Acta Derm Venereol (Stockh) 1997; 77: 281–284

Insulin Inhibits Tyrosinase Activity and 5-S-Cysteinyldopa Formation in Human Melanoma Cells

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The effects of insulin on melanogenesis were examined in human Swift melanoma cells. When these cells were grown in a chemically defined culture medium containing insulin (5 μg/ml), they showed a low pigmentation in association with a high activity of glutathione peroxidase (GPO) and a low activity of tyrosinase. In Eagle's minimum essential medium supplemented with foetal calf serum (EMEM-FCS), the Swift cells showed an intense pigmentation in association with a low GPO activity and a high tyrosinase activity. Modulation of GPO activity with sodium selenite had no effect on melanogenesis variables. In contrast, addition of insulin (5 μg/ml) to the EMEM medium led to a marked decrease in tyrosinase activity ($p<0.001$) and to a concomitant reduction in the levels of 5-S-cysteinyldopa ($p<0.01$). These results indicate that insulin inhibits the formation of 5-S-cysteinyldopa and that of melanin via the inhibition of tyrosinase activity. *Key words: cell culture; cysteine; glutathione; melanogenesis; sodium selenite.*

(Accepted February 3, 1997.)

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Melanogenesis is a mechanism dependent on the availability of tyrosine (1), the activity of tyrosinase and tyrosinase-related proteins (2), and the cellular concentration of thiols (3, 4). At low levels of cellular thiols, tyrosine oxidation by tyrosinase produces dark pigments (eumelanins). In contrast, interaction between thiols and tyrosine oxidation products leads to sulfur-containing reddish-brown phaeomelanin pigments (5). The switch from eumelanogenesis to phaeomelanogenesis could be caused by a modification of the ratio between cysteine and glutathione (GSH), as suggested in a recent study (6).

Pigment cells contain the phaeomelanin precursor 5-S-cysteinyldopa (5-S-CD) (7). Although GSH is the major storage form of cysteine in pigment cells (8), recent evidence indicates that 5-S-CD is formed by a direct reaction between free cysteine and dopaquinone. It has been shown that the amount of 5-S-CD in normal and malignant melanocytes correlates with the intracellular concentration of free cysteine (9, 10). In addition, a direct relationship has been observed between tyrosinase activity and the levels of 5-S-CD in melanoma cells (10). These data indicate that 5-S-CD genesis is modulated by tyrosinase activity and intracellular cysteine.

Human Swift melanoma cells are heavily pigmented and produce 5-S-CD when grown in essential medium containing foetal calf serum (10). Culture of these cells in serum-free chemically defined culture medium results, however, in decreased pigment formation. In this study, it is shown that insulin exerts profound effects on melanogenesis and that it could be one of the factors responsible for inhibition of pigment formation in serum-free culture medium.

MATERIAL AND METHODS

Cell culture

The Swift cell line was obtained from the Memorial Sloan-Kettering Cancer Center (New York, U.S.A.), through the courtesy of Dr. M. Eisinger. The cells were routinely maintained in Eagle's minimum essential medium (EMEM), containing 5% foetal calf serum (FCS), 2 mM L-glutamine, 1% non-essential amino acids, 100 units/ml penicillin, 0.1 mg/ml streptomycin sulfate and 2.5 μg/ml fungizone. This medium is referred to as EMEM-FCS medium. The Swift cells were also grown in a serum-free synthetic medium (GIBCO Keratinocyte™-SFM; Paisley, Scotland) containing 0.09 mM calcium chloride, bovine pituitary extract (50 μg protein/ml), 5 ng/ml human recombinant epidermal growth factor 1–53 (EGF 1–53) and 5 μg/ml human insulin. This medium is referred to as KSFM-BPE medium. All the cultures were kept at 37°C in an atmosphere of 5% CO_2/95% air.

Experimental design

The Swift cells were plated at a split ratio of 1:10 and grown for 6 days in EMEM-FCS or KSFM-BPE medium. The culture media were changed on days 3 and 5. Insulin (27 IU/mg protein) from bovine origin (Sigma, St. Louis, MO) was added to the EMEM-FCS medium on days 1, 3 and 5 at 5 μg/ml, final concentration. All the cultures were collected on day 6, 24 h after the last medium change.

