The effects of Vascular Endothelial Growth Factor on Human Melanocyte

Hyun Jung Kim

Department of Medicine

The Graduate School, Yonsei University

The effects of Vascular Endothelial Growth Factor on Human Melanocyte

Directed by Professor Seung Hun Lee

The Doctorial Dissertation

submitted to the Department of Medicine

the Graduate School of Yonsei University

in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

Hyunjung Kim

December 2009

This certifies that the Doctorial Dissertation of Hyun Jung Kim is approved.

Thesis Supervisor : **Prof.** ***Seung Hun Lee***

Thesis Committee Member : **Prof.** ***Kwan-Chul Tark***

Thesis Committee Member: **Prof.** ***Soo Chan Kim***

Thesis Committee Member: **Prof.** ***Jeong Taek Seo***

Thesis Committee Member: **Prof.** ***Jeong Hoon Kim***

The Graduate School

Yonsei University

December 2009

감사의 글

저의 부족한 능력에도 이 논문을 완성하게 해 준 모든 분들께 감사를 드립니다. 항상 지켜 봐 주시고 저의 피부과 연구의 좋은 등불이 되어 주신 이승헌 교수님께 감사의 말씀 올립니다. 모자란 저에게 또 하나의 완성을 만들어주신 탁관철 교수님, 김수찬 교수님, 서정택 교수님, 김정훈 교수님께 감사드립니다.

이 논문의 큰 힘이 되어준 아모레 퍼시픽의 남개원 책임 연구원, 엔프라니의 김진은 연구원, 네오팜의 정세규 박사님 그리고 무엇보다 항상 급박하게 돌아가는 저를 챙겨주는 우리 방 식구들, 안지원 연구원, 김지민 학생, 정민경 학생에게 고마운 마음 전합니다.

항상 열심히 일을 해 나갈 수 있게 도와주는 우리 가족, 나의 보물 혜리, 혜리 아빠, 하늘에서 저를 지켜 보고 보호 해주시는 우리 아빠, 그리고 항상 나의 모든 것인 우리 엄마께 모든 고마운 마음을 담아 드립니다.

저자 씀

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The Effects of

Vascular Endothelial Growth Factor on Human Melanocyte

Hyun Jung Kim

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Seung Hun Lee)

VEGF (Vascular endothelial growth factor) is a major angiogenetic factor expressed and secreted by epidermal keratinocytes. Melanocyte also expresses the VEGF receptors suggesting paracrine effects of keratinocyte derived VEGF on melanocyte. Melasma is a common acquired symmetrical hypermelanosis characterized by irregular light-brown to gray-brown macules and patches on the sun-exposed areas of the skin. An increased vascularity is the one of the major findings in melasma .VEGF (vascular endothelial growth factor) has been considered as a major angiogenetic factor in the lesion of melasma. We want to know that VEGF which is the representative angiogenic

factor, can also directly induce the melanogenesis and which signaling cascades are involved thorough VEGF. We evaluated the effects of VEGF on melanogenesis(expression of tyrosinase and MITF), proliferation and dendricity of human primary melanocyte and the melanin transfer from melanocyte to keratinocyte. We analyzed the VEGF effect on the proliferation of human primary melanocyte using cell counting and MTT assay and the increasing of dendricity. We also measured the melanin contents which induced by VEGF treatment. Western blotting for tyrosinase and MITF was used to detect the effect of VEGF on the melanogenesis. To evaluate the mechanisms related to the VEGF mediated effect on the melanocyte , cAMP and intracellular calcium level and phosphorylated Erk were measured. In addition to melanogenesis, the change of melanin transfer from melanocyte and keratinocyte by VEGF was measured by confocal microscopy using melanocyte and keratinocyte co-culture system.

VEGF might be the growth factor for melanocyte which is non-angiogenic cell that expresses VEGFR2(vascular endothelial growth factor receptor 2). It also affected on the melanin production and the increasing of dendricity.

VEGF stimulated the melanin synthesis through the up regulation of MITF and tyrosinase expression. The signaling pathway of VEGF induced melanogenesis involved various cascades such as the cAMP signaling, calcium signaling and MAP kinase pathway. VEGF also has an affect on the melanin transfer which might be mediated by the up-regulated PAR-2(protease activated receptor-2) on keratinocyte. This report suggests that keratinocyte derived VEGF in the skin of melasma patient can act not only on the increased vascularity which was shown in clinical expression of melasma, but also directly on the melanogenesis of melanocyte by paracrine effect. We also examined the function of VEGF as a melanogen in conjugation with its mitogenic properties in human epidermal melanocyte. These data suggested the possible use of VEGF can be the new target of molecule for melasma treatment.

Key words : vascular endothelial growth factor, melanocyte, keratinocyte, melanogenesis, melanin transfer, protease activated receptor-2, melasma

The Effects of

Vascular Endothelial Growth Factor on Human Melanocyte

Hyunjung Kim

Department of Medicine

The Graduate School, Yonsei University

(Directed by Professor Seung Hun Lee)

I. INTRODUCTION

Melasma is a common acquired symmetrical hypermelanosis characterized by irregular light-brown to gray-brown macules and patches on the sun-exposed areas of the skin. Melasma is most prevalent among young to middle aged oriental women especially in darker skin type[1]. Irregular light to dark brown macules or confluent patches on sun-exposed areas of the face, usually

in centrofacial, malar, or mandibular distribution are characteristic clinical findings[2]. The exact pathogenesis is still unknown. Genetic influences, exposure to UV radiation, pregnancy, hormonal therapies, cosmetics, phototoxic drugs, and antiseizure medications have been suggested as etiologic or aggravating factors[3,4] .

Melasma is characterized by epidermal hyperpigmentation which the amount of melanin was significantly increased in all epidermal layers in melasma skin, possibly caused both by an increased number of melanocytes and by an increased activity of melanogenic enzymes overlying dermal changes caused by solar radiation[5]. The melanocytes in the lesion of melasma also exhibited more dendrites[5]. Lesional melanocytes of melasma were filled with more mitochondria, Golgi apparatus, rough endoplasmic reticulum and ribosomes in the perikaryon than were melanocytes in normal skin, suggesting increased cell activity[5]. Melasma skin had more melanin in the whole epidermis, including the stratum corneum, whereas in normal skin the melanin pigment was mostly confined to the basal layer[5]. This suggests that an increased synthesis of melanosomes in the melanocytes, and increased transfer and

decreased degradation of melanosomes in the keratinocytes, are essential for the development of melasma[5].

In previous studies, the paracrine linkages among keratinocyte, fibroblast and melanocyte in increasing melanogenesis suggested as an important pathogenesis of melasma. Dermal changes, such as solar elastosis due to UV exposure, stem cell factor in melasma dermal skin and more increased vascularity in lesion than non lesional site may influence on the development of increased melanogenesis in melasma[5-7]. Kim et al [7] suggested that increased vascularity is one of the major findings in melasma and proposed VEGF (vascular endothelial growth factor) to be the major angiogenetic factor.

Vascular endothelial growth factor (VEGF) is a key molecule that orchestrates the formation and function of vascular networks. Impaired regulation of angiogenesis is implicated in a number of pathologic states[8]. Beyond tumor growth, excessive angiogenesis is associated with diabetic blindness, age-related macular degeneration, rheumatoid arthritis, psoriasis, and many other conditions[8]. Five distinct VEGF protein isoforms have been identified. They are VEGF121, VEGF145, VEGF165, VEGF189, and

VEGF206. The most commonly expressed proteins are VEGF121. Though the VEGF proteins are structurally similar, they are characterized by variation in function and differences in binding specificity[8,9] . VEGF-A, the major player among the VEGF family, binds and activates two tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR in humans/Flk-1 in mice) [9,10] . VEGF is produced in abundance by keratinocytes in the skin[11]. VEGF121, VEGF145, VEGF189 are the secreted forms of vascular endothelial growth factor by human keratinocytes[12].

Expression of the VEGF receptors was originally to be restricted to endothelial cells, but recent reports have demonstrated the presence of one or both of the receptors on various non-endothelial cell types[13-15]. Expression of VEGF receptor-1(VEGFR-1), but not VEGFR-2, was detected in normal human epidermal keratinocytes and VEGF stimulated the proliferation of keratinocyte by autocrine manner[16,17].

Melanocytes reside in the basal layer of the epidermis and each melanocyte is in contact with approximately 36 surrounding keratinocyte in a grouping termed the ‘epidermal melanin unit’ .Many keratinocyte-derived signaling

molecules and cytokines, for which melanocyte express receptors, affect melanocyte survival, migration, and melanogenesis. Normal human melanocytes constitutively express VEGF receptor-1 (VEGFR-1), VEGFR-2, and neuropilin-1. Furthermore, stimulation of melanocyte with VEGF165 isoform leads to phosphorylation of VEGFR-2, the receptor responsible for most of the VEGF-mediated effects in endothelial cells, suggesting that the receptor is functional[18].

