Endothelial-to-Mesenchymal Transition induced by Wnt 3a in Keloid pathogenesis

Won Jai Lee, MD, PhD1*, Ji Hun Park, MD2*, Jung U Shin, MD2, Hyun Noh, PhD1, Dae Hyun Lew, MD, PhD1, Woo Ick Yang, MD, PhD3, Chae Ok Yun, PhD4, Kwang Hoon Lee, MD, PhD2, and Ju Hee Lee, MD, PhD2§

1Institute for Human Tissue Restoration, Department of Plastic & Reconstructive Surgery; 2Department of Dermatology, Yonsei University College of Medicine, Seoul, Korea; 3Department of Pathology, Yonsei University College of Medicine, Seoul, Korea; 4Department of Bioengineering, College of Engineering, Hanyang University, Seoul, Korea

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A short running head: EndoMT in keloids and dermal microvascular endothelial cells

Key words: keloid, endothelial-to-mesenchymal transition, Wnt 3a

§Corresponding Author: Ju Hee Lee, MD, PhD

Department of Dermatology and Cutaneous Biology Research Institute,
Yonsei University College of Medicine
250 Seongsanno, Seodaemun-gu, Seoul, Korea
E-mail: juhee@yuhs.ac
Tel: 82-2-2228-2082
Fax: 82-2-393-9157

*These authors contributed equally to this work
Abstract

Endothelial-to-mesenchymal transition is a phenotypic conversion characterized by down-regulation of vascular endothelial markers and the acquisition of a mesenchymal phenotype. We hypothesized that keloid fibroblasts are of endothelial origin and that Endothelial-to-mesenchymal transition substantially contributes to collagen accumulation during the development and progression of keloids. Wnt-3a protein expression was examined using immunohistochemistry in keloid tissues. Human dermal microvascular endothelial cells were treated with Wnt-3a. mRNA and protein expression of endothelial (vascular endothelial cadherin) and mesenchymal (vimentin, slug, and α-SMA) cell markers were measured using real-time RT-PCR and immunocytochemistry, respectively. Additionally, co-expression of CD31, and endothelial cell marker, and vimentin in the vascular endothelium of keloid tissues was examined using immunofluorescence. Wnt-3a overexpression was observed in human keloid tissues. Wnt-3a treatment significantly reduced VE-cadherin mRNA expression and induced vimentin and slug mRNA expression in human dermal microvascular endothelial cells. Human dermal microvascular endothelial cells became spindle-shaped and exhibited reduced expression of CD31 and increased expression of vimentin, slug, and α-SMA. Moreover, co-expression of CD31 and vimentin was observed in the dermal vascular endothelium of keloid tissues from two patients with clinically-active keloids. In conclusion, transient conversion of human dermal microvascular endothelial cells to a mesenchymal phenotype may contribute to dermal fibrosis of keloid and hypertrophic scars.
INTRODUCTION

Keloids are a dermal fibroproliferative disorder resulting from imbalanced tissue homeostasis during normal wound healing and persistent collagen synthesis by abnormal dermal fibroblasts. This results in accumulation of excessive amounts of extracellular matrix (ECM) components, which can sometimes cause cosmetic and functional impairment. Although various mechanisms\textsuperscript{1-3} have been proposed to explain keloid pathogenesis, none have provided a comprehensive explanation. Keloids remain a difficult clinical problem. Although nearly every cell type has been implicated in the pathogenesis of fibrosis, dermal fibroblasts are likely the underlying cause of abnormal scars, including keloid and hypertrophic scars. Keloid fibroblasts (KFs) differ from normal dermal fibroblasts in several aspects. They are characterized by excessive synthesis of ECM components such as collagen, fibronectin, elastin, and proteoglycans, as well as altered expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Additionally, they react differently to metabolic regulating factors that are involved in apoptosis\textsuperscript{4} and ECM metabolism, as well as to glucocorticoids and growth factors.\textsuperscript{5,6}

