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Induction of TIMP-1 and HSP47 synthesis in primary keloid fibroblasts by exogenous nitric oxide

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KEYWORDS
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Tissue inhibitor of MMP (TIMP)-1

Summary

Background: The excessive accumulation of extracellular matrix is a hallmark of many fibrotic diseases, including the hypertrophic scar and keloid. Recent reports from this research team had shown that exogenous nitric oxide (NO) participates in the keloid formation; however, its role on the synthesis of fibrotic factor (TGF-β1, TIMP-1 and HSP47) in the keloid fibroblasts (KF) remained unclear.
Objective: In this study, to better define the potential effect of exogenous NO on the expression of fibrotic factors in KF, the enhancing effect of exogenous NO, released from a NO donor, on the synthesis of fibrotic factors in KF was investigated.
Methods: The seven primary KF cultures were set up to measure the effect of exogenous NO on enhancing the expression of fibrotic factor.
Results: Elevation of cellular cGMP levels was observed to be induced by NO or blocked by the hydrolysis activity of phosphodiesterase (PDE) by the PDE inhibitor. The elevated levels of cellular cGMP were noted to enhance the expression of TIMP-1 and HSP47 in KF. Exogenous NO was found to significantly accelerate the production of TIMP-1 and HSP47 in the seven primary KFs with a corresponding increase in the production of TGF-β1.
Conclusion: The results have led to a conclusion, that is: the excess collagen formations in the keloid lesion may be attributed to the NO/cGMP signal pathway by initiating a rapid increase in the expression of TGF-β1, TIMP-1 and HSP47 in the KF cells.

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1. Introduction

Keloid is characterized by an excess deposition of collagen. Late, It has become a spreading skin overgrowth and could constitute a nightmare for the plastic surgeon [1]. Keloids exhibit a high rate of collagen and proteoglycan accumulation by fibroblasts [2], which is typical in the early phase of a wound but not in a normal skin or the scars [3]. Collagen synthesis is very important for the healing of wounds, being synthesized and secreted by the fibroblast, in a 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 [4]. The biological molecule of nitric oxide (NO) was reportedly synthesized in the rodent wounds [5]. NO could be generated endogenously from L-arginine by the nitric oxide synthase (NOS) [6]. The inducible form NOS (iNOS) provides the production of NO by a calcium-independent process. iNOS is known to mediate a cytotoxic and cystostatic effect on the immune system, as NO possesses an anti-bacterial, anti-parasitic and anti-tumor properties [3]. Recently, this research group reported that the NO produced by iNOS was found to regulate collagen synthesis by playing a role in keloid formation [7]. Exogenous NO was observed to stimulate the expression of collagen type I and the production of TGF-β1 in the keloid fibroblast (KF) by the cGMP-dependent manner [8] and to enhance the synthesis of heat shock protein 47 in the normal human dermal fibroblast [9].

Transforming growth factor (TGF)-β plays a major role in the vascular response to an injury by controlling the turnover of both cellular proliferation and extracellular matrix though the SMAD-signaling pathway [10]. TGF-β is highly expressed in the damaged arteries, and TGF-β-dependent effects have been noted to play a role in the pathogenesis of atherosclerosis, coronary artery disease, transplant arteriosclerosis, hypertension, diabetes, myocardial remodeling and fibrotic disease [11]. TGF-β1 and its family members, β2 and β3, are one of the few cytokines known to stimulate the synthesis of collagen, and several studies have shown that it is up-regulated in the hypertrophic scars and in the keloids. It is also well known that TGF-β1 is an autocrine factor produced by the fibroblast, which is capable of upregulation its own synthesis [10,11].

Heat shock proteins (HSPs) function as a molecular chaperone in facilitating the folding, assembly and intracellular transport of proteins, and thus are essential for cellular function [12]. Among the HSP members, HSP47 (also designated as colligin) serves uniquely as a collagen-specific molecular chaperone [13]. The role of HSP47 has been also implicated in the pathogenesis of fibrotic disease. An increased expression of HSP47 has been demonstrated in the fibrosis of lung, kidney and liver [14] and in the keloid [15]. These finding may indicate that HSP47 is involved in the development of collagen accumulation in the keloid formation.

