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Exogenous nitric oxide stimulated collagen type I expression and TGF-β1 production in keloid fibroblasts by a cGMP-dependent manner

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Abstract

Keloids arise from the aberrant wound healing process and nitric oxide (NO) plays an important role in the inflammation stage of wound healing. In order to better define the potential effect of NO/cGMP signal pathway in the keloid pathogenesis, the enhancing effect of exogenous NO (released from NO donor) on collagen expression in the keloid fibroblast (KF) as well as on the induction of collagen type I protein and TGF-β1 expression in the KF were studied in this investigation. The DETA NONOate, an NO donor, was added to the KF, as the exogenous NO, to release NO in the culture medium. The expression of collagens was then determined by assaying the total soluble collagens and collagen type I in the KF. The cellular concentration of cGMP was measured by EIA in the KF. Exogenous NO was found to enhance the expression of collagens and elevate the cellular levels of cGMP. Moreover, to evaluate the effect of the elevated cellular cGMP levels on the expression of collagen and TGF-β1, both cGMP and TGF-β1 were measured by ELISA. The inhibitors for phosphodiesterase (PDE), such as IBMX (3-isobutyl-1-methylxanthine), Vinpocetine, EHNA, Milrinone and Zapriast, which have been reported to reduce the ability of PDE and subsequently produce an increase of cellular cGMP, induce the production of autocrine TGF-β1 as well as the synthesis of collagen in the KF. In this investigation, the inhibition of the PDE enzyme activity was observed to enhance the effect on the collagen synthesis, and was induced by exogenous NO. Taken together, these results have suggested that the NO/cGMP pathway could positively influence the progression of keloid formation, via the TGF-β1 expression in the KF.

Keywords: Nitric oxide (NO); Cyclic guanosine monophosphate (cGMP); Phosphodiesterase (PDE); Transforming growth factor-β1 (TGF-β1)

Keloids are the neoplastic growth resulting from an excessive response to a cutaneous injury. They are a pathological scar which has overgrown the original wound, over an extended time period, and caused some damage to the healthy dermal tissue [1]. Keloids and the hypertrophic scars are two kinds of abnormal scar formations in the skin. At the present time, the mechanisms for the formation remain unclear, but it has been thought to involve some local tissue factors in connection with a hereditary component in most of the cases [2]. Fibroblasts play an important part in the production and degradation of the extracellular matrix. They are also crucial in maintaining both the physiological properties and the structure of the tissue.

The patterns of the abnormal regulation of growth and matrix synthesis in the cultured keloid fibroblasts appear to reflect the in vivo phenotype and strongly support the use of keloid fibroblast as an in vitro model system to study the wound healing process of keloids [3]. Interactions between

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the epidermal keratinocytes and the dermal fibroblasts play an important role in regulating the repair processes of skin tissue [4]. Wound healing is a complex inflammatory response to an injury. Normal healing depends on a well balance of the promoting and the compromising factors. Part of the cell-to-cell and the cell-to-matrix interactions is mediated by some soluble mediators, such as the inflammatory cytokines, growth factors, nitric oxide (NO), and other factors [5], and the release cytokine [6]. Inflammatory cells have also been detected in the keloid scaring tissues and their responses have been implicated to play a potential role in the pathogenesis of keloids [7]. NO has been shown to contribute to cytotoxicity in cell proliferation during the inflammation stage of wound healing [8]. At the site of the inflammation, keratinocyte and macrophages, like neutrophils, are the major inflammatory cells infiltrating into the site. The inducible nitric oxide synthase was strongly expressed [9]. NO was noted to stimulate wound healing by increasing the formation of collagens and their mechanical strength [10–13,18]. NO, at a low–moderate concentration, was observed to act on the endothelial cells to stimulate angiogenesis by activating the activity of the soluble guanylyl cyclase (sGC), thereby increasing cGMP, which activates the cGMP-dependent protein kinases and the cGMP-dependent pathways [14].