Myofibroblasts are key mediators of fibrosis, but their origin is not completely clear. Although severe tissue fibrosis is widely accepted to be mediated by activation of resident dermal fibroblasts that proliferate and synthesize excessive ECM, in many tissues, myofibroblasts can also be derived from epithelial-to-mesenchymal transition (EMT),\textsuperscript{7-9} endothelial-to-mesenchymal transition (EndoMT),\textsuperscript{10-12} pericytes,\textsuperscript{13} and bone marrow mesenchymal precursors.\textsuperscript{14} Whether myofibroblasts of different cellular origins play distinct roles in the wound repair process and whether they participate in the normal repair response or fibrosis has not yet been elucidated.\textsuperscript{7}

EndoMT is a phenomenon that was recently reported to occur during embryonic cardiovascular development and has emerged as a possible mechanism of pathological fibrosis.\textsuperscript{13,14,17,18} EndoMT is a phenotypic conversion characterized by the down-regulation of vascular endothelial markers, such as cluster of differentiation 31 (CD31) and vascular endothelial cadherin (VE-cadherin), and acquisition of a mesenchymal or myofibroblastic phenotype that initiates expression of mesenchymal cell products, including $\alpha$-smooth muscle actin ($\alpha$-SMA), vimentin, type I collagen, and slug (Snail family transcription factor). Like EMT, EndoMT can be induced by transforming growth factor-\(\beta\) (TGF-\(\beta\)),\textsuperscript{13,14} other proinflammatory cytokines,\textsuperscript{19} and wingless protein (Wnt).\textsuperscript{20-22} Among these, the Wnt/\(\beta\)-catenin signaling pathway may activate gene expression that is crucial for EndoMT during cardiac endocushion
Several studies have demonstrated that EMT plays an important role in keloid or hypertrophic scar pathogenesis, but few studies on EndoMT in keloid or hypertrophic scar pathogenesis have been performed.

We hypothesized that KFs are of endothelial origin and that EndoMT substantially contributes to ECM accumulation during keloid development and progression. Therefore, EndoMT was induced by Wnt 3a in human dermal microvascular endothelial cells (HDMECs) in vitro, and expression of EndoMT-related genes was examined using qRT-PCR and immunocytochemistry. Additionally, EndoMT in active keloid tissues was investigated using immunofluorescence to identify co-localization of endothelial and mesenchymal cell markers.
METHODS

Keloid tissues

Keloid tissues (n=12) were obtained from inactive or active-stage keloid patients (Table 1) after obtaining informed consent. According to the clinical profiles, we categorized patients and five of 12 patients were categorized with clinically-active lesions (growth, pain, redness, and itching). Tissues were excised according to a protocol approved by the Yonsei University College of Medicine Institutional Review Board. All experiments involving humans adhered to the Declaration of Helsinki. Patients were excluded from the study if they were taking anti-fibrotic medication, showed signs of infection or bleeding diathesis, or had received continuous intra-lesional steroid injections less than 1 month prior to tissue sample excision.

HDMEC culture

HDMECs (Cambrex, Walkersville, MA, USA) were prepared and treated in a tissue incubator with 0.1% gelatin and microvascular endothelial cell media-2 (Cambrex), which contained human epidermal growth factor (EGF), hydrocortisone acetate, vascular endothelial growth factor (VEGF), human fibroblast growth factor-β (FGF-β), gentamicin, amphotericin B, 5% fetal bovine serum (FBS), R3-insulin growth factor-1 (IGF-1), and ascorbic acid. HDMECs were then treated with Wnt 3a (100 ng/ml; Cell Signaling Technology, Beverley, MA, USA) daily for 5 days, and phenotypic changes were examined as described below. Phase-contrast microscopy (Olympus, Tokyo, Japan) was used to examine the morphological changes. Experiments were conducted with HDMECs at passages 2 to 4.