Our previous study, the clinical reduction in melasma achieved by 578nm CuBr laser treatment as shown in MASI (Melasama assessment scoring index) and PGA(Physician gloval assessment) scores was statistically significant. The 578nm CuBr laser reduced both the size and number of vessels and also decreased the pigmentation in lesional skin. VEGF expressions in post treatment lesional skin also decreased compared to the pre-treatment skin. This clinical study can suggest CuBr 578nm laser is a new candidate for Melasma treatment. The mechanism of this treatment may be the reduction of the vascularity (size and number of dermal blood vessels) in melasma skin. The important mediator in improvement of melasma using yellow laser may

be the VEGF which can affect both angiogenesis and melanogenesis in melasma[19]

We hypothesized that the increased expression of VEGF from keratinocytes might affect the melanogenesis, proliferation and differentiation of melanocytes and the increased melanin transfer in addition to the increased vascularity shown in the melasma lesion of skin .

II. MATERIALS AND METHODS

1. Cell culture experiments

Normal human epidermal melanocytes and keratinocytes were isolated from neonatal foreskin, as described previously [20]. Neonatal melanocytes were grown in Medium 254 (Invitrogen Cell Culture, U.S.A) supplemented either with HMGS or with PMA-Free HMGS-2 (Invitrogen, Cell Culture, U.S.A). Neonatal keratinocytes were plated in serum-free EPiLife (Invitrogen, Cell Culture, U.S.A) with growth supplement (Defined Keratinocyte-SFM Growth Supplement, GIBCO).

2. Confocal microscopy

Monoclonal antibodies against VEGFR-2 (sc-6251, recognizes amino acids 1158–1345) and NP-1 (A-12, recognizes amino acids 570–855) , VEGF were obtained from R&D system, USA were used for confocal microscopy and western blotting. Cells were plated in wells of Lab-Tek 4-chamber slides and cultured as described above. The cells were fixed for 30 min with 10% buffered formalin (Poly Scientific R&D Corp.) and the slides

were then washed three times with 1× PBS. The nonspecific binding was blocked with Dako Cytomation Protein Block Serum-Free Ready-to-Use(Dako Cytomation, Carpinteria, CA, USA) for 15 min. Then, cells were incubated with the VEGFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at RT, respectively. Slides were washed with PBS and incubated with a donkey anti-goat antibody conjugated with FITC (1:100, Santa Cruz) for 30 min at RT. After washing, the slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and stored until use. Cells were examined and imaged with confocal microscope(Nikon C1 Plus, Nikon, Japan).

3. Cell proliferation and melanin content determination[21]

Melanocytes were plated in 60-mm culture dishes. Cells were stimulated with VEGF. After stimulation, melanocytes were collected using 0.25% trypsin-EDTA. After harvesting, the cell numbers were counted with a Coulter counter (Coulter, Electronics, Hialeah, FL, USA). MTT assay also has been used for study the proliferation of melanocyte. After cell counting, cells were spun down and the supernatant was discarded. The pellet was

solubilized in 1 N NaOH and incubated in 37℃ for 90 min. The optical densities were measured at 490 nm using an enzyme-linked immunosorbent assay reader, Model 680(Bio-Rad, Hercules, CA, USA).

4. Melanocyte dendricity assay[22]

Melanocytes were subcultured in 6 well plate (4×10^5 cells/well) and were treated .Following treatment, the image of cells was obtained using an inverted microscope. Dendrites-per cell was quantitated manually from the photographs.

5. Western blot analysis

The cultured melanocytes treated with VEGF with or without inhibitor were homogenized in ice-cold homogenization buffer containing 50 mM Tris-base (pH 7.4), 150 mM NaCl, 10 mM EDTA, 0.1% Tween-20, and protease inhibitors (0.1 mM PMSF, 5 μg/ml aprotinin, and 5 μg/ml leupeptin). Equal amounts of extracted proteins (30 μg) were resolved using 10% SDSPAGE and transferred to nitrocellulose membranes. The membranes were incubated with Erk,, phospho-Erk (rabbit polyclonal; Cell Signaling Technology, Beverly, MA), MITF (mouse monoclonal; Abcam,

Cambridge, UK) or tyrosinase (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA) antibody, diluted 1:1000 in blocking solution, overnight at 4℃. The membranes were further incubated with anti-rabbit or anti-mouse HRP-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and treated with an enhanced chemiluminescence's solution (Pierce, Rockford, IL). The signals were captured on an Image Reader. The protein bands were analyzed by densitometry.

6. cAMP immunoassay

Melanocytes were lysed in 0.1 M HCl to inhibit phosphodiesterase activity and centrifuged at 2,000 g for 15 min. The concentration of cAMP was measured using the cAMP assay kit in accordance with the manufactures instructions (Biomol International, Plymouth, PA, U.S.A) and expressed as pg per ml per 1×10^5 .

7. Measurement of intracellular calcium

Change in $[Ca^{2+}]_i$ in cells was measured by real-time fluorescence imaging of cells as described previously .Cells were grown on poly L-lysine coated glass cover slips and washed with Ringer's buffer pH 7.2. The fura-2-loaded

cells were mounted on the stage of an inverted microscope (Nikon, Tokyo, Japan) for imaging. The cells were superfused at a constant perfusion rate with the regular solution. The excitation wavelength was alternated between 340 and 380 nm, and the emission fluorescence was monitored at 510 nm with a CCD camera using MetaFluor system (Universal Imaging Co., Downingtown, PA). Fluorescence images were obtained at 4-s intervals. Background fluorescence was subtracted from the raw signals at each excitation wavelength, and the values of $[Ca^{2+}]_i$ were calculated from Grynkiewicz equation..

8. Melanosome transfer measurement[23,24]

Keratinocyte was detached using Trypsin-EDTA and plated in Chamber slide as 1×10^4/wells. After 24hr, Melanocyte was detached using Trypsin-EDTA and centrifuged in 1,500 rpm for 3min. After discarding the media, add the 2 μM contained 10 m HBSS for 30min in 37 ℃. Put the MC to the pre-prepared KC (5×10^3 /wells) and co-culture using media(HKGF contained EPiLife+ HMGF contained Medium 254 2:1 ratio). VEGF and VEGF antibody applied to this co-culture system. After 24 hours and 48 hours , the

media was discarded and the well was washed with PBS. 4 % . minutes. For double immunofluorescence, cells were incubated with the following primary antibodies: anti-tyrosinase polyclonal antibody (1:50 in PBS; C-19; Santa Cruz Biotechnology Inc.) and anti-human cytokeratin mAb (1:100 in PBS; clone MNF-116; Dako, Carpinteria, CA). The primary antibodies were visualized after appropriate washing with PBS, using the following secondary antibodies: rabbit anti-goat IgG-FITC (1:300 in PBS; Cappel Research Products, Durham, NC) and goat anti-mouse IgG-Texas Red (1:50 in PBS; Jackson ImmunoResearch Laboratories, West Grover, PA). Cells were examined in confocal microscopy.

III. RESULTS

1. The difference of melanin contents and VEGF and VEGFR expression in melasma.

To know the relationship of pigmentation level and the expression of VEGF in melasma skin, we checked the melanin content and VEGF expression. In melasam skin, we examined highly contained melanin in basal layer of keratinocyte(Fig.1(a)) in contrast to the nonlesional skin of melasma(Fig.1(b)). the characteristic pigment which was located in a 'cap' overlying the keratinocyte nuclei was examined in contrast to non-lesional skin The expression of VEGF showed similar pattern to melanin contents. The expression of VEGF on keratinocyte in melasma skin(Fig.1(c)) is more highly expressed then that of non-lesional skin(Fig.1(d)).

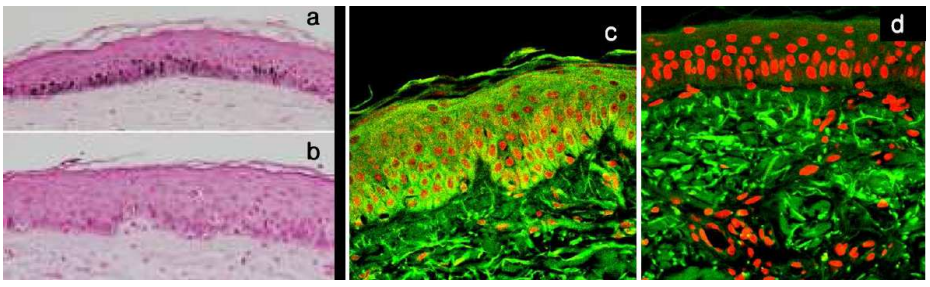


Figure 1. The difference of melanin contents in the lesional skin of

melasma patient (a) and non-lesional skin (b). The expression of VEGF on melasma lesional skin (c) and non-lesional skin (d). The expression levels of VEGF on lower epidermis are related to the amount of melanin contents in lesional skin (a) and non lesioal skin(b). Fontana-Masson stain in (a) and (b)(X400) and Confocal microscopy in (c) and (d) (green : FITC for VEGF or VEGFR2 , red : PI for nucleus) (X400) .

