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Regulation of Collagen Metabolism in Keratinocyte-Fibroblast Organotypic Co-culture by Pressure

Yang Liu[1,2,3], Baimei Liu[1,2,3], Chenchen Li[1,2,3] and Meiwen An[1,2,3,*]

[1]Institute of Applied Mechanics and Biomedical Engineering, Taiyuan University of Technology, Taiyuan 030024, China
[2]Shanxi Key Laboratory of Material Strength and Structural Impact, College of Mechanics, Taiyuan University of Technology, Taiyuan 030024, China
[3]National Demonstration Center for Experimental Mechanics Education, Taiyuan University of Technology, Taiyuan 030024, China

ABSTRACT

Hypertrophic scar (HS) is characterized by aberrant fibroblast phenotypes and excessive deposition of extracellular matrix. Pressure therapy is a common treatment to cure HS, which can regulate collagen synthesis and deposition. To investigate collagen metabolism in keratinocytes and fibroblasts co-culture under pressure, and study the role of IL-1α and MMP-3 in terms of regulating fibroblasts-keratinocytes collagen metabolism with pressure. Chitosan-gelatin scaffolds were fabricated by vacuum freeze drying. Keratinocytes and fibroblasts were seeding on scaffolds to form systems as: keratinocytes monoculture (KM), fibroblasts monoculture (FM), co-culture of keratinocytes and fibroblasts (KM&FM). Systems was treated with 3.4 kPa pressure for 1 day, systems without pressure was considered as control groups. The mRNA expression and protein secretion of supernatant were tested using Q-PCR and ELISA method, respectively. Co-culture KM&FM under 3.4 kPa pressure enhanced typeIcollagen and type III collagen mRNA expression and protein secretion from KM. It however reduced typeIcollagen and type III collagen mRNA expression and protein secretion from FM; it promoted mRNA expression and protein secretion of IL-1α from KM and enhanced expression of MMP-3 from FM. The MMP-3 mRNA expression and protein secretion from FM were respectively positively correlated with IL-1α from KM and negatively correlated with the expression of typeIcollagen and type III collagen of FM. The increased secretion of IL-1α from KM stimulated MMP-3 secretion from FM and promoted degradation of typeIcollagen and type III collagen from FM.

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Authors' Contribution
YL and MWA conceived and designed the experiments. BML and CCL contributed materials and analysis tools.

Key words
Co-culture, Fibroblast, Keratinocyte, Pressure.

INTRODUCTION

Hypertrophic scar (HS) is an abnormal connective tissue wound that results from trauma, inflammation, surgery, or burns (Ogawa, 2011; Darby *et al*., 2014; Cotellese *et al*., 2018; Babar and Naqi, 2017). Wound healing is a complex process which is determined by the balance between these internal and external forces, such as extracellular matrix (ECM) tension, stretching tension, compression and hydrostatic pressure (Cormac and Brendan, 2008; Agha *et al*., 2011; Ogawa *et al*., 2011). Moreover, regulation of collagen synthesis and deposition is a direct approach to control scar tissue formation. An understanding of the cellular and molecular events that are implicated in the development of these fibroproliferative disorders will allow for optimization of wound healing. In turn, treatment choices can be based on the most current scientific information available.

Tissue engineering skin grafting combined with pressure therapy has been widely used in wound treatment to control HS formation (Chuangsuwanich *et al*., 2014; Zhao *et al*., 2015). Studies have been reported to investigate the function of skin grafting and pressure treatment of HS in clinical applications (Chen *et al*., 2011; Verhaegen *et al*., 2011). Tissue materials composed of human skin cells with natural membranes have exhibited excellent clinical in treating HS (Chernoff *et al*., 2007). Application of these composite materials under the pressure of 15-25 mmHg (2.0-3.4kPa) has been suggested to improve their effectiveness (Lai *et al*., 2010). The mechanism of skin grafting combined with pressure therapy is unknown. Therefore, further studies of the therapy effects on scar tissues are needed.

During wound healing process, dermis fibroblasts are responsible for synthesis and deposition of extracellular matrix collagen (Karimi *et al*., 2013; Dobosz *et al*., 2014).