qRT-PCR analyses

Total RNA was prepared using the TRIzol® reagent (Gibco, Grand Island, NY, USA), and cDNA was prepared from total RNA (0.5 μg) using random priming and a first-strand cDNA synthesis kit (Promega Corp., Madison, WI, USA) under the following incubation conditions: 95°C for 5 min, 37°C for 2 h, and 75°C for 15 min. Applied Biosystems Taqman primer/probe kits [assay ID: Hs00195591_m1 (snail), Hs00950344_m1 (slug), Hs00185584_m1 (vimentin), and Hs00901463_m1 (VE-cadherin)] were used to analyze mRNA expression levels with an ABI Prism 7500 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Target mRNA levels were measured relative to an internal
glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control (assay ID: Hs99999905_m1, Applied Biosystems). For cDNA amplification, AmpliTaqGold DNA polymerase was activated by incubation for 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C plus 1 min at 60°C for each cycle. To measure cDNA levels, the threshold cycle at which fluorescence was first detected above baseline was used, and a standard curve was drawn between starting nucleic acid concentrations and the threshold cycle. mRNA expression levels were normalized to the levels of GAPDH. Relative quantitation was expressed as fold-induction compared with control conditions.

**Immunocytochemistry**

Cultured HDMECs were washed twice with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 15 min at room temperature, and permeabilized by incubating for 15 min in 0.01% Tween® 20 in PBS. Samples were blocked with 5% bovine serum albumin (BSA), followed by incubation overnight at 4°C with antibodies to CD31 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), α-SMA (1:100, Abcam, Cambridge, MA, USA), vimentin (1:100, Santa Cruz Biotechnology), and slug (1:100, Abcam). The next day, cells were washed with PBS and incubated for 2 h at room temperature with Alexa fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Life Technologies, Grand Island, NY, USA) and Alexa fluor 594-conjugated goat anti-mouse secondary antibody (Invitrogen). Cells were mounted onto slides using Vectashield® mounting media with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., Burlingame, CA, USA) and viewed using confocal microscopy (LSM700, Olympus Corp., Center Valley, PA, USA).

**Immunofluorescence assays and confocal microscopy of keloid tissues**

Tissues were cut into 10 µm-thick cross sections and fixed in 4% paraformaldehyde for 20 min at room temperature. Specimens were blocked for 1 h at room temperature in 5% goat serum in PBS-T (0.1% Tween® 20 in PBS) and incubated overnight at 4°C with primary antibodies: rabbit anti-CD31 (1:100, Cell Signaling Technology), mouse anti-α-SMA (1:100, Abcam), and mouse anti-vimentin (1:100, Santa Cruz Biotechnology). Slides were then rinsed three times (for 10 min each) in PBS-T. For double-labeling experiments, fluorescein isothiocyanate-conjugated anti-rabbit (1:250, Abcam) and Texas Red-conjugated anti-mouse (1:250, Abcam) secondary antibodies were used. Slides were rinsed in PBS-T for 10 min each,
and coverslipped with Vectashield® mounting media with DAPI (Vector Laboratories). Each tissue staining was independently analyzed by two blinded investigators using a scanning confocal microscope (LSM 700, Carl Zeiss Microscopy GmbH, Jena, Germany). Ten visual fields were analyzed for colocalization of endothelial and fibroblast markers.

Statistical analyses

Paired t-tests were performed to identify significant differences between distinct categories. Differences were considered statistically significant when the $p$ value was less than 0.05.
RESULTS

Clinical features and characteristics of keloid patients

A total of 12 patients (five men and seven women) were included in this study. The demographic characteristics of the patients, including descriptions of the clinical features of their keloids, are presented in Table 1. The mean age of patients was 38.6 years for men (range: 26 to 73 years) and 32.8 years for women (ages of the two female patients were 17 and 56 years).

Wnt 3a was over-expressed in keloid tissues.

Using H&E staining, we found that keloid tissue (n=5) had a dense and excessive deposition of collagen, which extended over the clinical keloid margin into the extra-lesional dermal tissue (Figure 1A). To evaluate the Wnt 3a expression pattern in keloid tissue, we performed immunohistochemical staining using an anti-Wnt3a monoclonal antibody. Compared to extra-lesional tissue, increased Wnt 3a immunopositivity was noted in the central and peripheral regions (Figure 1B). Wnt3a expression was quantitatively measured using MetaMorph® image analysis software (Universal Image Corp.) and found to be significantly increased in keloid regions compared to extra-lesional normal tissues (p <0.01) (Figure 1C).