Transforming growth factor, (TGF-β), plays a major role in the vascular response to an injury by controlling both the cellular proliferation and the extracellular matrix turnover though the SMAD-signaling pathway [15]. TGF-β was found highly expressed in the injured arteries, and TGF-β-dependent effects have been reported to play a role in the pathogenesis of atherosclerosis, coronary artery disease, transplant arteriosclerosis, hypertension, diabetes, myocardial remodeling and fibrotic disease [16]. TGF-β1 and its family members, β2 and β3, are one of the few cytokines known to stimulate collagen synthesis, 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 that is capable of up-regulating its own synthesis [17,34].

To better define the potential effects of NO signal pathway in the formation of keloids, the enhancing effect of the exogenous NO, released from a NO donor, on collagen expression in the keloid fibroblast (KF) as well as the effect of NO/cGMP on the induction of collagen type I protein and TGF-β1 expression in KF were examined. Moreover, the mechanism by which the collagen type I expression and TGF-β1 production in the keloid fibroblasts was stimulated by the exogenous nitric oxide, by a cGMP-dependent manner, was evaluated.

Materials and methods

A total of seven specimens of keloid scar were obtained from seven Taiwanese patients (four women and three men, ranging from 21 to 76 years old with a mean age of 45.0 years old). Keloids were each excised respectively from the chest of three patients, the ear lobe of three patients, and the shoulder region of one patient (with disease ranging from one to four years). Only the typical, clinically clear-cut cases, which extend beyond the original boundary of the wound were included in this study. None of these patients had received a treatment other than the pressure therapy. A written informed consent was given to all patients prior to the investigation and all procedures to be performed on the patients were reviewed and approved by the ethics boards at both Kaohsiung Veterans General Hospital and Taipei Medical University in adherence to the Helsinki Principles.

Cell culture

The primary keloid fibroblast cultures were established from the specimens of keloid scar obtained from the patients as described above. Tissue from the keloid 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 grown in tissue culture flasks. Primary keloid fibroblasts were grown in the Dulbecco's Modified Eagle Medium (Gibco/BRL, Gaithersburg, MD, USA) at 37 °C in an atmosphere of 5% CO_2. After quiescent by serum starvation for 24 h, the cells were ready for the experiments. All experiments were performed using third to fifth-passage cells. During these experiments, cultures showed no evidence of crisis or senescence.

Western blotting

Cells were harvested and frozen immediately in −70 °C. The cells were homogenized in 2× homogenizing buffer [a combination of: 20 mM of Tris/HCl (pH 8.0), 137 mM of NaCl, 10% of glycerol, 5 mM of EDTA, 1 mM of phenylmethylsulfonyl fluoride and 1.5 μg/mL of leupeptin], and the protease inhibitor cocktail (Roche, Germany). The cell lysates were then cleared by centrifugation. The amount of protein in the lysate was determined by Bradford method (Bio-Rad Protein Assay Kit, USA). The proteins were separated by using SDS–PAGE (10%) and transferred to a nitrocellulose membrane (Hybond, Amersham, USA) overnight. The membrane was blocked with a non-fat milk (5%) for overnight and incubated with the anti-β-actin (1:10,000; Sigma, USA), anti-human collagen type I (1:2000; Research Diagnostics Inc., USA) antibody for 2 h. The membrane was then washed and incubated with the anti-rabbit or mouse secondary antibody (1:5000; Santa Cruz Biotechnology, USA) for 1 h. Antigens were visualized, using chemiluminescence detection kit (ECLplus, Amersham, USA), and autoradiographic analysis, by the X-ray films (Fuji, Japan). Data from Western blot analysis were scanned and analyzed by NIH Image software. The relative levels of expression were determined by the expression ratio of Collagen I with β-actin.

Determination of total soluble collagen concentration

The supernatants from the culture medium were filtered through a membrane filter (with pore size of 0.45 μm). The

total soluble collagen concentrations were determined by using Sircol Collagen Assay kit (Sircol, USA).

