Role of Epidermal Vascular Endothelial Growth Factor in Melanosome Transfer through Upregulation of Protease-Activated Receptor-2

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Role of Epidermal Vascular Endothelial Growth Factor in Melanosome Transfer through Upregulation of Protease-Activated Receptor-2

Directed by Professor Seung Hun Lee

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This certifies that the Master's Thesis of
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<ABSTRACT>

Role of Epidermal Vascular Endothelial Growth Factor in Melanosome Transfer through Upregulation of Protease-Activated Receptor-2

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Melasma is a common acquired symmetrical hypermelanosis characterized by irregular light to dark brown macules and patches on sun-exposed areas of the skin. Histologically, melanosomes are increased in number and more widely dispersed in the keratinocytes of the lesional skin than perilesional normal skin. The major etiological factors are genetic background, exposure to ultraviolet radiation, pregnancy, hormonal therapies, cosmetics, phototoxic drugs, and antiseizure medications, although the pathogenesis of melasma is not fully understood. Recent studies found that epidermal vascular endothelial growth factor (VEGF) and dermal vessels are increased in melasma lesional skin compared to perilesional normal skin and suggested a possible role of dermal vessels and epidermal VEGF in the pathogenesis of melasma. Protease-activated receptor-2 (PAR-2) is seven transmembrane G protein-coupled receptors that are activated by serine protease cleavage. In the epidermis, PAR-2 is expressed in keratinocytes, but not in melanocytes, and is shown to involve in melanosome phagocytosis of keratinocytes. In our study, the melasma patient, who showed more prominent telangiectatic erythema confined to the hyperpigmented lesion, showed more intense PAR-2 staining intensity at melasma lesional skin. In

addition, positive correlation between PAR-2 and VEGF staining intensity was also observed in melasma lesional skin. To determine whether the expression of PAR-2 in the keratinocytes is regulated by VEGF, PAR-2 expression was measured after VEGF treatment in the mouse model and the cocultures using melanocytes and keratinocytes at 1:10 ratio. Intradermal injection of VEGF increased PAR-2 mRNA level of mouse skin. In cocultured cells, VEGF treatment increased mRNA and protein levels of PAR-2. In addition, PAR-2 agonist peptide and VEGF treatment also increased melanosome content of keratinocyte in cocultured melanocytes and keratinocytes. From these results, epidermal VEGF could be considered as one of the regulating molecule of the PAR-2 expression in epidermis and increased epidermal VEGF may have a role in melanosome transfer through upregulation of PAR-2 signaling.

Key words: melanosome transfer, VEGF, PAR-2

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I. INTRODUCTION

Melasma is a common acquired symmetrical hypermelanosis characterized by irregular light to dark brown macules and patches on sun-exposed areas of the skin. Histologically, melanosomes are increased in number and more widely dispersed in the keratinocytes of the lesional skin than perilesional normal skin. The number of epidermal melanocytes also increased in melasma lesional skin[1]. The major etiological factors are genetic background, exposure to ultraviolet radiation, pregnancy, hormonal therapies, cosmetics, phototoxic drugs, and antiseizure medications[2], although the pathogenesis of melasma is not fully understood. Recent studies have suggested a possible role of dermal vessels and vascular endothelial growth factor (VEGF) in the pathogenesis of melasma. Epidermal VEGF and dermal vessels are increased in melasma lesional skin compared to perilesional normal skin[3]. Copper bromide laser, which coagulates the vessels by inducing thermal energy and improves facial telangiectasia[4], induces not only clinical improvement of melasma but also decreased VEGF expression on the melasma lesional skin[5].

VEGF is an important cytokine in regulation of endothelial cell proliferation, migration and permeability during embryonic vasculogenesis and

physiological and pathological angiogenesis[6,7]. Recently, VEGF has been reported to have instrumental role in various conditions, such as wound healing, muscle growth, estrous cycle, lymphangiogenesis, formation of malignant tumor, and various inflammatory diseases[8]. Human VEGF is a homodimeric 36-46 kDa protein consisting of six differentially spliced variants, such as VEGF 121, VEGF 145, VEGF 165, VEGF 183, VEGF 189 and VEGF 206[9-12]. Recent reports revealed that VEGF receptors are expressed not only on the endothelial cells but also on the non-endothelial cell types, such as keratinocytes and melanocytes[13,14]. Among the various kinds of VEGF, VEGF 121 and VEGF 165 are synthesized and secreted mainly in normal keratinocyte[9,15]. Epidermal VEGF, which is thought to be constitutively produced by keratinocytes, is up-regulated in various skin conditions, such as psoriasis, wound healing, atopic dermatitis and other states of increased skin angiogenesis[16-18]. Based on those findings, increased VEGF in various skin conditions seems to have a role in the pathogenesis by autocrine or paracrine mode among keratinocytes and other types of cell in the skin.

Melanocyte, which synthesize the melanin, and keratinocyte, which receive and distribute melanin, are important cellular components in human skin pigmentation[19]. Melanosomes are specific melanin-containing intracellular organells, which share several features with lysosomes, are synthesized in melanocytes, and move from the perinuclear area to plasma membrane of melanocytes as they become more melanized. Melanosomes are classified into four distinct stages according to their degree of maturation. In maturation of melanosome, enzymatic components of melanosme, such as tyrosinase, tyrosinase-related protein 1, and dopachrome tautomerase, cooperate to synthesize two distinct types of melanins: black-brown eumelanins and yellow-reddish pheomelanins[20]. Then, matured melanosomes are transferred from melanocytes to keratinocytes by undetermined mechanism. Various hypothesis have been proposed to explain melanosome transfer, including

exocytosis, cytophagocytosis, fusion, and membrane vesicle transport[21]. Numerous factors are suggested to involve in melanosome transfer. Among these factors, Protease-activated receptor-2 (PAR-2) is relatively well known factor which involve melanosome transfer.

PAR-2 is a member of the PAR family consisted of four PARs, designated PAR1-4[22-25]. These are seven transmembrane G protein-coupled receptors that are activated by serine protease cleavage. Upon cleavage within the N-terminal extracellular part of the receptor, a peptide, which acts as a tethered ligand, activate receptor signaling[23,26,27]. Serine proteases, including thrombin, trypsin, and cathepsin G, could activate PARs, but thrombin could not activate PAR-2. PAR-2 has been shown to be activated by the following proteases: trypsin, mast cell tryptase, factor Xa, acrosin, gingipain, and neuronal serine proteases[28-30]. In addition to PAR-2 activation by protease cleavage, PAR-2 could also be activated by synthetic peptides that correspond to the N-terminal sequence of tethered ligand. PAR-2 is expressed in various tissues, including the gastrointestinal tract, pancreas, kidney, liver, lung, cardiovascular system, ovary, eye, brain, skin and in various types of cells, including the epithelial cells, endothelial cells, smooth muscle cells, neutrophils, neurons, and T cells[28,31]. PAR-2 activation in different tissues induces various physiological effects, including pancreatic and salivary exocrine secretion[32], aorta ring relaxation[33], and the secretion of pro-inflammatory cytokines from keratinocyte[34,35]. In the epidermis, PAR-2 is expressed in keratinocyte[36], but not in melanocyte[37,38], and is shown to involve in melanosome phagocytosis of keratinocytes[38] and epidermal permeability barrier homeostasis[39]. Some studies reported that pigmentation is regulated by PAR-2 signaling both in vitro and in vivo animal models including human skin[40-43]. In addition, downregulated PAR-2 expression was reported in vitiligo lesional skin, which is associated to melanin loss in keratinocytes, compared to perilesional normal skin[44].

