

Melanogenesis in Cultured Melanocytes can be Substantially Influenced by L-Tyrosine and L-Cysteine

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We investigated the effect of varying concentration of l-tyrosine and l-cysteine in culture medium on melanin production by human skin melanocytes (skin phototype II/III). In addition to the analyses of dopa oxidase activity and total melanin, pheomelanin production in the cells was assessed by high-performance liquid chromatography determinations of pheomelanin degradation products, 3-aminotyrosine and 4-amino-3-hydroxyphenylalanine. As another marker for pheomelanin, melanosomal sulfur was determined by the use of X-ray microanalysis. With varying concentration of both amino acids, profound changes in the pigmentation patterns of the melanocytes were observed. A high concentration of l-tyrosine (0.2 mM) was always connected with increased pigmentation. In combination with a low l-cysteine content we saw an increase in tyrosinase activity and the highest melanin content. At high concentrations of both

l-tyrosine and l-cysteine, the melanocytes showed reduced tyrosinase activity and they produced notably more pheomelanin. In case of the pheomelanin measurements by high-performance liquid chromatography and the sulfur detection with X-ray microanalysis, strongly increased concentrations were found when cells were maintained in high l-tyrosine medium as compared with those grown with low l-tyrosine. This was especially true for the combination with low l-cysteine showing that the l-tyrosine content of the medium strongly influences not only the eumelanin but also the pheomelanin production in the cultured melanocyte. It can be concluded that variations in the concentrations of l-tyrosine and l-cysteine in culture medium can be used to regulate the melanogenetic phenotype under *in vitro* conditions. **Key words:** pheomelanin/X-ray microanalysis. *J Invest Dermatol* 109:796-800, 1997

Pigment melanin is the main determinant of skin and hair color. The quantity and the chemical composition of this pigment play a decisive role in the skin competence to protect the body against ultraviolet radiation.

As far as the chemical composition of melanin is concerned, two distinct types of melanin are present in human skin: the red-yellow pheomelanin and the brown-black eumelanin. Although these two pigments utilize the same melanin precursor (l-tyrosine) and share the first steps of the melanogenic pathway, only eumelanin is directly built from o-dihydroxyphenolic and indolic precursors. Pheomelanin is formed by the conjugation of reactive phenolic intermediates with l-cysteine giving rise to benzothiazine derivatives as basic monomer units. The presence of sulfur in the molecular network of pheomelanin is therefore quite characteristic (Prota, 1980; Ito *et al*, 1988).

The importance of l-tyrosine as a substrate for pigment formation in the human skin and the role of sulfhydryl compounds in human pigmentation was recognized already a half century ago (Flesch and Rothman, 1948; Fitzpatrick *et al*, 1950).

The ratio between eumelanin and pheomelanin in the epidermis

may vary but the presence of eumelanin has been shown to roughly correspond to skin sun-sensitivity (skin phototypes): the higher the concentration of eumelanin, the less the sun-sensitivity (Thody *et al*, 1991). Persons with darker skin (higher proportion of eumelanin) are supposed to be better protected against the detrimental effect of ultraviolet radiation. This difference in sun-sensitivity can be explained by differences in the physical properties of eumelanin and pheomelanin. When ultraviolet irradiated, pheomelanin produces free radicals (Agin *et al*, 1980; Chedekel *et al*, 1980) whereas eumelanin is described to exhibit free-radical scavenging activity (Ezzahir, 1989). Data derived from *in vitro* experiments examining the protective role of melanin have, however, often been conflicting (Kitano and Hu, 1969; Hill and Hill, 1987; Niggli, 1990; Yohn *et al*, 1992; Kobayashi *et al*, 1993; Abdel-Malek, 1996).

In 1995, Hunt *et al* showed that the eumelanin/pheomelanin ratio of cultured melanocytes differed from that of the epidermis from which they were isolated. Generally, the ratios were lower in the cultured melanocytes than in the epidermis of their origin. These observations may have important implications for the use of cultured human melanocytes in the studies of pigmentation and it might be one of the reasons why in many studies conflicting results have been obtained.

To get more insight into the changes of melanin composition during culture we investigated the influence of variations of l-tyrosine and/or l-cysteine concentrations in culture medium on the course of melanogenesis in normal skin melanocytes. For this purpose we used a modification of a high-performance liquid chromatography (HPLC) method for determination of pheomelanin (Ito and Fujita, 1985). Furthermore, X-ray microanalysis was employed to assess the pheomelanin contents in melanosomes by the measurement of their sulfur

Manuscript received February 25, 1997; revised July 30, 1997; accepted for publication August 22, 1997.

