Mechanism of the Inhibitory Effect of Tranexamic Acid on Melanogenesis in Cultured Human Melanocytes in the Presence of Keratinocyte-conditioned Medium

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Tranexamic acid (TA) reduces hyperpigmentation in melasma patients, but the mechanism of its action is unknown. We have investigated the action of TA in human melanocyte cultures with or without keratinocyte-conditioned medium (KCM). In melanocyte cultures without KCM, TA in the concentration range of 0.5 mM to 5 mM did not reduce the activity of tyrosinase, a key enzyme of melanin synthesis, whereas it reduced the tyrosinase activity in the presence of KCM. These results indicate that TA inhibits melanin synthesis of melanocytes not by acting directly on melanocytes, but by inhibiting melanocyte activators contained in KCM. In fractionation studies of KCM, the stimulatory activity was predominantly contained in the fractions with an apparent molecular weight of 54000. Inhibition of the urokinase-type plasminogen activator (uPA) in KCM with specific anti-uPA antibody significantly decreased the KCM-induced increase of tyrosinase activity and inhibited the KCM-induced morphological changes of melanocytes. Our results suggest that TA inhibits melanin synthesis in melanocytes by interfering with the interaction of melanocytes and keratinocytes through inhibition of the plasminogen/plasmin system.

Key words —— tranexamic acid, melanocytes, urokinase-type plasminogen activator, melasma, depigmentation

INTRODUCTION

Melasma is an acquired symmetric hyperpigmentation characterized by irregular light- to gray-brown macules, especially on the face of women. Although the pathogenic mechanism is unknown, luteinizing hormone and progesterone may be involved, because melasma occasionally appears on women who take contraceptive pills, does not appear on women after menopause, and is related to pregnancy.[1,2] Moreover, it may be induced by exposure to ultraviolet (UV) radiation, especially UVA in sunlight.[3] Treatments include topical application of a depigmenting agent, hydroquinone[4] and chemical peeling using glycolic acid.[5] Recently, oral or topical administration of tranexamic acid (TA) has been used to reduce pigmentation in melasma.[6,7]

Plasminogen exists in human epidermal basal cells,[8] while cultured human keratinocytes produce plasminogen activator (PA),[9] and it is known that the level of serum PA is increased by administration of oral contraceptives or in pregnancy.[10] We considered that PA of keratinocyte origin might participate in the induction of hyperpigmentation, and hypothesized that TA acts upon this mechanism. Human keratinocytes contain both urokinase-type PA (uPA) and tissue-type PA (tPA)[9] but secrete only single-chain uPA (sc-uPA).[11] uPA has been implicated in keratinocyte growth, differentiation[12] and migration,[13] acting in an autocrine fashion. On the other hand, human melanocytes primarily produce tPA, but scarcely produce uPA.[14] Furthermore, TA does not inhibit melanin synthesis in pure melanocyte cultures. The relationship between uPA and melanogenesis and the mechanism of pigmentation inhibition by TA have not been investigated. Therefore, we have investigated melanocyte activation by keratinocytes, as well as the pharmacological effect of TA in the cultures, in order to clar-

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ify the mechanism of the effectiveness of TA in the treatment of hyperpigmentation of melasma.

MATERIALS AND METHODS

Materials —— Epidermal–growth factor (EGF), insulin and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Basal medium MCDB-153 was purchased from Kyokuto Pharmaceutical (Tokyo, Japan). Bovine pituitary extract (BPE) was from Clonetics (Palo Alto, CA, U.S.A.). Human anti-sc-uPA and anti-uPA antibodies (#390, #3471) were from American Diagnostica (Stamford, CT, U.S.A.). Other chemicals were purchased from Wako Pure Chemical (Osaka, Japan), unless otherwise noted.