The pattern of VEGFR expression also showed similar to that of VEGF. The expression of VEGFR on melasma lesion is slightly elevated than that of non-lesional skin (Figure 2).

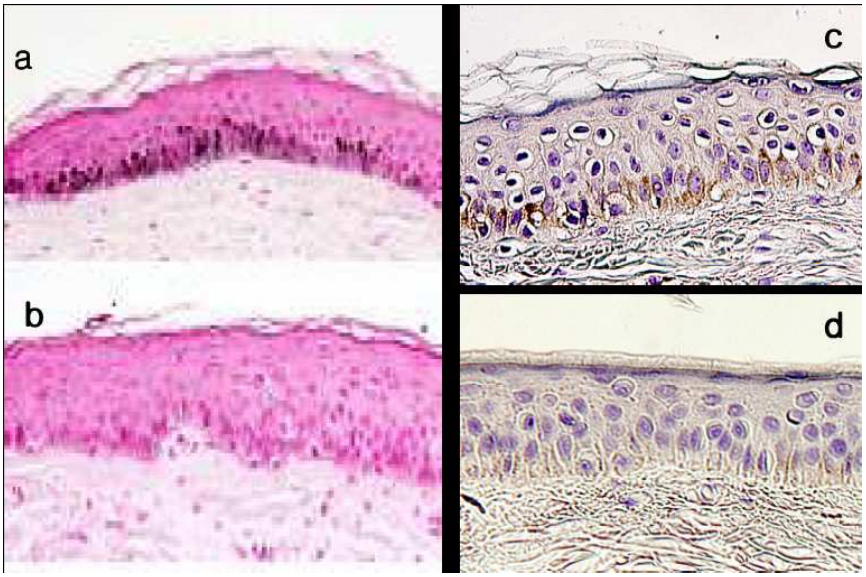


Figure 2. The difference of melanin contents in the lesional skin (a) and non-lesional skin (b) of melasma patient. The expression of VEGFR2 on melasma lesional skin (c) and non-lesional skin (d). The expression levels of VEGFR2 on epidermis are slightly elevated in lesional skin (c) and non lesioal skin(d).

2. The expression of VEGF and VEGF receptor on melanocyte

Normal human primary keratinocyte expressed the VEGF(A) (Fig.3(a)). We confirmed that human primary melanocyte expressed the VEGFR2(Fig..3(b)) and this VEGFR2 expression was up-regulated by the

VEGF treatment(Fig.3(c)).

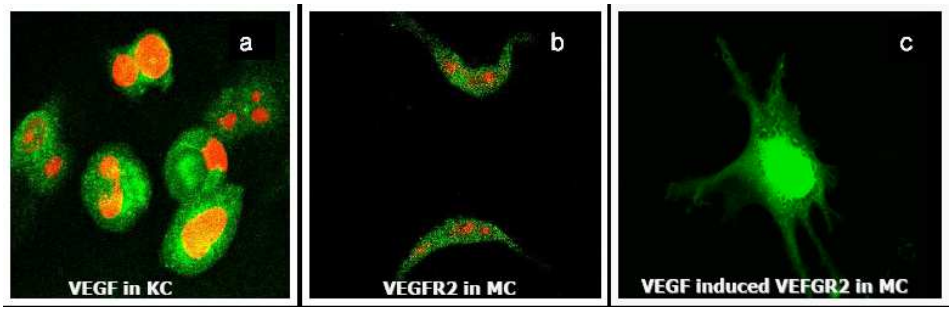


Figure 3. The expression of VEGF in human primary keratinocyte (a) and the VEGFR2 expression of human primary melanocyte (b,X600), and VEGF treated human primary melanocyte (C,X600). Confocal microscopy finding using VEGF and VEGFR2 antibody. (Green : FITC for VEGF or VEGFR2 , Red : PI for nucleus)

3. The proliferative effect of VEGF on melanocyte and keratinocyte

We assessed whether melanocytes are proliferated by VEGF using MTT assay. The VEGF(10 -100ng/ml) treatment for 48 hours induced 58.23%, 79.23 %, 112.34% increase in the melanocyte proliferation.(Fig.4(a)) The dose dependent effect of the VEGF on the proliferation of melanocytes(Fig.4(a)) was inhibited by the treatment of anti-VEGF antibody

(Bevacizumab) (Fig. 4(a)). The proliferative effect of VEGF on keratinocyte also checked (Fig. 4(b)). It also showed the dose dependent proliferative effect and inhibitory effect by VEGF antagonizing antibody (Fig. 4(b)). The result was summarized in Table 1.

The confocal microscopy showed the representative view of VEGF effect and VEGF antagonist on melanocyte (Fig. 4(c)).

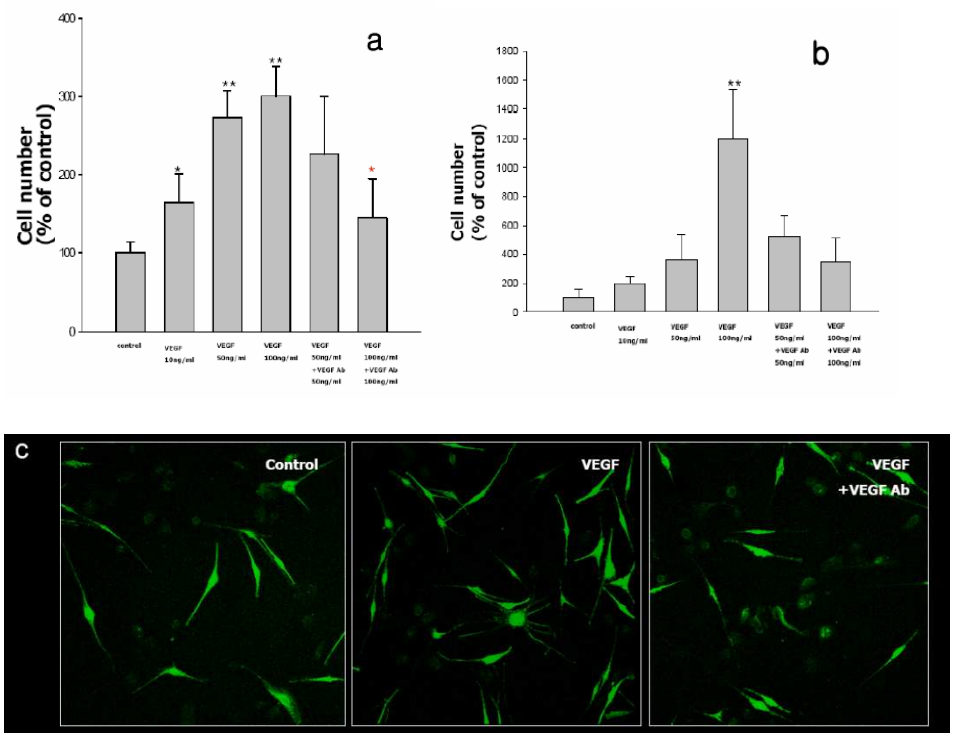


Figure 4. VEGF can induce the proliferation of melanocyte(a) and keratinocyte(b). The cell number of melanocyte which was treated

VEGF for 48hours showed dose dependent manner and the VEGF antagonistic antibody inhibited this proliferative effect (a). The proliferative effect of VEGF on keratinocyte also assessed (b). Those representative view of proliferative effect on melanocyte also examined in confocal microscopic study (c). (*p <0.05, **p<0.01 vs. control , *p <0.05 vs., VEGF treated group)

	Cell No.($x10^4$)
Untreated melanocyte(NC)-$10x10^4$	15±0.7
MC+VEGF10ng/ml	18.2±0.86 ^{c}
MC+VEGF50ng/ml	23.65±1.7**
MC+VEGF100ng/ml	25±1.9 **
MC+VEGF50ng/ml+ VEGF Ab50ng/ml	21.3±3.7
MC+VEGF100ng/ml+VEGF Ab100ng/ml	17.23±2.5 *
	Cell No.($x10^5$)
Untreated keratinocyte(KC)-$5x10^5$	7.1± 1.2
KC+VEGF10ng/ml	9.2±0.97
KC+VEGF50ng/ml	12.6±3.7
KC+VEGF100ng/ml	30.2±7.1**
KC+VEGF50ng/ml+ VEGF Ab50ng/ml	16.1±2.9
KC+VEGF100ng/ml+VEGF Ab100ng/ml	12.33±3.4

Table 1. The proliferative effect of VEGF on melanocyte and keratinocyte. (*p <0.05, **p<0.01 vs. control , *p <0.05 vs., VEGF treated group)

4. Effect of VEGF on melanin synthesis

To ascertain whether VEGF interferes with melanin production in vitro,

cultured melanocytes were treated with VEGF for 48 hours. VEGF altered the amount of melanin quantified spectrophotometrically (Fig.5). The dose dependent effect of the VEGF on the synthesis of melanin was inhibited by the treatment of anti-VEGF antibody(Bevacizumab)

