Effect of Trans-4-Aminomethylcyclohexanecarboxylic Acid on the Proliferation and Melanization in Cultured Normal Human Melanocytes

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Background: **Trans-4-aminomethylcyclohexanecarboxylic acid (tranexamic acid) has recently been reported to inhibit prostaglandin synthesis and hinder the pigmentation caused after UV radiation.**

Objective: **we evaluated the influence of tranexamic acid on the viability, morphogenesis and melanization of cultured normal human melanocytes.**

Method: **The cultured melanocytes from neonatal foreskin were exposed to UVB 20 mJ/cm^2, then treated with tranexamic acid [0.05 μg/ml, 0.05 μg/ml, and 0.5 μg/ml]. After 24 hours, the viability of melanocytes and the melanin concentration was measured. The number and length of the melanocytes' dendrites, and the expression level of tyrosinase, TRP-1 and TRP-2 were also evaluated.**

Results: **The viability of the melanocytes was decreased by tranexamic acid in a dose dependent manner ($p<0.05$). The increased melanin synthesis by UVB irradiation was decreased by tranexamic acid in a dose dependent manner ($p<0.05$). Also, the increased expressions of TRP-1, TRP-2 and tyrosinase after exposure to UV were statistically decreased by tranexamic acid in a dose dependent manner ($p<0.05$).**

Conclusion: **tranexamic acid may prevents UVB induced pigmentation.**

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Key Words: Melanocytes, Tranexamic acid, UVB

INTRODUCTION

Ultraviolet (UV) radiation induces a variety of responses, including tanning and inflammation in the human skin and may also act as a carcinogen[1]. Epidermal melanocytes, keratinocytes, and even other epidermal and dermal cells may be the target of UV light radiation[2,3], but melanocytes - the cells capable of synthesizing melanin - seem to be the first and the major target.

Pigmentation, due to synthesis and dispersion of melanin, protects the skin from harmful effects of sunlight[4], but unwanted hyperpigmentation can also produce a significant cosmetic and psychologic stress. Melanin is the major product of melanocytes and is the main determinant of differences in skin color. Pigmentation results from increased melanin production in melanocytes and dispersion to adjacent keratinocytes via dendrites of melanocytes.

Tyrosinase is a key enzyme involved in melanin production in mammals and consists of a signal peptide, and catalytic regions composed of two copper binding domains coordinated by histidine residues[5,6]. Tyrosinase-related protein 1 (TRP-1) and

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tyrosinase-related protein 2 (TRP-2) are involved in the regulation of melanin synthesis in mammals, too. Tyrosinase has an overall identity of 40 percent, with two these cDNAs encoding melanocyte specific proteins. Both TRP-1 and TRP-2 are known to stabilize tyrosinase and to increase its activity.

Trans-4-aminomethylcyclohexanecarboxylic acid (tranexamic acid) (Fig. 1), as a plasmin inhibitor has been used clinically for blocking abnormal fibrinolysis, and has anti-allergy and anti-inflammatory effects, primarily because of its ability to inhibit plasmin[7-12]. Tranexamic acid has been shown to reduce 5-lipoxygenase stimulation in human monocytes through a plasmin-inhibiting pathway[13]. The mechanisms of skin darkening due to UV include arachidonic acid (AA) metabolites such as prostaglandin E2 (PGE_2) and leukotriene C_4 (LTC_4)[14-16]. Tranexamic acid may decrease melanocyte tyrosinase activity by reducing the production of PGs through suppression of the UV-induced increase in epidermal plasmin activity[17]. In particular, tranexamic acid has recently been widely used to reduce skin darkening in mesotherapy.

The purpose of this study is to support the theoretical background of tranexamic acid mesotherapy in the treatment of skin pigmentation. Therefore, this study was performed to evaluate the effects of tranexamic acid on the changes of melanocyte morphology and melanogenesis in cultured normal human melanocytes exposed to UV irradiation.

MATERIALS AND METHOD

Normal human melanocytes

For harvesting normal human melanocytes, neonatal foreskin was obtained from neonatal circumcision specimens and then primary culture was carried out. Neonatal foreskin was chopped in 1 mm sized pieces and trypsinized in room temperature overnight. After vortexing vigorously, it was incubated for 5 minutes, then the supernatant was taken. It was plated on a 25 cm^2 culture flask and then incubated in 5% CO_2 at 37℃ ɔ melanocyte growth media (Cascade, Portland, USA). Cultured normal human melanocytes were irradiated by UVB 20 mJ/cm^2 and treated with trans-4-aminomethylcyclohexanecarboxylic acid (tranexamic acid) 0.05 μg, 5 μg, and 500 μg/ml, respectively and incubated for 24 hours.

