Endothelial-Mesenchymal Transition in the Keloid Pathogenesis

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<Abstract>

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Keloid is a fibroproliferative disorder characterized by abnormal fibrosis and increased extracellular matrix components deposition. The exact pathogenic mechanism of keloid formation is yet to be elusive, with a few known important involving factors, such as TGF-beta. Many articles have showed that fibroblasts originated from epithelial-mesenchymal transition (EMT) can be pivotal fibroblasts to form keloid tissues. More recently, several studies on the pathogenesis of fibrotic disorders are discovering the connection between endothelial mesenchymal transition (EndMT) and fibrosis. However, no article is found considering EndMT as the one of the factors in keloid pathogenesis, therefore the study on validating the involvement of EndMT in keloid tissue formation was set. Using confocal microscopy, cells in keloid tissue co-expressing platelet endothelial cell adhesion molecule-1 (PECAM-1, also known as CD31) and vimentin/ α-smooth muscle actin (α-SMA) simultaneously were recognized, and EndMT related markers were validated

by checking mRNA and protein level related to the keloid formation via quantitative real-time PCR and Western blot, respectively. Only in the active keloid tissue defined by clinical activity profiles, Snail expression was increased significantly, suggesting the involvement of EndMT in keloid pathogenesis. Hence, the EndMT can be suggested to act in the keloid pathogensis as importantly as EMT.

Key words: Keloid, Endothelial-mesenchymal transition

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I. INTRODUCTION

Keloid is a fibroproliferative disorder, resulting from the unbalanced homeostasis in normal wound healing, and is characterized by abnormal fibrosis and increased extracellular matrix components deposition. The exact pathogenic mechanism of keloid formation is yet to be elusive, but transforming growth factor beta 1 (TGF-beta 1), hypoxia, and hepatocyte growth factor (HGF)/C-Met system are known to be important factors[1-10]. These factors are known to be involved not only in keloid pathogenesis but also in many other fibrotic disorders such as fibrotic lung disease, systemic sclerosis, multifocal fibrosclerosis, and nephrogenic systemic fibrosis[11-12]. Among these fibrotic disorders, the role of epithelail-mesenchymal transition (EMT) is vigorously researched. More recently, it is a noticeable trend in

research fields to illuminate the relationships between fibrotic disorders and endothelial-mesenchymal transition (EndMT or EndoMT)[13-14]. Unlike to the other fibrotic disorders, however, study on keloid pathogenesis has high barriers to overcome in its lack of appropriate animal models, since humans are the only species known to develop keloids. The fact that EndMT only happens transiently, makes it even more difficult to study the involvement of EndMT in keloid pathogenesis.

It was, therefore, necessary to conduct a preliminary study to validate the possibility to capture the point of EndMT occurrence. Among 23 samples, only one weak positive result showed the EndMT moment, by following method of Zeisberg et al. [14] who suggested that the co-expression of fibroblast and endothelial cell marker is the sign of early stage of EndMT in their publication using confocal mciroscopy[15-19].

In the preliminary studies, positive but disappointing result had been obtained revealing only one weak positive finding out of 23 samples, in capturing of the EndMT moment. Therefore, the objective of this study was set to observe a more concrete evidence for the involvement of EndMT moment in the active keloid lesions.

II. PATIENTS AND METHODS

1. Patients

After receiving an approval of an institutional review board on the study protocol, consent forms of the participants who underwent excision treatment on their keloid lesions were received. In between December 31th, 2011 and October 30th, 2012, 6 patients were enrolled (4 males and 2 females). According to the clinical profiles, only one out of six patients is categorized as patient with clinically active lesion. Not only the patients who were on anti-fibrotic drugs or had an infection, or bleeding tendency, but also the patients who have received continuous intralesional steroid injection less than 1 month before the excision were excluded from the study. The age range of the subjects was from 23 to 73 years with an average 35.5 years.

2. Tissue preparation according to the clinical activity profiles

Patients' keloid tissues were obtained and if possible, subdivided into regions of active lesions, non-active lesions and normal skin lesions for analysis following our modified clinical activity profiles referring Schierle et al.[20]. All patients who underwent the procedures were explained to and subscribed the informed consents. Clinical activity was determined by the

following clinical activity profiles: growth, redness, pain, and itching. Specimens were taken from both an inactive and active region of the keloid lesion in addition to normal tissue, if the lesion is categorized as active status. Otherwise, only inactive keloid and normal tissues were obtained (figure 1).

