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The upregulation of heat shock protein 47 in human gingival fibroblasts stimulated with cyclosporine A

T.-Y. Chang[1], C.-H. Tsai[2], Y.-C. Chang[1,3]

[1]Graduate School of Dentistry, [2]Department of Oral Pathology, Chung Shan Medical University and [3]Department of Periodontics, Chung Shan Medical University Hospital, Taichung, Taiwan

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Background and Objective: Heat shock protein 47 (Hsp47), a collagen-specific molecular chaperone, is involved in the processing and/or secretion of procollagen. Heat shock protein 47 is consistently and dramatically upregulated in a variety of fibrotic diseases. The aim of this study was to compare Hsp47 expression in normal gingival tissues and cyclosporine A-induced gingival overgrowth specimens and further explore the potential mechanisms that may lead to induction of Hsp47 expression.

Material and Methods: Fifteen cyclosporine A-induced gingival overgrowth specimens and five normal gingival tissues were examined by immunohistochemistry. Western blot was used to investigate the effects of cyclosporine A on the expression of Hsp47 in human gingival fibroblasts. In addition, *Aggregatibacter actinomycetemcomitans*, interleukin-1α (IL-1α) and mitogen-activated protein kinase kinase (MEK) inhibitor U0126 were added to seek the possible regulatory mechanisms of Hsp47 expression.

Results: A significantly higher percentage of cells positively stained for Hsp47 was noted in the cyclosporine A-induced gingival overgrowth group than in the normal gingival group ($p < 0.05$). Expression of Hsp47 was observed mainly in the cytoplasm of fibroblasts, endothelial cells, epithelial cells and inflammatory cells. Expression of Hsp47 was significantly higher in cyclosporine A-induced gingival overgrowth specimens with higher levels of inflammatory infiltrates ($p < 0.05$). Cyclosporine A upregulated Hsp47 expression in human gingival fibroblasts in a dose-dependent manner ($p < 0.05$). The addition of *A. actinomycetemcomitans* or interlukin-1α significantly increased Hsp47 expression compared with cyclosporine A alone ($p < 0.05$). The MEK inhibitor U0126 was found to inhibit cyclosporine A-induced Hsp47 expression ($p < 0.05$).

Conclusion: Expression of Hsp47 is significantly upregulated in cyclosporine A-induced gingival overgrowth specimens, and Hsp47 expression induced by cyclosporine A in fibroblasts may be mediated by the MEK signal transduction pathway. The expression of Hsp47 could be significantly enhanced by *A. actinomycetemcomitans* and interlukin-1α.

Dr Yu-Chao Chang, DDS, MMS, PhD, Graduate School of Dentistry, Chung Shan Medical University, 110, Section 1, Chien-Kuo N Road, Taichung, Taiwan
Tel: +886 4 24718668 ext. 55011
Fax: +886 4 24759065
e-mail:cyc@csmu.edu.tw

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Gingival overgrowth is a common side-effect of the chronic use of the immunosuppressive drug cyclosporine A. Cyclosporine A is a cyclic polypeptide with potent immunosuppressive activity, used widely to prevent graft rejection in organ transplant patients and also in the treatment of autoimmune diseases such as rheumatoid arthritis and psoriasis (1). Cyclosporine A-induced gingival overgrowth is characterized by thickening of the gingival epithelium as well as a marked increase in the extracellular matrix of gingival connective tissue (2). The causative and underlying aetiological factors have been reviewed, and it has been demonstrated that local, systemic and genetic factors may also contribute to the development and progression of gingival overgrowth (3,4). Recently, our studies have shown that the upregulation of plasminogen activator inhibitor-1 (5), cystatin C (6) and lysyl oxidase (7) may contribute to the extracellular matrix accumulation in cyclosporine A-induced gingival overgrowth. However, the exact mechanism whereby cyclosporine A induces gingival overgrowth remains largely uncertain.

Heat shock protein 47 (Hsp47) is a 47 kDa collagen-binding heat shock protein, which belongs to the serine protease inhibitor (serpin) superfamily containing a serpin signature sequence (8). Heat shock protein 47 is known as a molecular chaperone that is specifically involved in the processing and/or secretion of collagen molecules (9). Heat shock protein 47 is expressed in the endoplasmic reticulum (ER) of cells producing type I collagen, and is actively involved in type I collagen biosynthesis (10). Heat shock protein 47 has been implicated in the pathogenesis of many fibrotic diseases. An increased expression of Hsp47 has been demonstrated in the keloids (11), human pulmonary fibrosis (12), bleomycin-induced pulmonary fibrosis (13) and oral submucous fibrosis (14).