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Abbreviations: AHP, 4-amino-3-hydroxyphenylalanine; AT, 3-aminotyrosine.

concentration (Inazu and Mishima, 1993). We show that eumelanogenesis and pheomelanogenesis are closely connected with each other and that the increased incorporation of L-tyrosine in the pigment polymer is always accompanied by enhanced incorporation of L-cysteine.

MATERIALS AND METHODS

Cell culture studies The melanocyte culture M9401 originating from newborn foreskin (white Caucasian, roughly skin phototype II/III) was used. The cells were grown by a modification (Smit *et al.*, 1995a) of the methods described by Eisinger and Marko (1982) and Halaban and Alfano (1984). The culture medium consisted of Ham's F-10 containing 1% Ultrosor-G, 16 nM 12-O-tetradecanoylphorbol-13-acetate, and 0.1 mM isobutylmethylxanthine. In the early stage of the culture, the cells were treated with geneticin to eliminate fibroblasts (Halaban and Alfano, 1984).

The melanocyte culture (M9401) was also grown in modified Ham's F-10 media. These media differed in concentrations of L-tyrosine and L-cysteine that were added to the basic medium lacking these amino acids (Life Technologies B.V. Breda, The Netherlands). The effects of different cysteine concentrations [1, 5, 10 and 15% of the regular (C+) 0.2 mM concentration] in Ham's F-10 medium were examined in the cultures. Starting from passage 13, the M9401 cells were grown in Ham's F-10 with four possible variations as outlined below.

Two concentrations of L-tyrosine were used: the basic concentration in Ham's F-10 medium (T-; 0.01 mM) and a 20-times higher concentration (T+; 0.2 mM). These were combined with the standard (C+; 0.2 mM) and a reduced (C-; 0.03 mM) concentration of L-cysteine. The four combinations are entitled T-C-, T-C+, T+C-, and T+C+.

Cells were harvested with 0.01% trypsin/0.02% ethylenediamine tetraacetic acid in phosphate-buffered saline containing 0.1% glucose. Detached cells were centrifuged at 1200 rpm in a IEC Centra-8 centrifuge. The supernatant was withdrawn and the cell pellet was washed once with phosphate-buffered saline and frozen at -20°C.

Protein determination A crude suspension of the cell pellets was made in distilled H₂O and the protein concentration was measured by the method of Lowry *et al.* (1951).

Tyrosinase Tyrosinase was determined as a dopa oxidase activity in the cell suspension according to Winder and Harris (1991).

Melanin measurements Total melanin in the same cell suspensions was measured spectrophotometrically after the solubilization of the pigment in 2N NaOH as described by Friedman and Gilchrist (1987). All the measurements were performed in duplicate.

Pheomelanin analysis was performed by a modification of the method of Ito and Fujita (1985). The hydrolysate obtained after overnight hydriodic acid (HI) hydrolysis of the sample at 130°C was dried under a stream of nitrogen, redissolved in 0.05 M Li phosphate solution (pH 4.0), and pipetted on an aromatic sulfonic acid column (J.T. Baker, Deventer, The Netherlands). After washing with one column volume of distilled H₂O, the pheomelanin products 3-aminotyrosine (AT) and 4-amino-3-hydroxyphenylalanine (AHP) were eluted with 2 ml 0.3 M KCl (pH 8.5). Next, 20–50 µl was injected with a Rheodyne 9125 injector and separated on a stainless steel reversed phase Supelco-sil LC-18-DB, 25 × 4.6 (i.d.) mm analytical column (Supelchem, Bornem, Belgium). The samples were eluted at a flow rate of 0.9 ml per min using a Gyncoetek isocratic pump, Model 300C (Separations, H.I. Ambacht, The Netherlands). The mobile phase consisted of 1% (vol/vol) methanol in 0.01 M phosphate buffer pH 5.7, 1 mM 1-octanesulfonic acid sodium salt, and 30 mg sodium-ethylenediamine tetraacetic acid per liter. Peaks were detected with an ESA model 5100 A Coulouchem electrochemical detector with an analytical cell model 5011 (InterScience, Breda, The Netherlands). The standards AT (Sigma) and AHP (kindly provided by Prof. Ito, Fujita Health University, Aichi, Japan) were used for the calibration.

Electron microscopy Electron microscopy was performed according to our standard procedure (Van der Meulen and Koerten, 1994). A portion of the cells were only fixed in glutaraldehyde and embedded. Sections (about 100 nm) of these samples were collected on aluminum grids and measured using a Tracor (TN) 2000 X-ray microanalyzer attached to a Philips EM 400 scanning transmission electron microscope (Koerten *et al.*, 1990). Of each sample 100 melanosomes were randomly selected, and X-ray point analyzes were performed at 80 kV, a 150 µm condensor aperture, and 100 s lifetime.

