Differential Expression of Extracellular Matrix–Associated Genes Between Dermal and Gingival Fibroblasts in Response to Arachidonic Acid

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The pattern of wound healing process differs markedly according to the cell types. Gingival wounds heal more rapidly without scar, however dermal wounds show collagen laid down in thick disorganized patterns and keloid formation. This has been suggested to be due to the presence of different ECM components and cytokines as well as growth factors. The purpose of this study was to examine the differential expression of genes in connection with keloid formation in gingival fibroblasts (hGFs) and dermal fibroblasts (hDFs) in response to inflammation. In this study, we investigated the differences between hGFs and hDFs in the expression and production of cyclooxygenase (COX–2), prostaglandins E2 (PGE2), transforming growth factor (TGF)–β, collagens, matrix metalloproteinases (MMPs), and tissue inhibitors of matrix metalloproteinases (TIMPs) which play important roles in collagen deposition in wound healing.

The hGFs and hDFs were primary cultured and allocated to arachidonic acid (AA) treatment group and control group. Protein and mRNA were extracted right after (0 hr) and 24 hr after AA treatment. At a defined concentration of AA in hGFs and hDFs, MTT assay was performed. The mRNA and protein expression levels of COX–2, TGF–β, collagen 1 and 3, MMP 1 and TIMP 1 were examined by Real-time PCR and Western blots. The amounts of PGE2 were measured by enzyme-linked immunosorbent assay (ELISA). The expression of COX–2 and TGF–β exhibited reduced levels in hGFs, but were increased in hDFs at 24 hr after AA treatment. Production of PGE2 was increased in hGFs and hDFs at right after AA treatment but, not changed at 24 hr after AA treatment. The protein and mRNA expression of collagen 1 and 3 were decreased in hGFs, whereas increased in hDFs at 24 hr AA treatment. Expression of MMP–1 protein was increased in hGFs at 24 hr but, was decreased in hDFs at 24 hr compared with that of control. The protein expression of TIMP–1 was decreased in hGFs but, was increased in hDFs at 24 hr compared with that of control. These observations demonstrate differential expression between gingival and dermal fibroblasts in regulation of collagenolytic capacity by extracellular matrix–associated genes in keloid formation associated with wound repair.

Key words: Arachidonic acid, Dermal and gingival fibroblasts, ECM–associated genes

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* This study was financially supported by Chonnam National University, 2009

I. INTRODUCTION

Wound healing represents an important physiological response to reestablish tissue integrity after
injury and involves a complex order of cellular and biochemical events\(^1\). Keloids are raised pathological scars that, over extended time periods, overgrow the margins of the original wound and cause damage to the healthy dermal tissue\(^2,3\). These disorders represent aberrations in the fundamental processes of wound healing, which include cell migration, cell proliferation, inflammation, increased synthesis of cytokines and extracellular matrix proteins, and defective remodeling\(^4\).

In wound healing in the skin, resident and migratory dermal fibroblasts not only synthesize granulation tissue but play a crucial part in remodelling the provisional extracellular matrix (ECM), the ultimate organization of the dermis, and appearance of the healed scar\(^5,6\). In contrast, oral mucosal wounds are characterized by rapid re-epithelialization and remodelling without scarring\(^7\). In vitro, oral mucosal fibroblasts exhibit a fetal phenotype with increased extracellular matrix reorganizational ability, migration and experimental wound repopulation when compared with dermal fibroblasts\(^7\). The reasons for these differences have been suggested to be due to differences in growth factor, cytokine and ECM synthesis in tissues\(^8,9\).

The extracellular matrix (ECM) is composed of collagens, proteoglycans, non-collagenous glycoproteins, glycosaminoglycans (GAGs) and elastic elements\(^10\). The major component of ECM is collagen\(^10\). During the wound healing process, it acts to modulate cell proliferation and migration and is important in the wound contraction process\(^11\). Of the many different types of collagen identified, keloid formation and remodeling process is related to type I and III collagen\(^11\). Keloid was increased collagen synthesis and decreased collagen breakdown in the keloid fibroblasts\(^12,13\). The matrix metalloproteinases (MMPs), which are involved in collagen's degradation, maintain collagen content including keloid formation in ECM remodeling\(^3\). MMPs are zinc dependent proteases that play a critical role in the turnover of ECM components including collagens, elastin, laminin, proteoglycans, fibronectin and other glycoproteins\(^14\). With inhibiting MMPs activities, TIMPs participate in remodeling of the ECM in tissue. The balance between MMPs and TIMPs activities is involved in both normal and pathological events such as tissue remodeling of wound healing\(^15\).