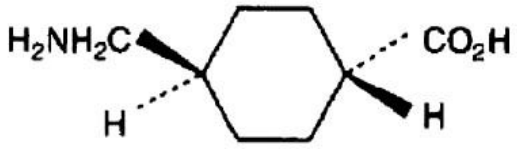

Fig. 1. Chemical structure of *trans-4-aminomethylclocyhexanecarboxylic* acid (tranexamic acid).

Ultraviolet B (UVB) irradiation

The dosage of irradiation was 20 mJ/cm^2, which was chosen based on preliminary data (Fig. 2) in this experiment. UVB irradiation was delivered with a Philips TL 20 W/12 (Eindhoven, Netherlands), a fluorescent bulb emitting a 280-320 nm wave with a peak at 313 nm wavelength. Before UVB irradiation, the medium was removed and covered with phosphate buffered saline (PBS). Irradiation output was monitored by means of a Waldmann UV-meter (Waldmann, Villigen-Schwenningen, Germany).

Trans-4-aminomethylcyclohexanecarboxylic acid (tranexamic acid) treatment

Tranexamic acid (Dai-ichi Pharmaceutical Korea, Seoul, Korea, Fig. 1) was dissolved in 10% ethanol, 10% 1,3-butanediol and 80% water. These solvents also used in control. The tranexamic acid treated 0.05 μg, 5 μg, and 500 μg/ml.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The MTT cell proliferation assay was carried out to measure the increase in cell numbers. Melanocytes were seeded 1×10^4 cells per 96 well plate. Cells were grown in 96 well plates in a humidified atmosphere. They were then incubated in 5% CO_2 at 37℃ ɔr 24 hours. After the incubation period, 10 μl of the MTT labeling reagent was added to each well and incubated for 4 hours in a humidified atmosphere. Then to each well 100 μl of the solubilization solution was added. The plate was then left to stand overnight in the incubator in a humidified atmosphere. A 570 nm wavelength was used to measure absorbance of the formazan product by ELISA reader (Bio-TEK instruments, CA, USA).

Melanin assay

Melanocytes were seeded 5×10^4 cells per 6 well plate. Melanocytes were grown in 6 well plates and incubated for 24 hours. To determine melanin

content, the remaining suspension was centrifuged at 1000 rpm for 3 minutes in a microcentrifuge. The resulting cell pellets were then dissolved in 0.1 µl of 1 M NaOH and diluted with 0.4 ml of water. Melanin content was then calculated by measuring the OD at 475 nm in comparison with a standard curve of synthetic melanin (Sigma, St. Louis, MO, USA). Melanin values were expressed as the amount of melanin per cell in picograms.

Measure of the morphologic changes of melanocytes

Cells were monitored by an inverted phase contrast microscope (Olympus, Tokyo, Japan) and photographs were taken to document morphological change. In addition, number and length of dendrites per cell were assessed in randomly selected five views.

Preparation of primer

We synthesized the PCR primer from the basis of Gene Bank data. Primers were chemically synthesized using DNA synthesizer (Pharmacia, Björgatan, Uppsala, Sweden). Their sequences were as follows :

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from normal human melanocytes using TRIZol reagent (Invitrogen, Carlsbad, CA, USA), then one millilitre of TRIZol reagent was added to the cultured dish. After 5 minutes at room temperature, 0.2 ml of chloroform per 1 ml of TRIZol reagent was added, the tubes were shaken vigorously by hand for 15 seconds and then incubated at 15℃ to 30℃ for 3 minutes. The mixtures were centrifuged at 12,000 rpm at 4℃ for 15 minutes, the upper aqueous phase transferred to a fresh tube, and the same amount of 2-propanol was added. After mixtures were incubated at 4℃ for 15 minutes, they were centrifuged with 12,000 rpm at 4℃ for 15 minutes. The supernatant was removed, then washed with 500 µl of 70% ethanol at 12,000 rpm at 4℃ for 5 minutes. The RNA pellet was then briefly dried. The purified RNA was dissolved in DEPC-DW 30 µl. Three µg of total cellular RNA was reverse transcripted at 42℃ for 30 minutes in a 20 µl volume containing 1 µl reverse transcriptase (TaKaRa, Shiga, Japan), 10 × buffer 2 µl, 10 mM dNTP 2 µl (dNTP mix), oligo dT primer 1 µl, RNase inhibitor 0.5 µl, 25 mM $MgCl_2$ 4 µl. Two µl of each cDNA sample from the RT-PCR was amplified in 25 µl containing 10 × buffer 2.5 µl, 25 mM $MgCl_2$ 2.5 µl and 10 pmol 0.75 µl primer. Thermal cycle profiles were as follows: 94℃ for 5 minutes; 35 cycles at 94℃ for 1 minutes, 59℃ for 1 minute, 72℃ for 1 minute, and a final extension step of 72℃ for 10 minutes.