* Corresponding author: zi_su_tai_yuan@163.com
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Keratinocytes strongly contribute to the modulation of dermal fibroblast collagen production and degradation. Type I, III collagen are the major collagen of the extracellular matrix and the major structural protein of scar tissue. Its metabolism is modulated by numerous cytokines including tumour necrosis factor-α (TNF-α) and matrix metalloproteinases (MMPs). Tissue engineered epidermal are always structured using epidermis keratinocyte which is able to synthesize cytokines to regulate collagen synthesis and secretion in dermis (Oh and Kim, 2011; Choi *et al*., 2014).

It has been reported that keratinocytes-fibroblast dependent production of TNF-α and MMP-3 could influence collagen secretion. IL-1α is a negative regulatory factor in HS formation, which can bind to fibroblasts receptors to restrain abnormal collagen accumulation (Niessen *et al*., 2004; Zhang *et al*., 2004; Xu *et al*., 2008). Matrix metalloproteinase-3 (MMP-3) could degrade typeI, III collagen. MMP-3 could also activate and associate with matrix metalloproteinase-1 (MMP-1). The effectiveness of the skin grafting combined with pressure therapy to treat HS will lead to fibroblast growth inhibition and collagen production decrease in dermis. This also leads to keratinocytes proliferation and an increase of collagen production in epidermis (Chernoff *et al*., 2007; Oh and Kim, 2011). The effects of pressure on fibroblast growth have been studied (Macintyre and Baird, 2006), but it is not clear how pressure affects the ECM protein expression and cytokine immunoassay in models of co-culture fibroblasts with keratinocytes in three-dimensional scaffolds.

In this study, we used our previously designed chitosan-gelatin scaffolds to culture keratinocytes and fibroblasts in three dimensions. A low pressure apparatus has been designed for this study. The purpose of this study was to investigate the effect of pressure on keratinocytes and fibroblasts collagen expression. Further research as to how the effect of IL-1α and MMP-3 on collagen metabolism in co-culture system is affected by exposure to 3.4 kPa pressure. This study will shed new light on how skin grafting combined with pressure therapy can be used to better treat hypertrophic scars.

MATERIALS AND METHODS

Fabrication of chitosan-gelatin scaffolds

Chitosan (Sigma, America) solution (2wt %) was prepared by dissolving chitosan in acetic acid (1wt %). Gelatin (Sigma, America) solution (2wt %) was prepared by dissolving gelatin in sterile water at 50°C. The two solutions were mixed with equal volume and cross-linked by 0.4% glutaraldehyde for 12 h at 25°C and frozen at -20°C for 24 h. The solution was then subjected to -80°C for 12 h using a Vacuum Freeze Drier (Beijing Fourth-ring Scientific Instruments Factory, China), followed by drying at -80°C for 24 h to generate a porous structure (Liu *et al*., 2013a, b, 2014). The resulting scaffolds were soaked in 1% NaOH for 2 h and deoxidated using 5% NaBr for 1 h, followed by further washing with sterile water. The chitosan-gelatin (CG) scaffolds were then freeze dried again. The structural morphology of CG scaffolds was examined by using scanning electron microscopy (SEM, TM-3000, Shanhai Liwan Analysis Instrument Co. Ltd., China) at an accelerating voltage of 15 kV. The average pore size of scaffold was determined by measuring 30 pores using Image-Pro Plus 5.1 (Olympus Co., Japan) (n =3). The CG scaffolds were then sterilized using γ ray to 25 kgy using for seeding cells.

Culture of keratinocytes and fibroblasts

Normal human skin specimens were obtained from circumcision surgery with the informed consent of the donors. Human keratinocytes and fibroblasts were cultured from the foreskin (Lan *et al*., 2014). Briefly, the skin specimens were washed using phosphate buffered saline (PBS) solution with 5 mg/ml gentamycin. The epidermis and dermis were separated by soaking the samples in a 0.2% type II dispase (Roche, Switzerland) solution for 12 h at 4°C overnight. To isolate the fibroblasts, dermis was shredded into small fragments and incubated in a 0.25% type II collagenase (Sigma) solution for 3 h at 37°C. The epidermis was shredded into small fragments and then incubated in a 0.25% trypsin (Sigma, America) plus 0.02% EDTA (Sigma, America) solution in PBS for 2 h to harvest keratinocytes. The isolated keratinocytes were cultivated in a serum-free keratinocyte growth medium (K-SFM, Gibco), and fibroblasts were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (HyClone, UT, USA) supplemented with 10% Fetal Calf Serum (FCS, Gibco, Canada). Cell images were photographed with an inverted microscope. The 4th passages of keratinocytes and fibroblasts were used for the pressure experiments.