RESULTS

Increased L-tyrosine concentration in culture medium enhances pigmentation of cultured melanocytes Our early observations

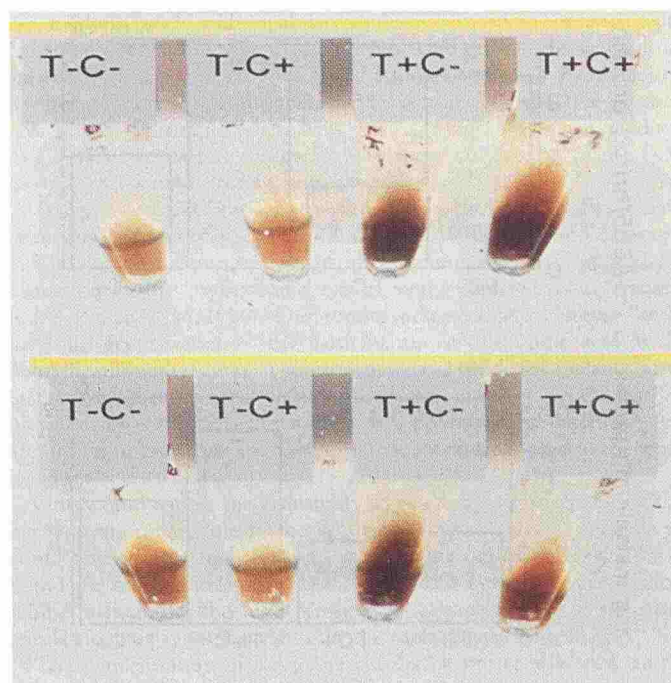


Figure 1. High concentration of L-tyrosine in culture medium leads to increased pigmentation of the cultured melanocytes. Minor differences can be observed as a result of varying L-cysteine levels. Melanocyte M9401 cell suspensions are shown of passages 13 (top) and 14 (bottom). The cells were grown in four different media: T-C-, T-C+, T+C-, and T+C+ [Ham's F-10 containing 0.01 mM or 0.2 mM L-tyrosine (T- and T+) and 0.03 mM or 0.2 mM L-cysteine (C- and C+)].

showed that the M9401 melanocyte cultures strongly increased their melanin production when they were maintained in DMEM medium that contains 40 times more tyrosine than Ham's F-10. The M9401 melanocytes were also cultured in Ham's F-10 medium with varying concentrations of L-tyrosine and L-cysteine. To maintain normal cell proliferation, the cysteine concentration could not be reduced below 10% (0.02 mM) of the standard concentration. Therefore, a medium containing 0.03 mM cysteine (15%) was used.

The culturing of the M9401 cells in Ham's F-10 medium with varying concentrations of tyrosine and cysteine led to profound changes of the pigmentation patterns already during the first passage when the cells were maintained in the four different media (passage 13, see Fig 1). It was evident that, again, the high concentration of tyrosine was connected with increased pigmentation. The result was reproducible in subsequent passages (see passage 14, Fig 1).

Pheomelanin/total melanin ratio in melanocytes cultured in high tyrosine medium can be modulated by varying L-cysteine concentrations

The concentration of cysteine in the medium also had a strong influence on the tyrosinase activity (Fig 2a). Cells grown in low-cysteine media exhibited higher tyrosinase activity than those grown in high-cysteine media. The high tyrosinase activity resulted in increased total melanin concentrations in these cells providing that sufficient tyrosine was present (T+C-, Fig 2b).

HPLC analysis of pheomelanin demonstrated that no distinct differences in pheomelanin contents were obtained when the cells were cultured in the media with low tyrosine (T-C-, 1.50 µg AT + AHP per mg protein *versus* T-C+, 1.41 µg AT + AHP per mg protein); however, the pheomelanin contents in the same cells grown in T+C- medium were higher (2.27 µg AT + AHT per mg protein) than that of the T-C+ medium. The highest quantity of pheomelanin was found in the cells cultured in high-tyrosine and high-cysteine medium (T+C+, 5.10 µg AT + AHP per mg protein, Fig 2c). Especially at the high tyrosine concentrations, a clear increase in the ratio of pheomelanin to total melanin was obtained in the cells cultured in the high-cysteine medium (T+C+) when compared with those maintained in the low-cysteine medium (T+C-, Fig 2d).