Previously, Martelli-Junior *et al.* (15) have demonstrated that Hsp47 is significantly upregulated in hereditary gingival fibromatosis compared with normal gingiva. These data suggest that Hsp47 might play an important role in the pathogenesis of cyclosporine A-induced gingival overgrowth. On the basis of these observations, the present work was undertaken to identify the *in situ* localization of Hsp47 expression in normal gingival tissues and cyclosporine A-induced gingival overgrowth specimens. In addition, western blot was used to determine the effects of cyclosporine A on the expression of Hsp47 in cultured human gingival fibroblasts (HGFs) *in vitro*. Drug-induced gingival overgrowth is exacerbated by plaque-induced gingival inflammation and/or any underlying periodontal diseases (4,16). Furthermore, the common periodontal pathogen *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) and proinflammatory cytokine interlukin-1α (IL-1α) were added to seek the possible regulatory mechanisms of Hsp47 expression in an inflammatory environment. In addition, mitogen-activated protein kinase kinase (MEK) inhibitor U0126 was added to investigate the possible mechanisms and their protective effects.

Material and methods

Tissue collection

The human biopsy materials used in this study were obtained under the guidelines of the Ethics Committee of the Chung Shan Medical University Hospital. Normal gingival tissue samples were obtained from five healthy individuals undergoing routine surgical crown lengthening, with little if any evidence of inflammation and no systemic medication. Redundant hyperplasic gingival biopsy materials were obtained from fifteen renal transplant patients receiving cyclosporine A therapy. These patients had been taking cyclosporine A for more than 1 year, and the dose had been adjusted to maintain stable serum levels of about 200 ng/mL. No sign of graft rejection was detected in these renal transplant patients. The samples were obtained during surgical removal of diseased gingiva as part of their routine clinical management, which also included intensive plaque control.

Immunohistochemistry

The surgically removed gingival tissues were fixed with 10% buffered formalin overnight and then the specimens were dehydrated in an ascending series of graded alcohols and embedded in paraffin. Five-micrometre-thick sections were stained with the monoclonal anti-Hsp47 antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) using a standard avidin–biotin–peroxidase complex method (5,6). Diaminobenzidine (Zymed, South San Francisco, CA, USA) was then used as the substrate to localize the antibody binding. Negative controls included serial sections from which either the primary or the secondary antibodies were excluded. The preparations were counterstained with haematoxylin, mounted with Permount (Merck, Darmstadt, Germany) and examined by light microscopy. Cells exhibiting a red-brown colour were counted as positive for the expression of Hsp47 in our samples. The sections were initially scanned at low power. Three high-power (×200) fields were randomly selected and Hsp47-labelled cells counted for each case.

One section from each cyclosporine A-induced gingival overgrowth specimen was stained with haematoxylin and eosin to evaluate the magnitude of inflammation at the histological level. Each specimen was graded at ×200 magnification as either grade low (inflammatory cells < 50% per field) or grade high (inflammatory cells > 50% per field). Grading of each specimen was based on the overall inflammatory condition in three consecutive microscopic fields starting from the epithelial–connective tissue border and proceeding gradually deeper into lamina propria. Most of the inflammatory cells present in the infiltrates represented in these specimens were lymphocytes.

Processed immunohistochemically for Hsp47 expression, sections graded as "low" were represented by positive stained cells less than 50%; sections graded "high" exhibited positive stained cells over 50% on 3 sections/tissue at 200× magnification.

Cell culture

Human gingival fibroblasts were cultured by using an explant technique as described previously (17,18). Samples from three healthy individuals undergoing the crown-lengthening procedure were selected for this study. The normal gingival tissue samples were minced using sterile techniques and washed twice in phosphate-buffered saline (PBS) supplemented with antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL of fungizone). Explants were placed into 60 mm Petri dishes and maintained in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Gibco Laboratories) and antibiotics as described above. Cell cultures between the third and eighth passages were used in this study.

