The Effect of Flashlamp Pulsed Dye Laser on the Expression of Connective Tissue Growth Factor in Keloids

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Objective: To investigate connective tissue growth factor (CTGF) expression before and after pulsed dye laser (PDL, 595 nm) treatment, and to better understand the mechanism of PDL treatment of keloids.

Method: Twenty-six patients with keloids were recruited for this study. For each patient, two keloids of similar anatomic location, duration, texture, and appearance were chosen for study; one of these keloids was treated and the other served as a control. Three sessions of PDL treatment, with pulse duration of 1.5 milliseconds, spot size 7 mm, DCD duration 20 milliseconds/delay 10 milliseconds and fluence of 10 J/cm^2, were performed on the keloids at 3- to 4-week intervals. Punch biopsies were performed both on the treated and untreated keloids prior to the first treatment and after the final treatment. The specimens underwent realtime polymerase chain reaction (PCR) and immunohistochemistry (IHC) to investigate the CTGF mRNA and protein expression after PDL treatment.

Results: According to realtime PCR, the CTGF mRNA was significantly down-regulated after PDL treatment in 80.77% of patients as compared to the control group. IHC investigation showed that after treatment the CTGF positive cells also significantly decreased in number as compared to the control group in 80.77% of patients. Using the Vancouver scar scale (VSS), there was an average decrease of 20.85 $\pm$ 12.33% after PDL treatment.

Conclusions: Pulsed dye laser treatment of keloids significantly down-regulates the expression of CTGF in most cases. This may partially explain the mechanism of action of PDL treatment of keloids. Lasers Surg. Med.

Key words: connective tissue growth factor; keloid; pulsed dye laser

INTRODUCTION

Keloids are an aberrant response to trauma, inflammation, surgery, or burns characterized by abundant deposition of collagen and glycoprotein [1]. They can also present spontaneously. In distinction to hypertrophic scars, keloids extend beyond the margin of the initial injury, and rarely improve with time. Keloids can also produce pruritus, pain, or pressure making them problematic for patients, who seek treatment. Disturbance of the complex wound-healing process may contribute to keloid formation [2,3].

Recent studies have investigated the influence of various growth factors on keloid formation, including transforming growth factor-β (TGF-β), connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF), heat shock protein-47 (HSP-47), Heat Shock Protein-27 (HSP-27), platelet-derived growth factor (PDGF), and insulin-like growth factors (IGF), among others. They have been found to play a pivotal role in fibroblast proliferation and collagen production [3–5].

CTGF is one of the major growth factors causing such fibrotic disorders as keloids, directly inducing fibroblast proliferation, and extracellular matrix (ECM) synthesis [6]. It has been shown that CTGF is mainly expressed in fibroblasts in the dermis and sometimes in epidermal basal cells as well. CTGF is not expressed in normal tissue, but it is specifically expressed in pathological fibrotic tissue such as keloids [4–6].

It is well-established that pulsed dye laser (PDL) treatment can be used to effectively treat keloids, relieving not only the symptoms such as redness, itching, and pain, but also the texture and pliability of keloids [3,7]. However, the mechanism of the laser treatment remains to be elucidated. Because it plays an important and specific role in keloid formation [4,5], CTGF is likely to be an important target of treatment. Therefore, the effect of PDL on CTGF expression needs to be further explored. To date, this growth factor's role in PDL treatment of keloids has not been investigated. The goal of the study is to detect the CTGF expression before and after PDL treatment, and

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thereby provide at least a partial explanation of the mechanism of action of PDL therapy of keloids.

MATERIAL AND METHODS

Patient Selection

Patients were recruited from the dermatology clinic in Huashan Hospital between June, 2009 and July, 2010. The diagnosis was based on clinical examination and pathological findings.

Inclusion criteria were as follows: Patients, ages 18–65, were required to have at least two similar appearing keloids in terms of anatomic location, duration, texture, and appearance. Alternatively, a patient could have one large keloid (two keloids merged into one) that could be divided easily into treatment and control sites. The biopsies were taken as far apart as possible. Exclusion criteria included: concomitant diagnosis of cancer, severe autoimmune disease, hemorrhagic disease or anticoagulant medication, psychosis, other systemic disease, history of keloid treatment 6 months prior to the study, active infection or inflammation in the area to be studied, pregnancy or breast feeding, or if the patient were undergoing a concomitant treatment of other skin lesions.

