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The change of break modulus drives human fibroblast differentiation in 3D collagen gels

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T TABLE OF CONTENTS

1. ABSTRACT

Extracellular matrix is one of the key environmental factors influencing cell survival and provides signals for cell morphological change, migration, proliferation and differentiation. However, the mechanism through which denatured collagen modulates the biological properties of fibroblasts, is unclear. We investigated the regulation of human fibroblast differentiation in vitro grown in collagen gels with different properties. The break modulus of collagen with denatured collagen and half-load normal collagen was reduced compared with that of normal collagen gel. Fibroblasts cultured in denatured collagen gels showed increased expression of matrix metalloproteinase9(MMP-9), tissue inhibitor of metalloproteinase 2(TIMP2), α-smooth muscle actin(α-SMA), osteoblast cadherin, phosphorylated Myosin phosphatase target subunit1(p-MYPT1), connective tissue growth factor, type I collagen, type III collagen, α-smooth muscle actin messenger RNA, RhoA, rho-associated protein kinase, and transforming growth factor β receptors 1 and 2 compared with that in cells cultured in normal collagen gel. But there was no significant difference regarding expression level between denatured collagen gel and half-load normal collagen gel .These findings suggest that the change in break modulus caused by decreasing normal collagen concentration may be the key factor inducing fibroblast differentiation.

2. INTRODUCTION

Hypertrophic scarring is an excessive healing response to trauma and leads to abnormal secretion and deposition of extracellular matrix (ECM). The formation of hypertrophic scars represents a challenge both in the diagnosis and for treatment (1). Human and animal models have shown that the degree of dermal tissue damage is a key in scar formation. Dermal tissue has a template function in repairing cells, inducing them to recover the normal biological behavior of the tissue. When the integrity and continuity of dermal tissue are compromised by trauma, the template function of the tissue is also impaired, leading to formation of a scar (2). Thus, scar formation can be prevented by retaining as much dermal tissue as possible or by replanting dermal tissue of sufficient thickness. Recently, Huang *et al*. (3,4) have identified that in deep second-degree burns wound surface repair and reduced scar formation can be achieved by partially preserving denatured dermis and grafting autologous thin skin . It is not clear as how dermal tissue participates in wound repair. A major question is whether scar formation after burning involves denatured ECM, or damage of the dermal template, or both. Fibroblasts are a key cell population in wound repair. Abnormal proliferation of fibroblasts during pathological scar formation is an important factor in causing scar hypertrophy and persistence. In the early stage

Table 1. Configuration of Different 3D Gels

	Group n (μl)	Group nd (μl)	Group 0.5n (μl)
DMEM (high glucose)	111	111	280.5
MEM	50	50	50
0.11 N NaOH	39	39	19.5
Normal collagen solution	300	150	150
Denatured collagen solution		150	
Cell suspension	500	500	500
Total volume	1000	1000	1000

of wound repair, fibroblasts are activating and differentiate into myofibroblasts. When fibroblasts transform into myofibroblasts, the latter cells migrate to the wound surface and produce a large amount of collagen, which helps to contract the wound surface and to accelerate repair. Fibrosis forms when the wound undergoes excessive healing. Activation of fibroblasts is the result of an interaction among cells, ECM, and cytokines. However, the mechanism through which denatured collagen modulates the biological properties of fibroblasts, remains unclear.

As an important environmental factor involved in cell survival, ECM provides signals for migration, proliferation, and differentiation and in modulation of cell morphology. When tissue is injured, the components and recognition sites and bioactive sites of the ECM become exposed and signals are sent to certain cells. This, in turn, regulates the biological function of cells at the wound surface (5).Collagen is a fundamental component of ECM. Collagen denaturation which occurs in burned tissuses can influence biological behavior of cells, such as macrophage adhesion (6), smooth muscle cell proliferation (4), and delaying aging of human embryonic lung fibroblasts (7). In addition, denatured collagen can increase phagocytosis of cells and promotes tissue remodeling (8). However, studies focusing on the influence of denatured collagen on scar formation are still rare. Collagen gel is a model for the *in vitro* study of the interaction between cells and ECM and the biological activity of cells in a three-dimensional (3D) environment (9). When cultivated in a collagen matrix, fibroblasts obtain a phenotype similar to that in tissue, which is significantly different from that in the monolayer culture. Recently, collagen gel containing fibroblasts has been widely applied in studies of wound repair and fibrosis (10,11).

