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석사학위 논문

Effects of Tranexamic Acid on the Activation of Autophagy System and the Production of Melanin in Cultured Melanoma Cells

조선대학교 대학원

생명과학과

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흑색종 세포에서 autophagy 활성화 및 멜라닌 생성에 미치는 tranexamic acid의 영향에 관한 연구

2017년 2월 24일

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이 논문을 이학 석사학위 신청 논문으로 제출함

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ABSTRACT

Effects of Tranexamic Acid on the Activation of Autophagy System and the Production of Melanin in Cultured Melanoma Cells

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Skin color is determined in accordance with the concentration and the distribution of melanin pigments in the skin interior, which are influenced by genetic factors and physiological or environmental conditions, such as ultra-violet (UV) light, fatigue and stress. The main skin color pigment, melanin is produced by the oxidation of the amino acid tyrosine in melanocytes. On the other hands, it is been well known that the cellular autophagy system plays an important role in removing wastable proteins (misfolded of aggregated) and eliminating dysfunctional organelles, including mitochondria, endoplasmic reticulum and peroxisomes. Recent studies show that the system also can be involved in the biogenesis of melanin and the degradation of melanosome, suggesting that the it's activation can be related to the fate of skin color by reducing the production of melanin pigments. However, there are still no direct evidences, showing a relationship between the activation of autophagy system and melanogenesis. The compound tranexamic acid (*trans*-4-aminomethyl-cyclohexanecarboxylic acid, TXA) routinely used by this study has been used as a hemostatic agent, due to its selective anti-plasmin effect. In recent, TAX is also being used as an agent to reduce the synthesis of melanin in melasma, although its action mechanism is not well known. Therefore, the present study was performed to examine the effects of TXA on the activation of autophagy system and the production of melanin in a cultured melanoma cell line called B16-F1. The results

obtained were as follows: The result of mushroom tyrosinase assay showed that TXA could directly inhibit the mushroom tyrosinase activity *in vitro* when L-tyrosine or L-DOPA (L-3,4-dihydroxyphenylalanine) was used as substrate. In addition, the rate of melanin synthesis could be decreased to approximately 46% by the treatment with TXA (4 ㎎/㎖) for 52 h in B16-F1 cells that were stimulated by α-MSH (α-melanocyte-stimulating hormone) to synthesize melanin. These results suggest that TXA can modulate the production of melanin by inhibiting tyrosinase. On the other hands, TXA could mediate the production of autophagy-related proteins, such as MAPKs (ERK and p38 *etc*), Beclin-1, Atg12, and LC3 I-II, whereas it could decrease the synthesis of mTOR complex, as judged by Western blottings. In addition, the confocal microscopic analysis clearly showed that TXA could lead the formation of autophagosomes in B16-F1 cells, as shown by an immunostaining with anti-LC3 antibody. To examine the effects of TXA on ERK1/2 signaling pathway and the production of melanogenesis-associated proteins, the expression levels of MAPKs (ERK and p38 *etc*), MITF (microphthalmia-associated transcription factor), tyrosinase, and TRP1 were also measured by Western blottings. The results showed that the compound clearly decreased the expression levels of MITF, tyrosinase, and TRP1/2 (tyrosinase-related protein 1 and 2), suggesting that it could decrease the production of melanin synthesis through by alleviating the production levels of tyrosinase and TRP1/2, which are all involved in the melanin synthesis, due to the reduced-MITF protein level. The involvement of autophagy activation by TXA in melanin synthesis also could be confirmed in B16-F1 cells transfected with 60 pmols of small interfering RNAs (siRNAs) for mTOR and Atg5. When the non-transfected cells were treated with TXA (0.1, 0.5, 1, 2, or 4 ㎎/㎖) for 52 h, there were clear decreases in melanin synthesis in a dose-dependent manner. However, when the cells that were transfected with siRNAs for mTOR or Atg5 were treated with 1 ㎎/㎖ of TXA for 52 h, the levels of melanin synthesis could be increased to approximately 20% and 40%, respectively, compared to that in non-transfected cells. In conclusion, all results obtained by this study suggest that TXA can inhibit tyrosinase activity directly and also reduce the melanin synthesis in skin by activating the ERK signaling pathway and the autophagy system. Therefore, the results demonstrate that TXA could be a potential agents for melasma treatment and also for a cosmetic compound for skin whitening.

Ⅰ. INTRODUCTION

The colors of the skin, hair, and eyes are derived from the biological pigmentation factor melanin, which is produced via melanogenesis within melanosomes (Kim *et al*., 2013). The amount of melanin production is influenced by various genetic background and environmental factors, including UV-light (Fig. 1). One of the roles of melanin is to protect the skin, tissues, and genes from UV-induced skin injury (Lim *et al*., 2009). However, the formation of excessive melanin in the skin causes hyperpigmentation, which can induce skin disorders such as melasma, freckles, and geriatric pigment spots (Ha *et al*., 2005). In response to UV-light, melanocyte immediates redistribution of preexisting melanin, followed by enhanced melanogenesis in epidermal melanocytes and increased transfer of melanin to epidermal keratinocytes (Archambault *et al*., 1995).

Melanins are produced as two different forms, that are eumelanin and pheomelanin. Tyrosinase and tyrosinase-related protein 1/2 (TRP1/2) are mainly involved in melanin synthesis. In the melanin synthetic pathway called melanogenesis, tyrosinase is a rate limiting enzyme that catalyzes tyrosine to L-DOPA (L-3,4-dihydroxyphenylalanin) and oxidizes it to dopaquinone (Fig. 2). Eumelanin is synthesised through TRP-2, which functions as a DOPA chrome tautomerase, catalyses the rearrangement of DOPA chrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA), and TRP-1 oxidizes DHICA to a carboxylated indole-quinone (Yokoyama *et al*., 1994). Pheomelanin is synthesised through the reaction of dopaquinone and cysteine. In addition, tyrosinase and TRP1/2 are transcriptionally regulated by microphthalmia-associated transcription factor (MITF), in melanocytes (Jeong *et al*., 2013). The phosphorylation of the MITF by extracellular signal-regulated kinase (ERK) 2 results in its degradation through the proteasome pathway (Kim *et al*., 2013).

Autophagy is catabolic procedure and allows the orderly degradation and recycling of organelles. Therefore, autophagy helps cell survival under stress conditions such as organelles damage and oxidative stress by supplying cellular energy sources. Nevertheless, the excessive activation of autophagy is also associated with cell death processes and various diseases (Fig. 3) (Kim *et al*., 2013; Moore 2015). The mammalian target of rapamycin complex 1 (mTORC1) protein kinase is an important regulator of autophagy (Racikumar *et al*., 2004).

Stress condition like nutrient deprivation leads to the inactivation of mTOR kinase, resulting in the activation of Unc-51-like kinases 1 and 2 (ULK1/2) (Jung *et al*., 2009). The active ULK1/2 complex then translocates to autophagosome nucleation centers and triggers consecutive stages (Karakas *et al*., 2014). The ubiquitin-like conjugation systems involved in autophagy covalently binds the ubiquitin-like protein Atg12 to Atg5. The resulting conjugate protein then binds Atg16L1 to form an E3-like complex, which functions as part of the second ubiquitin-like conjugation system (Hanada *et al*., 2007). The second system involves the conjugation of microtubllue-associated protein 1 light chain 3 (MAP1LC3) to the lipid phosphatidylethanolamine (PE). The lipid conjugation leads to the conversion of the soluble form of LC3-I to the autophagic-vesicle-associated form LC3-II (Kabeya *et al*., 2004). LC3-II contributes to the closure of phagophores to form autophagosome (Fujita *et al*., 2008).

Autophagosome may fuse directly with a lysosome to form an autophagolysosome (Fig. 4). Once the cargos are degrade, the products are released to the cytosol to be reused by the cell (Karakas *et al*., 2014). As described, autophagy system is involved in degrading unnecessary and wastable materials and dyfunctional organelles to maintain the cellular homeostasis, possible involvement of the system in melanin metabolism are still unclear.

Tranexamic acid (*trans*-4-amino-methylcyclohexanecarboxylic acid, TXA) is a plasmin inhibitor and has been used to treat heavy bleeding from trauma, surgery, and heavy menstrual bleeding. TXA is a synthetic derivative of the amino acid lysine and exerts its effects by reversibly blocking lysine binding sites on plasminogen molecules. TXA has emerged as a potential drug for treating melasma because it inhibits melanin synthesis by the inhibition of plasminogen/plasmin pathway, thereby blocking the interaction between melanocytes and keratinocytes (Fig 5) (Tse *et al*., 2013). As plasminogen also exists in cultured human keratinocyte are known to produce plasminogen activator, there is a basic principle that TXA will affect keratinocyte function and interaction (Kim *et al*., 2015). However, the accurate action mechanisms of TXA on melanogenesis and its associated pathways have not yet been studied.

This study was performed to analyze inhibitory effect of TXA on tyrosinase activity *in vitro* and on melanin synthesis in cultured melanoma cell line called B16-F1. In addition, the effect of TXA on the activation of autophagy system was examined in B16-F1 cells. Based on these results, this study describes the effect of TXA on the activation of autophagy system and the production of melanin in B16-F1 cell.

