Acidic catalase in human skin *in vivo*: a new marker of permanent damage

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Malignant melanoma incidence is increasing rapidly in Western countries. Its prevention requires a deep knowledge of the biological basis of the neoplasm leading to the identification of new biological risk markers. In in-vitro and ex-vivo models we demonstrated that catalase was modified not only in its activity but also in its charge properties after ultraviolet A irradiation through pheomelanin. Here we focus on the electrophoretic behaviour of catalase in the human skin *in vivo*, in association with cutaneous phototype. Zymographic analysis of the enzyme on skin biopsies from Caucasian population (phototype I–IV), collected from the trunk in autumn–winter, to exclude possible influences of an acute photoexposure, evidenced a protein doublet, representing the coexistence of two active isoforms of catalase with different charge properties. In the skin from low-phototype subjects, the percent contribution of the more acidic component of the doublet was prevalent, inversely correlated with total melanin concentration in hair, and associated with a high number of melanocytic nevi. In summary, this study shows for the first time the existence of an acidic catalase in association with clinically defined risk characteristics in low phototype skin *in vivo*, contributing to the knowledge of a new biochemical marker of cutaneous photosusceptibility. *Melanoma Res* 19:372–378 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Melanoma Research 2009, **19**:372–378

Keywords: cutaneous phototype, hair melanin, native catalase, skin biopsies

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Received 10 February 2009 **Accepted** 23 July 2009

Introduction

There is epidemiological evidence that melanotic skin cancer occurs with more frequency in individuals with a fair complexion, who have a high degree of freckling and a large quoted number of melanocytic nevi [1–5]. In addition, lifestyle influences, such as an intermittent ultraviolet radiation exposure, concomitant with sunburn, particularly during childhood, contribute significantly to the probability of inducing this neoplasm [6,7]. Clinically defined risk characteristics subtend, at the biochemical level, some molecular aspects only poorly known today, understanding of which could contribute to improving the prevention strategies of this neoplastic disease. In this regard, it has been shown that in-vitro primary cultures of human melanocytes from low phototype individuals possess lower concentrations of eumelanin [8,9] and proportionally higher levels of pheomelanin than high phototype ones, resulting in greater susceptibility to UV-mediated damage [10]. This increased ultraviolet (UV) susceptibility is due to the lower photoprotective capacity, caused by eumelanin deficiency and pheomelanin photosensitizing effect [11–13]. This latter aspect, attributing a key role to UV-generated free radicals in mediating cellular and tissue damages, focuses on the possible function of antioxidants, in association with melanins, in the protection against these deleterious effects [14–17].

The antioxidant defence system has to act in an integrated manner to minimize the damage caused by free radicals within the cell [18]. Among the reactive oxygen species, hydrogen peroxide (H_2O_2) plays a central role, both for its capacity to diffuse across cell membranes, reaching all cellular regions, and for its high reactivity [19]. This compound is generated by the dismutation of the superoxide anion through superoxide dismutase, it is then converted by catalases and peroxidases into water [20]. We decided to focus specifically on catalase because this was described as the main enzyme responsible for H_2O_2 decomposition into melanocytes [21]. Moreover, this enzyme is very susceptible to peroxidizing agents, such as its own substrate, and recovers slowly when damaged [22,23]. Thus, catalase can be considered as a useful marker of both acute and chronic oxidative stress. In the above-described experimental model, represented by primary cultures of human melanocytes, we recently found that catalase expression and activity were correlated with the total melanin content and with tyrosinase expression [10]. This study strengthened our previous observations, when we demonstrated a direct correlation between catalase activity and cutaneous phototype in primary melanocyte cultures [24] and three-dimensional epidermal reconstructions [25,26].

