

Disturbed melanin synthesis and chronic oxidative stress in dysplastic naevi

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Abstract

Dysplastic naevi (DN) are a known risk factor for malignant melanoma. Their occurrence is closely connected with the degree of skin pigmentation. People with a light complexion are more likely to develop DN than dark-skinned individuals. We examined the proposition that DN exhibit altered melanin formation, which may be involved in their malignant transformation. X-ray microanalysis was used to study the composition of melanosomes from DN and to compare the results with those obtained from melanomas, banal (dermal) naevi and normal cutaneous melanocytes. We analysed sulphur (an indicator of pheomelanin) and two metals, iron and calcium, involved in oxidative stress. FACS analysis of dihydrorhodamine-123-labelled cells was employed to quantify differences in the production of radical oxygen species in DN cells and normal skin melanocytes. A significantly higher sulphur content was found in melanosomes from DN cells and melanoma cells when compared with normal melanocytes and naevus cells from banal naevi. In addition, melanosomes of DN cells and melanoma cells contained higher amounts of iron and calcium. In the case of calcium, this was associated with a significantly elevated cytoplasmic concentration. FACS analysis showed that DN cells exhibited higher concentrations of radical oxygen species than normal skin melanocytes from the same individuals. We propose that increased pheomelanogenesis in DN cells is connected with oxidative imbalance, which is reflected by increased intracellular concentrations of reactive oxygen species and raised calcium and iron concentrations. We show that the metabolic alterations in DN cells resemble those found in melanoma cells. Our findings provide support for the idea that DN cells are true precursor lesions of melanoma.

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1. Introduction

A large number of pigmented cutaneous naevi (moles) is the most important risk factor for the development of cutaneous melanoma [1–3]. This applies particularly to larger naevi. Size is one of the criteria of the so-called dysplastic naevus (DN). There is compelling evidence from several epidemiological studies that DN are associated with greatly increased risk for melanoma [4]. In addition, a convincing demonstration that

these naevi are melanoma precursors is provided by the published cases in which invasive growth was shown to arise in pre-existing DN [5–7].

The occurrence of DN is closely connected with the grade of skin pigmentation. Individuals with red hair or fair skin type are more likely to develop DN. This is in agreement with findings by Weinstock *et al.* [8] that DN were more common in individuals with poor tanning ability. In the Japanese population the prevalence of DN is very low [9], and in black-skinned people DN do not occur. Although the prevalence of DN in white-skinned individuals is reportedly below 10% [10–12], some studies describe much higher percentages [13,14].

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The most striking histopathological changes in DN include proliferation and variable atypia of epidermal melanocytes, formation of irregular nests in the epidermal basal and suprabasal layers, and their interconnection (bridging). An additional feature is the presence of a dermal inflammatory host response. The atypical melanocytes exhibit morphological alterations in melanosomes, similar to those observed in melanoma cells [15].

The lack of clear objective standards to support the clinical and histological diagnosis of DN has caused their existence to remain a controversial issue, and the absence of data on metabolic abnormalities in DN cells has hampered the search for pathogenic mechanisms involved in the development of DN and malignant transformation. The only indication of disturbed metabolism in DN cells has been the finding of altered melanin composition [16].

The biogenesis of melanin in melanocytes takes place inside the melanosomes. Melanin in these organelles is more or less regularly distributed and attached to matrix proteins. The mechanism of melanin binding and the nature of matrix protein(s) are still not fully understood. The initial reaction in melanin formation is the enzymatic oxidation of L-tyrosine to dopaquinone [17] (Fig. 1). This reactive precursor is then either converted to monomers that polymerise to black/brown eumelanin or reacts with the –SH group of cysteine giving rise to cysteinyl-dopa, which undergoes cyclisation to benzo-

thiazine derivatives, the basic monomer units of red/light brown phaeomelanin. The detection of sulphur in melanin therefore indicates the presence of phaeomelanin. Recent work by Land and Riley [18] suggests that the intramelanosomal availability of cysteine may be a critical control point that determines the extent of eumelanin or phaeomelanin production. In addition, the reaction of dopaquinone with cysteine may lower the binding ability of melanin monomers and increase their leakage out of the melanosomes.

