Melanin from Epidermal Human Melanocytes: Study by Pyrolytic GC/MS

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Pigmentation of human skin is determined by the presence of melanin, the polymeric pigment that is produced in melanocytes and transferred to adjacent keratinocytes. Epidermal melanocytes produce two distinct types of melanin pigments: eumelanin, composed mainly of indole-type monomers, and pheomelanin that contains benzothiazine-type backbone. Eumelanin protects skin against UV-induced damages, whereas pheomelanin is believed to act as a potent UV photosensitizer and promote carcinogenesis. In this study, pyrolysis in combination with gas chromatography and mass spectrometry (Py-GC/MS) was applied for structural studies of the epidermal pigment isolated from the cultured human melanocytes. The analysis was preceded by investigations of DOPA-originated synthetic eumelanin and pheomelanin standards. This allowed determination of pyrolytic markers for both types of melanin pigments. To obtain additional information on the natural pigment structure, the samples were thermally degraded in the presence of tetramethylammonium hydroxide as the derivatizing agent. It was shown that the analyzed pigment from normal human epidermal melanocytes derived from moderately pigmented skin is of eumelanin type with little incorporation of a pheomelanin component. The results indicate that Py-GC/MS is a rapid and efficient technique for the differentiation of epidermal melanin types and may be an alternative to commonly used methods based on chemical degradation. (J Am Soc Mass Spectrom 2009, 20, 464–468) © 2009 Published by Elsevier Inc. on behalf of American Society for Mass Spectrometry

Pigmentation of human skin is determined by the presence of melanin, the polymeric pigment that is produced in melanocytes and transferred to adjacent keratinocytes [1]. Epidermal melanocytes produce two distinct types of melanin pigments: brown-to-black eumelanins, composed mainly of indole-type monomer units, and yellow-to-red pheomelanins containing structural subunits of benzothiazine and benzothiazole types [2]. Both melanin types can be synthesized by the same melanocyte within the specialized subcellular organelles termed melanosomes, where they are deposited on a protein framework [1, 3]. The common and obligatory step of both eumelanogenesis and pheomelanogenesis is tyrosinase-catalyzed hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (DOPA) and its further oxidation to dopaquinone catalyzed by the same enzyme. Biosynthesis of sulfur-containing pheomelanin requires the formation of cysteinyl conjugates of dopaquinone. The type of melanin produced in melanosomes and the number, size, and distribution of melanosomes within keratinocytes not only determine skin color, but also play an important role in photoprotection. Generally, light skin is associated with higher risk of photocarcinogenesis and pho-

toageing than dark skin [4]. Epidermal eumelanin protects skin against UV-induced damages acting as a sunscreen, free radical scavenger, and antioxidant. In contrast, pheomelanin is believed to act as a potent UV photosensitizer that generates reactive oxygen species after UV exposure and may promote carcinogenesis [5–8]. Besides genetic background, many other factors can influence the epidermal eumelanin-to-pheomelanin ratio, including UV radiation, cysteine and tyrosine availability, tyrosinase activity, or even melanosomal pH [1, 9].

Commonly used methods for the differentiation of melanin types are based on chemical degradation of the pigment, followed by HPLC analysis of the selected degradation products [10, 11]. Relatively poor reproducibility and insufficient yields of the degradation products are the main limitations of the methods. Furthermore, the compounds regarded as chemical markers of eu- and pheomelanins have rather little structural resemblance to the parent monomers of intact pigment and may be produced artificially due to possible alterations of melanin framework by sample treatment with the strong oxidizing or reducing agents [12, 13]. An alternative method of melanin compositional analysis and structural characterization is pyrolysis in combination with gas chromatography and mass spectrometry (Py-GC/MS). Apart from the very small amount of a sample to be analyzed without any pretreatment, the advantages

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of this technique versus that based on chemical degradation include high sensitivity, specificity and speed, and relatively low risk of possible artifacts [14]. Moreover, the same analysis may provide structural information on different components of a heterogeneous material.