Therefore, we hypothesized that PAR-2 has a role not only in hypopigmented skin disorder but also in hyperpigmented skin disorders, such as melasma, through upregulation of melanosome transfer. To test this hypothesis, a comparision of VEGF, PAR-2 expression, intensity of clinical telangiectatic erythema of melasma lesion, and melanin pigmentation among melasma lesional skin was done by using immunohistochemical staining and clinical photograph. To determine the relation between VEGF and PAR-2 expression, PAR-2 expression was measured after different amounts and durations of VEGF treatment in the mouse model and the cocultured mealanocytes and keratinocytes. Amount of melanin in keratinocyte also measured after VEGF and PAR-2 agonist peptide treatment in cocultured melanocytes and keratinocytes by electron microscope (EM).

II. MATERIALS AND METHODS

1. Antibodies and reagent

Rabbit primary antibodies, which were specific for human PAR-2 and VEGF, were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., CA, USA). Secondary goat anti-rabbit IgG-horseradish peroxidase and diaminobenzine were purchased from DakoCytomation (Glostrup, Denmark). Human primary keratinocytes and melanocytes were purchased from Gibco (Gibco Invitrogen corporation, Pasley, UK). Mouse and Human VEGF (VEGF-A_{165} for human and VEGF-A_{164} for mouse) were purchased from R&D Systems, Inc (R&D Systems, Inc., MN, USA). Human PAR-2 agonist peptide (AP) SLIGKV and scrambled control peptide ISLLRG were synthesized at Peptron, Inc (Peptron, Inc, Daejeon, Korea). All RT-PCR reagents were purchased from Invitrogen (Invitrogen Life Technologies, Carlsbad, CA, USA). All western blot reagents were purchased from Invitrogen (Invitrogen Life Technologies, Carlsbad, CA, USA). All reagents for immunohistochemistry were purchased from DakoCytomation (Glostrup, Denmark).

2. Patients

19 melasma patients, who were made diagnosis of melasma by histopathologic evaluation, were retrospectively evaluated for clinical telangiectatic erythema and PAR-2 staining intensity. After written informed consent was obtained, nine Korean patients (seven women and two men; mean age, 43.3 years; ranged, 34~56 years) clinically diagnosed with melasma were enrolled in this study between April 2010 and September 2010. Diagnosis of melasma was made through

physical examinations and histological examinations. Patients, who were treated with topical bleaching agents or topical steroids or phototherapy within 2 weeks before study entry, were excluded from the study. Patients who were treated with dermatological procedures, such as chemical peeling, laser treatment, and dermabrasion within 3 months before study entry were also been excluded from the study. The study protocol was approved by the Institutional Review Board of Gangnam Severance hospital. This study was conducted according to the Declaration of Helsinki Principles.

3. Animals

Four female C57BL/6 mice that were 5 weeks old were housed in a standard environment with temperature maintained at 22 ± 0.5, relative humidity at 60 ± 5% and a 12h/12h light and dark cycle.

4. Skin biopsy and tissue preparation

A 2mm punch biopsy specimen was obtained from the melasma lesional skin. Biopsy specimens from three patients were embedded in paraffin and six patients were prepared for frozen section.

5. Immunohistochemical staining of PAR-2 and VEGF

Frozen and paraffin-embedded punch biopsy tissues were cut into 4 μm sections. Both frozen and deparaffinized paraffin sections were processed for rehydration and air dried. Later the sections were incubated with a peroxidase-blocking reagent for 10 minutes in order to prevent endogenous peroxidase activity. After blocking the non-specific antibody binding by incubation with a serum free protein for 10 min at room temperature, the primary antibodies (1:300 dilution) of PAR-2 (Santa Cruz Biotechnology, Inc., CA, USA) and VEGF (Santa Cruz

Biotechnology, Inc., CA, USA) were applied for 1 hour at room temperature. Sections were then soaked with an horseradish peroxidase(HRP)-conjugated secondary antibody for 30 min at room temperature, staining for these proteins was detected using NovaRED (Vector, Burlingame, CA, USA) as a substrate. Between steps, the slides were rinsed for 10 minutes in 0.05M Tri-HCl buffer with 0.01% Triton-X-100. All sections were lightly counterstained with hematoxylin. Fontana-Masson staining was done to visualize melanin pigment.

6. Cell culture

Primary keratinocytes were maintained in Epilife (Gibco Invitrogen corporation, Pasley, UK) supplemented with human keratinocyte growth supplement (Gibco Invitrogen corporation, Pasley, UK) and primary melanocytes were maintained in Medium 254 (Gibco Invitrogen corporation, Pasley, UK) supplemented with human melanocyte growth supplement (Gibco Invitrogen corporation, Pasley, UK). To investigate the role of VEGF in the upregulation of PAR-2 expression and melanosome transfer from melanocyte to keratinocyte, co-culture experiment was established with human primary keratinocytes and melanocytes. In primary co-cultures, melanocytes and keratinocytes were seeded with a ratio of 1:10 and maintained in Medium 254 supplemented with human melanocyte growth supplement and Epilife with human keratinocyte growth supplement. Cells were plated at a density of 7.5×10^5 cells on the 60-mm dish.

7. VEGF and PAR-2 agonist peptide treatment

To determine whether PAR-2 expressions are regulated by VEGF, the expression of PAR-2 both at mRNA and protein levels of the co-culture

cells were measured at different time points after different doses of VEGF treatment. The effect of VEGF (100 nM) on the co-culture cells was determined by measuring PAR-2 expression at mRNA level and protein level using real-time quantative PCR (RT-PCR) at 3, 6, 12, 24h, and using western blotting at 6, 12, 24, 48h. Moreover the PAR-2 expression at mRNA and protein level after treating with different doses of VEGF (25, 50, 100, and 200nM) to co-culture cells for 48 hours was also determined.

In mouse model, intradermal injection of VEGF (1, 4, and 10μM) and PBS at dermis was done once a day for 3 days. PAR-2 mRNA level of mouse skin was measured using RT-PCR at one day after last injection.