3. Immunostains and confocal microscopy

We cut frozen tissues into 10 um-thick cross sections that would be fixed in 4% paraformaldehyde at room temperature for 20 min. Rinse slides three times in PBS-T (0.1% Tween 20) for 10 minutes each. Block specimen in 5% goat serum in PBS-T for 1 hr at room temperature. We incubated the sections with primary antibodies at 4℃ overnight. The primary antibodies were rabbit anti-CD31(1:100, Cell signaling), mouse anti–α-SMA(1:100, Abcam) and mouse anti-vimentin(1:100, Santacruz). Rinse three times in PBS-T for 10 minutes each. For double-labeling experiments, FITC-conjugated anti-rabbit (1:250, Abcam) and Texas Red-conjugated anti-mouse (1:250, Abcam) secondary antibodies were used. Rinse in PBS-T for 10 minutes each. Coverslip slides with vectashield (Vector) with DAPI. Each tissue staining was analyzed independently by two independent blinded investigators using a scanning confocal microscope (LSM 700, Zeiss). Ten

visual fields were analyzed for co-localization of endothelial and fibroblast markers.

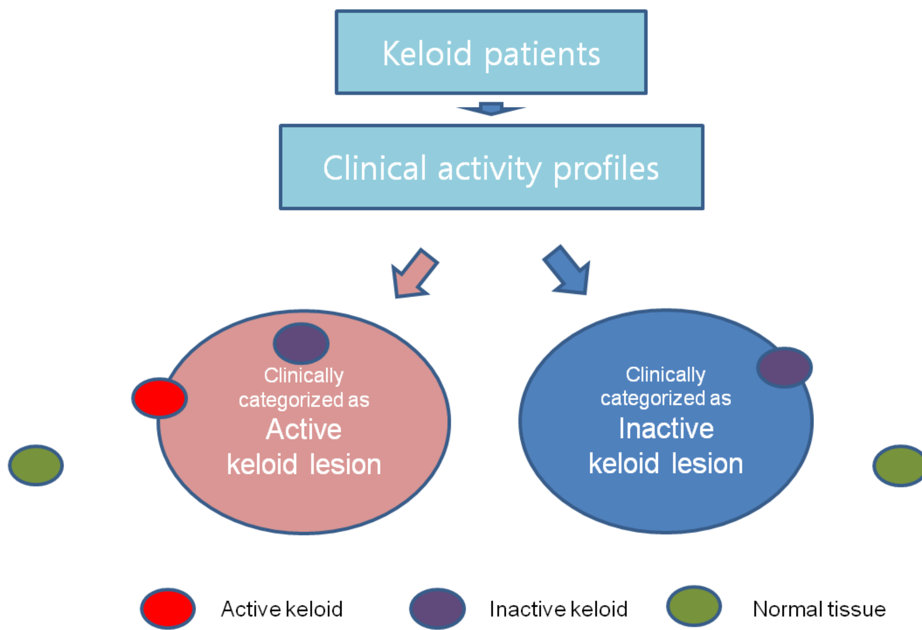

Figure 1. Tissue preparation sites according to the clinical activity.

4. Quantitative real-time PCR

Total RNA was extracted with RNeasy plus mini kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized using Transcriptor first strand cDNA synthesis kit (Roche) with 1 ug of total RNA. The real-time PCR was conducted in triplicate using TaqMan master mix (Applied Biosystems, Foster City, CA, USA) on the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). The mRNA expression levels were normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping genes. Snail, Slug, Vimentin, Twist were used with commercial TaqMan gene expression assays from Applied Biosystems. The accession assay numbers of above targets are as follows: Snail - Hs00195591_m1, Slug - Hs00950344_m1, Twist - Hs00361186_m1, and Vimentin - Hs00185584_m1.

5. Statistical analysis

The Mann-Whitney U test and Wilcoxon signed-rank test were performed to determine significant differences between distinct categories. SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses. Differences were considered statistically significant when the *p*-value was less than 0.05.