Here we examine the proposition that disturbed melanogenesis is involved in the process of DN formation and subsequent malignant transformation. We provide indications that DN cells synthesise more phaeomelanin than do normal melanocytes or dermal naevus cells and we propose that this is linked to an oxidative imbalance reflected by increased intracellular concentrations of reactive oxygen species and raised calcium and iron concentrations. We also show that some of the features found in DN cells are similar to those in melanoma cells. This supports the idea that dysplastic naevus cells are potential melanoma precursors.

2. Material and methods

2.1. Patients and specimens

The Ethical Committee of the hospital approved the study. Written permission to use histological material stored in the Department of Pathology was received from all patients. Sixteen skin specimens containing banal naevi, DN or melanoma were used for the analyses. Only lesions with typical macroscopic appearances were selected for the study. All the pigmented lesions had been excised under local anaesthesia, fixed in 7.5% formaldehyde, embedded in paraffin and processed for routine histological examination (haematoxylin and eosin staining). The adjacent piece of normal skin was used as a control. The histopathological diagnosis was made independently by two dermatopathologists. Six DN, six melanomas and four pigmented banal naevi (dermal type) were subjected to further analyses described below.

2.2. Tissue processing for electron microscopy

The paraffin was removed by melting at 70 °C and repeated extraction with xylene at the same temperature. After extraction the samples were transferred to a solution of 70% ethanol in water. The material was cut into small blocks (approximately 1 mm³) and dehydrated in ethanol to 100%. The tissue blocks were then embedded in Epon 812. Sections (200 nm thick) were cut on a Reichert Om U3 ultramicrotome and collected on aluminium grids.

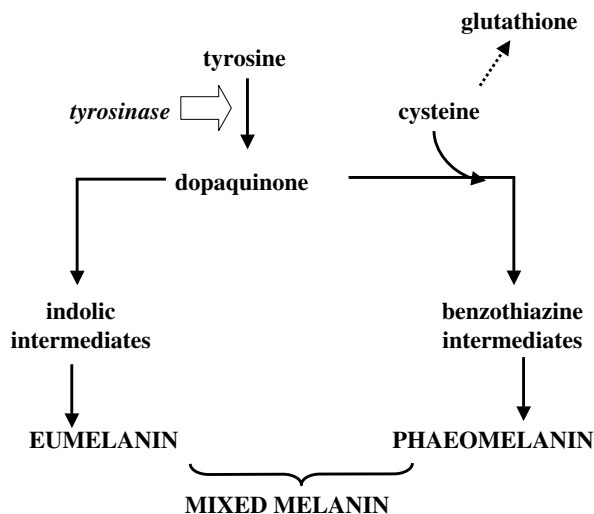


Fig. 1. A simplified scheme of melanogenesis showing the divergence between the synthesis of eumelanin and phaeomelanin. The enzyme tyrosinase catalyses the oxidation of tyrosine to reactive dopaquinone, which is normally converted to several indolic intermediates that polymerise with each other forming a polymer of eumelanin. In an alternative route, dopaquinone binds to the sulphur-containing amino acid, cysteine, leading to the formation of a series of benzothiazine derivatives, which undergo polymerisation to phaeomelanin. Normally, melanosomes contain a mixture of eumelanin and phaeomelanin.

2.3. Electron microscopy

X-ray microanalysis (XRMA) of the skin tissue section was performed using a Philips EM 420 transmission electron microscope (Philips Electron Optics, Eindhoven, The Netherlands) equipped with a scanning transmission electron-microscopy (STEM) unit and an EDAX-DX4 XRMA system. The microscope was operated at 80 kV at a magnification of 31000 \times , using spot size 3, a condenser diaphragm of 150 μ m, and a condenser lens current of 0.3 μ A. Each measurement was performed on the 200 nm-thick preparations during 100-s lifetime. The sample volume and analysis time were kept constant to guarantee the reproducibility and comparability of measurements. Results are expressed in counts. Each count is a constant number of X-ray photons detected by the X-ray detector. The continuous calibration was provided by an internal standard that was a part of an XRMA computer program. The counts were expressed in arbitrary units. The contents of some elements in the melanosomes were found to be under the detection limit.

