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学位論文

Tyrosinase Gene Transcription and Its Control by Melanogenic Inhibitors

色素細胞の内因性メラニン生成抑制物質によるタイロシネース遺伝子発現の制御

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Short title: Ty gene transcription and its control by MIs

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ABBREVIATIONS

AsA	ascorbic acid
DOC	sodium deoxycholate
dopa	3,4-dihydroxyphenylalanine
GSH	glutathione
LA	lactic acid
LGF	large granule fraction
MI	melanogenic inhibitor
ODS	octa-decyl silane
PB	phosphate buffer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
SDS	sodium dodecyl sulfate
SF	soluble fraction
TLC	thin layer chromatography
Ty	tyrosinase

SUMMARY

The levels of tyrosinase mRNA and tyrosinase activity were analyzed in two amelanotic melanoma cell lines, D_1178 (hamster origin) and G-361 (human origin). Neither tyrosinase mRNA nor tyrosinase activity were detected in D_1178 cells. On the other hand, both tyrosinase mRNA and weak tyrosinase activity were detected in G-361 cells. Assuming that the different types of melanogenic inhibitors affected melanogenesis in these two amelanotic melanoma cells in different manners, we performed a screening of melanogenic inhibitors in these two cell lines. As an isolated tyrosinase suppressive melanogenic inhibitor, ascorbic acid and glutathione were identified from D_1178 cells and G-361 cells, respectively. Furthermore, lactic acid was identified from D_1178 cells as an isolated tyrosinase non-suppressive melanogenic inhibitor. B-16 mouse melanotic melanoma cells were depigmented by treatment with lactic acid. The melanogenesis suppression by lactic acid in B-16 cells was found to be due to inhibition of tyrosinase gene expression.

INTRODUCTION

Melanogenesis in pigment cells includes various maturation steps and the translocation of tyrosinase (Ty) as well as the differentiation and melanization of premelanosomes[1-2]. In addition, it is considered that eu- and pheo-melanogenesis are controlled by the balance between Ty, which has been recognized as a key enzyme of melanogenesis, and naturally occurring melanogenic inhibitors (MIs)[3-5]. In addition to Ty inhibitors against isolated Ty, the depigmentation of pigmented cells by MIs is investigated by using *in vitro* cultured melanocytes or melanoma cell lines. Various MIs have been found and classified into the following two categories by Mishima [6]. One is a group of MIs which suppress isolated Ty such as ascorbic acid, kojic acid and indole blocking factor. The other is a group of MIs non-suppressive to isolated Ty. The latter is further divided into three subgroups. 1. Inhibitors of Ty synthesis in ribosomes such as biomein. 2. Inhibitors of Ty transfer to premelanosomes such as tunicamycin and glucosamine by interrupting the glycosylation of Ty. 3. Melanocyte cytotoxic inhibitors such as hydroquinone and azelaic acid.

Amelanotic melanoma cells were reported to occur

in the process of *in vivo* and *in vitro* passage of their parental melanotic melanoma cells [7]. It has been considered that naturally occurring MIs played some role in the occurrence of amelanotic melanoma cells[3-6]. The existence of naturally occurring MIs produced by the pigment cell itself within its cytoplasm was proven by Cooper[3] and Imokawa [4]. Kameyama [8] reported that naturally occurring MIs were produced in JB/MS-white cells cloned from parental JB/MS mouse melanotic melanoma cells. However, the naturally occurring MIs have not been identified yet. To identify these naturally occurring MIs produced in melanoma cells is considered to be important and useful in understanding the regulatory mechanism of Ty biosynthesis and that of melanogenesis with MIs.

We investigated the following three items; first, the levels of Ty mRNA and Ty activity were analyzed in two amelanotic melanoma cell lines. Second, three naturally occurring MIs were identified from two cell lines. One of them was newly identified as lactic acid (LA). Finally, the depigmenting mechanism of LA was studied. Details will be presented here.

MATERIALS AND METHODS

Cell lines Greene's D_1179, a hamster melanotic melanoma cell line, and Greene's D_1178, a hamster melanotic melanoma cell line obtained from the D_1179 cell line during the process of *in vivo* passage, were kindly provided from Dr. H.S.N. Greene [7]. G-361, a human amelanotic melanoma cell line, and B-16, a mouse melanotic melanoma cell line were obtained from American Type Culture Collection (Maryland, USA). Ihara, a human melanotic melanoma cell line, was established from a Japanese melanotic melanoma patient in Kobe University (Japan). These cell lines were cultured in Eagle's minimal essential medium (Nissui Pharmaceutical Co. Ltd., Japan) supplemented with 10% fetal calf serum (Gibco, USA), 2 mM glutamine, 4.7 mM HEPES and 24 mM sodium bicarbonate (E-MEM) at 37°C with 5% CO_2 / 95% air atmosphere.

Reagents Ascorbic acid, glutathione and lactic acid (special grade) were purchased from Wako Pure Chemical Industries Co. Ltd. (Osaka, Japan). Isolated Ty was prepared from B-16 cells according to the method of Seiji [9].

Cloning of mouse tyrosinase partial cDNA cDNA was synthesized from poly(A)$^+$RNA obtained from B-16 cells by using a cDNA synthesis kit (Amersham, USA). Primers for polymerase chain reaction (PCR) were synthesized using DNA synthesizer (Nippon Zeon Co., Ltd., Japan), and mouse tyrosinase partial cDNA (position 29 - 1162 from the nucleotide A of the ATG initiation codon according to the DNA sequence reported by Müller[10]) was amplified from the cDNA by PCR technique. The amplified mouse tyrosinase partial cDNA was ligated to SmaI site of plasmid vector pUC18, and then transformation of *E.coli* DH5α was performed. DNA sequencing was performed by the dideoxynucleotide chain-termination method described by Sanger[11].