All the patients signed the informed consent prior to the study, which was approved by the Huashan Hospital institutional review board.

Treatment Protocol

Two similar keloids were selected in most patients, and one big keloid in others. One keloid was randomly selected for treatment, and the other served as a control. Three sessions of full-lesion, single-pass PDL at 595 nm (Vbeam, Candela Corporation, Wayland MA) treatment was performed in the treatment group without pulse stacking with a pulse duration of 1.5 milliseconds, spot size 7 mm, DCD duration 20 milliseconds/delay 10 milliseconds and fluence of 10 J/cm^2 at 3- to 4-week intervals. No treatment was conducted on the control sites. Postoperatively antibiotic cream was applied to the treated side twice daily until complete healing.

At each subsequent visit, patients were asked to report on the adverse effects of the treatment, including blisters, erythema, hypopigmentation, hyperpigmentation, pruritus, pain, inflammatory, and worsening of the keloid.

Objective and Subjective Evaluations

All keloids were evaluated at the baseline and 3 weeks after the final treatment, biopsy was conducted on both the treated and control keloids at each time point. The specimen was divided into two parts, preserved by liquid nitrogen for realtime polymerase chain reaction (realtime PCR) investigation of CTGF mRNA expression and 10% formalin for immunohistochemistry (IHC) examination of CTGF expression.

Assessment of the scar itself was done by comparing before and after treatment by a blinded dermatologist according to the Vancouver scar scale (VSS, Table 1) which includes pigment, height, vascularity, and pliability of the keloid. A high score reflects a more severe keloid. Photographs were also taken at each visit.

Measurement of CTGF Expression

Realtime PCR. Connective tissue growth factor mRNA expression was measured by realtime PCR. Total RNA from tissue was extracted and purified by QIAGEN RNeasy Mini Kit. RNase-Free DNase i Set (Invitrogen, Carlsbad, CA) was used for DNase digestion during RNA purification. cDNA was synthesized from RNA with the SuperScript III First-Strand Synthesis System (Invitrogen) and PCR System 9700. We used TaKaRa Taq hot Start Version to explore the annealing temperature.

The real-time PCR was performed by using a QIAGEN QuantiTect SYBR Green PCR Kit and a 7500 Realtime PCR System (AB Applied Biosystems, Foster City, CA). CTGF Primers were synthesized by Sengon Biotech Company Limited (Shanghai). The primer sequences used in this study were as follows: forward, 5′-CGA CTG GAA GAC ACG TTT GG-3′; reverse, 5′-AGG CTT GGA GAT TTT GGG AG-3′. The realtime-PCR conditions were as follows: 20 minutes at 50°C (stage 1, carryover prevention), 15 minutes at 95°C (stage 2, PCR initial activation), and then 42 cycles of amplification for 30 seconds at 94°C, 30 seconds at 56°C, and 32 seconds at 72°C (stage 3, PCR). We performed a melting curve analysis of realtime PCR products. CTGF mRNA expression was evaluated.

H&E and IHC. Tissue sections (4 μm-thick) were subjected to hematoxylin and eosin (H & E) staining and Immunohistochemical staining (IHC) for CTGF. IHC staining was performed by using SABC Kit (Boster Bioengineering Co. Ltd, Wuhan, China), DAB staining kit (Boster Bio-engineering Co. Ltd), CTGF antibody (Abcam PLC., Cambridge, UK) and Goat polyclonal Secondary Antibody to Rabbit IgG (Abcam PLC.).

Sections were deparaffinized and rehydrated. Endogenous peroxidases were blocked by soaking slides in a solution of water and 30% H_2O_2 (10:1) for 15 minutes at room temperature. They were then washed three times with water, and washed once or twice with phosphate buffered

TABLE 1. Vancouver Scar Scale (VSS) [8,9]