The present study investigated the mechanism of scar formation in respect to the role that ECM plays. Complex factors participate in scar formation. A diversity of cells, cytokines, and ECM are all involved in such processes. To investigate the influence of denatured collagen on fibroblast cells in a 3D cultivation system, we prepared 3D gels with different natures and compared their effects on myofibroblast differentiation. This study aimed to explore the role of dermal collagen denaturation in scar formation and its possible mechanisms in burns.

3. MATERIALS AND METHODS

3.1. Cell culture

Human fibroblasts were obtained from Sciencell (Carlsbad, CA, USA) and grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (HyClone, Logan, UT, USA). These cells were maintained at 37°C in 5% CO_2. The culture medium was changed every 2 days, and the colonies were passaged every 7 days. The cells at passages 4-12 were analyzed in this study.

3.2. Preparation of denatured 3D collagen gel and 3D gels with different natures

Type I collagen solution was denatured through incubation in water at 50°C for 30 min (12) and then analyzed using atomic force microscopy and circular dichroism spectroscopy. The preparation of 3D gels with different natures is shown in Table 1. Group n contained 300 μL normal collagen solution, group nd contained 150 μL normal collagen and 150 μL denatured collagen solution, and group 0.5n contained 150 μL normal collagen (13).

3.3. Break modulus measurement of collagen gel

Collagen gels with different natures were prepared in 24-well plates. When the break modulus was assessed, collagen gel was removed from the 24-well plates to a mesh mold, and the maximum tolerated pressure was measured using a gas pressure meter.

3.4. Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen) according to manufacturer instructions and reverse transcribed. Messenger RNA (mRNA) expression of type I collagen, type III collagen, α-smooth muscle actin (SMA), RhoA, rho-associated protein kinase (ROCK), transforming growth factor (TGF) β receptor 1, and TGFβ receptor 2 was determined with real-time PCR using SYBR Green Master Mix (ABI, Foster City, CA, USA). The primers were as follows: type I collagen, 5′-AGGGCCAAGACGAAGACATC-3′ and 5′-GTCGGTGGGTGACTCTGAGC-3′; type III collagen, TTCATTTTCTCTG-3′; TGFβ receptor 1, 5′-TATGAGAGAATGTTGGTATG-3′ and 5′-CAATATCCTTCTGTTCCC-3′; TGFβ receptor 2, 5′-TCTTCATGTGTTCCTGTAG-3′ and 5′-CAGTAGAAGATGATGATGAC-3′; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-CAAGAAGGTGGTGAAGCAGG-3TTCATTTTCTCTG-3′; TGFβ receptor 1, 5′-TATGAGAGAATGTTGGTATG-3′ and 5′-CAATATCCTTCTGTTCCC-3′; TGFβ receptor 2, 5′-TCTTCATGTGTTCCTGTAG-3′ and 5′-CAGTAGAAGATGATGATGAC-3′; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-CAAGAAGGTGGTGAAGCAGG-3′ and 5′-AGTGGTCGTTGAGGGCAATG-3′. Data were collected and quantitatively analyzed on an ABI Prism 7500

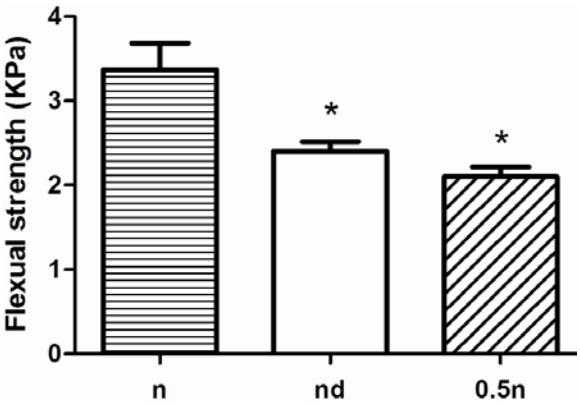

Figure 1. The break modulus of collagen gels in the three groups. The break modulus in Group nd was lower than that in Group n, while there was no significant difference between Groups nd and 0.5n. *Significant difference from native group; P<0.05. Group n: native collagen group (native type I collagen 1.5 mg/ml); Group nd: denatured collagen group (native type I collagen 0.75 mg/ml, denatured collagen 0.75 mg/ml); .Group 0.5n:half concentration group (native type I collagen 0.75mg/ml).