Determination of cellular cGMP concentration in keloid fibroblasts

The keloid fibroblast (KF) cells were seeded in the 96-well plates. After treating the cells with the exogenous nitric oxide, IBMX and the specific inhibitors for PDE (PDE I–V inhibitor), the KF cells were washed with PBS and the cellular cGMP concentrations in the KF cells were analyzed, using Enzyme Immunoassay Kit (Amersham, USA).

Enzyme-linked immunosorbent assay (ELISA)

The supernatants from the culture medium were filtered through a membrane filter (0.45 μm). Immunoassay was performed for TGF-β1 using the manufacturer's protocol (R&D Systems, USA).

Statistical analysis

All data were shown as the means (±SEM). Data were statistically analyzed by Student's *t*-test. *P* value less than 0.05 was considered significant.

Results

Exogenous nitric oxide and IBMX enhances collagen type I expression in keloid fibroblasts

Seven primary KF cultures were set up to determine whether or not the exogenous NO and IBMX have played a role in the synthesis of collagen type I. The DETA NONOate, an NO donor, was added, at a varying doses (0, 125, 250, 500 μM), as the exogenous NO, with or without IBMX (100 μM), to KF cells for 24 h. The results displayed in Fig. 1A show that the expression of collagen type I protein in the KF cells has been significantly increased after the treatment with DETA NONOate at various doses (black column; $^{*}P < 0.05$) as compared to the control (0 μM). Moreover, the collagen type I protein expression in the KF cells is further enhanced with the combination treatment with IBMX (white vs. black column, $^{\#}P < 0.05$).

Results in Fig. 1B also indicate that the secretion of soluble collagen into the KF cells culture medium also shows a dose-dependent increase as increasing the dose of DETA NONOate (black column). The combination treatment with IBMX (100 μM) also significantly enhances the secretion of soluble collagens as compared to that induced by DETA NONOate alone (white vs. black column, $^{\#}P < 0.05$). The results indicated that the treatment of KF cells with IBMX alone (without DETA NONOate) has yielded a 3-fold increase in the collagen type I protein expression (Fig. 1A) and soluble collagens (Fig. 1B), while the treatment with the combination of IBMX with DETA NONOate (at a concentration range of 125–500 μM) has produced a 1.4- to 1.75-fold increase in the expression of collagen type I protein (Fig. 1A) and a 1.3- to 1.67-fold increase in the production of soluble collagens (Fig. 1B), respectively as compared to the treatment with DETA NONOate alone in KF ($P < 0.05$). These results suggest that the exogenous NO (e.g., DETA NONOate) as well as the PDE inhibitor (e.g., IBMX), play an important role on collagen synthesis in the keloid fibroblast.

Nitric oxide enhances collagen expression via NO/cGMP pathway

The effect of the exogenous NO and IBMX on the cellular cGMP levels in the KF cells were also examined. The results outlined in Fig. 2A indicate that the concentrations of cellular cGMP in the KF cells has been found to be significantly increased after treating with the DETA NONOate at various doses (black column, $^{*}P < 0.05$) over the control (0 μM). Moreover, the levels of cellular cGMP in the KF cells treated with the combination of DETA NONOate and IBMX (100 μM) are higher than the treatment with DETA NONOate alone (white column vs. black column, $^{\#}P < 0.05$). A 3-fold increase in the cellular cGMP concentrations has been obtained in the KF cells treated with IBMX (100 μM) alone, while treatment with the combination of IBMX (100 μM) with the various concentrations (125, 250 and 500 μM) of DETA NONOate has produced a 1.6- to 1.7-fold increase in the levels of cellular cGMP in the KF cells over those attained by treatment with DETA NONOate alone. These findings suggest that the presence of IBMX, a PDE inhibitor, has further significantly enhanced the effect of DETA NONOate, an exogenous NO donor, on the cellular cGMP concentration.