Experimental evidence from in-vitro and ex-vivo models have shown that catalase can be also modified in its three-dimensional structure, generating a protein with more acidic properties in certain prooxidant environments [27–30]. In these conditions, the native enzyme is altered in the prosthetic group [27–29] and in some specific amino-acid residues, such as tryptophan and methionine, which, also being present on the protein surface, are responsible for the change in its electrophoretic behaviour [30,31]. The oxidative modification of these specific amino-acid residues has also been shown to be crucial for the correct three-dimensional structure of other enzymes such as acetylcholinesterase [32], dihydropteridine reductase [33], and pterin-4a-carbinolamine dehydratase [34]. It is noteworthy that in the case of catalase both the decrease of enzymatic activity and the alteration of the native enzyme charge properties occurred as distinct and not necessarily associated events [27–30]. To date, the charge properties of this enzyme have been studied neither in a more complex model, such as the skin *in vivo*, nor, apart from an acute photoexposure, in association with different phototypes [30].

The aim of the present study was to evaluate the charge properties of catalase in the human skin *in vivo* in correlation to the cutaneous phototype. Our analyses on skin bioptic fragments from a Caucasian population (phototype I–IV), collected from the trunk in autumn–winter to exclude the contribution of an acute photoexposure in mediating charge modifications of the enzyme, revealed the copresence of two active isoforms of catalase with different charge properties, in all the samples analysed. Furthermore, in skin fragments from low phototype individuals, the contribution of the more acidic component of this doublet was more relevant, and inversely associated with total melanin content in hair and directly correlated with the number of melanocytic nevi.

Methods

Participants and samples collection

Participants enrolled by the clinic of the San Gallicano Dermatological Institute received detailed information about the experimental study and provided written, informed consent. All the experimental procedures were approved by the Medical Ethic Committee of the institution, according to the Declaration of Helsinki principles. Recruited individuals ($n = 100$) were all Caucasian, 48 males and 52 females, aged from 15 to 69 years (mean age 39.25 ± 15.02 years), clinically classified as low (I–II, $n = 51$) or high (III–IV, $n = 49$) phototype according to the Fitzpatrick classification [35]. They presented no neoplastic skin disease, history of atopy or allergy, or inflammatory skin pathologies, and had received neither systemic medication nor topical treatment on the skin. Moreover, considering that melanoma incidence is more frequent in intermittently photo-exposed cutaneous regions [6,7], punch biopsies (6 mm) were collected from healthy skin on the trunk. To exclude the contribution of an acute photoexposure in mediating charge modifications of the catalase, all skin biopsies were collected during the autumn or winter. Hair from individuals were recruited only if not subjected to hairdye or chemical treatments.

Charge properties of catalase on polyacrylamide gels

Skin biopsies were homogenized with a Potter–Elvejeim homogenizer in physiologic buffer in the presence of a cocktail of protease inhibitors (Sigma-Aldrich Srl, Milan, Italy). Skin homogenates were centrifuged at 10 000 *g* for 10 min at 4°C. Discontinuous native minigels (8 × 9 cm and 0.75 nm thick) of 8% polyacrylamide (BIO-RAD Laboratories Srl, Milan, Italy) and 0.2% bis-acrylamide (Bio-Rad Laboratories S.r.l) were loaded with native supernatants of skin homogenates containing 2 μg of total proteins. Protein concentration was determined on the supernatants of skin homogenates by Bradford reagent (Sigma-Aldrich Srl). Gels were run at 150 V for 2 h 45 min at 4°C on a Miniprotean II Biorad apparatus (BIO-RAD Laboratories Srl). Native enzyme was detected by incubating the gels for 5 min in 5% aqueous methanol (Merck, KGaA, Darmstadt, Germany) and after rinsing in 10 mmol/l H_2O_2 (Merck KGaA). The gels were rinsed with tap water and then incubated in a 1 : 1 mixture of freshly prepared 2% potassium ferric cyanide and 2% ferric chloride (both from Sigma-Aldrich, Srl). Blue colour develops throughout the gel except where H_2O_2 was decomposed by catalase. Staining was stopped by soaking the gel in a 10% acetic acid (Merck) and 5% methanol solution [28]. Native catalase from U937 cells (2 μg of cell lysate) was used as a standard upper band, after freezing and thawing lysis in liquid nitrogen of cells in physiologic buffer containing a protease inhibitor cocktail (Sigma-Aldrich Srl). Lyophilized catalase from bovine liver (1 U of enzymatic activity), resuspended in physiologic buffer in the presence of the protease inhibitor cocktail (Sigma-Aldrich Srl), was employed as a standard lower band catalase.