Recently, we have successfully applied Py-GC/MS for structural investigation of natural neuromelanin from the human brain [15, 16]. In this study, we analyzed the thermal decomposition products of the pigment isolated from the cultured human epidermal melanocytes. The analysis was preceded by investigations of DOPA-originated synthetic pigments of eumelanin and pheomelanin type. Melanin samples were pyrolysed at 770 °C and the pyrolysis products separated by gas chromatography were identified by mass spectrometry. To obtain additional information on the natural pigment, a thermochemolysis (thermally assisted hydrolysis and methylation) method was applied. In this case, the samples were thermally degraded in the presence of tetramethylammonium hydroxide (TMAH).

Experimental

Materials and Reagents

All the chemicals used were of the highest purity available. L-3,4-Dihydroxyphenylalanine (L-DOPA), L-cysteine, mushroom tyrosinase (EC 1.14.18.1; 6680 U/mg of solid), and tetramethylammonium hydroxide pentahydrate (TMAH) were purchased from Sigma-Aldrich (Poole, UK). Methanol and hexane were from Merck (Darmstadt, Germany). Type I deionized water (NANOpure, bioresearch grade; Barnstead International, Division of Thermo Fisher Scientific, Waltham, MA, USA) was used. Ferromagnetic wires with a Curie-point temperature of 770 °C were obtained from Pye-Unicam Ltd. (Cambridge, UK).

Cell Culture

Normal human epidermal melanocytes, derived from moderately pigmented neonatal foreskin (HEMn-MP), were obtained from Cascade Biologics (Portland, OR, USA). The cells were cultured in Medium 254 supplemented with human melanocyte growth supplement (HMGS, Cascade Biologics) and gentamicin/amphotericin solution (Cascade Biologics), at 37 °C in a humidified incubator with 5% CO₂ and 95% air atmosphere. After reaching 80% confluence, melanocytes were subcultured by trypsinization.

For melanin isolation, HEMn-MP (fourth passage) were seeded onto 75-cm² tissue-culture flasks at a density of 5×10^3 cells/cm² and incubated for 15 days. The culture medium was changed every other day. In one flask, the cells were grown in the medium enriched with cysteine (0.1 mM). Confluent cultures were trypsinized and the detached melanocytes were collected

by centrifugation, washed with phosphate-buffered saline (PBS), and frozen at –70 °C.

Sample Preparation Procedures

Isolation of melanin from cultured melanocytes. Frozen melanocytes were thawed, rinsed with PBS, and lysed by incubation with 5 mL of 1% Triton X-100 in phosphate buffer (50 mM, pH 7.2) for 45 min at room temperature. The lysate was centrifuged and the remaining pellet, after washing with phosphate buffer, was resuspended in 8 mL of 5 mg/mL sodium dodecyl sulfate in Tris-HCl buffer (50 mM, pH 7.5) and incubated for 3 h at 37 °C in a shaking water bath. The insoluble melanin was separated by centrifugation; washed with 0.9% NaCl, deionized water (3 times), methanol (2 times), and hexane (2 times); and dried to a constant weight at 37 °C.

Preparation of melanin standards. Reference melanin samples were prepared by tyrosinase-catalyzed oxidation of L-DOPA (DOPA-melanin, eumelanin standard) or its equimolar mixture with L-cysteine (CysDOPA-melanin, pheomelanin standard). Melanin precursors were dissolved in phosphate buffer (50 mM, pH 6.8) to obtain the final concentration of 10 mM, then 100 U tyrosinase/mL were added and the reaction mixtures were incubated for 48 h at 37 °C with vigorous stirring and protection from light. The pigments formed were collected by centrifugation and washed several times with deionized water. To remove possible traces of tyrosinase, melanin standards were treated with sodium dodecyl sulfate and 0.9% NaCl according to the procedure described for natural pigment, then re washed with deionized water and dried to a constant weight at 37 °C.