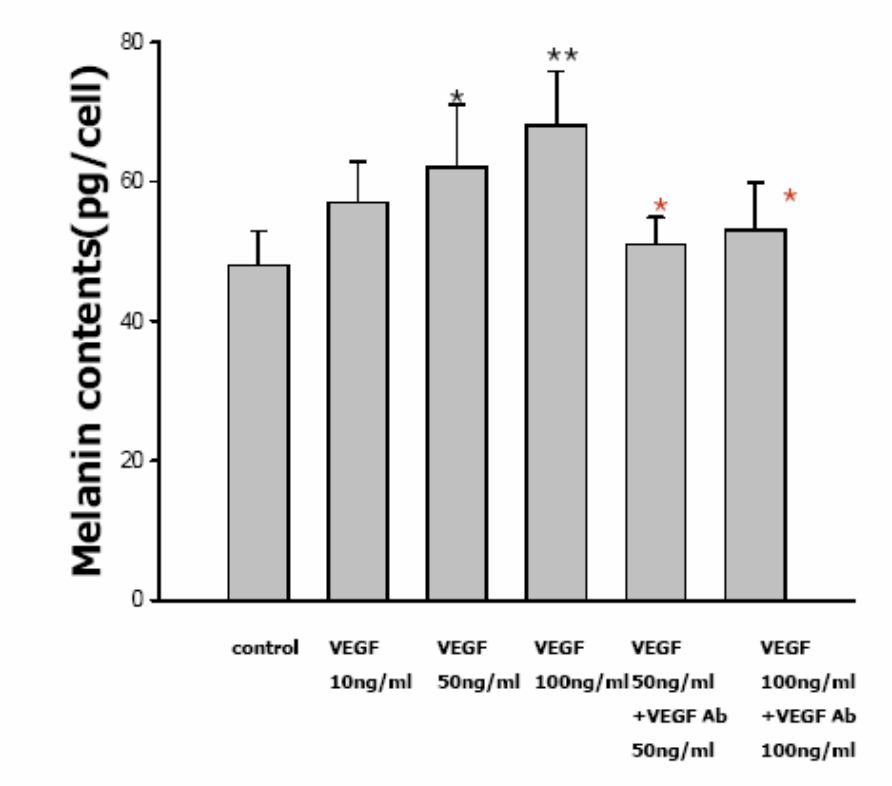
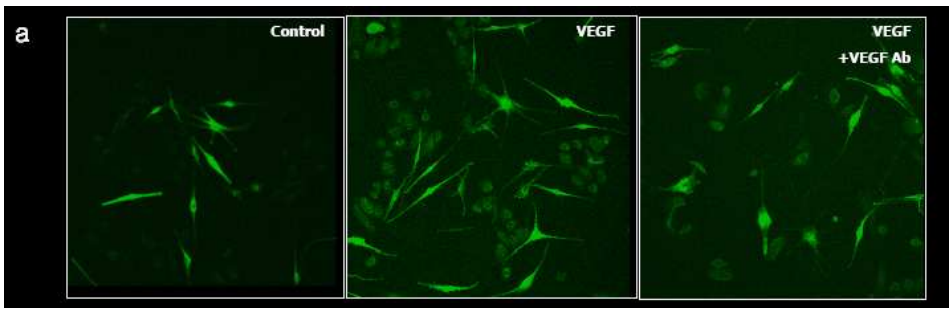


Figure 5. The VEGF affects on the melanin contents. The spectrophotemetically analyzed melanin contents showed that VEGF induced the increase of melanin and the VEGF antibody inhibited those effects. (*p <0.05, **p<0.01 vs. control , *p <0.05 vs., VEGF treated group)

5. Effect of VEGF on Dendricity of Melanocyte

To determine the effect of VEGF on melanocyte dendricity, cells were treated with VEGF and dendricity was quantitated 48 hours later. Figure 6(a) shows representative images of cells treated with VEGF (100 nM) compared with control cells. The majority of control cells exhibited a typical bipolar morphology, whereas VEGF treated cells had multiple dendrites. Quantitation of dendricity (Fig. 6(b)) showed that the percentage of cells with more than two dendrites was only 2% in control cells, but was 42% in cells treated with VEGF (100 nM). Co-incubation of the cells with Bevacizumab had inhibitory effect on VEGF-dependent increase in dendricity (Fig.6(b)). We also counted and the number of dendrite of melanocyte in 5 high power field (Fig.6(c)).



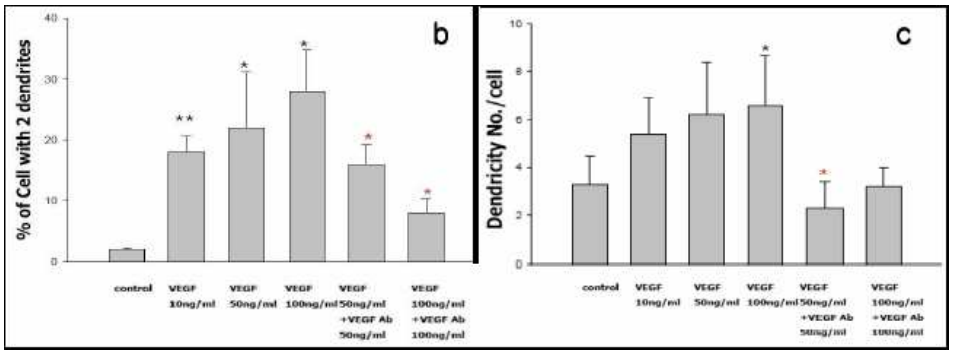


Figure 6. Human melanocytes become highly dendritic following treatment with VEGF(a). Quantitative analysis of dendricity in human melanocytes following treatment with VEGF for 48 hours. Results shown are percent of cells with more than two dendrites(b,c) VEGF induced a dose-dependent increase in the number of dendrites, which was statistically significant. In contrast, VEGF antibody had inhibitory effect on dendricity. The results represent the averaged results of four individual cultures±SEM. (*p <0.05, **p<0.01 vs. control , *p <0.05 vs. VEGF treated group)

6. Effect of VEGF on Tyrosinase and MITF expression

Melanin synthesis is an enzymatic cascade controlled by tyrosinase, Tyrp1 and dopachrome tautomerase. Tyrosinase is the key enzyme in this process as it controls the first and rate-limiting step, the hydroxylation of tyrosine to dihydroxyphenylalanine. The most important transcription factor in the regulation of both tyrosinase and Tyrp1 gene expression seems to be MITF which plays a critical role in regulating many aspects of melanocyte survival and differentiation[25]. For investigation of the mechanism of VEGF induced pigmentation, melanocytes were treated with 10,20nM VEGF for 48h. We found the expression of melanogenic factors, Intercellular MITF and tyrosinase were increased by VEGF treatment (Fig. 7.). VEGF antagonist inhibited the expression of melanogenic factors, MITF and tyrosinase. These results suggest that VEGF stimulates the melanin synthesis through the up regulation of MITF and tyrosinase expression.

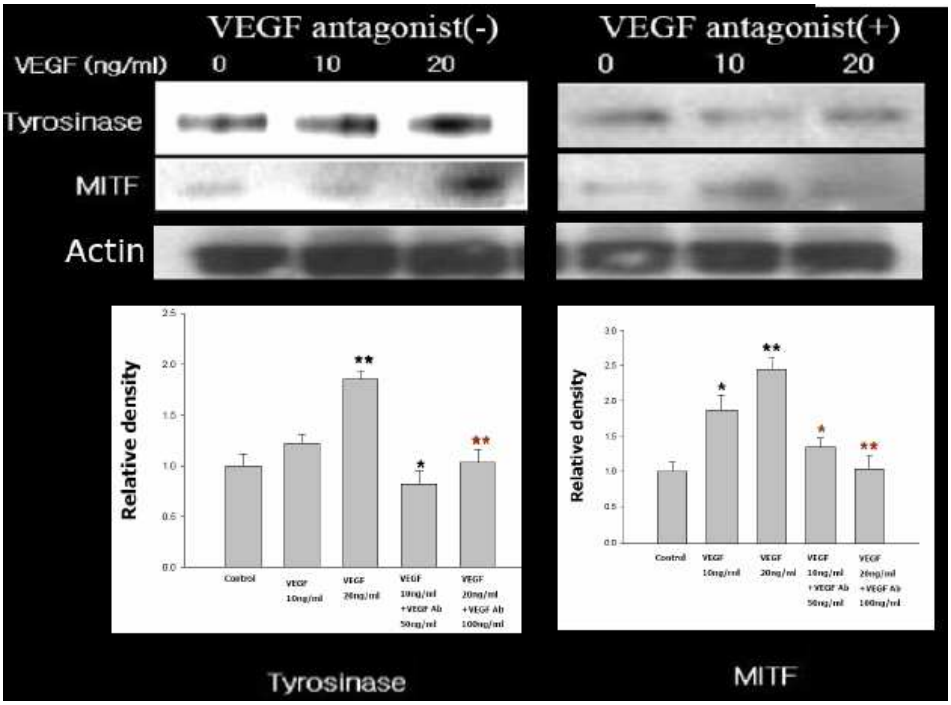


Figure 7. The expressions of intracellular tyrosinase and MITF and the inhibitory effect of VEGF antagonist on their expressions. Cellular lysates were analyzed by imuno-blotting with anti-tyrosinase and anti-MITF antibodies.