Western blot analysis

To denature proteins, native skin homogenates in physiological buffer (see above) were a mixed with 2X RIPA solution [36], supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). Proteins were then separated on a 10% acrylamide SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose (Amersham Biosciences, Milan, Italy). Membranes were blocked with 5% nonfat dry milk (Bio-Rad Laboratories Srl) in PBS containing 0.1% Tween 20 (Sigma-Aldrich Srl), and incubated overnight at 4°C with a mouse anticatalase monoclonal antibody (1 : 1000) (Sigma-Aldrich Srl). A secondary goat antimouse IgG HRP-conjugated antibody (1 : 3000) (DAKO Cytomation, Glostrup, Denmark) was employed. Membranes were

then washed and the specific band visualized by enhanced chemiluminescence reagent (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA).

Densitometric analysis

Densitometric analysis of native catalases was performed using a GS-800 Calibrated Image Densitometer (Bio-Rad Laboratories Srl). Obtained results are relative to three independent experiments.

Catalase activity

Supernatants of native skin homogenates were filtered on Microcon Amicon YM-100 centrifugal filter devices (Millipore, Bedford, Massachusetts, USA) to remove particles (such as the bulk of melanin) and molecules with a molecular weight under 100 kDa. The catalase activity of the filtered supernatants was the determined spectrophotometrically. Enzymatic activity was determined spectrophotometrically by the disappearance of H_2O_2 (10 mmol/l) [37]. After setting the baseline at 240 nm, against air, 2 ml of a solution of 10 mmol/l H_2O_2 (Merck) in 0.2 mol/l phosphate buffer pH 7.4, was put into a quartz cuvette. Thereafter, 10–50 μl of the supernatant of native skin homogenates was gently mixed with the buffer for 10 min with a tip. The kinetic of H_2O_2 consumption by catalase was then started and monitored at 240 nm for 2 min at 25°C. The H_2O_2 consumption/minute in the buffer was converted to units of enzymatic activity on the basis of a standard curve obtained testing scalar units of bovine catalase (Sigma-Aldrich, Srl). Units were normalized for the content of protein in the supernatant of skin homogenates determined by Bradford reagent (Sigma-Aldrich, Srl). Three tests were performed on each supernatant.

Spectrophotometric assay of total hair melanin

Aqueous suspensions of hair samples were prepared by homogenizing about 30 mg of hair in water at a concentration of 10 mg/ml with a glass homogenizer. For the spectrophotometric assay of total melanin aliquots of hair samples (100 μl) were heated in Soluene-350 for 45 min. The resulting solutions were analysed by measuring the absorbance at 500 nm [38]. The values of absorbance at 500 nm are referred to as total melanin in human hair. The amount of melanin is calculated by using a standard curve made with standard solutions of synthetic melanin (Sigma, St. Louis, Missouri, USA) treated as hair samples. Each measurement was performed in triplicate.

Statistical analysis

All the determinations have been expressed as average and standard deviations. Statistical differences were evaluated using Student's t-test. To evaluate the degree of proximity of the experimental points to the regression curve, the coefficient of determination r was employed.

Results

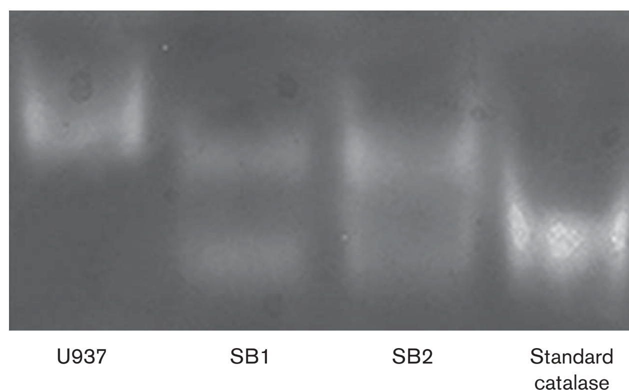
In the skin *in vivo*, two active isoforms of catalase with different charge properties exist