During the measurements, special care was taken that only randomly selected melanosomes localised around the junction portions of DN or melanoma cells were examined.

2.4. Cell cultures

Cell cultures from DN were established from skin excision material as follows. Epidermis was separated from the dermis after overnight incubation in phosphate-buffered saline (PBS) (pH 7.5) containing 0.05% EDTA and 0.25% trypsin at 4 °C. The naevus cells were suspended in Ham's F-10 melanocyte culture medium containing 1% Ultrosor-G (Gibco), 16 nM TPA, 0.1 mM IBMX and 1 nM cholera toxin. The same procedure and the same medium were used to isolate and grow normal melanocytes. An adjacent piece of normal skin (0.5–1.0 cm from the DN) was used as a source of normal melanocytes.

2.5. Fluorescent probe

DN cells and normal melanocytes from the same patients were plated on coverslips. The cell cultures from three patients were analysed. After attachment the cells were incubated with 1 μ mol dihydrorhodamine 123 (DHR 123) in culture medium for 1 h. The coverslips were examined microscopically with a Leitz Laborlux D fluorescent microscope (Leica, Rijswijk, NL). Oxidation of DHR123 was visualised with a standard blue-emission filter (KP500). Images were made by confocal microscopy using a Leica DM IRBE inverted microscope. The green fluorescein isothiocyanate excitation filter

(488 nm channel activated) was used and the emission of fluorescent oxidation products was measured in the range of 500–550 nm.

2.6. FACS analysis

For FACS analysis, cultured cells from three donors were incubated overnight with 1 μ M DHR123 and trypsinised before FACS measurements. Cells were maintained in PBS containing 1% bovine serum albumin. For quantification of cell fluorescence a Beckton–Dickinson FACScan was used with an argon-ion laser with excitation of 488 nm. Rhodamine-123 fluorescence was measured in FI-1 (530 nm, bandwidth 30 nm); 5000 events were recorded and analysed by *Cell Quest* software (Beckton–Dickinson).

2.7. Statistical analysis

Most of the presented data are shown as mean \pm SEM. All reported *P* values are two-tailed. *P* values of less than 0.05 were considered to indicate statistical significance. Variables obtained from cells of the same patient were compared by the non-parametric Wilcoxon signed-ranks test. To compare values derived from the measurement of different types of cells (normal melanocytes, DN cells, melanoma cells and dermal naevus cells) the non-parametric Mann–Whitney test was used. The data were processed with the software package *SPSS 10* (SPSS Inc., USA).

3. Results

Fig. 2 shows differences between the sulphur content of melanosomes from normal skin melanocytes, banal (dermal) naevi, DN and melanoma. The sulphur content in banal (dermal) naevi was similar to that of normal

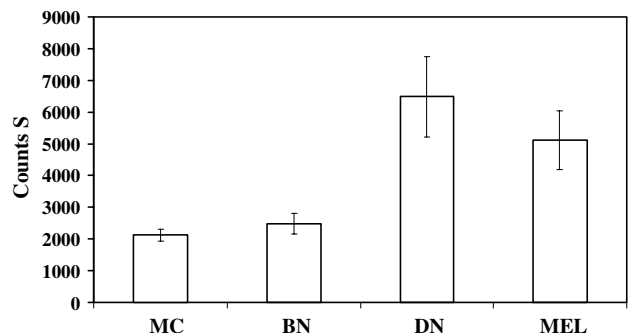


Fig. 2. Differences in the melanosomal sulphur content between 16 normal skin melanocytes (MC), four banal naevi (BN), six dysplastic (atypical) naevi (DN) and six melanomas (MEL). Fifty melanosomes were measured in each preparation. The values are shown as mean \pm SEM. Statistical comparison with MC (Mann–Whitney) provided the following *P* values: BN 0.038; DN <0.001; MEL <0.001.