Melanocyte Cultures —— Melanocytes isolated from the forearm of Asian volunteers were incubated in MCDB-153 medium containing 0.15 mM $CaCl_2$, 10 ng/ml EGF, 5 μg/ml insulin, 50 μg/ml BPE, 20 ng/ml of PMA and 5% FBS for 4 days, then further incubated for 3 more days in MCDB-153 medium without PMA. Melanocytes cultured in BPE-supplemented medium exhibited a predominantly polydendritic phenotype and continued to proliferate. In the study of melanocyte stimulation, BPE was eliminated, as it contains a potent melanocyte mitogen, which stimulates dendricity and melanization. The phenotype changed to a small and bipolar morphology during culture without BPE and PMA for more than 2 days. For the immunofluorescence study, cells were cultured in LabTek chamber slides in MCDB-153 without BPE and PMA for 2 days after the addition of keratinocyte-conditioned medium (KCM) or sc-uPA.

Keratinocyte Cultures —— Keratinocytes isolated from the forearm of Asian volunteers were cultured in serum-free medium MCDB-153 containing 0.15 mM $CaCl_2$, 10 ng/ml EGF, 5 μg/ml insulin and 50 μg/ml BPE for 7 days. Keratinocytes with a baseloid phenotype predominantly appeared, and did not stratify, but continued to proliferate. These cells were grown in 25 cm^2 flasks (Costar-Corning, Corning, NY, U.S.A.) to 70% to 80% confluence.

Immunofluorescence Staining and Morphometry —— Cultured melanocytes were fixed with methanol and acetone, then immunologically stained with a rat monoclonal antibody (TMH-1)[15] against tyrosinase-related protein-1 (TRP-1; a melanosomal protein which functions in eumelanin formation),[16] and a secondary antibody conjugated with fluorescein isothiocyanate (FITC; Biosource, Camarillo, CA, U.S.A.). Cellular morphology parameters, including number of dendrites, perimeter and area per cell, were determined using a fluorescence microscope and an image analyzer connected to a personal computer with the appropriate software, as previously described.[17]

Assay of Tyrosinase Activity —— Tyrosinase activity was assayed using L-β-3-(3,4-dihydroxyphenyl)alanine (L-dopa) as a substrate, as previously described.[18] Phosphate buffer (pH 7.2, 45 μl) containing 1% Triton-X was added to each well after removal of the culture medium. The cell membrane was destroyed by vibrating the plate for one minute, and the absorbance (475 nm) at zero time was measured with a micro plate reader, then 5 μl of 10 mM L-dopa solution was added to the well, and incubation was continued for 60 minutes at 37°C. The absorbance (475 nm) was then measured after vibrating the plate for one minute. The absorbance values were compared with a standard curve obtained with mushroom tyrosinase (Sigma-Aldrich); this curve was linear within the range of experimental values. The number of cells was estimated based on DNA assay with Hoechst 33258. The fluorescence intensities measured with a micro plate fluorescence reader were compared with a standard curve obtained with appropriate numbers of cells.

Preparation of KCM —— Keratinocytes were grown in 25 cm^2 culture flasks under sub-confluent conditions. The cells were washed with phosphate-buffered saline (PBS) and placed in 3 ml of MCDB-153 medium without BPE. After 24 hr, the medium was collected from the flasks, centrifuged at 1000 × g for 20 minutes to remove cellular debris, then frozen at −80°C until required. A 1 ml aliquot was taken for protein determination using the Bio-Rad protein assay (Bio-Rad Laboratories, Cambridge, MA, U.S.A.). The KCM was fractionated through a polysulfone membrane with a 10000 m.w. exclusion limit (Centricut 10, Kurabo, Osaka, Japan) by centrifugation. The retained fraction was diluted to the original volume with MCDB-153. KCM was sterilized by filtration through a 0.2 μm filter (Acrodisk, Gelman Sciences, Ann Arbor, MI, U.S.A.).

Molecular-Sieve Chromatography of KCM —— Approximately 100 ml of KCM was concentrated to 1 ml by ultrafiltration with a Centricon 10 microconcentrator and stored at −80°C. Ultrafiltered KCM was separated using Molecular-Sieve HPLC (TSK3000SWG column; Toyo Soda, Tokyo, Japan) that had been equilibrated in 1/10 PBS, pH 7.4, and 0.5 ml fractions were collected. Fractions were freeze-dried, resuspended in 0.2 ml of MCDB-153, then assayed for melanogenic activity after 2 days of incubation.