To determine whether melanosome transfer from melanocytes to keratinocytes is upregulated by PAR-2 activation and VEGF, we counted the number of melanosome in the cytoplasm of keratinocytes after VEGF and PAR-2 AP treatment to cocultured melanocytes and keratinocytes at 1:10 ratio using EM. Coculted cells are differently treated: 1) treating with PAR-2 AP (100 μM) for 48 hours after VEGF (100 nM) treatment for 48 hours, 2) treating with scrambled control peptide (100 μM) for 48 hours after VEGF (100 nM) treatment for 48 hours, 3) treating with PAR-2 AP (100 μM) for 48 hours after 48 hours without treatment, 4) treating with scrambled control peptide (100 μM) for 48 hours after 48 hours without treatment.

8. RT-PCR

Total RNA was isolated from each co-culture cells and mouse skin after VEGF treatment using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). One microgram of total RNA was reverse-transcribed with AMV reverse transcriptase (TaKaRa BIO INC.

Shiga, Japan). Pairs of primer for amplification of PAR-2 were designed using Primer Blast of NCBI. Real-time PCR amplifications were performed using Express SYBR GreenER Supermix with Premixed ROX (Invitrogen Life Technologies, Carlsbad, CA, USA) in a ABI 7300 (Applied Biosystems, CA, USA) following the manufacturer's protocol. The primer used for the RT-PCR of human and mouse PAR-2 is shown in Table I.

9. Western blot

Equal amounts of protein from each co-culture cells were loaded onto 12% SDS-polyacrylamide gels. After electrophoresis in slab gels, proteins were transferred onto nitrocellulose membranes (Invitrogen Life Technologies, Carlsbad, CA, USA) in a buffer containing 20 mM Tris, 20 mM glycine, and 20% methanol at a constant voltage of 100 V for 25 min. Residual binding sites were blocked, and the membrane was incubated with a PAR-2 monoclonal primary antibody (SAM-11; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a 1:1000 dilution. This antibody is directed to amino acids 37 to 50 (SLIGKVDGTSHVTG) of hPAR2. After further washing, the membrane was incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc., CA) at a 1:3000 dilution for 1 h. The membrane was developed using ECL Plus, and antibody detection was conducted with HyperFilm (GE Healthcare)

10. Electron microscope

After 96 hours of treatment, cocultured human primary keratinocytes and melanocytes were washed three times in PBS (pH 7.4) and collected in pellet using trypsin-EDTA. Pre-fixation was done with 2% glutaraldehyde in the same buffer for 24 hours at 4°C. After washing in

0.1M phosphate buffer for 2 hours, samples were post-fixed in 1% osmium tetroxide for 2 hours. After washing in 0.1M phosphate buffer for 10 minutes, dehydration and 10 minutes of infiltration on propylene oxide were done. Samples were embedded in 1:1 mixture of EPON mixture and propylene oxide at EM oven. After sectioning to 0.25μm semi-thin section using Ultra-microtome, staining with 1% Toluidine blue was done. After finding the area, where the cultured cells are seen, using light microscope, retrimming and sectioning to 80nm ultrathin section were done. Ultrathin sections were then stained with aqueous solutions of uranyl acetate (6%) and lead citrate and viewed and photographed in a JEOL JEM-1011 transmission EM. Ten photographes of each section were obtained for analysis. Number of melanosomes in keratinocyte was measured using Image J program.

11. Statistical analysis

Data were expressed as mean ± SE. Statistical significance ($P<0.05$ for all analysis) was assessed by ANOVA using Instat 3.05 (Graph-Pad Software, San Diego, CA, USA).

Table I. Primers used in the RT-PCR reactions

hPAR-2	5'-TGCTAGCAGCCTCTCTCTCC-3'	20mer
	5'-CCAGTGAGGACAGATGCAGA-3'	20mer
mPAR-2[45]	5’-TGGCCATTGGAGTCTTCCTGTT-3’	22mer
	5’-TAGCCCTCTGCCTTTTCTTCTC-3’	22mer

III. RESULTS

1. Melasma patient with prominent telangiectatic erythema on the lesion showed increased PAR-2 staining intensity compared to patient with less prominent telangiectatic erythema on the lesion.

In this study, nineteen Korean melasma patients were retrospectively evaluated. These patients showed light to dark brown irregular macules and patches mainly on their both cheeks. Telangiectatic erythema, which confined to melasma lesion, was seen variably. The telangiectatic erythmea of melasma patients was evaluated based on patient's photograph and graded the intensity of erythema with 1 to 6 scale. The PAR-2 expression of melasma lesional skin was measured by using immunohistochemical staining and graded intensity of PAR-2 staining with 1 to 4 scale. Patients, who showed clinically prominent telangiectatic erythema confined to hyperpigmented patches, revealed more intense PAR-2 staining intensity at their melasma lesional skin compared to patients who do not show prominent telangiectatic erythema. (Figure 1)