Matrix metalloproteinases (MMPs), which are zinc-containing Ca^{2+}-dependent endopeptidases, are known to maintain the homeostasis of cardiac structure by digesting the extracellular matrix (ECM) [16]. Dysregulation of MMPs is associated with the occurrence of various diseases, such as arthritis, cancer invasion and fibrotic diseases [17]. The tissue inhibitor of MMP (TIMP), an endogenous MMP inhibitor, which consists of four molecules (with 40—50% sequence homology) and inhibits the activity of various MMPs with differential affinities [18]. Loss of inhibitory control by TIMPs is associated with the heart failure [19], while the overexpression of TIMPs is associated with the fibrotic disease [20].

To better define the potential effect of the NO signal pathway on keloid formation, the KF isolated from the patients was used as a model system. In this report, the effect of the NO/cGMP signal pathway on inducing the expression of fibrotic factors (TGF-β1, TIMP-1 and HSP47) in the KF was examined. Moreover, the expression of collagen type I, as stimulated by the exogenous nitric oxide, and the increase of TGF-β1 in the NO/cGMP signal pathway could activate the SMAD-signal cascade, thereby increasing the expression of TIMP-1 and HSP47 in the KF cells were also evaluated.

2. Materials and methods

2.1. Subjects

A total of seven keloid scar specimens were obtained from seven Taiwanese patients (four women and three men with age range of 21—76 years and a mean age of 45.0 years). Skin tissues were excised from the chest of three patients, the ear lobe of three patients, and the shoulder of one patient (all have the disease for 1—6 months). Only the typical and clinically clear-cut cases, which had extended beyond the original boundary of the wound were included in this study. None of the patients had received any previous treatment other than the pressure therapy. A written informed consent was obtained from all the patients, and the procedures had received the approval of the ethics boards at both Kaohsiung Veterans General Hospital and Taipei Medical University in adherence to the Helsinki Principles.

2.2. Cell culture

A total of seven primary KF cultures were established from the clinical specimens of keloids scar skin described above. Dermis from the keloid 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 the tissue culture flasks. Cells were grown at 37 °C in the Dulbecco's Modified Eagle Medium (Gibco/BRL, Gaithersburg, MD, USA), supplemented with 20% and 10% (v/v) of fetal bovine serum (HyClone, USA) for the primary culture and the subsequent cultures, respectively, and 5 mg/mL of L-glutamine in an atmosphere of 5% CO_2. KF cells in the primary cultures were trypsinized with 0.05% trypsin/0.53 mM EDTA (Life Technologies, Inc., Carlsbad, CA) and passaged. All experiments were performed using the third to fifth-passage cells. During the experiments, the cultures were analyzed by flow cytometry (FACS Calibur, BD) and MTT test. The results showed no evidence of crisis or injury.

2.3. Western blot

The cells were harvested and frozen immediately to −70 °C. The frozen cells were homogenized in a homogenizing buffer: Tris/HCl (20 mM at pH 8.0) containing NaCl (137 mM), glycerol (10%), EDTA (5 mM), phenylmethylsulfonyl fluoride (1 mM), leupeptin (1.5 mg) and protease inhibitor cocktail. The cell lysate was cleared by centrifugation at 13,000 rpm for 15 min. The amount of protein in the lysate was determined using the Bradford method (Bio-Rad, USA). The total cellular levels of SMAD-2 and phospho-SMAD-2 in the total cellular extracts (60 μg) were analyzed by the Western blot analysis by the procedure described previously [21]. Total proteins were separated using 10% SDS-polyacrylamide gel, transferred onto a nitrocellular membrane (Schleicher & Schnell, Keene, NH, USA) in Tris (25 mM) containing 0.192 M of glycine (pH 8.3) and 20% of methanol at 30 V overnight. The membranes were then blocked with 5% non-fat milk in Tris—HCl (10 mM, pH 8.0) containing 150 mM of NaCl and 0.05% of Tween-20 overnight, and incubated with anti-SMAD-2 and anti-phosph-SMAD-2 (both from Cell Signal, Germany) for 2 h. The blots were further washed with Tris—HCl (pH 8.0) containing 150 mM of NaCl and 0.05% of Tween-20 for 3 times ×10 min each and incubated with the secondary antibody (Santa Cruz, USA) for 1 h. Antigens were visualized using chemiluminescence kit (ECL, Amersham, USA) followed by autoradiography (Hyperfilm, Amersham, USA).