Northern hybridization analysis Total RNA was extracted from cultured melanoma cells[12]. And then poly(A)$^+$RNA was further purified by using Oligotex-^{TM}dT30 (TAKARA SHUZO Co., Ltd., Japan). Poly(A)$^+$ RNA was fractionated on 1.2% formaldehyde denaturing agarose gel[12] and transferred to Hybond-N+ nylon membrane (Amersham) and hybridized with ^{32}P-labeled mouse tyrosinase cDNA probes under the condition of 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH7), 5 × Denhardt's solution (0.1% BSA, 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone), 50% formamide, 20

μg/ml salmon testes DNA. The membrane was then washed in 2 × SSC at room temperature for 5 min, and 2 times in 2 × SSC containing 0.1% SDS at 42°C for 15 min, and 0.1 × SSC containing 0.1% SDS at 42°C for 10 min. The membrane was then autoradiographed at -70°C. After the autoradiography, the previous probe was removed by treating the membrane in 0.1% SDS at 100°C boiled for 5 min and allow cool to room temperature. And the membranes was reprobed with β-actin DNA probe under the identical conditions to standardize mRNA levels. Density of the autoradiograms was measured using densitometer.

The assay for the Ty activity and Ty inhibitory activity Cell lysate of melanoma cells was prepared using Triton X-100 and subjected to Ty activity assay. The assay for Ty activity was carried out according to the modified method of Hamada[13] using L-3,4-dihydroxyphenylalanine (L-dopa) as a substrate under the incubating condition at 37°C. The initial rate of linear increase in absorbance at 475 nm, based on the formation of dopachrome, was measured using a spectrophotometer (UV-250, Shimadzu, Japan).

The assay for tyrosinase inhibitory activity was carried out according to the modified method of Imokawa[4] by measuring the extent of inhibition of the

activity of tyrosinase obtained from B-16 cells. The sample was mixed with tyrosinase and L-dopa solution, the tyrosinase activity was measured by the method described above.

Depigmenting assay The samples were added to E-MEM immediately after the seeding of the B-16 cells, and cultured at 37°C with 5% CO_2 / 95% air atmosphere for 5 days. The degree of depigmentation was observed macroscopically after packing by centrifugation of the B-16 cells into the capillary.

Purification of low molecular weight MIs D_1178 cells were propagated by transplanting to 4-week-old hamsters. The obtained D_1178 cells were homogenized in distilled water using POLITRON homogenizer (KINEMATICA GmbH, Switzerland). The homogenate was centrifuged at 12,000 × g for 1 hr. The supernatant was further ultra-filtrated to obtain diffusate (Mr<6,000) by ultrafiltrating module (Asahi Chemical Co., Ltd., Japan). The diffusate was applied to DEAE-Sephacel column. Non-adsorbed fraction which showed depigmenting effect on B-16 cells and inhibitory effect on isolated Ty was collected and concentrated by using a rotary evaporator. Then, the concentrated fraction was purified with the chromatography using TOYOPEARL

HW-40 gel and octa-decyl silane (ODS) reversed phase column.

G-361 cells were propagated by *in vivo* cell proliferation method using immunosuppressed hamsters [14,15]. The obtained G-361 cells were homogenized in 10 mM phosphate buffer (PB)(pH6.8) using POLITRON homogenizer. The homogenate was centrifuged at 11,000 × g for 10 min. The supernatant was added to 10 volume of methanol and centrifuged at 11,000 × g for 10 min. And the supernatant was evaporated. The residue was dissolved in distilled water and subjected to ODS reversed phase column chromatography.

Quantitative analysis of melanin content Both the quantity of eumelanin and pheomelanin were analyzed according to Ito's method[16]. Briefly, eumelanin content was determined as follows; cells were solubilized by Triton X-100 and then centrifuged. And the pellet was oxidized with potassium permanganate. The degradation product of pyrrole-2,3,5-tricarboxylic acid was analyzed by HPLC using ODS column with 6% MeOH in 0.1M potassium phosphate buffer (pH2.4) equipped with UV detector at 254 nm. For pheomelanin, cells were hydrolyzed with hydriodic acid. The degradation product of aminohydroxyphenylalanine was analyzed by HPLC using ODS column with 0.1 Sodium phosphate buffer

(pH4.0) containing 3% MeOH, 1 mM octanesulfonate and 0.1 mM Na_2EDTA equipped with electrochemical detector.

Electron microscopy B-16 cells were harvested and washed 3 times with phosphate buffered saline (PBS) and fixed with 2.5% glutaraldehyde in PBS for 1 hr at 4°C, washed twice with the PBS for 10 min. The cells were incubated in 0.1 M PB (pH6.8) containing 0.1% L-dopa for 16 hr at 37°C. And the cells were postfixed with 1% osmium tetroxide for 1 hr at 4°C, and dehydrated with graded ethanol and embedded in Spurr resin. Thin sections were cut with ultramicrotome (REICHERT-JUNG, Austria) and stained with lead citrate and uranyl acetate, and subjected to microscopic observation (JEM-100CX II, JEOL Ltd., Japan).