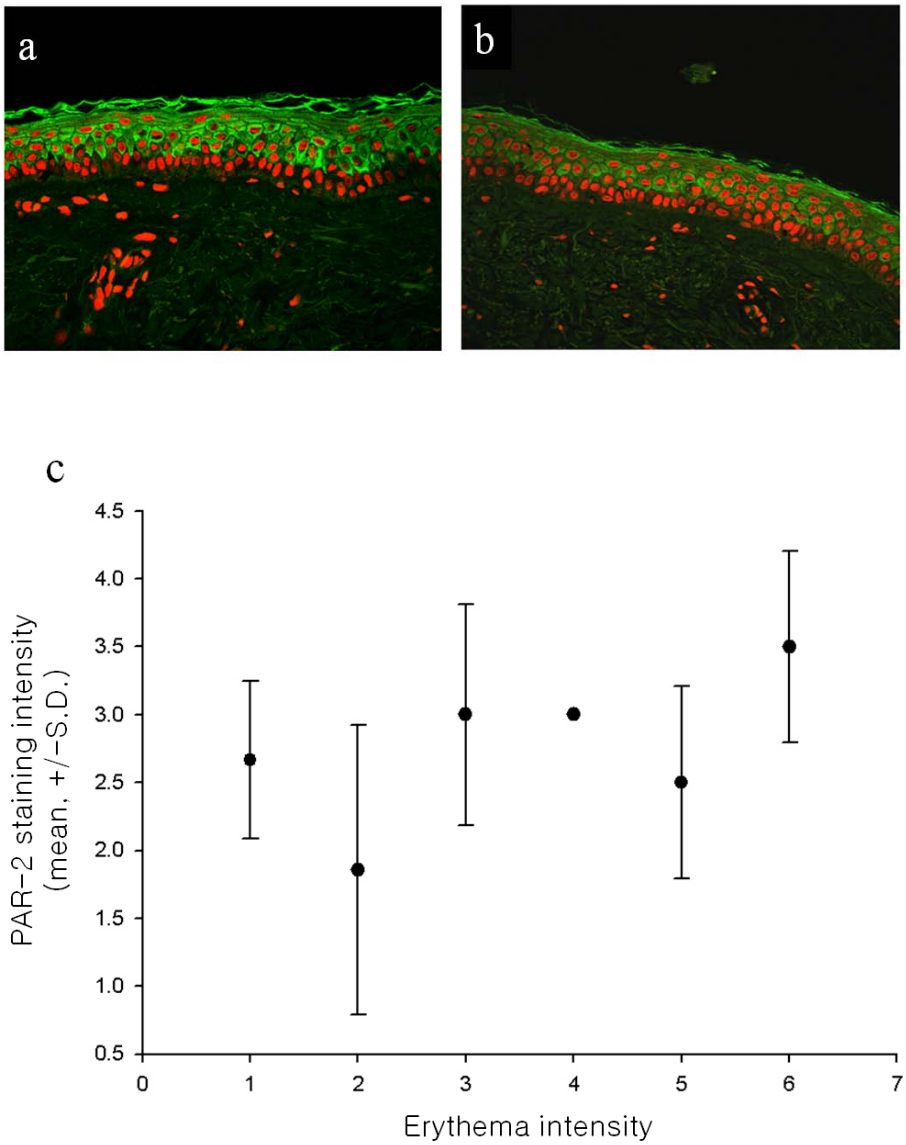

Figure 1. Comparison between the intensity of telangiectatic erythema and PAR-2 immunofluorescence staining of melasma patient's lesional skin (a) On the lesional skin of melasma patient who showed prominent telangiectatic erythema, increased PAR-2 immunofluorescence staining intensity was seen at the keratinocyte of suprabasal layer. (b) PAR-2 immunofluorescence

staining of patient with less prominent telangiectatic erythema showed decreased intensity compared to melasma patient with prominent telangiectatic erythema. (c) Patient with prominent telangiectatic erythema on the melasma lesion showed increased PAR-2 staining intensity compared to melasma patient with less prominent telangiectatic erythema

2. Staining intensity of PAR-2 and VEGF showed positive correlation at the lesional skin of melasma patients.

To measure the expression of VEGF and PAR-2 on the melasma lesional skin, immunohistochemical staining for VEGF and PAR-2 was done. Punch biopsy specimen from melasma lesional skin showed increased pigmentation not only basal layer but also suprabasal layer on Fontana-Masson staining. Although staining intensity of PAR-2 and VEGF was variable between the patients, positive correlation between PAR-2 and VEGF staining intensity was observe (Figure 2).

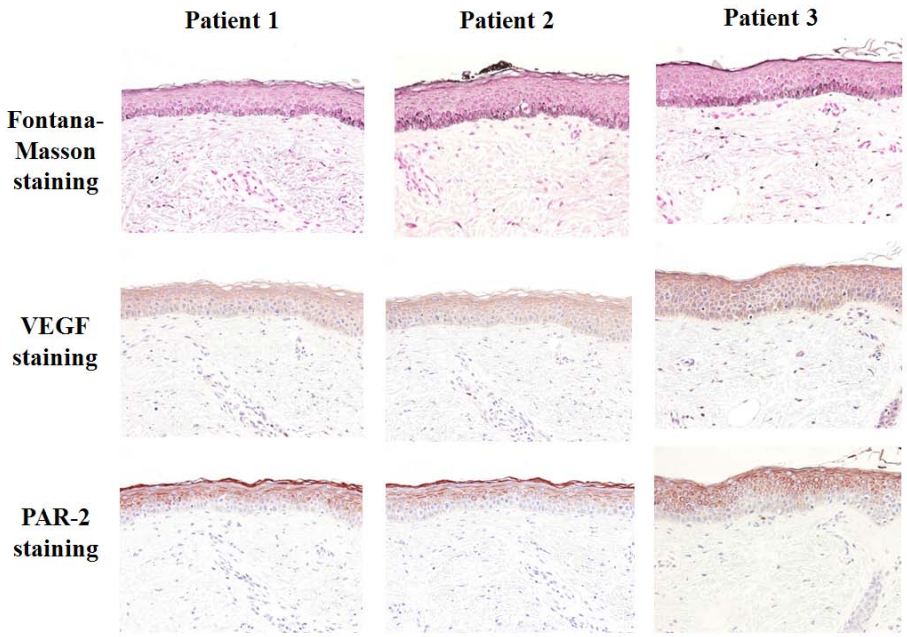

Figure 2. Fontana-Masson staining and immunohistochemical staining for PAR-2 and VEGF of melasma lesional skin. Fontana-Masson staining of melasma lesional skin showed increased pigmentation not only basal layer but also suprabasal layer. On VEGF staining, whole epidermal layers are

stained with VEGF, but staining intensity was most intense at granular layer. On PAR-2 staining, keratinocytes of suprabasal layers are stained with PAR-2 antibody. Although intensity of PAR-2 and VEGF staining was variable, positive correlation between PAR-2 and VEGF was observed.

3. mRNA levels of PAR-2 in co-cultured keratinocytes and melanocytes in different increased after different durations of VEGF treatment.

To investigate the relationship between PAR-2 expression and VEGF treatment, 100 nM of VEGF was treated on the co-cultured cells with melanocytes and keratinocytes at 1:10 ratio, and measure the mRNA expression levels of PAR-2 at different time points (3, 6, 12 and, 12h) using RT PCR. PAR-2 mRNA levels of the co-cultured cells increased 3 hours after 100 nM of VEGF treatment compared to control. After 3 hours of VEGF treatment, mRNA levels of PAR-2 increased about 1.5-fold, which was statistically significant (Figure 3).