2.4. Estimation of heat shock protein 47 (HSP47) in KF

The amount of HSP47 was determined by an in situ ELISA technique modified from a published method [9]. Briefly, KF cells were inoculated in the 96-well culture plates (at a density of 3×10^4 cells/well). After the treatment, the cells were first fixed with 10% formaldehyde and then blocked with 10% BSA for 2 h, after which 0.1 ml of anti-HSP47 antibody (Stressgen Bio, USA) solution in 0.5% BSA solution was added into each well and incubated at 37 °C for 2 h. The plate was then washed with PBST, and 0.15 ml of ABTS solution (0.3 mg/ml) in the phosphate—citrate buffer (0.1 M, pH 4), which contained hydrogen peroxide, was added to each well. After 30 min, absorbance at 405 nm in each well was measured using a microplate reader after mixing. The HSP47 content was expressed as the relative level of synthesis as compared with the control.

2.5. Immunoassay

The concentrations of TGF-β1 (R&D, USA) and TIMP-1 (Daiichi, Japan) in the conditioned medium from the KF culture were measured using an enzyme immunoassay (EIA). The supernatants from the culture media were filtered through a membrane filter (0.45 μm). Immunoassay was performed for TGF-β1 and TIMP-1, using the manufacturer's protocol. Experiments were performed in triplicate and the data were expressed as the mean (±S.E.M.).

2.6. Statistical analysis

All data were reported as the means (±S.E.M.) of four separate runs of experiment. The ANOVA test was employed for statistical analysis, with significant differences determined as $P < 0.05$.

3. Results

3.1. Elevation of TGF-β1, TIMP-1 and HSP47 expression in the KF cells by exogenous NO

To identify the role of exogenous NO on the synthesis of fibrotic factors (TGF-β1, TIMP-1 and HSP47) in the seven primary KFs, EIA analysis was carried out to determine the levels of TGF-β1 and TIMP-1 expression in the seven primary KF cells (by four separate runs of experiment for each primary cell). The amount of HSP47 was determined by an in situ ELISA technique. Seven primary cultures of the keloid fibroblast (KF) were set up to determine whether

exogenous NO has played a role in the synthesis of fibrotic factors. DETA NONOate, a NO donor, was added to KF, in varying doses (0, 125, 250, 500 μM) and a different time course (0, 6, 12, 24 h). The results summarized in Fig. 1 shows that exogenous NO has induced the expression of TGF-β1, TIMP-1 and HSP47 in the KF cells by a dose-dependent manner. Quantitative analysis of the results revealed that the expression of fibrotic factors in the KF has been significantly elevated after the addition of DETA NONOate (Fig. 1, versus the DETA NONOate (0 μM), as the control, $^{*}P < 0.05$). The synthesis of TGF-β1 has been significantly increased, by 43% (for the treatment with 250 μM of DETA NONOate) and by 76% (for treatment with 500 μM of DETA NONOate). The various concentrations of DETA NONOate has produced a 2.3- to 3.9-(TIMP-1) and 1.60- to 1.88-(HSP47)-fold increase in the KF cells over the control (0 μM DETA NONOate).

