Co-upregulation of Transforming Growth Factor Beta-1 and Nitric Oxide Synthase in Keloid by Comparison to Normal Human Skin - A Possible Role for TGFβ1 and NOS in Pathogenesis of Keloid

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Abstract

Keloid disease is a benign but progressive form of abnormal wound healing associated with skin fibrosis and can cause a major functional disability and morbidity. TGF beta (TGFβ) and Nitric Oxide (NO) are active biomarkers known to regulate phases of wound healing and have been implicated in pathogenesis of fibrotic disease. There are three isoforms of TGFβ (1, 2 and 3) TGFβ1 and 3 have a crucial role in fibrosis, with TGFβ1 profibrotic and TGFβ3 antifibrotic. NO is produced by Nitric Oxide Synthase (NOS) which exist in three isoforms, inducible NOS (iNOS), endothelial NOS (ecNOS) and neuronal NOS (nNOS). TGFβ isoforms and NO were found to be associated with fibrotic disorders affecting the skin. We hypothesis that the interaction between TGFβ and NO in keloid could promote the excessive collagen deposition associated with this disorder.

Using immunohistochemistry, we investigated the profile of TGFβ isoforms (TGFβ1, 3) and NOS isoforms (iNOS and ecNOS) in keloid tissues and normal human skin. The cellular distribution of all the isoforms were studied and the protein levels were assessed by using H-Scoring and Image J Scoring systems.

TGFβ1 showed wide cellular distribution in keloid both in the epidermal and dermal cells. There was significant upregulation (P<0.001) by comparison to normal skin. TGFβ3 showed limited expression in keloid and there was significant downregulation (P<0.03). iNOS and ecNOS showed significant upregulation in keloid by comparison to normal skin (P<0.01 and P<0.02) respectively. Interestingly, iNOS was expressed in the basal epidermal layer and in dermal connective tissue cells while ecNOS was solely expressed in vascular endothelial lining. Although it is documented that TGFβ has a negative feedback effect on iNOS, we have shown co-upregulation of TGFβ1 and iNOS in keloids. Thus, in keloid NO is as important as the profibrotic growth factor TGFβ1 and both could be working in coordination. Moreover, the lack of effective therapy for keloid could be because most of the therapeutic regimen target one factor whiles the other still in action. In conclusion, understanding the actions of TGFβ and NO in keloid disease could lead to the development of clinically useful combined anti-fibrotic agents.

Keywords: Wound repair; Scarring, Inflammatory mediators, Disorders

Abbreviations: TGFβ: Transforming Growth Factor β; NO: Nitric Oxide; NOS: Nitric Oxide Synthase; iNOS: Inducible Nitric Oxide Synthase; ecNOS: Endothelial Nitric Oxide Synthase and nNOS: Neuronal Nitric Oxide Synthase

Introduction

Wound healing involves integrated phases including haemostasis, inflammation and proliferation and remodelling. It should progress in a coordinated manner and requires the presence of various biological mediators and growth factors [1-7]. Disruption of this coordination results in abnormal healing; persistence inflammatory phase causes chronic wound [8] and persistence of remodelling phase causes excessive scarring [9]. Moreover, sustained release of cytokines results in continued cell proliferation and tissue remodelling [10] leading to keloid formation, one of the major abnormalities of wound healing [11,12].

Keloid research showed significant progress in the last few years [13-16] but there is still deficit in the literature regarding the mechanisms behind keloid development, recurrence and limited effective therapy. The available research data are mostly from in vitro studies and this often fails to represent in vivo pathophysiology. There is discrepancy in keloid research data, this is attributed to the fact that cells in vitro behave differently. In vitro studies demonstrate that keloid fibroblasts have the ability to express the alpha smooth muscle actin characteristic of myofibroblast [17], even though they do not express it in vivo and it has been suggested that this suppression is due to local factors found in vivo [17]. Moreover, keloid fibroblasts have been shown to produce the same amount of TGFβ as normal fibroblasts [18] although in vivo studies showed upregulation of TGFβ in keloid [19]. Interestingly, it has been found that keloid tissue does not survive transplantation [20] supporting the notion that keloids are a local phenomenon under control of several interacting bioactive molecules of which TGFβ and NO are crucial.

