Inhibition of MMPs Cat G and downregulates the signaling of TGF-β/Smad in chronic photodamaged human fibroblasts

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Abstract. – OBJECTIVE: To understand the action of Cathepsin G (Cat G) and matrix metalloproteases (MMPs) on the β/Smad pathway of transforming growth factor β (TGF-β) in chronically photodamaged human fibroblasts. Cat G plays a significant role in the process of skin photoaging and in collagen synthesis and degradation which is induced by UV irradiation. It could interact with TGF-β/Smad signaling. No available studies have thoroughly explored its molecular mechanisms of photoaging regulation.

PATIENTS AND METHODS: Fibroblasts were divided into 4 groups: (1) control, (2) UVA irradiation of 25 J/cm², (3) UVA irradiation of 25 J/cm² + MMPs inhibitor, and (4) 25 J/cm² UVA irradiation + Cat G inhibitor. All treatments were repeated daily for 21 days. Western blot and ELISA were employed to detect protein levels for Cat G, MMPs, and several smads.

RESULTS: Compared to UVA-irradiated cells, the addition of MMPs inhibitor downregulated the expression of smad2, smad3, and smad4 as well as TGF-β. The addition of Cat G inhibitor downregulated the expression of smad2, smad3, and smad4 as well as TGF-β. These data suggest that TGF-β/Smad signaling was decreased by inhibition of MMPs and Cat G decreased in chronically human fibroblasts which are photo-damaged.

CONCLUSIONS: These results may help expand our knowledge of mechanisms mediating photoaging and is possibly instrumental to the exploration of novel anti-photoaging treatments.

Key Words: Chronic photodamage, Cathepsin G, MMPs, TGF-β/Smad, Skin.

Introduction

Chromically photodamaged skin (photoaging) is caused by chronic ultraviolet (UV) exposure and accounts for most age-associated changes in skin appearance, such as increased wrinkles, reduced recoil capacity, increased fragility, and leathery appearance. The proposed mechanisms of skin photoaging include oxidative protein damage, mitochondrial DNA deletion, decorative telomere acceleration, membrane/nuclear signal alterations, and photoaging-dependent enzymes. Previous studies have confirmed the role of matrix metalloproteases (MMPs) in chronic photodamaged skin. Our previous work showed that the cathepsin (Cat) family (B, D, K, G) plays an important role in photoaging. As the relationship between cathepsins and MMPs in the mechanisms of photoaging was only superficially investigated, the functional interaction of these enzymes was still unknown.

Recent studies have shown that UV light-induced skin aging was not only associated with increased MMP and Cat G expression but also involved impaired TGF-β/Smad signaling. Based on recent literature and our previous researches, Cat G, MMPs, and TGF-β/Smad signaling played a role in collagen-I synthesis and degradation that is induced by UV light. However, the consequences of altering these critical and multi-functional cellular factors is not clear.

Here, we investigated the interaction of MMPs, Cat G, and the signaling of TGF-β/Smad in human fibroblasts that are chronically photodamaged. The results of this study can expand our comprehension of the underlying photoaging mechanisms.

Patients and Methods

Collection of Human Fibroblasts

The foreskin of healthy human donors was recruited to gain fibroblasts which were at the scale...
of age between three and five years old. Written consent was obtained from the guardians of each donor child. The study acquired the approval from the Medical Ethical Committee of the Third Affiliated Hospital of Sun Yat-sen University (No. [2009]2-2).

Culture and Treatment of Human Fibroblasts

Fibroblasts were cultured in DMEM (Dulbecco modified Eagle’s medium; Gibco/Brl, Eggenstein, Germany) with the supplementation of 10% fetal calf serum (Gibco/Brl, Eggenstein, Germany) and incubated in a humidified atmosphere with 5% CO₂ contained under 37°C. Media was refreshed weekly. Cell cultures were randomly divided into 4 groups: (1) control, (2) UVA irradiation of 25 J/cm², (3) MMPs inhibitor + UVA irradiation of 25 J/cm², as well as (4) Cat G inhibitor + 25 J/cm² UVA irradiation.

