Phaeomelanic Pigments from a Human Melanoma[1]

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Summary. The pigments in melanomas from 2 patients were studied with regard to solubility and chemical composition. Melanoma pigment from a patient with red-blonde hair was alkali-soluble and contained 9 or 10% sulfur and was thus of phaeomelanic type. Melanoma pigment from a patient with red-brown hair was insoluble in 0.2 *N* NaOH. Its sulfur content was 6%. This pigment was eumelanic with regard to solubility characteristics but the sulfur content was higher than previously observed for eumelanin.

It is now several years since it was shown that phaeomelanins, the pigments responsible for the distinctive colour of red hair, are chemically unrelated to eumelanins, since they contain sulfur and arise from a different metabolic pathway involving as a key step the 1,6-addition of cysteine to dopaquinone, produced by enzymatic oxidation of tyrosine. This reaction leads to the formation of 5-S-cysteinyldopa and 2-S-cysteinyldopa which by oxidation give rise to phaeomelanins[3-5]. Whereas this new metabolic pathway is well established with regard to hair pigments, little attention has been paid to the possibility that phaeomelanin formation may occur in certain melanomas. In fact, mammalian melanomas have been widely believed to produce eumelanin. This view has been challenged by the demonstration in melanoma of large amounts of 5-S-cysteinyldopa[6,7], which is one of the key intermediates in phaeomelanin formation.

Material and methods. Melanoma tissue was obtained from a liver metastasis at necropsy of a 41-year-old man who had red-blonde hair and freckles (H.N.). The patient had died from widespread metastasis of a skin melanoma. His urine had contained large amounts of 5-S-cysteinyldopa (124 mg/24 h). For comparison a melanoma metastasis from a patient with pathological but smaller amounts of 5-S-cysteinyldopa (7.0 mg/24 h) in the urine was also examined. The melanoma tissue was obtained from a gut metastasis of an eye melanoma present in a 45-year-old woman with red-brown hair and freckles (M.A.). 5-S-cysteinyldopa and dopa were determined by methods previously described[8,9].

Table I. Pigments and key intermediates of two human melanomas

	Red-blonde (H.N.)	Red-brown (M.A.)
5-S-cysteinyldopa (μg/g)	36	5.4
Dopa (μg/g)	3.4	8.9
Phaeomelanin (μg/g)	680	None
Eumelanin (μg/g)	None	8,600

Table II. Properties of the pigments isolated from two human melanomas (H.N. and M.A.)

Pigment	Colour	Solubility in 0.2 *N* NaOH	Composition (%)			
			C	H	N	S
HN-1	red-brown	+	44.1	3.7	9.4	8.8
HN-2	red-brown	+	42.6	3.2	8.2	10.1
M.A.	black	–	49.5	3.0	8.5	5.8

For the *isolation of pigments*, 50 g of each melanoma tissue was homogenized in 100 ml of 0.2 *N* HCl and centrifuged at 34,800 × *g* for 10 min. The acid-insoluble pigments were then treated in accordance with the procedures outlined below.

Melanoma pigments from the patient (H.N.) with red-blonde hair. The acid-insoluble fraction was suspended in *N* HCl (100 ml) and centrifuged at 34,800 × *g* for 15 min. Washing was repeated once with HCl and twice with H_2O. The supernatants were discarded, and the residue was treated twice with 100 ml 0.5 *N* NaOH at room temperature. Under these conditions most of the pigmented material was dissolved and the resulting solution, clarified by centrifugation, was adjusted to pH 1 with 6 *N* HCl. The precipitate containing the phaeomelanic pigments was collected by centrifugation and fractionated on 2 columns of Sephadex G 75 (2.6 × 77 cm) using 0.1 *M* Na_3PO_4 as eluent. 2 main phaeomelanin bands (HN-1 and HN-2) were collected, which appeared after 305 and 530 ml, respectively. They were dialyzed against water for 3 days, spun down, washed with water and acetone, and dried in vacuo over P_2O_5 to give 21 and 13 mg respectively.

Melanoma pigment from the patient (M.A.) with red-brown hair. The acid-insoluble dark material, washed repeatedly with *N* HCl, 0.5 *N* NaOH, and water, was suspended in 0.1 *M* phosphate buffer, pH 7.5, and treated with pronase and collagenase for 1 week. The mixture was then diluted with 100 ml of water and centrifuged at 400 *g* to give a dark sediment which was washed several times with *N* HCl, and finally with water and acetone. After drying, 1.0 g of a dark, amorphous powder, insoluble in any organic solvent, was obtained. To remove still more proteins, the pigment was treated by boiling 6 *N* HCl for 24 h. The pigment was collected by centrifugation, washed with water (3 times) and acetone (3 times), and dried, thus yielding 0.43 g of material.