III. RESULTS

1. Clinical features of keloid patients and their characteristics

A total 6 patients (4 men, 2 women) were included in this study. The demographic characteristics of the patients are presented in table 1. The mean age was 41.25 ± 21.67 and 24.00 ± 1.41 years for men and women respectively. The age ranged from 26 to 73 years for men and 23 to 25 years for women. Only one patient met our clinical activity profiles to be an active keloid lesion.

Table 1. Patient characteristics; Clinical activity is determined by the following criteria; 1.Growth 2. Redness 3. Pain 4. Itching.

Case No.	Sex	Age	Duration (years)	Sites	Clinical Activity
K1	F	23	2	Earlobe	Yes (growth,redness)
K2	M	73	30	Ant. Chest	No
K3	F	25	15	Lateral thigh	No (itching)
K4	M	29	3	Lt. forearm	No
K5	M	37	7	Rt. periauricular	No
K6	M	26	3	Earlobe	No

2. Confocal microscopy

Immunohistochemical staining results were viewed through confocal microscopy16- to capture the moment of EndMT via detecting successfully merged area of endothelial cell area, co-expressing all of endothelial cell marker (CD31) and mesenchymal marker (vimentin or α- SMA)[13-14,21].4',5'diamidino-2-phenylindole (DAPI) stain was used to validate the stained area are all located in the cellular regions.

Confocal microscopy visualization of CD31 and vimentin expression were performed with all of six tissue-samples. Only peripheral marginal area of K1 sample yielded a merged staining region in both staining (figure 2, A and figure 3). The other tissue samples, from K2 to K6 that are clinically inactive, however, did not show any merged area of positive CD31 and α-SMA or Vimentin throughout the serial section of the whole block (figure 2, B).

Same screening had been conducted to visualize the coexpression of CD31 and α-SMA, also yielded a same result of a positive finding on only in the peripheral marginal area of K1 sample (figure 3).

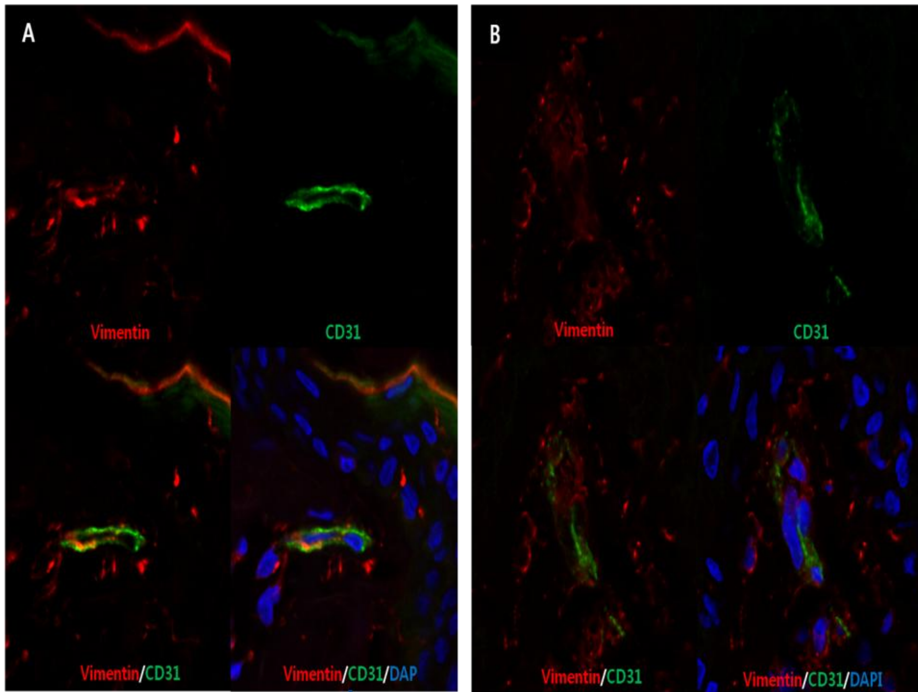

Figure 2. Confocal microscopy to immunofluorescence staining for Vimentin (red), CD31 (green), and DAPI (blue) (x100). A. Visualization in the peripheral growing area of the clinically active lesion (K1 sample). B. Visualization in the normal and inactive keloid lesions(K2 sample). K; keloid

