Biochemical and molecular studies of atypical nevi

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Increased Melanogenesis is a Risk Factor for Oxidative DNA Damage—Study on Cultured Melanocytes and Atypical Nevus Cells

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ABSTRACT

Melanin synthesis is an oxygen-dependent process that acts as a potential source of reactive oxygen species (ROS) inside pigment-forming cells. The synthesis of the lighter variant of melanin, pheomelanin, consumes cysteine and this may limit the capacity of the cellular antioxidative defense. We show that tyrosine-induced melanogenesis in cultured normal human melanocytes (NHM) is accompanied by increased production of ROS and decreased concentration of intracellular glutathione. Clinical atypical (dysplastic) nevi (DN) regularly contain more melanin than do normal melanocytes (MC). We also show that in these cultured DN cells three out of four exhibit elevated synthesis of pheomelanin and this is accompanied by their early senescence. By using various redox-sensitive molecular probes, we demonstrate that cultured DN cells produce significantly more ROS than do normal MC from the same donor. Our experiments employing single-cell gel electrophoresis (comet assay) usually reveal higher fragmentation of DNA in DN cells than in normal MC. Even if in some cases the normal alkaline comet assay shows no differences in DNA fragmentation between DN cells and normal MC, the use of the comet assay with formamidopyrimidine DNA glycosylase can disclose that the DNA of the cultured DN cells harbor more oxidative damage than the DNA of normal MC from the same person.

INTRODUCTION

Reactive oxygen species (ROS) are generated intracellularly as byproducts of the redox reactions in which oxygen is involved. Well known is the utilization of oxygen in mitochondria during the reaction resulting in the generation of energy (ATP) and water. The production of the pigment melanin also requires oxygen. The pigment-producing cells contain therefore additional sources of ROS— melanosomes. Melanin is often considered a protective polymer because it absorbs UV radiation and scavenges diverse radicals [2]. However, the production of this pigment involves oxygen-dependent generation of reactive ortho-dihydroxyindoles and –phenols and their respective (semi)quinones that polymerize with each other forming a polymer network. The redox-cycling of the ortho-dihydroxy compounds is known to result in the production of ROS [3,4]. In addition, the interaction of melanin and its precursors with UV radiation can lead to the generation of ROS [5]. This holds particularly true for pheomelanin that has been shown to function as a photosensitizer [6,7]. Melanin production can therefore be considered as both photoprotective and photosensitizing and melanin itself can act as a ‘‘two edged sword’’ [8]. We have recently described the photoprotective effect of melanin against UVB-induced pyrimidine dimers and 6-4 photoproducts [9]. The eumelanin concentration correlated better with DNA protection than did the pheomelanin concentration. In other experiments, we demonstrated that UVA irradiation caused more DNA single strand breaks if lightly pigmented cultured melanocytes (MC) were stimulated by tyrosine to increase pheomelanin synthesis [10]. The observation was also in agreement with the results of Marrot et al. who showed that UVA exposure of cultured MC with tyrosine-stimulated melanogenesis led to more extensive DNA fragmentation (assessed by comet assay) [11] and to additional generation of ROS [12].

The formation of pheomelanin may be hazardous for the cell not only because of the higher risk of photodamage but also because pheomelanin synthesis consumes cysteine, the essential component of glutathione. This can lower the capacity of antioxidant defense and increase the risk of oxidative damage in the cells with increased phaeomelanin production. During in vivo situation, normal skin MC transfer their melanosomes to the keratinocytes and in this way they can lower their own risk of oxidative stress. However, pigmented nevus cells lose their normal contact with keratinocytes and so the ongoing melanin synthesis may put them at risk of accumulated oxidative damage.

Dysplastic nevi (DN) are recognized precursors of melanoma. Recently it has been shown that these nevi synthesize more pheomelanin than do normal skin MC [13,14]. As mentioned above, this metabolic disturbance can make them predisposed to oxidative stress. Indeed, we have lately found evidence that nevus cells suffer from chronic oxidative stress, even without the influence of UV radiation. Our earlier work employing single-cell gel electrophoresis assay (‘‘comet assay’’) showed that cultured DN cells had significantly higher DNA fragmentation than normal MC or common nevus cells and that this difference could become more striking after UVB exposure [15].

In this study we demonstrate that the tyrosine-induced melanogenesis is accompanied by lowering glutathione content in normal MC isolated from normal skin. In addition, we show that the MC isolated from the normal skin of DN patients often synthesize less melanin than the DN cells of the same patient when cultured under the same circumstances and that in DN cells, especially pheomelanogenesis may be significantly increased. The measurement of oxidative stress using fluorescent molecular probes confirmed that the DN cells suffer from increased oxidative stress and this situation is reflected by an increase in oxidative DNA damage.