Keloid also show altered responses to various growth factors, although there is disagreement as to the extent of altered response to TGF-\(\beta\)^\(^4\). The healing wound contains a wide array of growth factors exerting different functions during the overlapping temporal processes of healing. These growth factors profoundly influence multiple processes including cell migration, cell proliferation, matrix production and degradation\(^4,16\).

Cyclooxygenase (COX-2) is a major marker of tissue inflammation that converts arachidonic acid into prostaglandins, such as PGE\(_2\)^\(^17\). There are two isoforms of COX, which are designated COX-1 and COX-2\(^18,19\). COX-1 is expressed constitutively in various cells and tissues, and is important in maintaining homeostasis. In contrast, COX-2 is induced in inflammatory cells and tissues by various stimuli including cytokines, suggesting that COX-2 has a key role in the process of inflammation. Increased PGE\(_2\) production in response to stimulation by proinflammatory cytokines coincides with the upregulation of COX-2 expression\(^20\). PGE\(_2\) is one of the major lipid mediators of pain and acute inflammation in tendons and other tissues\(^17\).

The pattern of wound healing process differs markedly according to the cell types. Gingival wounds heal more rapidly and without scar, however dermal wounds show collagen laid down in thick dis-
organized patterns and keloid formation. This has been suggested to be due to the presence of different growth factors, e.g., EGF, TGF-α, TGF-β and ECM components such as collagens, MMPs and TIMPs. However, the reasons for the keloids formation and wound contraction process of human dermal fibroblasts as compared with gingival fibroblasts have not been well investigated.

In the present study, arachidonic acid (AA), the precursor of COX-2, was used as an inflammatory stimulus, and the purpose of this study is to know the differences in expression of COX-2, PGE2, TGF-β, collagen 1 and 3, MMP-1, and TIMP-1 between dermal and gingival fibroblasts in response to the stimulation with arachidonic acid.

II. MATERIAL and METHOD

1. Cell Culture

Human gingival fibroblasts (hGFs) were obtained from patients for gingivectomy visiting Chonnam National University hospital and human skin fibroblasts (hSFs) were grown from foreskin dermis explants. The tissues were finely cut with scissors and were primary cultured. The hGFs were grown in alpha minimum essential medium (α-MEM) (GibcoBRL, UK) and hDFs were grown in Dulbecco's modified Eagle's medium ((DMEM; JBI, Korea) supplemented with % antibiotic-antimycotic solution (AAS; JBI, Korea) and 10% fetal bovine serum (JBI, Korea) at 37°C in 5% CO₂ humidified chamber. The cells were allowed to subculture for 3 days, and the medium was replaced with a fresh medium. Then cells were trypsinized using trypsin-EDTA solution (GibcoBRL, UK) and plated again. Three to eight passaged cells were used in the present study. Cells were seeded at 1 × 10⁵ cells/cm² in 6-well plates and grown for 24 hr in FBS-free media with 0.05% bovine serum albumin (BSA; sigma, USA).

2. Cell Treatment Condition

For individual experiments, the cells were seeded at 1 × 10⁵ cells/well in 6-well plates for real time PCR, Western blots and enzyme immuno assay. The cells were cultured for 1 day and quiescent cells were obtained by cultivation of 24 hr with FBS-free medium. Then, cells were treated with AA (arachidonic acid). There were three groups in this experiment, the first group is non-AA treatment, the second group is incubated for another 0 hr after AA treatment and the third group is incubated for another 24 hr after AA treatment.

