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Tyrosine-Induced melanogenesis Shows Differences in Morphologic and melanogenic Preferences of Melanosomes from Light and Dark Skin Types

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Abstract

The quality, quantity and distribution of melanosomes in epidermis play a crucial role in the determination of skin colour and its sensitivity to UV radiation. Melanocyte cultures originating from individuals with light and dark skin types were grown in media with varying concentration of L-tyrosine. Melanosomal melanin content and the size of the organelles were measured after subcellular fractionation. In light-skin type cells, increased melanin production resulted in a more elliptical shape of melanosomes. In melanosomes that constitutively produce more melanin, the tyrosine-induced melanogenesis caused enlargement in all dimensions. X-ray microanalysis provided evidence that the increase in sulfur content induced by high tyrosine concentration was more prominent in the melanosomes from light skin types. A ratio between pheomelanin and eumelanin found in light-skin type melanosomes by HPLC was increased more markedly than that in melanosomes from dark skin melanocytes. These findings suggest that the melanocytes of lightskinned individuals exhibit a preference for pheomelanogenesis. Pheomelanin production is a thiol-consuming process and that might increase the risk of oxidation stress in these cells. This fact, together with the limited ability of pheomelanin to absorb UV radiation may lead to an elevated skin cancer risk among light-skinned individuals.

Introduction

The color of the skin and its sensitivity to UV radiation is largely determined by the quantity, quality and epidermal distribution of melanosomes (1). These cytoplasmic organelles are produced by specialized cells, melanocytes, which are located on the basal lamina and project their dendrites into the epidermis. The melanocytes use their dendrites to maintain an intimate contact with surrounding keratinocytes and to transfer the melanosomes to them.

The melanosome is the site where the pigment melanin is synthesized. The melanin production may continue even after the transfer of melanosomes to keratinocytes. Whether this has any metabolic consequences for the hosting cells is not known.

Pigment production (melanogenesis) involves a chain of enzymatic and non-enzymatic reactions leading to the formation of phenolic and indolic intermediates that are characterized by their ability to polymerize [2]. The initial reaction in the melanogenic pathway is the enzymatic oxidation of L-tyrosine to dopaquinone [3], which is converted to eumelanin precursors by intramolecular cyclization giving rise to indolic monomers. Under normal circumstances, dopaquinone may also react with the thiol group of cysteine to form another type of melanin precursor, cysteinyldopa.

Cysteinyldopas may undergo cyclization of the cysteine side chain to form benzothiazine derivatives, the basic monomer units of pheomelanin. Recent work by Land and Riley has provided evidence that the availability of cysteine at the site of melanogenesis may play a central role in the determination of actual melanogenic route (i.e. eumelanogenesis or pheomelanogenesis) [4]. Individuals with a relatively higher proportion of pheomelanin in their skin (and hair) are at higher risk of developing skin cancer [5,6]. Thus, the composition of melanin in the melanosomes appears to be closely related to the risk of skin cancer.

Melanosome morphogenesis involves the assembly and organization of several elementary components, namely, structural proteins, tyrosinase, and other enzymes, regulatory factors and membranes [7]. Melanin can be found more or less regularly distributed and attached to a matrix protein. The mechanism of melanin binding and the nature of matrix protein(s) are still not fully elucidated. The prevailing type of melanogenesis (i.e. eu or pheomelanogenesis) has been reported to affect the morphology of melanosomes.

Melanosomes from melanocytes synthesizing pheomelanin have been described as having a spherical shape with spotty and microgranular melanization and lacking the lamellar internal structure typical of eumelanin-producing organelles. Such pheomelanosomes have been observed in human red hair [8], human melanoma tissue [9] and also in normal human melanocytes of the hair matrix [10]. Based on their experimental evidence, Borovansky et al. are of the opinion that the melanosomal ultrastructure is not determined by the type of melanin produced but rather by the conformation of the matrix proteins [11-13].