Analysis of Hsp47 expression

Cells arrested in G0 by serum deprivation (0.5% fetal calf serum; 48 h) were used in the experiments (5,6). Nearly confluent monolayers of HGFs were washed with serum-free Dulbecco's modified Eagle's medium and immediately thereafter exposed to various concentrations (100, 200 and 500 ng/mL) of cyclosporine A (Sigma, St Louis, MO, USA) after 24 h incubation period. Cultures without fetal calf serum were used as negative controls. Subsequently, *A. actinomycetemcomitans*, IL-1α or U0126 were added to dishes to test their regulatory effects over a 24 h incubation period with 200 ng/mL cyclosporine A. The preparation of *A. actinomycetemcomitans* and the concentrations of 10 ng/mL IL-1α and 23 μM U0126 were selected according to our previous studies (14,19).

Western blot

Briefly, cells were solubilized with sodium dodecyl sulphate–solubilization buffer [5 mM EDTA, 1 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5), 0.5% Trition X-100, 2 mM phenylmethysulphonyl fluoride and 1 mM *N*-ethylmaleimide] for 30 min on ice. Then the cell lysates were centrifuged at 12,000*g* at 4°C and the protein concentrations determined with Bradford reagent using bovine serum albumin as standards. Equivalent amounts of total protein per sample of cell extracts were run on 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immediately transferred to nitrocellulose membranes. The membranes were blocked with PBS containing 3% bovine serum albumin for 2 h, rinsed, and then incubated with primary antibodies anti-Hsp47 (1:1000 dilution) in PBS containing 0.05% Tween 20 for 2 h. After three washes with Tween 20 for 10 min, the membranes were incubated for 1 h with biotinylated secondary antibody diluted 1:1000 in the same buffer, washed again as described above and treated with 1:1000 streptavidin–peroxidase solution for 30 min. After a series of washing steps, the reactions were developed using diaminobenzidine. The intensities of the obtained bands were determined using a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, CA, USA). Each densitometric value was expressed as the mean ± SD.

Statistical analysis

Three replicates of each experiment were performed for each test. All assays were repeated three times to ensure reproducibility. Student's unpaired *t*-test was used to evaluate the differences between the cyclosporine A-induced gingival overgrowth group and control groups in the expression of Hsp47. The correlation between Hsp47 expression and the grade of inflammation in cyclosporine A-induced gingival overgrowth specimens was analysed using Fisher's exact test. The significance of the results obtained from control and cyclosporine A-treated HGFs was statistically analysed by one-way analysis of variance (ANOVA). Tests of differences between the treatments were analysed by Duncan's test. A p-value of < 0.05 was considered to be statistically significant.

Results

Figure 1A represents gingival tissue obtained from the normal gingival group, with faint Hsp47 expression. In the cyclosporine A-induced gingival overgrowth group, intensive red-brown staining for Hsp47 expression was observed, mainly in the cytoplasm of fibroblasts, epithelial cells, endothelial cells and inflammatory cells (Fig. 1B). A significantly higher percentage of cells positively stained for Hsp47 was noted in the cyclosporine A-induced gingival overgrowth group (85%) than in the normal gingival group (25%; $p < 0.05$; Fig. 1C).

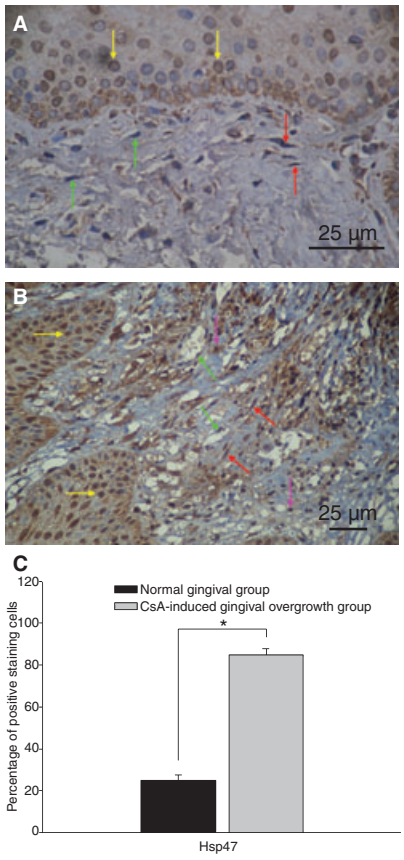

Fig. 1. Micrographs showing immunohistochemical staining for Hsp47 in gingival tissues from a normal human specimen (A) and from a cyclosporine A-induced gingival overgrowth specimen (B). Heat shock protein 47 was evident as an intense red-brown stain in the cytoplasm of fibroblasts (red arrows), epithelial cells (yellow arrows), endothelial cells (green arrows) and inflammatory cells (pink arrows). (C) The percentage of cells staining positively for Hsp47 is lower in the gingival tissues of the normal control group compared with the cyclosporine A-induced gingival overgrowth group (*$p < 0.05$).