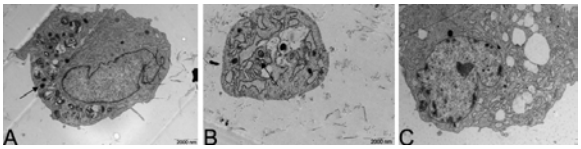

Figure 2. Ultrastructure of human fibroblasts. A: Fibroblast in native collagen gel (arrow indicates the lysosome); B: fibroblast in denatured collagen gel (arrow indicates endoplasmic reticulum); C: fibroblast in half-concentration native collagen gel.

(Applied Biosystems, Inc. USA) sequence detection system. Human GAPDH was used as an endogenous control for sample normalization.

3.5. Western blotting

Cells were harvested, pelleted through centrifugation, and resuspended in lysis buffer. Equal amounts of protein (20 μg) were loaded onto a 5% acrylamide stacking gel and separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis using a 10% separating gel. Following transfer of separated proteins, nitrocellulose membranes were blocked and probed overnight at 4°C with mouse anti-metalloproteinase (MMP)-2 monoclonal antibody (mAb), mouse anti-MMP-9 mAb, mouse anti-tissue inhibitor of MMP-2 (TIMP2) mAb, mouse anti-α-SMA mAb, mouse anti-osteoblast (OB)-cadherin mAb, mouse anti-p-MYPT1 mAb (1:1000; Abcam Inc., Cambridge, MA, USA), and mouse anti-connective tissue growth factor (CTGF) mAb (1:1000; Santa Cruz Biotech CA, USA). The membrane was then probed for 1 h at room temperature with goat anti-mouse peroxidase-conjugated immunoglobulin G (Kirkegaard & Perry Laboratories,USA), and the immunoreactivity was detected with chemiluminescence. For quantification of MMP-2, MMP-9, TIMP2, α-SMA, OB-cadherin, p-MYPT, and CTGF proteins, each band density was normalized to GAPDH protein.

3.6. Immunofluorescence staining

After culture in different 3D collagen gels for 3 days, the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 20 min. The cells were washed twice with PBS/0.1% Tween-20 to remove residual fixative and incubated in 0.05% Triton X-100 in PBS for 5 min prior to blocking in 4% normal goat serum in PBS for 30 min followed by incubation with antibody overnight at 4°C. Primary antibodies included α-SMA (Abcam Inc., 1:200) and OB-cadherin (Abcam Inc., 1:200). On the following day, the cells were washed three times with PBS and incubated with appropriate secondary antibody (Abcam Inc., 1:1000) in PBS for 2 h. After three washes with PBS for 5 min, the cells were mounted with anti-fade mounting 4, 6-diamidino-2-phenylindole/PBS and viewed on a microscope.

3.7. Statistical analysis

All statistical analyses were performed using SPSS version 16.0 (Chicago, IL, USA). Group data are expressed as Mean (SD). Significant differences between multiple groups were compared using one-way analysis of variance followed by the Student–Newman–Keuls test or the Games–Howell test. P values of <0.05 were considered statistically significant.

4. RESULTS

4.1. Mechanical properties of collagen gels

The break modulus of collagen gels in group nd (2.3 ± 0.2 kPa) was lower than that in group n (3.3 ± 0.5 kPa) ($P < 0.05$), while no significant difference was found between groups nd and 0.5n (Figure 1). These results showed that the denaturation of collagen gels could lead to changes in their mechanical properties.

4.2. Morphological analysis

Microscopic analysis revealed that the human fibroblasts in all three groups appeared spindle shaped when cultured in collagen gels. After 3 days of culture, the number of fibroblasts in groups nd and 0.5n was greater than that in group n.

The ultrastructure of human fibroblasts was analyzed with transmission electron microscope (Figure 2). Group n human fibroblasts secreted less collagen compared with those in groups 0.5n and nd, and the endoplasmic reticulum was less widespread. Group n had more lysosomes than groups 0.5n and nd did, suggesting senescence of cells in group n. In group nd, collagen secretion was greatest, the endoplasmic reticulum was most widespread, and the number of lysosomes was lowest among the three groups, suggesting strong synthesis and cell metabolism.