Pressure application

A pressure loading apparatus was fabricated to create positive pressure (Healey *et al*., 2003; Stanley *et al*., 2005). The air pressure in the device was supplied from a gas tank filled with 5% CO_2 and 95% air. The gas from the tank was filtered and sterilized and then fed into the test device through a hermetic box, which with an inlet valve and an outlet valve to adjust the pressure by using a manometer. The hermetic box was preheated to 37°C to maintain the gas at ambient temperature before it entered the test apparatus.

Organotypic co-culture of keratinocytes and fibroblasts under pressure

The CG scaffolds used for seeding keratinocytes or

fibroblasts were essentially cylinders with diameter about 5 mm and height about 2 mm. First, keratinocytes or fibroblasts were seeding, respectively on scaffolds at rate of 3×10^5 cells/scaffold for 2 days to form keratinocytes monoculture (KM) or fibroblasts monoculture (FM); the KM was cultivated with K-SFM and the FM using DMEM supplement with 10% FCS. The KM and the FM were took in one container to upper layer-under layer couture to form KM&FM for 12 h, with a medium mixed with DMEM plus 10% FCS and K-SFM in volume ratio of 1:1. Subsequently, the culturing apparatus was subjected to the 3.4 kPa gas pressure to the KM, FM and KM&FM cultures for 1 day. The KM, FM and KM&FM cultures without pressure were considered as control groups.

KM and FM can separated from KM&FM, eight groups were classified as: KM without pressure (group A), KM in KM&FM without pressure (group B); KM under 3.4 kPa pressure(group C); KM in KM&FM under 3.4 kPa pressure (group D); FM without pressure (group E), FM in KM&FM without pressure(group F); FM under 3.4 kPa pressure(group G); FM in KM&FM under 3.4 kPa pressure(group H)

Hematoxylin-eosin staining

Eight groups of specimens were collected and fixed with 4% paraformaldehyde for 24 h in preparation for HE staining to observate morphology of keratinocytes and fibroblasts on CG scaffold. Following fixing, dehydration, embedding in paraffin and deparaffinization, HE staining was performed according to instructions of manufacturer of the HE Staining Kit (Solarbio, Beijing, China).

RNA preparation and quantitative reverse transcription-PCR

Total RNA of eight groups was isolated using TRIzol Reagent (Gibco) and phase separation was invoked using chloroform. Isopropyl alcohol and ethanol were used to precipitate and washed the RNA. Then RNA was dissolved in RNase-free water. cDNA was synthesized using the reverse transcriptase kit (Fermentas, Australia). Quantitative real-time PCR for typeIcollagen, type III collagen, IL-1α and MMP-3 was performed using SYBR green reagents (Roche, Switzerland) (n=3, all analyses and calibrations were carried out three times). Primer sequence used for RT-PCR are in Table I. The expression of GAPDH was used to normalize samples. The denaturation and amplification conditions of the PCR thermal cycle protocol were: 94°C for 10 min followed by 45 cycles of 94°C for 15 sec and 60°C for 60 sec. The relative expression of target gene to GAPDH was calculated by $2^{-\Delta\Delta CT}$.

ELISA analyses

Six groups of supernatant were collected: group A, group B, group C, group E, group F and group G. Concentrations (typeIcollagen, type III collagen, IL-1α and MMP-3) released into medium was quantified using enzyme-linked immunosorbent assay (ELISA) employing ELISA kit (R&D Systems, Wiesbaden, Germany). Levels of each protein were measured using a microplate reader (Power Scan HT, Dainippon Pharmaceutical, Osaka, Japan) with 100 μm of medium supernatant. All analyses and calibrations were carried out with three times (n=3) and the results were averaged. All concentrations were documented as ng/ml.

Statistical analysis

All data were expressed as the mean standard deviation and were analyzed by analysis of variance (One-way ANOVA), $p<0.05$ was considered to be statistically significant.

Table I.- Primer sequence used for RT-PCR.