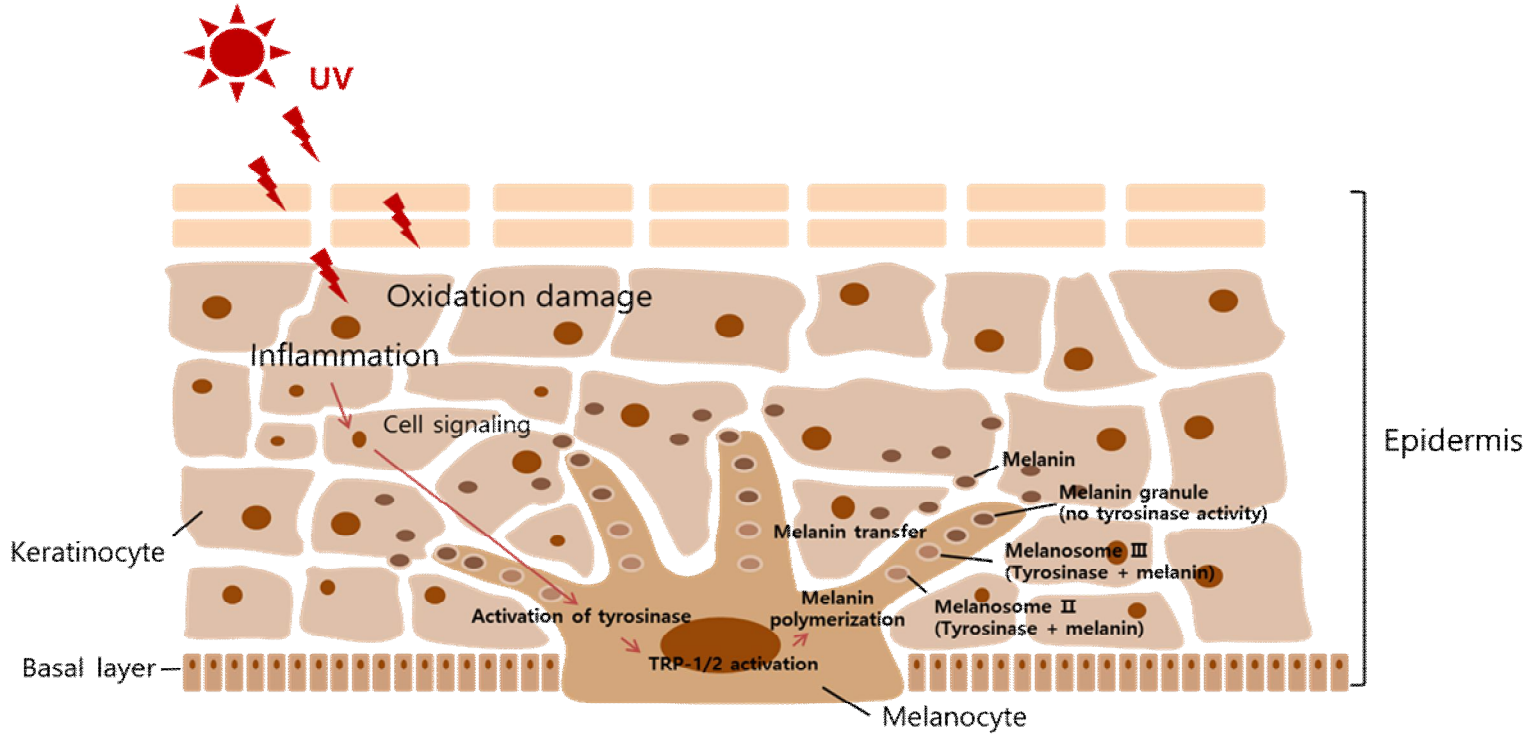

Fig. 1. Mechanism of melanin production in skin. In the skin, UV-light evokes melanogenesis. Melanin is produced by the oxidation of tyrosine by tyrosinase in melanocyte.

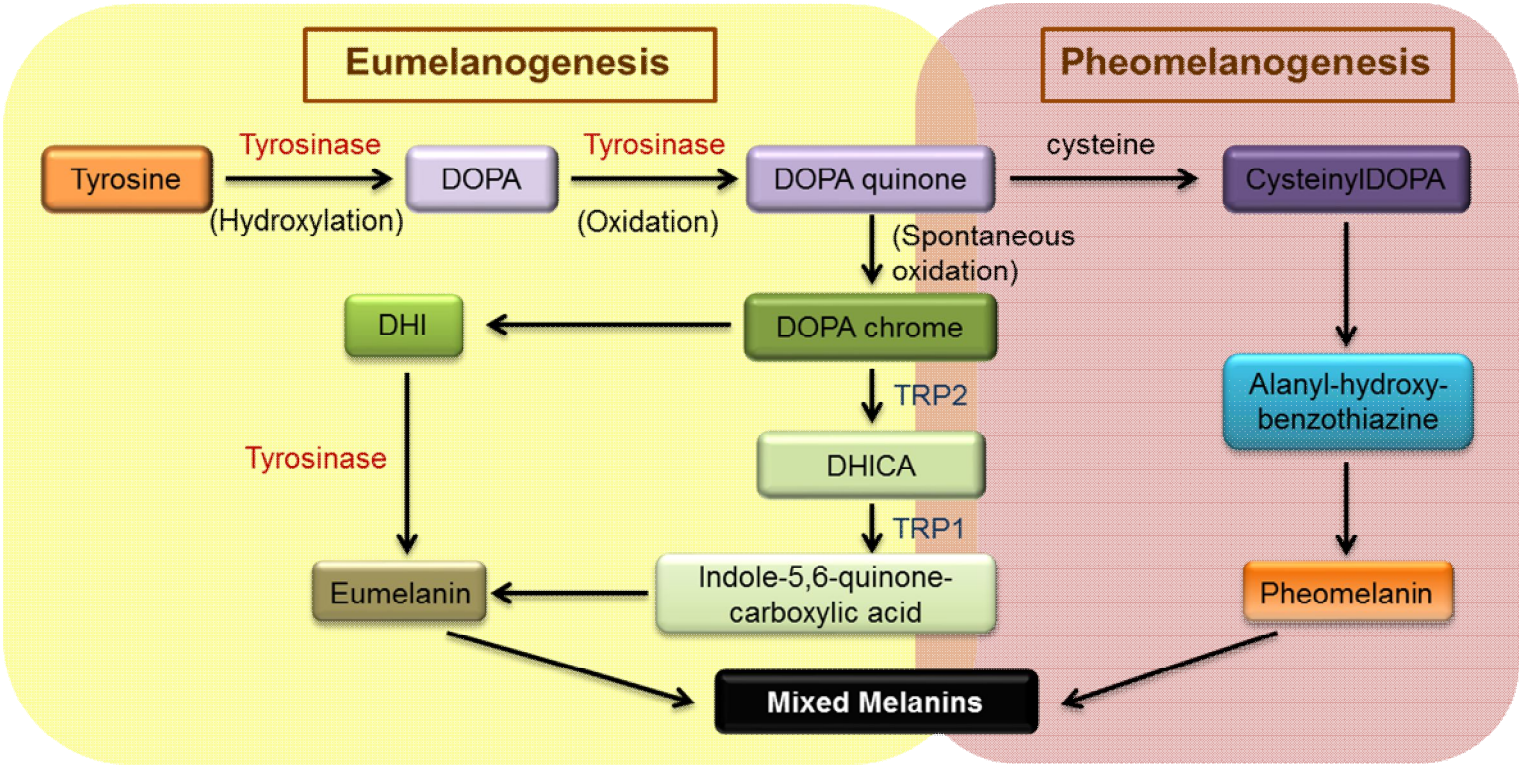

Fig. 2. The synthetic pathway of melanin. Melanogenesis begins with catalysis of the substrate tyrosine to produce DOPA by tyrosinase and partly tyrosinase hydroxylase. DOPA is converted to dopaquinone by tyrosinase. The dopaquinone is catalyzed and oxidized to eumelanin by TRP. Pheomelanin is synthesized through the reaction of dopaquinone and cysteine. DHICA, 5,6-dihydroxyin-dole-2-carboxylic acid; DOPA, 3,4-dihydroxyphenylalanin; TRP, tyrosinase-related protein. Modified from Yokoyama *et al.,* (1994).

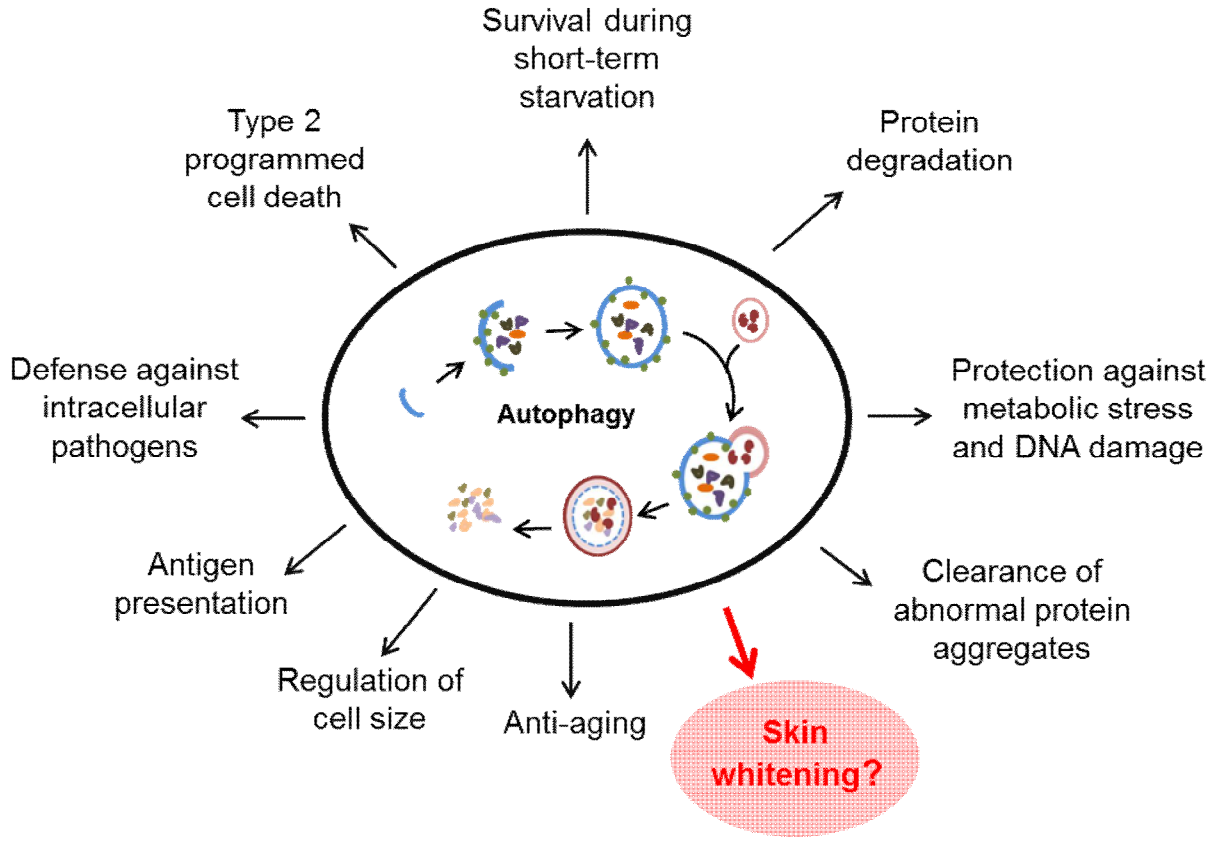

Fig. 3. Roles of autophagy system. Schematic diagram of the multiple cellular protective functions of autophagy in normal and dysfunctional cells. Modified from Moore (2015).