Immunoblotting —— KCM was collected over 20 hr periods and stored at −80°C. It was concentrated by centrifugation through a polysulfone membrane with a 10000 m.w. exclusion limit (Centricut 10), producing a 10-fold volume reduction. Samples (50 μl) were analyzed by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted using the mouse monoclonal sc-uPA antibody and an avidin-biotin-peroxidase complex reagent (Amersham International plc, Buckinghamshire, U.K.).

Assay of Plasminogen Activator —— KCM was collected for 20 hr periods, and centrifuged at 1000 × *g* for 20 minutes to afford a supernatant. Plasminogen activator activity was measured with an immunosorbent activity assay. Standard uPA and samples (10 μl) were added to 96-well plates coated with monoclonal antibody directed against uPA and the 54000-dalton form of two-chain uPA. Non-absorbed material was washed off, and the plates were incubated with or without purified plasmin solution for 10 minutes at room temperature. Then uPA was determined by the addition of a reagent containing plasminogen and a plasmin-sensitive chromogenic substrate (D-But-CHT-Lys-pNA; 100 μl). The uPA activates plasminogen to plasmin, which in turn cleaves the chromogenic substrate to yield a yellow color, which was quantitated by measuring the absorbance at 405 nm. The absorbance was proportional to the amount of sc-uPA present in the sample or standard.

Neutralization with Antibody —— An antibody against the A chain or B chain of uPA was incubated with KCM for 20 minutes at room temperature, and the treated KCM was added to human melanocyte culture.

Statistical Analyses —— In studies of cellular morphology, thirty individual cells were selected by random sampling in the fields and measured by morphometry. The statistical significance of differences was analyzed with Student's *t*-test (when the variance was equal) or Welch's *t*-test (when the variance was not equal). In biochemical studies, the results of three determinations were averaged for each point.

RESULTS

Effect of KCM on the Morphology and Melanogenic Activity of Human Melanocyte Cultures

Melanocytes grown in basal medium were mostly bipolar spindles [Fig. 1(A)], whereas those grown in the presence of KCM had a large and star-shaped polydendritic phenotype [Fig. 1(B)]. The amount of TRP-1 was also increased in the presence of KCM. Fig. 2 shows the changes in the tyrosinase activity and morphological features of melanocytes cultured in the presence or absence of KCM (15 μg protein/ml). Tyrosinase activity was increased significantly ($p < 0.05$) during culture of the melanocytes with KCM for 2 days [Fig. 2(A)]. The cell perimeter, the number of dendrites and the cell area were also increased significantly ($p < 0.01$) [Figs. 2(B), (C) and (D)]. KCM contained melanocyte-stimulating activity, as evidenced by the increase of tyrosinase and the alteration in the morphology of the melanocytes. These effects were observed with KCM from growing keratinocytes, but not in that from confluent cells.

Molecular-Sieve Chromatography of KCM

The fractionation of concentrated KCM using molecular-sieve chromatography afforded two peaks of tyrosinase-inducing activity. One peak cor-

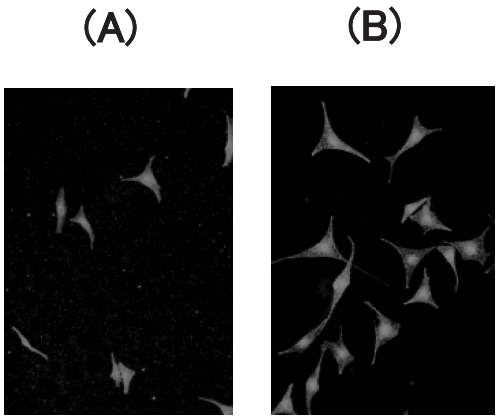

Fig. 1. FITC-TRP-1-immunostained Cultured Human Melanocytes Treated with KCM

Human melanocytes were cultured for 2 days in the absence (A) or presence (B) of KCM and stained as described in the text. Cultured melanocytes were immunostained with anti-TRP-1 antibody and FITC-conjugated secondary antibody.