MATERIALS AND METHODS

Cell culture studies

Normal human melanocyte (NHM) cultures were obtained from foreskin or biopsies of Caucasian donors of skin Types I and II. Melanocytes cultured from DN patients were established from DN excision material (skin Types II and III) as described earlier [13].

These cells were divided into DN MC isolated from the nevus part and the MC from the normal adjacent skin of the same patient. The diagnosis of DN was made by experienced dermatologists and confirmed by the histopathologic examination performed by two independent dermatopathologists. For the isolation of MC the skin was cut into pieces and incubated with dispase grade II (2.4 U ml^{-1}, Boehringer Mannheim) at 4° C overnight in order to separate the dermis and the epidermis. The epidermis was treated with 0.25% trypsin, 0.02% EDTA and 0.1% glucose in PBS (pH 7.5) at 37° C for 15–20 min until a single cell suspension was obtained. These cells were plated in Ham's F-10 culture medium containing 1% Ultroser-G, 16 nM 12-O-tetradecanoylphorbol-13-acetate (TPA), 0.1 mM 3-isobutyl-1-methylxanthine and 1 nM cholera toxin for culture of normal MC. After primary cultures were established, NHM cultures 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 [10,16]. Melanocytes from DN patients (MC and DN cells) were prepared in the same way except that the DN cells were isolated from both the dermal and epidermal part of the nevus skin.

In general, DN cells from the dermis were used in the experiments, although the epidermal nevus cells also often show differences from the MC of normal skin. Both MC and DN cells were grown in the same medium as above with the basal tyrosine concentration (1T medium). The use of TPA was avoided during the experiments regarding the measurement of intracellular oxidative stress with fluorescent probes.

Glutathione analysis

The dishes with the cultured cells were rinsed with PBS. The PBS was immediately removed and the dishes were put on ice for 10 min after which the remainder of PBS was removed.

Perchloric acid (3% vol／vol, 250 µL／dish) was added to precipitate the cellular protein. The cells were collected by scraping and the cell suspension was centrifuged (13 000 x g min^{-1}) for 2 min. The exact volume of the supernatant was measured in each sample. Additionally, 60 µL of 3 M potassium phosphate buffer (pH 13) was added to 200 µL of supernatant. After 10 min, the samples were neutralized by the addition of 90 µL 10% (vol／vol) $HClO_4$. Fifteen microliters of clear supernatant was injected into the flow-injection analysis system. Glutathione was determined using the method of Redegeld et al. [17].

Protein measurement

Protein concentration was measured by the method of Lowry et al. [18].

Melanin analysis

Eumelanin degradation by potassium permanganate leads to pyrrole tricarboxylic acid (PTCA) that can be quantified by HPLC [19]. We used our own modification of this method that was based on the HPLC separation of degradation products followed by fluorimetric detection [20].

Pheomelanin analysis was performed by hydroiodic hydrolysis of the melanin polymer and HPLC analysis of specific degradation products 4-amino-3-hydroxyphenylalanine (AHP) and 3-amino-L-tyrosine (AT) using a modified version [21] of the method originally described by Ito and Fujita [19].

Fluorescent probes and FACS analysis

Three different probes were used for the detection of ROS in the cells: dihydrorhodamine123 (DHR123), hydroethidine (HE) and 2,7-dihydrodichlorofluorescein diacetate (H2DCF-DA) (Molecular Probes, Leiden). DHR123 and H2DCF-DA are known for their specific reaction with hydrogen peroxide resulting in rhodamine123 and DCF products showing green fluorescence with mitochondrial and cytosolic localization, respectively. HE is converted by reaction with superoxide radicals resulting in the formation of ethidium ion that binds to DNA causing a red nuclear fluorescence [22]. For FACS analysis cultured cells were incubated overnight with one of the probes (1 µM DHR123, HE or H2DCF-DA) and trypsinized before FACS measurements. Cells were maintained in PBS containing 1% bovine serum albumin. For quantification of fluorescence of the cells a Beckton Dickinson FACScan was used with an Argon-Ion laser with excitation of 488 nm. The fluorescence of rhodamine123 and DCF was measured in Fl-1 (530 nm, bandwidth 30 nm) and ethidium (600 nm, bandwidth 30 nm) in Fl-3. The events were recorded and analyzed by Cell Quest software (Beckton Dickinson). The measurements were performed in 10 000 cells.