Table 1. Localization and number of cells expressing Hsp47 in five normal gingival specimens and 15 cyclosporine A-induced gingival overgrowth specimens

	Epithelial cells	Fibroblasts	Inflammatory cells	Endothelial cells
Normal gingival specimens	1 (20%)	0 (0%)	0 (0%)	2 (40%)
Cyclosporine A-induced gingival overgrowth specimens	13 (86.7%)	11 (73.3%)	15 (100%)	12 (80%)

As shown in Table 1, the rank orders with respect to Hsp47 positively stained cells in the cyclosporine A-induced gingival overgrowth group were as follows: inflammatory cells (100%) > epithelial cells (86.7%) > endothelial cells (80%) > fibroblasts (73.3%). Heat shock protein 47 positive staining in the normal gingival group was occasionally seen in endothelial and epithelial cells (Table 1). No expression of Hsp47 was detected in the negative control gingival specimens.

Among 15 cyclosporine A-induced gingival overgrowth specimens, five cases exhibited low inflammation and 10 cases exhibited severe inflammation (Table 2). Differences in Hsp47 expression between tissues with low and high levels of inflammation were subsequently analysed using Fisher's exact test. A significantly greater Hsp47 expression was noted in cyclosporine A-induced gingival overgrowth tissues with high levels of inflammation ($p = 0.017$). A power analysis for this result is 0.794.

To examine the effect of cyclosporine A on the Hsp47 expression, HGFs were treated with cyclosporine A. As a reference, the response of Hsp47 in HGFs treated with cyclosporine A was compared with their induction in cells cultured with serum-free medium. The effects of cyclosporine A on the Hsp47 protein expression in three cell strains from different subjects were similar, and their intracellular variations were limited.

Expression of Hsp47 in HGFs challenged with cyclosporine A was directly assessed in cell lysates using western blot analysis (Fig. 2A). Cyclosporine A was found to upregulate Hsp47 protein expression in a dose-dependent manner ($p < 0.05$). From the AlphaImager 2000, the levels of the Hsp47 protein increased about 2.4-, 3.8- and 5.1-fold after exposure to 100, 200 and 500 ng/mL cyclosporine A, respectively (Fig. 2B).

As shown in Fig. 3A, inflammatory mediators were found to increase the cyclosporine A-induced expression of Hsp47 ($p < 0.05$). The MEK inhibitor U0126 was found to inhibit cyclosporine A-induced Hsp47 expression ($p < 0.05$). The quantitative measurement of cyclosporine A-induced Hsp47 expression by the AlphaImager 2000 is shown in Fig. 3B. Interleuking-1α and *A. actinomycetemcomitans* were found to elevate Hsp47 expression about 1.4- and 2.5-fold, respectively, compared with cyclosporine A alone ($p < 0.05$). However, U0126 was found to significantly decrease the cyclosporine A-induced Hsp47 protein expression about 1.1-fold ($p < 0.05$).

Discussion

The characteristic main feature of drug-induced gingival overgrowth is an increase in connective tissue matrix

Table 2. Expression of Hsp47 in cyclosporine A-induced gingival overgrowth specimens with low or high levels of inflammation

	Inflammation high	Inflammation low
Hsp47 expression high	9	1
Hsp47 expression low	1	4

A significantly greater Hsp47 expression was noted in cyclosporine A-induced gingival overgrowth tissues with high levels of inflammation compared with tissues having low levels of inflammatory cell infiltrates by Fisher's exact test ($p = 0.017$). A power analysis is 0.794.

