

# Cellular Viability, Collagen Deposition, and Transforming Growth Factor $\beta$ 1 Production Among Ultraviolet B-Irradiated Keloid Fibroblasts

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## Abstract

**Background** A keloid is a fibrous tumor produced by fibroblast hyperproliferation and excessive collagen accumulation due to overproduction of transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1). High keloid incidence is found among individuals with unexposed skin, especially in Negroid and Mongoloid people with high melanin contents in their skin. Because melanin serves as an ultraviolet B (UVB) light absorber, it is assumed that lack of UVB light penetration may play a role in the keloid pathomechanism. This study aimed to evaluate the effect that UVB irradiation to monolayer keloid fibroblasts has on cell proliferation, collagen deposition, and TGF- $\beta$ 1 production.

**Methods** Keloid fibroblasts were isolated from five patients who underwent surgical treatment. Monolayer cultures of more than three passages of keloid fibroblast were exposed to various dosages of UVB irradiation. Cellular viabilities were measured by MTT assay. Collagen depositions were measured by Sirius Red assay for non-soluble collagen, and TGF- $\beta$ 1 production was measured by enzyme-linked immunoassay (ELISA). Data were analyzed by one-way analysis of variance (ANOVA).

**Results** Ultraviolet B 100 and 150 mJ/cm<sup>2</sup> were able to suppress keloid fibroblast viabilities and collagen accumulation significantly ( $P < 0.01$ ). Significant suppression of TGF- $\beta$ 1 production required UVB irradiation of 150 mJ/cm<sup>2</sup> ( $P < 0.01$ ).

**Conclusions** Lack of UVB skin penetration influences the keloid pathomechanism. Ultraviolet B irradiation with a minimal dosage of 150 mJ/cm<sup>2</sup> is a promising method of keloid prevention and treatment.

**Keywords** Cell viability · Collagen accumulation · Keloid fibroblast · TGF- $\beta$ 1 · UVB irradiation

A keloid is a fibrous tumor produced by excessive accumulation of connective tissue during the healing process. The pathogenesis of this disease is contributed by several factors: fibroblast hyperproliferation, increased collagen synthesis stimulated by keratinocytes and fibroblast release of transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) [1] and vascular endothelial growth factor (VEGF) [2], and reduction in collagen degradation resulting from the presence of a collagenase inhibitor [3] such as plasminogen activator inhibitor via inhibition of procollagenase activation [4].

Evidence has demonstrated high keloid incidence among Negroid [5, 6] and Mongoloid [7] populations and in the skin of unexposed regions such as the sternal and deltoid areas, back skin, and post-cesarean incision lines on the lower abdominal wall [8], suggesting that lack of solar radiation may play a role in the keloid pathomechanism. In addition, not only is the development of keloids more frequent among poststernotomy patients than in the common population (10–20% vs. 4.5–16%) [9], but keloids also commonly arise on the distal incision line out of the V-area, an area also protected from solar radiation.

It is well known that repeated and prolonged sun radiation influences the skin photoaging mechanism. Various findings have shown that solar radiation can inhibit skin fibroblast proliferation and collagen synthesis [10–12], can stimulate collagen degradation due to an increase in

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collagenase expression [13, 14] that degrades collagen material, and can inhibit further new collagen synthesis [15]. Studies of human skin have shown that the collagen content of exposed skin is 20% less than that of unexposed skin [12, 16].

The influence that lack of solar radiation has on the keloid pathomechanism became more definite after Blazić and Brajac [17] hypothesized that keloids might be caused by a failure of fibroblast senescence. Based on the fact that solar radiation is one of various factors contributing to aging skin, we hypothesized that lack of UVB skin penetration plays a significant role in the keloid pathomechanism. Therefore, we tried to irradiate fibroblast keloids by UVB light and observed keloid fibroblast proliferation rates, collagen accumulation, and TGF- $\beta$ 1 synthesis.

## Materials and Methods

### Experimental Design

Our experimental design can be observed based on the figures in the following sections.