3. MTT Assay

At a defined concentration of arachidonic acid, 3–(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; sigma, USA) assay was performed. To obtained the adequate treatment condition, various concentration of 200, 300, 400 μM and treatment time of 1 hr 30 min in hGFs and 50, 100, 150, 200 μM and treatment time of 1hr in hDFs were used. After this, the adequate condition was chosen for further experiments: AA was treated 300 μM for 1hr 30 min in hGFs and 200 μM for 1hr in hDFs. Cells were seeded in 96 well microtiter plates and cultured for 24 hr after arachidonic acid treatment for then rinsed twice with PBS, MTT (Molecular Probes) was diluted in PBS to a final concentration of 0.2 mg/ml, and sterile filtered through a 0.22 μm sterile filter. MTT solution 30 μl was added to each well and the cells were further incubated at 37°C for 3 hr. The formazan product was solubilized by addition of 50 μl
of dimethyl sulfoxide (DMSO; Calbiochem, USA). Optical density was measured at 570 nm and DMSO was used as a blank using an ELISER reader (ELx800 uv, BIO Tek instruments Inc., USA).

4. RNA Isolation and Real – Time PCR

The mRNA expression of COX–2, TGF–β, collagen 1 and 3, MMP–1 and TIMP–1 was determined by real time PCR. Total RNA was extracted using Trizol® (GibcoBRL, UK) reagent as usual. 1 μg of total RNA was reverse transcribed using RT Premix (Bioneer, Korea) according to the manufacturer’s protocol. The cDNA products were used for each PCR amplification. Primer sequences used in present study are listed in table 1. Exicycler™ Real Time Thermal Block (Bioneer, Korea) and its operation software (Bioneer, Korea) was used. Master mix (Bioneer, Korea) was used with the following reaction components: 5 μl of Greenstar™ PCR master mix, 2 μl of 10 mM MgCl₂, 1 μl of each primer, cDNA 2 μl, and RNAse-free water to make a final total volume of 20 μl. Then, we performed real-time PCR with 50 cycles as follows: 94℃ for 40 seconds, 56℃ for 40 seconds, and 72℃ for 1 minute 30 seconds. Annealing temperature and the number of cycles were optimized according to each primer pair. And 72℃ for 10 minutes, 1℃ hold time for 10 seconds between 50℃ to 94℃, finally store at 8℃. The samples were run on a Exicycler™ and the data was analyzed by the Exicycler™ software. All results are displayed as fold induction of target gene mRNA expression as determined by the ΔC₅₀ method.

5. Western Blots Analysis

The expression of TGF–β, collagen 1 and 3, MMP–1 and TIMP–1 was determined by Western blots. Cells were lysed in Tris–buffered saline (TBS) containing 0.1% sodium dodecyl sulfate (SDS), and the protein content of the lysates was determined by bovine serum albumin (Pierce, USA) as the standard. Cell lysates were adjusted to 10 μg of protein and were then applied to SDS–polyacrylamide gels (6–10%) for electrophoresis, as reported previously. Next, the proteins were electroblotted onto nitrocellulose membranes (Protran, pore size 0.45 μm, Schleicher & Schuell bioscience, Germany). After the membranes had been blocked in 10 mM TBS containing 0.1% Tween–20 (TBS–T) and 5% skimmed milk, the primary antibodies (Santa Cruz, USA) were added at a dilution of 1:500 (COX–2, MMP–1, TGF–β and TIMP–1) or 1:200 (collagen 1 and 3) in TBS–T, and incubation was performed for 1.5 hr. After the membranes had been washed with TBS–T, the secondary antibodies (HRP conjugated goat anti–rabbit antibody, HRP–conjugated goat anti–mouse antibody or HRP conjugated rabbit anti–goat antibody) (Santa Cruz, USA) were added (at a dilution of 1:10,000 or 1:5,000, respectively, in TBS–T) and incubation was performed for 1 hr. After further washing with TBST, protein bands were detected with an enhanced chemiluminescence Western blot analysis system (Amersham, USA).