Pyrolysis–Gas Chromatography/Mass Spectrometry

Melanin samples were placed on the tips of ferromagnetic wires and inserted into a Pye-Unicam Curie Point pyrolyzer type 795050 coupled directly to a Hewlett-Packard (Palo Alto, CA, USA) 5890 series II gas chromatograph interfaced with a Hewlett-Packard 5989A mass spectrometer. In thermochemolysis experiments, the wires were placed into the pyrolyzer immediately after covering the sample with a drop of methylating agent (10% TMAH in methanol). The pyrolysis cell was kept at 230 °C and the total pyrolysis time was 8 s. The thermal degradation products were separated on a fused-silica capillary column (60 m $\times$ 0.32 mm i.d., film thickness: 0.5 μ m) coated with a chemically bonded HP5-MS phase (5% diphenyl, 95% dimethyl polysiloxane). Helium was used as the carrier gas at a flow rate of 1.8 mL/min. The GC oven temperature was programmed from 40 °C (isothermal for 5 min) to 250 °C at a rate of 10 °C/min; the final temperature was held for 19 min. The mass spectrometer was operated in the electron ionization mode (70 eV). The ion source and the quadrupole analyzer were kept at 200 and 100 °C,

respectively. Mass spectra were recorded at m/z 15–200 (0–5 min) and m/z 33–500 (>5 min). A Hewlett-Packard ChemStation G1034C version C.02.00 software was used for data collection and mass spectra processing. Pyrolysis and thermochemolysis products of the analyzed melanin samples were identified by comparison of their mass spectra with the library standards (*Wiley Registry of Mass Spectral Data*, 7th Edition) and by interpretation of the recorded spectra considering known fragmentation patterns of structurally similar compounds.

Results and Discussion

The profiles of compounds formed during thermal degradation of synthetic DOPA-melanin and CysDOPA-melanin are shown in Figure 1a and b, respectively. Main pyrolysis products of the eumelanin standard were identified as benzene, pyrrole, phenol, indole, and their alkyl derivatives, which is fully consistent with the data reported earlier [15–18]. The pyrolysate of CysDOPA-melanin was dominated by sulfur-containing compounds, such as: thiophene, thiazole, and thiazolidine derivatives, hydroxybenzothiazole and 1,4-benzothiazine. Pyrrole, indole, and pyridine derivatives were also detected. Sulfur-containing heterocycles are typical products of thermally degraded pheomelanins. In our previous reports we have postulated to regard them as the pyrolytic markers of pheomelanin-type subunits in naturally occurring pigments [15, 16, 18]. Like the eumelanin markers, the pheomelanin markers also retain structural information about the parent monomer units, from which they arise. It seems, however, that the type and relative content of pheomelanin markers depend upon whether cysteinyl conjugates of DOPA or dopamine are the precursors of the studied pigment. For instance, thiazolidine derivative, the major decomposition product of CysDOPA-melanin, has never been detected in the pyrolysates of the pigments derived from cysteinyl dopamine, which are usually dominated by benzothiazine derivatives and contain large amounts of benzothiopyranoimidazoles [15, 19]. The latter compounds are in turn completely absent in the pyrolysates of CysDOPA-derived pigments.

As shown in Figure 1c, the pyrolytic pattern of the pigment isolated from the cultured melanocytes is similar to that of DOPA-melanin. The major pyrolysis product was identified as styrene. Other prominent peaks corresponded to pyrrole and its methyl derivatives, toluene, phenols, and indoles. The only pheomelanin markers present in the analyzed pyrolysate were thiazole and its methyl derivative, but the relative content of these compounds was not high. Additionally, some sulfur-containing low molecular weight gases with retention times <4 min were identified: hydrogen sulfide, carbonyl sulfide, and methanethiol (Figure 2b). The same compounds were formed during thermal decomposition of the DOPA-derived pheomelanin standard (Figure 2a). As we proposed previously, sulfur-containing

Figure 1. Chromatograms of the products formed during pyrolysis of (a) synthetic eumelanin standard (DOPA-melanin), (b) synthetic pheomelanin standard (CysDOPA-melanin), and (c) melanin isolated from the cultured human melanocytes. Peak designation: (1) benzene, (2) thiophene, (3) not identified (m/z 55, 54, 43), (4) not identified (m/z 43, 41, 39), (5) thiazole, (6) pyridine, (7) pyrrole, (8) toluene, (9) 2-methylthiazole, (10) 2-methylpyridine, (11) and (12) 2-methylpyrrole or 3-methylpyrrole, (13) not identified (m/z 42, 69, 68), (14) ethylbenzene, (15) 3-methylpyridine, (16) 4,5-dihydro-2-methylthiazole, (17) not identified (m/z 101, 86), (18) styrene, (19) 2-methylthiazolidine, (20) methylethenylbenzene, (21) phenol, (22) 5-ethyl-2-methylthiazole, (23) 5-ethyl-2-methylpyridine, (24) 4-ethyl-5-methylthiazole, (25) 4-methylphenol, (26) benzyl nitrile, (27) 3,4-dimethylphenol, (28) indole, (29) 4-hydroxybenzothiazole, (30) 3-methylindole, (31) 2H-1,4-benzothiazin-5-one, (32) 2,4-bis(1,1-dimethylethyl)phenol.