The effect of the exogenous NO donor and the combination of NO donor with the PDE inhibitor on the cellular cGMP concentrations was also studied as a function of time. For this study, the KF cells were treated with DETA NONOate, at 500 μM, with and without the presence of IBMX (100 μM). The highest concentration of cellular cGMP has been attained at 30 min after the DETA NONOate treatment (with and without the presence of IBMX). In the presence of IBMX, the cGMP concentration at the 30-min point following the treatment of DETA NONOate is 7.6-fold higher than that at time 0. The cellular cGMP concentration at 24 h was observed to decrease by 20% from the value obtained at 30-min. In the absence of IBMX, on the other hand, the cellular cGMP concentration was observed to increase by as much as 6.4-fold after treating with DETA NONOate for 30 min, which was then decreased by 53% at the 24 h-point. These findings suggest that IBMX, a PDE inhibitor, could promote the enhancing effect of DETA NONOate, an exogenous NO donor, on the cellular cGMP concentration in the KF cells.

To evaluate the relationship between cGMP and the expression of collagen type I protein, the effect of cGMP on

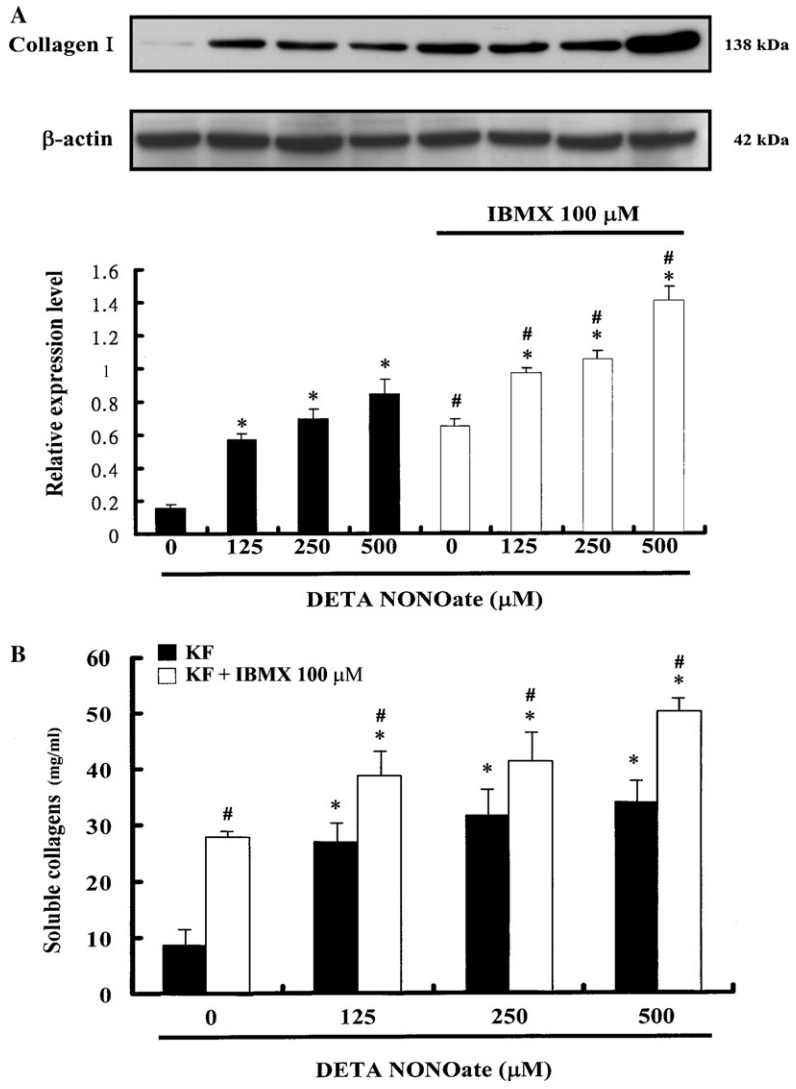

Fig. 1. Determination of collagen type I expression and total collagens secretion in the KF cells. (A) Western blot analysis of collagen type I protein expression and (B) determination of soluble collagens levels in the KF cells following the treatment of DETA NONOate at various concentrations with (white column) or without (black column) the presence of IBMX (100 μM). Quantitative analysis of collagen type I protein expression was performed and shown. The collagen type I protein expression was normalized by β-actin. (*$P < 0.05$, compared with DETA NONOate at 0 μM. #$P < 0.05$, compared with the KF cells treated with DETA NONOate at various respective concentrations without the presence of IBMX.) Data are shown as the mean (±SEM) of at least three separate experiments.