Score	0	1	2	3	4	5
Pigmentation	Normal	Slightly	Moderately	Severely		
Height	Normal	<1m m	1–2mm	2–4mm	>4mm	
Vascularity	Normal	Pink	Red	Purple		
Pliability:	Normal	Supple	Yielding	Firm	Adherent	contracture

saline (PBS) for antigen repairing. Five percent BSA was dropped for 20 minutes at room temperature and excess liquid was shaken and wiped off. Primary antibody was diluted at 4°C overnight, then washed for 2 minutes 3 times with PBS. Biotinylated Goat polyclonal Secondary Antibody IgG to Rabbit was added at 20–37°C 20 minutes, then washed for 2 minutes 3 times with PBS. SABC liquid was dropped at 20–37°C 20 minutes, then washed for 5 minutes 4 times with PBS. DAB stain was done with DAB staining kit to stain the slides. One milliliter of ddH_2O was mixed with one drop of solution A, one drop of solution B, and one drop of solution C, and the mix solution was added to the slides until there was a color change (approximately 5–30 minutes). Slides were drained and washed with ddH_2O. After they were counterstained with hematoxylin, they were dehydrated. The cover slip was then sealed. Three fields (250×) were selected in each section for image analysis. We used Image-Pro Plus 6.0 software to analyze the number of positive cells in each field and then calculated the average of the three fields as the final result. These pictures were taken by Olympus BH2 optical microscope and a Nikon 4500 digital camera.

Statistical Analysis

Data were analyzed by a paired *t*-test and Pearson correlation using Statistical Package for Social Sciences version 11.0 (SPSS Inc., Chicago, IL). The significance level was set at $P < 0.05$.

RESULTS

Twenty-six patients (14 females and 12 males; age 20–40, mean age 26.35 ± 6.50 years) were included in this study. Fourteen were of skin type III and 12 type IV. The duration of their keloids ranged from 5 to 240 months (mean ± SD: 54.56 ± 59.60 months). Keloids were mainly located in the chest or scapular areas, with the major cause for their presentation being folliculitis (22 patients, 84.62%). One patient suffered a keloid after carbon-dioxide laser treatment (one patient, 3.85%).

Real-Time PCR

According to our study CTGF mRNA expression (Fig. 1) was down-regulated in most cases and up-regulated in only a small portion of patients as listed in Table 2.

(1) CTGF mRNA was significantly down-regulated after treatment ($t = 0.00$, $P < 0.05$) as compared to the baseline in 21 cases (80.77%), the average degree of decline was 58 ± 29%. Meanwhile, CTGF mRNA level in the control group significantly increased ($t = 0.01$, $P < 0.05$) as compared to baseline, the average increase was 4.91 ± 6.35 times the pre-treatment. According to the Paired T-test, there was a significant difference between treated group and control group in terms of CTGF mRNA change. ($t = 0.00$, $P < 0.05$).
(2) CTGF mRNA levels increased in the rest of the 5 cases (19.23%) in the treated group, being 4.23 times the pre-treatment level on average. Meanwhile, CTGF mRNA level in the control group increased more significantly, i.e.,9.23 times the baseline on average.

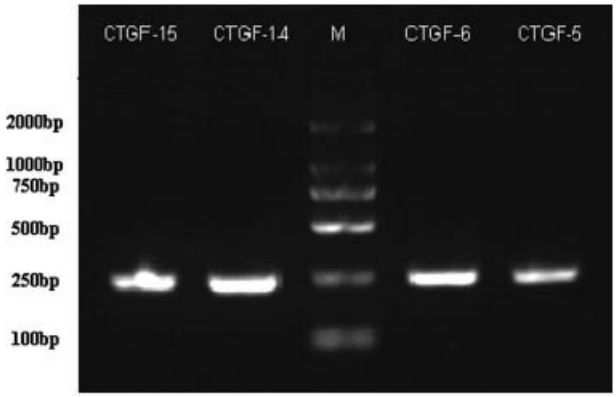

Fig. 1. The electrophoretogram shows the results of Realtime PCR before and after the treatment. The four bands are about 200 bp which is the same as our expectation.

The correlation between CTGF mRNA expression and the age of the patient and the duration of keloid.

As shown in Figure 2, there was negative correlation between post-treatment CTGF mRNA RQ/pre-treatment CTGF mRNA RQ and the age of the patients ($r = -0.442$, $P = 0.044$), which indicated older patients tended to have more down-regulation of CTGF mRNA after PDL irradiation. However, no significant correlation was observed between CTGF mRNA expression and the duration of keloid (Fig. 3).