Tyrosinase isozyme analysis Subcellular fractionation was performed by the method of Seiji[9]. Thus, B-16 cells suspended in 0.25 M sucrose were lysed by three cycles of freezing and thawing. The suspension obtained was first centrifuged at 700 × g for 10 min to remove nuclear debris. The supernatant was further centrifuged at 11,000 × g for 10 min to obtain a soluble fraction (SF) and large granule fraction (LGF) as a sediment. The LGF was suspended in 0.5% sodium deoxycholate (DOC) aqueous solution for 30 min at 4°C

and was centrifuged at 11,000 x g for 10 min. And the supernatant was collected. Acrylamide gel electrophoresis was carried out according to the method of Davis[17] and Iwata's DOC method[18]. In the Davis's method, the SF was applied to the 7.5% acrylamide gel column. Electrophoresis was carried out at pH8.3 in Tris-glycine buffer at 3 mA/cm gel. After electrophoresis, the gel was incubated with 0.1% L-dopa in 0.1 M PB (pH6.8) at 37°C for 3hrs. In the DOC method, the DOC solubilized LGF was applied to the 7.5% acrylamide gel column. 1 % sodium dodecyl sulfate (SDS) was added to the spacer gel and the electrode buffer, and 0.5% SDS was added to the separation gel. After electrophoresis, the gel was soaked in 0.1% L-dopa solution at 37°C for 3hrs. The dopa reactions were stopped by soaking in 10% acetic acid. Absorbance at 475 nm of dopa reacted bands was measured using densitometer.

RESULTS

Analyses of Ty mRNA expression and Ty activity in amelanotic melanoma cells The levels of Ty mRNA, Ty activity and melanin contents were determined in Greene's $D_1$178 hamster amelanotic melanoma cells, their parental $D_1$179 melanotic melanoma cells, G-361 human

amelanotic melanoma cells established from male Caucasian and Ihara human melanotic melanoma cells established from oriental female. The levels of Ty mRNA are rectified by ß-actin mRNA used as an internal standard. And Ty activity analyses on cell lysate of amelanotic melanoma cells were carried out before and after dialysis against 10 mM PB (pH 6.8). The results are summarized in Table I. In D_1178 cells, no Ty mRNA was detected. No Ty activity was found in the cell lysate before or after dialysis. Further, neither eu- nor pheo-melanin were detected in D_1178 cells. In G-361 cells, on the other hand, Ty mRNA was detected a half level compared to that of Ihara cells (Fig 1). Nevertheless, no Ty activity was detected before dialysis whereas 0.1 $mU/10^6$ cells of Ty activity was detected after dialysis of G-361 cell lysate, suggesting that a dialyzable low molecular weight Ty inhibitor may exist in the G-361 cell lysate. Furthermore, only 0.15 $\mu g/10^6$ cells of pheomelanin was detected in G-361 cells but no eumelanin was found. In melanotic melanoma cell lines, D_1179 and Ihara, Ty mRNA and Ty activity were detected and both eu- and pheo-melanin were detected. These results indicate that different types of MIs play a role for melanogenesis regulation in these two amelanotic melanoma cell lines.

Identification of naturally occurring MIs in amelanotic melanoma cell lines The following two assays were applied for purification of naturally occurring MIs in amelanotic melanoma cells. One was the inhibition assay for isolated Ty, and the other was the depigmentation assay using *in vitro* cultured B-16 cells as target cells. MIs from D_1178 cells were purified as described in "Materials and Methods". The fraction obtained after HW-40 gel filtration, which had both depigmenting activity and Ty inhibitory activity, was subjected to ODS column chromatography. The elution profile is shown in Fig 2. Two types of MIs, named α1 inhibitor and α2 inhibitor, were isolated from D_1178 cells. α1 inhibitor had inhibitory activity against isolated Ty. α2 inhibitor showed depigmenting activity to B-16 cells but no inhibitory activity against isolated Ty. The identification of α1 inhibitor was performed in Toray Research Center, Inc. (Japan) using FAB-MS and NMR analyses. The molecular weight of α1 inhibitor was 176 dalton in FAB-MS analysis and the spectrum of α1 inhibitor in NMR analysis coincided with that of ascorbic acid (AsA) (data not shown). In addition, the tyrosinase inhibitory activity of α1 inhibitor was lost after the treatment of ascorbate oxidase. So, α1 inhibitor was identified as AsA. In order to identify α2 inhibitor, thin-layer

chromatography (TLC) was performed. The α2 inhibitor was indicated to be organic acid with carboxyl-residue, because it was stained with bromcresol green in TLC. A comparative study employing α2 inhibitor and well known 10 organic acids by HPLC analysis revealed that the retention time of α2 inhibitor coincided with that of lactic acid (LA). To confirm that α2 inhibitor is LA, α2 inhibitor was treated with lactate dehydrogenase before HPLC analysis. The peak corresponding to LA was decreased and the peak of pyruvic acid was newly appeared (Fig 3). Thus, α2 inhibitor was identified as LA.

The homogenate of G-361 cells was centrifuged, and the obtained supernatant was further purified by ODS column chromatography. The fraction whose retention time was different from those of AsA and LA in ODS column chromatography showed inhibitory activity to isolated Ty. This naturally occurring MI was named α3 inhibitor. The α3 inhibitor, detected as reducing substance by electrochemical detector, was identified as glutathione (GSH) because of coincidence of the retention time in the HPLC analysis (Data not shown).

Depigmenting effect of LA and Ty inhibitory activity of AsA, GSH and LA To confirm our findings described above, the following experiments were performed using commercially available AsA, GSH and LA. The effect of LA on B-16 cells was analyzed. B-16 cells were cultured in the presence of 10, 20 and 40mM LA (pH adjusted to 7.2) for 5 days, the cells were depigmented in dose dependent manner as shown in Fig 4 *A*.

Electron microscopic observation was performed on the B-16 cells depigmented by 40 mM LA treatment. Melanosomes in the B-16 cells depigmented by LA-treatment decreased in number significantly compared to those of the control cells (Fig 4 *B,C*).

The inhibitory effects of AsA, GSH and LA on isolated Ty are shown in Fig 5. AsA and GSH inhibited the activity of isolated Ty in the manner which gave lag time in the early reaction stage of dopachrome formation. However, LA did not inhibit isolated Ty activity.