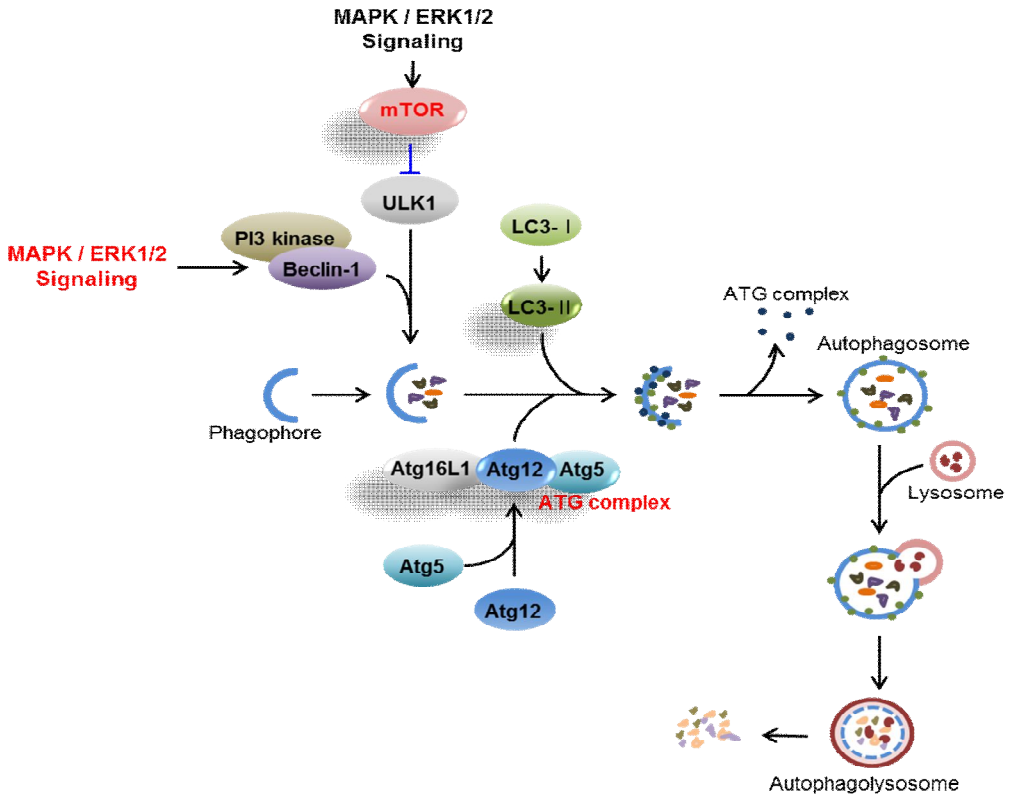

Fig. 4. Autophagy signaling pathway. The mTOR is a major controller for autophagy. Upon stimulatory signals, mTOR is inactivated and the ULK complex is hypophosphorylated and relocalizes to the phagophore, along with PIP3, produced by class III PI3K complex. Next, two conjugation systems, the ATG complex and LC3-II are added to the phagophore membrane. The membrane grows to enwrap a protein of the cytosol, forming an autophagosome. In this final step of the process, lysosomes fuse with the autophagosome, resulting in the degradation of the vesicle contents. Modified from Jung *et al*., (2009) and Fujita *et al.,* (2008).

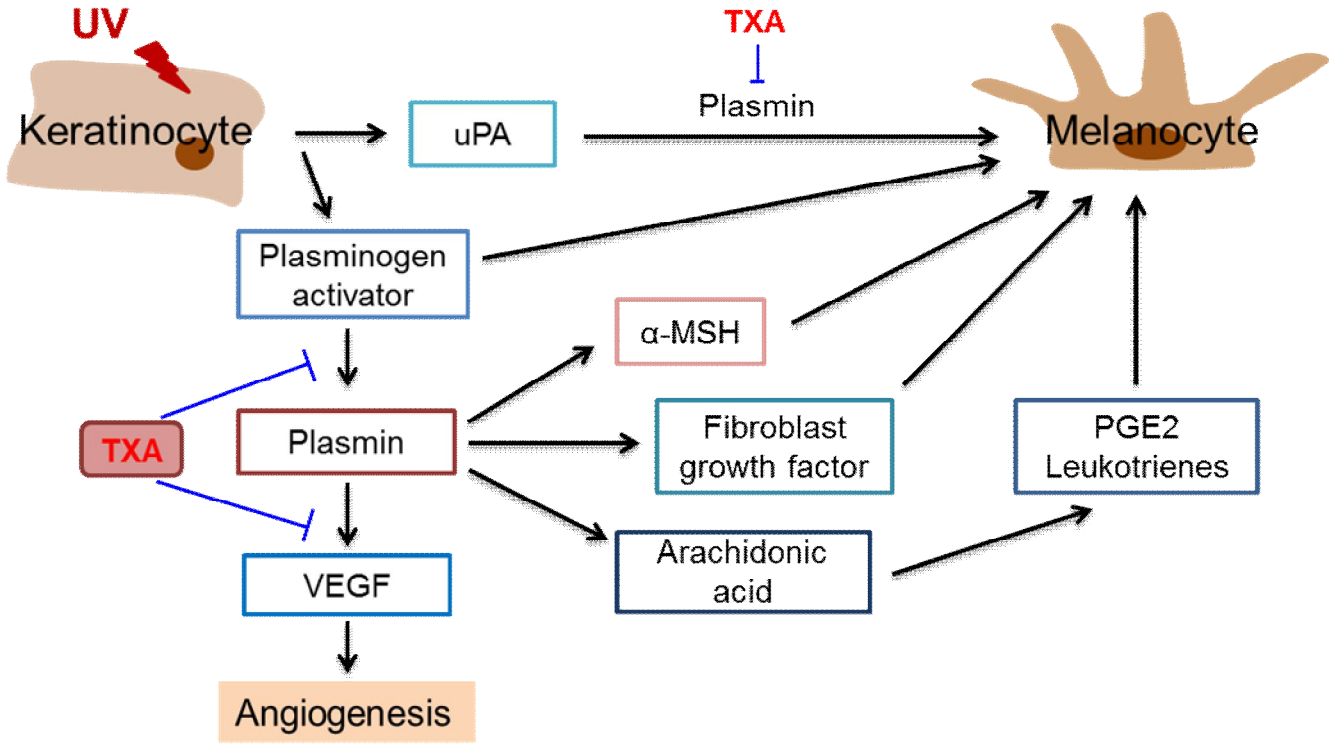

Fig. 5. Pathogenesis of melasma and tranexamic acid (TXA). Tranexamic acid has emerged as a potential drug for treating melasma because it inhibits melanin synthesis by the inhibition of plasminogen/plasmin pathway, thereby blocking the interaction between melanocytes and keratinocytes. α-MSH, α-melanocyte-stimulating hormone; PGE_2, prostagandin E_2; uPA, urokinase-type plasminogen activator; VEGF, vascular endothelial growth factor.

Ⅱ. MATERIALS AND METHODS

Ⅱ-1. Materials

Tranexamic acid (TXA) was donated from a Korean company Hugel (Hugel, Inc., Korea). Mushroom tyrosinase, L-tyrosine, L-DOPA, arbutin, poly-L-lysine(0.01% solution), 1% penicillin-streptomycin, and other chemicals were purchased from Sigma-Aldrich (St. Louis Mo, USA). Dulbecco's modified eagle's medium (DMEM) was from Lonza (USA). Antibodies raised against MAP-LC3β, GAPDH, tyrosinase, and TRP1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA., USA) and beclin-1, Atg12, phospho-Erk1/2, p44/42 MAP kinase, phospho-p38 MAP kinase, p38 MAPK, MITF, phospho-mTOR, and mTOR were from Cell Signaling (Cell Signaling Technology, Inc., USA).