TGFβ and NOS isoforms are known biomarkers implicated in pathogenesis of scarring and are of therapeutic importance in fibrotic conditions [21,22]. TGFβ1 and 3 have different biological activities in wound healing, TGFβ1 promotes fibrosis and scar formation [23-26] whereas TGFβ3 has been shown to be either scar inducing [27] or...

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reducing [28] depending on the study. Due to its profibrotic activity, TGFβ1 was proposed to have a major role in pathogenesis of keloid. TGFβ isoform expression was studied in keloids and showed increased TGFβ1 with no changes in TGFβ3 expression, relative to normal [23,24].

NO is generated by nitric oxide synthase (NOS) [29]. NOS present in three isoforms; inducible NOS (iNOS) and endothelial (ecNOS) and neuronal NOS (nNOS) [18]. NO plays important role in wound healing [17] through enhancing collagen synthesis [30] and epithelialization [31] and could affect the fate of wound healing causing abnormal healing. Our previous research showed upregulation of NO during the inflammatory stage of normal wound healing but not remodelling stage [32] and showed persistence upregulation in chronic wound [8].

The relative balance of iNOS and its control by TGFβ are critically important in wound healing. TGFβ has been shown to suppress iNOS expression [33-35] and anti-TGFβ antibody has been shown to block the suppression of iNOS in the vasculature [36]. Moreover, in an endotoxin model of septic shock, TGFβ treatment markedly reduced iNOS mRNA in several organs and blocked the lipopolysaccharide-induced hypotension [37]. TGFβ and NOS isoforms have been extensively studied in wound healing. But, to the best of our knowledge the interrelation between these mediators was overlooked in keloid due to lack of the keloid animal model and the difficulty getting human keloid samples.

Materials and Methods

Specimens

Keloid specimens (14 samples) were retrieved from the archives of Histopathology Lab from Department of Histology and Department of Pathology, Minia Faculty of Medicine. They were obtained from randomly selected patients who underwent surgical excision of keloids in the department of surgery, Minia Faculty of Medicine during the period from 2006-2011. Normal human skin specimens (9 samples) were obtained during routine surgery when excision of skin was required in the surgical procedure or from the edge of the surgical incision.

Immunohistochemical staining

Immunohistochemical staining was performed according to a previously published protocol [8]. Sections were deparaffinized, hydrated then washed in 0.1 M phosphate buffer saline (PBS). Sections were then treated with trypsin 0.01% for 10 minutes at 37°C then washed with PBS for 5 minutes. Endogenous peroxidases were quenched by treatment with 0.5% H2O2 in methanol and non-specific binding was blocked in normal goat serum diluted 1:50 in 0.1 M PBS. Sections were incubated in the diluted primary antibody of interest overnight at 4°C. Sections were washed and incubated in biotinylated goat anti-rabbit secondary antibody (Vector laboratory:1:2000) for 30 minutes. The substrate, diaminobenzidine tetrahydrochloride in distilled water (Sigma, Poole, UK), was added for the appropriate period (5-10 min). Positive cells were labelled brown. For the negative control, primary antiserum was replaced with normal serum of the host species of the secondary antibody. Specimens were viewed using a Leica DMRB microscope and images were captured using a Spot RT Slider digital camera (Image Solutions) using Spot RT software run on a PC. The antibodies used were: Monoclonal mouse anti-human ecNOS ([1:400, Transduction laboratories], polyclonal rabbit anti-human iNOS (1:1000 Transduction laboratories), polyclonal rabbit anti-TGFβ1 (1:500 ABCAM) and polyclonal rabbit anti-TGFβ3 (1:200 ABCAM).