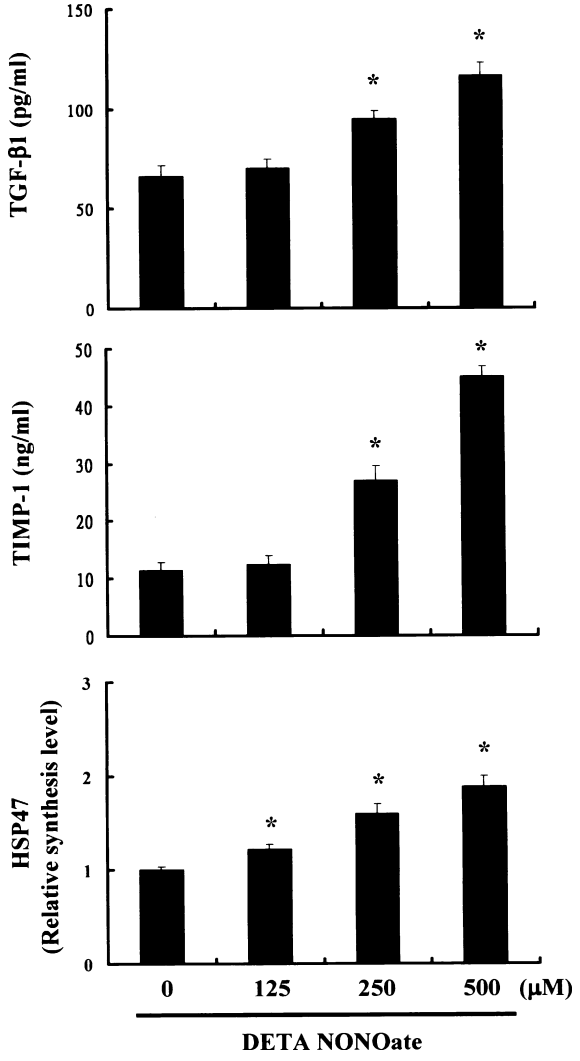

Fig. 1 Synthesis of fibrotic factors (TGF-β1, TIMP-1 and HSP47) in the KF cells as a function of the concentration of DETA NONOate incorporated. EIA analysis was used to determine the levels of TGF-β1 and TIMP-1 expression in the seven primary KF cells. The amount of HSP47 was determined by an in situ ELISA technique. The primary KF cultures ($n = 7$) were set up to determine whether exogenous NO played any role in the synthesis of fibrotic factors. The varying doses (0, 125, 250, 500 μM) of DETA NONOate, a NO donor, was added into the KF. The columns each represents the expression of TGF-β1, TIMP-1 and HSP47 in the KF cells. All data are the mean (±S.E.M.) of four separate experiments. The ANOVA with post hoc was employed for statistical analysis, with significant differences determined as $^{*}P < 0.05$.

The effect of exogenous NO on the expression of Fibrotic factors was also investigated in a range of time-courses. The results outlined in Fig. 2 shows that exogenous NO has induced the highest expression of fibrotic factor protein in the KF at 24 h.

Addition of DETA NONOate for 6—24 h has resulted in an 3—4.5-fold increase in the expression of TIMP-1 as compared to time-0 control ($^{*}P < 0.05$). On the other hand, the elevation of TGF-β1 expression has been raised from 56% to 89% when compared to the time-0 control. The expression of HSP47 was elevated from a 1.54- to 1.98-fold increase as compared to the time 0 control (Fig. 2).

3.2. Elevation of TIMP-1 and HSP47 expression in KF cells by cellular cGMP

Research findings reported earlier from this lab suggested that exogenous NO stimulates the expression of collagen type I and TGF-β1 in the KF by a cGMP-dependent manner [8]. To evaluate the role of the cellular cGMP elevation in the synthesis of TIMP-1 and HSP47 in the seven primary KFs, EIA analysis was used to determine the levels of TIMP-1 expression in the KF cells and the amount of HSP47 was determined by an in situ ELISA technique (four separate runs of experiment were performed for each of the KF cells). The KF cultures were set up to determine whether the cellular cGMP played a role in the synthesis of fibrotic factors. The NO donor DETA NONOate (500 μM), a non-selective PDE inhibitor:IBMX (100 μM), and three PDE-specific inhibitors (Vinpocetine: PDE I inhibitor; EHNA: PDE II inhibitor; Zapriast: PDE V inhibitor) at a level of 100 μM was added to the KF.