For the UVA treatment, fibroblasts were irradiated with UVA (UVASUN3000, Munich, Germany) with a wavelength ranging from 360 to 420 nm and irradiance of 5.2 mW/cm², and the irradiation dose reached 25 J/cm² daily for 21 days. For MMPs inhibitor treatment, fibroblasts were incubated with 16 µM MMPs inhibitor II (57157, Sellek Chemical, Houston, TX, USA) for 24 h after 21 days of UVA irradiation. For Cat G inhibition, fibroblasts were incubated with 10 µM Cat G inhibitor (SC-221399, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 24 h after 21 days of UVA irradiation.

Western Blot Analysis

Fibroblasts were collected after 21 days of treatment and homogenates were prepared after the cells were split in BSA and PMSF solution. The modified Lowry protein assay kit (Pierce, Rockford, IL, USA) was used to quantify protein. After electrophoresis and electrobloptting, PVDF membranes were incubated with primary mouse anti-Cat G (ab50845, 1: 50) and rabbit anti-TGF-β (ab53169, 1: 1,000) IgG antibodies from Abcam (Cambridge, MA, USA), rabbit anti-smad2 (CST5339s, 1:1,000), rabbit anti-smad3 (CST9523s, 1:1,000), and rabbit anti-smad4 (CST9518s, 1:1,000). IgG antibodies from Cell Signaling Technology (Danvers, MA, USA) at 4°C overnight, followed by rabbit Anti-Mouse IgG- HRP or goat anti-rabbit-HRP secondary antibodies (Southern Biotech, Birmingham, AL, USA, 1:10,000) in 1 h under room temperature. We used the ECL detection kit (Fermentas China, Shenzhen, China) in consistence with the manufacturer’s instructions to develop the signal in the membrane.

ELISA

After 21 days of treatment, fibroblasts were collected. The expression of MMP-1, MMP-2, and MMP-3 and the activity were tested using the Human Pro-MMP Immunoassay Quantikine ELISA (R&D Systems, Minneapolis, MN, USA). A spectrophotometer (Spectrophotometer SP-830 Plus, Barnstead International, Dubuque, IA, USA) was employed to monitor absorbance at 450 nm. The concentration of standard MMPs measured was then used to prepare calibration curves.

Statistical Analysis

The experiments were replicated at least three times. Mean ± SE is used as the form to express all data. SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was employed to perform statistic analyses. Normal distribution test and t-test analysis were run to compare data. p<0.05 is considered to be statistically significant.

Results

Changes of TGF-β/Smad Pathway, Cat G, and MMPs by UVA Irradiation

After subjecting human fibroblasts to 21-day 25 J/cm² UVA treatment, the activation of the TGF-β/Smad signaling pathway was examined through looking at the levels of several smads (Figure 1). Smad2 increased by 2.1 ± 0.59 times compared to the control. The levels of smad3, smad4, and TGF-β decreased by 75.8 ± 9.2% (p<0.01), 91.8 ± 1.1% (p<0.01), and 91.8 ± 1.1% (p<0.01), respectively.
12.9% \( (p=0.06) \), and 0.92 ± 0.23% \( (p<0.01) \) of the control, respectively (Figure 1). After the UVA treatment, Cat G expression increased by 1.2 ± 0.5 \( (p<0.01) \) times of the control (Figure 3).

ELISA suggested that the expression of MMP-1, MMP-2, as well as MMP-3 increased by 1.1 ± 0.4 \( (p=0.03) \), 1.1 ± 0.3 \( (p=0.03) \), and 1.39 ± 0.22 \( (p=0.02) \) times compared with the control, respectively (Figure 5).

**MMPs Inhibition**

After confirming the activation of TGF-β/Smad signaling, we next repeated the UVA treatment and added MMPs inhibitor. Compared to the UVA group, TGF-β expression, smad2, smad3, and smad4 MMPs inhibitor + UVA group decreased to 69.8 ± 5.1% \( (p=0.02) \), 0.18 ± 0.02% \( (p=0.04) \), 22.5 ± 3.2% \( (p<0.01) \), and 67.1 ± 5.1% \( (p<0.01) \), respectively (Figures 1 and 2). 24 hours after adding the MMPs inhibitor, Cat G expression decreased by 31.7 ± 8.9% \( (p<0.01) \) of the control. After adding the MMPs inhibitor II, the expression of MMP-1, MMP-2, and MMP-3 decreased to 58.9 ± 9.6% \( (p=0.01) \), compared to Cat G inhibitor), 55.2 ± 9.1% (compared with Cat G inhibitor, \( p>0.05 \)) and 25.9 ± 5.7% (compared with Cat G inhibitor, \( p>0.05 \)) of the control respectively (Figure 5).