Some authors even consider the possibility that each melanin-producing cell may contain two populations of melanosomes--eumelanosomes and pheomelanosomes [9,10]. Until now, melanin analyses of pigment-producing cells have been performed on the cellular or tissue level. In our recent work, we have shown that melanocytes cultured in media with increased tyrosine concentrations manifest enhanced melanogenesis and exhibit a preference for eu- or pheomelanogenesis [14]. At least a part of the observed effect on melanogenesis of tyrosine supplementation of the growth medium might be due to increased number of melanosomes. In the present communication we concentrate on establishing whether there are also changes in the melanosomes themselves. Our results show that melanosomes from melanocytes originating from light and dark skin types differ in favoring eu- or pheomelanin synthesis. Furthermore, we found that the tyrosine-induced increase of melanin influenced melanosomal size and shape, especially of those originating from the light skin types.

Materials and Methods

Cell culture studies

After receiving informed consent and approval by local ethics committee, biopsies were taken by several volunteers with different skin types. Melanocytes were isolated from skin biopsies of two individuals with light skin type (I/II) and two with a dark skin (both type VI). Biopsies were incubated overnight at 4° C in PBS (pH 7.5) containing 0.25% trypsin, 0.02% EDTA, and 0.1% glucose. The epidermis was separated from the dermis and epidermal cells were suspended in Ham's F-10 culture medium containing 1% Ultroser-G, 16 nM TPA, 0.1 mM IBMX, and 1 nM cholera toxin. After the primary cultures were established, cells were routinely maintained in the medium either with the basal concentration of L-tyrosine (0.01 mM, 1T) or with a 20-times increased L-tyrosine concentration (0.2 mM, 20T) used for tyrosine induced melanogenesis as described in our previous work [15,16]. In described experiments three cultures were from passage 12 and one culture was passage 7. The cells were in contact with higher tyrosine concentration for two passages (approximately 2 x 15 = 30 d).

Isolation of large granular fraction

Six Petri dishes (64 cm^2, Greiner) with confluent monolayers of melanocytes were used for each culture and cells were collected in 0.25 M sucrose using a rubber policeman. The melanocytes were homogenized 35 times with a 10 mL homogenizer and centrifuged at 600 x g for 5 min.

The supernatant was removed and the formed pellet was resuspended, homogenized and centrifuged for a second time.

The post-nuclear supernatant was transferred to a Sorvall DuPont 15 mL Cortex tube and centrifuged in a Sorvall RC58 centrifuge at 10.000 x g for 10 min in order to obtain a large granular fraction containing melanosomes (Dupont Instruments, Wilmington, DE). The resulting pellet was resuspended in 0.5 ml of 0.25 M sucrose.

100 µl was used for electron microscopy and the remaining 400 µl for duplicate HPLC analysis of both pheo- and eumelanin. All steps were performed at 4° C.

Electron microscopy

Transmission electron microscopy

Electron microscopy of melanocyte cultures was carried out with a Philips EM 400 transmission electron microscope as described previously [15] (Philips Electron Optics, Eindhoven, The Netherlands).

Determination of shape parameters of isolated melanosomes

Grids were incubated with melanosomes fixed in 1.5% glutaraldehyde in 0.14 M cacodylate buffer (pH=7.3). They were dried and evaporated under argon with gold using a Balzers Union Med 010, for 3 min at 40 mA. The samples were visualized with a Philips SEM 525M and electron micrographs were taken with a Cline Rollex 56 x 72 Camera (Philips Electron Optics, Eindhoven, The Netherlands). Shape parameters of the randomly selected melanosomes were evaluated using Videoplan release 2.1 software.

Data were statistically analyzed by Student's t-test (independent samples test). A minimum of 25 randomly chosen melanosomes was measured.