responded to a molecular weight of about 300000 and the other to a molecular weight of about 60000 (Fig. 3). The purified factor had an estimated molecular mass of 54000 based on SDS/PAGE under reducing and nonreducing conditions. From these data, the factor appears to consist of a single polypeptide chain with a molecular mass of 54000. Based on the immunological characteristics, it was identified as sc-uPA (Fig. 3).

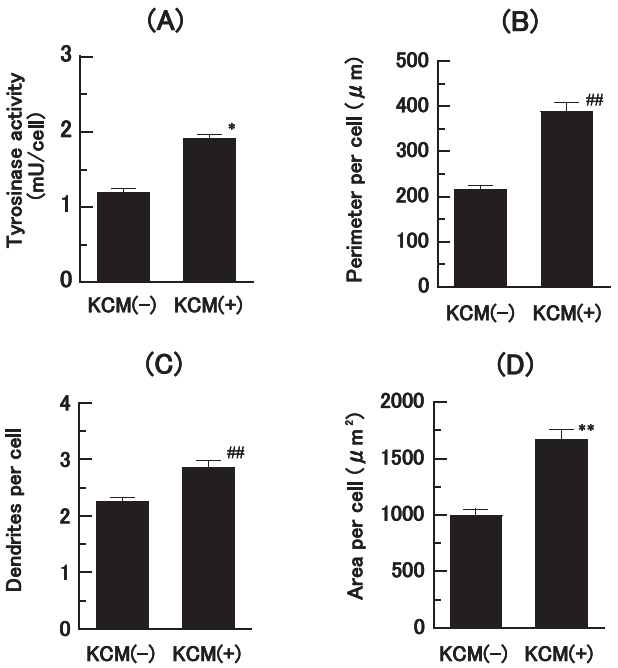

Fig. 2. Effects of KCM on Tyrosinase Activity and Morphological Features of Human Melanocytes *In Vitro*

Human melanocytes were cultured for 2 days in the absence or presence of KCM. Changes per cell in (A) tyrosinase activity, (B) perimeter, (C) number of dendrites, and (D) area are shown. $^{*}p < 0.05$ by Student's *t*-test, $^{**}p < 0.01$ by Student's *t*-test, $^{\#\#}p < 0.01$ by Welch's *t*-test. Bars represent the standard error of the mean.

Amount of Sc-uPA in the Supernatant of Sub-confluent and Confluent Keratinocytes

We measured the amount of sc-uPA in the culture medium of keratinocytes by examining the change in uPA activity when plasmin was added to the supernatant. The value was 74.9 ± 0.7 (ng/ml) in the presence of plasmin, but 0.35 ± 0.28 (ng/ml) in its absence for sub-confluent keratinocytes. The amount was only 8.4 ± 0.2 (ng/ml) in the presence of plasmin in the case of confluent keratinocytes.

Effect of sc-uPA on Human Melanocyte Cultures

Next, sc-uPA was added to human melanocyte cultures and the effects on the tyrosinase activity and morphological features of melanocytes were examined. Treatment of human melanocytes with sc-uPA (0.1–100 ng/ml) for 2 days resulted in a dose-dependent increase in the tyrosinase activity [Fig. 4(A)]. The treatment of the cells with 1 ng/ml, 10 ng/ml or 100 ng/ml sc-uPA resulted in a significant increase in the tyrosinase activity ($p < 0.05$, $p < 0.01$ and $p < 0.05$, respectively). Further, treatment of the melanocytes with sc-uPA (100 ng/ml) resulted in a significant enlargement of the cell perimeter and cell area, as well as an increase of dendrites [$p < 0.01$, $p < 0.01$ and $p < 0.01$, respectively; Figs. 4(B), (C) and (D)].