6. ELISA for Measuring PGE2 Levels in Media

An enzyme immuno assay kit (R&D Systems Inc., Minneapolis MN USA) was used to measure the PGE₂ production levels in the medium. Briefly, 100 μl of either the medium from the sample group or the standard solution was loaded into each well of an antibody coated 96–well plate. Then, 50 μl of PGE₂ conjugate (PGE₂ conjugated to alkaline phosphatase) and 50 μl of PGE₂ antibody (mouse monoclonal antibody to PGE₂) solution were added into the wells and incubated at 4℃ for 18 to 24 hr. After incubation,
the solution was removed and the wells were washed four times with wash buffer. Next, 200 μl of pNPP (p-Nitrophenyl Phosphate) substrate solution was added to each well and incubated at 37°C for 1 hr. Following the addition of 50 μl of stop solution (Trisodium Phosphate solution), the absorbance was measured at 405 nm by a microplate reader (Spectra MAX 190, Molecular Devices, Sunnyvale, CA) and was then converted to PGE$_2$ concentrations using a standard curve.

III. Results

1. MTT Assay for Choose the Adequate Concentration of AA

To determine the optimum concentration of AA, we examined the cell viability of various concentration of arachidonic acid in hGFs and hDFs by MTT assay. The hGFs were exhibited the 50% cell viability at 300 μM, but the hDFs were at 200 μM. Microscopic finding were revealed reduced viable cells in hGFs and hDFs compared with control after AA treatment.

2. The Expression of COX−2 and PGE2 Activity After AA Treatment

The mRNA expression of COX−2 was significantly 2 fold increased in the second group, but the third group was decreased in hGFs compared with control. In contrast, mRNA expression of COX−2 was not increased in the second group of hDFs but, significantly increased in the third group.

Production of prostaglandin E$_2$ was markedly 10 fold increased both in the second and the third group of hGFs compared with control. Also, similar to hGFs, hDFs were increased by 10 fold in the second and the third group compared with control.

3. The mRNA and Protein Expression of TGF−β After AA Treatment

The mRNA expression of TGF−β in hGFs was not significantly affected in the second and the third group compared with that of control. However, in hDFs, it’s expression was increased in the third group compared with that of control. The protein expression of TGF−β was significantly decreased in the second and third group compared with control in hGFs. The protein expression of TGF−β in hDFs was decreased in the second group, but was significantly increased in the third group compared with that of control.

4. The mRNA and Protein Expression of Collagen 1 and 3 After AA Treatment

The expression of collagen 1 and 3 mRNA was decreased in the third group of hGFs but was increased in the third group of hDFs compared with the control. Especially, expression of collagen 3 mRNA show greatly increased. The protein expression of collagen 1 and 3 was in accordance with the mRNA expression. In hGFs, expression of collagen 1 and 3 protein was decreased in the third group compared with the second group. In hDFs, expression of collagen 3 was increased in the third group compared with that of control.

5. The mRNA and Protein Expression of MMP−1 After AA Treatment

The expression of MMP−1 mRNA was increased in the third group of both in hGFs and hDFs. Interestingly, it’s was increased by 2 fold, but was
Fig. 1. The effect of various concentrations of arachidonic acid in hGFs and hDFs. 300 μM AA was treated in hGFs for 1 hr 30 min and 200 μM AA was treated in hDF for 1 hr. Cell viability was assessed by MTT assay (A). Morphology of gingival and dermal fibroblast after arachidonic acid treatment, Cells were photographed using a camera attached to an inverted microscope (×100) (B).

Fig. 2. The mRNA expression of COX-2 (A) and production of PGE2 (B) in hGFs and hDFs after AA treatment. The cells with 24 hr starvation were incubated with arachidonic acid and then, samples were acquired at 0 and 24 hr respectively, 300 μM AA was treated in hGFs for 1 hr 30 min and 200 μM AA was treated in hDF for 1 hr. The control group was not treated with AA, The mRNA expression of COX-2 was determined by real–time PCR (A). The PGE2 activity was measured by enzyme–linked immunosorbent assay (B).

Fig. 3. The expression of TGF-β in hGFs and hDFs after AA treatment. The cells with 24 hr starvation were incubated with arachidonic acid and then, samples were acquired at 0 and 24 hr respectively, 300 μM AA was treated in hGFs for 1 hr 30 min and 200 μM AA was treated in hDF for 1 hr. The control group was not treated with AA, The mRNA expression of TGF-β was determined by real–time PCR (A). The protein expression of TGF-β was determined by Western blot (B). Band intensities were quantified by scion image and determined by sigma plot.

