Research Article

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Identification of the role of TG2 on the expression of TGF-β, TIMP-1 and TIMP-2 in aged skin

https://doi.org/10.1515/tjb-2023-0235
Received November 4, 2023; accepted January 4, 2024;
published online February 12, 2024

Abstract

Objectives: Transglutaminase 2 (TG2) is a unique protein having enzymatic and nonenzymatic functions that have been implicated in various biological and pathological processes such as cell survival and apoptosis, cell signaling, differentiation, adhesion and migration, wound healing and inflammation. As reported in previous studies, TG2 expression and activity increase by age suggesting that TG2 possibly has roles in cellular aging process. In this study, we aimed to explore the role of TG2 in chronological skin aging through its impact on the expression of some important extracellular matrix (ECM) proteins including TGF-β, TIMP-1 and TIMP-2.

Methods: We have compared TG2 expression and activity in young and *in vitro* chronologically aged human dermal fibroblasts via Western blot and *in situ* TG2 activity assays. Afterwards, we inhibited TG2 expression via siRNA transfection and activity via active site inhibitor of TG2 separately in aged dermal fibroblasts and monitored the expression levels of TGF-β, TIMP-1 and TIMP-2 in these cells by Western blot and compared to that of untreated control cells.

Results: We obtained evidence that both TG2 expression and activity increase in aged cells. However, protein levels of TGF-β, TIMP-1 and TIMP-2 do not exhibit any significant difference in TG2 downregulated or TG2 activity inhibited aged cells compared to control cells.

Conclusions: Our results indicate that changes in the expression and activity of TG2 in (*in vitro*) chronologically aged human dermal fibroblasts do not impact the expression patterns of TGF-β, TIMP-1 and TIMP-2 proteins.

Keywords: skin aging; dermal fibroblasts; extracellular matrix; transglutaminase 2; crosslinking activity

Introduction

The skin provides a protective barrier between the human body and extrinsic environment [1]. It also has roles like sensation, secretion, and regulation of body temperature [2]. Skin aging is stimulated by both intrinsic and extrinsic factors. Intrinsic skin aging is regulated by genetic factors whereas extrinsic skin aging mainly occurs due to exposure to sunlight via UV radiation and also to other environmental factors such as cigarette smoking and air pollution [3]. Both intrinsic and extrinsic aging leads to the formation of dry and dysfunctional skin and enhances the risks of skin diseases [4]. In aging skin, proliferation rate and number of fibroblasts are decreased, the levels of collagen, especially type I and III, and elastin in extracellular matrix (ECM) are reduced which in turn results in the thinning of dermis, increased wrinkles and the loss of elasticity [5]. Matrix metalloproteinases (MMPs) are proteolytic enzymes which catalyze degradation of numerous proteins in ECM. In the aging process of skin, the expression of MMPs is increased while there is a decrease in the expression of tissue inhibitor of metalloproteinases (TIMPs) [6]. Transforming growth factor-β (TGF-β) regulates ECM homeostasis by stimulating ECM production and inhibiting ECM degradation via Smad signaling network. In aged skin, TGF-β signaling pathway is impaired by elevated cellular levels of ROS which results in substantial inhibition of type Iα1 procollagen protein production and gene expression [7].

Transglutaminase 2 (TG2) is a distinguished member of the transglutaminase family of enzymes especially with

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its multifunctional nature. TG2 catalyses protein-protein cross-linking reactions, post-translational modifications of proteins, amine incorporation, and site-specific deamidation [8, 9]. In addition to its conserved 3D structure, TG2 possesses other unique protein sequences including intrinsically disordered regions and short linear motifs [10]. Intrinsically disordered regions (IDRs) are polypeptide segments composed of insufficient hydrophobic amino acids and higher proportion of polar or charged amino acids [11]. Therefore, under physiological conditions IDRs exhibit different conformations in dynamic equilibrium and allow TG2 to ideally interact with various protein partners. These interactions can be either enzymatic or non-enzymatic interactions and contribute to diversity of TG2 functions [12]. In addition to its previously mentioned enzymatic activities, non-enzymatic interactions of TG2 enables it to be involved in various adapter and signaling functions both in intracellular and extracellular environment [13]. Various studies in human tissue and a range of animal studies revealed that protein expression and catalytic activity of TG2 increase by age [14, 15]. The elevated levels of TG2 in vitro aged human dermal fibroblasts suggested the strong possibility of a role for TG2 in the aging process [14]. In the present study, we aimed to identify the possible influence of TG2 on chronologically aged dermal fibroblasts. For this purpose, we used cells from passage numbers 26 to 32 as aged cells [14], inhibited the enzymatic activity of TG2 and downregulated its protein expression in different experimental setups. Then we evaluated the effect of the changes in the activity and protein expression of TG2 on the expression levels of some important ECM components including TGF-β, TIMP-1 and TIMP-2.