**Cat G Inhibition**

After confirming the activation of Cat G and TGF-β/Smad signaling, we next repeated the UVA treatment and added Cat G inhibitor. Compared to the UVA group, TGF-β expression, smad2, smad3, and smad4 Cat G inhibitor + UVA group decreased to 55.4 ± 7.3% \( (p<0.01) \), 0.18 ± 0.04% \( (p=0.04) \), 7.5 ± 1.9% \( (p<0.01) \), and 5.2 ± 1.4% \( (p<0.01) \) of the control, respectively (Figures 1 and 2). 24 hours after Cat

![Figure 2](image2.png)

**Figure 2.** Quantification of Western-blot values for the TGF-β/Smad pathway. Compared to the UVA-irradiated cells, expression of TGF-β, smad2, smad3, as well as smad4 in the UVA + MMP inhibitor, decreased to 69.8 ± 5.1% \( (p=0.02) \), 0.18 ± 0.02% \( (p=0.04) \), 22.5 ± 3.2% \( (p<0.01) \) and 67.1 ± 5.1% \( (p<0.01) \). In the UVA + Ca G inhibitor, TGF-β, smad2, smad3, and smad4 expression decreased to 55.4 ± 7.3% \( (p<0.01) \), 0.18 ± 0.04% \( (p=0.04) \), 7.5 ± 1.9% \( (p<0.01) \) and 5.2 ± 1.4% \( (p<0.01) \).

![Figure 3](image3.png)

**Figure 3.** The Western blot analysis of Cat G. After 21 days of UVA treatment, fibroblasts of the four groups were collected and Cat G was quantified. Electrophoretogram showed that Cat G was elevated in the UVA irradiated cells versus the control group. Cat G was significantly reduced in the UVA + MMP inhibitor and UVA + Cat G inhibitor cells than the UVA and control groups.
G inhibitor II treatment, Cat G expression decreased to 17.18 ± 3.57% (p<0.01) of the control (Figures 3 and 4). MMP-1, MMP-2, and MMP-3 levels decreased to 98.6 ± 19.1% (p=0.03), 57.1 ± 12.4% (p<0.01), and 27.5 ± 6.5% (p<0.01) of the control, respectively (Figure 5).

**Discussion**

Chronic skin photodamage (photoaged skin) is a major extrinsic cause of skin aging. The mechanism of photoaging is still not fully understood, but the role of proteases, especially MMPs, is widely accepted. Yaar and Gilchrest have found other proteases such as elastase, lysozyme, elafin, and α-1-antitrypsin participate in photoaging. Our previous studies found changes of Cat G in photoaged skin and fibroblasts. Based on these findings, we wondered which of these enzymes plays a more relevant role in photoaging and whether there are interactions or cross-talk among them.

MMPs, Cat G, and the TGF-β/Smad pathway are powerful contributors to human skin photoaging through UV-induced collagen synthesis and degradation. The up-regulation of MMPs,

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**Figure 4.** Quantification of Western blot values for Cat G. After 21 days UVA irradiation, Cat G expression increased to 1.2 ± 0.5 (p<0.01) times of control. 24 h after adding MMPs inhibition, Cat G expression decreased to 31.7 ± 8.9% (p<0.01) of control. 24 h after Cat G inhibitor treatment, Cat G expression decreased to 17.18 ± 3.57% (p<0.01) of control.

**Figure 5.** ELISA results. After 21 days UVA irradiation, ELISA suggested that MMP-1, MMP-2, as well as MMP-3 expression increased to 1.1 ± 0.4 times (p=0.03), 1.1 ± 0.3 times (p=0.03) and 1.39 ± 0.22 times (p=0.02) of the control. After adding Cat G inhibitor, expression of MMP-1, MMP-2, and MMP-3 decreased to 98.6 ± 19.1% (p=0.03), 57.1 ± 12.4% (p<0.01) and 27.5 ± 6.5% (p<0.01) of control. After adding MMPs inhibitor, MMP-1, MMP-2, and MMP-3 expression decreased to 58.9 ± 9.6% (p=0.01 compared to Cat G inhibitor group), 55.2 ± 9.1% (p>0.05, compared to Cat G inhibitor group) and 25.9 ± 5.7% (p>0.05, versus Cat G inhibitor group) of control.
encompassing collagenase-1 (MMP-1), gelatinase A (MMP-2), as well as stromelysin-1 (MMP-3), exerts a crucial role in degrading connective tissue such as collagen and elastin. MMPs-mediated collagen destruction partly accounts for connective tissue damage that occurs during skin photoaging. Our previous studies found that expression of Cat G was up-regulated in the photoaged skin and fibroblasts. Activation of Cat G might lead to the matrix disorders of photoaged skin presented as wrinkles and coarseness on the skin surface.