7. Effect of VEGF on intracellular cAMP signaling

MITF expression can be mediated by the cAMP dependent mechanism during melanogenesis[26]. For revealing the cAMP relation to the VEGF

induced MITF expression, we assayed the cAMP after the treatment of VEGF and antagonist. When human melanocytes were incubated with 10 nM ET-1 for the 39min, the peak level of cyclic AMP was examined[27]. In our study, the treatment of VEGF can induce the cAMP in human melanocyte and anti-VEGF antagonist reversed the increased cAMP level due to the action of VEGF (Fig.8(a)). The VEGF effect on HM3KO cell is less significant (Fig.8(b)).

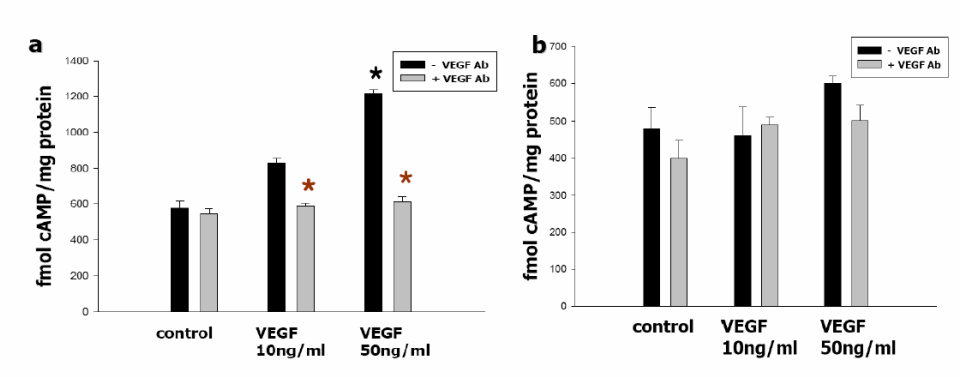
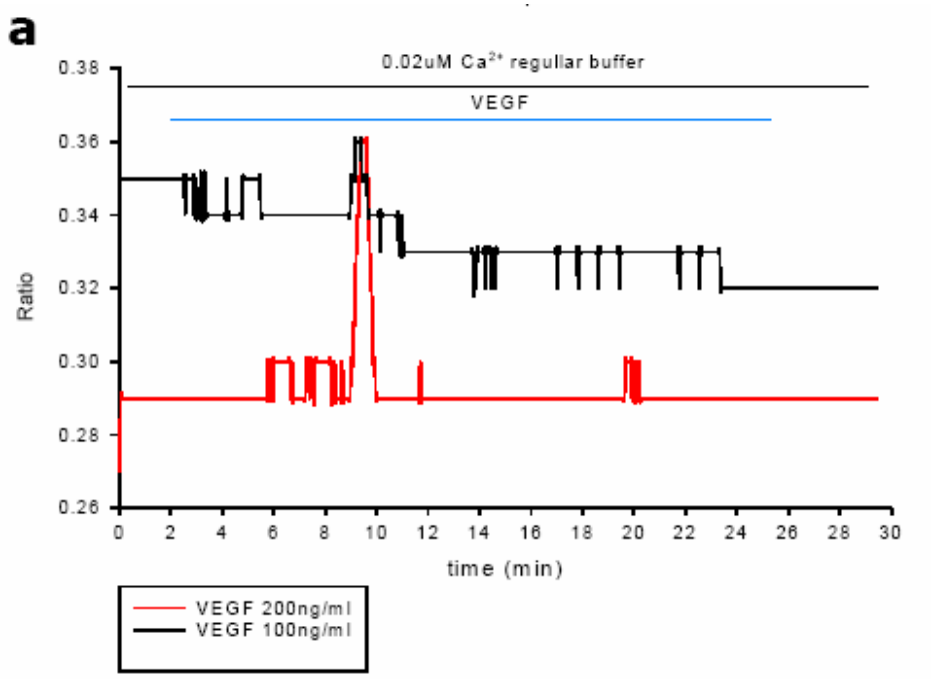


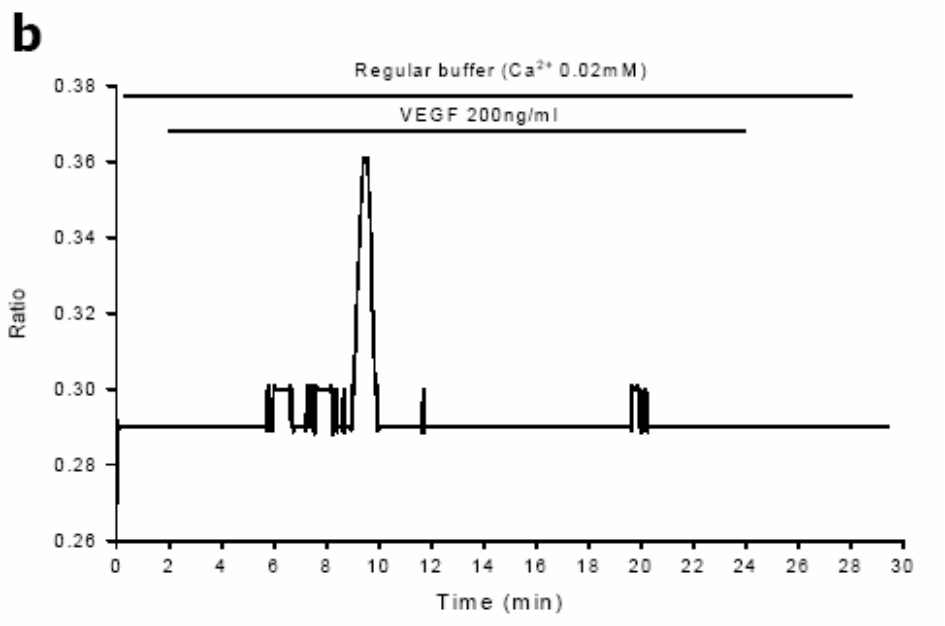
Figure 8. Effect of VEGF on intracellular cAMP. Normal human melanocyte(a) and HM3 KO cell (b) was treated with different concentration of VEGF for 30min, and then intracellular cAMP was determined. Treatment with 10ng/ml but not 100ng/ml VEGF on primary melanocyte caused a significant increase in cAMP level. VEGF

antibody can inhibited the elevation of cAMP due to VEGF totally. The VEGF effect on HM3KO cell is less significant . Treatment with 50ng/ml caused an increase in cAMP level in HM3KO cell , but there was statistically no difference.

8. [Ca 2+]*i* after VEGF stimulation

Melanocytes are neural-derived cells. VEGF signaling regulates hippocampal neurons by elevating the intracellular calcium level and the activation of calcium/calmodulin protein kinase II[28]. The endothelin induced melanogenesis was related to the elevation of intracellular calcium level via PKA signaling[27] VEGF induced mobilization of intracellular calcium in cultured human melanocytes (Fig.8(a,b))and VEGF antagonist suppressed the VEGF induced accumulation of intracellular calcium(Fig.8(b,c)).


a
0.02uM Ca2+ regullar buffer
VEGF
Ratio
time (min)
VEGF 200ng/ml
VEGF 100ng/ml


b
Regular buffer (Ca2+ 0.02mM)
VEGF 200ng/ml
Ratio
Time (min)

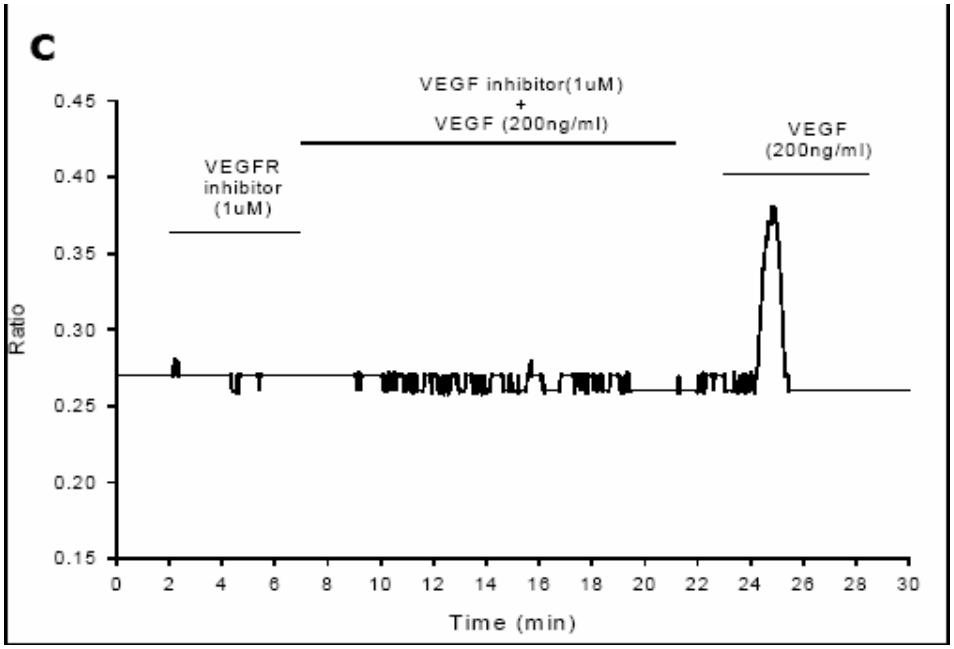


Figure 9. [Ca $^{2+}$]*i* after VEGF stimulation. Immediate increase in [Ca $^{2+}$]*i* in response to VEGF in human primary melanocyte.(a,b). [Ca $^{2+}$]*i* after VEGF antagonist treatment and VEGF stimulation.(c) VEGF antagonist prevented the [Ca^{2+}]*i* increase evoked by VEGF. This calcium signaling is specifically mediated by VEGF.