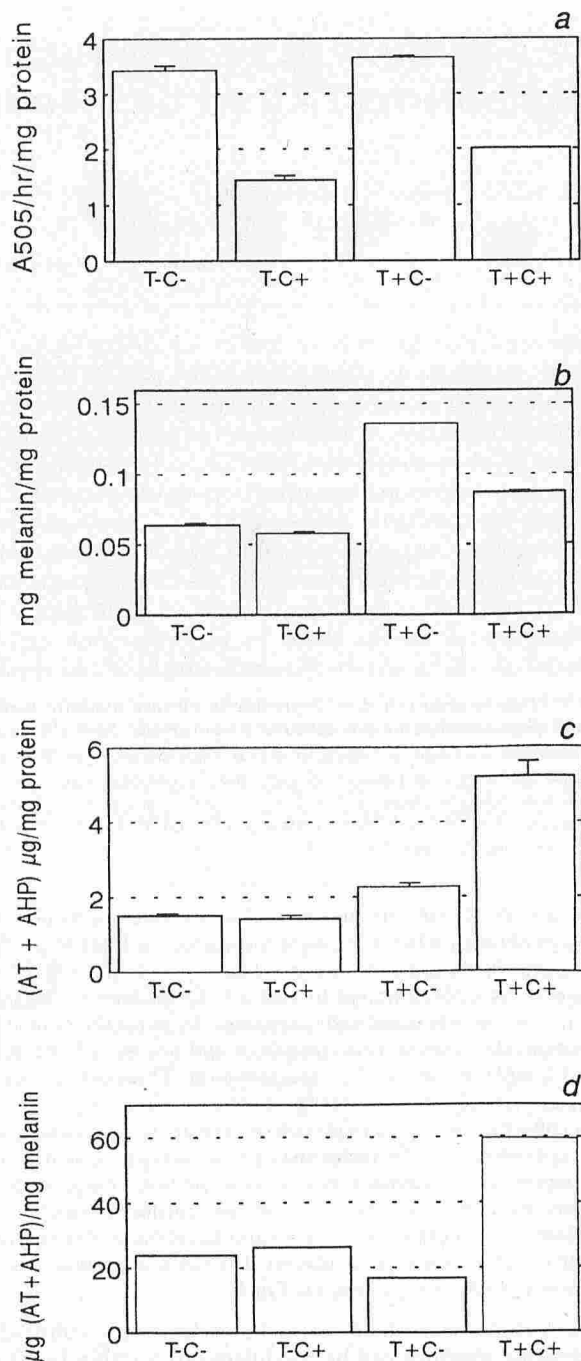


Figure 2. Varying l-tyrosine and l-cysteine concentrations in culture media of M9401 melanocytes strongly influences melanin production in the cells. In the homogenates of the melanocyte suspensions of passage 13 (as shown in Fig 1), tyrosinase activity (a), total melanin (b), and pheomelanin (c) contents were measured as described in *Materials and Methods*. The ratio of pheomelanin and total melanin (d) for the melanocytes cultured in the four media (T-C-, T-C+, T+C-, and T+C+) illustrates the differences of melanin composition obtained. The bars indicate the range in duplicate measurements.

The results of the tyrosinase, total melanin, and pheomelanin measurements in the samples of passage 13 of the M9401 melanocytes were exactly the same as those obtained from the samples of the next passage (results not shown, for macroscopic comparison see Fig 1).

Melanosomal density and sulfur content are elevated in melanocytes cultured in medium containing high l-tyrosine The results of transmission electron microscopy were in agreement with the macroscopic observations and the analytical HPLC measurements. As shown in Fig 3, melanosomes of cells grown in