Many studies have indicated key roles of intracellular signaling pathways in skin photoaging. The TGF-β/Smad pathway is important for cell proliferation, differentiation, and death. The beginning phase of the TGF-β/Smad signaling cascade is impaired by UV light irradiation. Intrinsically and UV-induced skin aging is associated with impaired TGF-β signaling. TGF-β/Smad signaling play important roles in extracellular matrix remodeling characteristic of chronic photodamaged skin. TGF-β induction of Adam12 and Timp-1 requires the Smad pathway and non-Smad signaling for remodeling and regulating the extracellular matrix. Here we found that after 21 days of UVA irradiation, TGF-β, smad1, smad2, smad3, MMP-1, MMP-2, MMP-3, and Cat G expression and activity were all elevated. These data reconfirmed the role of TGF-β/Smad signaling, Cat G, as well as MMPs in skin chronic photodamaging mechanisms.

The impact of TGF-β/Smad signaling and MMPs in chronically UV damaged skin is well documented. MMPs belong to the family of endoproteases which require zinc with a wide variety of substrate specificities degrading extracellular matrix proteins, a characteristic of chronic photodamaged skin. Interaction of MMPs and TGF-β had been studied in different models. TGF-β can inhibit the specific enzymes expression with the involvement of breakdown of collagen, encompassing MMP-1 and MMP-3. Levels of pSmad2 are lower in photoaged forearm skin with the comparison with the matched intrinsically aged skin, suggesting that UV-induced downregulation of TbRII and smad7 overexpression inhibit TGF-β activity. Both the UVA-induced transcription of the TGF-β/Smad signaling cascade and MMPs expression was regulated by chito-oligomers treatment in UVA-irradiated dermal fibroblasts. UVB-induced ROS can upregulate TGF-β biosynthesis and activation of TGF-β through increased activity of MMP-2 and MMP-9. Our study found that MMPs inhibition also downregulated TGF-β, smad2, smad3, and smad4 expression. This indicated that in chronic UV-damaged skin the increasing MMPs may influence signal of cell proliferation, differentiation, and death by inducing TGF-β/Smad signaling.

Recent literature suggested that UVA activated several signaling pathways, including TGF-β/Smad, and regulated enzyme activity. Inhibition of Cat G in a murine bone invasion model reduced TGF-β signaling, which subsequently reduced tumor vascularity. Here we found that the interaction of MMPs, Cat G, as well as TGF-β/Smad in fibroblasts that are chronically photodamaged. Cat G downregulation decreased MMPs expression and modified the TGF-β/Smad signaling in chronic UVA-irradiated fibroblasts. Inhibition of MMPs also decreased Cat G expression and changed TGF-β/Smad signaling. Our results indicated that the TGF-β/Smad pathway was influenced by both Cat G and MMPs in skin chronic photodamaging mechanisms.

Cat G is a protease that participates in proteolytic cascades, proteolytically destructive processes, inflammation, tumor invasion, and metastases. MMP-1, MMP-2, MMP-3, and Cat G are up-regulated in chronic photodamaged cells. As early as 1989, Okada et al reported that Cat G activated MMP-1, MMP-2, and MMP-3. Son et al confirmed this by demonstrating that Cat G degraded fibronectin (Fn) into fragments that increased MMP-1 mRNA and protein levels as well as MMP-2 activity in NHFs. Here, we found that Cat G inhibition decreased MMPs expression and activity in chronic UVA-irradiation fibroblasts. Both MMPs and Cat G played important roles degrading connective tissue such as collagen and elastin, and their interaction may contribute to the dermal changes, particularly those implicated in wrinkling.

**Conclusions**

MMPs and Cat G activity have a regulatory effect on TGF-β/Smad signaling. The interaction between Cat G and MMPs in chronically photodamaged human fibroblasts is involved in the underlying mechanism. These results may help to expand our knowledge of the cellular factors implicated in the skin photoaging.

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Conflict of interest
The authors declare no conflicts of interest.

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