Glutathione reductase (GR) and glutathione peroxidase (GPO)

The cells were detached with 0.02% EDTA and suspended in cold 0.125 M phosphate buffer, containing 0.625 mM EDTA, pH 7.2. The homogenates were sonicated and centrifuged at 12,000 g. GR activity was determined according to the method of Goldberg & Spooner (11). GPO activity was measured as described by Paglia & Valentine (12). One mU of GR or GPO is defined as the catalytic amount which oxidizes one nmol of NADPH per minute.

Tyrosinase activity

Cell extracts for the determination of tyrosinase activity were prepared in cold 0.1 M phosphate buffer, pH 6.8, containing 0.5% Triton X 100. The dopa oxidase activity of tyrosinase was assessed at 37°C by measuring dopachrome formation at 475 nm in the presence of 2 mM L-dopa (13). The data were corrected for L-dopa autoxidation. One mU of tyrosinase activity corresponds to one nmol of dopachrome formed per minute.

HPLC analysis of cysteine and GSH

All solvents used for thiol extraction and analysis were degassed with helium (purity 99.99%). The cells were extracted with 0.25 M perchloric acid. Cysteine and GSH were determined in the 12,000 g supernatant by high performance liquid chromatography (HPLC) with electrochemical detection. The detector LC-4C, from Bioanalytical Systems (BAS, West Lafayette, IN, U.S.A.), was equipped with a Hg-Au electrode set at a working potential of +150 mV versus an Ag/AgCl reference electrode (14). The analytical column (100 × 3.2 mm) consisted of a Phase II ODS 3 μm (BAS). The mobile phase (flow rate 0.7 ml/min) was a mixture of 0.1 M monochloroacetic acid and 3.3 mM 1-heptanesulfonic acid adjusted to pH 2.6.

 ISSN 0001-5555

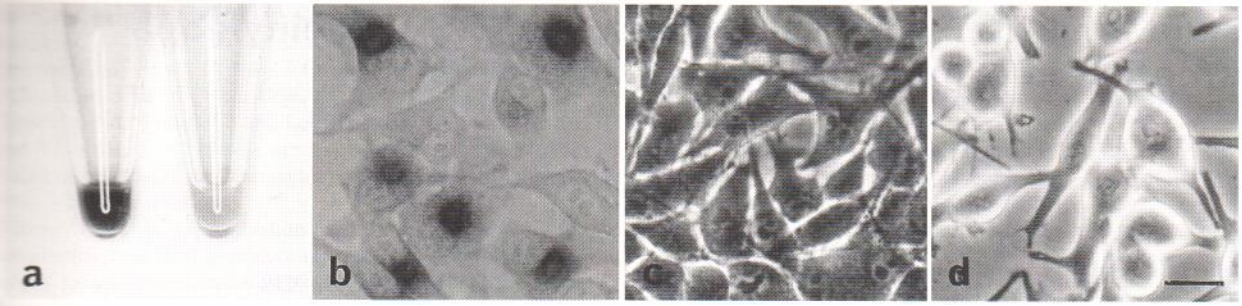

Fig. 1. Inhibition of melanogenesis in serum-free chemically defined culture medium containing insulin. (*a*) Swift cells grown in EMEM-FCS (*left*) and in KSFM-BPE (*right*) media. (*b*) Dense melanin pigmentation around the nucleus of the Swift cells grown in EMEM-FCS medium. (*c, d*). Optimal and suboptimal growth of the Swift cells in EMEM-FCS and KSFM-BPE, respectively. (*Bar* = 25 μm).

HPLC analysis of 5-S-CD

5-S-CD was determined in cell extracts (0.25 M perchloric acid) and in culture supernatants by HPLC with electrochemical detection, using a glassy carbon electrode operating at +750 mV against an Ag/AgCl reference electrode (15). Culture supernatants were adsorbed on acid-washed activated alumina (7). A column (100 × 3.2 mm) of Phase II ODS 3 μm was eluted with 0.1 M phosphate, 0.1 mM EDTA, 73 mM methanesulphonic acid pH 3, at a flow rate of 0.7 ml/min.

Protein measurements

Proteins were determined with the Folin phenol reagent (16). The protein precipitates obtained with 0.25 M perchloric acid were previously dissolved in 0.05 M sodium carbonate.

Calculations and statistics

Each experimental protocol was repeated at least three times. Results are expressed as nmol of cysteine, GSH or 5-S-CD or as mU of enzyme activity per mg protein. The statistical significance of the data was evaluated using the Student's *t*-test.