Our previous observations on in-vitro primary cultures of human melanocytes and an ex-vivo three-dimensional model of epidermal reconstructs showed alterations in catalase charge properties after prooxidant conditions associated with the presence of pheomelanin in the samples [30]. Considering that melanoma incidence is more frequent in intermittently photoexposed cutaneous regions [4–7], we here evaluated the charge properties of native catalase in the skin from in-vivo biopsies, collected from the trunk. To exclude the contribution of an acute photoexposure in mediating charge modifications of the enzyme, all skin biopsies were collected during the autumn or winter. Surprisingly, in all the samples analysed ($n = 10$), native catalase appeared as a protein doublet (Fig. 1), which can be accounted for by the coexistence of two active isoforms with different charge properties.

In the skin *in vivo*, two coexisting differently charged isoforms of catalase show the same molecular weight

To understand whether the two components of the doublet were molecular weight and/or charge isoforms of catalase, the enzyme was analysed in a further 10 skin biopsies, both in native and denatured conditions (Fig. 2). Also in these samples, as in the previous ones, zymographic analysis showed the native enzyme as a protein doublet (Fig. 2a); meanwhile, after its denaturation in SDS and western blot analysis, catalase appeared as an unique protein band, with a molecular weight of 60 kDa, corresponding to the monomeric form (Fig. 2b).

Fig. 1



In the skin *in vivo*, two active isoforms of catalase with different charge properties coexist. Skin in-vivo biopsies were processed and analysed for zymography on undenaturating native polyacrylamide gel electrophoresis, as described in Methods. SB1 and SB2, native catalase from two in-vivo skin biopsies (2 μg of proteins in each lane) from different participants, which were representative for the presence of the active enzyme as a double band; standard catalase, catalase from bovine liver (1 U of enzymatic activity) as a standard lower band catalase; U937, native catalase from U937 cells (2 μg of protein cell lysate) as a standard upper band.

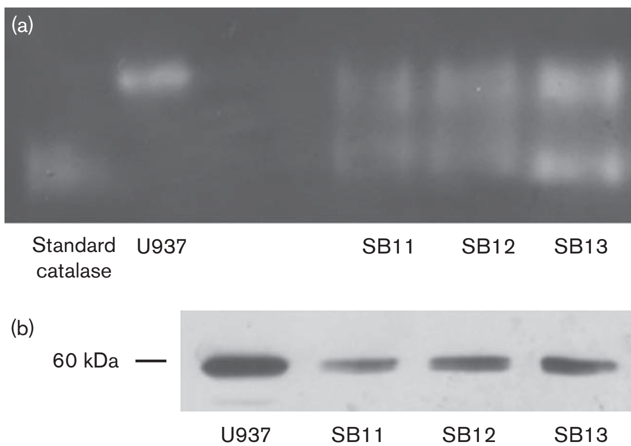


Fig. 2

In the skin *in vivo*, two coexistent, differently charged isoforms of catalase show the same molecular weight. Skin in-vivo biopsies were processed and analyzed for zymography and western blot as described in Methods. (a) Representative zymographic analysis of catalase from three in-vivo skin biopsies (2 μg of protein in each lane) from different participants is shown. (b) Representative western blot analysis is shown. Thirty micrograms of total proteins were loaded in each electrophoretic lane, and the enzyme detection was then performed. In these denatured conditions, catalase appeared in SB11, SB12 and SB13 samples such as in U937 cells, as an unique protein band with molecular weight of 60 kDa. SB11, SB12, SB13, native catalase from three in-vivo skin biopsies (2 μg of proteins in each electrophoretic lane) from different participants; standard catalase, catalase from bovine liver (1 U of enzymatic activity) as a standard lower band catalase; U937, native catalase from U937 cells (2 μg of protein cell lysate) as a standard upper band.