Fig. 4. The expression of collagen 1 and 3 in hGFs and hDFs after AA treatment. The cells with 24 hr starvation were incubated with arachidonic acid and then, samples were acquired at 0 and 24 hr respectively, 300 μM AA was treated in hGFs for 1 hr 30 min and 200 μM AA was treated in hDF for 1 hr. The control group was not treated with AA, The mRNA expression of collagen 1 and 3 was determined by real–time PCR (A). The protein expression of collagen 1 and 3 was determined by Western blot (B). Band intensities were quantified by scion image and determined by sigma plot.
significantly increased by 10 fold compared with that of control. The expression of MMP-1 protein was also increased in the third group of hGFs. However, expression of MMP-1 protein decreased both in the second and the third group compared with that of control but, it's was not significantly affected.

6. The mRNA and Protein Expression of TIMP-1 After AA Treatment

The expression of TIMP-1 mRNA was increased in the third group of hGFs and hDFs compared with that of control. However, protein expression of TIMP-1 was decreased in hGFs compared with that of control and was increased in the third group of hDFs compared with that of control.

IV. DISCUSSION

This study showed the differential expression of genes in connection with keloid formation in gingival and dermal fibroblasts. We demonstrated the differential expression of cytokines, growth factors, MMPs and TIMPs that are known to influence the collagen deposition in the wound healing process.

In most cells, including macrophages, COX-1 expression is constitutive, whereas COX-2 expression is induced in response to a variety of inflammatory mediators, COX-2 regulates ECM degradation and tissue remodeling observed in inflammation diseases. Several studies have demonstrated prostaglandin E synthesis and MMP activity by increased COX-2. In the present study, COX-2 expression was elevated by arachidonic acid in gingival and dermal fibroblasts. The mRNA expression of COX-2 was increased in the second group, but decreased in the third group of hGFs. In contrast to hGFs, COX-2 expression was peaked in the third group of hDFs. These results suggested that hGFs rapidly respond to inflammation in comparison with the hDFs.

PGE, which is one of the most abundant prostaglandins in many tissues, mediates inflammation. Previous studies showed that fibroblasts can produce high levels of PGE in response to mechanical loading. In the present study, activity of prostaglandin E was markedly increased in the second and the third group in gingival and dermal fibroblasts, but there were no significant between both of them.

Several studies have supported the role of TGF-β as an important pathological factor in excessive scar formation and making it a potential drug target for scar reduction therapy. TGF-β induces fibroblast contraction, which is implicated in wound healing and keloid formation. TGF-β stimulates formation of collagen 1 and 3 and reduces degradation via stimulating the TIMPs together with a suppression of MMPs, thus favoring ECM and especially of collagen. Some studies have suggested that keloid scarring may be caused by fibroblasts that secrete elevated levels of TGF-β. The result of the present study show decreased expression of mRNA and protein of TGF-β in hGFs, whereas increased in hDFs in the third group. This is suggested that hDFs may be further regulated by TGF-β in the process of inflammation than hGFs.

Proliferation of fibroblasts, overproduction of collagens and contraction are the characteristics of keloids. In the present study, the hGFs were decreased expression of collagen 1 and 3 but, in the hDFs, its were increased. These results suggested that collagens were further deposited the hDFs compared with that of hGFs. Accordingly, regulation of collagen deposition differs in gingival and dermal fibroblasts.

In important regulators of tissue remodeling, ma-
Fig. 5. The expression of MMP-1 in hGFs and hDFs after AA treatment. The cells with 24 hr starvation were incubated with arachidonic acid and then, samples were acquired at 0 and 24 hr respectively. 300 μM AA was treated in hGFs for 1 hr 30 min and 200 μM AA was treated in hDF for 1 hr. The control group was not treated with AA. The mRNA expression of MMP-1 was determined by real-time PCR (A). The protein expression of MMP-1 was determined by Western blot (B). Band intensities were quantified by scion image and determined by sigma plot.