Single cell gel electrophoresis (comet) assay

The comet assay was carried out as described by Marrot et al. [23]. Cells were embedded in an agarose-PBS microgel and placed in lysis buffer (Lysis Solution, Trevigen, Inc., MD) at 4° C for 1.5 h and then washed and equilibrated in alkaline buffer (0.3 M NaOH, 1 mM EDTA) for 30 min to unwind DNA.

Electrophoresis was performed at 25 V and 300 mA for 20 min in the same buffer in the presence of 1% dimethyl sulfoxide. After neutralization in Tris buffer (pH 7.5), DNA was stained with ethidium bromide (2 μg mL^{-1}) and the comets were examined and photographed using a fluorescence microscope. For quantification, a mean tail moment for 50 analyzed nuclei was calculated using software image analysis (Comet 4.1; Kinetic Imaging, Silver Spring, MD). For the detection of oxidized purines, agarose slides were preincubated with the enzyme formamidopyrimidine [fapy]-DNA glycosylase (FPG) (a gift of Dr. S. Boiteux, CEA, Fontenay aux Roses, France) before DNA unwinding in alkaline buffer [24]. The measurements including 50 nuclei were performed at least twice.

RESULTS

As shown in Fig. 4.1, the incubation of NHM from lighter skin types in a medium with high tyrosine content (20T) increased melanin production. Although there were some variations in the final melanin content, the increased melanogenesis was in all cases accompanied by an increase in DHR123 fluorescence (a marker of oxidative stress) and decreased intracellular glutathione. Under our experimental conditions, the cells with increased melanin formation exhibited approximately 20% rise in DHR123 fluorescence and 40% drop in intracellular glutathione level when compared with the control cells grown in the same medium with basal L-tyrosine concentration (1T).

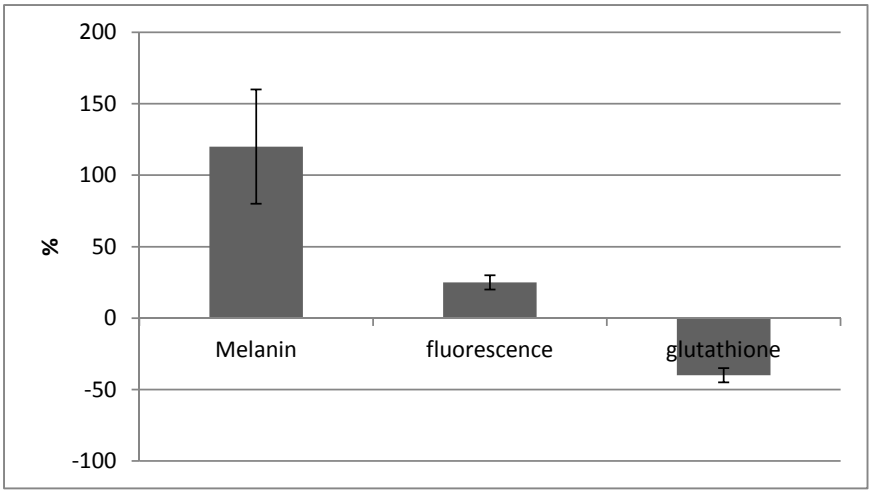

Figure 4.1: Tyrosine-induced melanogenesis causes an increase in melanin content and is accompanied by increased rhodamine123 fluorescence ($P < 0.05$) and decreased total glutathione concentrations ($P < 0.03$). Four different normal human melanocyte cultures were used isolated from normal skin and grown in medium with basal (1T) and increased L-tyrosine (20T). Values are shown as mean ± SD of relative changes.

When culturing normal MC and DN cells originating from the same individuals, a clear difference in pigmentation is usually observed. In many cases the nevus cells are more pigmented than the normal MC and the pigmentation differences remain during many passages. Table 1 shows the results of melanin measurements in cultures of normal and atypical nevus cells derived from four different patients. In three of them, both pheomelanin (AT + AHP) and eumelanin (PTCA) were increased in the atypical nevus cells (cultures 2–4). Culture 3 showed the most striking difference in pigmentation (Fig.4. 2) and especially the pheomelanin content of the cells was elevated. In those three cultures of nevus cells the pheomelanin／eumelanin ratio was 2.0–3.9 times higher than in normal MC (Table 1). In addition, cultured DN cells exhibited reduced lifespan with early signs of senescence (see for example culture 4, Fig.4. 3). A similar result was obtained for culture 3 (Table 1) that showed senescence at passage 13 for the DN cells whereas the MC still normally proliferated at passage 19.