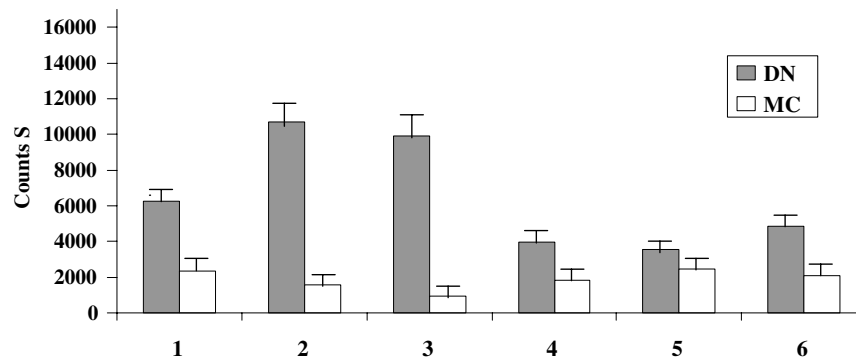


Fig. 3. Differences between the melanosomal sulphur content in normal melanocytes (MC) and dysplastic naevi (DN) of the same patients. Each column represents the measurements of 50 melanosomes. The values are shown as means \pm SEM. All differences between normal melanocytes (MC) and DN cells of the same patients were statistically significant (Wilcoxon; $P < 0.05$).

melanocytes. There was a significantly higher sulphur content in the melanosomes from DN and melanoma cells. Fig. 3 illustrates the differences between the sulphur content of melanosomes from DN and from normal melanocytes of the same patients. In spite of large variations between different naevi there was always a significant increase of melanosomal sulphur in DN cells. Naevi 3 and 4 were removed from the skin of two members of melanoma-prone families (FAMMM syndrome). Naevi 5 and 6 were possibly in earlier stages of development, as they had been removed from the skin of children under the age of 10 years.

XRMA revealed differences in the melanosomal content of iron (Fig. 4). The highest iron concentration was found in the melanosomes of melanoma cells, followed by those in DN. These values were significantly different from those found in normal melanocytes and dermal naevus cells. It is worthy of note that in the cells with low malignant potential (intradermal naevi) the concentration of melanosomal iron was the lowest. The large variations in the values of elements measured in DN cells (and melanoma cells) are probably due to the randomly selected melanosomes that can exist in various stages of maturity and pigmentation. When we deliberately analysed melanosomes with striking morphological alterations we obtained higher values for all three elements than when we applied a strict random selection (results not shown).

We also found differences in the melanosomal content of calcium (Fig. 5). Melanoma melanosomes exhibited the highest calcium concentration, followed by those in DN cells. Due to the large number of negatively charged carboxyl and hydroxyl groups, melanin binds cations that enter the melanosomes and the increased melanosomal iron and calcium may reflect elevated concentrations in the cytoplasm of these cells. This apparent correlation was confirmed by XRMA measurements of calcium in the cytoplasm of normal melanocytes and DN cells from the same patients (Fig. 6).

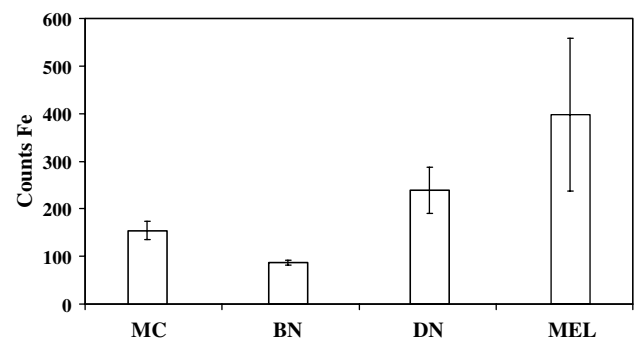


Fig. 4. Differences in the melanosomal iron content between 16 normal skin melanocytes (MC), four banal naevi (BN), six dysplastic (atypical) naevi (DN) and six melanomas (MEL). Fifty melanosomes were analysed in each preparation. The values are shown as means \pm SEM. Statistical comparison (Mann–Whitney) with MC led to the following P values: BN 0.006; DN 0.043; MEL 0.039.

