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REDUCTION IN EUMELANIN BY THE ACTIVATION OF GLUTATHIONE REDUCTASE AND γ-GLUTAMYL TRANSPEPTIDASE AFTER EXPOSURE TO A DEPIGMENTING CHEMICAL

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Abstract—Topical application of 4-tertiary butyl catechol (TBC) causes vitiligo in the skin of man and animals, and previous electron microscopic studies showed pheomelanin formation in the affected areas. In the present study, we investigated changes of enzyme activities, eumelanin content and amount of sulfur in tissue cultured human melanoma cells exposed to the depigmenting chemical. TBC enhanced glutathione reductase activity without changing the eumelanin content by 24 hr after exposure and subsequently (by 42 hr) increased γ-glutamyl transpeptidase activity and sulfur content in the cells with a decrease in eumelanin content. It is suggested that this chemical alters the types of melanin formed by modulation of these enzyme activities.

Geschwind [1] and his collaborators have induced eumelanin in genotypically yellow mice, producing pheomelanin by injection of melanocyte-stimulating hormone (MSH). Others [2, 3] have also observed that the shift from production of eumelanin to pheomelanin, or vice versa, occurs in a single melanocyte which has been stimulated by various methods. However, specific factors which promoted the changes in melanogenesis have remained unknown. Recently, Nishimura *et al.* [4] demonstrated that topical application of 4-tertiary butyl catechol (TBC) on mouse melanocytes engaged in eumelanin formation resulted in the appearance of pheomelanosomes. These findings offered a new experimental approach for the investigation of the control mechanisms of melanogenesis.

Prota [5] reviewed possible biochemical mechanisms that may be involved in the formation of eumelanin, pheomelanin and a mixture of the two. He suggested that glutathione reductase (GR) may trigger generation of reduced glutathione (GSH) and that γ-glutamyl transpeptidase (GGTP) was involved in the formation of cysteinyldopa. Direct measurement of GSH and oxidized glutathione (GSSG) in different colored skin of the tortoiseshell guinea pig was done by Benedetto *et al.* [6]. The results showed that the level of GSH was proportional to pheomelanin production and the ratio of GSH to GSSG was higher in skin with pheomelanin than in skin with eumelanin, indirectly supporting Prota's view. In this study, we designed experiments to test whether there is any correlation between the enzyme activities which involve GSH and cysteinyldopa formation and the eumelanin and sulfur contents of tissue cultured melanoma cells after exposure to TBC.

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MATERIALS AND METHODS

Cell culture. A human melanoma cell line (M_3) maintained in our laboratory was used. Cells 3–4 × 10^5) were seeded into 75 cm^2 tissue culture flasks and cultured with Dulbecco's Modified Eagle's Medium (DME) containing 10% fetal bovine serum (FBS) and penicillin-streptomycin (Grand Island Biological Co., Grand Island, NY). They were incubated in the humidified atmosphere of 5% CO_2/95% air at 37°. Cells were detached from the flask with 2 mM EDTA in phosphate (20 mM) buffered saline and serially passed. Cell numbers were counted in isotonic diluent (Fisher Scientific Co., Fairlawn, NJ) using an electronic model ZBI Coulter Counter (Coulter Electronics Inc., Hialeah, FL). Cell viability was determined from the proportion of the cells excluding 0.04% trypan blue in saline.

Chemical treatment of cells. TBC (Aldrich Chemical Co., Milwaukee, WI) dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO) at the concentration of 10^{-1} M was further diluted to 10^{-4} M with Basal Medium (Eagle's) (BME). At day 6 of culture, DME was decanted and the cells were incubated for 2 hr in TBC containing BME or BME containing 0.1% DMSO at 37°. The media with the chemicals was discarded and replaced by BME containing FBS to continue the experiment.