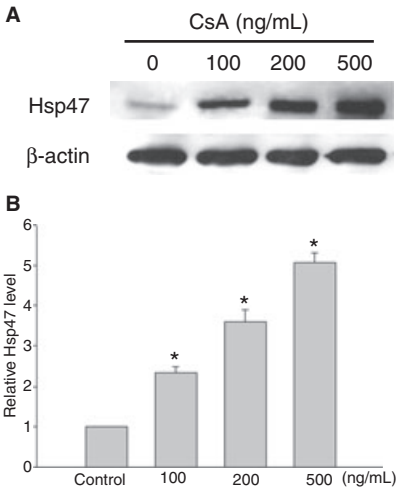

Fig. 2. (A) Expression of Hsp47 protein by HGFs in the presence of 100, 200 and 500 ng/mL cyclosporine A. Cells were exposed to cyclosporine A for a 24 h incubation period. β-Actin was assessed in order to monitor equal protein loading. (B) Levels of Hsp47 protein treated with cyclosporine A measured by the AlphaImager 2000. The relative level of Hsp47 protein expression was normalized to the β-actin signal and the control was set as 1.0. Optical density values represent the means ± SD of three different HGF strains. *$p < 0.05$ significant difference from control values.

(20). Many studies have shown that cyclosporine A can alter the extracellular turnover of collagen (21). Heat shock protein 47 is a collagen-binding protein that resides in the endoplasmic reticulum of collagen-producing cells. Available information suggests that Hsp47 is involved in the correct folding of triple-helical procollagen, and it is believed to assist in transporting procollagen from the endoplasmic reticulum into the Golgi complex. The expression of Hsp47 always increases in fibrotic diseases. Unlike most molecular chaperones, which recognize several target proteins, collagen is the only substrate protein for Hsp47 (22).

To the best of our knowledge, the present study is the first to show that Hsp47 expression is upregulated in cyclosporine A-induced gingival overgrowth specimens compared with normal gingival tissues. Heat shock protein 47-labelled cells were detected in fibroblasts, epithelial cells, inflammatory cells and endothelial cells.

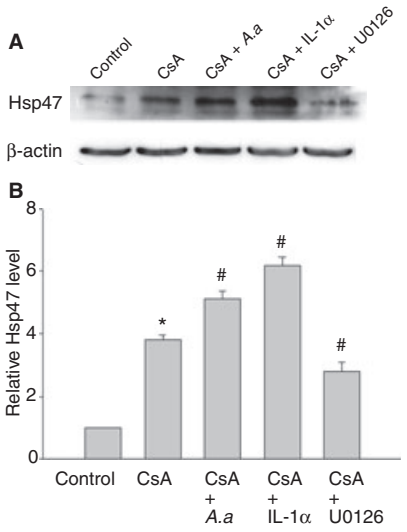

Fig. 3. (A) Effects of interleukin-1α (IL-1α), *A. actinomycetemcomitans* (*A.a*) and U0126 on cyclosporine A-induced Hsp47 protein expression in HGFs. Cells were co-cultured with IL-1α, *A. actinomycetemcomitans* or U0126 in the presence of 200 ng/mL cyclosporine A. β-Actin was assessed in order to monitor equal protein loading. (B) Levels of Hsp47 protein treated with cyclosporine A and IL-1α, *A. actinomycetemcomitans* or U0126 measured by AlphaImager 2000. Optical density values represent the means ± SD of three different HGF strains. Experiments were performed in triplicate. $*p < 0.05$ significant difference from control values; $\#p < 0.05$ significant difference between cyclosporine A alone and cyclosporine A with IL-1α, *A. actinomycetemcomitans* or U0126.

Fibroblasts have been classically described as engineers and architects of the connective tissue matrix, and thus excessive accumulation of extracellular matrix and hypercellularity in cyclosporine A-induced gingival overgrowth has been postulated to result from hyperactivity of these cells (23). Studies have expanded the function of fibroblasts to include synthesis of various mediators of inflammation (24). Thus, gingival fibroblasts were selected for the present experiments to investigate the possible mechanism of cyclosporine A-induced gingival overgrowth.

In this study, cyclosporine A was found to elevate Hsp47 protein expression in HGFs. Similar results were found by Martelli-Junior *et al.* (15), who found that Hsp47 is significantly upregulated in hereditary gingival fibromatosis. Consistently, Hsp47 has been shown to be upregulated in cyclosporine A-induced rat vascular fibrosis (25). These findings suggest that one of the pathogenic mechanisms of cyclosporine A-induced gingival overgrowth may be the induction of Hsp47 expression by resident cells in response to the cyclosporine A challenge. The accumulation of extracellular matrix components in gingival connective tissue may be stimulated by Hsp47.