Double immunofluorescence

Immunohistochemical staining was performed according to a previously published protocol [38]. Sections were prepared and incubated with antibody to iNOS (1:1000) for 1 hour at room temperature. Then, they were washed and incubated for a further 30 minutes with TRITC a conjugated goat anti-rabbit secondary antibody diluted 1:200 in TBS. Sections were incubated with an antibody to ecNOS (1:400) for 1 hour at room temperature. Then, they were washed and incubated with FITC conjugated goat anti-mouse secondary antibody 1:100 in TBS for 30 minutes at room temperature. Sections were then mounted in polyvinyl alcohol. Viewed using the Leica DMRB microscope operating in fluorescence mode with appropriate filter sets and images were captured as above.

TGFβ1 and 3 assessment using H-Score

H Score is a semi-quantitative method used for assessing immunoreactivity on immunoperoxidase stained sections. It assesses both the density of the staining and the surface is covered by the staining. Sections were scored in the field of a 20x objective using bright field microscopy. TGFβ1 and 3 immunoreactivities were assessed in 10 adjacent areas from each section. In each case 6 sections were scored and the distance between sections were 150 µ. Staining intensity was assessed as: strong [3], medium (2), weak (1) and none (0) over the percentage area of each staining intensity. H scores were calculated by multiplying the percentage area by the intensity grade (H score range 0-300). Each section was assessed by two histologist scorers and a consensus agreed. Dr. Abd El-Aleem was trained on using H scoring by DR Abed M Zaitoun a consultant pathologist at the University of Nottingham, Department of Cellular Pathology.

iNOS and ecNOS assessment using Image J software

Image J software (developed at US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij/) was used to assess the immunoreactivity on immunofluorescence stained slides. iNOS and ecNOS were assessed by measuring the surface area covered by the positive staining. Assessment was done in 10 adjacent areas from each section. In each case 6 sections were scored and the distance between sections were 150 µ. The analysis is automated and was performed by the software. Before starting the analysis, the setting was adjusted on a test image and the same setting parameters were used throughout the whole experiment on all images from normal and keloid tissues. Figure 1 demonstrates the method of assessing and scoring the staining. Briefly, image (Original) was imported to image J and then converted to adjust the colour threshold to pick only the immunostaining and exclude any other staining. The selected structures were outlined, counted and the total surface area of the immunoreactivity was calculated. The result from the assessed images showing the measurements of the immunoreactivity were exported to an excel sheet to be processed for statistical analysis.

Statistics

Statistical analyses were performed using IBM SPSS statistical package. Results were expressed as the mean±SEM. The Mann-Whitney U-test was used, with P<0.05 being considered as statistically significant.
Results

Upregulation of TGFβ1 in keloid tissues by comparison to normal human skin

TGFβ1 was expressed in the normal human skin (Figure 2A) and in keloid tissues (Figure 2B) both in the dermis and epidermis. In normal human skin immunoreactivity density was mild to moderate and was in few dermal connective tissue cells, perivascular cells and vascular endothelial lining (Figure 2A-2C). In keloid tissues, immunoreactivity was dense and widely distributed in the dermis (Figure 2D-2F). The main cellular source was keratinocytes (Figure 2D) in the epidermis. In the dermis, TGFβ1 was expressed in various dermal structures including sebaceous glands (Figure 2D), inflammatory cell infiltrate (Figure 2E), blood cells and vascular endothelial lining (Figure 2F). TGFβ1 was expressed both in the cytoplasm and nuclei. H scoring showed significant (P<0.0001) upregulation of TGFβ1 in keloid tissues by comparison to normal human skin (Figure 3).