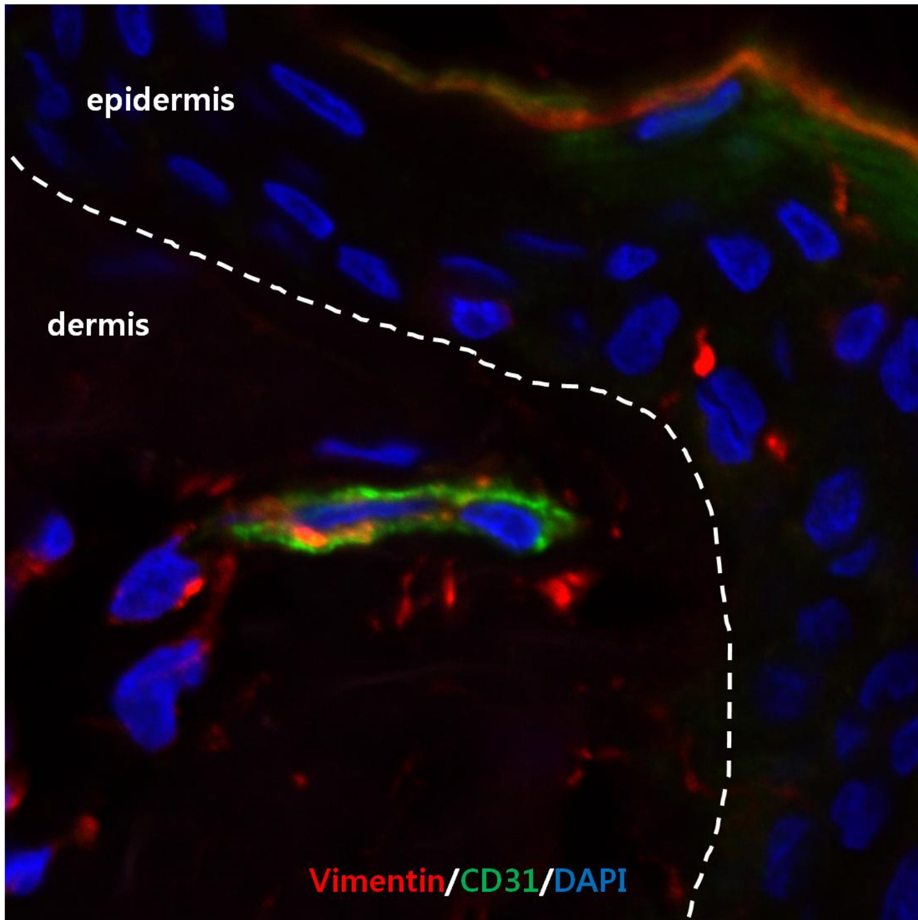

Figure 3. Confocal microscopy to immunofluorescence staining for Vimentin (red), CD31 (green), and DAPI (blue) (x100,K1 sample). K; keloid)