Materials and methods

Cell culture

Human neonatal foreskin (dermal) fibroblasts (BJ ATCC® CRL-2522™) were cultured in DMEM (ThermoFisher, 41965039) medium supplemented with 10 % fetal bovine serum (FBS) (Thermo Fisher, 10270106), and penicillin/streptomycin antibiotics (ThermoFisher, 15140122) at 37 °C in an atmosphere of 5 % CO_2. Human dermal fibroblasts were chronologically aged by *in vitro* cell culturing. Some of them were maintained and collected for experiments and some of them were stored in liquid nitrogen for further use. In the experimental design, two cell groups were used: cells from passage number 4–10 were used as the young group and 26–32 were used as chronologically aged group. In a previous study [14], the passage number >26 is affirmed as aged cells via morphological differences in the cells and the reduced rate of proliferation, typical aging indicators.

Western blot analysis

Cells were lysed in the RIPA buffer (Thermo Fisher, 89900). The lysates were then centrifuged at 10,000 rpm for 15 min and analyzed by SDS-PAGE. Protein samples were then transferred electrophoretically onto PVDF membranes. After blocking with 5 % nonfat dry milk in Tris-buffered saline containing 0.1 % Tween 20 (TTBS) for 1 h at room temperature, the blots were incubated with primary antibodies (TG2 CUB 1:2,000, anti-TGF-β antibody 10 µg/mL, anti-TIMP-1 antibody 4 µg/mL, anti-TIMP-2 antibody 1:1,000 and anti-GAPDH antibody 1:2,000) in the blocking solution overnight. After washing three times with TTBS, the blots were further incubated for 1 h with secondary antibodies (anti-mouse and anti-rabbit antibodies) in the blocking solution. Following the same washing steps with TTBS, ECL detection system (Pierce ECL Western Blotting Substrate) was used to visualize the blots following the manufacturer's protocol.

In situ TG2 activity assay

In situ TG2 activity [via 5-(Biotinamido) pentylamine (BPA) incorporation] assay was carried out as described previously [16] with some modifications. This assay was carried out to visualize TG2 activity and also the inhibition of its activity by ZDON in human dermal fibroblasts. In order to inhibit TG2 activity, the human dermal fibroblasts were treated with 50 µM ZDON (Zedira), active site TG2 inhibitor, for 21 h (optimized). Next, the ZDON treated and untreated cells were lysed with RIPA buffer and by sonication. Protein in the supernatant was quantified with Bradford. Accordingly, 100 µg of cell lysates were treated with 1 mM BPA (5-(biotinamido) pentylamine) and 5 mM $CaCl_2$ in the Eppendorf centrifuge tubes for 1 h at 37 °C and then Western blot was performed with these lysates. The blots were incubated with streptavidin conjugated HRP and HRP substrate. BPA incorporated cellular proteins (TG2 activity) were visualized.

WST1 assay

Cell viability of the ZDON treated and untreated dermal fibroblasts were determined using WST1 assay according to manufacturer's protocol (Abcam). Cells were cultured (5×10^4/well) in a 96-well plate including 100 µL/well culture medium for 24 h and then treated with ZDON (50 µM) for 21 h. Treated and untreated cells were incubated with 10 µL WST1 solution for 4 h and absorbance was measured at 450 nm using a microtiter plate reader.

siRNA transfection

Chronologically aged human dermal fibroblasts were transfected with 50 nM, TG2 specific siRNA (ThermoFisher, AM16708) using Lipofectamine™ 2000 Transfection Reagent (ThermoFisher) following the manufacturer's protocol. Scrambled RNA-transfected cells were used as a control.