Gene	Primer sequence	Size (bp)
COL1α1	F: AGCCTGGGGCAAGACAGTGATT R: TTGCTTGTCTGTTTCCGGGTTG	207
COL3α1	F: TCGAACACGCAAGGCTGTGAG R: TGTCGGTCACTTGCACTGGTTGA	201
IL-1α	F: AGGCATCCTCCACAATAGCAGACA R: TCGGACCAATTACTGGCTCAAGGT	184
MMP-3	F: ATTCCATGGAGCCAGGCTTTC R: CATTTGGGTCAAACTCCAACTGTG	138
GAPDH	F: CGCTCCTCCTGTTCGAC R: TTAAAAGCAGCCCTGGTGAC	217

RESULTS

The morphology of CG scaffolds and cell morphology on CG scaffold

The structural morphology of CG scaffold was uniform 3D open cell foam as shown in Figure 1A. The pore size varied from 100 μm to 350 μm. The original circular or polygonal generation of keratinocytes presents as a typical stone (Fig. 1B). Fibroblasts characterized by long spindle, with a typical swirling direction (Fig. 1C).

The HE stains of keratinocytes and fibroblasts on CG scaffold are shown in Figure 1D and E, representing by group B (KM in KM&FM without pressure) and group F (FM in KM&FM without pressure). It can be seen form HE pictures that scaffold was stain to red, cell nucleus and cytoplasmic ribosome was dyed to violet. Keratinocytes growed around pores of scaffold, spread as circular or polygonal morphology to perform multilayer growth and fusion with scaffold. Also, fibroblasts present as long spindle bunch to perform multilayer growth and fusion with scaffold. It was suggested that CG scaffold was

suitable for culturing keratinocytes and fibroblasts.

Co-culture keratinocytes and fibroblasts under pressure regulated expressions of type I, III collagen mRNA and proteins in KM and FM

The results shown in Figure 2A demonstrated that type I collagen mRNA expression in KM has markedly increased with the stimulation of co-culture and pressure. Compared with group A, type I collagen expression of group B increased significantly ($P<0.05$), also in comparison with group C, type I collagen expression of group D was significantly increased ($P<0.05$), suggesting that co-culture of KM with FM can promote type I collagen mRNA expression in KM. Type I collagen mRNA expression of group C increased significantly ($P<0.05$) in contrast to that of group A, and similarily, that of group D was increased significantly ($P<0.05$) in contrast to group B, suggesting that 3.4 kPa pressure can obviously promote type I collagen mRNA expression in KM.

However, Figure 2B demonstrated that type I collagen mRNA expression in FM has markedly decreased due to co-culture and pressure stimulation. Compared with group E, type I collagen expression of group G significantly decreased ($P<0.05$), and likewise in comparison to group F, type I collagen expression of group H was significantly decreased ($P<0.05$), suggesting that the co-culture of FM with KM can obviously restrain type I collagen mRNA expression in FM. Type I collagen mRNA expression of group G decreased markedly ($P<0.05$) in contrast to group E and meanwhile mRNA expression of group H increased significantly ($P<0.05$) in contrast to group F, suggesting that 3.4 kPa pressure can decrease type I collagen mRNA expression in FM.

Moreover, the type III collagen mRNA expression of the eight groups was statistically different (Fig. 2C, D): the co-culture of KM with FM appears to promote type III collagen mRNA expression in KM, but restrain type III mRNA collagen mRNA expression in FM. 3.4 kPa pressure promote type III collagen mRNA expression in KM, obviously decrease the type III collagen mRNA expression in FM.

It was found that typeI, III collagen concentrations of the six groups were statistically varied (Fig. 2E, F). Compared with group A and group B, group E and group G, typeI, III collagen concentrations of group D and F was increased, it suggest that 3.4 kPa pressure promotes typeI, III collagen synthesis of KM, however, 3.4 kPa pressure obviously retrain typeI, III collagen synthesis of FM. From the typeI, III collagen concentration ofthe six groups, it appeared that the co-culture of KM with FM regulated the synthesis of typeI, III collagen. The typeI, III collagen concentration of group F decreased in contrast to group B ($P<0.05$), it appeared that 3.4 kPa pressure effect retrained typeI, III collagen synthesis of co-cultured KM with FM.