collagen expression was further investigated. The effect of the various specific inhibitors for PDE (e.g., Vinpocetine: PDE I inhibitor, EHNA: PDE II inhibitor, Milrinone: PDE III inhibitor, Rolipram: PDE IV inhibitor, Zapriast: PDE V inhibitor) on the activity of cGMP and the expression of collagen type I protein in the KF was compared in Fig. 3A. The cellular cGMP concentrations were observed to increase after treatment with Vinpocetine (by 3.6-fold), EHNA (by 6.3-fold), Milrinone (by 1.6-fold) and Zapriast (by 3.5-fold) (vs. the control; *$P < 0.05$). To further determine the effects of cGMP on the expression of collagen type I protein in the KF cells, Western blotting was performed using the antibody specific for collagen type I. As shown by the results in Fig. 3B, a significant expression of collagen type I protein was also noted in the treatment with Vinpocetine, EHNA, Milrinone and Zapriast. It is interesting to note that there is a similar trend between the activity of cGMP and the expression of collagen type I protein in the KF cells following the treatment with the various PDE-specific inhibitors. The results of quantitative analysis indicated that the collagen type I protein expression has been elevated by 5-, 6.5-, 2- and 4.8-fold for Vinpocetine, EHNA, Milrinone and Zapriast, respectively. These results imply that the elevation in the concentrations of cellular cGMP could play an important role in the accumulation of keloid collagen.

Cellular cGMP induces autocrine TGF-β1 expression

Since TGF-β has been shown to increase the production of extracellular matrix protein in various systems, it is thus hypothesized in this investigation that the elevated

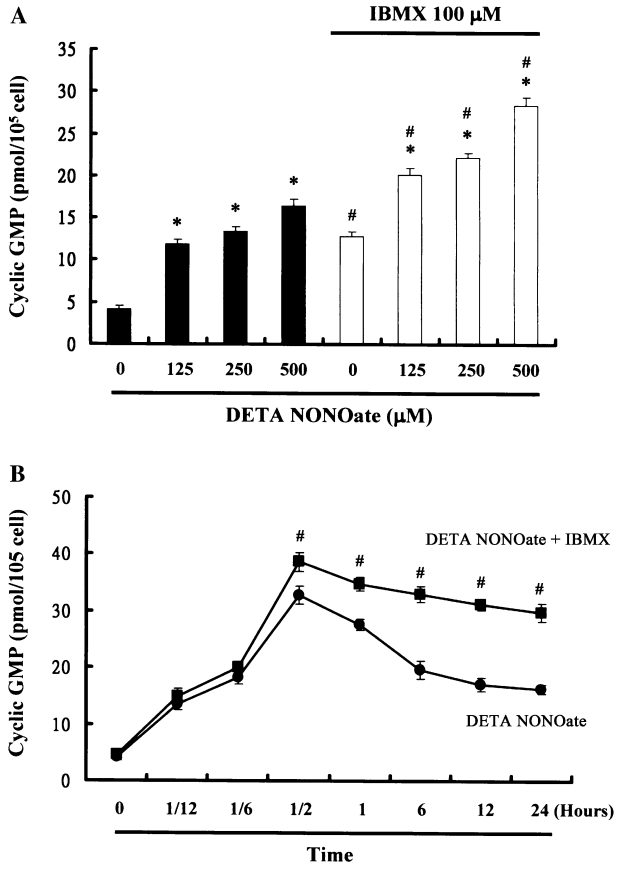

Fig. 2. Determination of cellular cGMP concentrations in the KF cells. (A) Enzyme immunoassay analysis of cellular cGMP concentrations in the KF cells following the treatment of DETA NONOate at various concentrations with (white column) or without (black column) the presence of IBMX (100 μM). Quantitative analysis of cellular cGMP concentrations was shown. (*$P < 0.05$, compared with DETA NONOate at 0 μM. #$P < 0.05$, compared with the KF cells treated with DETA NONOate at various respective concentrations without the presence of IBMX.) (B) Determination of cGMP levels in the KF cells as a function of time following the treatment of DETA NONOate (500 μM) with (square) or without (circle) the presence of IBMX (100 μM). (#$P < 0.05$, compared with the KF cells treated with DETA NONOate without the presence of IBMX.). Data are shown as the mean (±SEM) of at least three separate experiments.