Fig. 6. The expression of TIMP-1 in hGFs and hDFs after AA treatment. The cells with 24 hr starvation were incubated with arachidonic acid and then, samples were acquired at 0 and 24 hr respectively. 300 μM AA was treated in hGFs for 1 hr 30 min and 200 μM AA was treated in hDF for 1 hr. The control group was not treated with AA. The mRNA expression of TIMP-1 was determined by real-time PCR (A). The protein expression of TIMP-1 was determined by Western blot (B). Band intensities were quantified by scion image and determined by sigma plot.

Table 1. Primer sequences used in the present study.

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<th>Primers</th>
<th>Sequences</th>
<th>References</th>
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<td>COX-2</td>
<td>F:5’-TTCAAAATGAGATTGTGGGAA-3’ R:5’-AGATCATCTCTGCTGACTTCTT-3’</td>
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<td>TGF-β</td>
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<td>Collagen 1</td>
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<tr>
<td>TIMP 1</td>
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<td>NM_003254</td>
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Trix metalloproteinases (MMPs) degrade extracellular matrices and are responsible for excessive connective tissue breakdown in inflammatory disorders. Of the MMPs family, interstitial collagenase (MMP-1) is the key enzyme responsible for degrading type I and III collagen because the initial breakdown of fibrillar collagen network is mediated primarily by MMP-1. Also, MMP-1 gene is regulated by a variety of factors including cytokines, COX-2 expression precedes the MMP-1 induction. Thus far, little is known about the collagenase in keloid tissues, TIMPs are inhibitors of MMPs found in most tissues and among
them TIMP-1 inhibits most of MMPs. By inhibiting MMPs activities, they participate in tissue remodeling of the extracellular matrix ECM. Recent studies pointed out that TIMP-1 might act as a cell survival factor\textsuperscript{28}.

In the present study, the protein expression of MMP-1 was increased in hGFs and decreased in hDFs. Also, the protein expression of TIMP-1 were decreased in hGFs but, hDFs were increased in th third group compared with that of control. These result suggested that controlling collagens formation were affected by up-regulation of MMP-1 and suppression of TIMP-1 in hGFs, but by suppression of MMP-1 and up-regulation of TIMP-1 in hDFs.

In summary, based on this study, it is clear that significant differences exist in the expression of COX-2, TGF-β, collagen 1 and 3, MMP-1 and TIMP-1 between gingival and dermal fibroblasts. This might indicate that scarless wound healing may be not a function of the action of one single actor, but rather a tightly controlled balance various factors.

V. CONCLUSION

Gingival wounds heal more rapidly and with scarless, however dermal wound show collagen laid down in thick disorganized patterns and keloid formation. This has been suggested to be due to the presence of different growth factor, cytokines and ECM components as collagens, MMPs and TIMPs.

The purpose of this study was to determine the differential expression of genes in connection with keloid formation in hGFs and hDFs after inflammation. In this study, we investigated the differences in the expression and production of COX-2, PGE\textsubscript{2}, TGF-β, MMPs, and TIMPs to influence the collagen deposition in keloid formation between the hGFs and hDFs.

The hGFs and hDFs were primary cultured and allocated to three groups in this experiment, the first group is non-AA treatment, the second group is AA treatment and the third group. To determine the concentration of AA, MTT assay was performed. The mRNA and protein expression levels of COX-2, TGF-β, collagens 1 and 3, MMP-1 and TIMP-1 were examined by Real-time PCR and Western blots. The amounts of PGE\textsubscript{2} was measured by enzyme-linked immunosorbent assay.

The expression of COX-2 and TGF-β exhibited reduced levels in hGFs, but were increased in the third group of hDFs. Production of PGE\textsubscript{2} was increased both in hGFs and hDFs in the second group but, not changed in the third group. The protein and mRNA expression of collagen 1 and 3 was decreased in hGFs, whereas increased in hDFs in the third group. Expression of MMP-1 protein was increased in hGFs but, was decreased in hDFs in the third group compared with that of control. The protein expression of TIMP-1 was decreased in hGFs but, were increased in hDFs in the third group compared with that of control.

In conclusion, based on this study, it is clear that significant differences exist in the expression of COX-2, TGF-β, collagen 1 and 3, MMP-1 and TIMP-1 between gingival and dermal fibroblasts.

Also, this might indicate that scarless wound healing may be a function of the action of one single actor, but rather a tightly controlled balance various factors.

VI. REFERENCES

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