Measurement of eumelanin and sulfur content. For eumelanin, protein in 2 × 10^6 cells from 1- to 8-day cultures was precipitated with 5% trichloroacetic acid (TCA) and washed with 0.2 N NaOH. The alkali-insoluble fraction was dissolved at 37° in 2 ml of Soluene-100 (Packard Instrument Co., Downers Grove, IL), and the absorbance at 400 nm was read by a Perkin–Elmer spectrophotometer (model 550) [7, 8]. Measurement of sulfur was done in the cells cultured for 8 days after seeding. Protein in

Table 1. Numbers and viability of human melanoma (M_3) cells in culture immediately or 42 hr after exposure to 10^{-4} M TBC or solvent for 2 hr*

Chemical	Cell number ($\times 10^5$) 0 hr	Cell number ($\times 10^5$) 42 hr	Cell viability (%) 0 hr	Cell viability (%) 42 hr
None	88.3 ± 12.6	133.3 ± 20.8	91.0 ± 3.2	90.3 ± 3.0
DMSO	91.5 ± 18.1	121.7 ± 23.5	88.5 ± 2.6	85.6 ± 2.9
TBC	80.6 ± 22.3	141.6 ± 19.1	91.8 ± 4.0	88.6 ± 4.0

* Values are expressed as means ± S.D.; N = 4.

5×10^7 cells was similarly precipitated with TCA, washed in water, and lyophilized. The sample was heated first in nitric acid and then in hydrogen peroxide for combustion. Water was added and pH was adjusted to make a final volume of 5 ml at pH 4.0. Barium chloranilate in acetate buffer, pH 4.0, was added and centrifuged. The absorbance at 327.5 nm in the supernatant fraction was read spectrophotometrically [9]. Sulfur content was expressed as $\mu g/5 \times 10^7$ cells.

Enzyme assays. The cells were scraped off from the flasks at 0, 6, 24 or 42 hr after the 2-hr treatments with BME containing TBC–DMSO or DMSO alone. They were homogenized at 4° in 3 ml of 50 mM Tris–HCl buffer, pH 8.0, containing 25 mM sucrose using a Polytron homogenizer three times for 15 sec at maximum intensity. Aliquots (1.5 ml) were used for GGTP assay and the other 1.5 ml for GR assay after centrifugation at 105,000 *g* for 30 min at 4° and separation of the supernatant fraction.

For GGTP, the cell homogenate (0.1 ml) was added to 0.9 ml of the transpeptidase substrate solution containing 5 mM L-α-glutamyl-*p*-nitroanilide, 20 mM glycylglycine, 75 mM NaCl and 50 mM Tris–HCl buffer, pH 8.0, and incubated for 1 hr at 37° with constant stirring. The reaction was terminated by the addition of 0.2 ml of glacial acetic acid, and the absorbance of *p*-nitroaniline released was measured spectrophotometrically at 410 nm [10]. The activity was expressed as μmoles of *p*-nitroaniline released per min per mg protein. Protein concentration was determined by the method of Lowry *et al.* [11]. For GR, the supernatant fraction (0.25 ml) was added to 2.25 ml of a reaction mixture consisting of 0.1 M Tris–HCl buffer, pH 7.0, 0.5 mM NADPH, 1 mM EDTA, 0.6 mM oxidized glutathione and 0.02% bovine serum albumin. The oxidation rate of the cofactor NADPH was measured by a decrease in absorption at 340 nm using the spectrophotometer connected to a Perkin–Elmer recorder (model 56) [12, 13]. The activity was expressed as μmoles of NADPH oxidised per min per mg protein at 37°.