Statistical analysis

Results are indicated as mean ± standard error of mean (SEM). Experimental groups were compared by Student's t-test. $p<0.05$ was implicated as statistically significant.

Results

TG2 activity and protein expression in young and chronologically aged human dermal fibroblasts

Previous studies indicated that the protein expression and catalytic activity of TG2 increase by age in various tissues [14, 15]. In order to confirm this phenomenon in human skin fibroblasts, we have examined the protein expression and activity of TG2 in young and chronologically aged fibroblasts. For this aim, the human dermal fibroblasts were aged by multiple passages and cells from different passages (passage no 26–32) were collected for examination. Aged cells were larger and exhibited reduced rate of proliferation compared to younger ones as indicated in previous studies [14]. Firstly, TG2 protein expression in young (passage no 4 and 10) and aged fibroblasts (passage no 26 and 32) were determined by Western blot analysis. Aged fibroblasts show evidently increased levels of TG2 expression compared to their young counterparts (Figure 1A). Then, the enzymatic activity of TG2 was analysed in young and aged fibroblasts by *in situ* TG2 activity assay and its activity of cells from passage 26 were found to be greater than that of young fibroblasts from passage 10 (Figure 1B). These results confirm that the protein expression and catalytic activity of TG2 increase by age in human dermal fibroblasts.

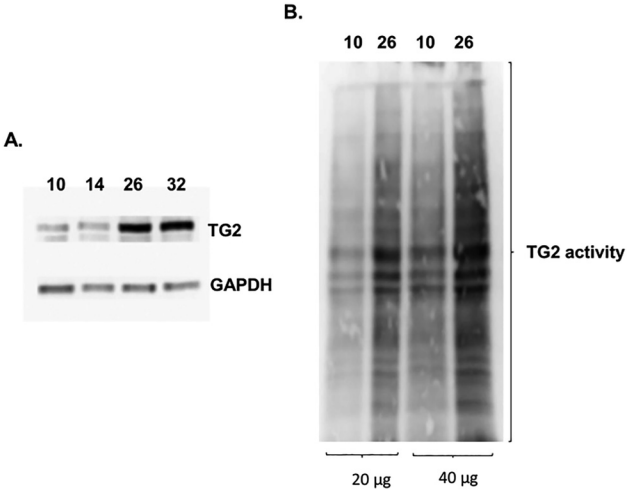

Figure 1: The protein expression and activity of TG2 in young and chronologically aged human dermal fibroblasts. (A) TG2 amounts were determined in young (passage no 4 and 10) and aged fibroblasts (passage no 26 and 32) by Western blot analysis using monoclonal anti TG2 antibody (1:2,000) and anti-GAPDH antibody (1:2,000). (B) The enzymatic activity of TG2 in the cells from passages 10 and 26 was determined by *in situ* TG2 activity assay using HRP-streptavidin antibody (1:2,000) and BPA incorporated cellular proteins were visualized. Similar results were obtained in three independent experiments.

Inhibition of TG2 activity by ZDON did not influence the expression levels of TGF-β, TIMP-1 and TIMP-2 in aged fibroblasts

As mentioned before, the crosslinking activity of TG2 plays important roles in various biological and pathological processes. Crosslinking activity of TG2 could influence the expression profiles of some proteins by the formation of cross-linked and inactivated transcription factors and thus repressing the expression of their responsive genes [13]. Since we confirmed that TG2 activity increases in aged fibroblasts we decided to evaluate the possible effect of TG2 activity on the protein levels of TGF-β, TIMP-1 and TIMP-2. For this aim, TG2 activity was inhibited in aged fibroblasts using a TG2 specific active site inhibitor, ZDON. Inhibition of TG2 activity was visualized by *in situ* TG2 activity assay and subsequent Western blot analysis (Figure 2A). The expression levels of TGF-β, TIMP-1 and TIMP-2 were then examined in ZDON treated and untreated (control) cells by Western blot analysis and results suggested that inhibition of TG2 activity by ZDON did not have any visible effect on the protein levels of TGF-β, TIMP-1 and TIMP-2 (Figure 2B). Cell viability assay result also suggested that ZDON did not have any cytotoxic effect on human dermal fibroblasts (Figure 2C).