Electrophoresis

The products were run on 1.5% agarose gel containing 1 µg ethidium bromide per millimeter. 20 µl of reaction mixture was mixed with a loading buffer separated by electrophoresis for 15 minutes at 100 voltages and visualized by UV transillumination.

Quantitative analysis

Quantitative analysis of RT-PCR products was accomplished by computerized optical densitometry of the bands.

primer	Forword	Reverse
Tyrosinase(Tys)	5'-TTGGCAGAT GTCTGTA GCC - 3'	5'-AGGCATTGTGCATGCT GCTT - 3'
Tyrosinase related protein-1(TRP-1)	5'-AGAGATGAT CGCGAG GTCTG - 3'	5'-CTGTGCCATGTGAGA AAAGC - 3'
Tyrosinase related protein-2(TRP-2)	5'-GAGGTGCGAGCCGAC ACAAG - 3'	5'-CGGTGCCAGGTA ACA AATGC- 3'
GAPDH	5'- CCACCCATGGCAAAT TCC ATG GCA -3'	5'-GGTGCTGCTTGTTAG GAGGTCAAGTAAAGG GC -3'

Table 1. Effect of UVB and tranexamic acid on the melanocytes viability (*P<0.05)

	Control	UVB	UVB + T 0.05 μg	UVB + T 5 μg	UVB + T 0.5 mg
No. of melanocytes	22.860 ± 3.21	250.10 ± 10.05	200.05 ± 10.00*	186.33 ± 11.85*	113.33 ± 11.55*

*: Statistically significant compared to UVB irradiated group only ($p<0.05$)
T: Tranexamic acid

Table 2. Effect of uvb and tranexamic acid on melanin concentration (*p<0.05) (unit = pg/cell)

	Control	UVB	UVB + T 0.05 μg	UVB + T 5 μg	UVB + T 0.5 mg
Melanin amount	1.70 ± 0.25	3.63 ± 0.25	2.40 ± 0.10*	2.17 ± 0.06*	1.83 ± 0.06*

*: Statistically significant compared to UVB irradiated group only ($p<0.05$)
T: Tranexamic acid

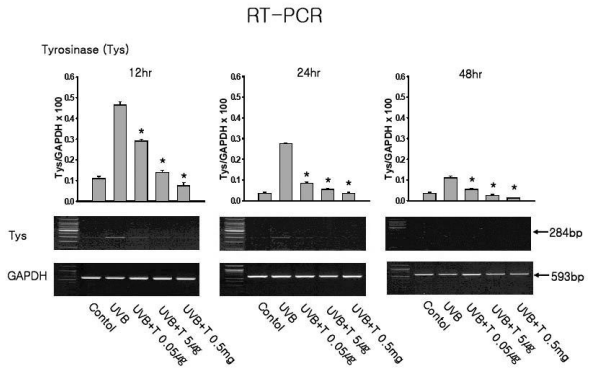

Fig. 2. Effect of tranexamic acid on tyrosinase activity in cultured normal human melanocytes. The tyrosinase activity was increased by UVB after 12 hours, 24 hours, and 48 hours. The tyrosinase was decreased by tranexamic acid in a dose dependent manner.
*: Statistically significant compared to UVB irradiated group only ($p<0.05$)
T: Tranexamic acid

Statistical analysis

Each experiment was run at least 3 times. Data was expressed as means ± SD and analysed by a paired analysis of variance or One-Way ANOVA.

RESULTS

Viability of melanocytes

UVB irradiation did not affect the viability of melanocytes. But, after treatment with tranexamic acid, the viability of melanocytes was statistically decreased in a dose dependent manner [200.05 ± 10.00, 186.33 ± 11.85, 113.33 ± 11.55, respectively] in comparison with UVB irradiation only [250.10 ± 10.05] (Table 1).

Melanin assay

Melanin content was calculated by measuring by the OD at 475 nm and compared with a standard curve of synthetic melanin. Melanin synthesis was statistically significantly increased by UVB radiation [3.63 ± 0.25 pg/ml] and significantly decreased by tranexamic acid in a dose dependent manner [2.40 ± 0.10 pg/ml, 2.17 ± 0.06 pg/ml, 1.83 ± 0.06 pg/ml, respectively] (Table 2).