Data analysis. The values in TBC-treated cells are presented as the percentage of those obtained in DMSO-treated cells. At least four sets of experi-

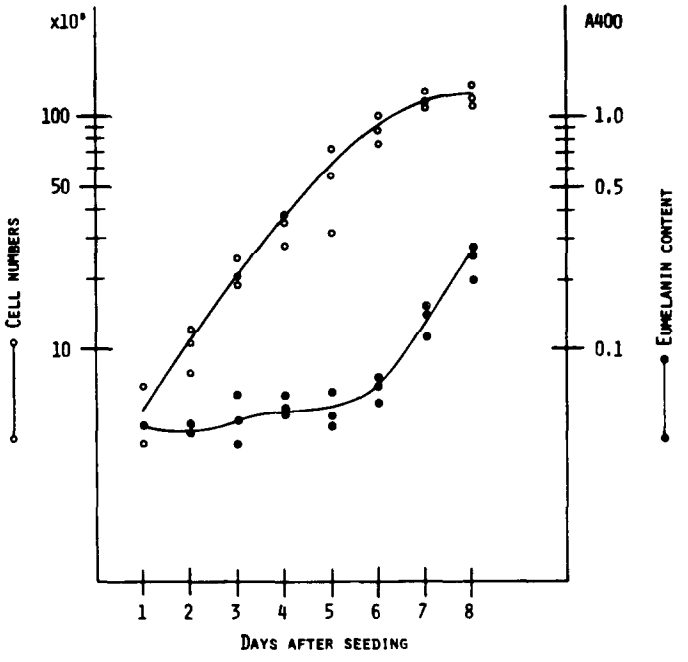

Fig. 1. Growth curve and changes in eumelanin content of human melanoma (M_3) cells in culture. The cell number (○—○) increased rapidly while the melanin content (●—●) slowly increased during the first 6 days after the seeding of 3–4 × 10^5 cells in a 75 cm^2 tissue culture flask.

Table 2. Reduction of the eumelanin increase in human melanoma (M_3) cells in culture resulting from exposure to 10^{-4} M TBC or solvent for 2 hr*

	Inhibition of eumelanin ($A_{400}/2 \times 10^6$)			
	Hr after TBC treatment			
Chemical	0	6	24	42
DMSO	0.07 ± 0.01	0.07 ± 0.02	0.14 ± 0.02	0.32 ± 0.04†
TBC	0.06 ± 0.02	0.07 ± 0.02	0.16 ± 0.03	0.22 ± 0.02†

* Values are expressed as means ± S.D.; N = 4.
† $P < 0.02$.

ments were done and mean ± S.D. calculated; Student's *t*-test and analysis of variance were used to compare the significance of the differences.

RESULTS

Cell growth and viability. About 90% (91.0 ± 3.2%) of the cells excluded trypan blue dye and were considered viable at all time periods studied. Figure 1 shows a typical growth curve of the M_3 cell line after the subculture of 3–4 × 10^5 cells. Exponential proliferation was seen during the first 6 days with growth reaching a plateau by day 8. TBC and DMSO treatment on day 6 after seeding did not cause immediate (0 hr) or late (42 hr) effects on either the cell number or cell viability (Table 1).

Eumelanin and sulfur contents and cell color. The eumelanin content was low and absorption at 400 nm remained constant (0.06 ± 0.02) during the first 6 days after subculture. It rapidly increased after day 7, attaining about a 5-fold increase (0.30 ± 0.03) by day 8 (Fig. 1). The DMSO treatment did not affect the value significantly. TBC treatment of the cells on day 6 for 2 hr did not reflect on the eumelanin content of the cells up to 24 hr later, but did cause a reduction in the increase of the eumelanin content 42 hr later (8 days after seeding) (Table 2). Cell color was compared by day 8 after making cell pellets of TBC-treated and nontreated cells by centrifugation. Significant lightening of the cell color was noted macroscopically. Sulfur content measured at the same experimental period showed 153.7 ± 5.0 μg/5 × 10^7 cells in TBC-treated cells as compared with 134.7 ± 1.8 μg/5 × 10^7 cells in DMSO-treated cells.