Figure 1 presents the experimental design. Keloid material was isolated from male patients who had combined keloid surgery in which keloids were diagnosed as minor based on the keloid classification of Butler et al. [18]. Three patients had recurrent anterior chest keloids, and two patients had persistent keloids in the deltoid areas. All had experienced keloids for more than 2 years. More than 1 year previously, three of the patients had undergone an elliptical excision followed by triamcinolone injection, and the remainder had received a triamcinolone injection for more than 3 months.

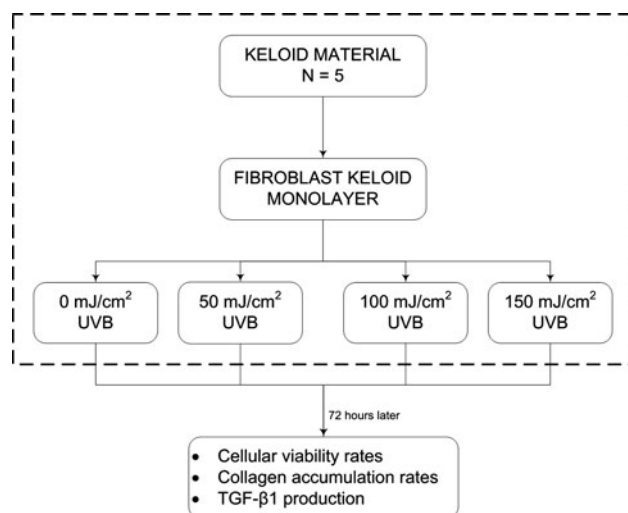


**Fig. 1** Experimental design

### Keloid Fibroblast Isolation and Cultures

Because keloid material is resistant against collagenase activities, we performed explantation techniques in primary keloid fibroblast cell cultivation. Briefly, keloid materials were rinsed in phosphate-buffered saline (PBS), cut into 3-mm<sup>3</sup> pieces, seeded onto a 75-mm culture flask, and soaked with a small amount of Dulbecco modified Eagle medium (DMEM) complete media (DMEM-Gibco, Grand Island, NY + 10% fetal bovine serum-Gibco, Grand Island, NY + 100  $\mu$ g – 100 U/ml penicillin-streptomycin Gibco, Grand Island, NY + 2.5  $\mu$ g/ml Fungizone-Gibco, Grand Island, NY) overnight. After the attachment was checked, medium was aspirated and replaced by 10 ml of freshly complete medium. Keloid fibroblast growth was continued by replacement of the complete medium every 3 days, and once 80–90% confluence was reached, the cells were trypsinized to obtain fibroblast keloid subcultures. More than a third of the passages of keloid fibroblast were kept in liquid nitrogen.

### Experiments

#### *UVB Irradiation: Keloid Fibroblast Viabilities and Collagen Accumulations*

After reculture of five frozen keloid fibroblast passage 3 of different sources in different flasks, the 90% confluence cells were harvested, and 200  $\mu$ l of  $2 \times 10^5$  cells suspension/ml/sources were seeded onto each well of twenty 96-multiwell plates in quadruplicating procedures. After overnight incubation, the medium was aspirated, and the cells were washed three times with 200  $\mu$ l of PBS. The cells then were soaked in 200  $\mu$ l of PBS, and various dosages of UVB irradiation were performed using a computerized UVB source (SUN PAS-UVB) produced by the Indonesian Academy of Science (LIPI).

The first plate was designed as an nonirradiated group. The second disk and others were designed to be irradiated by UVB with dosages of: 50, 100, and 150 mJ/cm<sup>2</sup>, consecutively. For 3 h after irradiation, the nonirradiated and irradiated plates were incubated in a carbon dioxide (CO<sub>2</sub>) incubator. After that, PBS was removed carefully, then replaced by complete medium, and the plates were incubated for 72 h.

We performed UVB irradiation with TGF- $\beta$ 1-releasing activities after carrying out UVB irradiation on keloid fibroblast proliferation and collagen accumulation.