Morphologic changes of melanocyte dendrites

Cells were monitored by an inverted phase contrast microscope and photographs were taken to detect morphological change such as number and length of dendrites. The number and length of dendrites did not show any significant change caused by UVB or tranexamic acid (Table 3, 4).

Table 3. Effect of UVB and tranexamic acid on the number of melanocyte dendrites

	Control	UVB	UVB + T 0.05 μg	UVB + T 5 μg	UVB + T 0.5 mg
No. of dendrites	3.43 ± 0.06	3.90 ± 0.10	3.50 ± 0.10	3.43 ± 0.06	3.40 ± 0.10

T: Tranexamic acid
#Cells were monitored by an inverted phase contrast microscope and photographs were taken to document morphological changes. In addition, number of melanocyte dendrites per one melanocyte was assessed in randomly selected five views.

Table 4. Effect of UVB and tranexamic acid on the length of melanocyte dendrites

	Control	UVB	UVB+ T 0.05μg	UVB+ T 5μg	UVB+ T 0.5mg
nm	6.37 ± 0.15	4.33 ± 0.15	5.67 ± 0.06	5.57 ± 0.06	5.50 ± 0.10

T: Tranexamic acid
#Cells were monitored by an inverted phase contrast microscope and photographs were taken to document morphological changes. In addition, the lengths of melanocyte dendrites was assessed in randomly selected five views and averaged.

Expression of tyrosinase, TRP-1, and TRP-2

After UVB 20 mJ/cm^2 irradiation and treatment with tranexamic acid [0.05 μg, 5 μg, and 500 μg/ml], the expression of tyrosinase mRNA were upregulated by UVB irradiation and that of tyrosinase mRNA were statistically significantly decreased by tranexamic acid in a dose dependent manner at 12, 24, and 48 hours (Fig. 2).

After UVB 20 mJ/cm^2 irradiation and treatment with tranexamic acid [0.05 μg, 5 μg, and 500 μg/ml], the expression of TRP-1 mRNA were upregulated by UVB irradiation and that of TRP-1 mRNA were statistically significantly decreased by tranexamic acid in a dose dependent manner at 12, 24, and 48 hours (Fig. 3).

After UVB 20 mJ/cm^2 irradiation and treatment with tranexamic acid [0.05 μg, 5 μg, and 500 μg/ml], the expressions of TRP-2 mRNA were upregulated by UVB irradiation and that of TRP-2 mRNA were statistically significantly decreased by tranexamic acid in a dose dependent manner at 12, 24, and 48 hours (Fig. 4).

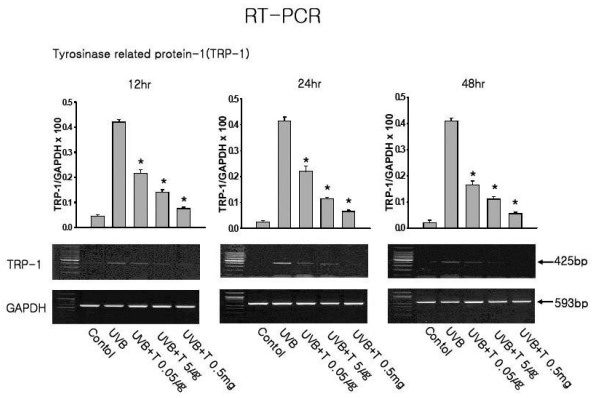

Fig. 3. Effect of tranexamic acid on TRP-1 activity in cultured normal human melanocytes. The tyrosinase activity was increased by UVB after 12 hours, 24 hours, and 48 hours. The TRP-1 was decreased by tranexamic acid in a dose dependent manner.
*: Statistically significant compared to UVB irradiated group only ($p<0.05$)
T: Tranexamic acid

DISCUSSION

The epidermal melanin unit in human skin is composed of melanocytes and keratinocytes in a 1 : 40 ratio. Normal human melanocytes (NHM), located in the basal layer of the epidermis, produce melanin-loaded melanosomes. Melanin determines the characteristic color of the skin, as well as exerts its photoprotective properties. The melanocytes'