Downregulation of TG2 expression by siRNA did not affect the expression levels of TGF-β, TIMP-1 and TIMP-2 in aged fibroblasts

In addition to its enzymatic activity, TG2 also plays roles as a scaffold or adaptor protein in various signalling pathways regulating the expression of some proteins. As it was confirmed previously, the protein expression of TG2 was elevated in aged dermal fibroblasts compared to that of young fibroblasts. In this regard, the possible influence of TG2 expression on the protein levels of TGF-β, TIMP-1 and TIMP-2 was evaluated. For this purpose, we downregulated TG2 in aged fibroblasts using TG2 specific siRNA and expression levels of TGF-β, TIMP-1 and TIMP-2 were determined in these cells and compared to that of scRNA transfected control cells by Western blot analysis. The results indicated that TG2 expression levels do not have any visible effect on the expression of TGF-β, TIMP-1 and TIMP-2 proteins (Figure 3).

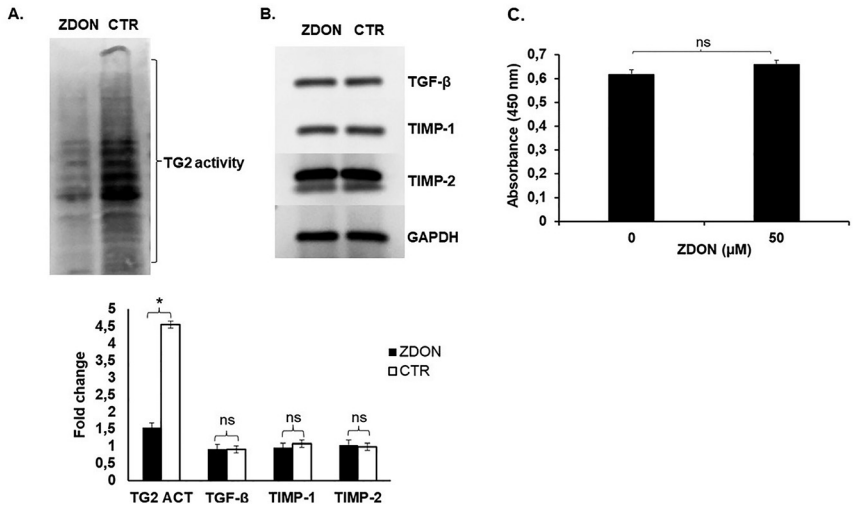

Figure 2: The protein expression of TGF-β, TIMP-1 and TIMP-2 in ZDON treated human dermal fibroblasts. (A) Chronologically aged human dermal fibroblasts (passage no 26) were treated with TG2 active site inhibitor ZDON (50 μM) for 21 h and *in situ* TG2 activity assay was performed as described in materials and methods. Untreated cells were used as control (CTR). BPA incorporated cellular proteins (TG2 activity) were visualized (TG2 ACT: TG2 activity). (B) The protein levels of TGF-β, TIMP-1 and TIMP-2 were determined in ZDON treated and untreated (CTR) cells using anti-TGF-β antibody (10 μg/mL), anti-TIMP-1 antibody (4 μg/mL), anti-TIMP-2 antibody (1:1,000) and anti-GAPDH antibody (1:2,000). (C) Cell viability of ZDON treated and untreated human dermal fibroblasts were also visualized. All experiments were repeated at least three times with the cells from passage no 26 and 32. Similar results were obtained. Data are represented as means ± SEM, *p<0.05, ns, not significant.