#### *Keloid Fibroblast Proliferation Assay*

Cellular viabilities were measured by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)—MP

Biomedicals, Inc. Solon, OH—assay. Briefly, all the medium of the plate designed for MTT assay was aspirated and then rinsed by PBS 10 min each three times. Next, 200  $\mu$ l of fresh medium was filled into each well, and 50  $\mu$ l of 5-mg/ml MTT solution was added. The plate then was covered by aluminum foil and incubated for 6 h. After aspiration of the solution, 200  $\mu$ l of dimethyl sulfoxide (DMSO) was filled into each well followed by the addition of 25  $\mu$ l of glycine buffer on the top of the DMSO. The plate then was read at 570 nm by a plate reader.

### Keloid Fibroblast Collagen Accumulation

Collagen deposition was measured based on the insoluble collagen assay adopted from Tullberg-Reinert and Jundt [19] with a little modification. Briefly, after washing of the wells with PBS, cells were fixed with Bouin solution for 1 h and rinsed with tap water until all the yellow color was completely gone. The plates then were dried at room temperature overnight.

For 1 h, 200  $\mu$ l Sirius Red diluted in saturated picric acid was added. Unbinding Sirius Red then was removed by washing with 200  $\mu$ l of 0.1 N HCl until clear supernatant was observed. Sirius Red on the well bases then was removed by dilution with 200  $\mu$ l of 0.5 N NaOH, aspirated-refilled into new plates and read at 550 nm.

### Keloid Fibroblast Releasing TGF- $\beta$ 1

Measurement of TGF- $\beta$ 1 was performed using TGF- $\beta$ 1 ELISA from a Bender MedSystems kit (Cat. no. BMS249/2 and BMS249/2TEN) based on the manufacturer's protocol. Briefly, 20  $\mu$ l of fibroblast keloid culture supernatant was diluted with 180  $\mu$ l of assay buffer. Next, 100  $\mu$ l of pre-treated sample in duplicate was added to the sample wells. A 50- $\mu$ l HRP (Horse raddish peroxidase) conjugated solution then was added to each well, covered by adhesive film, and incubated for 4 h on microplate shakers. After that, every well was washed three times by wash buffer. Then 100  $\mu$ l of TMB (3,3',5,5'-tetramethyl benzidine) substrate solution was added to all the wells and incubated for 10 min, after which the reaction was stopped by adding 100  $\mu$ l of stop solution. The plates then were read at 450 nm. By using a standard curve, levels of TGF- $\beta$ 1 were determined.

## Results

Five different sources of keloid fibroblast monolayer cultivation treated by various dosages of UVB irradiation were used, and the results are presented in Figs. 1, 2, 3.

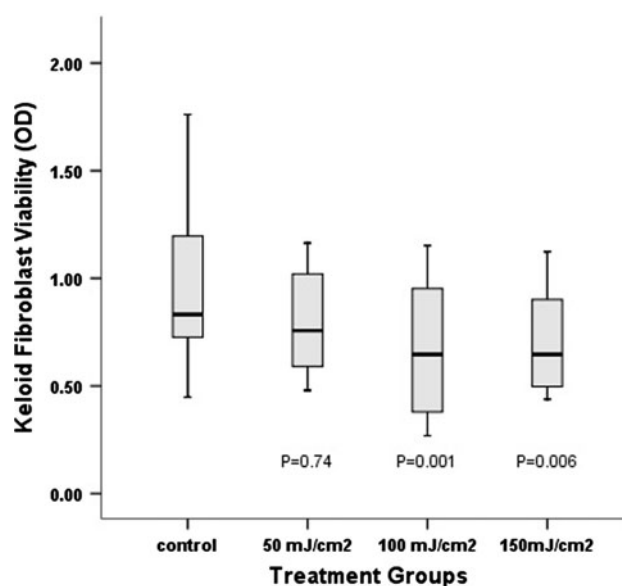


**Fig. 2** The effect of ultraviolet B (UVB) on keloid fibroblast viability

The Effect of UVB on Keloid Fibroblast Viabilities (Fig. 2)

A significant reduction in keloid fibroblast viability ( $P < 0.01$ ) was found among the groups treated with 100 and 150 mJ/cm<sup>2</sup> of UVB.