XRMA of melanosomes

Isolated melanosomes were fixed in 1.5% glutaraldehyde in 0.14 M cacodylate buffer (pH=7.3) for 5 min. Immediately after fixation aluminum grids were placed in the solution with the melanosomes, dried and stored at room temperature. XRMA of randomly chosen melanosomes was performed with a Philips TEM-420 apparatus equipped with a CM 20 detector and EDAX DX4 v2.20, rom version 2.91 and rom version 3.04 software. Melanosomes were measured with 80 kV at a magnification of 31,000x, using a spot size of 3, a condenser diaphragm of 150 mm, and condenser amperage of -0.3 mA.

The sample volume and analysis time were kept constant to guarantee the reproducibility and comparability of measurements. Results are expressed in counts. Each count comprises a constant number of X-ray photons detected by the X-ray detector. Continuous calibration was provided by an internal standard. Counts are expressed in arbitrary units.

Melanin analysis

Eumelanin degradation by potassium permanganate leads to pyrrole-2,3,5-tricarboxylic acid (PTCA) that can be measured by HPLC [17]. We used our own modification of this method that was based on the HPLC separation followed by fluorimetric detection [18]. Sepia melanin from Sigma was used as an eumelanin standard.

The PTCA yield from this melanin source was 0.4%. The pheomelanin analysis of melanosomal fractions was performed by the hydroiodic hydrolysis of melanin polymer and HPLC analysis of specific degradation products 4-amino-3-hydroxyphenylalanine (AHP) and 3-amino-L-tyrosine (AT) using a modified version of the method originally described by Ito and Fujita [17,19]. PheoMelanInk (Mel-Co, Whittier, CA) was used as a pheomelanin standard. The yield of AHP and AT from PheoMelanInk was 1.4%. The calculation of the relative representation of pheomelanin and eumelanin was done according to the following formula:

$$Pheomelanin = \frac{((AHP + AT) * \frac{100}{1.4}))}{((AHP + AT) * \frac{100}{1.4})) + (PTCA * \frac{100}{0.4})}$$

$$Eumelanin = 1 - pheomelanin$$

Results

XRMA shows an increase of sulfur content in melanosomes isolated from cells grown in the high-tyrosine medium.

The higher tyrosine concentration (0.2 mM) in the culture medium caused a rise in electron density of the melanosomes in both types of melanocytes. X-ray micro analysis (XRMA) was utilized to determine the amount of sulfur in situ in isolated melanosomes. As can be seen from figure 2.1 there was an increase in the sulfur content of melanosomes isolated from cells grown in high-tyrosine medium. This increase was relatively more prominent in the melanosomes from light skin types when compared with melanosomes from dark skin types. The figure also shows that the content of sulfur in the melanosomes of darker skin types under basal conditions was relatively high. This corresponds to a high total level of pigment in these organelles.

HPLC analysis of melanin shows differences in the melanogenic preferences of melanosomes from dark and light skin types

High-performance liquid chromatography (HPLC) analysis of pheomelanin and eumelanin in isolated melanosomes was performed by measurement of specific degradation products of both types of melanin. As expected, melanosomes from light skin types contained less pigment than did the melanosomes from dark individuals. In addition, both types of melanosome showed increased melanin content as a result of higher tyrosine concentration in the culture medium. There was, however, a difference in the preference of melanosomes to synthesize eumelanin or pheomelanin (figure 2.2).

The pheomelanin/ eumelanin ratio in the melanosomal population originating from light skin types was higher when compared with those originating from a dark skin. This supports the notion that there are intrinsic differences in the ability of melanosomes from dark- and light-skinned individuals to synthesize eumelanin and pheomelanin.