Immunohistochemistry

Positive cells were stained brown, mainly distributed in the basal layer of epidermis and dermis. Data of CTGF positive cells in the treated and control group are summarized in Table 3. According to our investigation, positive

TABLE 2. Changes of CTGF mRNA

	Group (1)		Group (2)	
	Treated group	Control group	Treated group	Control group
Pre-treatment average of mRNA RQ	1	0.58	1	0.67
Post-treatment average of RQ	0.42	1.94	4.84	6.27
Average of change (post-treatment/ pre-treatment ± standard error)	0.42 ± 0.29	4.91 ± 6.35	4.23 ± 3.58	9.23 ± 4.03

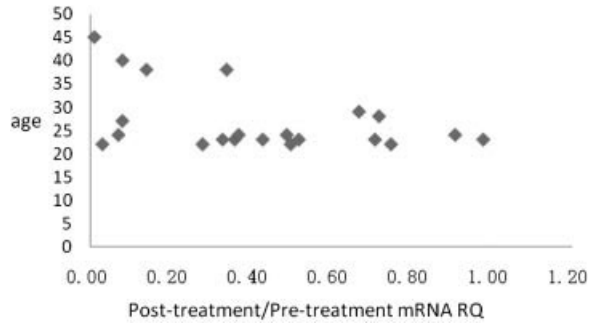

Fig. 2. The data in Figure 2 indicates a negative correlation. The coefficient r is -0.442 ($P = 0.044$).

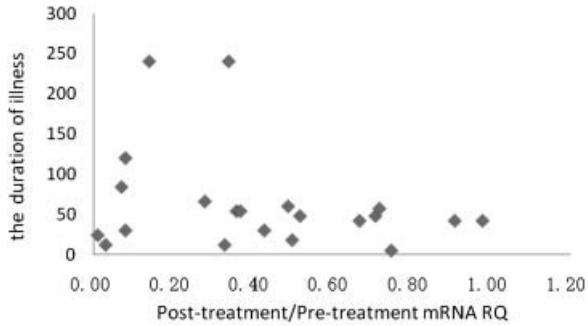

Fig. 3. No trend line can be seen. The data appear to be uncorrelated ($r = -0.256$, $P = 0.262$).

cells/field decreased after treatment in most cases and increased in a small portion of patients.

(1) CTGF positive cells/field significantly decreased in number after treatment ($t = 0.00$, $P < 0.05$) in 21 cases (80.77%) as compared to baseline, decreasing by $70 \pm 21.39\%$ (Fig. 4) on the average. Meanwhile, CTGF positive cells/field in the control group significantly increased ($t = 0.01$, $P < 0.05$) in number by 1.32 ± 0.52 times on average as compared to baseline. According to paired t-test for CTGF positive cells/field changes between treated group and control group, there was a significant difference. ($t = 0.00$, $P < 0.05$).
(2) In three cases (11.54%) in the treated group, the average number of CTGF positive cells/field increased by 1.38 times as compared to baseline. However, CTGF positive cells/field in the control group increased more significantly by 3.71 times.
(3) In the rest of the other two cases (7.69%) the average number of CTGF positive cells/filed rose by 5.41 times as compared to pre-treatment, more significantly than that of the control group which was 1.19 times on the average.

H&E Staining

(1) Pre-treatment: There was significant collagen proliferation in the dermis. In some areas, collagen mass and homogenization could be observed. Fibroblasts were interspersed irregularly in collagen bundles (Fig. 5).
(2) Post PDL treatment: Collagen proliferation was reduced in the dermis. Fibroblast cells increased and distributed more regularly in collagen bundles, in parallel to the skin surface (Fig. 6).

VSS Score

The VSS scores significantly decreased ($t = 0.00$, $P < 0.05$) after treatment by $20.85 \pm 12.33\%$ on the average. Improvement of scar texture, alleviation of symptoms, and reduction of redness primarily contributed to the decrease of VSS score.

Side-Effects

Six patients (22.22%) had blisters after laser therapy. Hyperpigmentation occurred in 13 patients (48.15%), resolving over time. In this study there was no hypopigmentation, infection, or keloid worsening.

DISCUSSION

Keloids are a pathological scar with a genetic predisposition. They occur more frequently in patients with darker Fitzpatrick skin phototypes such as Asians, Africans, and Hispanics. The incidence of keloids overall is 4.6–16%, and there is no difference in the incidence between males and females [1].