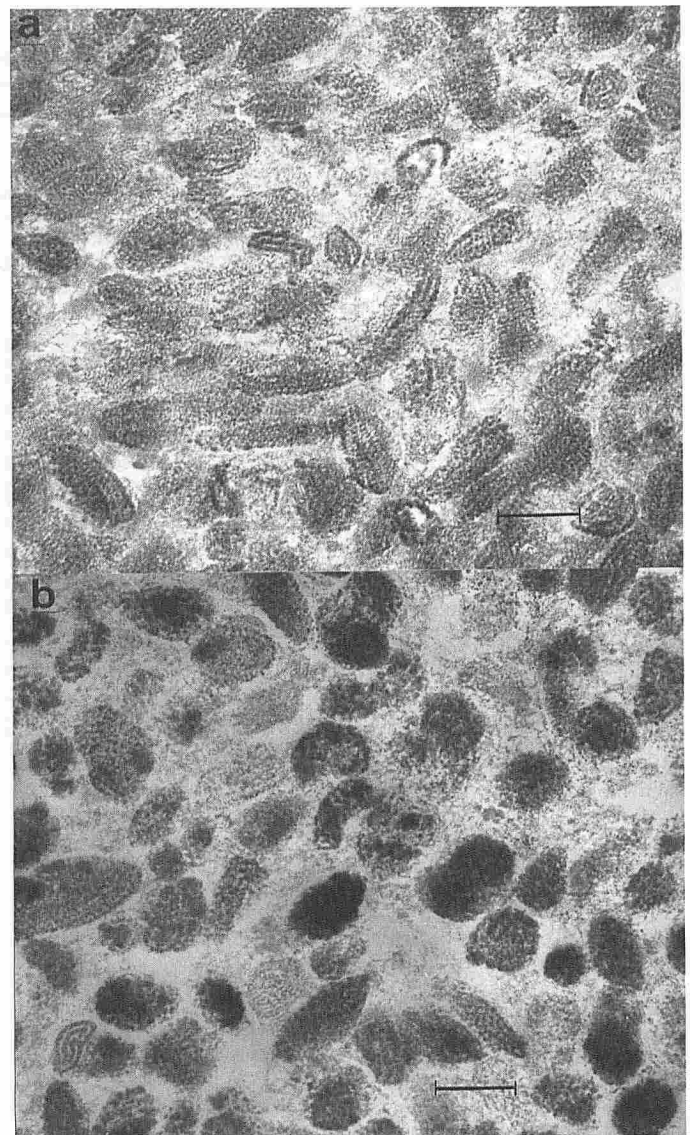


Figure 3. Electron density of the melanosomes is increased in melanocytes cultured with high l-tyrosine concentration in the culture medium. Electron micrographs of details of melanocytes (M9401) grown in medium with (a) low l-tyrosine and low l-cysteine (T-C-) and (b) high l-tyrosine and high l-cysteine (T+C+). A clearly larger number of melanosomes exhibited higher electron density when the cells were grown in T+C+ medium. Scale bars, 0.25 μ m.

medium supplemented with a high concentration of both amino acids (T+C+) were more electron dense than those cultured in T-C- medium.

X-ray microanalysis focussed on the quantitation of sulfur as a specific marker of pheomelanin in melanosomes. Using this method it was shown that the average sulfur counts were higher in melanosomes from cells grown in high-tyrosine media (T+C+ and T+C-) than in those from cells grown in media with a low tyrosine concentration (Fig 4). Repeated measurements showed that at the same concentration of cysteine [and comparable tyrosinase activity (Fig 2)], the availability of tyrosine was a determining factor for the incorporation of sulfur into the melanosomes (compare, for example, T+C- with T-C- in Fig 4). The distribution of sulfur counts in the randomly analyzed melanosomes of these cultures are shown in Fig 5. It can be seen that in the medium with high tyrosine content an increased incorporation of sulfur into the melanosomes took place. From the melanosomes of the cells cultured in T+C- medium, 60% showed sulfur counts higher than 1000. In contrast, when the cells were grown in tyrosine-low medium (T-C-) only 30% of the analyzed melanosomes showed such

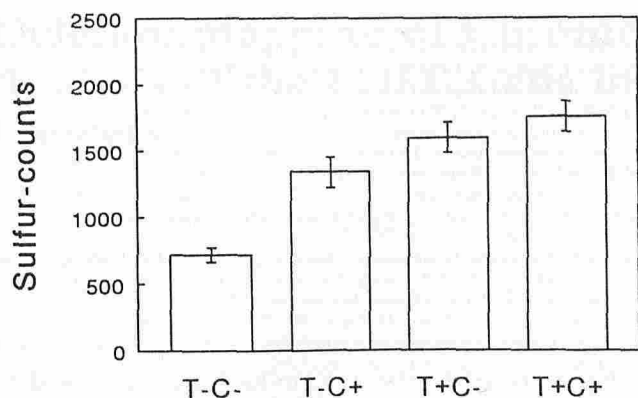


Figure 4. Melanosomal sulfur content is strongly affected by the concentration of l-tyrosine in the culture medium. X-ray microanalysis of melanosomal sulfur counts in 100 randomly chosen melanosomes of the M9401 cells cultured in the media (T-C-, T-C+, T+C-, and T+C+ as in Fig 1). Data are expressed as means \pm SEM, $n = 100$.