Downregulation of TGFβ3 keloid tissues by comparison to normal human skin

TGFβ3 was expressed in normal human skin (Figure 4A) and keloid tissues (Figure 4C); the immunoreactivity was almost the same density. In normal human skin (Figure 4A and 4B) and keloid (Figure 4C-4E), immunoreactivity was seen in the epidermis, dermal blood vessels and dermal connective tissue cells. Interestingly, in keloid, connective tissue cells showed characteristic cellular localisation limited to the submembranal cytoplasmic compartment with depletion from the perinuclear cytoplasmic compartment (Figure 4E). H scoring showed significant (P<0.0001) downregulation of TGFβ3 in keloid tissues by comparison to normal human skin. (Figure 5).
showed significant (P<0.03) downregulation of TGFβ3 in keloid tissues by comparison to normal human skin (Figure 5).

**Upregulation of iNOS and ecNOS in keloid by comparison to normal skin**

iNOS was expressed in normal human skin and keloid tissues (Figure 6). In normal human skin, the expression was mainly in the dermal connective tissue cells (Figure 6A and 6B). In keloid, the expression was in the dermal connective tissue cells and in the basal epidermal layer (Figure 6C and 6D). By running double immunofluorescence to colocalise iNOS and ecNOS, we have shown that in normal skin iNOS is expressed mainly in the dermis, in connective tissue cells and blood cells most probably blood monocytes and that ecNOS is expressed solely in vascular endothelial lining (Figure 6C). However, in keloid iNOS was expressed both in the epidermis and in the dermis (Figure 7A-7C). Double immunofluorescent showed that most of the connective tissue cells which express iNOS are macrophages (Figure 7D-7F). Assessment
of immunoreactivity showed significant upregulation of iNOS and ecNOS (P<0.01 and P<0.02 respectively) in keloid by comparison to normal human skin (Figure 8).

Discussion
Keloid is an abnormal tissue repair following trauma to the skin. This disorder is unique to humans and there are no animal models. Keloid is characterised by several lesional features including excessive collagen deposition [39-41], lack of fibroblast-myofibroblast transition, thickening of the epidermis and high vascularity. In this study, we demonstrated upregulation of TGFβ-1 and NO producing enzymes [iNOS and ecNOS] in keloid by comparison to normal human skin. Here we introduce biological interpretation of these features in relation to our results:

TGFβ upregulation and its role in excessive collagen deposition in keloid
We demonstrated high levels of TGFβ1 but low levels of TGFβ3 in keloid tissues, this upregulation could account for the excessive collagen deposition in keloid. It is well documented that TGFβ isoforms regulate collagen syntheses and turnover [42-45] and they have been postulated in pathogenesis of fibrotic disorders [46]. TGFβ1 has a profibrotic activity and TGFβ3 has antifibrotic effect [28]. Our result is in line with this, as we have shown upregulation of the profibrotic TGFβ1 and downregulation of the antifibrotic TGFβ3. The profibrotic activity of TGFβ1 could be due to its ability to induce matrix deposition and production of protease inhibitors, which inhibit the enzymatic breakdown of collagen [47]. We have shown expression of TGFβ1 in endothelial cells, this in turn could activate the adjacent fibroblasts to produce high levels of TGFβ1 and collagen [42]. Therefore, it was suggested that the way for treatment of fibrotic conditions is either by blocking the effects of the profibrotic TGFβ1 or administration of the antifibrotic TGFβ3 [28]. Our in vivo results in this study support this suggestion.

NO upregulation and its role in excess collagen deposition in keloid
We demonstrated upregulation of iNOS and ecNOS in keloid
tissues by comparison to the normal human skin; both enzymes are contributing to the high NO production in keloids. High NO levels could be contributing to excessive collagen deposition. There are enormous evidences that NO is an important factor in collagen metabolism [48-50] and remodelling phase of wound healing [51]. Moreover, exposure of keloid fibroblast to NO increased collagen expression [52]. The primary source of NO in healing wounds is iNOS activity [53], however in our study we show that eNOS could be contributing to NO production in keloid. It was shown that human dermal fibroblasts express both eNOS and iNOS, thus both are important in remodelling phase of wound healing [50,54]. These evidences highlight a potentially important role for NO in excess collagen synthesis and keloid pathogenesis. We conclude that, in keloids high levels of TGFβ1 and NO could be contributing to excessive collagen deposition.