In the skin *in vivo*, the acidic isoform of catalase is more expressed in low phototype participants

To investigate whether, in the skin *in vivo*, the contribution of the acidic isoform of catalase was proportionally more relevant in the fair skin type (phototype I and II) compared with the darker one (phototype III and IV), we analysed the charge properties of the protein on cutaneous biopsies collected from the trunk of two groups of participants, who were clinically classified as low-phototype subjects (LPS: phototype I and II, $n = 23$) or high-phototype subjects (HPS: phototype III and IV, $n = 26$) respectively. The densitometric analysis of the percent contribution of two catalase charge isoforms showed a higher expression of the more acidic component of the doublet (lower band) in LPS biopsies (55% ± 8), than in HPS group (33% ± 9) (Fig. 3; $P < 0.0001$). Neither the sex nor age of the participants influenced the charge properties of the catalase. The parallel analyses of catalase activity did not reveal significant differences in total activity of the enzyme in the two groups (167.42 ± 33.54 U/mg protein in HPS vs. 172.74 ± 52.02 U/mg protein in LPS).

In the skin *in vivo*, the acidic catalase percentage is inversely correlated with the hair melanin concentration and is associated with a high number of nevi

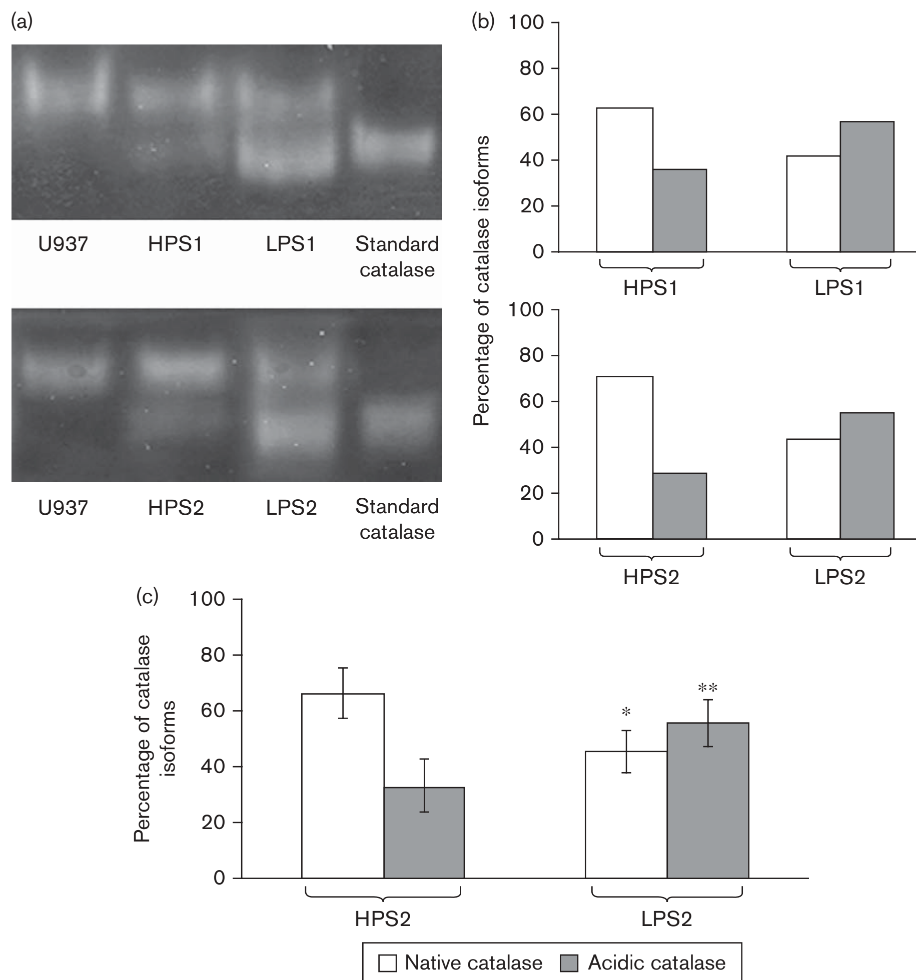
In accordance with some studies that, in hair, identified melanins and melanogenic products as biogenetic markers for predicting individuals at high risk for skin cancer and melanoma [39–41], in 31 new samples we evaluated the possible relationship between lower catalase band percentage and the content of melanin in hair, and we observed the existence of an inverse correlation between the two considered parameters ($r = -0.65$) (Fig. 4). On the same 31 cases, we performed a clinical retrospective evaluation for melanocytic nevi lesions on the trunk, subdividing the individuals in two groups: ≥ 25 or < 25 nevi, respectively. On the whole, the participants classified as ≥ 25 showed lower levels of total melanin in hair and a higher content of acidic catalase in the skin than the other group analysed (Fig. 4).