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Results. Evidence has been obtained that the two melanoma tissues examined differed both with regard to the concentration of the pigment key intermediates and the type of pigments produced (Tables I and II). In particular the melanoma metastasis from the woman with red-brown hair contained only a dark insoluble pigment with rather high sulfur content. By contrast, the melanoma tissue from the patient with red-blonde hair contained a mixture of alkali-soluble pigments.

Purification of the alkali-soluble fraction on Sephadex G 75 column led to the isolation of 2 reddish-brown polymeric pigments containing nitrogen and sulfur in a ratio characteristic of phaeomelanins isolated from red hair and feathers.

Discussion. Most of our knowledge on mammalian melanins derives from studies on hair shown to contain 2 separate but biogenetically interrelated classes of pigments, the dark, insoluble eumelanins derived from enzymic oxidation of tyrosine, and the alkali-soluble phaeomelanins, ranging from yellow to reddish-brown, which arise from a deviation of the eumelanin pathway by intervention of cysteine.

The chemical nature of melanins has been subject to little attention in current studies on melanomas. The results reported in this study provide evidence that the melanoma from the red-blonde patient was pigmented by phaeomelanins. The melanin present in the melanoma of the patient with red-brown hair illustrates the difficulties involved in classifying melanins. The pigment resembles the eumelanins with respect to colour and insolubility in alkalies. It cannot, however, be regarded as a typical eumelanin because of its high sulfur content (5,8%), which is incompatible with a polymer formed only by tyrosine and related metabolites. Unfortunately, the small amount of material available precluded further experiments to gain information on the structure of this melanin pigment. The fact that human malignant melanocytes may produce different types of pigments has important implications for pigment cell biology, and may provide a chemical basis for the classification of melanomas.

It has previously been demonstrated that 5-S-cysteinyldopa, a key intermediate in phaeomelanin formation, is present in substantial amounts in many different melanomas irrespective of the type of pigmentation[10,11]. The relationship between 5-S-cysteinyldopa content and pigment formation in melanoma has not been defined, however. As shown in Table I, both melanomas examined contained 5-S-cysteinyldopa although in different amounts. In the patient with red-blonde hair, the high content of 5-S-cysteinyldopa is quite consistent with the presence of phaeomelanin-forming melanocytes. The presence of smaller amounts of this metabolite in the other melanoma can also be explained in terms of pigment formation by assuming that the sulfur-containing 'eumelanin' produced in the melanocytes arises by a copolymerization process involving both dopa and cysteinyldopa intermediates. So far the content of sulfur in eumelanin has been attributed to SH bindings of the pigment to protein[12]. However, the high sulfur content found in the insoluble melanoma pigment from the patient with red-brown hair certainly cannot be explained as deriving from sulfur of proteins.

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Resistance of Purified Cholera Toxin to Enzymatic Treatment with Pancreatic Elastase and Papain

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Summary. Treatment of Cholera toxin with pancreatic elastase and papain in vitro showed a high resistance of the toxin molecule to these enzymes, under non-denaturing conditions or in the presence of 2 *M* urea. These experiments support the hypothesis of a particularly stable molecular structure of the toxin, as an explanation of its activity in the intestinal lumen where the pancreatic proteases are active.

The exotoxin produced by *Vibrio cholerae* (Choleragen) is a protein of molecular weight 84,000, constituted of two different types of subunits[1], and it exerts its toxic activities in the intestinal lumen where the pancreatic proteases are active. The activity of the toxin in the presence of proteolytic enzymes could be explained by a particular molecular structure of the toxin itself, resistant to the enzymes, as well as to an increased Choleragen production by the *Vibrio*, prevailing over the inactivation by proteases[2].

In order to ascertain the first hypothesis, we tried to digest the toxin with trypsin and chymotryps in in vitro experiments, and we demonstrated the resistance of the molecule to these enzymes[3]. To explore further this aspect of the problem, we studied the treatment of Choleragen with pancreatic elastase, another enzyme present in the intestinal lumen with differing specificity in comparison with the enzymes previously mentioned.

Non-physiological conditions, such as digestion with papain or the presence of 2 *M* urea, were also studied, in order to evidence a particularly stable molecular structure of the Cholera toxin.

Materials and methods. Highly purified Cholera toxin was prepared according to Saletti et al.[4]. The toxin was characterized[4] by chemico-physical, immunological and biological methods: polyacrylamide gel electrophoresis and immunodiffusion on agar against a specific antiserum

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