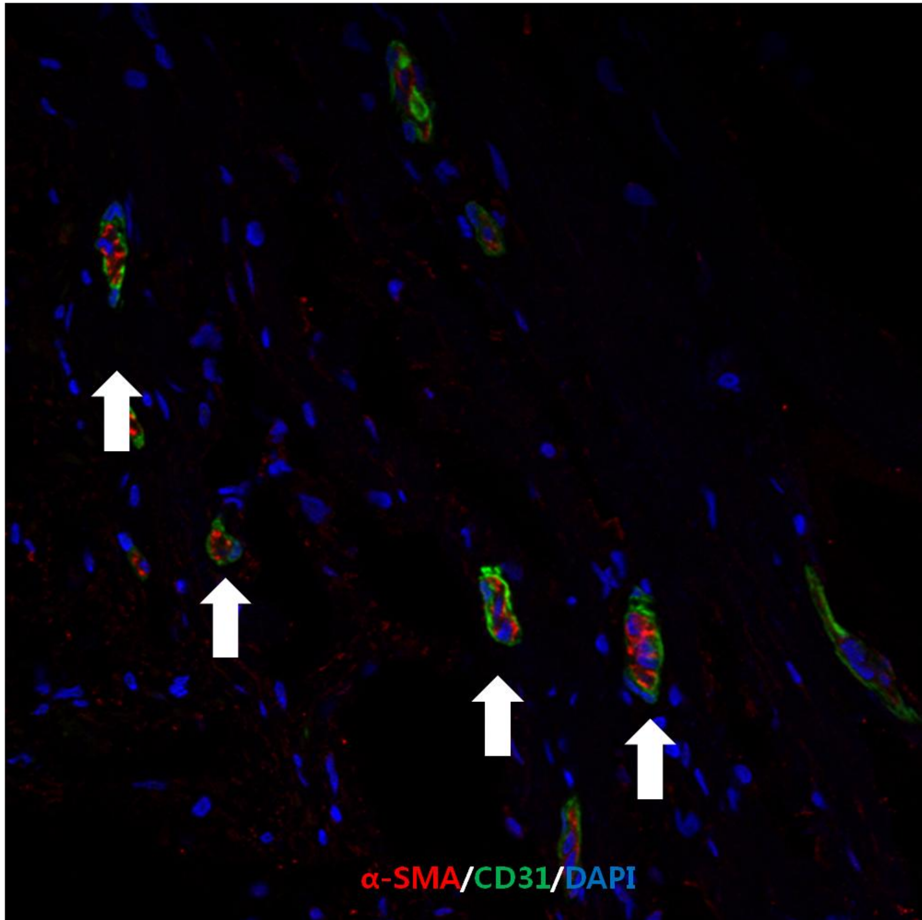

Figure 4. Confocal microscopy to immunofluorescence staining for α-SMA (red), CD31 (green), and DAPI (blue) (x100,K1 sample). K; keloid

4. Quantitative RT-PCR

Expression of EndMT related genes were measured by quantitative RT-PCR. According to the former EndMT studies[2,4,13-14], following mRNA expression were measured: snail, slug, twist and vimentin. Only the K1 sample out of all 6 samples was able to be divided into an active keloid lesion, and inactive lesion and normal tissue. The other 5 samples which were categorized as clinically inactive were divided into keloid lesion and normal lesion. In K1, result showed significant increase (74.12 folds increase) of Snail expression in the active keloid region comparing to the normal and inactive keloid region. (figure 5) Other keloid tissues showed little differences among the samples. (Data not shown) Overall, EndMT-related genes-expressions are increased except Slug in average of total samples. (figure 6).

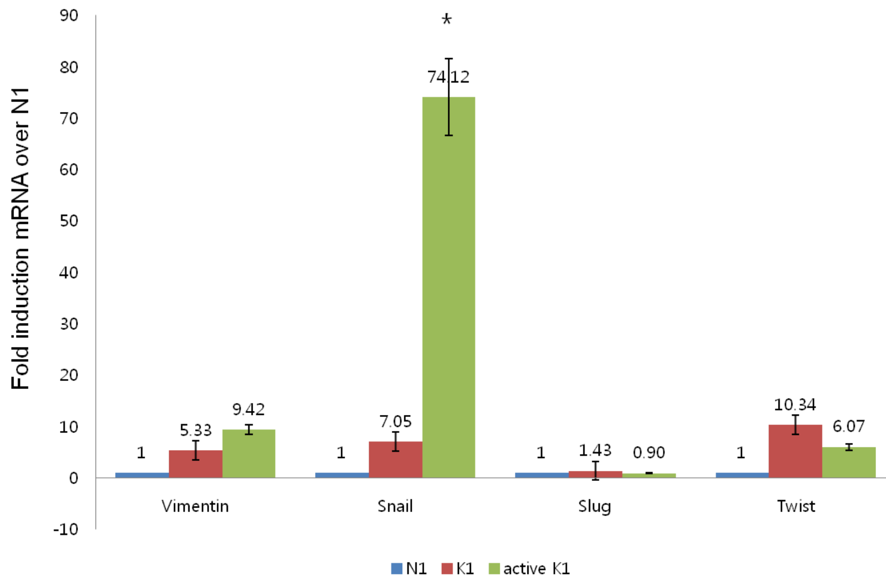

Figure 5. Quantitative RT-PCR on EndMT-related gene expressions of K 1 tissue sample was performed. K, keloid; N, normal