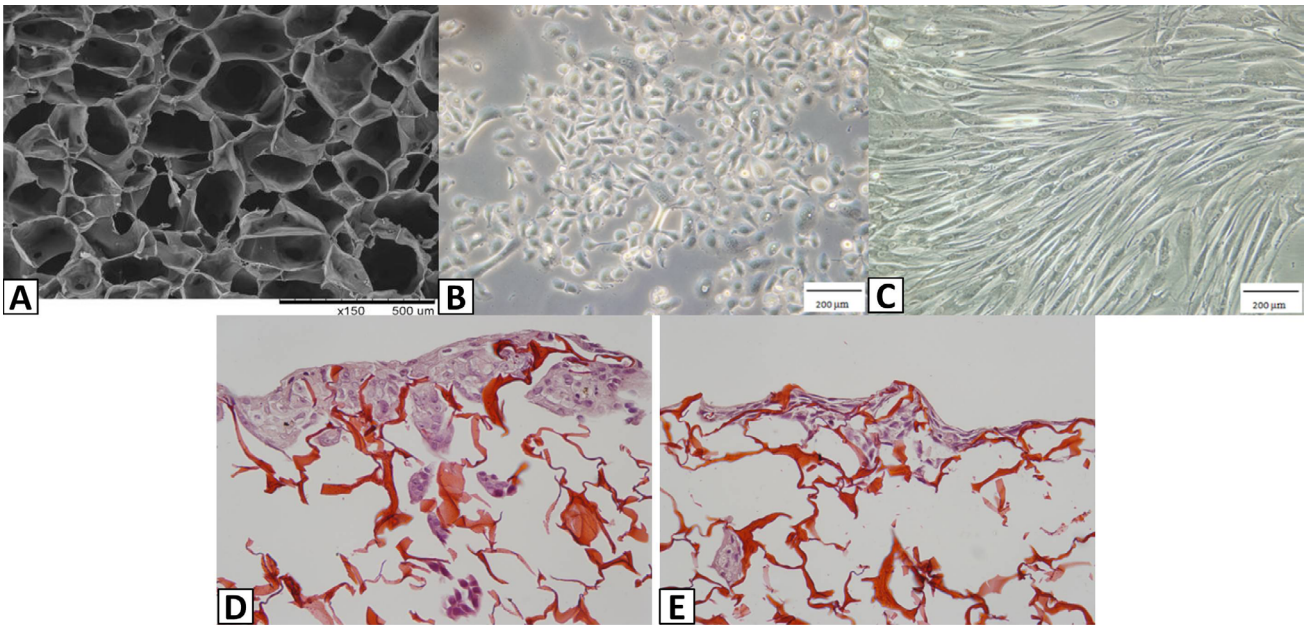

Fig. 1. A, SEM morphology of CG three-dimensional scaffolds; B, keratinocytes; C, Fusion of fibroblasts; D keratinocytes morphology on CG scaffolds, HE stains of group B (KM in KM&FM without pressure); E, fibroblasts morphology on CG scaffolds, HE stains of group F (FM in KM&FM without pressure).