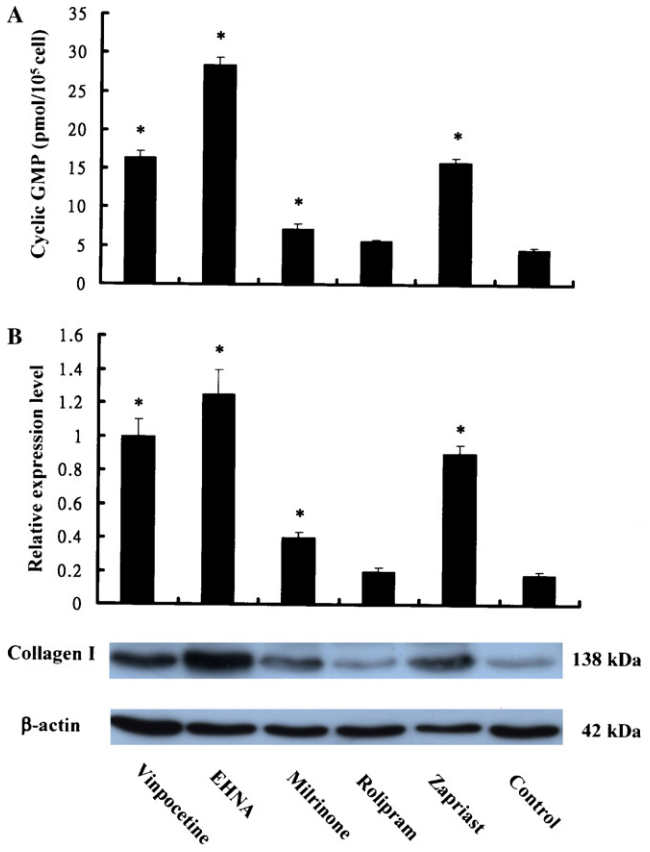

Fig. 3. Determination of cellular cGMP concentrations and collagen type I protein expression in the KF cells treated with the PDE-specific (type I–V) inhibitors. (A) Enzyme immunoassay analysis of cellular cGMP concentrations and (B) collagen type I protein expression in the KF cells following the treatment of various PDE-specific inhibitors (Vinpocetine: PDE I inhibitor, EHNA: PDE II inhibitor, Milrinone: PDE III inhibitor, Rolipram: PDE IV inhibitor, Zapriast: PDE V inhibitor) at 100 μM. Quantitative analysis of cellular cGMP concentrations was performed (*$P < 0.05$, compared with the KF control value). Data are shown as the mean (±SEM) of at least three separate experiments.

synthesis of collagen type I in the KF by cGMP, via the expression of TGF-β1 is the underlying mechanism. To identify the roles of cGMP in the TGF-β1 expression, the ELISA analysis was used to determine the production of TGF-β1 in the culture medium. The results in Fig. 4 indicate that the concentration of TGF-β1 has been significantly elevated by treating with DETA NONOate (500 μM) and PDE inhibitors (at 100 μM), such as IBMX, Vinpocetine, EHNA, Milrinone and Zapriast. The concentrations of TGF-β1 in the KF cells treated with DETA NONOate, IBMX, Vinpocetine, EHNA, Milrinone and Zapriast have been significantly increased over that in the control group (by 2-, 3-, 2.7-, 3-, 1.3- and 2.1-fold, respectively). These results demonstrate that the exogenous NO-stimulated elevation of cellular cGMP could be an important factor for the TGF-β induced-accumulation of keloid collagen.