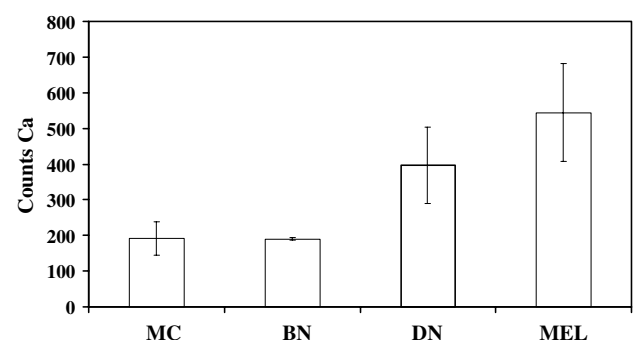


Fig. 5. Differences in the melanosomal calcium content between 16 normal skin melanocytes (MC), four banal naevi (BN), six dysplastic (atypical) naevi (DN) and six melanomas (MEL). Fifty melanosomes were analysed in each preparation. The values are expressed as means \pm SEM. Statistical comparison (Mann–Whitney) with MC provided the following P values: BN 0.109; DN 0.052; MEL 0.008.

Release of calcium and iron from intracellular reserves is known to occur under conditions of oxidative stress [19,20]. We examined the proposition that DN

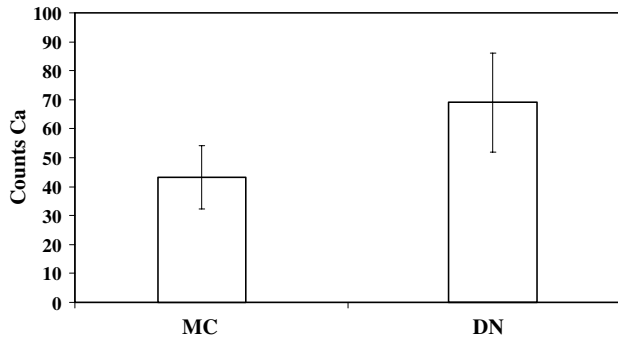


Fig. 6. Differences in the cytoplasmic concentration of calcium in six normal melanocytes (MC) and dysplastic naevi (DN) of the same patients. The values are shown as means \pm SEM. $P = 0.046$ (Wilcoxon).