The cause of keloids remains unclear. Genetic immune dysfunction, collagen over-expression, and changes in the internal environment are all possible causes [1]. Studies suggest that keloids are associated with: human leukocyte antigen, high incidences of allergic diatheses, and a high level of serum immunoglobulin E [1,10]. Therefore, it is very likely that the formation of keloids has a strong

TABLE 3. Average of Positive Cells/View

	Group 1		Group 2		Group 3	
	Treatment group	Control group	Treatment group	Control group	Treatment group	Control group
Pre-treatment average of positive cells	22	25	17	18	10	29
Post-treatment average of positive cells	15	31	22	42	10	42
Average of (post-treatment/pre-treatment) positive cells	0.7	1.33	1.38	3.71	5.41	1.19

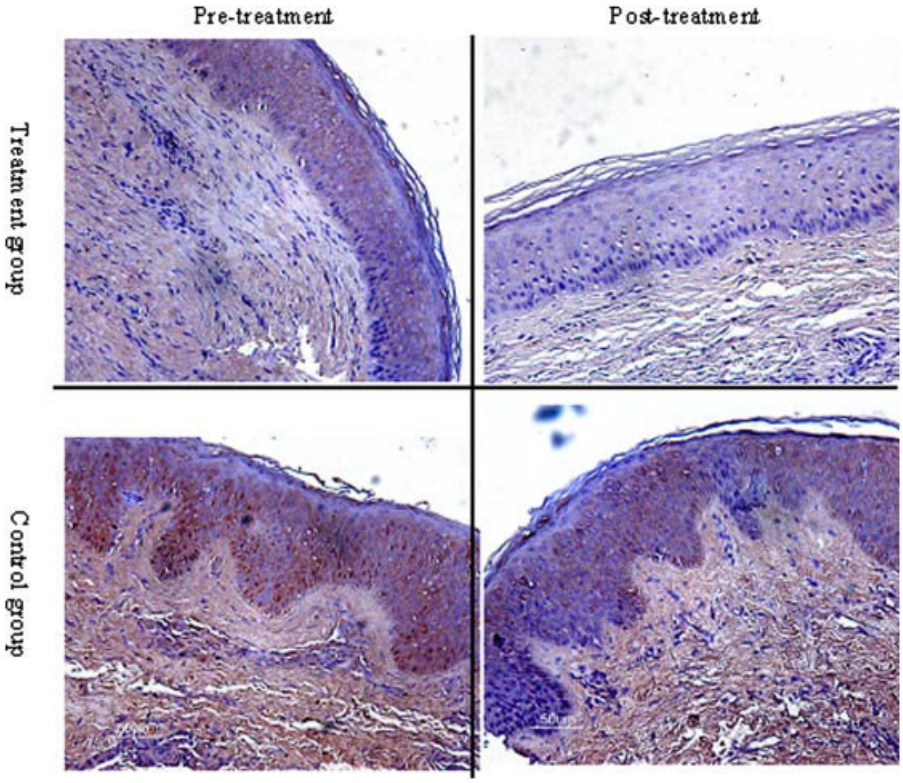

Fig. 4. IHC staining of CTGF in keloid tissue before and after laser treatment in the treatment and control groups. The patient was a 23-year-old male who had a keloid for 4 years. Positive cells were stained brown. CTGF positive cells significantly decreased after treatment in the treated group. There is no difference before and after treatment in the control group.

underlying genetic component that may alter an affected individual's immune response [1,11,12].

It is recognized that keloid formation is primarily due to the shift of collagen equilibrium toward more collagen deposition [12]. The collagen has a stronger, self-proliferation capability, while at the same time the concentration of collagenase inhibitor is increased as well [1,13]. Consequently, the ultimate result is more collagen synthesis and less degradation [1,11,12].

Increased CTGF growth factor activity has been considered to play an important role in the pathogenesis of keloids. CTGF is associated with fibrotic disease and is highly correlated with TGF-β. CTGF encodes a 38 kda cysteine-rich secreted polypeptide [12]. As a member of