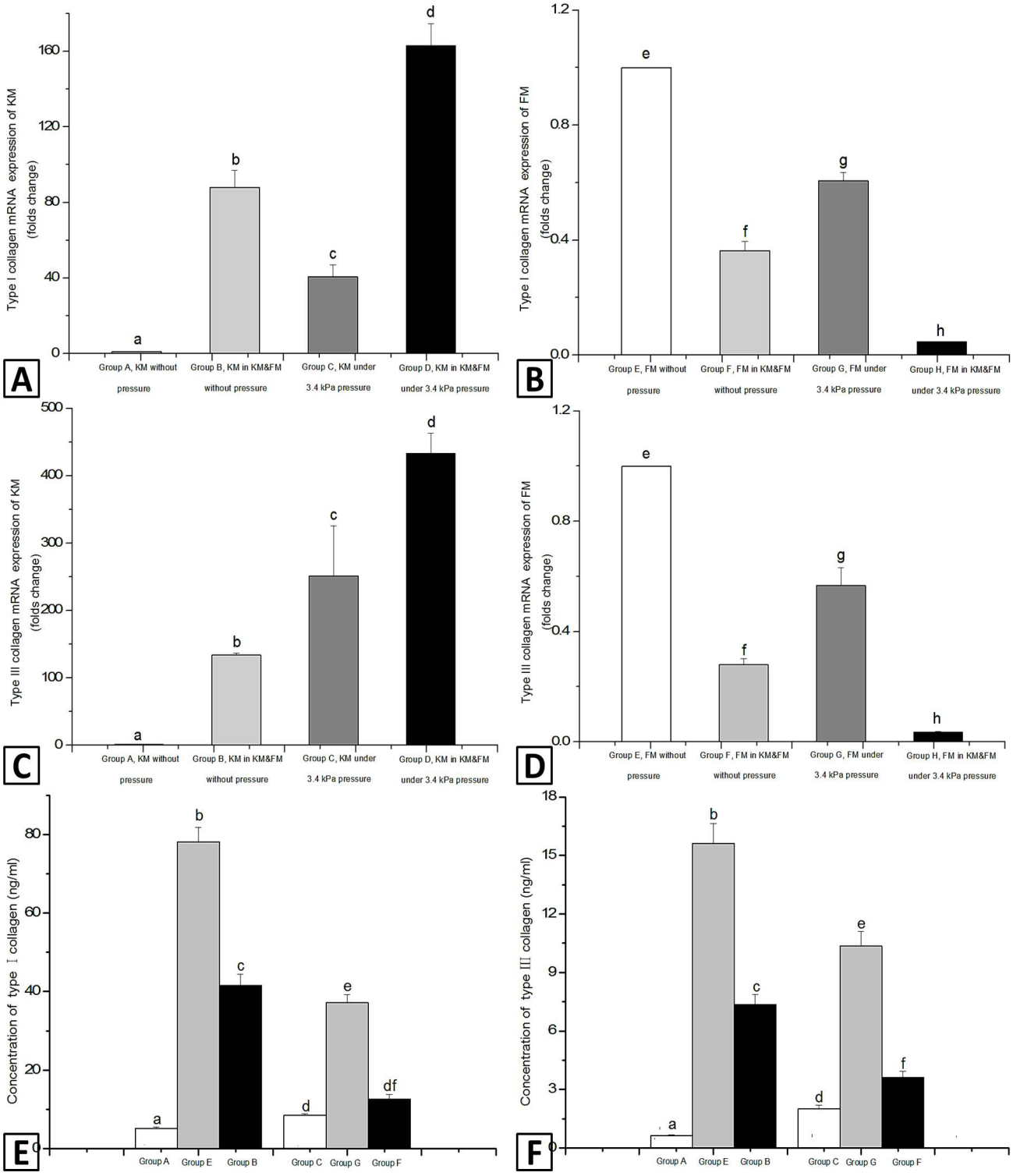

Fig. 2. A, the comparation expression of type I collagen mRNA in KM; B, type III collagen mRNA expression in KM; C, type I collagen mRNA expression in FM; D, type III collagen mRNA expression in FM; E, production of type I collagen; F, production of type III collagen: a to d shows significant difference of protein expression of KM (**P*<0.05), and e and f shows significant difference of protein expression of FM (**P*<0.05). n=3.

