM DETA NONOate and harvested at different time points. (A) RT-PCR analysis of collagen type I mRNA expression in KF. Note the peak expression at 24 h after DETA NONOate treatment. (B) Calibration of the collagen type I RNA expression versus β-actin mRNA expression in KF. Note collagen type I RNA expression in KF was significantly higher in 6–24 h post-treatment compared to Time 0 control ($^*P < 0.01$, $n = 3$). (C) Western blot analysis of collagen type I protein expression in KF after DETA NONOate treatment. Note the collagen type I protein expression was enhanced in KF and the expression peaked at 24 h post-treatment. (D) Calibration of the collagen type I protein expression versus β-actin protein expression in KF. Note collagen type I protein expression in KF was significantly higher in 24 h post-treatment when compared to time 0 control ($^*P < 0.01$, $n = 3$).

numerous macromolecules, and may play a multifunctional role in tissue regeneration and maintenance [29]. The basal layer consisted of several types cells, one of them being keratinocytes. Keratinocytes are known to produce a wide variety of cytokines which are believed to play a significant role in cutaneous inflammatory and immunologic reactions [30]. Over the years, increased numbers of studies have pointed out a role for the NOS pathway in the regulation of skin function and homeostasis. The homeostatic activities of NOS in human skin include regulation of vasodilatation, melanogenesis, and protection against invading pathogens [21]. In response to challenge with microbes or microbial-derived substances, the production of NO and skin inflammatory cytokines occur in keratinocytes [31,32]. However, there is little data available on iNOS regulation in keratinocytes, even though it can play a role in keratinocyte proliferation, and differentiation during inflammation following normal scars [33,34]. Another report showed that epidermal keratinocyte-produced NO plays a significant but incompletely understood role in fibroblast function and cutaneous wound collagen synthesis [35]. Our data revealed that iNOS was expressed mainly in basal layer keratinocytes in keloid tissue wound repair. We hypothesized that the NO produced from iNOS in the basal layer may act via diffusion or microcirculations to KF. The paracrine activities of NO enhanced the collagen I synthesis in KF. We used the exogenous NO by NO donor release as the paracrine NO which comes from the basal layer to the KF in vitro model. The exogenous NO enhanced the collagen type I expression in KF. The NO produced by iNOS in the basal layer of keloid tissues may play both roles in keratinocyte proliferation and differentiation, and may also induce the overexpresssion of collagens in KF.

It has been suggested that wound healing processes may augment collagen accumulation. Generation of NO from the iNOS gene was found to be associated with the cutaneous wound healing process [36]. Dietary arginine was found to enhance wound healing in normal but not in iNOS knockout mice [37]. Inhibition of NO reduced the accumulation of collagen in wounds created in the mouse model [38]. Direct transfection of the iNOS gene also enhanced collagen accumulation in cutaneous wounds in rats [39]. NOS inhibitor L-NAME attenuated the reduction of collagen mRNA in bradykinin treated rat cardiac fibroblast [40]. Furthermore, recent studies also hypothesize excess collagen production in keloid lesions is attributed to higher levels of NO [41,42]. However, the results were without direct evidences of the effects of NO in keloid formation. In this report, we hypothesized that exogenous NO might influence the fibroblast to synthesize collagen.

In our in vitro model, we added NO donor DETA NONOate to mimic NO production and secretion by iNOS. Our results showed that exogenous NO enhanced both mRNA and protein expressions of collagen type I gene in KF but not in NSSF. The different activities of KF and NSSF in response to NO may provide a new strategy for keloid therapy. Fibroblast from keloid scars produce more collagen I on exogenous NO stimulation than healthy fibroblasts and may account for the florid scaring in keloids.

Our results indicate that NO production results from elevated iNOS expression in the basal layer of keloid tissue. The NO was then secreted into the dermal fibroblasts via diffusion or microcirculations to induce higher collagen synthesis in KF. In conclusion, our study showed that the iNOS expression during the wound healing process may play an important role in keloid formation.

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