Expression of EndoMT-related genes in Wnt 3a-treated HDMECs

Expression of EndoMT-related genes was measured using qRT-PCR. VE-cadherin was used as a marker of vascular endothelial cells, and vimentin and slug were used as markers of myofibroblastic cells. Treatment of HDMECs with Wnt 3a (100 ng/ml) significantly reduced VE-cadherin mRNA expression and induced vimentin and slug mRNA expression (Figure 2). The mRNA levels of VE-cadherin in HDMECs were decreased by 15.2% (p <0.01; Fig. 1) compared to non-Wnt 3a-treated cells. However, vimentin and slug mRNA expression was increased by 49.2% and 56.7%, respectively, compared with Wnt 3a-treated HDMECs (p <0.01). These results suggest that long-term culture (>5 days) of HDMECs with Wnt 3a resulted in acquisition of a fibroblast-like phenotype.

Immunocytochemistry analyses of Wnt 3a-treated HDMECs
We used immunocytochemistry to examine whether Wnt 3a-treated HDMECs convert to mesenchymal phenotype. Following treatment for 5 days with Wnt 3a (100 ng/ml), the cellular morphology of HDMECs changed to sheet- and spindle-like (Figure 3A). Spindle-shaped HDMECs exhibited reduced expression of the epithelial cell marker CD31 and increased expression of the mesenchymal markers vimentin, α-SMA, and slug proteins (Figure 3B, middle panel). These results are similar to that of TGF-β-treated HDMECs (Figure 3B, lower panel), and indicate that Wnt 3a induced mesenchymal phenotypes in HDMECs. These results are also consistent with our qRT-PCR results.

**CD31 and vimentin are co-expressed in the vascular endothelium of keloid tissues**

The ages of the keloid scars in the patients ranged from 2 to 30 years. Moreover, the keloid scars were in the growth phase based on clinical features such as growth, redness, pain, itching, and consistency compared to the surrounding skin (Figure 4A and 5A). Surgical specimens of both keloid tissue and adjacent normal tissue were collected (n=12). Endothelial cells were identified by CD31 expression (green fluorescence) and mesenchymal features were identified by vimentin expression (red fluorescence), which were primarily localized in cells in the dermis of normal skin. DAPI staining was used to confirm that all stained areas were cellular. Co-expression of CD31 and vimentin was observed at the dermal vascular endothelium of keloid tissues (Figure 4B and 5B, white arrows). In addition, CD31 and vimentin co-localization was observed in five tissue samples from patients whose keloid lesions were categorized as clinically active. The other tissue samples did not show colocalization of CD31 and vimentin throughout serial sections of the entire blocks (data not shown).
DISCUSSION

EndoMT is a specific type of EMT. It is a complex biological process induced by members of the TGF-β family in which endothelial cells adopt a mesenchymal or myofibroblastic phenotype, acquire motile and contractile properties, and initiate expression of mesenchymal cell products (such as α-SMA and type I collagen). These cells also become capable of migrating into surrounding tissues. Recently, it was reported that EndoMT may also play an important role in tissue regeneration, cancer metastasis, and pathological fibrosis of organs such as the heart, lung, liver, and kidney. Additionally, previous research has suggested that microvessel endothelial cells may function in the fibrosis of hypertrophic scar development. However, few studies have focused on EndoMT as a factor in keloid pathogenesis. In our study, immunofluorescence analyses revealed co-localization of the epithelial marker CD31 and the mesenchymal marker vimentin in the dermal vasculature of keloid tissue (Figures 4 and 5).

To investigate our hypothesis that dermal fibrosis of keloids may be of endothelial origin and that EndoMT contributes to keloid pathogenesis, we induced EndoMT in HDMECs using Wnt 3a. As demonstrated by both mRNA (Figure 2) and protein levels (Figure 3), treatment of HDMECs with Wnt 3a significantly reduced VE-cadherin and CD31 expression (endothelial cell markers) and increased vimentin, slug, and α-SMA expression (mesenchymal cell markers). Moreover, CD31 and vimentin co-localization was observed in the dermal vascular endothelium of clinically-active keloid tissues.