Tyrosine-induced melanogenesis affects the shape of the melanosomes isolated from the same cultures

Morphometric measurements on the isolated melanosomes revealed that the tyrosine-induced enhancement of melanogenesis affected the shape of the melanosomes originating from the same cultures (Table 2.I). Increased pigmentation in the light skin type melanosomes was accompanied by their elongation and a reduction in width. In the case of melanosomes from dark skin type melanocytes, enlargement was found for both the average length and width. Thus, the shape factor calculated as a ratio between the melanosomal length and width, only changed in melanosomes of the light skin type cells (figure 2.3A). Calculation of the surface area of the melanosomes showed that, although enhanced melanogenesis changed the shape of light skin type melanosomes, there was no significant change of the surface of these organelles (figure 2.3B). From these measurements it can be concluded that the increased production of melanin in melanosomes of light skin type resulted in more elliptical forms, whereas melanosomes isolated from the dark skin type melanocytes increased in volume but did not change in shape.

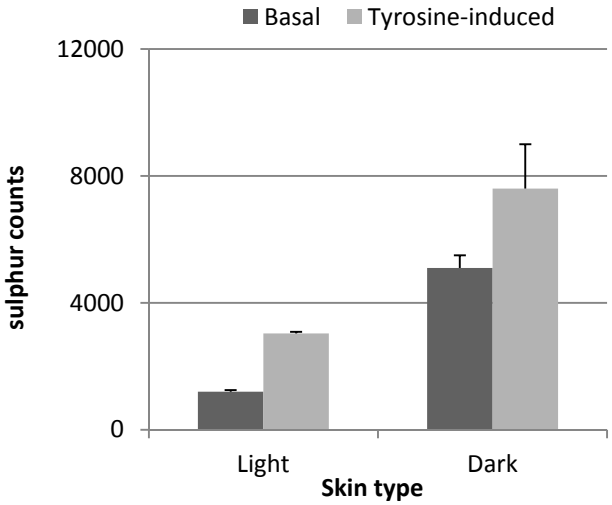

Figure 2.1: Tyrosine-induced melanogenesis in culture medium leads to elevated content of melanosomal sulfur. Melanosomes were isolated from the cultured melanocytes originating from two light and two dark skin types and maintained in medium with lower (0.01 mM, basal) and higher (0.2 mM) tyrosine concentration. The increase in tyrosine induced sulfur concentration was higher in light skin type melanosomes (x 2.6) than in dark skin type melanosomes (x 1.5). Each column represents the results of measurements in 50 isolated melanosomes (mean ± SD).

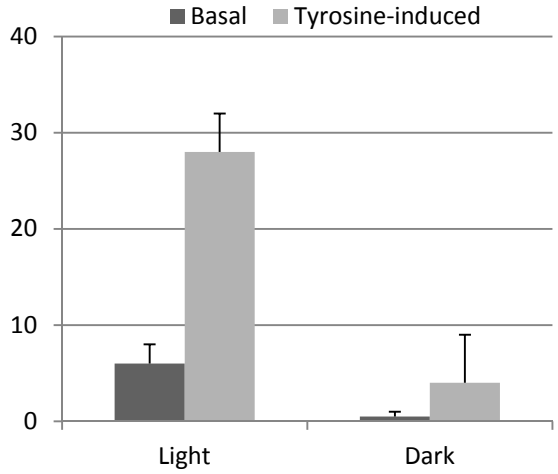

Figure 2.2: Tyrosine-induced melanogenesis reveals differences in the preference for eumelanin and pheomelanin synthesis at the melanosomal level. The ratio between pheomelanin and eumelanin (expressed as AHP + AT/PTCA ratio) is higher in light skin type melanosomes than in those isolated from dark skin melanocytes. Upon tyrosine-induced pigment production the ratio increased x 4.5 in light skin type melanosomes and x 3.5 in those isolated from dark skin type melanocytes. Each column represents the results of calculations of pheomelanin/eumelanin ratio based on measurements carried out on material from two different cultures.