Ⅱ-2. Mushroom tyrosinase activity assay

Mushroom tyrosinase assay was performed with L-tyrosine as a substrate for tyrosinase activity assay in a test tube (Jeong *et al*., 2013). The reaction mixtures were prepared by adding 240 ㎕ of TXA (10, 30, 50, 60, and 80 ㎎/㎖) or arbutin (0.2, 0.4, 0.8, 1, and 2 ㎎/㎖) diluted in 0.1 M potassium phosphate buffer (pH 6.8), 20 ㎕ of 1,500 U/㎖ mushroom tyrosinase, and 40 ㎕ of 1.5 mM L-tyrosine. The mixture was incubated for 15 min at 37°C and then measured absorbance at 490 nm. Arbutin was used as a positive control. The inhibition rate of tyrosinase activity was calculated using the following formula:

Inhibition rate of tyrosinase activity (%) = 100 - (S-B) / (C-B) × 100

in which,

S: A_{490} from TXA plus tyrosinase in buffer

B: A_{490} from buffer only

C: A_{490} from tyrosinase only in buffer

Ⅱ-3. DOPA oxidation inhibition assay

The inhibitory effect of tranexamic acid on L-DOPA oxidation was determined according to the methods described above in mushroom tyrosinase activity assay with a minor modification. The reaction mixtures were prepared by adding 450 ㎕ of TXA (10, 30, 50, 60, and 80 ㎎/㎖) or arbutin (0.2, 0.5, and 1 ㎎/㎖) diluted in 0.1 M potassium phosphate buffer (pH 6.8) and 25 ㎕ of 1,500 U/㎖ mushroom tyrosinase. The mixture was incubated for 6 min at 37°C. Then, 25 ㎕ of 2 mM L-DOPA added to initiate the reaction. The mixture was incubated for 1 min at 25°C and then measured absorbance at 475 nm. Arbutin was used as a positive control. The inhibition rate of tyrosinase activity was calculated using the following formula:

Inhibition rate of tyrosinase activity (%) = 100 - (S-B) / (C-B) × 100

in which,

S: A_{475} from TXA plus tyrosinase in buffer

B: A_{475} from buffer only

C: A_{475} from tyrosinase only in buffer

Ⅱ-4. Cell culture

Murine melanoma cells (B16-F1) routinely used by the present study were purchased from the Korean Cell Line Bank (KCLB; Seoul, Korea). The cells were

cultured in dulbecco's modified eagle's medium (DMEM; Lonza, Swiss) containing 10% fetal bovine serum (FBS; ATLAS, USA) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Luis MO, USA) at 37°C in 5% CO_2. The culture medium was changed every 2 days (Lim *et al*., 2009). The cells were harvested by trypsin-ethylenediaminetetraacetic acid (EDTA) (Welegen, Daegu, Korea) when the cells were approximately 70% confluent.

Ⅱ-5. Cell viability assay

Cell viability was evaluated using the CellTiter 96® Non-Radioactive cell proliferation assay (Promega; USA). B16-F1 cells were cultured at a density of 0.5×10^5 cells/well in 96-well plates. After culturing for 24 h, cells were treated with various concentrations of TXA (0.1 ~ 10 ㎎/㎖), arbutin (0.1 ~ 10 ㎎/㎖), and TGF-β (0.00002 ~ 0.2 ng/㎖) for 24 h. At the end of the incubation, 15 ㎕ of MTT dye solution was added to each well and then incubated at 37°C in 5% CO_2 for 4 h. It was added 100 ㎕ of the Solubilization Solution/Stop Mix to each well. One hour after addition of the Solubilization Solution/Stop Mix, the contents of the wells mixed to get a uniformly colored solution. Mixing could be done using a multichannel pipette. It was measured the absorbance at 570 nm wavelength using a 96-well plate reader.

Ⅱ-6. Western blot analysis

B16-F1 cells were seeded at a density of 0.5×10^5 cells/well in 6-well culture plates. After incubating for 24 h to induce autophagy, the cells were treated with

TXA (1 ㎎/㎖) or TGF-β (10 ng/㎖) at 37°C in 5% CO_2 for 15 min. In addition, to induce melanogenesis, the cells were treated with 10 nM α-MSH with 1 ㎎/㎖ TXA or 0.2 ㎎/㎖ arbutin for 24 h at 37°C in 5% CO_2. The protein extracts were isolated from the cells using a ProNA CETi lysis buffer (TransLab, Korea), in which, protein concentrations were measured by Bradford assay. Fifty ㎍ of proteins were boiled at 100°C for 3 min and then loaded onto 8%, 12%, or 15% polyacrylamide gel (Kim *et al*., 2013). After the electrophoresis, proteins were transferred onto PVDF membrane (Bio-Rad, Hercules, CA., USA). The membrane was blocked with 5% skim milk in TBS-T (250 mM Tris-HCl, 1.5 mM NaCl with 0.1% Tween 20; pH 8.0) at room temperature (RT) for 2 h. The membrane was then incubated with specific primary antibodies (1:500 in the blocking buffer) overnight at 4°C. And washed six times with TBS-T buffer. The membrane was then incubated with HRP-conjugated secondary antibodies (1:4000 in the blocking buffer) at RT for 2 h and washed five times with TBS-T buffer. The signals were detected using EZ-Western Lumi Plus system (DAEILLAB SERVICE co., Ltd., Seoul, Korea).

Ⅱ-7. Immnostaining for confocal microscopic analysis

B16-F1 cells were seeded at a density of 0.1×10^5 cells/well in 12-well culture plates. The cells were grown on poly-L-lysine (0.01% solution, Sigma-Aldrich, St. Luis MO, USA)-coated glass coverslips in 12-well culture plates. After culturing for 24 h, the cells were treated with 10 nM α-MSH with 1 ㎎/㎖ TXA or 10 ng/㎖ TGF-β for 15 min or 30 min at 37°C in 5% CO_2. The cells were washed with PBS, fixed with 3.7% formaldehyde in PBS for 20 min at RT. And washed three times with PBS, The cells were then treated with 0.1% TritonX-100 in PBS for 10 min. And washed three times with PBS. The cells were then blocked with 1% BSA in PBS for RT at 20 min. And washed three times with PBS. The cells were then

incubated overnight with primary antibody mouse anti-MAP LC3 that diluted 1:50 in PBS. After incubating for overnight at 4°C, and washed with PBS. The cells were then incubated secondary antibody Alexa Fluor 488 goat anti-mouse IgG (diluted 1:200 with PBS; Invitrogen, USA) for 1 h at RT. And washed three times with PBS, mounted on a slide with 5 ㎕ 4' 6-diamidino-2-phenylindole (DAPI). Confocal imaging was performed with a Zeiss LSM-510 microscope (confocal laser scanning microscopy)

Ⅱ-8. Transfection of siRNA into B16-F1 cells

B16-F1 cells were transfected with 60 pmols each of mTOR and Atg5 small interfering RNA (siRNA; Santa cruz, USA) with siRNA transfection reagent and suspended in siRNA transfection medium (serum-free medium) at 37°C in 5% CO_2 for 24 h. Control and transfected cells were used as described previously (Rai *et al*., 2013).

Ⅱ-9. Melanin content measurement

B16-F1 cells were cultured at a density of 0.5×10^5 cells/well in 6-well plates using the DMEM without phenol red (WELGENE, Daegu, Korea). After culturing for 24 h, cells were treated with 10 nM α-MSH (Sigma-Aldrich, St. Luis MO, USA) alone and 10 nM α-MSH with various concentrations of TXA (0.1, 0.5, 1, 2, and 4 ㎎/㎖) for 52 h at 37°C in 5% CO_2. The cell-free medium was collected after centrifugation for 4 min at 13,000 rpm. The extracellular melanin content was measured by analyzing the absorbance at 490 nm. For estimation, a standard graph was made using synthetic melanin (0 to 50 ㎍/㎖; Sigma-Aldrich, St. Luis MO, USA).

Ⅲ. RESULTS AND DISCUSSION

Ⅲ-1. Inhibitory effect of tranexamic acid on mushroom tyrosinase activity *in vitro*.

To investigate the inhibitory effect of TXA on mushroom tyrosinase activity *in vitro*, a reaction mixture composed of L-tyrosine (1.5 mM), mushroom tyrosinase (1,500 U/㎖), TXA (10, 30, 50, 60 or 80 ㎎/㎖) or arbutin (0.2, 0.4, 0.8, 1, or 2 ㎎/㎖) that used as a positive control was incubated for 15 min at 37°C and then the resulting degree of tyrosine hydroxylation was measured by analyzing the absorbance at 490 nm. As shown in Fig. 6, the tyrosinase activity decreased by TXA (80 ㎎/㎖) to approximately 13% in a dose-dependent manner, compared to that of non-treated control. This result suggests that TXA can directly inhibit the tyrosinase activity.

Ⅲ-2. Inhibitory effect of tranexamic acid on the oxidation of L-DOPA

To observe the inhibitory effect of TXA on the oxidation of L-DOPA by mushroom tyrosinase activity *in vitro*, a reaction mixture composed of mushroom tyrosinase (1,500 U/㎖), TXA (10, 30, 50, 60 or 80 ㎎/㎖) or arbutin (0.2, 0.5, or 1 ㎎/㎖) was pre-incubated at 37°C for 6 min and the reaction was continued for 1 min at 25°C after adding 2 mM of L-DOPA. The resulting oxidation degree of L-DOPA was measured by analyzing the absorbance at 475 nm. As shown in Fig. 7, the tyrosinase activity decreased by 80 ㎎/㎖ of TXA to approximately 20% in a dose-dependent manner, compared to that of non-treated control. This result confirms once again that TXA can directly inhibit the tyrosinase activity.

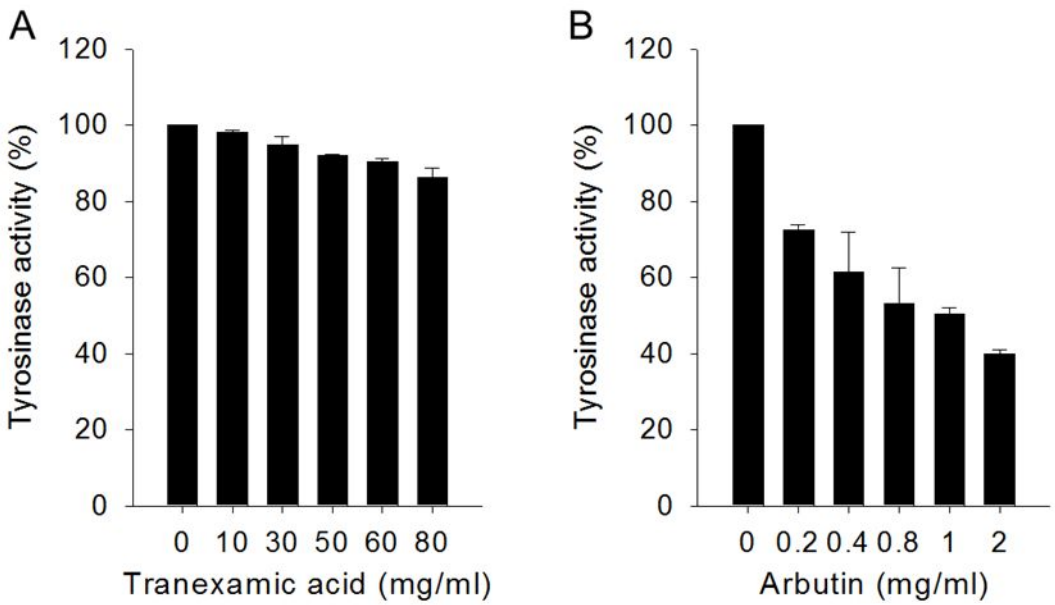

Fig. 6. Effect of tranexamic acid on mushroom tyrosinase activity. Different concentrations of TXA (10, 30, 50, 60 or 80 ㎎/㎖) were incubated with L-tyrosine (1.5 mM) and mushroom tyrosinase (1,500 U/㎖) at 37°C for 15 min *in vitro* and absorbances at 490 nm were measured as described in Materials and Methods. (A) Inhibitory effect of TXA on mushroom tyrosinase activity. (B) Inhibitory effect of arbutin (0.2, 0.4, 0.8, 1, or 2 ㎎/㎖) on mushroom tyrosinase activity, which was used as a positive control.