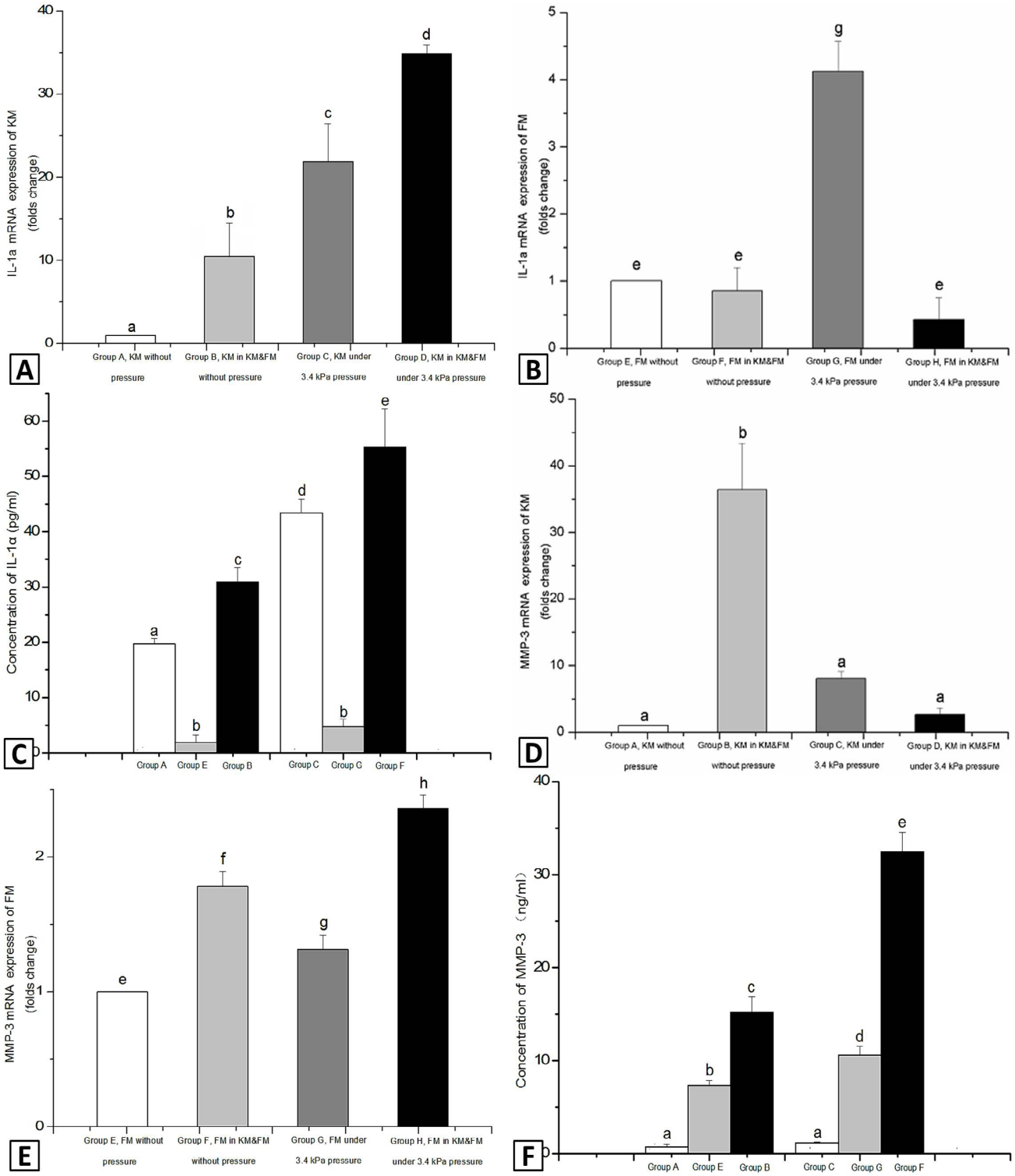

Fig. 3. A, the comparation expression of IL-1α mRNA in KM; B, the comparation expression of IL-1α mRNA in FM; C, the production of IL-1α; D, the comparation expression of MMP-3 mRNA in KM; E, the comparation expression of MMP-3 mRNA in FM; F, the production of MMP-3: a to d shows significant difference of protein concentration of KM (**P*<0.05), and e and f shows significant difference of protein concentration of FM (**P*<0.05). n=3.

Co-culture cells under pressure promoted expressions of IL-1αmRNA and proteins in KM

It can be seen that IL-1α mRNA expression in FM is not obviously, however, IL-1α mRNA expression of KM was statistically different (Fig. 3A, B). The co-culture of KM with FM appears to promote IL-1α mRNA expression of KM, and 3.4 kPa pressure promote IL-1α mRNA expression in KM. Also it can be found that IL-1α concentration of KM changed obviously (Fig. 3C). The co-culture of KM with FM and 3.4 kPa pressure promoted IL-1α secretion of KM.

Co-culture cells under pressure promoted expressions of MMP-3mRNA and proteins in FM

The results suggest that MMP-3 mRNA expression in FM was statistically different (Fig. 3D, E). The co-culture of FM with KM appears to promote MMP-3 mRNA expression in FM, and 3.4 kPa pressure promote MMP-3 mRNA expression in FM. Also it can be found that the MMP-3 concentration of FM was obvious varied (Fig. 3F). The application of co-culture and pressure promoted MMP-3 secretion of FM.

Relationship of IL-1α and MMP-3 expressions with collagen metabolism

A positive correlation was found between MMP-3 mRNA expressions in FM with IL-1α mRNA expression in KM (correlation coefficient was 0.734). There appeared to be negative correlation between the MMP-3 mRNA expression in FM with type I, III collagen mRNA expression in FM (correlation coefficient were -0.952 and -0.976, respectively). Also, there was a positive correlation between MMP-3 secretion of FM with IL-1α secretion of KM (correlation coefficient was 0.804), but a negative correlation between MMP-3 secretion of FM with type I, III collagen secretion of FM (correlation coefficient were -0.755 and -0.944, respectively).