Discussion

The previous report from this laboratory has suggested that NO derived from the wound healing could augment the collagen accumulation in the formation of keloid scar. The exogenous NO was found to induce collagen synthesis in the keloid fibroblast, but not in the site-matched normal skin fibroblasts [18]. The generation of NO from iNOS has been shown to be required for the healing process of a cutaneous wound [19]. In the mice model, the inhibition of NO was observed to reduce the accumulation of collagen in the wound areas [20]. Supplement dietary arginine was found to enhance wound healing in the normal but not in the iNOS-knockout mice [21]. Although the NOS inhibitor, L-NAME, was noted to attenuate the reduction of collagen mRNA expression after the bradykinin treatment in the rat cardiac fibroblast [22]. The direct application of nitric oxide inhibitor, at a low dose, to patient has been shown to help the surgical excision of keloid formation [23]. Recent studies have also provided

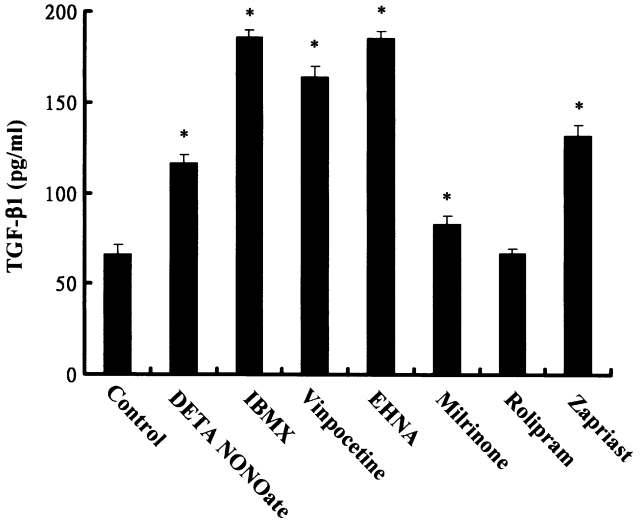

Fig. 4. Determination of autocrine TGF-β1 production in the KF cells treated with PDE-specific inhibitors. ELISA analysis of TGF-β1 production in the KF cells following the treatment of PDE-specific inhibitors (Vinpocetine, EHNA, Milrinone, Rolipram, Zapriast) at 100 μM. Quantitative analysis of TGF-β1 production was performed (*$P < 0.05$, compared with the KF control value). Data are shown as the mean (±SEM) of at least three separate experiments.

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 [18,24]. However, no direct evidence has been reported on the effect of exogenous NO-stimulated cGMP on the keloid formation. In this study, further evidence has been obtained and demonstrated that the increased levels of cGMP, as stimulated by the exogenous NO or PDE inhibitor, has induced an excess synthesis of collagen and an increased expression of TGF-β1 in the KF. In order to validate the hypothesis that the exogenous NO-stimulated cellular cGMP is playing an important role in the collagen synthesis of keloid formation, experiments which utilized the nitric oxide donor (DETA NONOate) as an exogenous NO source were carried in the KF cells. The KF cells were treated with NO donor at varying concentrations to mimic the production of NO by the epidermal keratinocyte or macrophage in vivo. A dose-dependent increase in the expression and secretion of collagen protein in the KF cells was observed. The treatment of DETA NONOate in the combination with IBMX has further significantly enhanced the expression of collagen type I and the secretion of soluble collagens, as induced by the exogenous NO. The IBMX was also observed to enhance the production of collagen type I protein and soluble collagens (Fig. 1). The nonselective phosphodiesterase inhibitor (e.g., IBMX) was found to enhance the effect of NO on the cellular cGMP levels in the KF cells (Fig. 2). These results could support the hypothesis that the elevated levels of cellular cGMP play an important role in the expression and production of collagen protein in the KF cells.