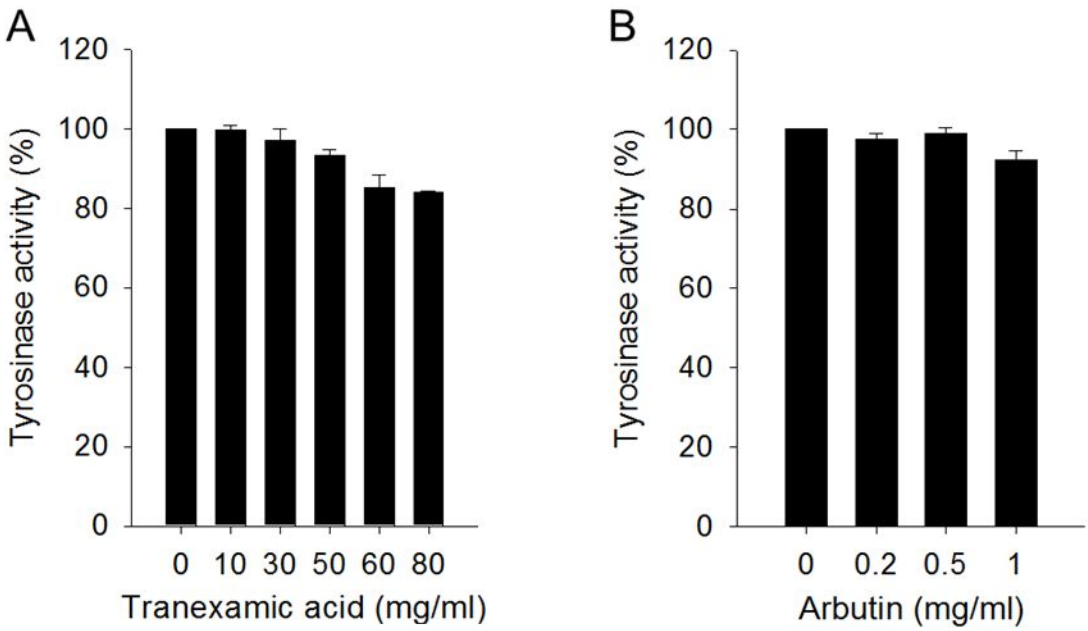

Fig. 7. Effect of tranexamic acid on mushroom tyrosinase activity. Different concentrations of TXA (10, 30, 50, 60 or 80 ㎎/㎖) were pre-incubated with mushroom tyrosinase (1,500 U/㎖) for 6 min at 37°C and the reaction was continued for 1 min at 25°C after adding 2 mM of L-DOPA. The enzyme activities were measured at A_{475} described in Materials and Methods. (A) Inhibitory effect of TXA on mushroom tyrosinase activity. (B) Inhibitory effect of arbutin (0.2, 0.5, and 1 ㎎/㎖) on mushroom tyrosinase activity, which was used as a positive control.

III-3. Effect of tranexamic acid on cytotoxicity on B16-F1 cells

To assess the cytotoxic effect of TXA on B16-F1 cells, MTT assay was performed, in which cells were treated with various concentrations of TXA (0.1, 0.5, 1, 5, and 1 ㎎/㎖) for 24 h. As shown in Fig. 8, TXA did not show cytotoxic effects up to 5 ㎎/㎖ and the cells proliferation decreased by 10 ㎎/㎖ of TXA to approximately 20%, compared to non-treated control. All further experiments were performed in the presence of 1 ㎎/㎖ TXA on B16-F1 cells if there are no comments.

III-4. Activation of autophagy system by tranexamic acid in B16-F1 cells

It has been known that a kinase mTOR is a critical regulator of autophagy induction, in that the positive regulation of mTOR (through Akt and MAPK signaling) suppresses the system, whereas the negative regulation of the kinase (by AMPK and p53 signaling pathway) promotes it (Alers *et al*,. 2012). Especially ERK1/2 pathway plays a key role in regulating autophagy (Wu *et al*., 2011). In this study, the effect of TXA on the activation of autophagy system was examined in B16-F1 cells. When B16-F1 cells were treated with TXA (1 ㎎/㎖) or TGF-β (10 ng/㎖) for 15 min or 30 min, the expression levels of phospho-p38, phospho-ERK1/2, Beclin-1, Atg12, and LC3 were increased to approximately 46%, 10%, 48%, 126%, and 49%, respectively, compared to non-treated control, as revealed by Western blottings (Fig. 9). However, the expression level of phospho-mTOR that is a negative regulator for

autophagy system was decreased to about 65% by TXA. These results suggest that TXA can induce the up-regulation of autophagy-related proteins (Beclin-1, Atg12, and LC3) expressions in B16-F1 cells by activating ERK1/2 signaling pathway.

III-5. Formation of autophagosomes by tranexamic acid

The activation of autophagy begins with the formation of phagophores (also called isolation membranes). The phagophores expand and capture cargos inside and then form autophagosome to be fused with a lysosome (Zhou *et al*., 2015). Atg proteins control the process forming autophagosomes through Atg12-Atg5 and LC3-II (Atg8-II) complexes, during which Atg12 is conjugated to Atg5 in a ubiquitin-like reaction that requires Atg7 and Atg10 (Hanada *et al*., 2007). The Atg12-Atg5 conjugate then interacts noncovalently with Atg16 to form a large complex (Hanada *et al*., 2007). In the process, LC3-I is conjugated to a lipid phosphatidylethanolamine (PE) in a ubiquitin-like reaction that requires Atg7 and Atg3 and the resulting lipidated form of LC3 (also known as LC3-II that is a prominent marker molecule routinely used for the detection of autophagosome) is attached to the autophagosome membrane (Hanada *et al*., 2007).

In this study, the ability of TXA to form autophagosomes was observed by confocal microscopy after immunostaining with anti-LC3 antibody. As shown in Fig. 10, the autophagosomes could form in the cells treated with TXA (1 ㎎/㎖), TGF-β (10 ng/㎖) or α-MSH (10 nM) for 15 min or 30 min. This result suggests that TXA can lead the formation of autophagosome.

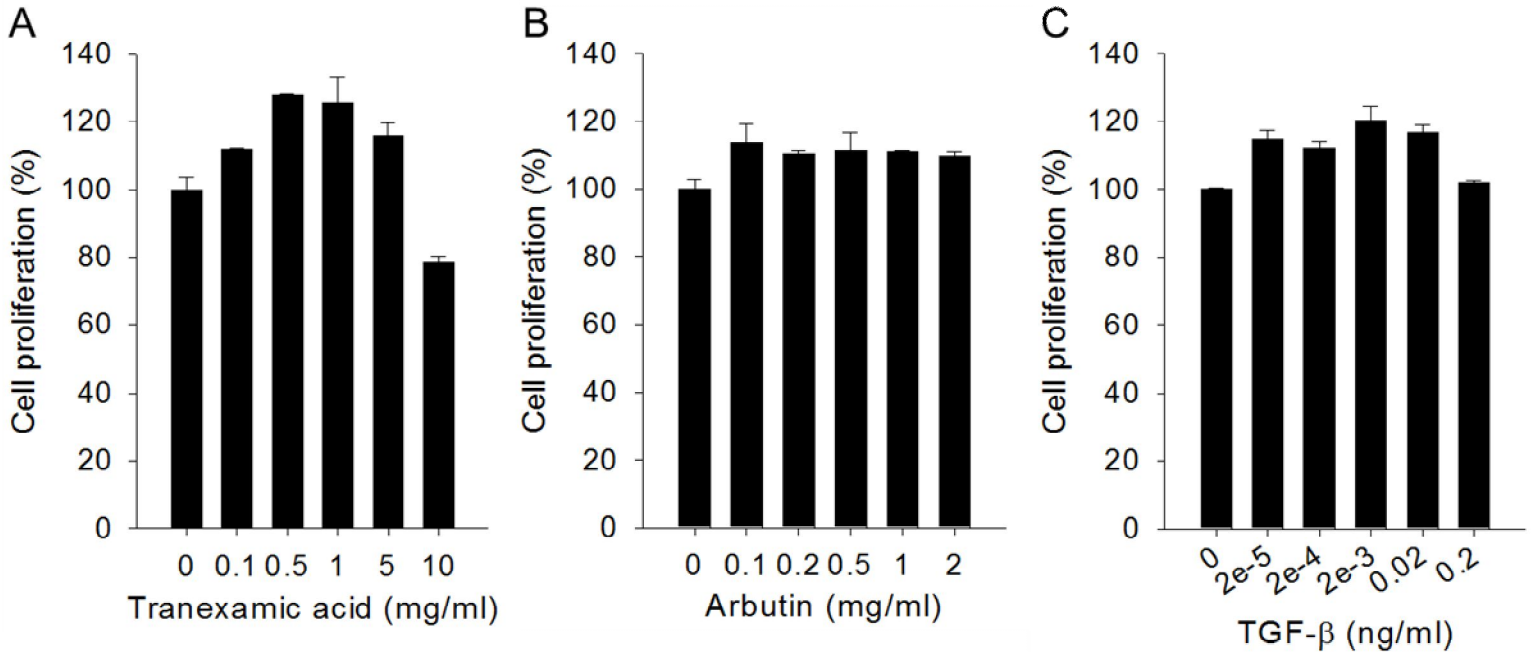

Fig. 8. Effect of tranexamic acid on cytotoxicity on B16-F1 cells. B16-F1 cells were treated with various doses of TXA (0.1 ~ 10 ㎎/㎖) (A), arbutin (0.1 ~ 2 ㎎/㎖) (B), and TGF-β (0.00002 ~ 0.2 ng/㎖) for 24 h and the cell survivals were examined by MTT assay as described in Materials and Methods. TGF-β was used as a positive control.