TGFB and NO effect on fibroblast-myofibroblast transition in keloid

In normal wound healing, there is transition of fibroblasts to contractile fibroblasts [myofibroblasts], however, this phenomenon is supressed and there is absence of myofibroblasts in keloid [53,55-58]. Transition of fibroblast to myofibroblasts is inhibited by NO [53] and is enhanced by TGFB. Therefore, high NO production in keloid account for the absence of myofibroblasts and the inhibiting effect of NO on fibroblast transition overcome the stimulating effect of TGFB.

NO up regulation and its role in epidermal thickening in keloid

Keloid is associated with a thickened epidermis and increase keratinocyte population [17,59]. This could be attributed to the proliferative effect of NO on keratinocytes [60]. In this study, we have shown upregulation of iNOS in the basal epidermal layer, this could be explained by cause-effect relation between NO and melanocytes. In wound region melanocytes are destroyed and do not re-generate causing reduction of melanin in wound region [61], however keratinocytes regenerate and continue to produce NO [62]. Melanin absence from the wound space implies that there is additional UVB stimulation of keratinocytes to produce more NO [62]. NO activates tyrosinase, one of the main enzymes responsible for the biosynthesis of melanin [63], therefore, excessive NO production by keratinocytes could be a compensatory mechanism to enhance melanin synthesis in wound space. This facts about NO role in melanin synthesis, suggests that we would expect to observe higher levels of NO associated with darker skin pigmentation, to account for the extra melanin observed. Interestingly, majority of keloids are found to occur in deeply pigmented skin [64]. Therefore, high NO in dark skin could be a predisposing factor to keloid formation.

NO upregulation and its role in high vascularity in keloid

Keloid exhibits a high vascularity but microvessels are partially or fully occluded [17,59] and this was attributed to endothelial cell proliferation [65-67] NO promotes endothelial cell proliferation [68-72]. We demonstrated upregulation of eNOS that contribute to NO production [69]. Also, NO was found to be linked with the growth-promoting effects of vascular endothelial growth factor [VEGF] [70]. This strongly suggests that high NO levels in keloid could account for the endothelial cell proliferation and blood vessel occlusion via growth factor upregulation.

NO and the therapeutic effect of steroids in keloid

To date there is no entirely effective treatment for keloid. One of the existing therapies involves excision combined with intralesional administration of corticosteroids. This has a high response rate, but recurrence is still common [73]. Corticosteroids are known to suppress iNOS but not eNOS [74]. Thus, they could be blocking NO produced by iNOS but not NO produced by eNOS and this could account for their partial effectiveness in treatment of keloid. This support our hypothesis, that eNOS is as important as iNOS in keloid pathogenesis. Therefore, administration of NO inhibitor such as L-NG-Monomethyl-L-arginine, monoacacetate salt (NMMA) would be more effective [75].

TGFB down regulate NO but this may be different in keloid because of the transient nature of this cytokine and the fact that NO production in wound continue until healing is complete [53]. Moreover, TGFB downregulate NO production through feedback inhibition of iNOS [58] but it may not affect NO production from eNOS. Also, it is model dependant and cell type dependent.

Conclusion

Our results support Campane et al., 2006 hypothesis that upregulation of TGFB1 expression may be necessary but is not sufficient for excessive scarring. Therefore, balance between TGFB and NO could have much importance. We conclude that, TGFB and NO could be affecting keloid cells through autocrine and paracrine effects and could jointly play a role in pathogenesis of keloid.

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Conflict of Interest

There is no conflict of interest.

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