4.3. mRNA expression of α-SMA and types I and III collagen in human fibroblasts

We used quantitative real-time PCR to measure mRNA expression of α-SMA and types I and III collagen in human fibroblasts when cultured for 3 days

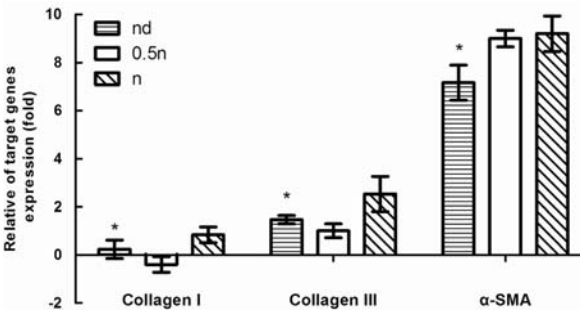

Figure 3. Delta CT value levels for types I and III collagen and α-SMA in the three groups. Expression of α-SMA mRNA in Groups nd and 0.5n was increased, compared with Group n. In Group nd, mRNA expression for types I and III collagen was increased when compared with Group n. *P<0.05 compared with Group n; **P<0.05 compared with Group 0.5n.

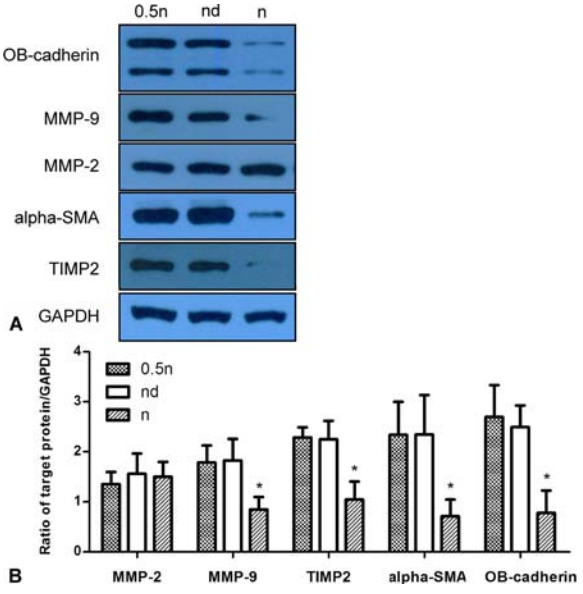

Figure 4. Protein expression of MMP-2, MMP-9, TIMP2, α-SMA and OB-cadherin in the three groups. The protein expression of MMP-2, MMP-9, TIMP2, α-SMA and OB-cadherin in human fibroblasts was analyzed by western boltting when cultured in 3D collagen gels with different nature for 3 days. Protein expression of MMP9, TIMP2, α-SMA and OB-cadherin was increased in Group nd compared with that in Group n.

in 3D collagen gels with different natures. Expression of α-SMA mRNA in groups nd was significantly increased. And the mRNA expression of types I and III collagen was increased compared with that in group n, but no significant difference is found when comparing versus group 0.5n (Figure 3).

4.4. Protein expression of MMP-2, MMP-9, TIMP2, α-SMA, and OB-cadherin in human fibroblasts

We used western blotting to measure protein expression of MMP-2, MMP-9, TIMP2, α-SMA, and OB-Cadherin in human fibroblasts cultured for 3 days in 3D collagen gels with different natures. Protein expression of MMP9, TIMP2, α-SMA, and OB-cadherin was increased in group nd compared with that in group n (all $P < 0.05$). However, no difference expression was observed between group nd and group 0.5n. (Figure 4)

4.5. Immunofluorescence staining of α-SMA and OB-cadherin in human fibroblasts

Immunofluorescence staining of α-SMA showed that in group n, the dendrites of human fibroblasts were small and the shape of most cells was narrow and long, whereas in groups nd and 0.5 n, cells were stretched with coarse dendrites (Figure 5). OB-cadherin-positive cells were observed in groups nd and 0.5 n, whereas OB-cadherin-positive cells were rare in group n (Figure 6). These results showed that the collagen gels in groups nd and 0.5 n induced differentiation of human fibroblasts.

4.6. mRNA and protein expression of the TGF/RhoA/ROCK pathway

We used quantitative real-time PCR to measure mRNA expression of RhoA, ROCK, and TGFβ receptors 1 and 2 in human fibroblasts cultured for 3 days in 3D collagen gels with different natures. mRNA expression of RhoA, ROCK, and TGFβ receptors 1 and 2 in group nd was higher than that in group n (all $P < 0.05$; Figure 7), whereas little difference was found between groups nd and 0.5n. Protein expression of p-MYPT and CTGF was analyzed with western blotting. Expression of both proteins was increased in group nd compared with that in group n (all $P < 0.05$; Figure 8), but little differences were found between groups nd and 0.5n

5. DISCUSSION

The present study characterized the differentiation, activity, and function of human fibroblasts cultured in denatured collagen gel. We show that the break modulus of denatured collagen gel and half-load normal collagen gel was decreased compared with that of normal collagen gel. In group nd, the expression of MMP9, TIMP2, α-SMA, OB-cadherin, p-MYPT1, CTGF, types I and III collagen, α-SMA mRNA, RhoA, ROCK, and TGFβ receptors 1 and 2 was all increased compared with that in the control group n, but no significant difference was found betweem group n and 0.5n.