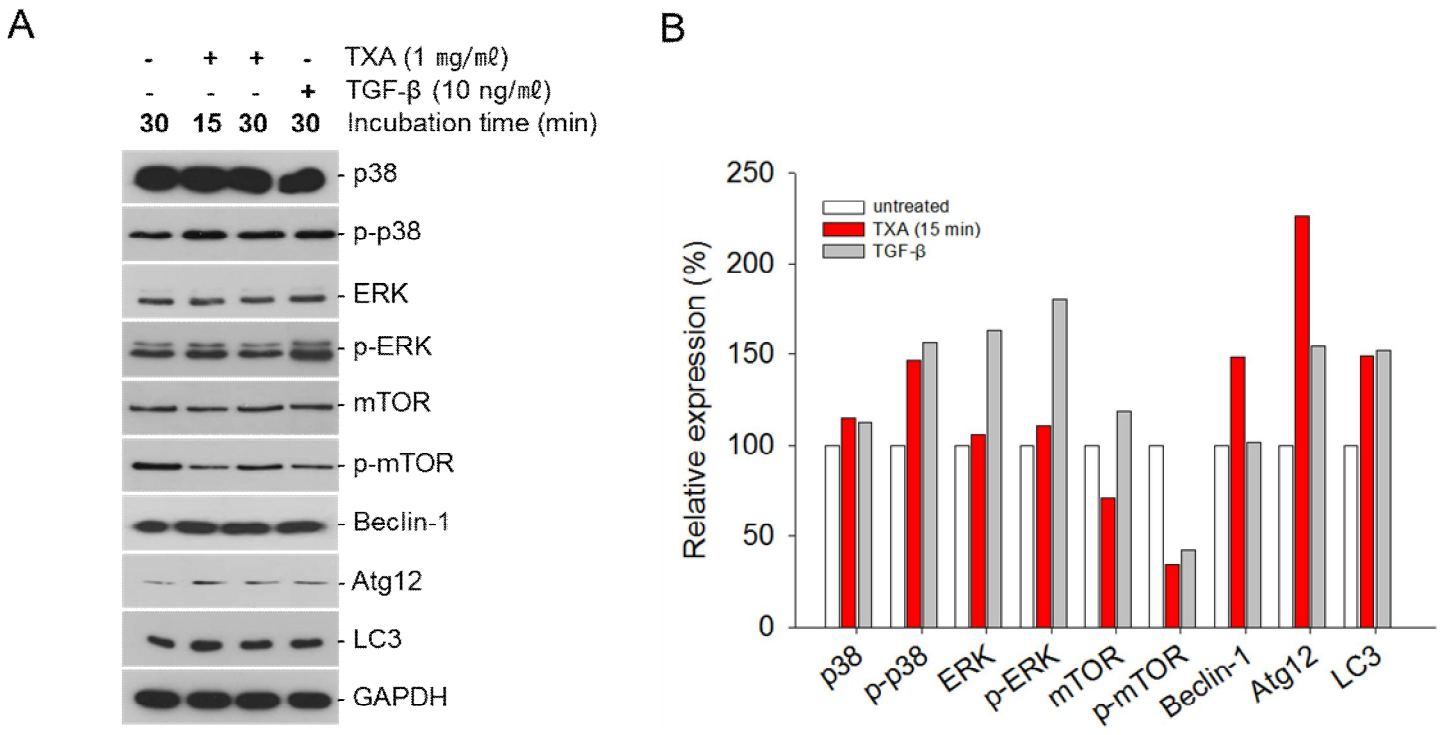

Fig. 9. Tranexamic acid induces autophagy system through the activation of MAPK/Erk1/2 signaling pathway. (A) B16-F1 cells were treated with TXA (1 mg/mℓ) for 15 min and 30 min. Proteins from each sample were separated by SDS-PAGE and Western blottings were performed with antibodies raised against p38, p-p38, ERK1/2, p-ERK1/2, mTOR, p-mTOR, Beclin-1, Atg12, LC3, and GAPDH. TGF-β was used as a positive control. (B) The histograms show the relative expression rates. Each value was calculated as the ratio of signal intensity, compared to that of GAPDH.

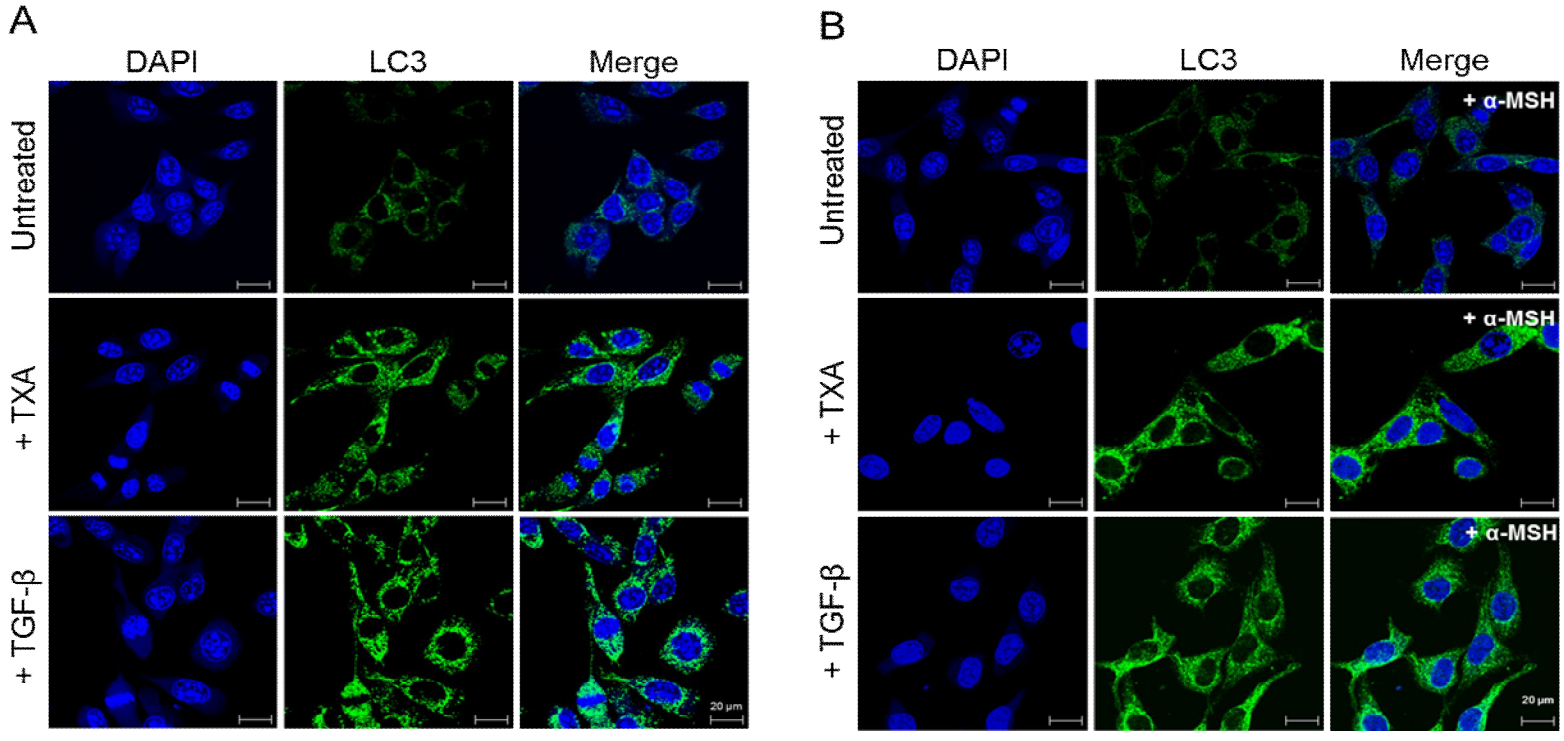

Fig. 10. Confocal microscopic analysis of the induction of autophagy by tranexamic acid in B16-F1 cells. (A) The cells treated with TXA (1 ㎎/㎖) for 15 min or TGF-β (10 ng/㎖) for 30 min were stained with anti-LC3 antibody labeled with fluorescein and the green fluorescences emitted were observed with confocal microscope. (B) The cells treated with TXA (1 ㎎/㎖) for 15 min or TGF-β (10 ng/㎖) for 30 min in the presence of α-MSH (10 nM) were stained with the same anti-LC3 antibody and the fluorescences were observed with confocal microscope.