The results compared in Fig. 3 indicates that the cellular cGMP-stimulating agent (NO donor and PDE inhibitors) has induced the expression of TIMP-1 and HSP47 in the KF cells at 24 h ($^{*}P < 0.05$ versus the control). Quantitative analysis of the results reveals that the expression of TIMP-1 and HSP47 has been significantly elevated in the KF following addition of the cellular cGMP-stimulating agent. The PDE inhibitors, on the other hand, have been noted to block

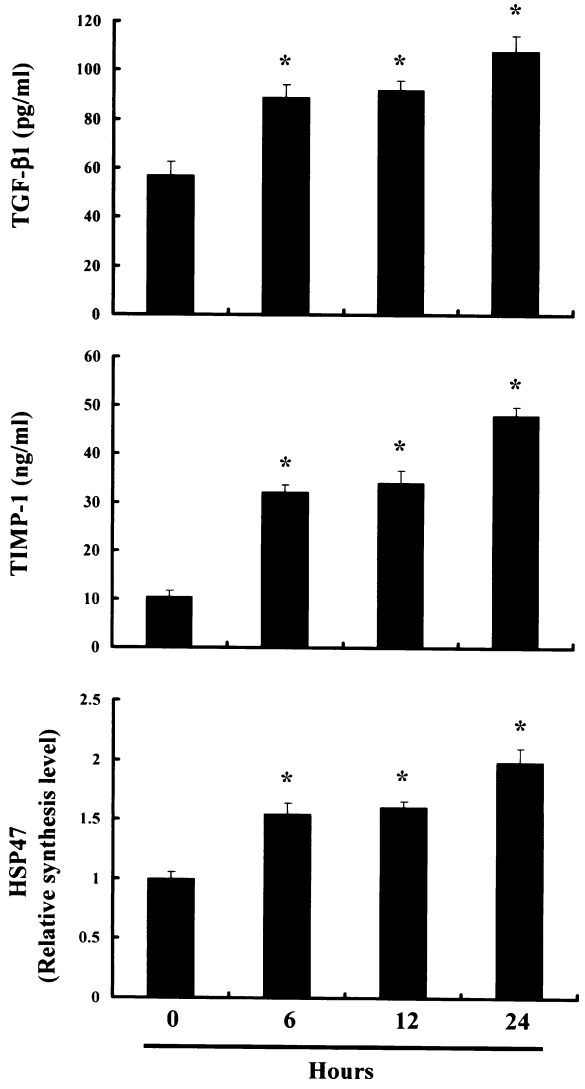

Fig. 2 Time course for the synthesis of fibrotic factors (TGF-β1, TIMP-1 and HSP47) in the KF cells after incorporation of DETA NONOate. EIA analysis was used to determine the levels of TGF-β1 and TIMP-1 expression in the seven primary KF cells. The amount of HSP47 was determined by an in situ ELISA technique. The primary KF cultures ($n = 7$) were set up to determine whether exogenous NO played any role in the synthesis of fibrotic factors. The KF was incubated with DETA NONOate (500 μM), an NO donor, for a varying duration (0, 6, 12, 24 h) and harvested. The columns each represent the expression of TGF-β1, TIMP-1 and HSP47 in the KF cells. All data represent the mean (±S.E.M.) of four separate experiments. The ANOVA with post hoc was employed for statistical analysis, with significant differences determined as $^{*}P < 0.05$.