Many reports indicate that TGF-β, which plays a crucial role in keloids, is a potent inducer of EndoMT. Recently, the Wnt/β-catenin signaling pathway was found to play critical role in development and adult tissue homeostasis. Pathologically-activated canonical Wnt signaling has been implicated in abnormal wound repair and various fibrotic diseases, including keloids or hypertrophic scars. Activation of Wnt/β-catenin signaling in fibroblasts enhances proliferative and migratory activity, as well as matrix production. There are reports that Wnt 3a, Wnt 4, and Wnt 5a function in tissue fibrosis. Recently, canonical Wnt/β-catenin signaling was reported to be necessary for TGF-β-mediated fibrosis.

There are reports regarding the role of Wnt pathway activation in the phenotypic conversion of EMT. Moreover, Wnt/β-catenin signaling may activate expression of genes that are crucial for EndoMT in cardiac endocushion development. In our study, based on the role of Wnt 3a as a fibrotic...
molecule, we examined the association of increased Wnt 3a expression and EndoMT in keloids. Notably, in vitro and clinical data showed that Wnt 3a induced myofibroblastic features in HDMECs and mesenchymal features in endothelial cells in keloid tissues.

Among keloid tissues from 12 patients, five samples, harvested from patients with active clinical symptoms such as pain, itching, and growth, exhibited co-expression of CD31 and vimentin in the dermal vasculature. Several studies have shown that co-expression of mesenchymal and endothelial cell markers is a sign of early-stage EndoMT. Additionally, EMT might be highly localized and transient or limited to specific steps in cancer, which further complicates clinical analyses of this process. Therefore, the negative results in seven stable keloid samples can be explained by early and transient expression of these markers during EndoMT. Specifically, if the HDMEC conversion to mesenchymal phenotype is sustained at the early stage, pathological dermal fibrosis (such as keloids and hypertrophic scars) could develop. All patients received surgical treatment for keloids and hypertrophic scars, and recurrence of keloids was observed in four patients. Among these four patients (K7, 9, 10, and 12 in Table 1), three had clinically active keloids and evidence of EndoMT. However, one patient had a stable keloid and less symptomatic characteristics. These results suggest that clinical activity and keloid severity may be related to EndoMT. However, limitations of this study include the relatively small sample size and that age- and sex-matched sampling was not accomplished. Future research efforts should incorporate serial observations with larger sample sizes and age- and sex-matched samples.

Chronic and dysregulated inflammation plays a major role in the development of keloids and hypertrophic scars. Significant changes in macrophage and mast cell numbers, as well as abnormal distribution of these cells, have been reported in keloids. TGF-β, the pro-fibrotic cytokine, is secreted by tissue-infiltrating chronic inflammatory cells and initiates EndoMT. Fibroblasts that are phenotypically altered by EndoMT then migrate into the interstitium and participate in tissue fibrosis. However, keloids have complex etiologies and multifactorial predisposing factors, including skin tension, wound infection, racial differences, and genetic predisposition. TGF-β alone induces a scattering, spindle-like cell phenotype that is fully reversible after TGF-β withdrawal. Aberrant and continuous signaling from pathways such as TGF-β, Wnt/β-catenin, and other growth factors are involved in keloid pathogenesis.

We examined in vitro EndoMT induction mediated by Wnt 3a in HDMECs and found that Wnt 3a significantly reduced VE-cadherin mRNA expression and induced vimentin and slug mRNA expression.
We used immunofluorescence to confirm co-localization of both endothelial and mesenchymal cell markers in the dermal vasculature, indicating the presence of EndoMT in active keloid tissues. Therefore, we suggest that transient conversion of HDMECs to a mesenchymal phenotype may contribute to dermal fibrosis of the keloids and hypertrophic scars.
Acknowledgments

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Conflict of Interest

No authors have conflicts of interest that could potentially influence the described research.
Abbreviations