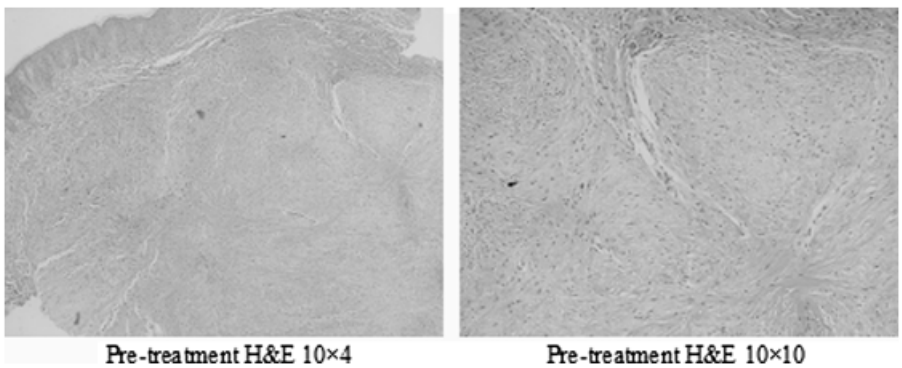

Fig. 5. An H&E section of pre-treatment lesion.

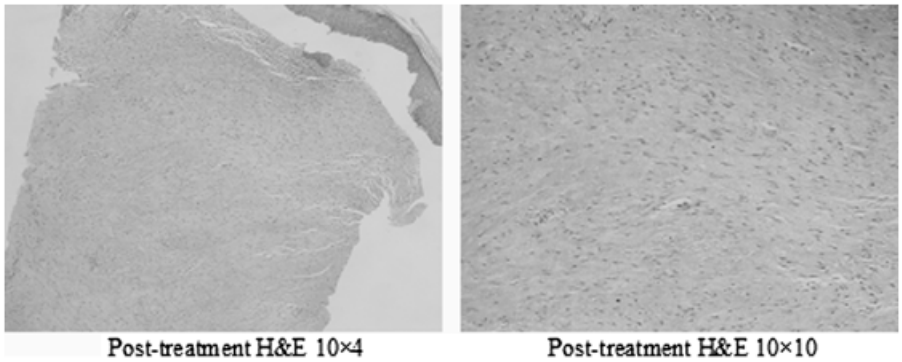

Fig. 6. An H&E section of post-treatment lesion.

the CCN (CTGF, Cyr 61, and Nov) family of secreted proteins, CTGF plays an important role in bone growth, angiogenesis, cell migration, and cell adhesion [11,12]. CTGF, a downstream factor of TGF-β, directly or indirectly enhances production of collagen, fibronectin, ECM components, and integrin. CTGF can also promote fibroblast growth and angiogenesis [5]. CTGF transcription and translation stimulates fibroblast proliferation and collagen-based ECM deposition [11]. Proliferation of fibroblasts will, in turn, produce more CTGF [3]. This positive feedback mechanism eventually results in a large amount of fibrotic tissue thereby leading to scar formation [5].

Treatment modalities of keloids include: intralesional injections of corticosteroids and 5-fluourouracil, PDL, ablative and non-ablative fractional resurfacing, cryosurgery, radiotherapy, pressure therapy, silicone gel sheeting, and excisional surgery [3]. These methods can provide some improvement, but also produce some unwanted side effects; recurrence is not uncommon. Compared with other treatments, PDL therapy can significantly improve pain and itch, soften scar tissue, and improve scar pliability. Typically, side effects are temporary and minor. There have been several reports of keloid improvement after PDL therapy using low to moderate fluences [14–16].

A study by Kuo et al. [14] showed that 86% of keloid patients had 50% remission after 1–11 PDL treatments, only 13.3% of patients had no significant regression. Moreover, a study by Manuskiatti et al. demonstrated that the texture of scar improved after PDL treatment in 12 of 19 patients (including keloids and hypertrophic scars). In most of these patients, keloid volume was shown to be reduced after treatment [17]. In our study, improvement of scar texture, improvement of pain and pruritus symptoms, and decreased erythema were observed after PDL treatment, again demonstrating the efficacy of PDL therapy of keloids.