RESULTS

Effect of culture conditions on melanogenesis

The phenotypic characteristics of the Swift cells were found to be modulated by the culture conditions. When cultured in the EMEM-FCS medium, the cells showed a heavy pigmentation (Fig. 1*a*), consisting of numerous melanin granules that were densely packed around the nucleus (Fig. 1*b*). In contrast, when cultured in the KSFM-BPE medium, the Swift cells showed a low pigmentation (Fig. 1*a*) and displayed a rounded or elongated morphology (Fig. 1*d*). The culture conditions also affected the growth rate of the cells. Only those cells grown in the EMEM-FCS medium formed confluent monolayers (Fig. 1*c, d*).

As shown in Table I, the Swift cells grown in the EMEM-FCS medium showed a higher tyrosinase activity than those grown in the KSFM-BPE medium ($p < 0.001$). In contrast, they exhibited a lower glutathione reductase (GR) and glutathione peroxidase (GPO) activity. The levels of GSH did not differ significantly between the cells grown in the two different culture systems.

Inhibition of melanogenesis by insulin

The KSFM-BPE medium contains various proteinic additives, including EGF, bovine pituitary extract, transferrin and insulin. Among these components, only insulin was found to inhibit melanogenesis in the Swift cells (Table II). The addition of insulin to the EMEM-FCS medium for 6 days decreased tyrosinase activity by 61% ($p < 0.001$) but had no effect on the activities of GPO and GR. The decrease in tyrosinase activity was associated with a decrease in cell pigmentation.

Insulin addition to the EMEM-FCS medium also induced a strong reduction in the Swift cells and supernatant levels of 5-S-CD (Table III). To see whether this effect of insulin was related to a modification of the cell levels of cysteine and GSH, the latter were also determined. As shown in Table III, addition of insulin did not change the thiol levels in the Swift cells.

Effects of calcium and selenite on melanogenesis

The KSFM-BPE medium contains only 0.09 mM calcium, while the EMEM-FCS medium contains 1.8 mM calcium (17). It has been reported that calcium ions modulate melanogenesis in cultured melanoma cells (18, 19). To investigate whether the relative lack of calcium was responsible for the inhibition of melanogenesis, the KSFM-BPE medium was supplemented with calcium chloride (0.5–2 mM) separately and in combina-

Table I. *Effect of the culture conditions on tyrosinase activity and glutathione-related variables of human Swift melanoma cells*

The Swift cells were grown for 6 days with medium changes on days 3 and 5.

Growth medium	Tyrosinase (mU/mg protein)	GR[a] (mU/mg protein)	GPO[b] (mU/mg protein)	GSH (nmol/mg protein)
EMEM-FCS	6.7 ± 2.0 (12)	55 ± 6 (7)	32 ± 6 (12)	65 ± 10 (11)
KSFM-BPE	0.8 ± 0.2*** (15)	94 ± 7*** (15)	105 ± 8*** (15)	56 ± 6 (12)

Mean ± SD (number of separate experiments). Cells grown in KSFM-BPE versus EMEM-FCS: *** $p < 0.001$. [a] GR: glutathione reductase. [b] GPO: glutathione peroxidase.

Table II. *Effect of insulin on the enzymatic activities of tyrosinase, glutathione reductase (GR) and glutathione peroxidase (GPO) in Swift cells*

The cells were grown for 6 days in EMEM-FCS medium supplemented or not with 5 μg/ml insulin.

Treatment	Tyrosinase	GR	GPO
	(mU/mg protein)		
None	7.0±0.1	51±3	29±3
Insulin (5 μg/ml)	2.7±0.6***	48±5	34±4

Values are means±SD from three experiments. Cells grown in the presence versus absence of insulin: *** $p<0.001$.

tion with FCS (5%). These conditions had no effect on tyrosinase activity or on pigment formation in the Swift cells (data not shown).

Selenite is a common additive in chemically defined synthetic media (20). Since the enzyme GPO contains selenocysteine at its active site (21), it was hypothesized that selenite could be responsible for the stimulation of GPO activity and for the associated inhibition of tyrosinase activity. To test this hypothesis, sodium selenite (5 ng/ml) was added to the EMEM-FCS medium. Under these conditions, the Swift cells showed a twofold increase in GPO activity (70±9 versus 29±3 mU/mg protein, mean±SD, $p<0.001$). However, tyrosinase activity and pigment formation were not affected (data not shown).