III-6. Inhibitory effect of tranexamic acid on melanin synthesis in B16-F1 cells

It has been reported that TXA is a inhibitor against urokinase-type plasminogen activator (uPA) and can inhibit melanin synthesis in melanocytes by interfering with the interaction of melanocytes and keratinocytes (Higashi 1998; Maeda *et al*., 2007). Therefore, it is basically expected that the compound can reduce the degree of melanin pigmentation in skin. To investigate the inhibitory effect of TXA on melanin production, B16-F1 cells were treated with α-MSH (10 nM) alone or α-MSH (10 nM) with TXA (0.1, 0.5, 1, 2, or 4 ㎎/㎖) for 52 h. As shown in Fig. 11, the rate of melanin synthesis could be decreased dose-dependently by the treatment of TXA, in which 4 ㎎/㎖ of TXA decreased to about 46%, compared to that in α-MSH only. This result suggests that TXA can directly inhibit the melanin synthesis in cell.

III-7. Inhibitory effect of tranexamic acid on melanogenesis in B16-F1 cells

It was reported that phospho-ERK1/2 results in degradation of MITF, which down-regulates the expression of melanogenesis-associated proteins such as tyrosinase and TRP1/2, leading the suppression of melanogenesis (Lee *et al*., 2015). In this study, the activation ability of TXA to ERK signaling pathway and its effects on the expressions of MITF, tyrosinase, and TRP-1 were examined using Western blottings (Fig. 12). As shown in Figs. 12A and B, the expression levels of ERK1/2 and p-ERK1/2 were increased to 18% and 5%, respectively, in B16-F1 cells treated with 1 ㎎/㎖ of TXA for 24 h. In contrast, the levels of MITF, tyrosinase, and TRP 1 were significantly decreased to 17%, 14%, and 5%, respectively, in the cells

treated with 1 ㎎/㎖ of TXA for 24 h, compared to that of α-MSH only (Figs. 12A and B). These results suggest that TXA has synergic dual functions to exert an anti-melanogenetic effect: 1) it can activate ERK signaling pathway, which leads the degradation of MITF that down-regulates the expression of melanogenesis-associated proteins such as tyrosinase and TRP1/2; 2) it can also directly suppress the productions of proteins/enzymes, including MITF, tyrosinase, and TRP 1/2.

III-8. Effect of siRNAs against mTOR and Atg5 on the production of melanin synthesis

All the results demonstrate that TXA could activate the autophagy system by up-regulating the autophagy-related proteins, but inhibit the melanogenesis by stimulating the ERK signaling pathway and also by up-regulating the melanogenesis-associated proteins. However, there was still necessary to confirm that TXA could activate the autophagy system that eventually leads to the reduction of melanin synthesis. Therefore, the reverse genetic approaches using small interfering RNAs (siRNAs) against mTOR and Atg5 mRNAs were applied to reveal a relationship between the activation of autophagy system and the inhibition of the production of melanin by TXA (Fig. 13). When non-transfected B16-F1 cells were treated with TXA (0.1, 0.5, 1, 2, or 4 ㎎/㎖) for 52 h, there were clear decreases in melanin synthesis in a dose-dependent manner. However, when the cells transfected with siRNAs for mTOR or Atg5 were treated with 1 ㎎/㎖ of TXA for 52 h, the levels of melanin synthesis could be clearly increased to approximately 20% and 40%, respectively, compared to that in non-transfected cells (Figs. 13A and B). These results clearly show that TXA can regulate the melanin synthesis in B16-F1 cells through by the activation of autophagy system.

Collectively, all results obtained by this study suggest that 1) TXA itself can be a inhibitor for tyrosinase activity; 2) it can activate the autophagy system by up-regulating the autophagy-related proteins, including Beclin-1, Atg12, and LC3 through by inhibiting the expression level of mTOR; and finally 3) it can activate ERK signaling pathway, which results in the degradation of MITF that down-regulates the expression of melanogenesis-associated proteins such as tyrosinase and TRP1/2, together with its direct suppressive role in the productions of proteins/enzymes, including MITF, tyrosinase, and TRP 1/2 (Fig. 14). Therefore, the results demonstrate that TXA could be a potential agents for melasma treatment and also for a cosmetic compound for skin whitening.

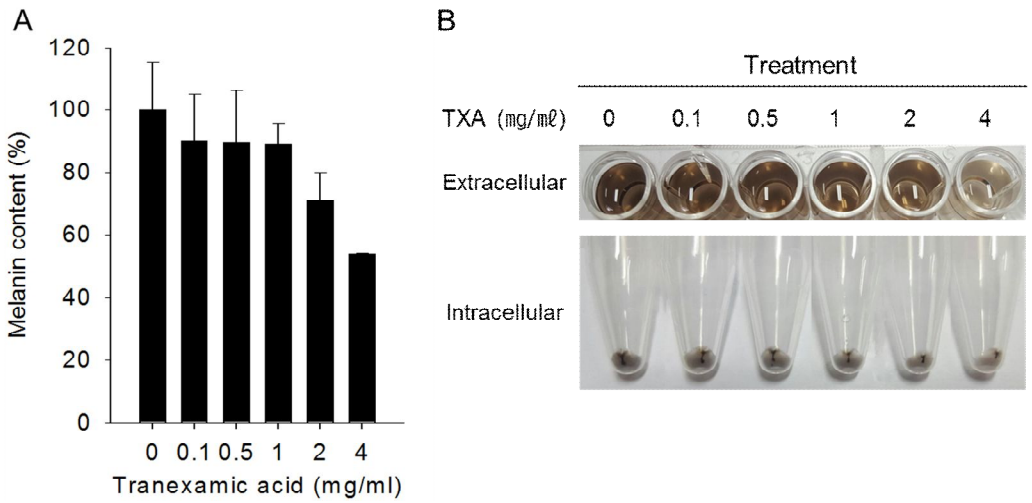

Fig. 11. Inhibition of melanin synthesis by tranexamic acid in B16-F1 cells. (A) B16-F1 cells were co-treated with α-MSH (10 nM) and tranexamic acid (0.1~4 ㎎/㎖) for 52 h and the amounts of melanin were examined by measuring absorbance at 490 nm. (B) Photographs showing the inhibitory effects of TXA on melanin production, on which the words, extracellular and intracellular mean the culture supernatant and cell precipitate, respectively.

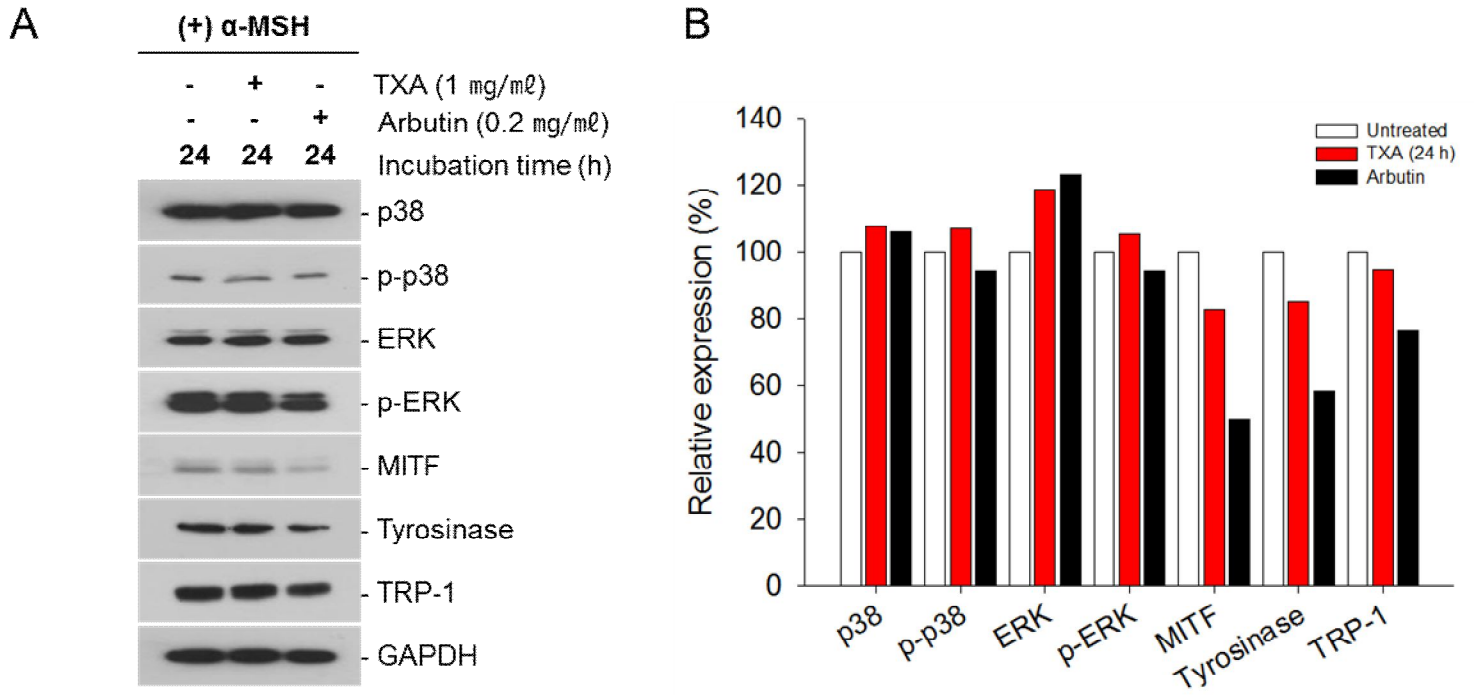

Fig. 12. Effect of tranexamic acid on ERK1/2 pathway and melanogenesis-associated protein expression. (A) B16-F1 cells were treated with TXA (1 ㎎/㎖) for 24 h. Proteins from each sample were separated by SDS-PAGE and estern blottings were performed with antibodies raised against p38, p-p38, ERK1/2, p-ERK1/2, MITF (microphthalmia-associated transcription factor), tyrosinase, TRP-1, and GAPDH. Arbutin (0.2 ㎎/㎖) was used as a positive control. (B) The histograms show the relative rates of expressions. Each value was calculated as the ratio of signal intensity, compared to that of GAPDH.