Tyrosine-induced melanogenesis affects the shape of the melanosomes isolated from the same cultures

Morphometric measurements on the isolated melanosomes revealed that the tyrosine-induced enhancement of melanogenesis affected the shape of the melanosomes originating
from the same cultures (Table 2.1). Increased pigmentation in the light skin type melanosomes was accompanied by their elongation and a reduction in width. In the case of melanosomes from dark skin type melanocytes, enlargement was found for both the average length and width. Thus, the shape factor calculated as a ratio between the melanosomal length and width, only changed in melanosomes of the light skin type cells (figure 2.3A). Calculation of the surface area of the melanosomes showed that, although enhanced melanogenesis changed the shape of light skin type melanosomes, there was no significant change of the surface of these organelles (figure 2.3B).

Table 2.1: Differences in length and width of melanosomes*

	Low tyrosine	High tyrosine	P-value
Skin type I			
Length (µm)	0.623 ± 0.158	0.699 ± 0.155	0.015
Width (µm)	0.433 ± 0.094	0.376 ± 0.078	0.001
Skin type VI			
Length (µm)	0.556 ± 0.097	0.694 ± 0.172	0.001
Width (µm)	0.322 ± 0.087	0.372 ± 0.076	0.037

*Melanosomes were isolated from melanocytes of light and dark skin types that were cultured in Ham's F-10 medium with low (0.01 mM) and high (0.2 mM) tyrosine concentration.

From these measurements it can be concluded that the increased production of melanin in melanosomes of light skin type resulted in more elliptical forms, whereas melanosomes isolated from the dark skin type melanocytes increased in volume but did not change in shape.