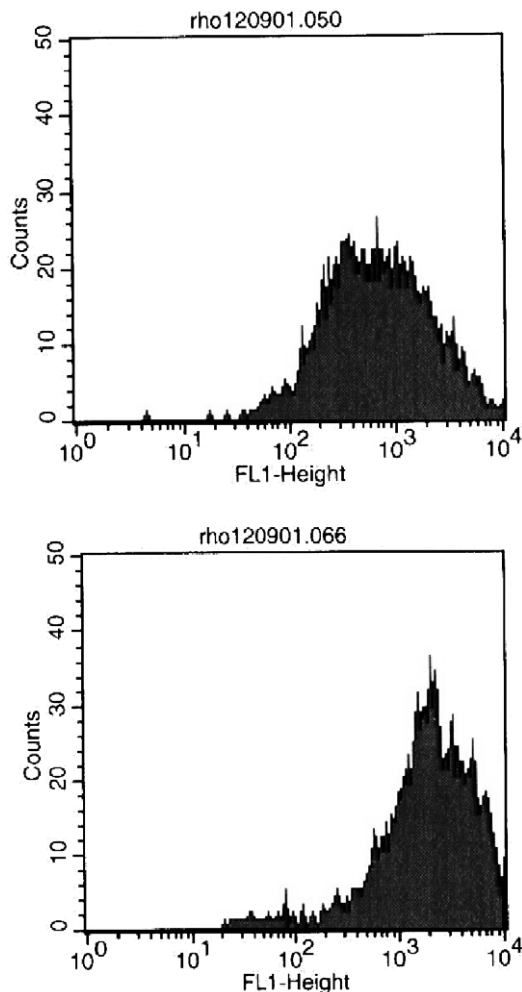


Fig. 7. A typical example of the histograms of dihydrorhodamine fluorescence in cultured normal melanocytes (upper) and DN cells (below) after overnight preincubation with 1 μ M DHR123 ($n = 5000$). The cells originated from the same patient and were cultured under the same conditions. The vertical axis shows cell counts and the horizontal axis fluorescence intensity. The quantitative analysis indicates 2.5 times higher production of reactive oxygen species in DN cells (mean 2506.57 counts) than in the normal melanocytes (mean 1008.4 counts).

cells are exposed to such oxidative imbalance by utilising the redox-sensitive molecular probe DHR 123 in cultured cells [21]. This mitochondrion-selective dye diffuses into cells and is oxidised, primarily by hydrogen peroxide, to green fluorescent rhodamine-123. In order to quantify differences between normal melanocytes and DN cells from the same donor, FACS analysis of the cultured cells was performed. A typical result for FACS analysis of cultured normal melanocytes and DN cells preincubated with DHR 123 is shown in Fig. 7; DN cells exhibited 2.5 times higher fluorescence than normal melanocytes. From these results we conclude that DN cells are subjected to chronic oxidative stress.

4. Discussion

According to Traupe and co-workers the mutation rate in patients with DN syndrome is exceedingly high and can only be explained by a non-hereditary defect, i.e. by the occurrence of 'postnatal' (somatic) mutations in the melanocytes [22]. Theoretically, two mechanisms would bring about the required increase in the mutation rate in a 'mutator strain' of cells: (a) reduced efficiency of DNA repair or (b) an increase in intrinsic DNA damage.

A general repair deficiency, like that seen in the patients with xeroderma pigmentosum, is not likely, since the occurrence of other epidermal tumours is not elevated. We propose that DN cells exhibit a raised mutation rate as the result of increased intrinsic generation of oxidising agents or diminished cellular antioxidant defences, or a combination of both.

Melanin synthesis is a source of oxidation products in the cell. Although confinement of this process to membrane-limited organelles provides some protection against reactive metabolites, the leakage of some melanogenic intermediates poses a potential threat to the cell [23,24]. Leaking melanin precursors are detected in small amounts in the urine of healthy persons and in elevated amounts in patients with disseminated melanoma [25,26].

It is known that catecholamines and their oxidation products are toxic to mitochondria, causing the release of Ca^{2+} and cytochrome *c*, osmotic swelling and loss of oxidative phosphorylation [27,28]. We suggest that the structurally similar melanin precursors have the same effect on the mitochondria of pigment cells. We have observed multiple melanosomal abnormalities in our material and the presence of numerous swollen mitochondria in DN cells has been described by Langer *et al.* [29]. Our results with the fluorescent redox indicator DHR 123 demonstrate that there is an increased level of reactive oxygen species in these cells.

The presence in cells of transition metals, such as iron, presents a significant risk of structural damage due to their interaction with hydrogen peroxide to yield extremely toxic hydroxyl radicals [30]. To abrogate this toxicity the concentration of free cytoplasmic iron is kept very low and the majority of cellular iron is stored in ferritin. However, some agents are able to liberate iron from ferritin and, as we have shown, these include melanogenic precursors such as 5,6-dihydroxyindole-2-carboxylic acid and 5-*S*-cysteinyl-dopa [31]. These observations are supported by work of Tanaka *et al.* [32] using similar *o*-dihydroxyphenolic (catecholic) compounds for iron liberation.

Iron is known to associate readily with DNA and, in the presence of reactive oxygen species such as hydrogen peroxide, gives rise to several types of DNA damage that result in a higher degree of DNA fragmentation [30,33]. Using the single-cell gel electrophoresis assay ('comet assay') we have previously demonstrated that cultured DN cells exhibit significantly higher DNA fragmentation than normal melanocytes or common naevus cells [34]. We propose that the chronic accumulation of iron in DN cells (and melanoma cells) may be implicated in their increased mutation rates.