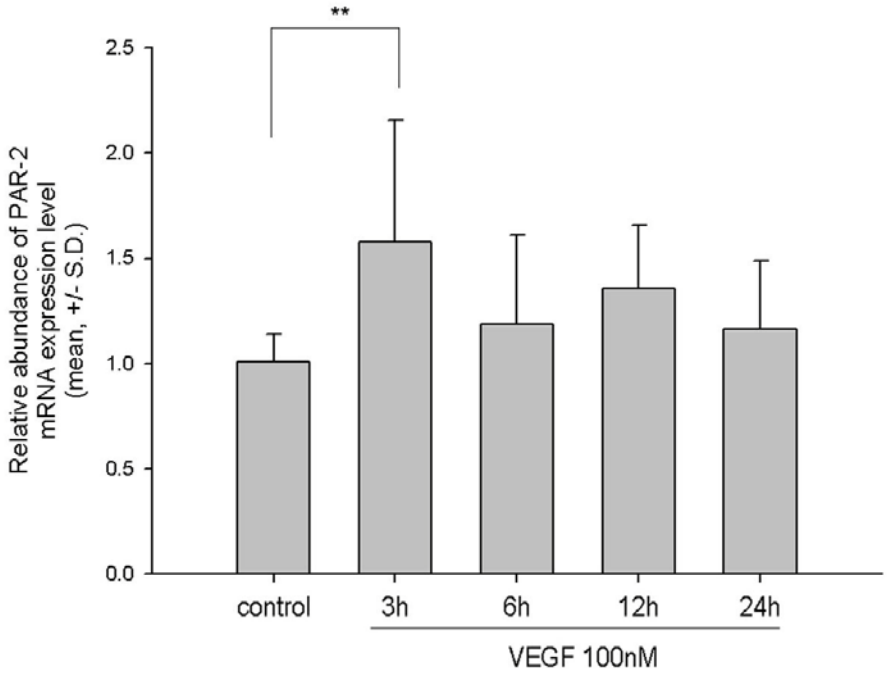

Figure 3. PAR-2 mRNA levels of co-cultured melanocytes and keratinocytesis at 1:10 ratio increased after different durations of VEGF treatment. VEGF (100 nM) was treated on co-cultured cells with melanocytes and keratinocytes at 1:10 ratio. The mRNA levels of PAR-2 at different time points (3, 6, 12 and, 24 hours) were measured using RT-PCR. mRNA of PAR-2 increased about 1.5-fold after 3 hours of VEGF treatment (100 nM), which was statistically significant. ** $p<0.01$.

4. VEGF increases mRNA levels of PAR-2 in co-cultured keratinocytes and melanocytes at different concentrations.

To investigate the relationship between PAR-2 expression and VEGF, different doses of VEGF were treated for 24 hours on the co-cultured cells with melanocytes and keratinocytes at 1:10 ratio, and the mRNA levels were measured. PAR-2 mRNA levels of co-cultured cells were increased after VEGF treatment from 100nM compared to control. mRNA levels of PAR-2 are significantly increased after treatment with 100 nM and 200 nM of VEGF (Figure 4).

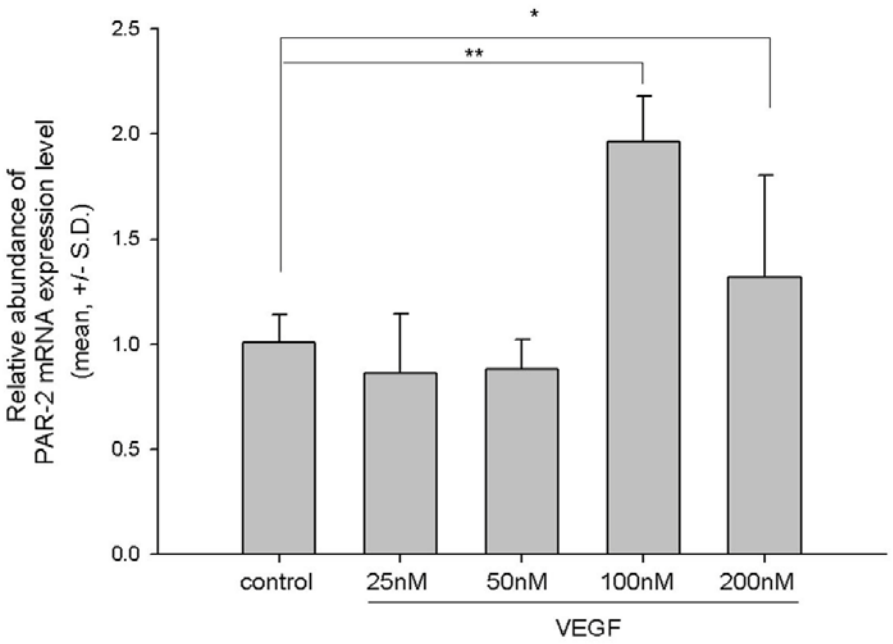

Figure 4. PAR-2 mRNA levels of co-cultured melanocytes and keratinocytes at 1:10 ratio increased after different concentrations of VEGF treatment. VEGF was treated with different doses (25, 50, 100 and, 200 nM) on co-cultured melanocytes and keratinocytes at 1:10 ratio. The mRNA levels of PAR-2 at 24 hours after VEGF treatment were significantly increased after 100 nM and 200 nM of VEGF treatment. *$p<0.05$, **$p<0.01$.

5. VEGF increases protein levels of PAR-2 in co-cultured keratinocytes and melanocytes.

To investigate the relationship between VEGF and PAR-2 expression, the protein levels of PAR-2 after VEGF treatment was measured using western blot. Protein levels of PAR-2 are increased after VEGF treatment (Figure 5a and 5b).

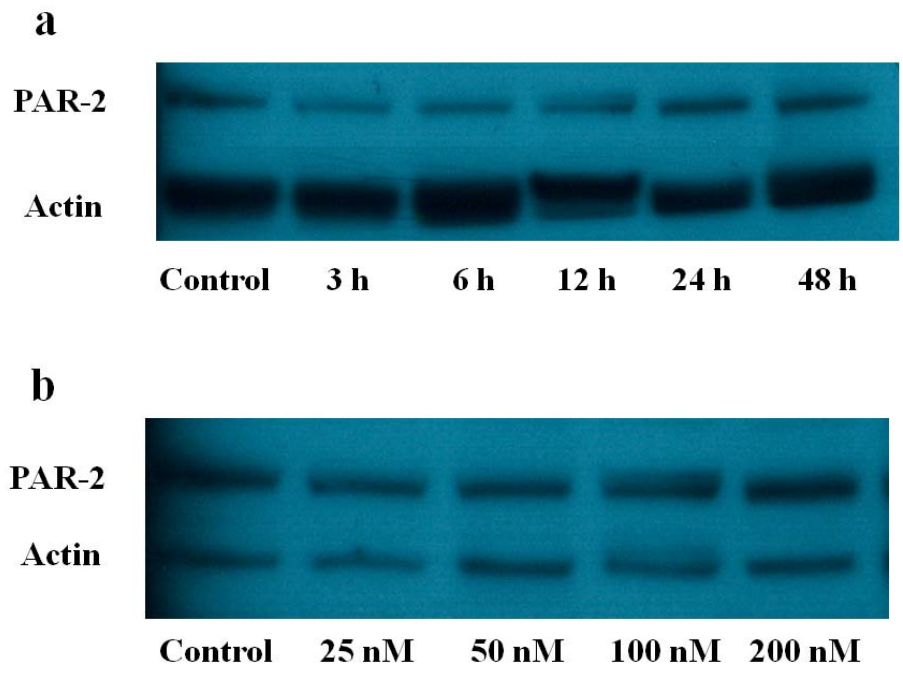

Figure 5. PAR-2 protein levels of co-cultured melanocytes and keratinocytesis at 1:10 ratio increased after VEGF treatment. VEGF treatment with different doses and durations on the co-cultured cells with melanocytes and keratinocytes at 1:10 ratio was measured using western blot. (a) Protein levels of PAR-2 are significantly increased at 24 hours and 48 hours of VEGF treatment compared to control. (b) Protein levels of

PAR-2 are increased after 100nM and 200nM of VEGF treatment compared to control.