Analyses of depigmenting effect of LA on cultured B-16 cells In order to investigate the depigmenting effect of LA on *in vitro* cultured B-16 cells, we performed the analyses on Ty mRNA level, Ty activity and pattern of Ty isozyme in B-16 cells cultured with or without 40mM LA for 5 days. The results are summarized in Table II.

Northern hybridization analysis revealed that the level of Ty mRNA in LA-treated B-16 cells was reduced to 60% of that of the untreated control cells (Fig 6). Ty activity in both SF and LGF were reduced to 13% and 24%, respectively, by treatment with 40mM LA. Ty isozyme levels in SF and LGF were suppressed to 37-62% by treatment with LA as compared with those in untreated controls.

^{3}H-uptake tests using [methy-^{3}H]-thymidine, [5,6-^{3}H]-uridine and ^{3}H-labeled amino acid mixtures were performed on B-16 cells cultured for 5 days with or without 40 mM LA. No significant difference of ^{3}H-uptake was observed between the treated and non-treated B-16 cells (Data not shown).

DISCUSSION

The melanogenesis in pigment cells has been considered to be controlled by the balance between Ty and naturally occurring MIs[3-5]. And the existence of naturally occurring MIs in pigment cells was proven by Couper[3] and Imokawa[4]. Kameyama[8] reported that nonpigmented cells which were cloned from its parental pigmented JB/MS cells produced potent and stable MIs of Ty suppressive type. However, naturally occurring MIs produced in melanoma cells themselves have not been

identified and the mechanism of their depigmenting action for cultured pigment cells has not been elucidated. In order to elucidate the melanogenesis controlled by naturally occurring MIs, we analyzed the levels of Ty mRNA, Ty activity and melanin contents in two amelanotic melanoma cell lines, D_1178 and G-361. The results of these analyses on the two amelanotic melanoma cell lines were compared with each other. The differences in levels of Ty mRNA, Ty activity and melanin contents were found between D_1178 and G-361 cells (Table 1). The results suggest that different types of MIs might be produced in these two cell lines. The analysis on MIs in D_1178 cells, in which neither Ty mRNA nor Ty activity was detected, revealed that two types of MIs were produced in D_1178 cells; one was LA and the other was AsA. In addition, we identified GSH as a MI in G-361 cells in which only a slight presence of pheomelanin was determined compared to melanotic melanoma cells (Table I). Both GSH and AsA inhibited isolated Ty activity in the manner which gave lag time in early reaction stage of dopachrome formation and resulted in inducing the depigmentation of pigment cells. GSH is reported as a key molecule which induces pheo-melanogenesis[19, 20]. The result that only pheomelanin was detected in G-361 cells established from male Caucasian coincides with the reported GSH

action. Furthermore, it is interesting that GSH, not AsA, was found as a Ty suppressive MI in human cell lines. This might be due to the fact that AsA is not able to be synthesized in human body because of lacking L-gulono-γ-lactone oxidase.

Lactic acid identified as a MI of isolated Ty non-suppressive type, showed depigmenting effect on cultured B-16 cells and the melanosomes were decreased in number significantly in the depigmented B-16 cells. Furthermore, T2, T1 and T3 isozyme levels decreased equally and the reduction of Ty mRNA was also observed in LA-treated B-16 cells. The results suggest that depigmenting effect of LA was due to the inhibition of Ty gene transcription but not due to interruption of Ty maturation. In order to confirm that LA was not cytotoxic for target cells, we examined total DNA, RNA and protein syntheses in B-16 cells by ^{3}H-uptake tests using [methyl-^{3}H]-thymidine, [5,6-^{3}H]-uridine and ^{3}H-labeled amino acid mixtures, respectively. In the LA-treated B-16 cells, no reduction of ^{3}H-uptake was observed in total DNA, RNA and protein syntheses (data not shown). The results suggest that reduction of Ty mRNA and Ty activity levels in the LA-treated B-16 cells might not be due to cytotoxicity of LA. We conclude that LA, discovered as a naturally occurring melanogenic inhibitor in Ty mRNA non-expressed $D_1$178

cells, suppressed the transcription of Ty gene and resulting in suppression of *de novo* synthesis of melanin in pigment cells.

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LEGENDS FOR FIGURES

Figure 1. Northern hybridization analysis of melanoma cells. Five micrograms of poly(A)$^+$RNA from D_1178 (*lane* 1), D_1179 (*lane* 2), G-361 (*lane* 3), and Ihara (*lane* 4) melanoma cells were electrophoresed on a 1.2% formaldehyde denaturing agarose gel, blotted and hybridized with the mouse tyrosinase cDNA probe (*A*) and rehybridized with chicken β-actin DNA probe as an internal standard (*B*).

Figure 2. ODS reversed phase column chromatogram of α_1 and α_2 inhibitors from D_1178 cells. The homogenate of D_1178 cells was ultrafiltrated and the obtained diffusate was further purified by DEAE-Sephacel column chromatography and TOYOPEARL HW-40 gel filtration. The fraction, which had both depigmenting effect on B-16 cells and inhibitory effect on isolated tyrosinase, was collected. And the fraction was further purified by HPLC using ODS reversed phase column (VYDAC, particle size 5 μm, column size 25 cm × 4.6 mm) detected with UV detector of absorbance at 215 nm. The mobile phase was 50 mM PB (pH 2.5) and the flow rate was 0.5 ml/min. The major two peaks were observed.