Discussion

Considering the increasing incidence of melanoma in Western countries [1–7], the possibility of preventing it necessitates a deep knowledge of the biological basis of the disease, leading to both the definition of clinical parameters indicative of relevant molecular alterations in the skin and to the identification of biochemical risk markers. The current study of catalase focuses mainly on the charge properties of this enzyme, and showed that its acidic isoform is particularly expressed in the low phototypes biopsies, in association with both a high number of melanocytic nevi and a low concentration of melanin in the hair of the participants analysed. We collected skin samples from the trunk of the participants with different cutaneous phototypes (from I to IV), as a seat of intermittent UV exposure, more frequently associated with the development of melanoma [6,7]. To exclude the contribution of an acute photoexposure in mediating alterations on catalase, we performed the skin biopsies in autumn–winter.

We have previously demonstrated, using an in-vitro model, that native catalase shifted to a more acidic isoform when irradiated with ultraviolet A (UVA) through synthetic pheomelanin [30]. The same catalase charge modification was also reproduced, on an ex-vivo model of pheomelanic three-dimensional epidermal reconstruct, after an acute UVA-exposure [30]. In both the models, catalase charge alteration was an event distinct from the alteration of the activity [30]. Here we report that a catalase with acidic properties is detectable *in vivo*, in intermittently photoexposed skin, independently from an acute photoexposure. This conformer was more represented in the skin from participants with a fair complexion and phenotype characteristics of cutaneous photosusceptibility. The in-vivo data confirm the results from the in-vitro model [30], as both the studies associate the presence of an acidic catalase isoform with a pheomelanic phenotype. Although the transition in the charge properties of catalase we observed *in vitro* was clearly associated with the photosensitizing properties of pheomelanin after acute UVA exposure [30], the skin

Fig. 3

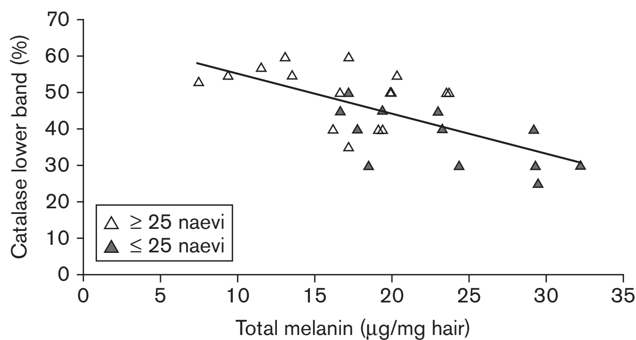


In the skin *in vivo* the acidic isoform of catalase is more expressed in low-phototype subjects (LPS). Representative zymographic analysis of catalase on skin biopsies from two groups of participants, which were clinically classified as high-phototype subjects (HPS: phototypes III and IV) or LPS (phototypes I and II) respectively, collected from the same skin areas (trunk) with the modality described in Methods. (a) 2 μg of total proteins from U937 cells and tissues homogenates and 1 U of catalase from bovine liver (standard catalase) were loaded in each electrophoretic lane, and the run was performed on undenaturating native polyacrylamide gel electrophoresis, as described in Methods. HPS1 and 2, such as LPS1 and 2, were four different samples representative for the different proportional contribution of native and acidic catalase in the HPS and LPS, respectively. (b) Densitometric analysis of the proportional contribution of upper and lower band of catalase in the four samples considered on the left side the figure. (c) Densitometric analysis of the proportional contribution of upper and lower band of catalase in all the skin biopsies analysed (HPS: phototypes III and IV, $n = 26$; LPS: phototypes I and II, $n = 23$). The data represent the mean ± SD. *,** $P < 0.0001$ respect to HPS.