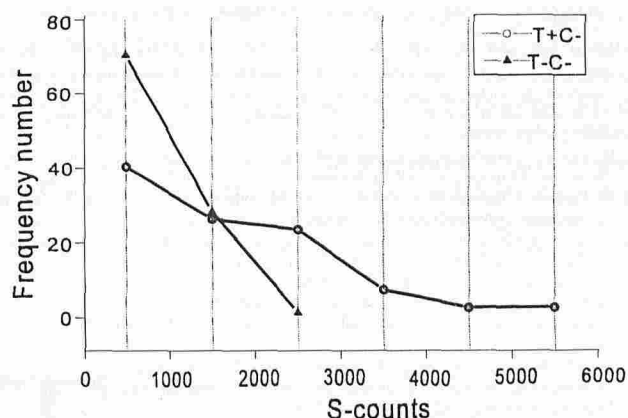


Figure 5. Melanocytes cultured with high l-tyrosine concentration contain increased numbers of high-sulfur-containing melanosomes. Frequency distribution was determined for the melanosomal sulfur counts obtained with X-ray microanalysis in melanocyte cultures (M9401) grown in the media differing in l-tyrosine concentrations, with low l-cysteine concentration (T-C- and T+C-). The data are expressed as number of melanosomes in particular ranges of sulfur counts, i.e., 0–1000, 1000–2000, etc.

high count rates. From the cells cultured in high-cysteine medium (T-C+ and T+C+), sulfur count rates higher than 1000 were found in, respectively, 52 and 72% of the melanosomes (not shown).

DISCUSSION

The melanin synthesis in skin melanocytes is under genetic control. This control is maintained also when the cells are removed from their natural environment and grown in culture.

Melanin production in melanocytes from different skin phototypes strongly differ, which is in agreement with the *in vivo* situation and can be clearly seen on the appearance of the cell pellets (not shown). In spite of this, the pigmentation characteristics of the cultured melanocytes are not fully comparable with that in the *in vivo* situation. It has already been shown in hamster melanoma cells that the increased concentration of l-tyrosine or the addition of l-DOPA to Ham's F-10 medium results in a rapid increase of melanin formation. In this model, a positive regulatory role of the two melanogenic substrates was proposed because supplementing the media with these two compounds led to the increase of tyrosinase activity (Slominski *et al.*, 1988).

In our model we confirm that the availability of l-tyrosine also plays an important role in the melanin formation in normal cultured melanocytes. Media with a high tyrosine concentration induce a distinct increase in pigmentation. In the M9401 melanocyte cultures, however, no elevation of the tyrosinase activity was found. The strong increase of pigmentation in cultured melanocytes due to supplementation of

the medium with additional l-tyrosine is also in accordance with previous observations when melanocytes were grown in a cell culture fermenter (Smit *et al.*, 1995b). Furthermore, increased pigmentation has been found in all our melanocyte cultures, irrespective of their skin phototype origin when cells were grown in DMEM medium (0.4 mM l-tyrosine) as compared with those cultured in Ham's F-10 medium (0.01 mM) (results not shown).

Transport mechanisms for l-tyrosine across the cell membrane have been described previously (Guidotti *et al.*, 1978; Saga and Shimojo, 1982) and the consumption of this amino acid for (eu)melanin formation in the melanosome was recently depicted as a "tyrosine sink" (Potterf *et al.*, 1996). Our results indicate that a cysteine "sink" may also occur owing to the participation of this amino acid in the detoxification of tyrosinase products generated inside the melanosomes. Such a mechanism can explain why the pigment production of melanocytes is strongly directed towards pheomelanin when the cells are grown in high-cysteine (MCDB 153) medium as used in the study by Hunt *et al.* (1995).

One would expect the availability of l-cysteine to be the factor that plays an important role in the synthesis of pheomelanin. Indeed, we could observe that high tyrosine and cysteine concentrations led to a high level of pheomelanin. The reduction of l-cysteine concentration, whilst maintaining the high tyrosine concentration, led to the more eumelanogenic type of melanin in the melanocytes (Fig 2).

The concentration of l-cysteine also had a strong influence on the tyrosinase activity. The melanocytes grown in low-cysteine media exhibited increased tyrosinase activities when compared with those grown in high-cysteine media. This is in agreement with the experiments of Seiji *et al.* (1969) who demonstrated the direct inhibition of tyrosinase by cysteine under *in vitro* conditions. The inactivation of the enzyme by the interaction of sulfhydryl compounds has been demonstrated by different authors (Jergil *et al.*, 1984; Jara *et al.*, 1988; del Marmol *et al.*, 1993). The regulation role of sulfhydryl compounds has already been described by Flesch and Rothman in 1948.

Recently, del Marmol *et al.* (1996) have shown that by the depletion of cysteine in (Ham's F-10) medium in two melanoma cell lines the tyrosinase activity was increased, and that this was accompanied by an increased eumelanin production. We have now clearly shown that, also in cultured melanocytes, melanogenesis can be directed towards the eumelanogenic pathway by the reduction of l-cysteine and simultaneous increase of l-tyrosine concentration.