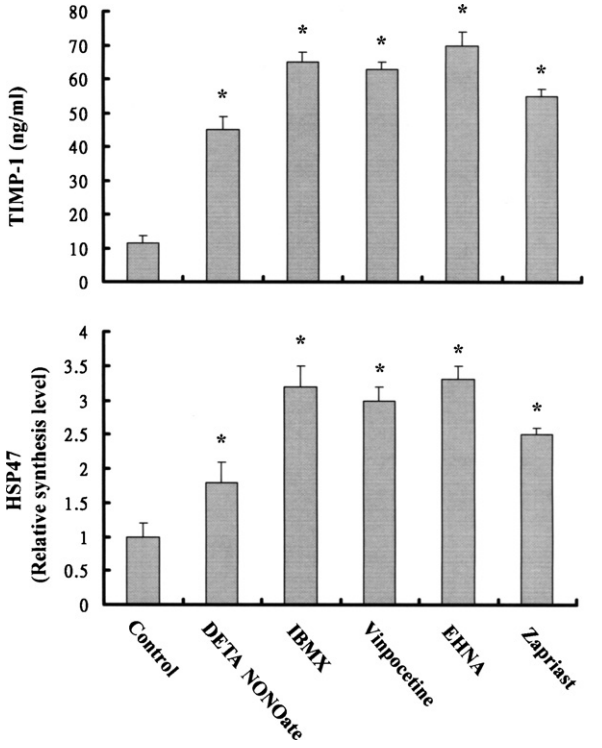

Fig. 3 Determination of the expression of TIMP-1 and HSP47 protein in the KF cells treated with cGMP-stimulating agents. EIA analysis was used to determine the levels of TIMP-1 expression in the seven primary KF cells. The amount of HSP47 was determined by an in situ ELISA technique. The KF cells were treated with DETA NONOate (500 μM) alone or in combination with IBMX (100 μM), an non-specific PDE inhibitor, or one of the PDE-specific inhibitors (Vinpocetine 100 μM: PDE I inhibitor; EHNA 100 μM: PDE II inhibitor; Zapriast 100 μM: PDE V inhibitor) and the expression of TIMP-1 and HSP47 was analyzed quantitatively. The columns each represent the expression of TIMP-1 and HSP47 in the KF cells ($^{*}P < 0.05$, compared to the control value). All data represent the mean (±S.E.M.) of four separate experiments.

the PDE hydrolysis activity and to increase the levels of cellular cGMP in the KF [8]. Various PDE-specific inhibitors have been found to yield an increase of 6.0—6.8-fold (for TIMP-1) and of 2.7—3.2-fold (for HSP47) in the KF cells (compared to the control). These results have demonstrated that the elevation in the cellular cGMP levels induces the expression of fibrotic factor in the KF. The NO/cGMP signal pathway may play an important role in the synthesis of fibrotic factors, which contributes to collagen accumulation in the keloid fibroblast.

3.3. TGF-β1 induces TIMP-1 and HSP47 expression in KF cells

To identify the roles of TGF-β1 in the synthesis of TIMP-1 and HSP47 in the seven primary KFs, EIA analysis was used in this series of studies to determine the levels of TIMP-1 expression in the KF cells while the amount of HSP47 was determined by an in situ ELISA technique (by four separate runs of experiment for each primary KF). Recombinant

human TGF-β1 (Merck USA) was added to the KF at varying doses (0, 0.1, 1.0 ng/ml). The results outlined in Fig. 4A illustrates that TGF-β1 was found to induce the expression of both TIMP-1 and HSP47 in the KF cells by a dose-dependent manner ($^{*}P < 0.05$ versus the control—TGF-β1 at 0 ng/ml). The addition of various concentrations of TGFβ1 has produced an increase of 2.2—2.7-fold (for TIMP-1) and 1.5—2.7-fold (for HSP47) in the KF cells, as compared to the control (TGF-β1, 0 ng/ml). Thus, the neutralization of TGF-β1 by an anti-TGF-β1 antibody (0.2 μg/ml), the expression of TIMP and HSP47 has been noted to be reduced ($^{**}P < 0.05$ versus the control of TGF-β1 at 1 ng/ml). These results have suggested that TGF-β1 could induce the expression of TIMP-1 and HSP47 proteins in the KFs.