BSA, bovine serum albumin
CD31, cluster of differentiation 31
DAPI, 4',6-diamidino-2-phenylindole
ECM, extracellular matrix
EGF, human epidermal growth factor
EMT, epithelial-to-mesenchymal transition
EndoMT, endothelial-to-mesenchymal transition
FBS, fetal bovine serum
FGF-b, human fibroblast growth factor-b
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
HDMECs, human dermal microvascular endothelial cells
IGF-1, insulin growth factor-1
KF, Keloid fibroblasts
MMPs, matrix metalloproteinases
PBS, phosphate-buffered saline
Slug, Snail family transcription factor
TGF-β, transforming growth factor-β
TIMP, tissue inhibitors of metalloproteinases
VE-cadherin, vascular endothelial cadherin
VEGF, vascular endothelial growth factor
Wnt, wingless protein
α-SMA, α-smooth muscle actin
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Table 1. Patient demographic information and descriptions of their keloids

<table>
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Abbreviations: Lt, left; Rt, right; EndoMT, endothelial-to-mesenchymal transition, Lat, lateral; Ant, anterior
Figure 1. Histological analyses of keloids and adjacent normal dermal tissues. (A) Using H&E staining, we found that keloid tissue had a dense and excessive deposition of collagen. (B) Immunohistochemical studies (n=7) showed that Wnt 3a expression within the deep dermal layer of keloid tissues was higher compared to the normal adjacent dermis. (C) Wnt 3a expression was semi-quantitatively measured using MetaMorph® image analysis software (Universal Image Corp.) and was significantly increased compared to the adjacent normal area. (200×, **p <0.01)

Figure 2. Effects of Wnt 3a on HDMEC mRNA expression. HDMECs were treated with Wnt 3a (100 ng/ml) daily for 5 days, and phenotypic changes were examined using qRT-PCR as described in Materials and Methods. Treatment of HDMECs with Wnt 3a reduced VE-cadherin mRNA expression and induced vimentin and slug mRNA expression. Data represent four independent experiments and are the mean ± SD of four per group. (*p <0.05; **p <0.01)

Figure 3. Phenotypic alterations of HDMECs following Wnt 3a (100 ng/ml) and TGF-β (10 ng/ml) treatment. (A) Morphologic changes of HDMECs after treatment with Wnt 3a for 5 days. The transformation of endothelial cells to a spindle-shaped phenotype was examined (400×). (B) EndoMT was demonstrated by the expression of α-SMA, vimentin, and slug (red immunofluorescence) in cells cultured for 5 days. The expression of CD31 (green), which is predominantly in endothelial cells, decreased following Wnt 3a treatment. Nuclei were stained blue with DAPI. Wnt 3a (middle panel), similar to TGF-β (lower panel), induced myofibroblastic features in HDVECs.

Figure 4. EndoMT characteristics in keloid tissues. Co-localization of CD31 and mesenchymal markers such as vimentin was observed in the dermal vascular endothelium of keloid tissues. Immunofluorescence staining of CD31 (green) and vimentin (red) in cryosections of keloid tissues from a 23-year-old woman who had a keloid scar on her earlobe (patient K1). (A) This patient reported keloid growth, redness, and moderate tenderness, which are indicative of clinically-active keloids. (B) Co-expression of CD31 and vimentin was observed in the peripheral margin of this keloid tissue. White arrows indicate CD31/vimentin double-positive cells (200×).
**Figure 5.** EndoMT characteristics in keloid tissues. Co-localization of CD31 (green immunofluorescence) and mesenchymal markers such as vimentin (red) was observed in the dermal vascular endothelium of keloid tissues, indicated by the white arrow. (A) A 50-year-old man had an elevated and contractile keloid scar on the anterior chest. The patient suffered from pain, tenderness, and a tightening sensation, symptoms indicative of clinically-active keloids. (B) The panels are merged images in which the expression of CD31 is green and the expression of vimentin is red. Nuclei were stained with DAPI (blue). Co-expression of CD31 and vimentin was observed in the dermal vascular endothelium of the keloid scar (white arrows) (400×). The patients had the keloid surgically excised, but it recurred after 6 months.
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254x400mm (195 x 195 DPI)
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