The cause of scar formation is complex, and the ECM, plays an important role in the wound repair. There have been three models for in vitro FB three-dimensional cultivation: 1) Floating collagen matrices, 2) Anchored collagen matrices and 3) stress relaxation.matrices. The phenotype of fibroblasts changed in different gel models. Floating collagen could not induce the differentiation of fibroblasts into myofibroblasts, whereas fibroblasts in anchored collagen matrices developed tonofilaments similar to those in myofibroblasts and finally acquired the matrix structure of granulation tissue (14). These findings indicate that the mechanical characteristics of tissues can regulate the synthesis, remodeling, and production of ECM. The biological characteristics of human fibroblasts in anchored collagen matrices were confirmed in the present study.

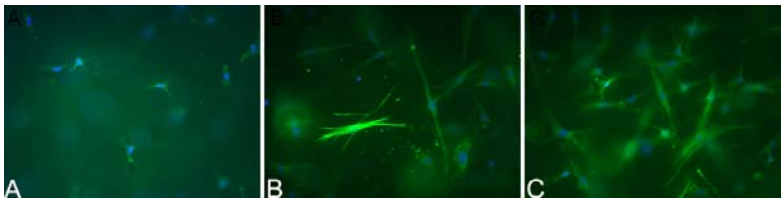

Figure 5. Immunofluorescence staining of α-SMA. A: α-SMA immunofluorescence staining of fibroblasts on native collagen gel; ×200. B: α-SMA immunofluorescence staining of fibroblasts on Group 0.5n collagen gel; ×200. C: α-SMA immunofluorescence staining of fibroblasts in Group nd collagen gel; ×200.

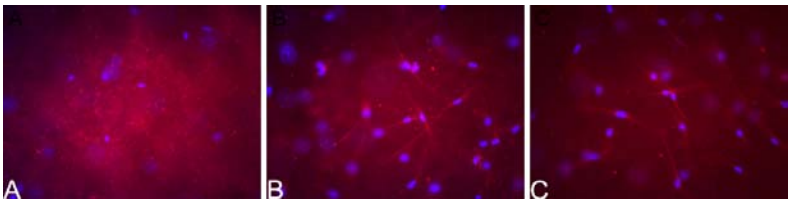

Figure 6. Immunofluorescence staining of OB-cadherin. A: OB-cadherin immunofluorescence staining of fibroblasts on native collagen gel; ×200 (OB-cadherin positive cells were rare). B: OB-cadherin immunofluorescence staining of fibroblasts on Group 0.5n collagen gel; ×200. C: OB-cadherin immunofluorescence staining of fibroblasts on Group nd collagen gel; ×200.

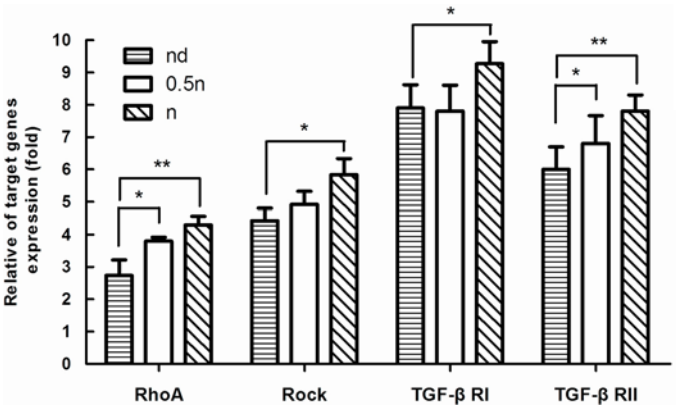

Figure 7. Delta CT value levels for RhoA, ROCK, and TGFβ receptors 1 and 2 in three groups. mRNA expression for RhoA, ROCK, and TGFβ receptors 1 and 2. mRNA expression was higher in Group nd than that in Group n, while no differences were found between Groups nd and 0.5n. *P<0.05 compared with Group n; **P<0.05 compared with Group 0.5n.