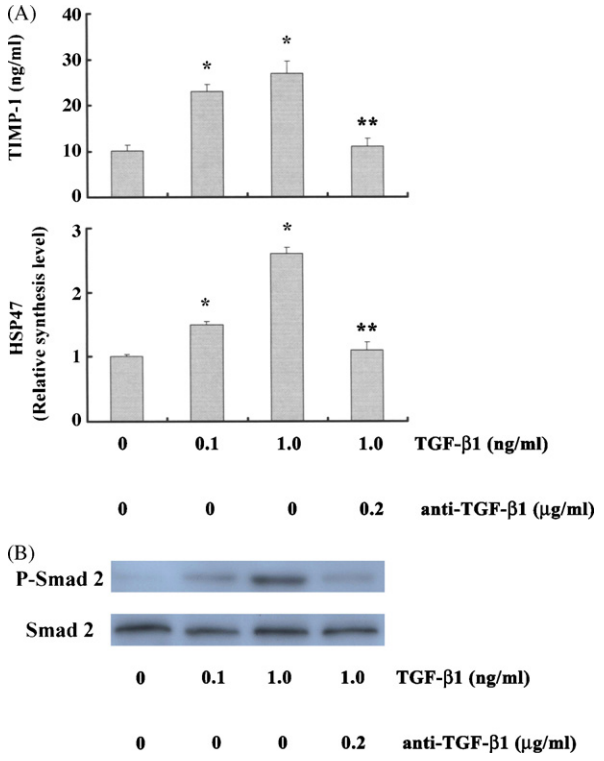

Fig. 4 The TGF-β1-induced expression of TIMP-1 and HSP47 via the TGF-β/SMAD cascade pathway. EIA analysis was used to determine the levels of TIMP-1 expression in the KF cells. The amount of HSP47 was determined by an in situ ELISA technique. The KF cells were treated with TGF-β1 (0, 0.1, 1.0 ng/ml) alone or in combination with an anti-TGF-β1 antibody (0.2 μg/ml). (A) Determination of the synthesis of TIMP-1 and HSP47 in the KF and the expression of TIMP-1 and HSP47 was analyzed quantitatively. The columns each represented the expression of TIMP-1 and HSP47 in the KF cells ($^{*}P < 0.05$, compared with the control value at TGF-β1, 0 ng/ml. $^{**}P < 0.05$, compared with the value at TGF-β1, 1 ng/ml). All data represent the mean (±S.E.M.) of three separate experiments. (B) The Western blot analysis of the p-SMAD 2 and SMAD 2 expression in the KF, which had been treated with TGF-β1, at various concentrations, and neutralized with an anti-TGF-β1 antibody.

3.4. Exogenous NO activates TIMP-1 and HSP47 expression in KF via TGF-β1/SMAD-2 signal pathway

The results have shown that the NO/cGMP signal pathway enhances the expression of TGF-β1, TIMP-1 and HSP47 in the KFs. Moreover, it has been observed that TGF-β1 induces the expression of TIMP-1 and HSP47 in the KF. To identify the role of the TGF-β1/SMAD-2 signal pathway on the expression of TIMP-1 and HSP47 in the KF cells, the varying doses (0, 0.1, 1 ng/ml) of recombinant human TGF-β1 was added to the KF. The results summarized in Fig. 4B shows that TGF-β1 appears to induce the production of phoph-SMAD-2 (p-SMAD-2) in the KF cells by a dose-dependent manner (versus the control of TGF-β1 at 0 ng/ml). The neutralization of TGF-β1 by the anti-TGF-β1 antibody (0.2 μg/ml) has reduced the p-SMAD-2 production in the KF (versus the control of TGF-β1, 1 ng/ml).

Collectively, these data demonstrate that NO/cGMP signal pathway can activate the SMAD-signal cascade, through an initiation of a rapid increase of TGF-β1, thereby increasing the expression of both TIMP-1 and HSP47 in the KF cells.

4. Discussion

The results reported from this research team suggested that the NO derived from the wound healing could be an argument to the accumulation of collagen in the formation of keloid scars [7]. Moreover, the research findings from this lab showed that exogenous NO could stimulate the expression of collagen type I and TGF-β1 in KF by a cGMP-dependent manner [8]. The generation of NO from the iNOS has been shown to be required in the healing process of cutaneous wound [22]. Recent studies also provided evidence that the excess production of collagen in the keloid lesions could be attributed to the presence of a higher-than-normal level of NO [23]. However, no direct evidence has been shown on the effect of the exogenous NO-stimulated cGMP on the expression of fibrotic factors in the KF. In this study, further evidence has been produced to demonstrate that the increased levels of cGMP, stimulated by exogenous NO, has induced the synthesis of TGF-β1, TIMP and HSP47 in the KFs.