9. Effect of VEGF on the melanin transfer from melanocyte to keranitocyte

The melanosome transfer from the melanocyte to keratinocyte is the final step for pigmentation in epidermis. To check the effect of VEGF on melanin transfer , we used the melanocyte and keratinocyte co-culture system. VEGF induced more melanosomal transfer than normal (Fig. 10.(b)) and VEGF-antibody controlled group. (Fig. 10.(a.c))

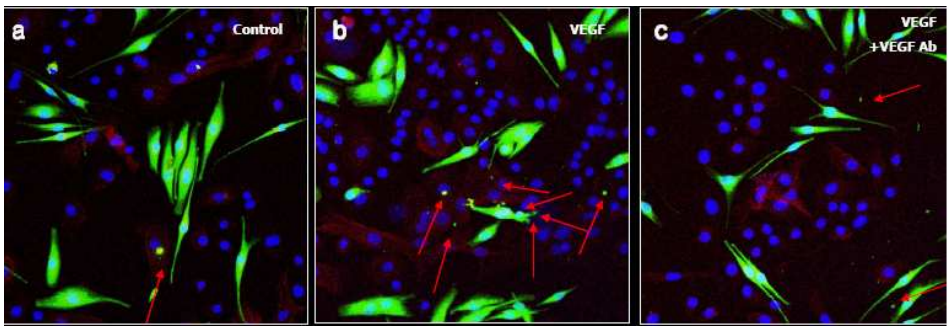
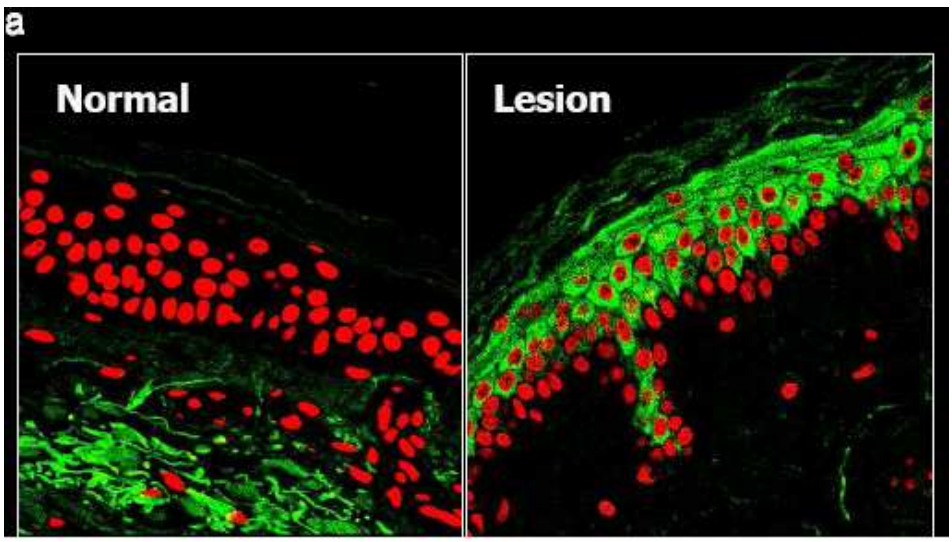
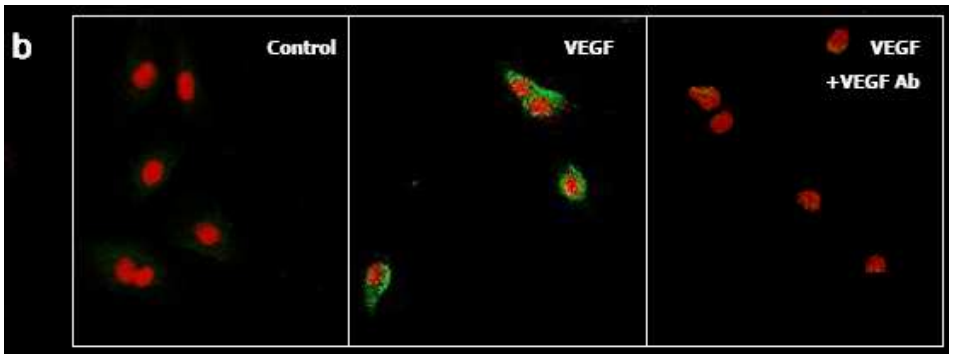


Figure 10. Effect of VEGF on the melanin transfer from melanocyte to keranitocyte. VEGF 10nM treatment can induce the increasing melanin transfer (b) than control(a). The VEGF antagonist neutralized the VEGF effect on the melanin transfer (c). (red arrow: transferred melanosom from melanocoyte(green for MITF) to kenatinocyte(red-for cytokeratin)

10. Expression of PAR2 in melasma skin and VEGF induced PAR2 expression in KC

One of the transfer mechanism is related to the PAR-2(Protease mediated receptor 2).We checked the PAR2 expression in the lesion of melasma skin. The prominent expression of PAR2 was examined in lesional skin (Fig. 11.(a)) in contrast to normal. skin. We also examined the VEGF effect on PAR2 expression of keratinocyte in protein level (Fig. 11.(b)) and mRNA level (Fig. 11.(C)). The VEGF antagonist inhibit the VEGF inducible effect on PAR-2.





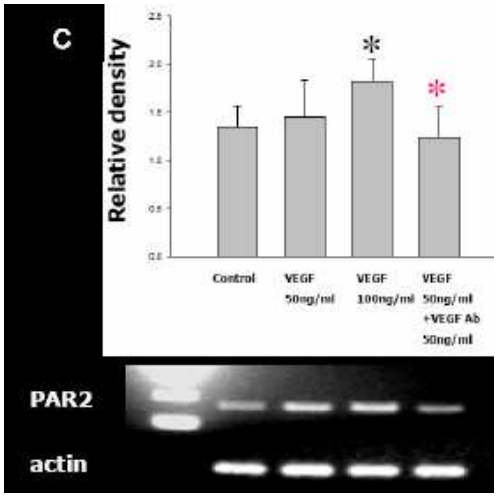


Figure 11. The prominent expression of PAR-2 in the lesion of melasma skin than normal skin, Confocal microscopy(x400) (a). The VEGF induced the PAR-2 expression on keratinocyte in protein level (b) and mRNA level (c)

IV. DISCUSSION

Melasma is a complex, multifactorial pigmentary disease whose pathogenesis is not yet fully elucidated. Clinical observations such as the increased vascularity suggest a role for the angiogenetic factor in the pathogenic mechanism of melasma[7]. Kim et al[7] suggested the VEGF as such angiogenetic factor. In addition to that clinical observation, our previous study, the yellow laser which can control the vascularity and pigmentation in the skin of melasma patient also control the level of VEGF[19]. UV radiation has been considered the aggravating factor for melasma[3] and can up-regulate the expression of VEGF and down regulated those of thrombospondin-1[29,30]. Major effect of UV irradiation on the skin induce the increased pigmentation thorough melanocyte proliferation and survival both directly and indirectly through its effect on keratinocyte , inducing the synthesis and secretion of paracrine keratinocyte factors[31]. These paracrine factors are bFGF, ET-1, Il-1alpha/beta,ACTH, alpha-MSH, PGE2/PGF2a, GM-CSF, NO, TNF-alpha, NGF and they affect on the

melanocyte proliferation , increase of dendricity, melanogenesis and melanosomal transfer. In our study, VEGF has own melanogenic activity with mitogenetic activity on melanocyte. It also induced the melanin transfer effectively. We suggested that the VEGF as a new paracrine factor from keratinocyte which can be related to UV induced pigmentation. Melasma can be explained by epidermal hyperpigmentation, possibly caused both by an increased number of melanocytes and by an increased activity of melanogenic enzymes overlying dermal changes caused by solar radiation[5]. We suggested that the increased vascularity in refractory and aggravating melasma might be related to VEGF which induced by UV radiation and has both activity as angiogenetic factor and melanogenetic factor and that anti-VEGF therapy can be the new target for melasma treatment. Retinoids can be the good strategy on the base of our hypothesis. Tretinoin in a fixed, triple-combination therapy (hydroquinone 4%/tretinoin 0.05%/fluocinolone acetonide 0.01%) for the treatment of melasma has been considered with evidence based grade B[32]. tRA pretreatment significantly inhibits UV-induced VEGF overexpression and has

antiangiogenic effect[33]. We want to add the anti-melanogenic effect of retioids via anti-VEGF mechanism.