The Effect of UVB on Collagen Accumulation (Fig. 3)

A significant reduction in keloid fibroblast collagen accumulation ( $P < 0.01$ ) was found among the groups treated with 100 and 150 mJ/cm<sup>2</sup> of UVB.

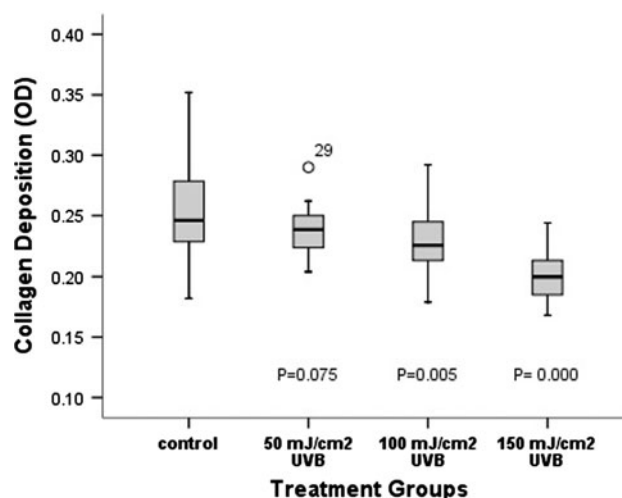


**Fig. 3** The effect of ultraviolet B (UVB) on collagen accumulation

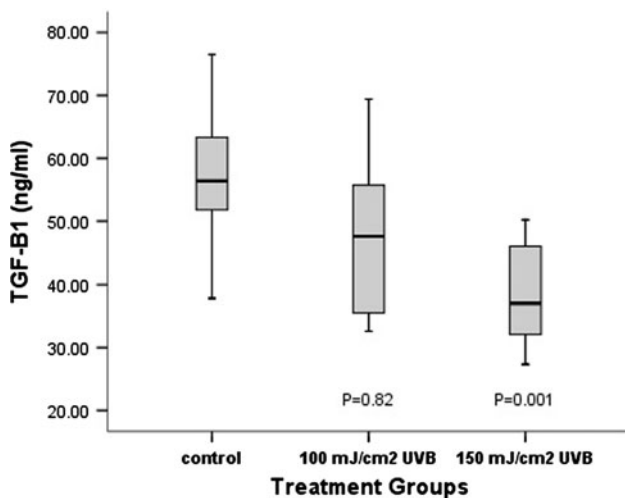


**Fig. 4** The effect of ultraviolet B (UVB) on transforming growth factor  $\beta 1$  (TGF- $\beta 1$ ) production

#### The Effect of UVB on TGF- $\beta 1$ Production (Fig. 4)

A significant reduction of keloid fibroblast TGF- $\beta 1$  synthesis ( $P < 0.01$ ) was found among the groups treated with 150 mJ/cm<sup>2</sup> of UVB.

## Discussion

Blazić and Brajac [17] hypothesized that keloids are caused by a failure of fibroblast senescence. On the other hand, solar radiation, especially UVB radiation, was one of various factors contributing to the photoaging process. The current study showed that UVB radiation in dosages of 100 and 150 mJ/cm<sup>2</sup> was able to suppress fibroblast keloid viability as well as collagen accumulation significantly (Figs. 1, 2). The lower dosage of UVB irradiation (50 mJ/m<sup>2</sup>) did not have a significant effect. However, in TGF- $\beta 1$  releasing keloid fibroblast only 150 mJ/cm<sup>2</sup> was able to suppress TGF- $\beta 1$  production significantly (Fig. 3). This finding was similar to that of Xu et al. [20], who reported that such dosages could suppress normal skin fibroblast viability.

In fibroblast keloid proliferation, TGF- $\beta 1$  plays a very significant role. Kamamoto et al. [21] showed that fibroblast proliferation of normal skin in medium enriched by TGF- $\beta 1$  equaled fibroblast keloid proliferation in regular medium. Our result showed that 150 mJ/cm<sup>2</sup> of UVB irradiation suppressed TGF- $\beta 1$  production. Dai et al. [22] reported that UVB irradiation on normal skin mice could cause a decrease in TGF- $\beta$  and TGF- $\beta$  receptor expression. Dosages of both 100 and 150 mJ/cm<sup>2</sup> suppressed fibroblast keloid viabilities.