In order to prove the validity of our hypothesis, that is: exogenous NO stimulates the synthesis of the cellular cGMP-inducing fibrotic factors in the KF. The

nitric oxide donor (DETA NONOate) was used as an exogenous NO source for the KF cells. The KF cells were treated with the NO donor, at a varying concentrations (to mimic the production of NO by the epidermal keratinocyte or macrophage in vivo). A dose-dependent as well as a time course-dependent increases have been observed in the expression and the secretion of TGF-β1, TIMP and HSP47 in the KF cells (Figs. 1 and 2). The previously studies obtained from this research group suggested that several cellular cGMP-stimulating agent (NO donor and PDE inhibitor) have been found to enhance the production of collagen type I protein and soluble collagens in the KF [8]. The NO-inducing cGMP signal is conveyed intracellularly by the activation of several effectors molecules: cGMP-dependent protein kinases, cGMP regulated phosphodiesterase, and cGMP-gated ion channels [24]. The second messenger, cAMP and cGMP, are hydrolyzed by phosphodiesterase (PDE), which could be divided into at least 11 superfamilies of structurally and functionally related enzymes [25]. In our studies, the role of cGMP in the exogenous NO-enhancing expression of TGF-1, TIMP, and HSP47 was investigated. The cellular cGMP concentrations in the KF cells were noted to be increased by the treatment with both NO donor and PDE inhibitors [8].

The superfamilies of PDE are known to have a different activity in the regulation of cellular cGMP and cAMP. In order to prove the validity of our hypothesis, that is: cellular cGMP plays an important role in the expression and production of fibrotic factors, several PDE-specific inhibitors (Vinpocetine: PDE I inhibitor; EHNA: PDE II inhibitor; Zapriast: PDE V inhibitor) were used in our studies to identify the role of cellular cGMP in the synthesis of fibrotic factors. The results obtained have shown that the inhibition of PDE activity by the PDE-specific inhibitors (I, II and V) has elevated not only the levels of cellular cGMP and the expression of collagen type I protein [8], but also increased the synthesis of fibrotic factors in the KF (Fig. 3).

The TGF-β is known to have a promoting effect on extracellular matrix in the various cells by stimulating the synthesis of matrix proteins. An important physiologic feature of TGF-β includes the de novo synthesis of extracellular matrix proteins and to inhibit the expression of matrix metalloproteinases [26]. Lastly, an increased expression of TGF-β isoforms has been documented in the fibrotic cells [27] and keloid [8]. The inorganic molecule NO has also been studied as a potent factor in the collagen synthesis during the healing of wounds [28,29]. The exogenous administration of NO was noted to act on the numerous cell types, including fibroblasts, via the cGMP-dependent pathway. However, no direct evidence was shown on the effect of the exogenous NO-stimulating cGMP on the transactivation of TGF-β. In this study, we provided further evidence that the increased level of cellular cGMP, stimulated by exogenous NO, has induced the production and transactivation of autocrine TGF-β in the KF. The TGF-β1 was observed to induce the expression of TIMP-1 and HSP47 protein in the KF ($^{*}P < 0.05$ versus the control, Fig. 4A,) via the TGF-β1/SMAD signal pathway (Fig. 4B).

Based on these observations, NO/cGMP pathway could positively influence the progression of keloid formation via the TGF-β1/SMAD signal cascade by increasing the expression of TIMP-1 and HSP47 in the keloid fibroblasts. A better understanding of the underlying molecular mechanisms will greatly improve our current knowledge of the keloid formation, which in turn could also help to elucidate the pathology of keloid, and have the potential of leading us to the development of an effective and a specific drug for the therapy of keloid.

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