DISCUSSION

In the present study, insulin was found to inhibit melanogenesis in a human melanoma cell line. This effect was observed by growing the cells in the presence of insulin, using two different culture media. The results also show that a serum-free chemically defined culture medium, containing insulin, induced depigmentation in human melanoma cells.

The mechanism by which insulin inhibited melanogenesis in the human Swift melanoma cells involves inhibition of the activity of tyrosinase. A similar effect has been reported in Cloudman S91 mouse melanoma cells (22, 23). Thiol compounds are known to inhibit the activity of isolated tyrosinase through reduction of copper atoms at the active site of the enzyme (24, 25). As shown by our results, intracellular cysteine and GSH were not modulated by insulin, which is supportive of a thiol-independent effect.

Tyrosine and dopa oxidation to melanin by the enzyme tyrosinase requires molecular oxygen (24, 26). Therefore, it may be questioned whether insulin modulates oxygen levels in human melanoma cells. Insulin is known to stimulate the transport and metabolism of glucose in Harding-Passey melanoma cells (27), which could affect melanogenesis indirectly. On the other hand, it has been found that hydrogen peroxide induces the activity of tyrosinase in melanoma cells (28). A decreased production of melanin could therefore be related to a modification of the redox balance of pigment cells. In support of this hypothesis is the finding that insulin can normalize the circulating levels of ascorbate, alpha-tocopherol and retinol when administered to streptozotocin diabetic rats (29).

Table III. *Effect of insulin on 5-S-CD and thiol levels*

The Swift cells were grown for 6 days in EMEM-FCS medium supplemented or not with 5 μg/ml insulin.

Treatment	ic 5-S-CD[a]	ec 5-S-CD[b]	Cysteine	GSH
	(nmol/mg protein)			
None	3.0±0.2	0.50±0.10	0.8±0.2	52±4
Insulin (5 μg/ml)	1.4±0.2***	0.20±0.04**	1.0±0.2	48±3

Values are means±SD from three experiments. Cells grown in the presence versus absence of insulin: ** $p<0.01$, *** $p<0.001$. [a] ic 5-S-CD: intracellular level of 5-S-cysteinyldopa. [b] ec 5-S-CD: extracellular level of 5-S-cysteinyldopa.

Whether insulin modifies the expression of melanogenic proteins other than tyrosinase remains an open question. It has been shown in recent years that tyrosinase-related protein 2 (TRP-2) catalyzes dopachrome rearrangement to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (30) and that tyrosinase-related protein 1 (TRP-1) may be involved in DHICA oxidation (31). In addition, it has been found that TRP-1 expression correlates with eumelanin formation in cultured melanoma cells (32). Since insulin inhibits melanin formation in Swift melanoma cells, there is a possibility that insulin acts not only on tyrosinase but also on TRP-1 expression.

We have recently shown that 5-S-CD production in human melanoma cells is modulated by the activity of tyrosinase (10). Tyrosinase catalyzes the hydroxylation and oxidation of tyrosine to dopaquinone, which is the immediate precursor of 5-S-CD (24). Therefore, the insulin-induced inhibition of tyrosinase activity may be responsible for the observed decrease in 5-S-CD formation. This hypothesis is further substantiated by the finding that the levels of cysteine were not modified in insulin-treated melanoma cells compared to control cells. Recent data indicate that addition of cysteine to dopaquinone could be the main reaction leading to 5-S-CD in normal and malignant melanocytes (9, 10).

Insulin has been found to be a strong growth factor for primary and metastatic cell lines of human melanoma (33, 34). In addition, insulin and insulin-like growth factors I and II are known to induce migration of melanoma cells in vitro (35). As shown in this study, melanin formation in human melanoma cells is significantly modulated by insulin. Taken together, these data indicate that insulin in culture medium could be a major factor controlling the growth and differentiation of human malignant melanoma cells.

In conclusion, the results indicate that insulin inhibits the activity of tyrosinase in human melanoma cells, and that this effect is responsible for the decreased formation of 5-S-CD and melanin in these cells.

ACKNOWLEDGEMENT

The authors would like to thank the University Department of Dermatology, Lausanne (Prof. R. Panizzon) for supporting this research.

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