There are various cellular mechanism for melanogenesis[26,32]. Activation of tyrosinase by PKC-β. UV irradiation, as well as activated cell surface receptors after binding with their specific ligands (ET-1/ET-1Receptor and Norepinephrine/a1-AR), releases DAG from the cell membrane. Inactive PKC- β is activated by DAG and activated PKC- β (PKC- βa) binds to RACK-I. The PKC-βa/RACK-I complex translocates to melanosome and then phosphorylates serine residues on tyrosinase. This phosphorylation activates the enzyme. UVirradiation upregulates the levels of a-MSH,ACTH and their cognate receptor MC1R to stimulate cAMP production. Intracellular level of cAMP can also be increased after keratinocyte-released epinephrine binds to its cell surface receptor b2-AR on melanocytes. Steel factor (stem cell factor) by binding to c-Kit leads to receptor autophosphorylation and activation of MAP kinases. MAP kinases phosphorylate (activate) MITF, leading to transcription of the melanogenic enzymes tyrosinase, TRP-1, TRP-2 and PKC-b. NO activates the enzyme

guanylate cyclase that increases the intracellular level of cGMP. UV irradiation, by decreasing the level of BMP receptors, prevents BMP-4 mediated inhibition on melanogenesis. Like endothelin-1, VEGF has both melanogenic and mitogenic effect on the melanocyte. It also used the cAMP signaling and intracellular calcium elevation. Melanocyte Further study needed to continue for revealing the exact mechanism of VEGF induced melanogenesis. MAP kinase pathway can be related to the VEGF induced melanogenesis. VEGF might induce the phosphorylation of Erk (data not shown). Further study might uncover the relation of VEGF signaling on MAP kinase pathway. Developmentally, melanoblasts are derived from the neural crest, so it is not surprising that similar signaling can be examined. In recent study, VEGF signaling regulates hippocampal neurons by elevation of intracellular calcium and activation of calcium/calmodulin protein kinase II[28]. In our study , VEGF also might regulate signaling using elevation of intracellular calcium in melanocyte. Further detailed mechanism related VEGF mediated calcium signaling in melanocyte must be studied.

In conclusion, keratinocyte derived VEGF in the skin of melasma patient

can be act not only on the increased vascularity which was shown in clinical expression of melasma, but also on the melanogenesis in melanocyte by paracrine effect. This is first report that show the function of VEGF as a melanogens in conjugation with its mitogenic properties in human epidermal melanocyte using various signaling pathway. This data suggested the possibility that the VEGF can be the new target of melasma treatment.

V. CONCLUSION

In this study we examined the Direct actions of VEGF on human melanocyte in the view of the induction of Proliferation , the increase of Dendricity , Melanogenesis and Melanosomal transfer via PAR-2 . Our study also suggest the this VEGF action used various cellular mechanism in melanocyte for example cAMP signaling and calcium signaling , but the exact cellular mechanism should be further evaluated. Our study can be the new support the hypothesis for vascular Melasma which shows increased VEGF and pigmentation.

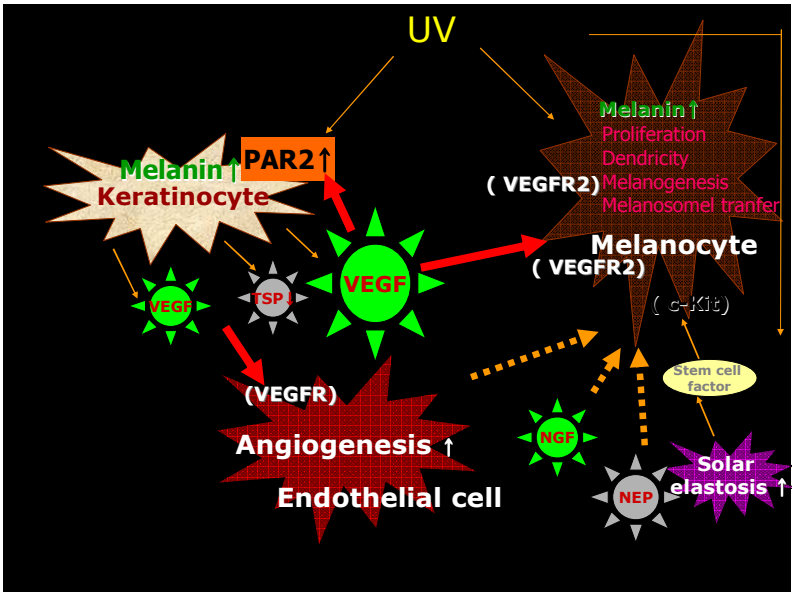


Figure 12. New hypothesis for the pathogenesis of melasma. The increased expression of VEGF affects on not only the angiogenesis , but also directly the increased pigmentation through the effects on the melanocyte and keratinocytes which showed the up-regulated PAR-2 expression by VEGF

REFERENCE

1. Jean L Bolognia JLJ, Ronald P Rapini. Dermatology, 2nd edn. spain MOSBY ELSEVIER. 2008.

2. Grimes PE. Melasma. Etiologic and therapeutic considerations. Archives of dermatology 1995; 131: 1453-7.

3. Prignano F, Ortonne JP, Buggiani G et al. Therapeutical approaches in melasma. Dermatologic clinics 2007; 25: 337-42, viii.

4. Kang WH, Yoon KH, Lee ES et al. Melasma: histopathological characteristics in 56 Korean patients. British journal of dermatology 2002; 146: 228-37.

5 .Kang HY, Hwang JS, Lee JY et al. The dermal stem cell factor and c-kit are overexpressed in melasma. British journal of dermatology 2006; 154: 1094-9.

6. Kim EH, Kim YC, Lee ES et al. The vascular characteristics of melasma. Journal of dermatological science 2007; 46: 111-6.

7. Birk DM, Barbato J, Mureebe L et al. Current insights on the biology and clinical aspects of VEGF regulation. Vasc Endovascular Surg 2008; 42: 517-

30.

8. Shibuya M, Claesson-Welsh L. Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. Exp Cell Res 2006; 312: 549-60.

9. Shibuya M. Vascular endothelial growth factor (VEGF)-Receptor2: its biological functions, major signaling pathway, and specific ligand VEGF-E. Endothelium 2006; 13: 63-9.

10. Brown LF, Yeo KT, Berse B et al. Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing. J Exp Med 1992; 176: 1375-9.

11. Ballaun C, Weninger W, Uthman A et al. Human keratinocytes express the three major splice forms of vascular endothelial growth factor. The Journal of investigative dermatology 1995; 104: 7-10.

12. Brown LF, Detmar M, Tognazzi K et al. Uterine smooth muscle cells express functional receptors (flt-1 and KDR) for vascular permeability factor/vascular endothelial growth factor. Lab Invest 1997; 76: 245-55.

13. Zhu Y, Jin K, Mao XO et al. Vascular endothelial growth factor promotes

proliferation of cortical neuron precursors by regulating E2F expression. FASEB J 2003; 17: 186-93.

14. Takagi H, King GL, Aiello LP. Identification and characterization of vascular endothelial growth factor receptor (Flt) in bovine retinal pericytes. Diabetes 1996; 45: 1016-23.

15. Man X, Yang X, Cai S et al. Immunolocalization and expression of vascular endothelial growth factor receptors (VEGFRs) and neuropilins (NRPs) on keratinocytes in human epidermis. Molecular medicine 2006; 12: 127-36.

16. Wilgus TA, Matthies AM, Radek KA et al. Novel function for vascular endothelial growth factor receptor-1 on epidermal keratinocytes. The American Journal of Pathology 2005; 167: 1257-66.

17. Kim EJ, Park HY, Yaar M et al. Modulation of vascular endothelial growth factor receptors in melanocytes. Experimental dermatology 2005; 14: 625-33.

18. Gilchrest BA, Calhoun JK, Maciag T. Attachment and growth of human keratinocytes in a serum-free environment. Journal of cellular physiology 1982; 112: 197-206.

19. Hakozaki T, Minwalla L, Zhuang J et al. The effect of niacinamide on

reducing cutaneous pigmentation and suppression of melanosome transfer. British journal of dermatology 2002; 147: 20-31.