low molecular weight gases arise principally from the fragmentation of cysteinyl side chains of pheomelanin-type units being incorporated into the pigment structure in uncyclized form [15, 16].

Figure 2. Extracted ion chromatograms (EICs) of low molecular sulfur-containing compounds formed during thermal degradation of (a) pheomelanin standard (CysDOPA-melanin), (b) melanin isolated from the human melanocytes cultured under normal conditions, and (c) melanin isolated from the melanocytes cultured in cysteine-enriched medium.

We also pyrolyzed the pigment samples obtained from the cells growing in cysteine-enriched medium. It has been reported that eumelanin to pheomelanin ratio in cultured melanocytes depends strongly upon cysteine and tyrosine concentrations [20]. Surprisingly, although the culture conditions used should promote pheomelanogenesis, no increase of the heterocyclic pheomelanin markers was observed in the pyrolysate, and the only detectable change was the higher relative abundance of methanethiol (Figure 2c).

To obtain additional structural information on the analyzed epidermal pigment, in particular on its possible nonmelanin components, the pigment sample was

pyrolyzed in the presence of the derivatizing reagent, TMAH. The pyrolysate generated under such conditions is a result of various chemical reactions, including thermally assisted base hydrolysis, pyrolytic bond cleavage, and methylation of functional groups. TMAH thermochemolysis is a well-established and very useful technique for determination of fatty acids in samples of various types, including waxes, polymers, sediments, or even whole bacteria [21–24]. Recently, we used this technique for structural investigation of the lipid component of the human neuromelanin [15, 16]. In contrast to the human brain pigment, TMAH thermochemolysate of the studied epidermal melanin did not contain any methyl esters of higher fatty acids, which indicates that the pigment is devoid of a lipid component. As shown in Figure 3, the profile of TMAH thermochemolysis products of the studied pigment is completely different compared with that obtained by conventional pyrolysis. The most prominent peaks were identified as the methyl ester of *N,N*-dimethylated amino acid glycine and amine derivatives multiply methylated at their nitrogen atoms. At least several possible sources of these compounds should be taken into consideration. First, they can be the decomposition products of melanosomal protein matrix residue closely associated with the isolated pigment. Methylated derivatives of glycine and other amino acids have been reported to be formed with high efficiency during TMAH-thermochemolysis of amino acid mixtures, and bovine albumin [25]. Accordingly, it has been concluded that TMAH reactivity is high enough to hydrolyze and derivatize numerous peptide bonds in a protein chain. Our previous experiments also revealed the presence of the methyl ester of *N,N*-dimethylglycine among TMAH-thermochemolysis products of natural neuromelanin from human brains, which provided another evidence on a protein compo-

Figure 3. Chromatogram of the TMAH thermochemolysis products of melanin isolated from the cultured human melanocytes. Peak designation: (1) carbonic acid, dimethyl ester, (2) 2-methylpropanenitrile, (3) methoxy-acetic acid, methyl ester, (4) styrene, (5) 2-methoxypropanoic acid, methyl ester, (6) methyl dimethylcarbamate, (7) *N,N*-dimethylglycine, methyl ester, (8) *N,N*-dimethyl-2-butanamine, (9) toluene, (10) 1-methoxy-4-methylbenzene, (11) *N,N,N',N'*-tetramethyl-1,2-ethanamine, (12) not identified (*m/z* 116, 128, 173), (13) not identified (*m/z* 116, 128, 173) (14) 4,6-di(1,1-dimethylethyl)-2-methylphenol.

nent being an integral part of the analyzed pigment [15, 16]. Another possible source of N-methylated derivatives of glycine and short-chain aliphatic amines may be the fragmentation of alanyl and cysteinyl side chains of uncyclized melanin monomers, as we have observed high levels of these compounds among the thermochemolysis products of protein-free eumelanin and pheomelanin standards (data not shown). Unfortunately, scant amounts of the discussed thermochemolysis products were also identified in the pyrolysate of a pure TMAH, suggesting the limited suitability of this methylating reagent in melanin analysis. To overcome this problem, we are currently evaluating the usefulness of other derivatizing reagents in structural characterization of natural and synthetic melanin pigments.