The binding of Hsp47 to the nascent type I procollagen peptides prevents premature folding and aggregation of procollagen chains (26). Steady-state levels of Hsp47 and type I collagen are increased in a co-ordinated fashion in tissues undergoing pathological fibrosis (27). Schincaglia *et al.* (28) have shown that cyclosporine A can increase type I procollagen production and mRNA level in HGFs. In the present study, cyclosporine A was found to increase Hsp47 protein expression in HGFs. On the basis of the data reported above, we suggest that the cyclosporine A-induced gingival overgrowth could be due to the type I collagen deposition. The interaction between Hsp47 and type I collagen is worth further investigation.

Cyclosporine A-induced gingival overgrowth has been associated with poor oral hygiene. It has been reported that bacterial plaque and the resulting gingival inflammation are factors that promote gingival overgrowth significantly (29). Therefore, it is likely that the mechanism of cyclosporine A-induced gingival overgrowth *in vivo* does involves not only direct effects of cyclosporine A on HGFs, but also complex interactions with inflammation-promoting constituents of the gingival milieu, such as the constant challenge by bacterial plaque or the presence of inflammatory cytokines. In human periodontitis, certain species of gram-negative bacteria harboured in periodontal pockets play a major part in the pathogenesis of the disease. Among these periodontopathic bacteria, *A. actinomycetemcomitans* has been implicated as an aetiological agent in adult periodontitis. Interleukin-1α is known to be present in inflammation and can up-regulate the levels of different matrix-degrading proteinases by gingival fibroblasts. Interleukin-1α seems to be directly relevant to periodontal destruction, such as periodontal attachment loss, destruction of collagen and alveolar bone resorption (30). In the present study, the expression of Hsp47 increased with the grade of inflammation in cyclosporine A-induced gingival overgrowth specimens. Addition of the predominant periodontal pathogen *A. actinomycetemcomitans* or the pro-inflammatory cytokine IL-1α significantly increased the expression of Hsp47 compared with the addition of cyclosporine A alone. Taken together, our results suggest that cyclosporine A may predispose to fibrosis via Hsp47 overexpression in inflammatory environment.

Mitogen-activated protein kinases (MAPKs) are a unique family of serine/threonine kinases that are activated via reversible phosphorylation and mediate signal transduction for multiple extracellular stimuli. Recently, cyclosporine A was found to induce extracellular signal-regulated kinase and p38 MAPK activation in HGFs (31). Consistently, in the present study, the MEK inhibitor U0126 was found to decrease cyclosporine A-induced Hsp47 expression in HGFs. Therefore, cyclosporine A-induced gingival overgrowth may be involved in MAPK pathways. The MEK signal transduction mechanism may be a novel molecular target for the prevention of gingival overgrowth in cyclosporine A-treated patients.

No effective antifibrotic therapy has been available that can be used for patients with fibrotic diseases, including cyclosporine A-induced gingival overgrowth. Although several important fibrogenic molecules have been identified, some of these molecules are not suitable for the therapeutic targets because they may act on other body parts. However, unlike most fibrogenic molecules, which recognize several target proteins, collagen is the only substrate protein for Hsp47. This

characteristic of Hsp47 may allow it to be a potential target for the development of antifibrotic therapy. Moreover, Hagiwara *et al*. (32,33) have found that Hsp47 antisense oligonucleotide inhibition of Hsp47 improves paraquat-induced pulmonary fibrosis and bleomycin-induced pulmonary fibrosis pathology in rats. Thus, based on this experimental evidence, Hsp47 may be suitable as an antifibrotic therapeutic target to prevent or delay cyclosporine A-induced gingival overgrowth.

As far as we know, this is the first attempt to evaluate the role of Hsp47 expression in HGFs stimulated with cyclosporine A. Data from our *in vitro* experiments showed that cyclosporine A was capable of stimulating Hsp47 expression in HGFs. This suggests that one of the pathogenic mechanisms of cyclosporine A-induced gingival overgrowth *in vivo* may be the synthesis of Hsp47 by resident cells in response to cyclosporine A challenge. In addition, Hsp47 was inhibited by U0126. However, more detailed studies should be undertaken to clarify the agents that can regulate Hsp47 *in vitro* and *in vivo*.

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