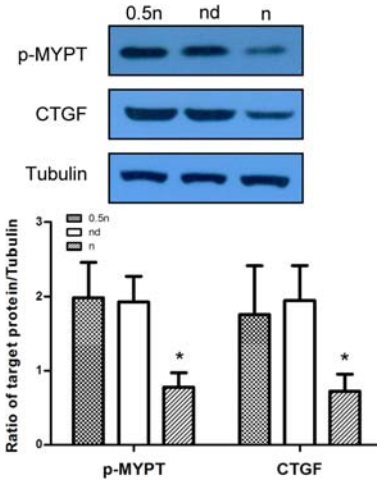

Figure 8. Protein expression of p-MYPT and CTGF. Expression of both proteins was increased in Group nd when compared with that in Group n, but no differences were found between Groups nd and 0.5n. *P<0.05.

α-SMA is a marker of fibroblast differentiation and induces strong contraction forces in cells. Physiologically, α-SMA is expressed only in vascular smooth muscle and muscular tissue, but its pathologic presence has been described in many tissues (15). Cadherins are transmembrane adhesion proteins that connect and stabilize the contact between cells through interaction with the cytoskeleton (16). Both N-cadherin and OB-cadherin play key roles in the function of fibroblastss.N-cadherin is expressed in normal fibroblasts, and OB-cadherin is produced in myofibroblasts (17,18). Thus, α-SMA and OB-cadherin are considered markers of myofibroblast differentiation. Moreover, the synthesis and secretion of myofibroblasts are so strong that a large amount of collagen (mainly type III), cytokines, and enzymes are produced and secreted. The data presented here showed that in denatured collagen gel (group nd), levels of types I and III collagen, MMP9 and TIMP2 protein and mRNA increased. These results suggest that fibroblasts exhibit more active remodeling in denatured collagen gel. Moreover, the ultrastructure of cell in group nd differed from that in group n, and the expression of α-SMA and OB-cadherin was higher on denatured collagen gel compared with that on normal collagen gel. These findings suggest that fibroblasts can be induced to differentiate to myofibroblasts when cultured in denatured collagen gel.

Fibroblast morphology and markers of myofibroblast differentiation in group 0.5n did not differ significantly from those in group nd. In the present study, the break modulus of collagen gel in group nd was significantly decreased compared with that of group n but was similar to that of group 0.5 n. This result suggested that the denaturation of collagen gel could lead to decreased concentration of normal collagen and a change in its break modulus, then enhanced collagen remodeling and myofibroblast differentiation.

In Group nd, levels of RhoA, ROCK, TGFβ receptors 1 and 2, p-MYPT and CTGF were higher than those in group n but did not differ from those in group 0.5 n. Rho protein is a small G protein that participates as a signal converter or molecular switch in the assembly of tensile fibers and formation of adhesion plaques in cells. ROCK interacts with Rho proteins in a GTP-dependent manner to regulate cells biologically via modulation of the cytoskeleton (19–21). Thus, the Rho/ROCK pathway participates in signal transduction of actin. Studies have shown that the Rho/ROCK signaling pathway participates in fibrosis of various organs such as lung and liver (22–24). CTGF acts as an effector molecule, downstream of the Rho/ROCK pathway, stimulates the synthesis of ECM, and is involved in pathological fibrosis. CTGF is a low-affinity insulin-like growth factor binding protein, which moderates angiogenesis and promotes cell mitosis, chemotaxis, adhesion, and proliferation. In addition, scar formation after burns is associated with endogenous growth factors, among which TGFβ1 is crucial. The present study suggests that culture in 3D denatured collagen gel promotes changes in gel mechanics, activates the Rho/ROCK signaling pathway, and increases TGF β receptor expression, resulting in myofibroblast differentiation.

6. CONCLUSION

ECM is a template for proper cell function, and it is conducive to the recovery of cell biological behavior . Multiple studies have shown that collagen denaturing may influence the biological behavior of cells on the plate culture..But in 3D cultivation system, the present study showed that denatured collagen is not the major factor in inducing fibroblast differentiation, but rather the change in break modulus which is likely caused by decreasing normal collagen concentration. Preserving normal break modulus of dermal tissues may help in reducing scarring.

7. ACKNOWLEDGEMENTS

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Abbreviations: TGF-β: transforming growth factor, α-SMA: α-smooth muscle actin, ECM: extracellular matrix, 3D: three-dimensional

Key Words: Three-dimensional collagen gel, Denatured Collagen, Fibroblast Cells

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