Figure 3. Identification of α_2 inhibitor as lactic acid by the treatment with lactate dehydrogenase (LDH). α_2 inhibitor purified by ODS column chromatography was mixed with NAD and LDH. The mixture was incubated for 15 min at 25°C. The samples before and after the LDH treatment were applied to G-oligo-PW column (TOSOH, column size 30 cm × 7.8 mm). The mobile phase was 0.1 M PB (pH 6.8) and the flow rate was 0.5 ml/min. The peak of α_2 inhibitor was decreased and the peak of pyrubic acid was newly observed.

Figure 4. Depigmentation of B-16 cells by lactic acid. B-16 cells were cultured with 0 mM, 10 mM, 20 mM and 40 mM lactic acid for 5 days. *A*, The treated cells were harvested and packed into the capillary. B-16 cells were depigmented in dose dependent manner. *B*, Electron micrograph of control B-16 cells. *C*, Electron micrograph of B-16 cells treated with 40 mM lactic acid. Bar; 1μm. The melanosomes in B-16 cells were decreased in number by the treatment of lactic acid.

Figure 5. Inhibitory effects of AsA, GSH and LA on isolated Ty. Ty from B-16 cells and L-dopa solution were mixed with 40 mM lactic acid (LA), 0.15 mM ascorbic acid (AsA) or 0.15 mM glutathione (GSH), respectively. And the initial increase rate of absorbance at 475 nm, based on the formation of dopachrome, was measured under the incubating condition at 37°C.

Figure 6. Northern hybridization analysis of B-16 cells treated with 40 mM lactic acid. Five micrograms of poly(A)+RNA from cultured B-16 cells (*lane* 1) and cultured in the presence of 40 mM lactic acid (*lane* 2) for 5 days were electrophoresed on a 1.2% formaldehyde denaturing agarose gel, blotted and hybridized with the mouse tyrosinase cDNA probe (*A*), and rehybridized with chicken β-actin DNA probe as an internal standard (*B*).

Table I. Tyrosinase mRNA levels and activities in melanoma cells

cells	mRNA(%)	Tyrosinase Activity (mU/10^6 cells)		Melanin (μg/10^6 cells)	
		cell ext.	after dialysis	Eu-	Pheo-
D_1 178 (AM)	< 0.1[a]	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]
D_1 179(MM)	100	23.3	n.t.[c]	6.2	1.0
G-361 (AM)	50[d]	N.D.[b]	0.1	N.D.[b]	0.15
Ihara (MM)	100	2.0	n.t.[c]	1.7	1.4

[a] Relative percentage of mRNA to D_1 179 mRNA.

[b] Not detectable.

[c] Not tested.

[d] Relative percentage of mRNA to Ihara mRNA.

Table II. Effect of lactic acid on melanogenesis in B-16 cells

Treatment	Depigmentation	Tyrosinase						Eumelanin
		mRNA(%)[a]	Activity (mU/10^6 cells)		Isozyme(%)[a]			(μg/10^6 cells)
					SF[b]	LGF[c]		
			SF[b]	LGF[c]	T_2	T_1	T_3	
Non(control)	No	100	0.30	0.58	100	100	100	9.5
LA 40mM	Yes	60	0.04	0.14	45	37	62	2.4

[a] Relative percent to the control values.

[b] Supernatant after centrifugation of cell lysate at 11,000 × g.

[c] Precipitate after centrifugation of cell lysate at 11,000 × g.

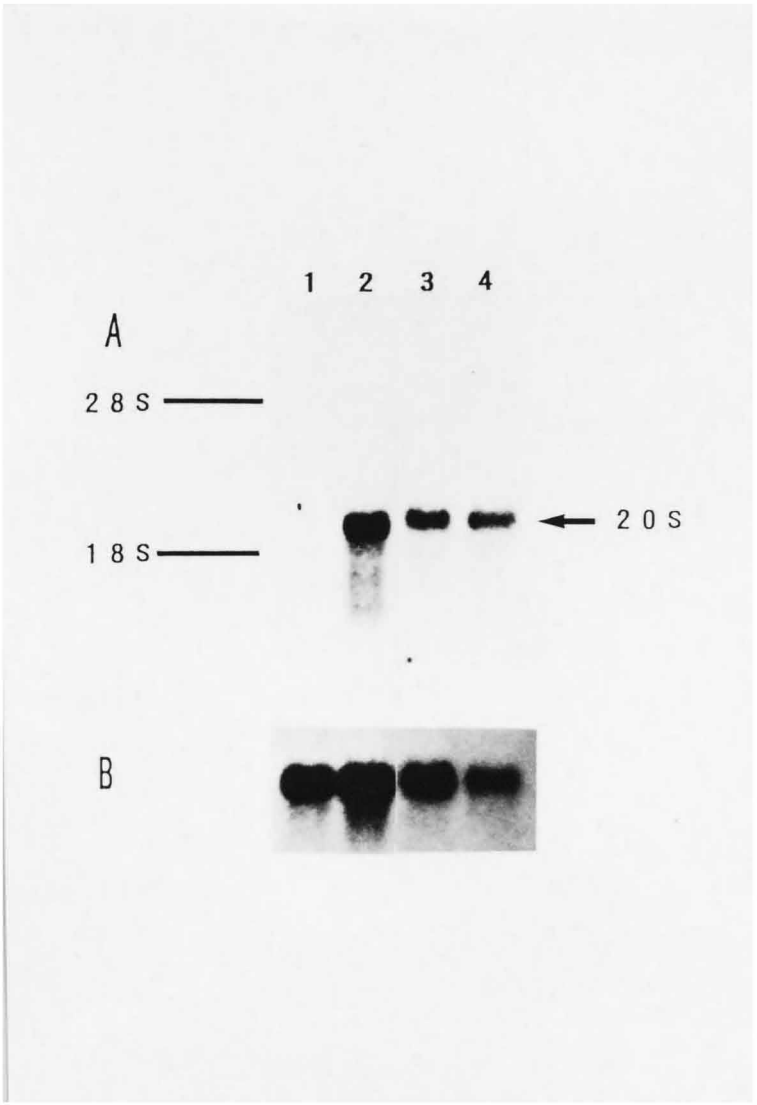


Figure 1. Northern hybridization analysis of melanoma cells.