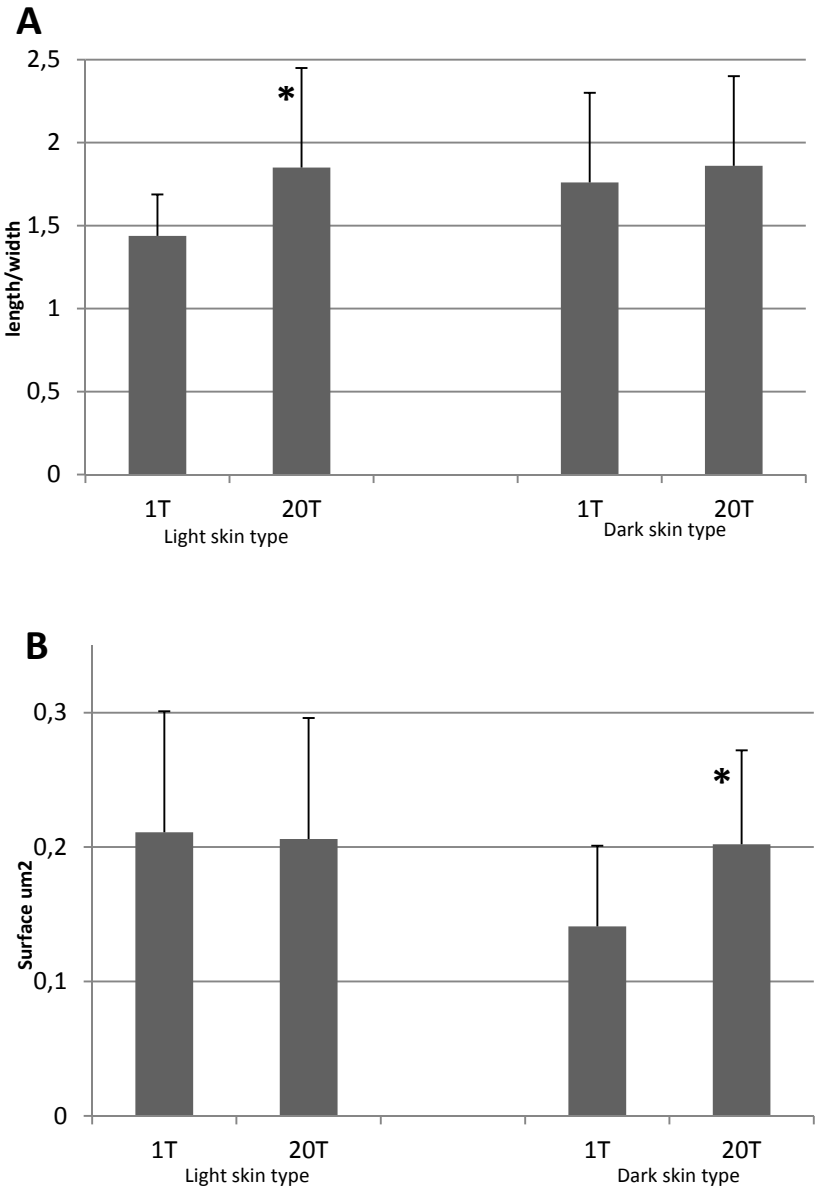

Figure 2.3: Morphomethric measurements show differences in the length/width ratio and the shape of isolated melanosomes after tyrosine induced melanogenesis. Morphometric measurements were performed on melanosomes isolated from melanocytes of light (I) and dark (VI) skin types cultured in Ham's F-10 medium with lower (0.01 mM, 1T) and higher (0.2 mM, 20T) tyrosine concentration (see also Table I). A shape factor (length/width ratio) (A) and surface area (B) were calculated. A significant increase (p*o0.001) in length/width ratio was* found in the light skin type melanosomes (A) and a significantly enlarged surface area (p¼0.003) (B) in melanosomes originating from dark skin type melanocytes. Each column shows the results (mean SD) of the measurement of 25 individual melanosomes.

Discussion

The concentration of melanosomes in normal cutaneous melanocytes has not yet been reported. In human melanomas, melanosomes can represent as much as 40% of dry tumor weight [20,21]. Obviously, such a high proportion of organelles containing active melanogenic ‘‘machinery’’ may interfere with intracellular metabolism. The skin of individuals with high risk of skin cancer contains smaller amount of melanin with relatively higher proportion of pheomelanin. Since the synthesis of melanin is entirely restricted to melanosomes, the detailed knowledge of mechanisms involved in eu- and pheomelanogenesis regulation on subcellular level is of crucial importance.

Utilizing the approach of tyrosine-induced melanogenesis in vitro we have previously demonstrated at the cellular level that there is a difference in pheomelanin and eumelanin synthesis between melanocytes from light and dark skinned donors [22]. Our present results provide further support for this observation indicating that preferential
pheomelanogenesis in light skin type melanocytes takes place at the melanosomal level and is not a reflection of non specific extramelanosomal melanin deposition.

Tyrosine-induced melanogenesis caused the rise of melanin content in the melanosomes of all cultures examined. Eumelanin production was measured by a HPLC technique, which makes it possible to quantify a degradation product of eumelanin (PTCA). Pheomelanogenesis was assessed by two different techniques: (1) HPLC determination of AT +AHP and (2) XRMA measurement of melanosomal sulfur content.

Although the HPLC method is specific for pheomelanin, XRMA measures total intramelanosomal sulfur. In addition to pheomelanin, this determination may, at least theoretically, involve other substances like cysteine, methionine, cystathione, taurine, thiamines, or sulfates. These compounds may be present as a part of various melanosomal structures or as free substances.

Employing both techniques we were able to record an increase of pheomelanin in the melanosomes after the tyrosine-induced melanogenesis. Knowing the analytical yields of melanin degradation products (see Material and Methods) we were able to estimate the proportion of pheo- and eumelanin in the isolated melanosomes (figure 2.4). The results of this calculation are also supporting the view that melanosomes from light-phototype melanocytes favour pheomelanogenesis. One may also notice relatively high representation of pheomelanin in all types of melanosomes. The phenomenon of cultured melanocytes synthesizing more pheomelanin relative to the epidermis or to co-cultures with keratinocytes is well known [23,24]. This artificial imbalance in mono-cultures, however, did not prevent us from finding differences in melanogenic behavior of melanosomes from light and dark skin types.

We think that the final proportion of pheomelanin in light-skin melanosomes might reach its maximum after the tyrosine induction and could not increase more.