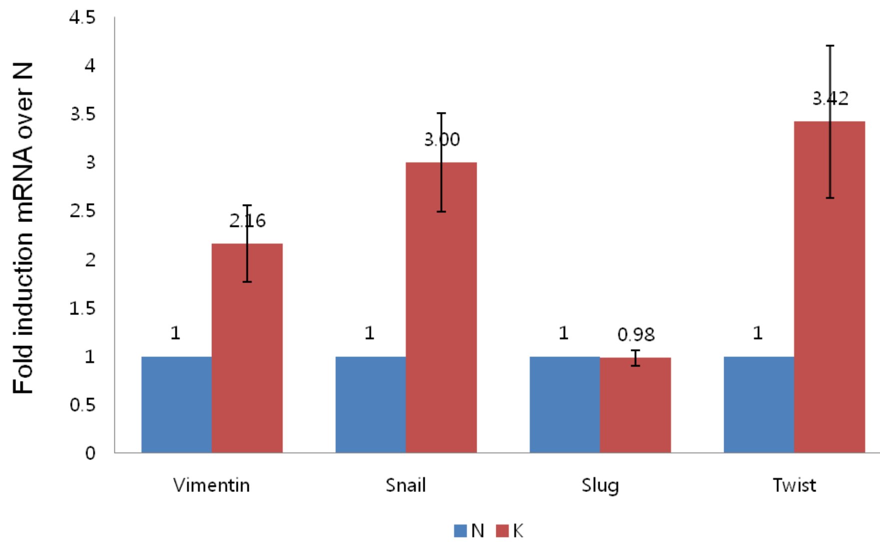

Figure 6. Quantitative RT-PCR on EndMT-related gene expressions of K1 to K6 was performed. K, keloid; N, normal

IV. DISCUSSION

In this study, one out of six samples was found to reveal the positive confocal microscopic result by showing merged area of CD31(PECAM1) and vimentin, and also CD31(PECAM1) and α-smooth muscle actin (α-SMA) simultaneously. From the quantitative RT-PCR result which showed the increased Snail expression, was also found with the same sample tissue. Therefore, the interpretation of finding evidence of EndMT involvement in the keloid pathogenesis is possible from these two accordance results. Possible notion of the EndMT involvement in keloid pathogenesis can be explained by following consideration.

First, among the many fibrotic diseases, keloid is a representative fibrotic cutaneous disease, therefore it is possible to accord with other fibrotic disease pathogenesis in which recently Zeisberg et al.[13-14] proved that EndMT mechanism is involved.

Second, by knowing the fact that TGF-beta is involved in keloid pathogenesis[9], publication showed EndMT can be induced by TGF-beta and its downstream effector molecules such as Smad family[11] is supporting the notion of EndMT involvement in keloid pathogenesis.

Third, from the fact that decreased blood vessel and increased myofibrobalst in the keloid was found by Appleton I et al.[22] who suggested the clinical color

of keloid lesion is not by increased vessels or melanin, but by myofibrotic component of keloid itself, it is possible to make the hypothesis that hypoxic environmental change of tissue due to EndMT can lead the ignition of keloid pathogenesis. This concept is supported by several publications dealt the relationships between the hypoxic environment and EMT with similar concept where hypoxic environment is found to increase the mesenchymal transition markers[5,23-26].

Among the 6 samples, the reason why only one sample could show the positive was reviewed. Best explanation of it is that the transient nature of EndMT[10], thus not always detectable. It should be considered, however, the result was led because only one tissue sample was from the patient whose keloid lesion is categorized as clinically active. Clincal activity profiles were used in this study according to Schierle HP et al.[20] They proved the amount of androgen receptors are significantly different between clinically active keloid lesion and normal tissue, using same clinical activity profiles of this study. They noticed even the keloid lesion is clinically active, it is still needed to be divided into major and minor active lesion, but actual method to differentiate the region of tissue sampling was not mentioned. From result of this study, it is possible to hypothesize that growth profile is most important to differentiate the keloid lesion whether clinically active or inactive since the only the tissue

with growth profile could yield the positive result of this study. In addition the hypothesis that the periphery of the active lesion where shows growth profile is a best site of tissue gathering for the tissue analysis .This hypothesis is to be evaluated and validated with more studies, but if the role EndMT in keloid pathogenesis is found to be cleared with the further experiments, this simple clinical activity profiles could help to tell which patients will be tried to use experimental treatment such as Snail inhibitor on his keloid with higher chance of yielding better outcomes.