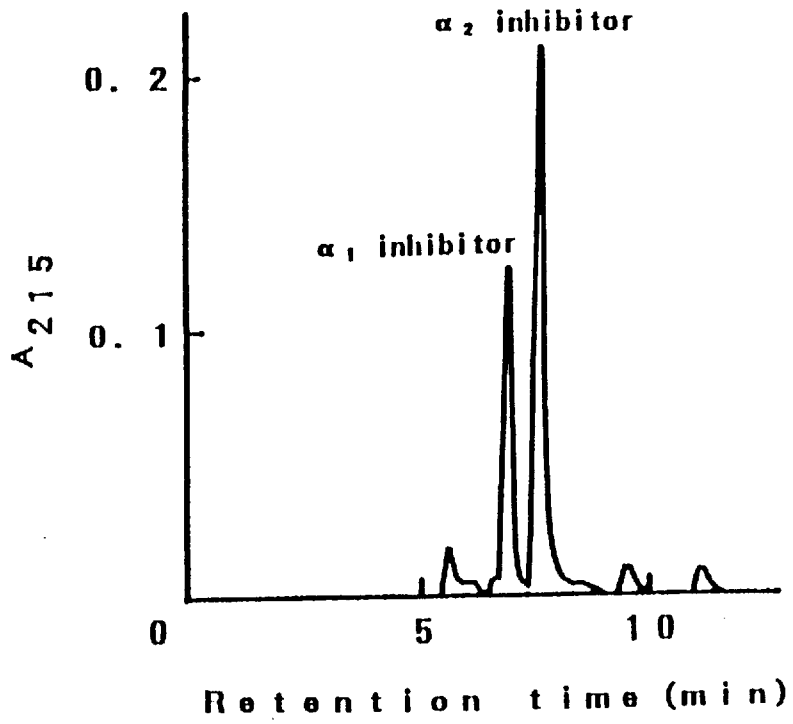


Figure 2. ODS reversed phase column chromatogram of α_1 and α_2 inhibitors from D_1178 cells.