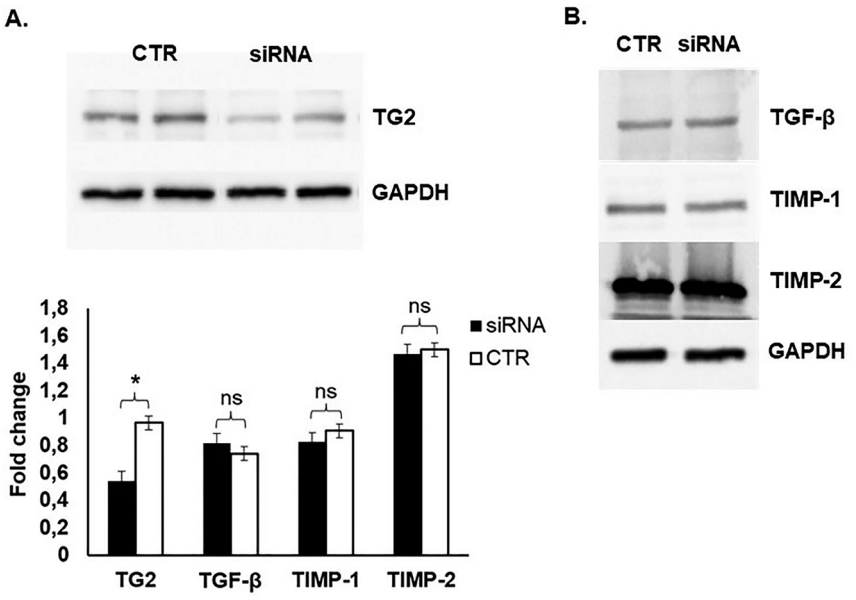

Figure 3: The protein expression of TGF-β, TIMP-1 and TIMP-2 in TG2 downregulated human dermal fibroblasts. (A) Chronologically aged human dermal fibroblasts (passage no 26) were transfected with TG2 specific siRNA (50 nM) as described in materials and methods and scRNA transfected cells were used as control (CTR). TG2 and GAPDH antibodies were used in 1:2,000 ratio. Two lanes for each siRNA and CTR represent TG2 bands in parallel loadings from the same lysate. (B) The protein levels of TGF-β, TIMP-1 and TIMP-2 were determined in siRNA transfected (TG2 downregulated) cells and compared with scRNA transfected control cells (CTR) using anti-TGF-β antibody (10 μg/mL), anti-TIMP-1 antibody (4 μg/mL), anti-TIMP-2 antibody (1:1,000) and anti-GAPDH antibody (1:2,000). All experiments were repeated at least three times with the cells from passage no 26 and 32. Similar results were obtained. Data are represented as means ± SEM, *p<0.05, ns, not significant.

Discussion

Skin aging is a highly complex and inevitable process induced by inside and outside factors [17, 18]. In both aging processes, skin dermis undergoes structural changes leading to decrease in the structural integrity and loss of physiological function [19]. TGF-β is the major player of ECM homeostasis and maintains the structural and mechanical integrity of dermis. It regulates ECM production and degradation by upregulation of ECM genes including collagens, fibronectin, decorin and TIMPs and by downregulation of MMPs [20].

Transglutaminase 2 (TG2) is the most abundant and most studied member of the transglutaminase enzyme family (TG1–7, F13, and Band 4.2). It catalyses Ca^{2+}-dependent transamidation reactions and Ca^{2+}-independent enzymatic and nonenzymatic functions [10]. TG2 exhibits a wide range of substrate specificity and shows flexible interaction with diverse proteins which contribute to its multiple biological functions [21]. Previous studies in human and animal tissues indicated that TG2 expression and/or transglutaminase activity increase with age in absence of pathology. For example, TG2 activity increases with age in normal knee meniscus tissue and chondrocytes [14]. Total TG2 levels were reported to be enhanced in old pig articular chondrocytes compared to young ones [22] and also in the macrophages of old vs. young mice [23]. Elevated TG2 expression and activity were observed in rat liver and brain of old animals and also in old vs. young human dermal fibroblasts [14]. It was demonstrated that inhibition of TG2 activity in fibroblasts downregulated the TGF-β mediated expression and activity of MMP-2 and MMP-9 while upregulating that of MMP-1a and MMP-13 and enhanced the migration and invasion ability of fibroblasts [24]. In a recent study, TG2 knockdown in cultured neonatal rat ventricular fibroblasts resulted in increased proliferation of these cells and also elevated the expression of TGF-β, indicating the functional importance of TG2 in myocardial ECM homeostasis [25].