This study has double edged points of advantages and disadvantages of using tissue quantitative RT-PCR. Its advantage side is to perform experiments done with *in vivo* tissue. However, its disadvantage side is on the compounding factors that both EndMT and EMT can be the origin of mesenchylmal transition markers, because all tissue samples contained both of their epidermal and dermal components. Therefore, the interpretation should be made with caution that mesenchymal transition marker-expression can either from the EndMT and EMT. Attempts to eliminate epidermal portion in the analysis process and the result confocal microscopic data showed EndMT region in the dermal area only prefers that the result is obtained by solely from EndMT. For the future studies, method to adjust the influence of EMT should be better considered.

Limitations of this study lies in the small sample size and the fact that age-sex matched sampling was not able to meet. Therefore for the future studies, serial observations with bigger sample sized and also applying the age-sex matched sampling conditions is needed for the better interpretation.

V. CONCLUSION

In this study, six keloid patients were enrolled during 1 year of study duration to retrieve evidences of EndMT involvement in keloid pathogenesis. Despite the considerable small number of enrolled patients as the major limitation of this study, that fact that finding a keloid sample yielded a positive result of EndMT nature, confirmed by confocal microscopy and quantitative RT-PCR, only in the peripheral region of clinically active keloid is recognized. In addition, among the clinical activity profiles, the importance of growth profile is also noticed.

Therefore, the fact that EndMT can be a meaningful part of active keloid pathogenesis is cautiously suggested, and the need of proving the clinical activity profile in keloid research is also reminded.

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ABSTRACT (IN KOREAN)

켈로이드 형성에서의 내피-간충직 세포 전환

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박지훈

켈로이드는 상처회복과정에서 발생하는 비정상적인 섬유화와 다양한 세포외기질의 과다한 발현과 축적을 특징으로 하는 섬유 증식성 질환이다. 현재까지도 정확한 발생기전은 밝혀지지 않은 상태이지만, TGF 베타와 같은 중요한 작용 인자에 대해서는 연구가 진행되어있는 상태이며, 많은 문헌에서 상피-간충직 세포 전환을 통해 발생한 섬유아세포가 켈로이드 조직의 형성에 중요한 역할을 할 수 있음을 보여준 바 있다. 최근에는, 몇몇의 연구가 여러가지 섬유화질환에서의 섬유화와 내피-간충직 세포 전환과의 관계를 보여준 바 있다. 하지만, 켈로이드 조직에서의 내피-간충직 세포 전환의 관계를 다룬 보고는 거의 없어, 이에, 켈로이드 조직 형성에 있어서의 내피-간충직 세포 전환의 관련성을 입증하기 위한 연구를

진행하였다. Confocal 현미경을 통하여 켈로이드 조직에서 platelet endothelial cell adhesion molecule-1 (PECAM-1, 또는 CD31)과 fibroblast-specific protein 1 (FSP1)을 동시에 발현하는 부위를 찾아내었으며, 또한, Snail expression과 단백질의 발현 정도를 각각 quantitatitve RT PCR과 Westernblot을 통하여 나타내었다. 임상적 활성도 기준을 따라 분류한 활성적인 켈로이드 조직에서는 내피-간충직 세포 전환에 중요하게 작용하는 Snail의 발현이 비활성적 켈로이드 조직이나 정상조직에 비해 증가하는 것을 확인함으로서 내피-간충직 세포 전환이 켈로이드 형성에 실제 관여함을 보여 주었으며, 더불어 이러한 현상은 활성적인 켈로이드 조직에서만 관찰될 수 있음을 확인하였다. 따라서 저자는 이 실험을 통하여, 켈로이드 형성과정에서 내피-간충직 세포전환이 중요한 역할을 하고 있음과 더불어, 각 병변의 임상적인 활성도 파악이 켈로이드 연구에 중요한 조건임을 조심스럽게 제안하는 바이다.

핵심되는 말: 켈로이드, 내피-중간엽 세포 전환