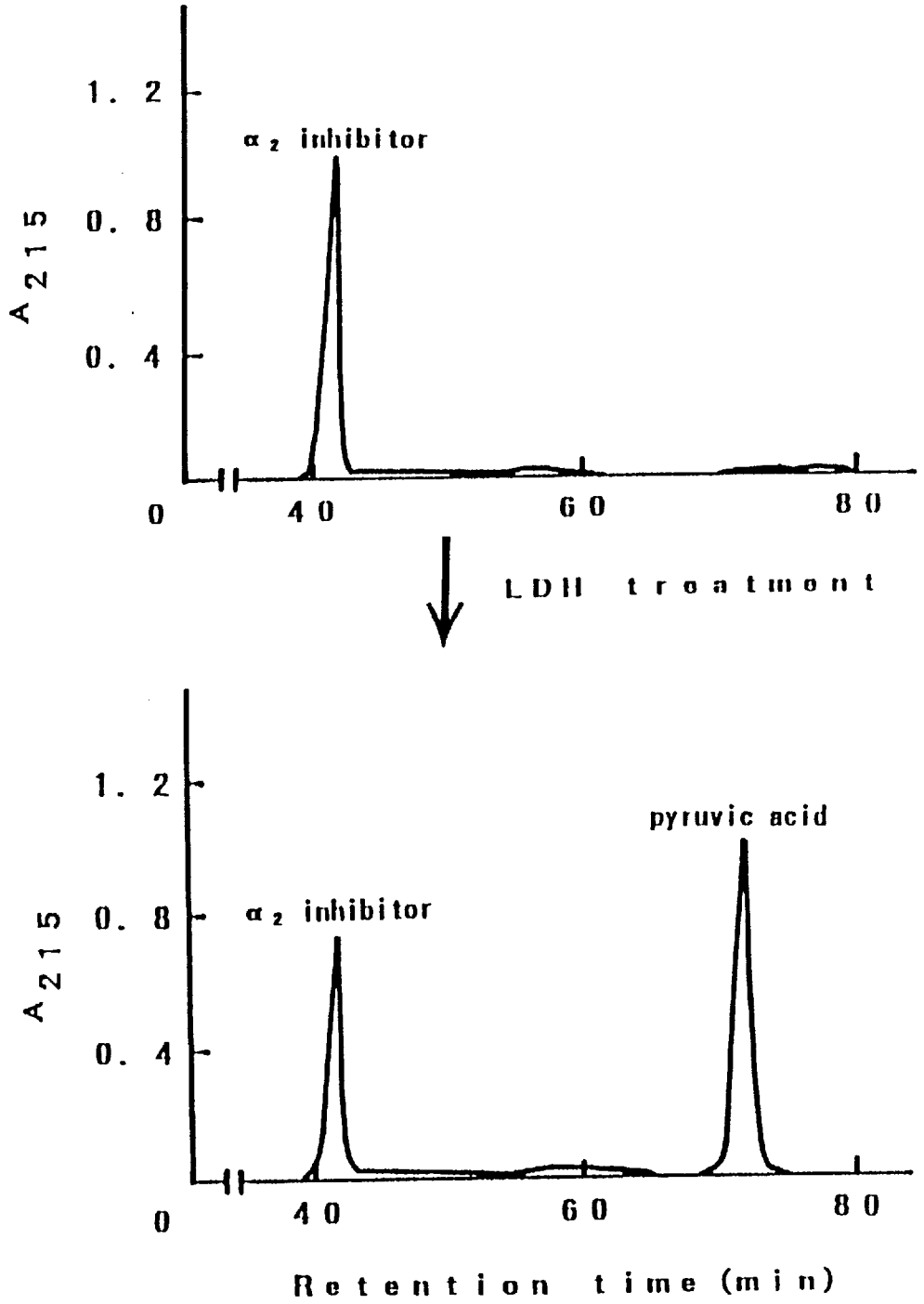
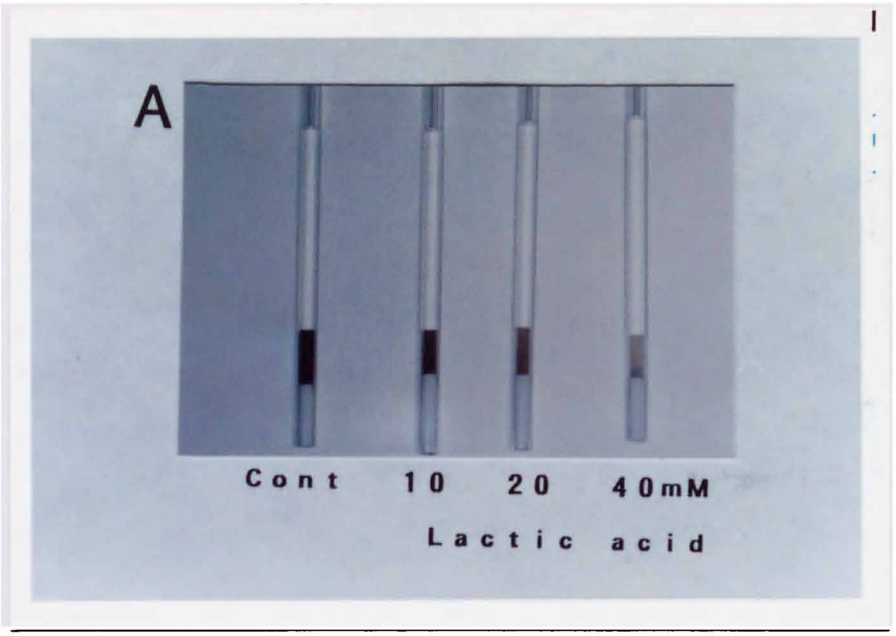
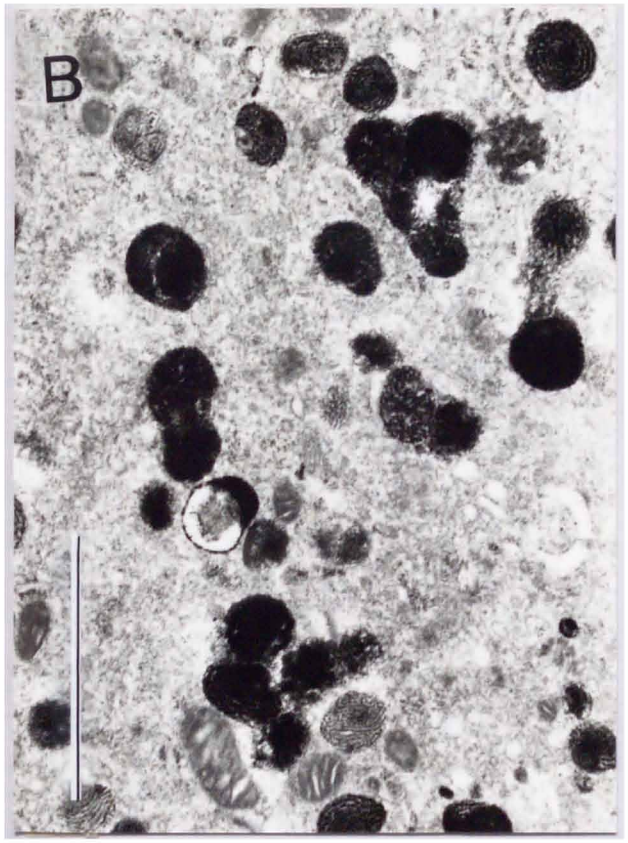


Figure 3. Identification of α_2 inhibitor as lactic acid by the treatment with lactate dehydrogenase (LDH).





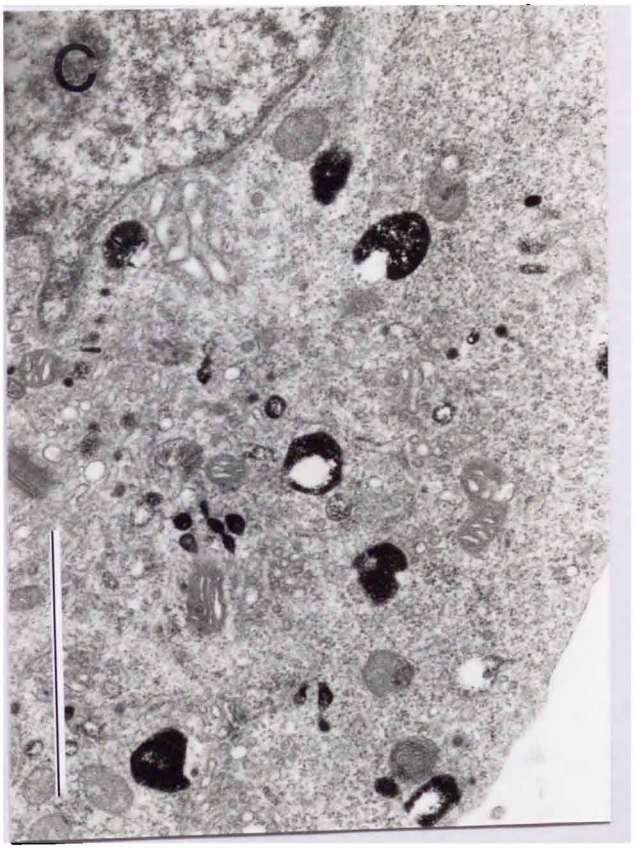


Figure 4. Depigmentation of B-16 cells by lactic acid.