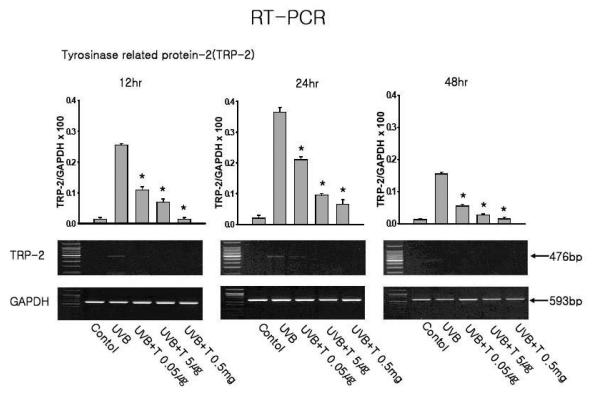

Fig. 4. Effect of tranexamic acid on TRP-2 activity in cultured normal human melanocytes. The tyrosinase activity was increased by UVB after 12 hours, 24 hours, and 48 hours. The TRP-2 was decreased by tranexamic acid, in a dose dependent manner.
*: Statistically significant compared to UVB irradiated group only ($p<0.05$)
T: Tranexamic acid

activation by UV causes an increase of melanin synthesis. It also transfers melanin to adjacent keratinocytes and leads to darkening of skin color[1,3].

Skin hyperpigmentation can be dependent on either an increased number of melanocytes or activity of melanogenic enzymes, such as tyrosinase[5,6]. The latter plays a critical role in the biosynthesis of melanin and is considered to be the key enzyme in coloring of skin, hair, eyes, and food browning[18]. Therefore, tyrosinase activity can be shown through tyrosinase, TRP-1 and TRP-2 mRNA expression. Only tyrosinase can induce melanin biosynthesis when expressed in the heterologous cellular system, even in the absence of any other melanocyte specific proteins. Other proteins, such as TRP-1 and TRP-2, can stabilize tyrosinase in the endoplasmic reticulum (ER). In cells lacking melanosome, melanosomal proteins are delivered to the lysosomes and both TRP-1 and TRP-2, which are a kind of melanosomal protein, are found to stabilize tyrosinase and to increase its activity[19]. This suggests the presence of a melanogenic complex that might not only be important for enzymatic optimization, but also for the trafficking of the proteins out of the ER. A high molecular weight complex containing tyrosinase, TRP-1, and TRP-2 has been identified, but understanding the significance of this complex requires further studies[20,21].

Although the mechanism of skin darkening due to ultraviolet is not yet fully understood, a number of factors including arachidonic acid (AA) metabolites such as prostaglandin E2 (PGE2), hormones such as melanocyte-stimulating hormone, stem cell factor, basic fibroblast growth factor, diacylglycerol and endothelin may be involved in skin darkening[14-16,22-25]. Furthermore, recently it has been reported that phospholipases contribute to the release of arachidonic acid and lyso-phospholipid, increasing melanogenesis[17,24]. One approach to preventing pigmentation following UV exposure is inhibition of the lipid mediators, such as PGE_2, that are synthesized and released after exposure to UV radiation.

Recently, according to the report by Maeda et al., tranexamic acid inhibits UV-induced plasmin activity in keratinocytes by preventing the binding of plasminogen to the keratinocytes, which ultimately results in less free AA and a diminished ability to produce AA metabolites, such as PGE_2 and LTC_4[15-17,26]. Tranexamic acid may decrease melanocyte tyrosinase activity by reducing the production of AA metabolites through suppression of the UV-induced increase in epidermal plasmin activity[15-17,27]. In our results, not only melanin synthesis but also expressions of tyrosinase, TRP-1 and TRP-2 mRNA have been upregulated by UVB irradiation and have been downregulated by the tranexamic acid in a dose dependent manner.

In previous studies, UVB induced significant changes indicative of activity, namely increased dendricity and cell flattening, but there was no report that tranexamic acid might change melanocyte morphology[28]. In this study, UVB irradiation and tranexamic acid did not change melanocyte morphology, melanocyte dendricity and dendrites length. Viability of melanocytes did however decrease due to tranexamic acid in a dose dependent manner. We thought that it was caused by an apoptosis mechanism from the direct effect of tranexamic acid.

In our results, treatment with tranexamic acid inhibited tyrosinase expression, and melanocyte count. Melanin content levels were equivalent to those measured in the unstimulated control group. These results are consistent with the finding that tranexamic acid attenuated AA-induced skin pigmentation in cultured normal human melanocytes. Under this theoretical background, we suggest that

application of tranexamic acid may result in a decrease of UV-induced pigmentation. These results revealed that tranexamic acid may decrease tyrosinase activity of melanocytes by reducing mRNA expression of tyrosinase, TRP-1, and TRP-2.

In dermatologic fields, mesotherapy using tranexamic acid has become one of the treatment modalities for melasma and postinflammatory hyperpigmentation. However, there have been few theoretical backgrounds until now. These results suggest that treatment with tranexamic acid for skin darkening will be one type of therapy.

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