GR and GGTP activity. No changes in the enzyme activities were detected during the first 6 hr after TBC treatment. However, 24 hr after treatment an increase in GR activity (118.3 ± 4.0% of DMSO-treated cells) was seen and 42 hr after treatment both GR (132.5 ± 3.0% of DMSO-treated cells) and GGTP (131.4 ± 2.4% of DMSO-treated cells) activities were increased (Fig. 2).

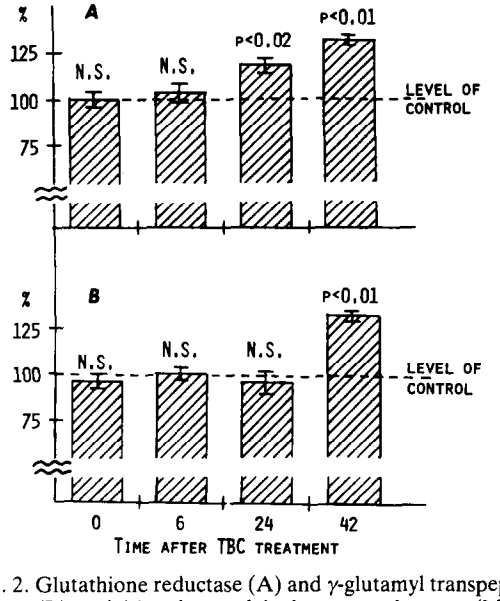

Fig. 2. Glutathione reductase (A) and γ-glutamyl transpeptidase (B) activities detected in human melanoma (M_3) cells immediately or 6, 24 and 42 hr after exposure to 10^{-4} M TBC. They were compared to the activities measured in nontreated cells taken at the same culture periods.

DISCUSSION

Preliminary studies were conducted with several different concentrations of TBC and different treatment times. The concentration of 1 × 10^{-4} M TBC was chosen because this was the highest concentration found which was not cytolytic to M_3 human melanoma cells 2 hr of exposure and the total number of viable cells remained unchanged after replacement with the media without TBC. We consider that the conditions we used in the study may simulate *in vivo* studies in hairless mouse skin. Topical application of TBC for a shorter duration neither lysis of melanocytes nor color changes but does form pheomelanosomes, while a longer exposure to the same dose of the drug causes a reduction in the melanocyte number and a lightening of the color [4, 14]. In addition, James *et al.* [15] clinically observed that the severity of leukoderma has a good correlation with the amount of exposure to a depigmenting chemical.

There were no detectable changes during the first 6 hr after TBC treatment, but elevation of GR activity was noted by 24 hr after discontinuation of TBC. Eumelanin content increased in these cells to the degree as seen in control cells and the GGTP activity also remained the same. GR generates GSH and maintains a steady state of the glutathione level which regulates functions involved in cell survival [16]. It is difficult to postulate the mechanisms by which TBC stimulated GR activity within this time interval. TBC, an antioxidant, may have changed first one or more step(s) of the metabolism in melanoma cells, including elevation of GSH, and

then most likely changed GR activity indirectly. Studies investigating the direct effects of TBC on the levels of GSH and GSSG in melanoma cells are in progress.

GR continued to be elevated in these TBC-treated cells for at least another 20 hr. The additional change that we detected was an increase in GGTP and lightening of cell color by 42 hr after discontinuation of TBC treatment. GGTP is one of the hydrolytic enzymes considered to function in the conversion of glutathionedopa to cysteinyldopa [5, 17, 18]. The increase of the substrate, glutathionedopa, may have promoted GGTP activity subsequently, because GGTP catalyzes the transfer reaction of the γ-glutamyl group of GSH to various amino acid acceptors [19, 20]. Significant elevation of GGTP has been reported in patients with various lever diseases [21–23], after drug administration [24, 25] and in several types of malignancy [26–28]. Activation of the enzyme in melanocytes, therefore, is not necessarily a specific indicator of pheomelanogenesis. However, reduction of eumelanin and increase of sulfur content occurred simultaneously with the increase of GGTP, and ultrastructural study of TBC-treated melanocytes demonstrated that TBC could cause pheomelanosome formation *in vivo* [4] and *in vitro* [29]. Thus, detection of increased GR and GGTP activities in the present study was considered to indicate a shift in melanogenesis toward sulfur-rich melanins. It appears that modulation of melanocyte function occurs with TBC, resulting in a reproducible and time-dependent alteration in specific enzyme activity and the induction of pheomelanin synthesis. Application of this experimental system is expected to achieve a better understanding of the control mechanisms in melanogenesis.