Conclusions

Since epidermal eumelanin-to-pheomelanin ratio affects susceptibility of human skin to UV-induced damages, its determination may be of clinical importance. The results presented here indicate that pyrolysis in combination with gas chromatography and mass spectrometry is a rapid and efficient technique for the differentiation of epidermal melanin types and may be an alternative to commonly used methods based on chemical degradation. It has been shown that the pigment isolated from the cultured normal human melanocytes derived from moderately pigmented skin is of eumelanin type with little incorporation of pheomelanin units.

Acknowledgments

This work was supported by Medical University of Silesia in Katowice, Poland.

References

- Slominski, A.; Tobin, D. J.; Shibahara, S.; Wortsman, J. Melanin Pigmentation in Mammalian Skin and Its Hormonal Regulation. *Physiol. Rev.* **2004**, *84*, 1155–1228.
- Ito, S. A Chemist's View of Melanogenesis. *Pigment Cell Res.* **2003**, *16*, 230–236.
- Wakamatsu, K.; Kavanagh, R.; Kadekar, A. L.; Terzieva, S.; Sturm, R. A.; Leachman, S.; Abdel-Malek, Z.; Ito, S. Diversity of Pigmentation in Cultured Human Melanocytes is Due to Differences in the Type as Well as Quantity of Melanin. *Pigment Cell Res.* **2006**, *19*, 154–162.
- Gilchrist, B. A.; Eller, M. S.; Geller, A. C.; Yaar, M. The Pathogenesis of Melanoma Induced by Ultraviolet Radiation. *N. Engl. J. Med.* **1999**, *340*, 1341–1348.
- Brenner, M.; Hearing, V. J. The Protective Role of Melanin Against UV Damage in Human Skin. *Photochem. Photobiol.* **2008**, *84*, 539–549.
- Wenczl, E.; Van der Schans, G. P.; Roza, L.; Kolb, R. M.; Timmerman, A. J.; Smit, N. P. M.; Pavel, S.; Schothorst, A. A. (Pheo)melanin Photosensitizes UVA-Induced DNA Damage in Cultured Human Melanocytes. *J. Invest. Dermatol.* **1998**, *111*, 678–682.
- Vincensi, M. R.; d'Ischia, M.; Napolitano, A.; Procaccini, E. M.; Riccio, G.; Monfrecola, G.; Santoianni, P.; Prota, G. Pheomelanin versus Eumelanin as a Chemical Indicator of Ultraviolet Sensitivity in Fair-Skinned Subjects at Risk for Melanoma: A Pilot Study. *Melanoma Res.* **1998**, *8*, 53–58.
- Takeuchi, S.; Zhang, W.; Wakamatsu, K.; Ito, S.; Hearing, V. J.; Kraemer, K. H.; Brash, D. E. Melanin Acts as a Potent UVB Photosensitizer to Cause an Atypical Mode of Cell Death in Murine Skin. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 15076–15081.
- Hennessy, A.; Oh, C.; Diffey, B.; Wakamatsu, K.; Ito, S.; Rees, J. Eumelanin and Pheomelanin Concentrations in Human Epidermis Before and After UVB Irradiation. *Pigment Cell Res.* **2005**, *18*, 220–223.
- Ito, S.; Wakamatsu, K. Quantitative Analysis of Eumelanin and Pheomelanin in Humans, Mice, and Other Animals: A Comparative Review. *Pigment Cell Res.* **2003**, *16*, 523–531.
- Panzella, L.; Manini, P.; Monfrecola, G.; d'Ischia, M.; Napolitano, A. An Easy-to-Run Method for Routine Analysis of Eumelanin and Pheomelanin in Pigmented Tissues. *Pigment Cell Res.* **2006**, *20*, 128–133.