Recently, the effects of thiolmodulating agents and melanin precursors on the formation of 5-S-cysteinylDOPA in four different melanoma cell lines has been described and related to the pigmentation and biosynthesis of glutathione in these cell lines (Benathan, 1996). Glutathione is the compound known to fulfil many protective functions in the cell. Theoretically, this tripeptide could also be a source of sulfur in melanosomes when incorporated. Ito *et al.* have described that the reaction of glutathione and l-DOPA does not result in the formation of benzothiazine precursors of pheomelanin but forms 7-glutathionyl-5,6-dihydroxyindole units. This could serve as an alternative route for the incorporation of sulfur from glutathione into melanin *in vivo* (Ito *et al.*, 1988). According to the work of Seiji *et al.*, however, it seems very unlikely that the glutathione molecule itself can enter the melanosomes (Seiji *et al.*, 1969).

The detection of sulfur by X-ray microanalysis has been useful as a tool to distinguish eumelanogenic and pheomelanogenic melanosomes (Inazu and Mishima, 1993). Our results confirm that the sulfur concentration was the highest in melanosomes of cells grown in T+C+ medium. The same cells also exhibited the highest pheomelanin content. The lowest values for sulfur concentration were measured in the cells grown in T-C- medium that also showed a low level of pheomelanin. The differences found in melanosomal sulfur contents, however, were not as strong as those of the pheomelanin levels when measured by HPLC. This may be due to the experimental differences because X-ray microanalysis may detect sulfur in individual melanosomes, not only from pheomelanin but also from l-cysteine, whereas the AT + AHP values are directly and thus specifically inferred from the pheomelanin polymer.

L-cysteine availability undoubtedly plays an important role in pheo-

melanin production; however, our HPLC measurements also show that the synthesis of pheomelanin depends on the magnitude of the tyrosine incorporation (compare T-C- with T+C-, and T-C+ with T+C+ in **Fig 2**). These conclusions drawn from the HPLC analysis were confirmed by transmission electron microscopy and X-ray microanalysis of the melanosomes (**Fig 4**) documenting an increased number of melanosomes with higher sulfur counts in the medium with high tyrosine (**Fig 5**).

Our results demonstrate that the nature of pigmentation in cultured melanocytes is dependent not only on l-tyrosine and l-cysteine concentration but also on their mutual ratio. This observation may be of great importance in studies related to melanin pigmentation.

This work was supported by a grant from the Netherlands Cancer Society (RUL 94-756).