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REFERENCES

1. I. I. Geschwind, *Endocrinology* **79**, 1165 (1966).
2. T. Sakuri, H. Ochiai and T. Takeuchi, *Dev. Biol.* **47**, 466 (1975).
3. T. Ikejima and T. Takeuchi, *Jap. J. Genet.* **55**, 283 (1980).
4. M. Nishimura, G. A. Gellin, S. Hoshino, J. H. Epstein, W. L. Epstein and K. Fukuyama, *Anat. Rec.* **202**, 193 (1982).
5. G. Prota, *J. invest. Derm.* **75**, 122 (1980).
6. J-P. Benedetto, J-P. Ortonne, C. Voulot, C. Khatchadourian, G. Prota and J. Thivolet, *J. invest. Derm.* **77**, 402 (1981).
7. A. Oikawa and M. Nakayasu, *Yale J. Biol. Med.* **46**, 500 (1973).
8. A. Oikawa and M. Nakayasu, *Analyt. Biochem* **63**, 634 (1975).
9. B. Spencer, *Biochem. J.* **75**, 435 (1960).
10. S. S. Tate and A. Meister, *J. biol. Chem.* **249**, 7593 (1974).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
12. E. Racker, *J. biol. Chem.* **217**, 855 (1955).
13. I. Carlberg and B. Mannervik, *J. biol. Chem.* **250**, 5475 (1975).
14. S. Hoshino, M. Nishimura, K. Fukuyama, G. A. Gellin and J. H. Epstein, *J. invest. Derm.* **76**, 231 (1981).
15. O. James, R. W. Mayes and C. J. Stevenson, *Lancet* **2**, 1217 (1977).
16. N. S. Kosower and E. M. Kosower, *Int. Rev. Cytol.* **54**, 109 (1978).
17. G. Agrup, B. Falck, B-M. Kennedy, H. Rorsman, A-M. Rosengren and E. Rosengren, *Acta derm.-vener., Stockh.* **55**, 1 (1975).
18. G. Agrup, B. Falck, H. Rorsman, A-M. Rosengren and E. Rosengren, *Acta derm.-vener., Stockh.* **57**, 221 (1977).
19. F. Binkley, *J. biol. Chem.* **236**, 1075 (1961).
20. M. Orlowski and A. Meister, *J. biol. Chem.* **240**, 338 (1965).
21. E. Szczeklik, M. Orlowski and A. Szewczuk, *Gastroenterology* **41**, 353 (1961).
22. M. Orlowski and A. Szczeklik, *Clinica chim. Acta* **15**, 387 (1967).
23. G. Lum and S. R. Gambino, *Clin. Chem.* **18**, 358 (1972).
24. S. B. Rosalki, D. Tarlow and D. Rau, *Lancet* **2**, 376 (1971).
25. S. B. Rosalki and D. Rau, *Clinica chim. Acta* **39**, 41 (1972).
26. J. Dawson, G. D. Smith, J. Boak and T. J. Peters, *Clinica chim. Acta* **96**, 37 (1979).
27. J. L. Murray, M. P. Lerner and R. E. Nordquist, *Cancer*, **49**, 1439 (1982).
28. F. Hu, *J. invest. Derm.* **79**, 57 (1982).
29. J. D-S. Mansur, K. Fukuyama, G. A. Gellin and W. L. Epstein, *J. invest. Derm.* **70**, 275 (1978).