Inhibition of Melanocyte-stimulating Activity in KCM by Anti-uPA Antibody

The activating activity in KCM was assessed in the presence of antibody against uPA. Normal human melanocytes were cultured for 2 days with KCM which previously incubated for 20 minutes with anti-uPA, or pre-immune immunoglobulin G

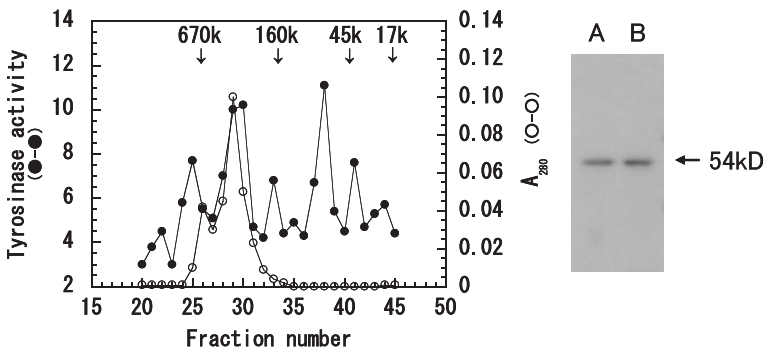

Fig. 3. Molecular Sieve Chromatography of KCM and Immunoblotting Analysis of Sc-uPA in KCM

Concentrated KCM without FBS and BPE was separated by gel-filtration HPLC and 1.0 ml fractions were collected. Fractions were lyophilized and resuspended in 0.2 ml of MCDB-153. Tyrosinase activity was assayed after 2 days of culture. After separation of the fractionated sample (lane A) and purified sc-uPA (54 kD form; 200 ng, lane B) on SDS-PAGE, the bands were transferred to nitrocellulose and immunoblotted with anti-uPA antibody, and detected using a peroxidase system.

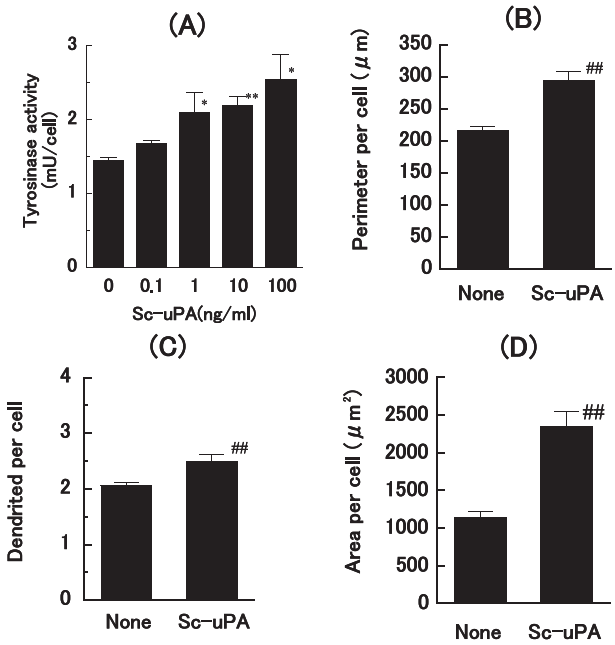

Fig. 4. Effects of Sc-uPA on Tyrosinase Activity and Morphological Features of Human Melanocyte Cultures

Human melanocytes were cultured for 2 days in the absence or presence of sc-uPA. Changes per cell in (A) tyrosinase activity, (B) perimeter, (C) number of dendrites, and (D) area are shown. Bars represent standard deviation. $^{*}p < 0.05$ by Student's *t*-test, $^{**}p < 0.01$ by Student's *t*-test, $^{\#\#}p < 0.01$ by Welch's *t*-test.