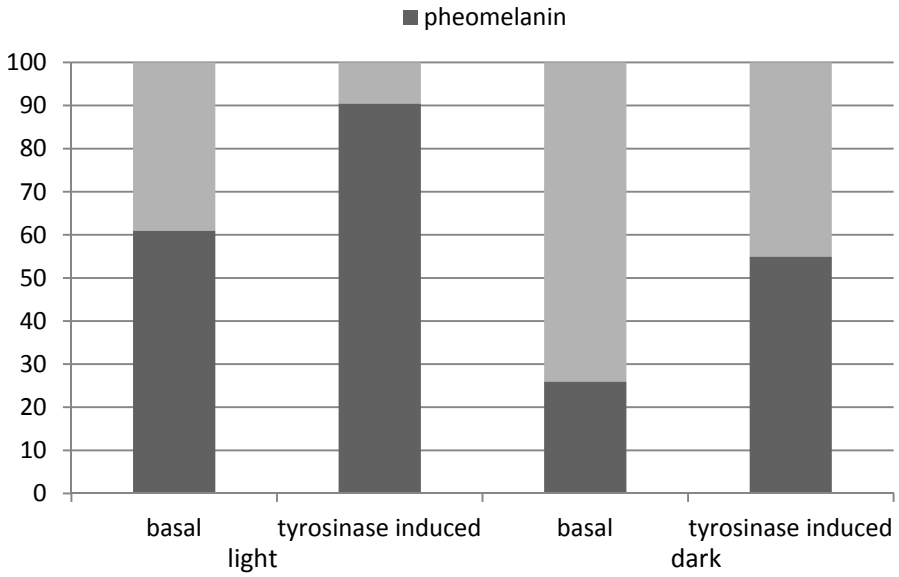

Figure 2.4: Melanosomes from light skin type melanocytes exhibit preference for pheomelanin synthesis. The calculation of the relative representation of pheomelanin and eumelanin in isolated melanosomes illustrates roughly differences in the preference for pheomelanin or eumelanin synthesis in the melanosomes isolated from light and dark skin type melanocytes. Although the pheomelanogenesis was induced by increased tyrosine in medium in both types of melanocytes, the achieved proportion of pheomelanin was much higher in light skin type melanosomes.

Our morphometric measurements on the isolated melanosomes of the same cultures revealed that, as a result of increased pigmentation, the melanosomes of skin type I melanocytes changed to a more ellipsoidal shape similar to that of skin type VI melanocytes. In the melanosomes that constitutively produce more melanin (such as those from dark skin type individuals) the additional pigment caused general enlargement.

The widely accepted concept of morphogenesis of melanosomes has been based largely on observations made on animal models such as chicken feathers and mouse hair [25,26].

One may expect morphological differences between the melanosomes of animal and human origin. In addition, the existence of some morphological differences between the follicular and epidermal melanosomes in humans is also likely.

So-called ‘‘pheomelanosomes’’ are spherical and do not form a matrix like that seen in eumelanosomes. We have not been able to find support for the existence of two populations of melanosomes in our melanocytes originating from the epidermis of individuals with different skin types. We believe that all melanosomes originating from human epidermal melanocytes contain a mixture of eu- and pheomelanin.

The results presented support our previous observations that melanosomes originating from light skin type melanocytes exhibit a preference for pheomelanogenesis. It is not clear whether this fact can be ascribed to some difference in the intracellular metabolism or to some intrinsic diversity in melanosomes from diverse skin types. The latter assumption seems to be more probable.

Pheomelanin formation is a cysteine-consuming process. Since cysteine (especially as a part of glutathione molecule) plays an important role in protecting cells against oxidative imbalance, the consumption of cysteine compounds during pheomelanogenesis might increase risk of oxidative stress in these cells. This fact, together with the limited ability of pheomelanin to protect the skin against UV radiation, may lead to an elevated skin cancer risk among the light-skinned population.

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