The classical signal transduction pathway ascribed to NO is the activation of guanylyl cyclase (GC) and a subsequent increase in cGMP levels in the smooth muscle cells [25]. The NO-sensitive GC is generally accepted as the most important receptor for the signal molecule NO. Stimulation of GC by its physiological activator NO leads to a tremendous (up to 200-fold) increase in its catalytic rate. The NO-induced cGMP signal is conveyed intracellular by the activation of several effectors molecules: cGMP-dependent protein kinases, cGMP-regulated phosphodiesterase, and cGMP-gated ion channels [26]. The secondary messenger, cAMP and cGMP, are hydrolyzed by phosphodiesterase (PDE), which can be divided into at least 11 superfamilies of structurally- and functionally related enzymes [27]. In the current study, the role of cGMP in the exogenous NO-enhanced collagen overexpression was investigated. The cellular cGMP concentrations were observed to be increased by both the exogenous NO and the PDE inhibitor treatments in the KF cells. The highest levels of cellular cGMP concentration in the KF cells were observed at 30 min (Fig. 2B). At 24 h, the cellular cGMP levels of the DETA NONOate-treated KF were reduced by 54% as compared to the treatment with the combination of DETA NONOate and IBMX. These results have provided further evidence that combination IBMX with exogenous NO has produced a higher collagen expression in the KF (Fig. 1).

The PDE superfamilies have different activities in the regulation of cellular cGMP and cAMP. In order to prove our hypothesis, that is: The cellular cGMP plays an important role in the keloid's collagen expression and accumulation, five PDE-specific inhibitors (Vinpocetine: PDE I inhibitor, EHNA: PDE II inhibitor, Milrinone: PDE III inhibitor, Rolipram: PDE IV inhibitor, Zapriast: PDE V inhibitor) were used to investigate the role of cGMP or cAMP in the KF's collagen synthesis. The PDE I and II have both a hydrolytic activity in both cGMP and cAMP. The PDE III and IV have a hydrolytic activity in cAMP but not in cGMP, while the PDE V has a hydrolytic activity in cGMP, but not in cAMP. As shown by the results in Fig. 3, the hypothesis has been found to be supported by the observations that the inhibition of PDE activity by the PDE-specific inhibitors (I, II, III and V) has elevated the levels of cellular cGMP and the expression of collagen type I protein in the KF.

The effect of TGF-β in the various cells has been associated with the promotion of extracellular matrix by stimulating the synthesis of matrix proteins. An important physiology feature of TGF-β includes the de novo synthesis of extracellular matrix proteins and the inhibition of the matrix metalloproteinase's expression [28]. Recently, an increased expression of TGF-β isoforms has been documented in the fibrotic cells [29] and in the keloid [16]. By contrast, the inorganic molecule NO has been studied as a potent factor in the collagen synthesis during a wound healing [30,31]. The NO was noted to up-regulate the expression of TGF-β inducible early response 1 gene (TIEG1), which is known to play a pivotal role in the TGF-β-regulated cell growth control, apoptosis and

fibrosis factor expression [32,33]. However, the recombinant TGF-β1 has been reported to repress NOS activity and nitrite production in the cultured dermal fibroblasts by the inhibition of nitric oxide signaling [34]. The results indicated that TGF-β1 inhibits endogenous NO signal pathway in collagen synthesis. This disagreement could be resulted from the different sources of NO donor used (endogenous NO in the previous report vs. exogenous NO in the present investigation) as well as the difference in the cells isolated and processing used. In the numerous types of cells, including fibroblasts, the exogenous administration of NO, acting via the cGMP-dependent pathway. No direct evidence has been obtained, however, concerning the effect of the exogenous NO-stimulated cGMP on the production of TGF-β. In this study, we have provided further evidence which indicates that the increased cGMP levels as stimulated by the exogenous NO, has induced the production of autocrine TGF-β in KF (vs. control; $*P < 0.05$). Similar results were also observed following the administration of PDE inhibitor (Fig. 4). On the basis of these observations, it is thus confirmed that the NO/cGMP pathway could positively influence the progression of keloid formation via the expression of TGF-β1 in the keloid fibroblasts. These findings help to elucidate the pathology of keloid, and have the potential of leading to the discovery of an effective and specific drug useful for keloid therapy.

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