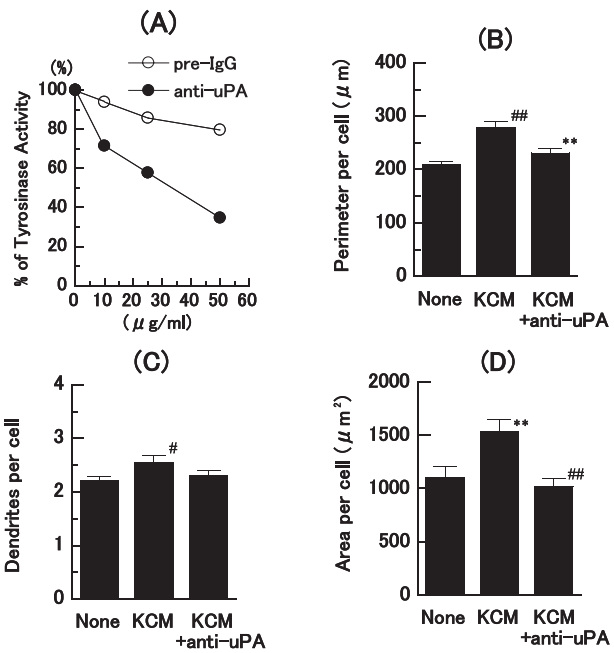

Fig. 5. Neutralization of Melanocyte-stimulating Activity in KCM by Anti-uPA Antibody

Changes per cell in (A) tyrosinase activity, (B) perimeter, (C) number of dendrites, and (D) area are shown. Human melanocytes were cultured for 2 days with KCM previously incubated for 20 minutes with anti-uPA antibody, or pre-IgG. Anti-uPA antibody was used at 50 μg/ml in the morphological studies. $^{**}p < 0.01$ *vs.* none or KCM by Student's *t*-test, $^{\#}p < 0.05$ *vs.* none by Welch's *t*-test, $^{\#\#}p < 0.01$ *vs.* KCM by Welch *t*-test. Bars represent the standard error of the mean.

(pre-IgG) in the concentration range of 0.5 μg/ml to 50 μg/ml. Figure 5 demonstrates that increase of tyrosinase-inducing activity by KCM was dose-dependently inhibited by the addition of anti-uPA antibody. The increases of cell perimeter and cell area were also significantly ($p < 0.01$) inhibited by 50 μg/ml of anti-uPA antibody. The number of cells was not affected by the addition of anti-uPA antibody.

Inhibition of the Activation of Melanocytes in KCM by TA

We examined whether the tyrosinase-inducing activity of KCM was inhibited by TA. The tyrosinase-inducing activity by KCM was significantly ($p < 0.05$) inhibited by 1 mM and 5 mM TA without affecting cell viability (Fig. 6).

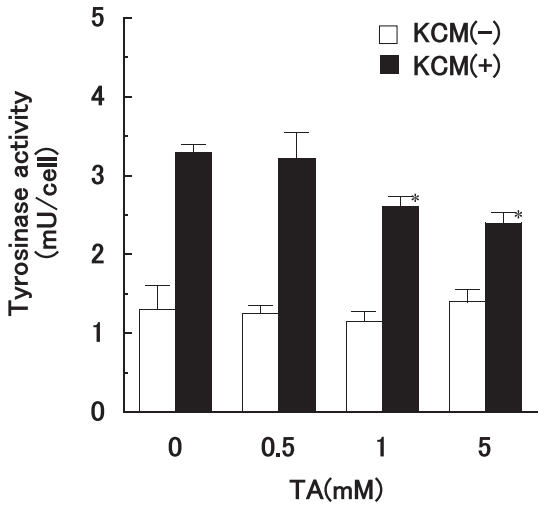

Fig. 6. The Suppression of Tyrosinase Activity in KCM by TA

Tyrosinase activity was assayed after culture of melanocytes for 2 days in the presence or absence of KCM. TA was added to the culture system at the concentration of 0 (control), 0.5, 1 or 5 mM. $^{*}p < 0.05$ *vs.* control by Student's *t*-test. Bars represent the standard error of the mean.