- Di Donato, P.; Napolitano, A. 1,4-Benzothiazines as Key Intermediates in the Biosynthesis of Red Hair Pigment Pheomelanins. *Pigment Cell Res.* **2003**, *16*, 532–539.
- Wakamatsu, K.; Ito, S. Advanced Chemical Methods in Melanin Determination. *Pigment Cell Res.* **2002**, *15*, 174–183.
- Dworzański, J. P.; Meuzelaar, H. L. C. Pyrolysis Mass Spectrometry, Methods. In *Encyclopedia of Spectroscopy and Spectrometry*, Lindon, J. C.; Tranter, G. E.; Holmes, J. L., Eds.; Academic Press: San Diego, CA, 2000; p. 1906.
- Dzierżęga-Lęcznar, A.; Kurkiewicz, S.; Stepień, K.; Chodurek, E.; Wilczok, T.; Arzberger, T.; Riederer, P.; Gerlach, M. GC/MS Analysis of Thermally Degraded Neuromelanin from the Human Substantia Nigra. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 920–926.
- Dzierżęga-Lęcznar, A.; Kurkiewicz, S.; Stepień, K.; Chodurek, E.; Riederer, P.; Gerlach, M. Structural Investigations of Neuromelanin by Pyrolysis–Gas Chromatography/Mass Spectrometry. *J. Neural Transm.* **2006**, *113*, 729–734.
- Dworzański, J. P. Pyrolysis–Gas Chromatography of Natural and Synthetic Melanins. *J. Anal. Appl. Pyrolysis* **1983**, *5*, 69–79.
- Dzierżęga-Lęcznar, A.; Chodurek, E.; Stepień, K.; Wilczok, T. Pyrolysis–Gas Chromatography–Mass Spectrometry of Synthetic Neuromelanins. *J. Anal. Appl. Pyrolysis* **2002**, *22*, 239–248.
- Dzierżęga-Lęcznar, A.; Stepień, K.; Chodurek, E.; Kurkiewicz, S.; Świątkowska, L.; Wilczok, T. Pyrolysis–Gas Chromatography/Mass Spectrometry of Peroxynitrite-Treated Melanins. *J. Anal. Appl. Pyrolysis* **2003**, *30*, 457–467.
- Smit, N. P. M.; van der Meulen, H.; Koerten, H. K.; Kolb, R. M.; Mommaas, A. M.; Lentjes, E. G. W. M.; Pavel, S. Melanogenesis in Cultured Melanocytes Can Be Substantially Influenced by L-Tyrosine and L-Cysteine. *J. Invest. Dermatol.* **1997**, *109*, 796–800.
- Asperger, A.; Engewald, W.; Fabian, G. Thermally Assisted Hydrolysis and Methylation—A Simple and Rapid Online Derivatization Method for the Gas Chromatographic Analysis of Natural Waxes. *J. Anal. Appl. Pyrolysis* **2001**, *21*, 91–109.
- del Río, J. C.; Hatcher, P. G. Analysis of Aliphatic Biopolymers Using Thermochemolysis with Tetramethylammonium Hydroxide (TMAH) and Gas Chromatography–Mass Spectrometry. *Org. Geochem.* **1998**, *29*, 1441–1451.
- Pulchan, K. J.; Helleur, R.; Abrajano, T. A. TMAH Thermochemolysis Characterization of Marine Sedimentary Organic Matter in a Newfoundland Fjord. *Org. Geochem.* **2003**, *34*, 305–317.
- Dworzański, J. P.; Berwald, L.; Meuzelaar, H. L. C. Pyrolytic Methylation–Gas Chromatography of Whole Bacterial Cells for Rapid Profiling of Cellular Fatty Acids. *Appl. Environ. Microbiol.* **1990**, *56*, 1717–1724.
- Zang, X.; Brown, J. C.; van Heemst, J. D. H.; Palumbo, A.; Hatcher, P. G. Characterization of Amino Acids and Proteinaceous Materials Using Online Tetramethylammonium Hydroxide (TMAH) Thermochemolysis and Gas Chromatography–Mass Spectrometry Technique. *J. Anal. Appl. Pyrolysis* **2001**, *21*, 181–193.