REFERENCES

- Abdel-Malek Z: Differential responses of human melanocytes with different melanin contents to UVB light and the role of the cAMP pathway in the UVB-induced melanogenesis. *Photochem Photobiol* 63:42S-43S, 1996
- Agin PP, Sayre RM, Chedekel MR: Photodegradation of pheomelanin: an *in vitro* model. *Photochem Photobiol* 31:359-362, 1980
- Benathan M: Modulation of 5-S-cysteinyldopa formation by tyrosinase activity and intracellular thiols in human melanoma cells. *Melanoma Res* 6:183-189, 1996
- Chedekel MR, Agin PP, Sayre RM: Photochemistry of pheomelanin: action spectrum for superoxide production. *Photochem Photobiol* 31:553-555, 1980
- Eisinger M, Marko O: Selective proliferation of normal human melanocytes *in vitro* in the presence of phorbol ester and cholera toxin. *Proc Natl Acad Sci USA* 79:2018-2022, 1982
- Ezzahir A: The influence of melanins on the photoperoxidation of lipids. *J Photochem Photobiol* 3:341-349, 1989
- Fitzpatrick TB, Becker SW, Lerner AB: Tyrosinase in human skin: demonstration of its presence and of its role in human melanin formation. *Science* 112:223-225, 1950
- Flesch P, Rothman S: Role of sulfhydryl compounds in pigmentation. *Science* 108:505-506, 1948
- Friedman PS, Gilchrist BA: Ultraviolet radiation directly induces pigment production by cultured human melanocytes. *J Cell Physiol* 133:88-94, 1987
- Guidotti G, Borghetti AF, Gazzola GC: The regulation of amino acid transport in animal cells. *Biochem Biophys Acta* 515:329-366, 1978
- Halaban R, Alfano FD: Selective elimination of fibroblasts from cultures of normal human melanocytes. *In Vitro* 20:447-450, 1984
- Hill HZ, Hill GJ: Eumelanin causes DNA strand breaks and kills cells. *Pigment Cell Res* 1:163-170, 1987
- Hunt G, Kyne S, Ito S, Wakamatsu K, Todd C, Thody AJ: Eumelanin and pheomelanin contents of human epidermis and cultured melanocytes. *Pigment Cell Res* 8:202-208, 1995
- Inazu M, Mishima Y: Detection of eumelanogenic and pheomelanogenic melanosomes in the same normal human melanocyte. *J Invest Dermatol* 100:1725-1755, 1993
- Ito S, Fujita K: Microanalysis of eumelanin and pheomelanin in hair and melanomas by chemical degradation and liquid chromatography. *Anal Biochem* 144:527-536, 1985
- Ito S, Imai Y, Jimbow K, Fujita K: Incorporation of sulfhydryl compounds into melanins *in vitro*. *Biochim Biophys Acta* 964:1-7, 1988
- Jara JR, Aroca P, Solano F, Martinez JH, Lozano JA: The role of sulfhydryl compounds in mammalian melanogenesis: the effect of cysteine and glutathione upon tyrosinase and the intermediates of the pathway. *Biochim Biophys Acta* 967:296-303, 1988
- Jergil B, Lindbladh C, Rorsman H, Rosengren E: Inactivation of human tyrosinase by cysteine. Protection by dopa and tyrosine. *Acta Derm Venereol* 64:155-182, 1984
- Kitano Y, Hu F: The effects of ultraviolet light on mammalian pigment cells *in vitro*. *J Invest Dermatol* 52:25-31, 1969
- Kobayashi N, Muramatsu T, Yamashina Y, Shirai T, Ohnishi T, Mori T: Melanin reduces ultraviolet-induced DNA damage formation and killing rate in cultured human melanoma cells. *J Invest Dermatol* 101:685-689, 1993
- Koerten HK, Hazekamp J, Kroon M, Daems W: Th: Asbestos body formation and iron accumulation in mouse peritoneal granulomas after the introduction of crocidolite asbestos fibers. *Am J Pathol* 136:141-157, 1990
- Lowry DH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurements with the Folin phenol reagent. *J Biol Chem* 193:265-275, 1951
- del Marmol V, Solano F, Sels A, Huez G, Libert A, Lejeune F, Ghanem G: Glutathione depletion increases tyrosinase activity in human melanoma cells. *J Invest Dermatol* 101:871-874, 1993
- del Marmol V, Ito S, Bouchard B, Libert A, Wakamatsu K, Ghanem G, Solano F: Cysteine deprivation promotes eumelanogenesis in human melanoma cells. *J Invest Dermatol* 107:698-702, 1996
- Niggli HJ: Comparative studies on the correlation between pyrimidine dimer formation and tyrosinase activity in Cloudman S91 melanoma cells after ultraviolet-irradiation. *Photochem Photobiol* 52:519-524, 1990
- Potterf SB, Muller J, Bernardini I, Tietze F, Kobayashi T, Hearing VJ, Gahl WA: Characterization of a melanosomal transport system in murine melanocytes mediating entry of the melanogenic substrate tyrosine. *J Biol Chem* 271:4002-4008, 1996
- Prota G: Recent advances in the chemistry of melanogenesis in mammals. *J Invest Dermatol* 75:122-127, 1980
- Saga K, Simojō T: Studies of the transport of tyrosine, leucine, and methionine in cultured B-16 mouse melanoma cells. *J Biochem* 92:343-355, 1982
- Seiji M, Yoshida T, Itakura H, Toshikatsu I: Inhibition of melanin formation by sulphhydryl compounds. *J Invest Dermatol* 52:280-286, 1969
- Slominski A, Moellmann G, Kuklinska E, Bomirski A, Pawelek J: Positive regulation of melanin pigmentation by two key substrates of the melanogenic pathway, l-tyrosine and l-dopa. *J Cell Sci* 89:287-296, 1988
- Smit NPM, Westerhof W, Menko WJ, Verbeek NM, Pavel S: Stimulation of cultured melanocytes in medium containing a serum substitute: Ultraser-G. *Pigment Cell Res* 8:19-27, 1995a
- Smit NPM, Westerhof W, Smit W, Nanninga B, Pavel S: Some aspects of melanin formation of melanocytes cultured on collagen-coated microcarrier beads. *Pigment Cell Res* 8:89-96, 1995b
- Thody AJ, Higgins EM, Wakamatsu K, Ito S, Burchill SA, Marks JM: Pheomelanin as well as eumelanin is present in human epidermis. *J Invest Dermatol* 97:340-344, 1991
- Van der Meulen J, Koerten HK: Inflammatory response and degradation of three types of calcium phosphate ceramic in a non-osseous environment. *J Biomed Mat Res* 28:1455-1463, 1994
- Winder AJ, Harris H: New assay for the tyrosine hydroxylase and dopa oxidase activities of tyrosinase. *Eur J Biochem* 198:317-326, 1991
- Yohn JJ, Lyons MB, Norris DA: Cultured human melanocytes from black and white donors have different sunlight and ultraviolet A radiation sensitivities. *J Invest Dermatol* 99:454-459, 1992