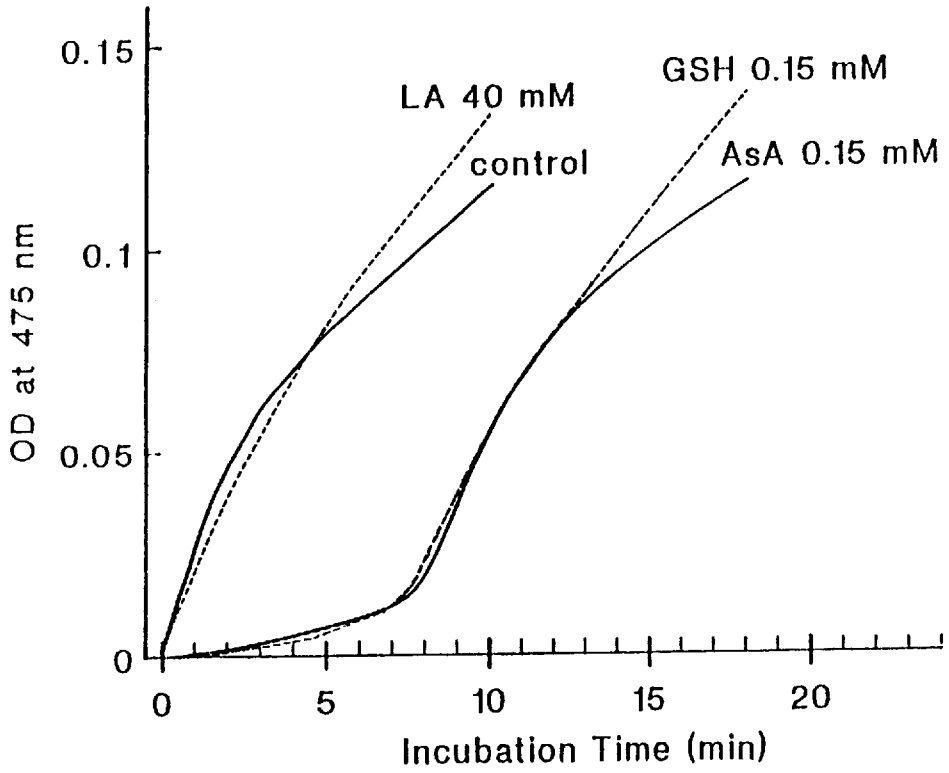


Figure 5. Inhibitory effects of AsA, GSH and LA on isolated Ty.

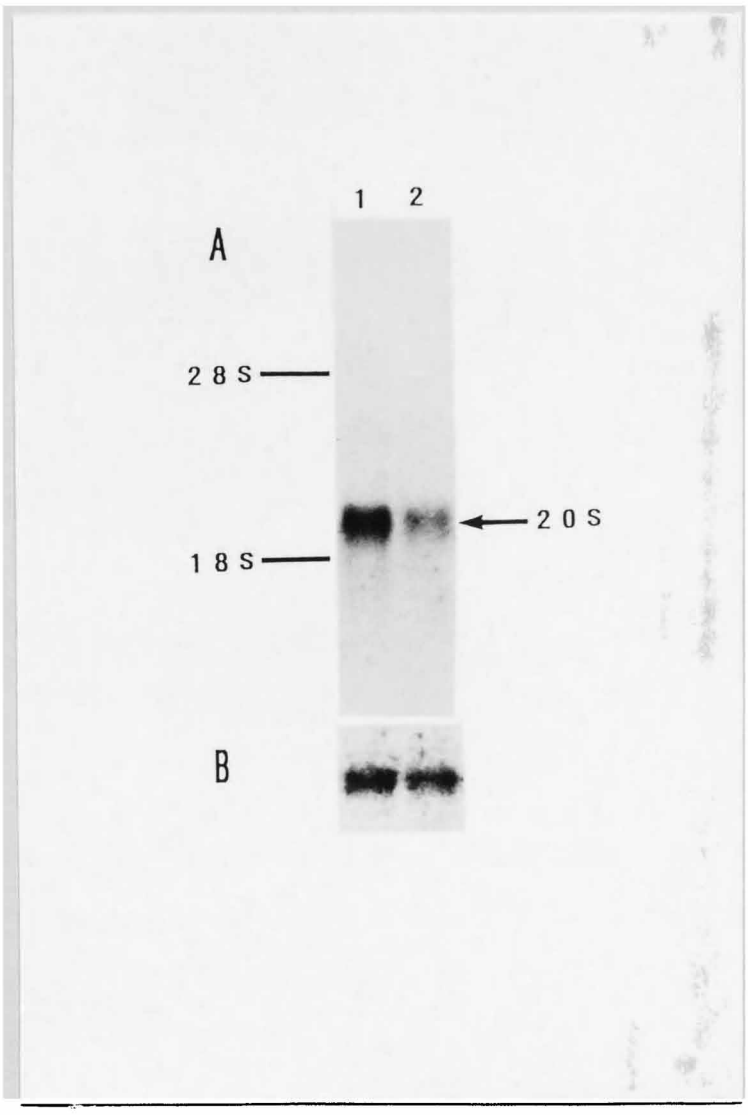


Figure 6. Northern hybridization analysis of B-16 cells treated with 40 mM lactic acid.