One of the predisposing factors associated with the development of DN is a low degree of skin pigmentation. We have shown that the melanocytes of light-skinned individuals synthesise relatively more phaeomelanin [35]. This could be regarded a precondition for naevus formation. In this study we found a significantly higher sulphur content in the melanosomes of DN cells and melanoma cells when compared with the melanosomes of normal melanocytes and banal naevi. Although we cannot rule out the possibility that the increase in sulphur was associated with greater synthesis of total melanin, our results are consistent with other evidence that phaeomelanin synthesis is favoured in DN cells [16,36].

Phaeomelanin synthesis is a cysteine-consuming process. This amino acid, being a part of the glutathione molecule, acts as a part of the defence system against intracellular oxidation processes. We and others have shown that the availability of cysteine is of critical importance for phaeomelanin synthesis [35,37]. There is also evidence that glutathione, considered the most important intracellular antioxidant, can be depleted during phaeomelanin production [37]. We propose, therefore, that the susceptibility of melanocytes to oxidative stress associated with their principal metabolic function of pigment synthesis is enhanced by phaeomelanogenesis. Part of this predisposition is determined by inherited skin colour, but in DN cells there is a further shift towards phaeomelanin formation that lowers the antioxidant defences of these cells, leading to increased amounts of genetic damage, thus accounting for their elevated mutability and susceptibility to malignant

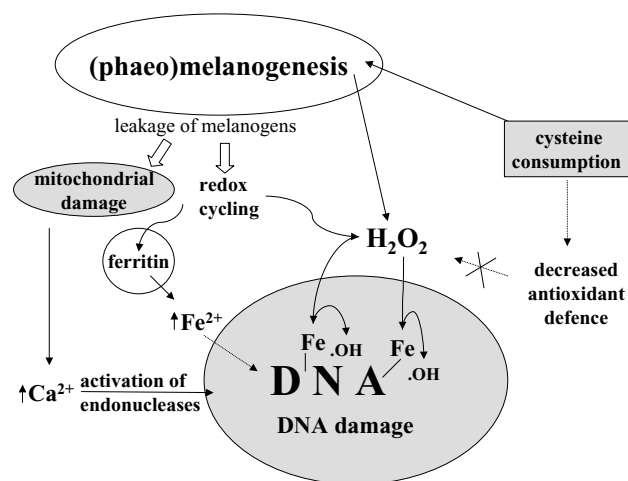


Fig. 8. Proposed scheme of pathways involved in increased mutagenesis in (phaeo)melanin-producing cells. Phaeomelanogenesis is a cysteine-consuming metabolic route that can lower the ability of cells to cope with oxidative stress. This process is also a source of hydrogen peroxide production. Leaking reactive melanin precursors (melanogens) are toxic to mitochondria and are responsible for the elevation of intracellular calcium. This increases the risk of DNA fragmentation. Melanogens also undergo redox cycling (another source of hydrogen peroxide) and they release iron from ferritin stores. The binding of iron to DNA and the interaction with hydrogen peroxide leads to oxidative DNA damage and the increased risk of mutations.

transformation. As we have documented on a unique patient with a p16 deletion and with an oxidative imbalance due to glucose-6-phosphate deficiency, the combination of such metabolic alterations may increase the risk of melanoma enormously [38].

The contribution of ultraviolet (UV) radiation to the malignant transformation of pigment cells might proceed along the same pathways. Since UV radiation stimulates melanogenesis, the pigment cells favouring phaeomelanin production may face all the disadvantages connected with this pathway (Fig. 8). In addition, as UV radiation (especially its UVA part) generates the production of hydrogen peroxide and radical oxygen species [39,40], it has a damaging effect on mitochondria [41] and releases iron from its stores [42,43]. The role of UV radiation in the malignant transformation of melanocytes and naevus cells can therefore be seen in connection with the amplification of existing oxidative imbalances in (phaeo)melanin-producing cells.

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