In the present study, the differences in TG2, both in amounts and activity, were investigated in young and old human foreskin fibroblast cultures. Our results indicate that the amount of TG2 protein increases in the aged cells, which correlates well with TG2 activity as analysed by Western blot (Figure 1). The increase in TG2 levels and activity in aged cells suggest that TG2 possibly has an active role in the skin aging process. Since the possible influence of TG2 in chronological skin aging has not been studied so far, we further investigated the role of TG2 in this process. TG2 possesses specific structural features which enables it to interact with multiple partners inside and outside the cells. Enzymatic and non-enzymatic interactions of TG2 contribute to its diverse biological functions [13]. For instance, cell-surface TG2 interacts with fibronectin and regulates the cell adhesion, migration, and formation of focal adhesions in human erythroleukemia, fibroblasts and endothelial cells [26]. On the other hand, under specific circumstances cytoplasmic TG2 translocates to the nucleus and makes enzymatic and non-enzymatic interactions [27] which enable TG2 to be involved in gene expression of transcriptional factors and some other proteins including histones, hypoxia inducible factor (HIF) 1 and Sp1 via these interactions [28].

Further, in order to elucidate the possible influence of TG2 on expression levels of some important ECM proteins, we first inhibited the activity of TG2 in aged dermal fibroblasts with a specific active site inhibitor ZDON and evaluated the protein levels of TGF-β, TIMP-1 and TIMP-2 in these cells compared to untreated control cells. Our results indicate that the inhibition of TG2 activity did not affect the protein expressions of these ECM components (Figure 2). We also downregulated TG2 expression via siRNA transfection and checked the expression levels of the same ECM proteins. Our results show that reduction of TG2 expression also has no impact on the protein levels of TGF-β, TIMP-1 and TIMP-2 (Figure 3). These results suggest that, alterations of both TG2 expression and activity in aged dermal fibroblasts do not affect the protein levels of these ECM components.

As mentioned previously, the number of dermal fibroblasts decrease in the process of skin aging which results in reduced production of extracellular matrix proteins especially collagens and thus leads to the formation of dysfunctional skin. Elevated levels and activity of TG2 have been previously found to be correlated with enhanced apoptosis in various cells and tissues including CD4+ T lymphocytes and neuroblastoma cell lines [29–31] whereas inhibition of TG2 resulted in reduced apoptosis [32]. Considering these results, the role of TG2 accumulation and its increased activity on skin aging could be also further evaluated through its possible effect on the survival of dermal fibroblasts. TG2 has the ability to crosslink a variety of proteins inside and outside the cells. Some of the important substrates of TG2 in ECM include collagen, elastin, fibronectin, fibrinogen and laminin [33, 34]. Increased expression and activity of TG2 leads to accumulation of crosslinks and decrease in solubility of collagen and other ECM proteins in aged skin cells [35, 36]. Enhanced crosslinking of ECM proteins results in impaired matrix turnover which contributes to the aging process. These phenomena might be the other possible mechanisms through which TG2 is involved in skin aging.

In summary, our results indicate that protein levels and enzyme activity of TG2 increase in chronologically aged dermal fibroblasts. In addition, we observed that inhibition of TG2 activity and downregulation of its expression in these

cells did not influence the protein levels of TGF-β, TIMP-1 and TIMP-2. The current study allows to conclude that, TG2 does not influence the aging process of dermal fibroblasts through the expressions of these proteins. Considering the great diversity of its structure and aforementioned roles of TG2 including its impact on gene exressions and cell survivals, the possible role of TG2 in chronological skin aging could be further investigated in the future through its impact on the expression of other ECM proteins and metalloproteinases, or on the proliferation of human dermal fibroblasts.

Research ethics: Not applicable.
Informed consent: Not applicable.
Author contributions: All experiments were performed by Elvan Ergülen and the results were evaluated by Elvan Ergülen and Gül Akdoğan. Gül Akdoğan is the coordinatior of the study. She participated in the design of the study and helped to draft the manuscript. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.
Competing interests: Authors state no conflict of interest.
Research funding: This study was supported by Scientific Research Projects funds of İzmir university of Economics.
Data availability: The authors confirm that the data supporting the findings of this study are available within the article.

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