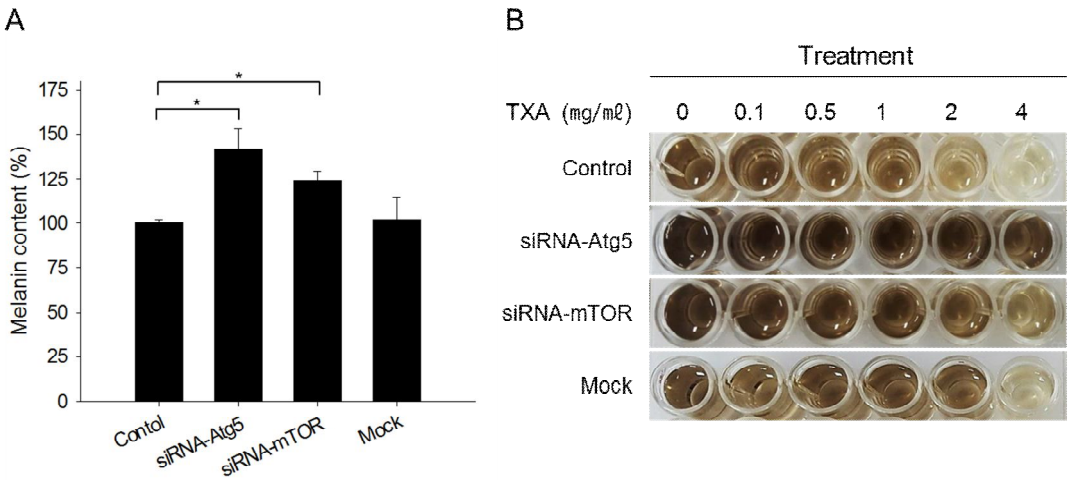

Fig. 13. Effects of siRNAs for mTOR, Atg5, and non-specific control on the melanin production in B16-F1 cells. B16-F1 cells were transfected with siRNAs specific to mTOR, Atg5, or non-specific control (Mock) siRNA. (A) The melanin content were calculated by measuring the absorbance at 490 nm. Data were shown as mean ± SEM (*$p<0.0001$). (B) Photographs showing the inhibitory effects of the corresponding siRNAs melanin production.

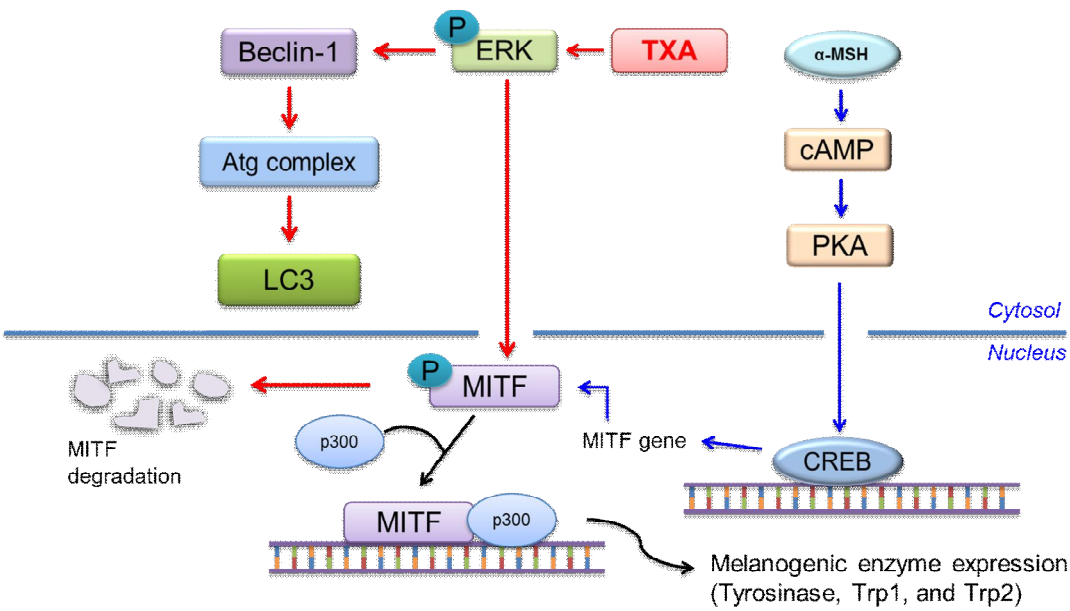

Fig. 14. Tranexamic acid can induce the activation of autophagy system and lead skin whitening through by ERK phosphorylation. TXA can activate ERK signalling pathway, which induces the activation of autophagy system and leads the degradation of MITF that down-regulates the expression of melanogenesis-associated proteins such as tyrosinase and TRP1/2.

Ⅳ. 초록

흑색종 세포에서 **autophagy** 활성화 및 멜라닌 생성에 미치는 **tranexamic acid**의 영향에 관한 연구

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피부색깔을 결정하는 멜라닌(melanin) 색소의 생성은 유전적 요인, 환경적 요인(자외선 등) 및 생리적 조건(피로 및 스트레스 등)에 의해 영향을 받는다. 멜라노사이트(melanocyte)에서 생성되는 흑갈색 색소인 멜라닌은 타이로시나아제(tyrosinase)에 의해 아미노산인 타이로신(tyrosine)이 차례로 L-3,4-dihydroxy-phenylalanine(L-DOPA)과 도파퀴논(dopaquinone)으로 전환 되는 산화반응을 통해 생성된다. 최근의 연구결과들은 멜라닌의 분해 및 생합성이 autophagy system에 의해 영향을 받을 수 있음을 보여주고 있으나, 이에 대한 자세한 기작연구는 매우 미흡한 실정이다. 한편, 지혈제로 사용되는 트라넥사민산(tranexamic acid; *trans*-4-amino-methylcyclohexanecarboxylic acid, TXA)은 케라티노사이트(keratinocyte)에서 melanocyte로의 플라스민(plasmin)에 의한 멜라닌 생성을 억제하는 효능이 있는 것으로 알려지면서 최근에는 기미 치료제로도 사용되고 있다. 그러나 이는 TXA가 멜라닌 확산을 일시적으로 차단한다는 연구결과에 근거 한 것이며, 멜라닌 생성에 미치는 TXA의 작용기작은 알려진 바가 없다. 따라서 본 연구에서는 TXA에 의한 autophagy 활성화 및 멜라닌 합성 억제기작 연구를 통해 TXA의 미백 효능 여부를 분석하고자 하였다. L-tyrosine과 L-DOPA를 기질로 버섯 tyrosinase 활성에 미치는 TXA의 영향을 분석한 결과, TXA는 tyrosinase 활성을 직접적으로 억제시킬 수 있음을 확인하였다. 또한 α-MSH(α-melanocyte-stimulating hormone) (10 nM)를 처리하여

멜라닌 생성을 촉진 시킨 흑색종 세포주(B16-F1)에 TXA(4 ㎎/㎖)를 52시간 동안 처리할 경우, 멜라닌 생성률이 약 46% 저해됨을 확인하였다. 이러한 결과는 TXA가 tyrosinase 활성을 직접 억제할 수 있을 뿐 아니라 세포에서도 tyrosinase 활성을 억제함으로써 멜라닌 생성을 억제할 수 있음을 보여주는 것이다. 본 연구에서는 또한 autophagy 활성화에 미치는 TXA의 영향을 규명하기 위하여 B16-F1 세포에 TXA (1 ㎎/㎖)를 15분 동안 처리한 후, autophagy 유도 단백질들의 발현량을 Western blotting으로 분석하였다. 그 결과, MAPK(ERK 및 p38 등), Beclin-1, Atg12 그리고 LC3의 단백질 발현량은 증가되었으나, autophagy의 음성조절자(negative regulator)로 알려진 mTOR의 단백질 발현량은 감소됨을 확인하였다. 이러한 결과는 TXA는 MAPK들을 활성화 시키는 반면, mTOR의 발현은 감소시킴으로써 autophagy system을 활성화시킬 수 있음을 시사하는 것이다. 이러한 TXA의 autophagy 활성화 특성은 항-LC3 항체로 immunostaining한 세포의 confocal 현미경 관찰로도 재확인하였다. 실제로 B16-F1 세포에 TXA를 처리하면 autophagosme들이 관찰되었다. 본 연구에서는 또한, TXA를 처리한 B16-F1 세포의 멜라닌 생성양상을 항-MAPK, -MITF, -tyrosinase 및 -TRP1 항체를 사용한 Western blotting으로 분석하였다. 그 결과, MITF, tyrosinase 및 TRP1의 발현량이 모두 감소됨을 확인하였다. 이러한 결과는 TXA에 의해 MITF 단백질 발현량이 감소됨으로써 멜라닌 합성에 관여하는 tyrosinase와 TRP1의 단백질 발현량이 감소되었음을 시사하는 것이다. 또한 멜라닌 생성에 미치는 autophagy 활성화의 영향을 분석하기 위해 mTOR 및 Atg5에 대한 small interfering RNAs(siRNA)를 B16-F1 세포에 transfection한 후 멜라닌 생성을 분석하였다. 해당 siRNA들에 의해 mTOR와 Atg5의 발현이 억제된 B16-F1 세포에 TXA(0.1, 0.5, 1, 2, or 4 ㎎/㎖)를 52시간 동안 처리한후 멜라닌 생성량을 측정한 결과, 멜라닌 생성량이 각각 20% 및 40% 정도 증가되었음을 확인하였다. 이러한 결과는 TXA는 autophagy system을 활성화시킴으로써 멜라닌 생성을 억제할 수 있음을 제시하는 것이다. 이상의 결과들은 TXA가 tyrosinase를 직접적으로 저해시킬 수 있을 뿐만 아니라 ERK 세포신호 전달계를 통해 autophagy 활성화를 유도하며, MITF 인산화를 통해 단백질 분해를 촉진시켜 멜라닌 합성에 관여하는 단백질의 발현량을 감소시킬 수 있음을 시사하는 것이다. 결론적으로, 본 연구결과들은 TXA가 피부세포의 항상성에 중요한 역할을 하는 autophagy의 활성화를 제어할 수 있는 항노화 및 미백 치료제로 사용될 가능성을 보여주고 있다.

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