Kuo et al. [18] indentified that the regression of keloids after PDL treatment was associated with the down-regulation of TGF-β1 expression and up-regulation of MMP-13 activity. However, the effect of PDL treatment on the expression of CTGF in keloid has not been reported, and remains to be elucidated. It is believed that the formation of keloid is associated with a range of growth factors. Among them, CTGF plays an important role on the formation and development of a keloid [3–5]. CTGF is not expressed in normal skin, but is specifically expressed in pathologic scar tissue, and its expression in fibroblasts is increased in proportion to the proliferation of the keloid [19]. CTGF is a highly specific cytokine that can reflect the disease activity and proliferation. It is different from TGF-β, in that the biochemical function of CTGF is more specific. Although TGF-β and CTGF can promote the keloid development, blocking TGF-β will attenuate both normal and pathologic scarring, while blocking CTGF will only attenuate pathologic scarring. [11]. Therefore, CTGF can be regarded an independent mediator of scarring and fibrosis. [20]. Down-regulation of CTGF is likely to reverse the formation of fibrosis, which is very important to the treatment of keloids. Thus, it would be expected that CTGF is an important target in the treatment of keloids.

In our study, it has been shown that in 80.77% of keloid patients, CTGF mRNA expression was down-regulated significantly by 58% after three PDL treatments. In contrast, CTGF mRNA level in the control group increased by an average of 4.91 times. Although CTGF mRNA in the rest of five patients (19.23%) increased by an average degree of 4.23 times after three PDL treatment, such increase in the control group was even higher (9.23 times). The results indicate that CTGF mRNA of keloid can be down-regulated by PDL treatment. At least the high expression of CTGF mRNA can be inhibited to a less degree. Even in the five patients of up-regulated CTGF mRNA after PDL therapy, the up-regulation was much higher in the control group than in the treated group. Additionally, this number was also higher than the rest of the control. It seems that the effect of CTGF mRNA down-regulation may be related with keloid activity. In other words, if the keloid is less active, the down-regulation of CTGF expression may be more obvious. The level of CTGF expression is consistent with the severity of keloid, and therefore more significant down-regulation should lead to a better clinical response. Moreover, the down-regulation of CTGF mRNA was negatively correlated with the age of the patients

according to our study, suggesting that older patients might have better treatment outcome. Meanwhile, there is no significant correlation among therapeutic efficacy of PDL on keloid and duration of illness. However, further investigation on a large sample is still required. Antibiotic cream was applied to the treated sites after the PDL treatment twice daily to reduce the risk of infection but was not applied to the control sites, because there is currently no data or research supporting the idea that the use of topical antibiotics can affect the CTGF expression. Moreover keloid with any sign of active infection was excluded from our study.

The clinical signs of keloids were improved after three PDL treatments and this is reflected by the decreased VSS score. This likely reflects keloid CTGF mRNA down-regulation after treatment. Consistent with prior keloid studies, keloid symptoms including pain and itch improved as well [3]. IHC results are basically the same as Realtime PCR results. In most cases (80.77%), the number of CTGF positive cells/field significantly decreased after PDL treatment and increased in the control group. This indicated that keloid CTGF expression can be down-regulated after PDL treatment at protein level.

According to IHC investigation, in two cases the number of CTGF positive cells of the treatment group increased more than the control group after treatment. The corresponding result of mRNA expression is down-regulation in the treatment group in one case and slight up-regulation in another case, while there was significant up-regulation in control group. Such inconsistency between Real-time PCR and IHC investigation may be due to the selection of field under microscope. Real-time PCR is a more precise method to measure CTGF mRNA expression, and the result might be more reliable.

It has been shown that PDL treatment can down-regulate CTGF expression or at least inhibit CTGF high expression at both the transcriptional and translational levels. Thus, it is very likely to be one of the mechanisms of therapeutic efficacy for PDL on keloids. Remission of a keloid is possible after PDL treatment. The relationship between the down-regulation effect of PDL and the number of treatments as well as the parameter of treatments needs further study in order to establish appropriate settings for optimal treatment. Because PDL can down-regulate the expression of CTGF, it stands to reason that other disease processes in which CTGF is highly expressed might also be treated with PDL. Moreover, a better understanding of CTGF, which is an important target of keloid treatment, is likely to provide the basis for combined therapies as well.

According to our study, PDL is an effective and safe treatment for keloids. The effects are limited in the setting of an actively proliferative keloid or a large keloid that is treated by PDL alone. It is thus reasonable to combine PDL with other treatment modalities to achieve potentially synergistic effects on CTGF expression. In this way, CTGF expression will be down-regulated more significantly and the maximum therapeutic benefit will be achieved.

CONCLUSION

Pulsed dye laser can effectively down-regulate the expression of CTGF, which may be one of the main mechanisms of keloid treatment. Further study is required to indicate differential effect on CTGF expression by using different PDL parameters.

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