DISCUSSION

TA has been used clinically for over 30 years to treat abnormal bleeding, as well as skin diseases such as eczema, hives, drug-induced irritation, and toxicodermia, via internal administra-

tion,[19] and also orally to treat itching, swelling, and erythema.[20] It is a representative ω-amino acid-type anti-plasmin agent that has a highly specific action on the fibrinolytic system,[20] blocking the conversion of plasminogen to plasmin by inhibiting PA action through the formation of a reversible complex with plasminogen.[19] TA is also thought to form a reversible complex with plasmin, inhibiting the reaction with fibrin.[19]

Our study indicates that sc-uPA (1–100 ng/ml) stimulates tyrosinase activity and increases the cell size of melanocytes in the physiologically significant concentration range, because the amount of sc-uPA in the culture medium of keratinocytes was 74.9 ± 0.7 ng/ml in the presence of plasmin in sub-confluent keratinocytes. Therefore, sc-uPA may be involved in the paracrine regulation of melanocytes, as well as in autocrine regulation of keratinocytes. The concentration of TA that inhibited melanocyte activity was 1–5 mM in this experiment. This is more than 100 times the concentration (ID_{50} = 0.05 μM) required to show anti-plasmin action *in vitro*.[20] It is therefore thought that TA acted by suppressing sc-uPA binding to cell membrane receptors, not through the protease activity inhibition. Sc-uPA and tc-uPA have the receptor-binding site, EGF-like growth factor domain, and Kringle domain at the N-terminus and can be purified on a lysine affinity column, so it is considered that there is a lysine-binding site at the Kringle domain.[21] TA is a lysine derivative and, thus, is expected to bind readily to these lysine-binding sites, resulting in a change of the configuration. On the other hand, sc-uPA does not have protease activity, but protease activity is generated after its conversion to tc-uPA by plasmin. The stimulatory effect of KCM on melanocytes was inhibited by the addition of TA. This is consistent with the idea that TA inhibits the action of sc-uPA on melanocytes by binding to the lysine-binding site. Specific sc-uPA/tc-uPA receptors were recently detected in several cell types,[22] and shown to bind sc-uPA/tc-uPA at the growth factor domain with high affinity.[23] Therefore, sc-uPA/tc-uPA produced by keratinocytes may be involved in regulating the activity of melanocytes through specific receptors on the melanocytes via a receptor-mediated signal transduction pathway. Sc-uPA levels were higher in sub-confluent than in confluent keratinocyte cultures, in agreement with a report that uPA mRNA levels are increased in quiescent cultured keratinocytes upon growth stimulation.[24] KCM from the growth phase was found to induce tyrosinase activity more strongly than that from the confluent phase in human melanocyte cultures. Thus, the growth of keratinocytes surrounding melanocytes may play an important role in melanin synthesis.

We found that KCM stimulated the melanocyte activity, and this effect was suppressed by anti-uPA antibody, implying that uPA is one of the intrinsic activating factors for human pigmentation. Plasmin activates precursors of secretory phospholipase A_2,[25] which participates in the production of arachidonic acid (a precursor of melanogenic factors, such as prostaglandins[26] and leukotrienes[27]). Plasmin also participates in the release of basic fibroblast growth factor (bFGF), which is potent melanocyte growth factor.[28] In addition, plasmin is reported to participate in cytokine expression and activation, production of active peptides, and phosphorylation of transcription factors.[29–32] It is thought that plasmin promotes melanin synthesis via these melanocyte-keratinocyte interactions. Because TA inhibits the production of plasmin by inhibiting the activity of plasminogen activator, the effectiveness of TA in reducing pigmentation is thought to involve inhibition of melanocyte activation by plasminogen activator. However, it remains to be established whether other melanogenic factors, such as melanocyte-stimulating factor (MSH)[33] and stem cell factor (SCF),[34] are also produced in the skin of melasma patients.

In summary, sc-uPA generated by keratinocytes increases the activity of melanocytes *in vitro*, and blockade of this effect may be the mechanism through which TA reduces hyperpigmentation of melasma patients.

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