6. Administration of intracutaneous VEGF increases mRNA levels of PAR-2 in mouse model.

To verify the effect of VEGF in upregulation of PAR-2 expression in keratinocyte, intradermal injection of VEGF-A164 (1, 4, and 10μM) and PBS was done on C57BL/6 mice once a day for 3 days. PAR-2 mRNA levels of mouse skin were measured using RT-PCR. mRNA levels of PAR-2 were significantly increased after 4 and 10 μM VEGF intradermal injection in mouse skin. (Figure 6)

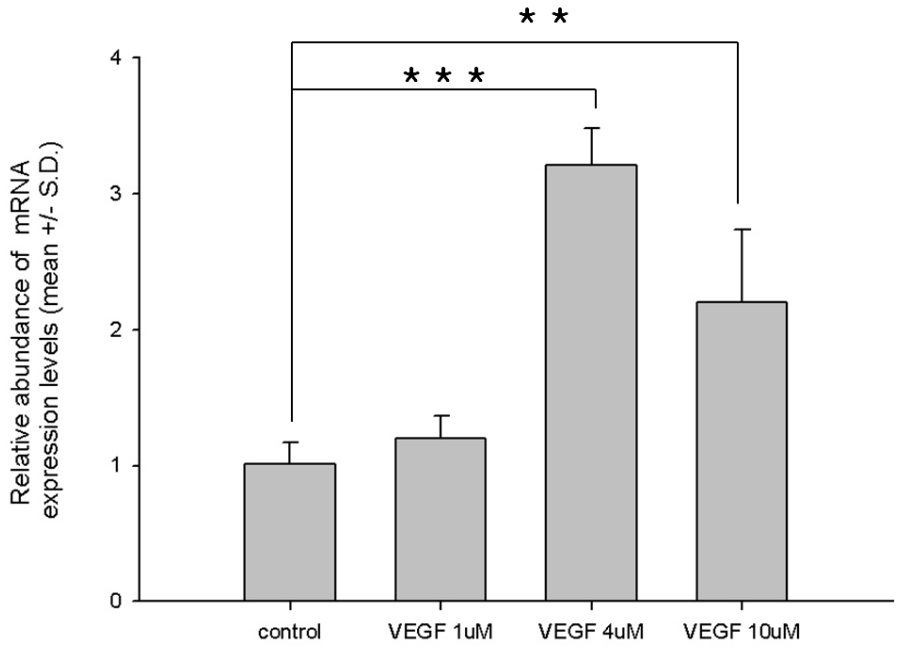

Figure 6. Administration of intracutaneous VEGF increased the expression of PAR-2 mRNA in mouse epidermis. Different doses (1, 4, and 10μM) of VEGF and PBS intradermal injection on C57BL/6 mice were done once a day for 3 days. Then, PAR-2 mRNA levels of mouse skin were measured using RT-PCR. VEGF treated mouse skin showed increased PAR-2 mRNA levels compared to control (PBS treated mouse skin). Increase of PAR-2 mRNA level at 4 and 10μM treated skin showed statistical significance compared to control. $^{**}p<0.01$; $^{***}p<0.001$.

7. PAR-2 agonist peptide increases the melanosome amounts of keratinocyte in co-cultued keratinocytes and melanocytes.

To determine the effect of VEGF and PAR-2 activation in melanosome transfer from melanocyte to keratinocyte, melanosome amount of keratinocyte are measured after VEGF and PAR-2 AP treatment in cocultured melanocytes and keratinocytes at 1:10 ratio using EM. Coculted cells are differently treated: 1) treating with PAR-2 AP (100 μM) for 48 hours after VEGF (100 nM) treatment for 48 hours, 2) treating with scrambled control peptide (100 μM) for 48 hours after VEGF (100 nM) treatment for 48 hours, 3) treating with PAR-2 AP (100 μM) for 48 hours after 48 hours without treatment, 4) treating with scrambled control peptide (100 μM) for 48 hours after 48 hours without treatment. Based on randomly taken photograph, number of melanosome was measured. Mean number of melanosome in keratinocyte area are 5.1 in SP treated cocultured cells, 16.6 in AP treated cocultured cells, 7.3 in VEGF and SP treated cocultured cells and 6.2 in VEGF and AP treated cocultured cells. Difference of melanosome amount between AP and SP treated cocultured cells are statistical significant (Figure 7 and 8).

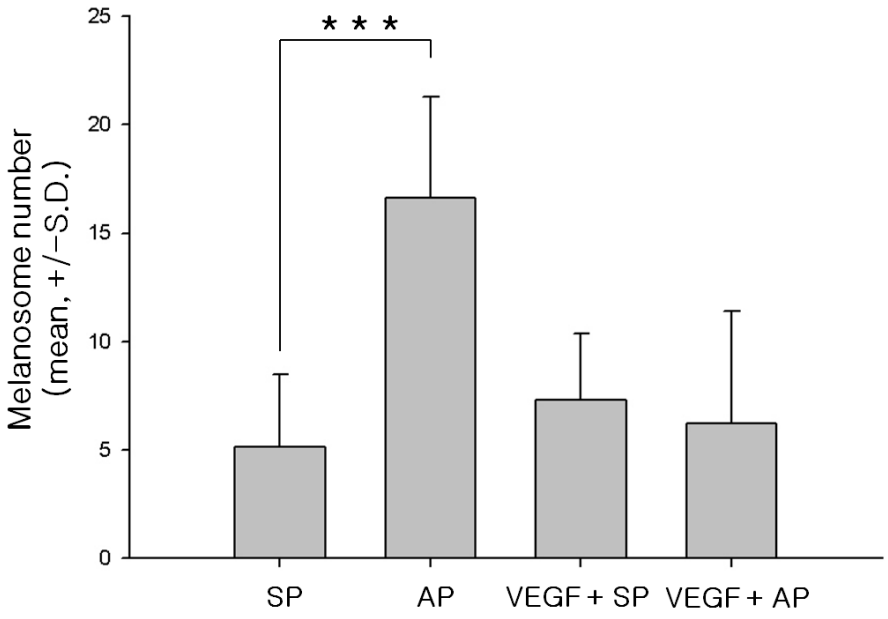

Figure 7. Amounts of melanosome in keratinocytes are increased after PAR-2 agonist peptides treatment in co-cultured melanocytes and keratinocytes at 1:10 ratio. Coculted cells are differently treated: 1) treating with PAR-2 AP (100 μM) for 48 hours after VEGF (100 nM) treatment for 48 hours, 2) treating with scrambled control peptide (100 μM) for 48 hours after VEGF (100 nM) treatment for 48 hours, 3) treating with PAR-2 AP (100 μM) for 48 hours after 48 hours without treatment, 4) treating with scrambled control peptide (100 μM) for 48 hours after 48 hours without treatment. Based on randomly taken photograph, number of melanosome was measured using ImageJ program. Mean number of melanosome in keratinocyte area are 5.1 in SP treated cocultured cells, 16.6 in AP treated cocultured cells, 7.3 in VEGF and SP treated cocultured cells and 6.2 in VEGF and AP treated cocultured cells. Difference of melanosome between AP and SP treated cocultured cells are statistical significant ***$p<0.001$.