In fibroblast keloid collagen synthesis, TGF- $\beta 1$  has a very significant role. Addition of exogenous TGF- $\beta 1$  in fibroblast

keloid culture resulted in collagen synthesis 12 times greater than that of normal fibroblasts [23]. Analysis of TGF- $\beta 1$  receptors showed that keloids expressed TGF- $\beta 1$  receptors at higher levels than hypertrophic scar or normal skin [24]. Our results showed that UVB dosage suppressing TGF- $\beta 1$  was similar to the UVB dosage suppressing collagen accumulation. Suppression of TGF- $\beta 1$  activity has been considered important in inhibiting collagen synthesis. Chu et al. [25] showed that introduction of a truncated TGF- $\beta 1$  receptor gene into a vector successfully trapped TGF- $\beta 1$ , consecutively inhibited keloid fibroblast proliferation, and inhibited keloid fibroblasts in collagen synthesis.

Besides TGF- $\beta 1$ , VEGF also has an important role in fibroblast keloid collagen synthesis. Our previous research showed that UVB in dosages of 75 and 100 mJ/cm<sup>2</sup> also suppressed VEGF expression immunohistochemically [26]. Compared with these results, suppression of TGF- $\beta 1$  requires a higher dose of UVB than suppression of VEGF.

Another cytokine contributing to tissue fibrosis is tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) [27], which also was found to be elevated among black patients with keloids [28]. However, a study showed that apoptosis-related genes, including TNF receptor 1-associated protein, in keloid tissues are downregulated [29], so we did not measure TNF- $\alpha$  levels in our study.

Strong evidence suggests a genetic susceptibility in individuals affected by keloids. A study in Nigeria concluded the predisposition to keloid formation is inherited as an autosomal recessive character [30]. A genome-wide linkage search for genes predisposing to keloid formation among Japanese family indicated that the responsible genes were located on 2q23 compared with a linkage to 7p11 among an African-American family [31], and that it was similar in the Chinese population [32]. However, neither locus is the locus for the TGF- $\beta 1$  gene because the TGF- $\beta 1$  gene is located on 19q13 [33]. This discrepancy leads us to take a second environmental way such as considering ultraviolet radiation as the influence on keloid formation.

Some questions were raised regarding the lack of UVB skin penetration in the pathomechanism of keloids, especially in the earlobe and cheek area, where keloids also were commonly found [34]. These areas were considered to be human body surface areas with low predicted effective radiation and low projected area factors according to the study of Tanabe et al. [35]. In addition, study on manikins exposed to solar radiation showed that tree-shade skin areas were able to reduce UV dosimetry measured by polysulphone dosimeters [36]. We speculate that tree shade is similar to hair-style shade of affected people in that it can reduce UV energy on the earlobe and cheek areas.

The lack of UVB skin penetration is only one of various factors contributing to keloid formation. Ogawa [37] declared his hypothesis that keloids and hypertrophic scars

result from an excessive responsiveness of either dermal cell mechanoreceptors or mechanosensitive nociceptors of sensory fibers in the skin. That excessive responsiveness may stimulate TGF- $\beta$  production and subsequent abundant collagen synthesis, contributing to specific anatomic keloid predilection. However, because UVB induces skin aging and may attenuate the skin's elasticity, UVB radiation is a promising method for keloid therapy and prevention.

Based on the aforementioned findings, an explanation for the high incidence of keloids among Negroid and Mongoloid people with high melanin skin content and also the prevalence of keloids among protected areas of post-sternotomy lines may be related to low UVB skin penetration. A clinical trial using a higher UVB skin penetration, reaching at least 150 mJ/cm<sup>2</sup>, is reasonable for the prevention of keloid formation in the remodeling phase of wound healing for suspected patients. A thin epidermal layer covering the fibrous material of keloid lesions should be considered an important factor in successful keloid phototherapy.

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**Conflict of interest** None.

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