DISCUSSION

It is a common method to combine epidermis transplantation and pressure to treat HS. In this process, epidermis thickenede step by step until the occurrence of re-epithelization with dermis decreased to normal levels. And meanwhile the regulation of extracellular matrix metabolism and cytokine expression by HKC and HFB under pressure was involved in this process. To better understand this process, more in-depth research is needed to determine the influence of pressure and HKC and HFB on extracellular matrix and cytokine expression. Understanding the mechanism of HS treatment using epidermis transplantation combined with pressure to remove scar can provide a valuable reference for clinical therapy. In this paper, a system of co-culture of HKC and HFB under 3.4 kPa pressure were formed to determine the effects on type I collagen, type III collagen, IL-1α and MMP-3 mRNA expression and the protein concentration of culture supernatant.

3.4 kPa pressure together with co-culturing of KM with FM appeared to promote type I, III collagen mRNA expression in KM and increase the concentration of the KM supernatant. However, it appeared to inhibit type I, III collagen mRNA expression in FM and decrease the concentration in the FM supernatant. These changes are good for epidermis extracellular matrix deposition and dermis extracellular matrix degradation, which is advantageous for the epidermis re-epithelization and scar dermis reconstitution.

It was also found that the regulation of HKC and HFB on extracellular collagen metabolism was obvious in the process of wound healing (Ghaffari *et al*., 2009; Ranzato *et al*., 2010; Gu *et al*., 2010), which is developed due to cell cytokines (Shephard *et al*., 2004; Werner *et al*., 2007). IL-1α can affect the extracellular collagen synthesis of HFB negatively, but stimulate MMP-3 synthesis from HFB (Daniel *et al*., 2002). MMP-3 is an important collagen protein enzyme, the IL-1α secretion of HKC and MMP-3 secretion of HFB may be associated with the extracellular matrix collagen remodeling of HFB, through the co-culture of HKC with HFB. In this experiment, IL-1α was synthesized by KM and MMP-3 was synthesized primarily by FM. 3.4 kPa pressure and co-culture of KM with FM appeared to promote IL-1α mRNA expression, protein synthesis of KM, FM and MMP-3 mRNA expression. There is a positive correlation between the MMP-3 mRNA and protein synthesis of FM in reference to IL-1α of KM, indicating that the high expression IL-1α of KM may promote the high expression of MMP-3 of FM. And meanwhile, the MMP-3 mRNA expression was negatively related to protein synthesis of FM and type I, III collagen mRNA expression, which indicates that the high MMP-3 expression of FM may lead to the weaker expression of type I, III collagen mRNA and decreased protein synthesis of FM.

By structuring a model of the 3D co-culture KM and FM under pressure, it was concluded that: KM and FM could regulate extracellular matrix type I, III collagen metabolism. Co-culture of KM and FM at an air pressure of 3.4 kPa promotes epidermis extracellular matrix deposition and dermis extracellular matrix degradation. The high expression IL-1α of KM may promote the high expression of MMP-3 of FM to regulate the extracellular matrix type I, III collagen metabolism of FM. It may be the possible mechanism to quickly promote the healing of a scar, which can help the epidermis continuously thicken and the dermis matrix degrade. So it is of clinical value to

apply transplanted tissue-engineered epidermis combined with pressure to treat scar, following complete resection the HS tissue.

It reported that MAPK signaling pathway participate in collagen production of normal human skin fibroblasts (Lan *et al*., 2014), IL-1α and pressure could stimulate MAPK signaling (Shiratsuchi *et al*., 2005; Ma *et al*., 2015). It is warranted further experiment about role of MAPK signaling on collagen metabolism in 3D co-culture KM and FM under pressure.

CONCLUSIONS

Co-culture KM&FM under 3.4 kPa pressure regulated collagen metabolism of KM and FM. The increased secretion of IL-1α from KM stimulated MMP-3 secretion from FM and promoted degradation of type I collagen and type III collagen from FM.

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Statement of conflict of interest

The authors declare no conflict of interest.

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