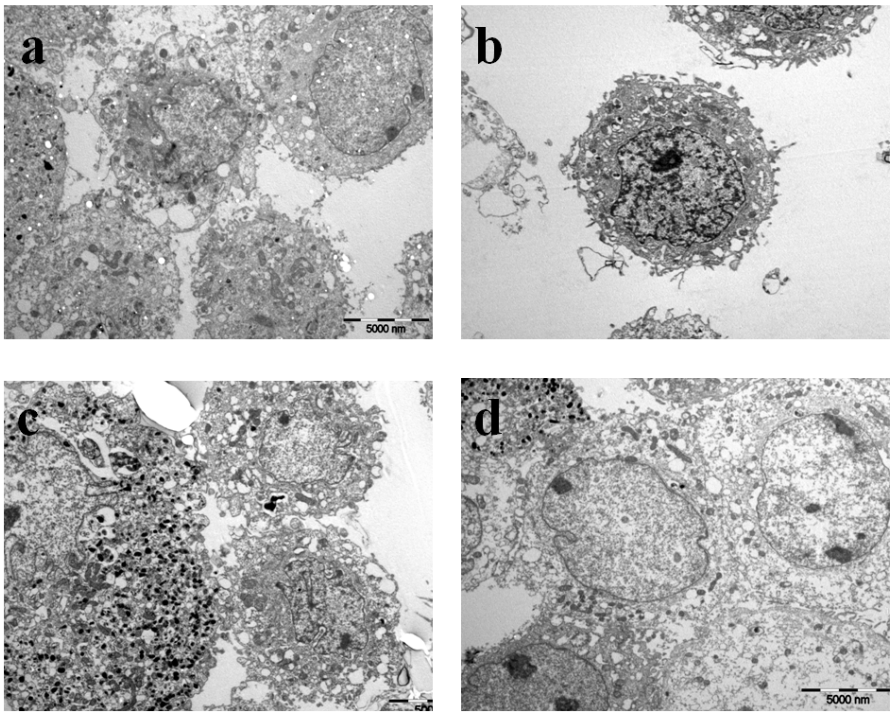

Figure 8. Electron microscope findings of co-cultured melanocytes and keratinocytes after treating different combinations of VEGF and PAR-2 agonist peptide (a) EM findings of SP treated cocultured cells. Melanosomes are rarely seen on the kratinocyte, which is located near the melanocyte. (b) Several melanosomes are seen on the cytoplasmic area of keratinocyte in AP treated cocultured cells. (c) Some melanosomes, which are located in keratinocytes, are seen in VEGF and SP treated cocultured cells. (d) VEGF and AP treated cocultured cells also showed some melanosomes in keratinocytes.

IV. DISCUSSION

Melasma lesional skin shows increased melanin pigment on the keratinocytes of whole epidermis, including the stratum corneum, whereas in normal skin the melanin pigment was mostly confined to the basal layer[1]. Melanin pigments are synthesized at melanocytes, which is normally present on the basal layer, and transferred to keratinocytes. Previous report have demonstrated that lesional melasma skin shows not only increased melanin pigment in epidermal keratinocytes but also increased epidermal VEGF expression and increased dermal vasculature compared to perilesional normal skin. Furthermore, in this study, number of vessels had a positive relationship with pigmentation in melasma lesional skin of melasma patient. And, they also found that melasma lesion is clinically characterized not only hyperpigmented patches but also telangiectatic erythema confined to the melasma lesional skin. Based on these findings, authors suggested that increased epidermal VEGF may have a role in increased dermal vasculature of melasma lesional skin and increased vascularity and epidermal VEGF are one of the major findings of melasma and may have a role in pathogenesis of melasma[3].

There are several findings which suggest that VEGF has an effect on the melanogenesis. Human melanocytes express VEGF receptors[14]. And, VEGF is known to stimulate the release of arachidonic acid and activates phospholipase A2[46]. Metabolites of arachidonic acid pathway had been reported to have a possible effect on the melanogenesis.

In our investigation, we retrospectively evaluated telangiectatic erythema and PAR-2 expression of 19 melasma patients. The telangiectatic erythmea of melasma patients was evaluated based on patient's photograph and graded the intensity of erythema with 1 to 6 scale. The PAR-2 expression of melasma lesional skin was measured by using immunohistochemical staining and graded intensity of PAR-2 staining with 1 to 4 scale. In this study, the

patients, who showed clinically prominent telangiectatic erythema confined to hyperpigmented patches, revealed more intense PAR-2 staining intensity at their melasma lesional skin compared to patients who do not show prominent telangiectatic erythema confined to hyperpigmented lesion. PAR-2 was expressed mainly on the suprabasal keratinocytes, where the increased melanin pigment is seen in histological evaluation of melasma lesional skin. In addition, PAR-2 staining intensity and VEGF staining intensity in melasma lesional skin showed positive correlation. To elucidate the relationship between epidermal VEGF and PAR-2, VEGF with different doses and durations was treated to co-cultured cells with melanocytes and keratinocytes and measured PAR-2 expression both at mRNA level and protein level using RT-PCR and western blot. Results showed that VEGF induced increased PAR-2 expression both at mRNA and protein level. To verify the role of VEGF in upregulation of PAR-2 expression, PAR-2 mRNA levels of mouse skin were measured after intradermal injection of VEGF. In this mouse model, mRNA level of PAR-2 also increased after intradermal VEGF injection. Then, to determine the effect of PAR-2 activation and VEGF on melanosome transfer from melanocyte to keratinocyte, amount of melanosome was measured after VEGF and PAR-2 AP treatment using EM. In this study, melanosome of keratinocytes is increased at VEGF and/or PAR-2 AP treated cocultured cells compared to SP treated cocultured cells. Difference between AP treated cocultured cells and SP treated cocultured cells are statistically significant, but others are not.