20. Kawasaki A, Kumasaka M, Satoh A et al. Mitf contributes to melanosome distribution and melanophore dendricity. Pigment cell & melanoma research 2008; 21: 56-62.

21. Cardinali G, Bolasco G, Aspite N et al. Melanosome transfer promoted by keratinocyte growth factor in light and dark skin-derived keratinocytes. The Journal of investigative dermatology 2008; 128: 558-67.

22. Berens W, Van Den Bossche K, Yoon T et al. Different approaches for assaying melanosome transfer. Pigment cell research 2005; 18: 370-81.

23. Gaggioli C, Busc R, Abbe P et al. Microphthalmia-associated transcription factor (MITF) is required but is not sufficient to induce the expression of melanogenic genes. Pigment cell research 2003; 16: 374-82.

24. Park HY, Kosmadaki M, Yaar M et al. Cellular mechanisms regulating human melanogenesis. Cellular and molecular life sciences 2009; 66: 1493-506.

25. Imokawa G, Yada Y, Kimura M. Signalling mechanisms of endothelin-

induced mitogenesis and melanogenesis in human melanocytes. The Biochemical journal 1996; 314: 305-12.

26. Kim BW, Choi M, Kim Y et al. Vascular endothelial growth factor (VEGF) signaling regulates hippocampal neurons by elevation of intracellular calcium and activation of calcium/calmodulin protein kinase II and mammalian target of rapamycin. Cellular signalling 2008; 20: 714-25.

27. HyunJung Kim SL. Copper bromide 578nm Dual/Yellow laser for the new treatment of Melasma. dermatol surgery 2008; submitted.

28. Yano K, Kajiya K, Ishiwata M et al. Ultraviolet B-induced skin angiogenesis is associated with a switch in the balance of vascular endothelial growth factor and thrombospondin-1 expression. J Invest Dermatol 2004; 122: 201-8.

29. Yano K, Kadoya K, Kajiya K et al. Ultraviolet B irradiation of human skin induces an angiogenic switch that is mediated by up regulation of vascular endothelial growth factor and by downregulation of thrombospondin-1. British journal of dermatology 2005; 152: 115-21.

30. Park HY, Pongpudpunth, M., Lee, J., Yaar, M. Biology of Melanocytes,In Fitzpatrick s Dermatology in General Medicine. In, 7 edn.: Wolff, K.,

Goldsmith, L. A., Katz, S. I.,Gilchrest, B. A., Paller, A. S., Leffell, D. J. 2007.

31. Kang HY, Valerio L, Bahadoran P et al. The role of topical retinoids in the treatment of pigmentary disorders: an evidence-based review. American Journal of Clinical Dermatology 2009; 10: 251-60.

32. Kim M, Kim YK, Eun HC et al. All-trans retinoic acid antagonizes UV-induced VEGF production and angiogenesis via the inhibition of ERK activation in human skin keratinocytes. The Journal of investigative dermatology 2006; 126: 2697-706.

< ABSTRACT(IN KOREAN)>

혈관내피형성성장인자가 인간 멜라닌세포에 미치는 영향

지도 교수 이 승 헌

연세대학교 대학원 의학과

김 현 정

혈관내피형성성장인자(vascular endothelial growth factor)는 주로 표피각질형성세포에서 형성되어서 분비되는 혈관 형성에 기여하는 성장인자이다. 멜라닌세포는 이에 대한 수용체를 발현하고 있어 각질형성세포에서 분비하는 혈관내피성장인자의 영향을 받을 가능성을 제시하고 있으나 그 영향에 대한 연구는 구체화되지 않았다. 동양인에게서 흔한 색소성 병변의 하나인 기미 병변에서 관찰되는 혈관의 증식과 혈관내피성장인자의 과발현은 혈관내피성장인자의 혈관 형성에 기여하는 것 이외에 직접적으로 색소 형성에 기여할 가능성을 생각해 볼 수 있다. 이번 연구에서는 혈관내피형성성장인자가 직접

적으로 멜라닌 세포 및 멜라닌 세포에 미치는 다양한 영향에 대해 살펴보고자 한다. 색소가 증가하는 과정은 직접적으로 멜라닌 과립의 증가, 멜라닌 세포의 증식, 멜라닌 과립을 효과적으로 전달할 수 있도록 dendicity의 증가, 멜라닌 세포에서 각질형성세포로의 멜라닌 소체의 전달 증가로 요약할 수 있다. 이번 연구는 이 과정들에 혈관내피형성성장인자가 미치는 영향과 이 과정에서 관여할 수 있는 세포 내 신호전달과정에 대해서 살펴보고자 하였다. 멜라닌 증식에 미치는 직접적인 영향을 혈관내피형성성장인자 처리 후 멜라닌 세포의 수의 관찰과 MTT assay를 통하여 관찰하였고 , 직접적으로 멜라닌 과립의 증가를 측정하였다. 또한 멜라닌 세포의 형태 변화를 관찰하여 혈관내피형성인자가 멜라닌세포의 dendricity에 미치는 영향을 알아보았다. 멜라닌형성과정의 대표적인 지표인 tyrosinase와 MITF의 발현량을 관찰하여 멜라닌형성에 미치는 과정을 분자적인 측면에서 알아보았다. 멜라닌 세포가 만들어낸 멜라닌 소체는 궁극적으로 각질형성세포에 전달됨으로써 전체적인 색소형성에 관여하게 된다. 혈관내피형성성장인자 처리 후 멜라닌세포에서 각질형성세포로의 멜라닌 소체의 전달 정도를 관찰하였고 이러한 전달과정에

기여한다고 알려져 있는 PAR-2(Protease activated receptor -2) 의 발현을 기미 병변과 혈관내피형성인자를 처리한 각질형성세포에서의 변화를 통해 관찰하였다. 이러한 모든 과정에 관여할 것으로 생각되는 세포 내 신호전달체계를 알기 위해 멜라닌 세포에 혈관내피형성인자 처리 후 cAMP 의 변화 정도, intracellular calcium level의 변화 phosphorylated Erk의 발현을 측정하였다. 이러한 모든 실험이 혈관내피형성성장인자 특이 발현임을 이에 대한 중화 항체를 사용함으로 확인하였다.

이 연구를 통해 혈관내피형성성장인자는 혈관내피세포가 아닌 세포 중의 하나이며 이에 대한 수용체를 함유한 멜라닌세포에 영향을 미침을 할 수 있었으며 이는 기존에 알려진 혈관내피형성성장인자의 혈관 형성 외 또 하나의 새로운 역할을 함을 시사한다. 혈관내피형성성장인자는 멜라닌세포의 증식을 촉진하며 이는 멜라닌 과립의 증가를 보이게 된다. 멜라닌 과립을 효과적으로 멜라닌 세포 내에서 전달하거나 각질형성세포로 전달하기 위해서는 멜라닌 세포가 형태학적으로 dendrite가 증가하여야 하는데 혈관내피형성성장인자는 이 또한 증가시킴을 알 수 있다. 혈관내피형성성장인자는 멜라닌형성에

중요한 기전인 MITF 및 tyrosinase의 발현을 증가시키는 효과를 보인다. 멜라닌이 증가되는 마지막 단계로 생각되는 멜라닌세포에서 각질형성세포로의 멜라닌 소체의 전달 증가 또한 혈관내피형성성장인자에 의해 유도되며 이 과정은 혈관내피형성인자가 각질형성세포의 PAR-2의 발현을 증가시키는 과정이 연관되었을 것으로 관찰되었으며 실제 기미 환자의 병변에서도 PAR-2의 발현이 증가됨을 통해 이의 연관성을 유추할 수 있었다. 이러한 일련의 과정에 관여하는 세포 내 신호 전달체계는 cAMP signaling, Ca signaling 그리고 MAP kinase pathway 로 생각되나 구체적인 과정은 향후 추가실험을 통해서 밝혀야 할 것이다. 이 연구를 통해서 정확한 기전이 알려져 있지 않은 기미의 병인의 하나로 혈관내피형성인자를 고려할 수 있게 되었다. 기미에서 관찰되는 증가된 멜라닌 세포 수의 증가.. dendrite 의 증가. 멜라닌 소체의 전달 증가는 이번 연구에서 밝혀진 바와 같이 혈관내피형성성장인자에 의해서도 유도되며 임상적으로 관찰되는 기미 병변에서의 혈관의 증가와 색소의 증가 모두를 유도할 수 있다는 측면에서 그 중요성을 강조할 수 있으며 이는 향후 기미의 치료의 새로운 방법으로 직접적, 간접적으로 혈관내피형성성장인자의

역할을 감소시키는 방법들을 제시할 수 있게 된다. . 또한 기미 병변에서 관찰되는 PAR-2의 발현 증가는 향후 PAR-2의 역할을 감소시키는 치료 제재들의 중요성을 제시하는 바이다.

핵심 되는 말 : 혈관내피형성성장인자, 멜라닌세포, 각질형성세포, 멜라닌형성, 멜라닌 전달, Protease activated receptor-2, 기미