in vivo was not photoexposed at the time of catalase zymographic analysis, and any alteration in the elecrtophoretic behaviour of the enzyme, induced in response to an acute photoexposure, was overwhelmed by the natural turnover of the protein. Therefore, to explain the phenomenon we describe here, a reasonable hypothesis is to consider an influence, which is still operative at the moment of catalase analysis.

In the past, only sporadic studies, on in-vitro models, analysed the transitional charge modifications of catalase to an active more acidic conformer, but always after an exposure to acute stimuli [27–29]. More recently, other experimental evidence associated the same enzymatic alteration to other prooxidant environmental influences, not necessarily involving photosensitizing events: catalase charge modifications have been described in association with specific phase of fungine cell-cycle development [42], and acidic catalase was the only one present in the extracellular environments [31]. On this basis, we do not exclude the possibility that catalase charge alterations in the skin *in vivo* could derive from thickening of the acellular stratum corneum. In fact, a swelling of this epidermal layer has been observed in response to an acute UV exposure, subsisting for months in in-vivo skin models [43]. However, there is no direct evidence, at the moment, of a more pronounced corneum thickening in relationship with the low phototype after UV-exposure,

Fig. 4



In the skin *in vivo* the acidic catalase percentage is inversely correlated with the hair melanin concentration and associated with a high number of nevi. Skin in-vivo biopsies and hair from different participants ($n = 31$) were processed and analysed for zymography on un-denaturating native polyacrylamide gel electrophoresis and melanin determination, as described in Methods. Total melanin in hair was inversely correlated to the percentage of catalase lower band ($r = -0.65$). The participants classified as ≥ 25 nevi (open triangles; $n = 18$) showed lower levels of total melanin in hair and a higher percent content of acidic catalase in the skin in comparison with the other group (≤ 25 nevi; black triangles; $n = 13$).

even if it is possible to envisage such a phenomenon as a protective strategy for a skin deficient in melanin content.

In the past, we showed that the change in the electrophoretic behaviour of native catalase was a consequence of the modification in some amino-acid residues on the protein surface, which are oxidized in the presence of specific prooxidant stimuli [30]. In this regard, oxidative environmental conditions are more frequent in fair skin, mainly dependent on pheomelanogenesis, which generate a significant amount of reactive oxygen species [11,13,30,44,45]. Moreover, the acidic catalase was particularly expressed in participants with a high number of nevi, considered a clinical risk parameter for melanoma susceptibility [1–3,6,7], further supporting the relevance of this biochemical parameter. It is also possible to envisage that catalase charge modifications, due to a prooxidant environment, could be associated with other permanent alterations, such as DNA damage, leading to the first steps of carcinogenesis. A more in-depth analysis of the synthesis of pheomelanin and its intermediates in association with the biological modification of catalase in the skin could be relevant. Nevertheless, in the absence of specific information about this point, it is possible to envisage the total melanin in the hair as a more plausible quantifiable marker of cutaneous susceptibility in comparison with a merely clinical evaluation of skin susceptibility. However, a direct correlation between pheomelanin and total melanin has been described in the past, at least on primary cultures of human melanocytes, where the amount of pheomelanin, compared with total melanin is proportionally higher in lightly pigmented melanocytes and decreases progressively with the increase of pigmentation. As such, the differences in pigmentation in the cultures seemed to arise primarily from to the eumelanin content [8,10].

In conclusion, we believe that the presence of an acidic isoform of catalase is a footprint of a chronic and persistent damaging condition that can be amplified by UV exposure through the induction of pheomelanogenesis and the generation of reactive oxygen species.

Acknowledgement

This study has been supported by the grant from Italian Ministry of Health n.07/01/G/71 "Cell signaling in human melanoma: identification of genetic and epigenetic mechanism potentially relevant for clinical management".

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