To date, PAR-2 activation has been investigated in various fields. Role of PAR-2 in upregulating phagocytic activity of keratinocyte are well known. But, these findings have been investigated in vitro and animal models, and it’s implications in human hyperpigmented skin disorders are not elucidated. Furthermore, expression of PAR-2 on the keratinocyte may be regulated by numerous factors, but regulating factors of PAR-2 mRNA expression are not

fully elucidated yet except ultraviolet radiation[47]. This study hypothesized that VEGF, which is observed to be increased in melasma patient lesion, but it's role in pigmentation is not known yet, may have a role in melanosome transfer, partially by upregulation of PAR-2. In our study, PAR-2 expression was more intense in melasma patient who showed more prominent telangiectatic erythema on the lesion and VEGF expression showed positive correlation with PAR-2 expression in melasma lesional skin. In addition, PAR-2 mRNA and protein levels are increased after VEGF treatment in vitro and animal model. And, amounts of melanosome are also increased in keratinocytes after VEGF and/or PAR-2 AP treatment in cocultured melanocytes and keratinocytes. Based on these findings, VEGF could be suggested to have a role in melanosome transfer partially through upregulation of PAR-2 expression. Because hyperpigmentory skin disorder, such as melasma, is divided into several subtypes, numerous factors may differently involve in the pathogenesis of the melasma. Based on our findings, VEGF may involve in melanosome transfer through upregulation of PAR-2 especially in melasma which have prominent telangiectatic erythema on the lesion.

Our study has several limitations. Number of enrolled patients in our study was too small to find statistical significance between VEGF and PAR-2 expression. Because murine epidermis is devoid of melanocytes except for follicular melanocytes present in dermis, our mouse model did not fully represent the effect of increased VEGF as human skin. Our study did not count amount of melanosome in keratinocyte which located around melanocyte. To determine the role of VEGF and PAR-2 activation in melanosome transfer from melanocyte to keratinocyte more accurately, further experiment with cell culture model, which is more identical to human skin, such as skin equivalent, are necessary.

V. CONCLUSION

The aim of this study is to identify the role of VEGF and PAR-2 in melanosome transfer. The summary of the results are described below.

1. PAR-2 expression increased in melasma lesional skin of patient who showed more prominent telangiectatic erythema on the lesion. PAR-2 expression and VEGF expression showed positive correlation in lesional skin of melasma patients.

2. VEGF upregulated PAR-2 expression in mouse skin and co-cultured cells with keratinocytes and melanocytes.

3. PAR-2 AP and VEGF increased amount of melanosome in keratinocyte of cocultured melanocytes and keratinocytes at 1:10 ratio. Difference of melanosome amount between SP treated cocultured cells and AP treated cocultured cells was statistically significant.

In conclusion, the increased epidermal VEGF may involve in melanosome transfer at least partially through upregulation of PAR-2.

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ABSTRACT(IN KOREAN)

표피 혈관내피 성장인자의 protease-activated receptor-2 발현 증가를 통한 멜라노좀 전달에 미치는 영향

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이의형

기미는 주로 여성의 일광 노출부위에 후천적으로 발생하는 과색소성 질환으로 조직학적으로 각질형성세포의 멜라닌 색소 증가 소견이 표피의 모든 층에서 관찰된다. 기미의 발생과 관하여 유전적 요인, 자외선, 호르몬 등의 요인들이 원인으로 언급되고 있으나 정확한 기전에 대해서는 아직 밝혀지지 않았다. 최근 기미 병변이 주변의 비병변에 비해 표피의 VEGF 발현과 진피의 혈관이 증가되어 있음이 보고되었으며 이러한 현상이 기미의 발생에 관련이 있을 것으로 생각되고 있다. Protease-activated receptor-2 (PAR-2)는 seven transmembrane G protein-coupled receptor로 세린 단백분해효소에 의해 활성화되어 세포 내부로 신호를 전달하게 된다. PAR-2는 다양한 장기 및 세포에 존재하는 것으로 알려졌으며 각 장기에서 다양한 역할을 하는 것으로 보고 되고 있다. 특히 표피의 각질형성세포에 존재하는 PAR-2는 피부 투과 장벽의 항상성 조절뿐만 아니라 멜라닌의 멜라닌 세포에서 각질형성세포로의 이동을 조절하는 것으로 알려져 있다.

기미 환자를 대상으로 후향적으로 진행한 연구에서 모세혈관확장을 동반한 홍반이 기미 병변 주변에 뚜렷하게 나타나는 환자에서

기미 병변 조직의 PAR-2 발현이 상대적으로 증가되어 있음을 확인하였다. 또한 기미 환자의 병변 조직에서 PAR-2의 발현과 혈관내피 성장인자의 발현간에 상관성이 관찰되었다. 혈관내피 성장인자와 PAR-2간의 관련성을 알기 위해 각질형성세포와 멜라닌 세포로 구성된 공조 배양에 혈관내피 성장인자를 다양한 농도 및 기간 동안 처리한 후 PAR-2의 mRNA와 단백질 양을 RT-PCR과 western blot을 통하여 측정하였다. PAR-2 mRNA의 발현은 100nM의 혈관내피 성장인자 3시간 처리 군에서 대조군에 비해 통계적으로 의미있는 증가를 보였다. 또한 100nM과 200nM의 혈관내피 성장인자 24시간 처리 군에서 PAR-2의 mRNA 발현이 대조군에 비해 통계적으로 의미 있는 증가를 보였다. 쥐를 통한 동물 실험에서 혈관 내피 성장인자를 진피 내에 주사하였을 때 쥐 피부의 PAR-2 mRNA의 발현이 대조군에 비해 증가하였으며 특히 4μM과 10μM의 혈관내피 성장인자를 처리하였을 때 대조군에 비해 통계적으로 의미 있는 증가를 보였다. 혈관내피 성장인자와 PAR-2의 활성화가 멜라노좀의 각질형성세포로의 이동에 미치는 영향을 확인하기 위해서 각질형성세포와 멜라닌 세포로 구성된 공조 배양에 혈관 내피 성장인자와 PAR-2의 활성화 펩타이드를 처리한 후 전자현미경을 통해 각질형성 세포 내부의 멜라노좀의 수를 측정하였다. 이 결과 혈관내피 성장인자 및 PAR-2의 활성화 펩타이드를 처리한 군에서 대조군에 비하여 각질형성 세포 안의 멜라노좀 수가 증가하였다. 이러한 결과를 토대로 표피의 혈관내피인자가 각질형성 세포의 PAR-2의 발현 증가에 영향을 주며, 이를 통하여 멜라노좀의 멜라닌 세포로부터 각질형성세포로의 이동에 영향을 줄 것이라고 추정해 볼 수 있겠다.

핵심되는 말: 멜라노좀 이동, 혈관내피 성장인자, PAR-2