Table 1. Ratio of pheomelanin (AT + AHP) and eumelanin (PTCA) concentrations and pheomelanin／eumelanin ratio in cultured dysplastic nevus cells and normal melanocytes from four different patients grown under the same conditions.

Culture	**AT+AHP** **DN/normal**	**PTCA** **DN/normal**	**AT + AHP/PCTA** **DN/normal**
1	0.29	0.40	0.72
2	2.47	1.22	2.03
3	14.67	3.37	3.94
4	3.69	1.07	3.44

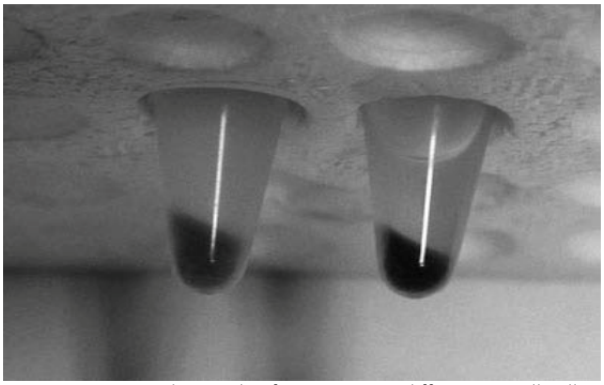

Figure 4.2: A typical example of pigmentation difference—cell pellets of pigment cells isolated from the central part of a dysplastic nevus (right) and from the adjacent normal skin (left) and cultured under the same conditions. The samples correspond to that of culture 3 in Table 1.

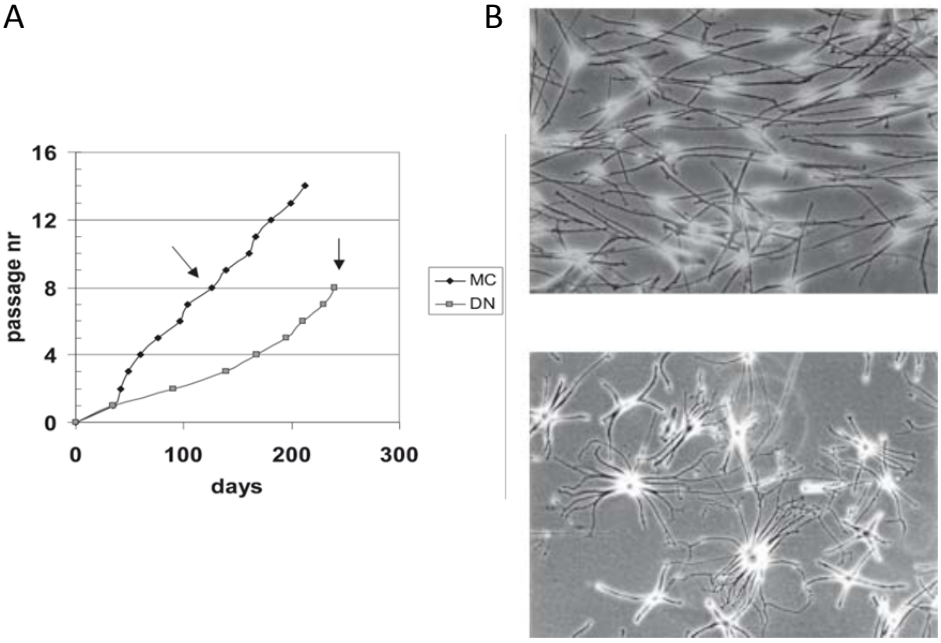

Figure 4.3: (A) A representative graph showing the difference in the growth abilities of cultured dysplastic nevus (DN) cells and normal skin melanocytes (MC) originating from the same person and cultured under the same conditions. Whereas the DN cells did not grow beyond passage 8, the normal MC did not show any sign of delayed growth at passage 14 (results are shown for culture 4 in Table 1). (B) Cultures of DN cells (bottom) and normal melanocytes (top), both from passage 8, exhibit morphologic differences. The nevus cells typically show a more senescent, highly dendritic phenotype whereas the normal MC remains mainly bipolar (culture 4, Table 1).

In a subsequent series of experiments with ROS production in the normal MC and DN, cells of the same culture (no. 4) were measured using three different fluorescent probes. The incubation with HE or H2DCF-DA resulted in a relatively low fluorescence in the cells. Still, a 117% and 82% increase in fluorescence was found for the DN cells compared to the corresponding MC, when HE and H2DCF-DA were used, respectively. The highest level of intracellular fluorescence was achieved when DHR123 was incubated with cultured cells overnight. Figure 4.4 shows the differences in the fluorescence of rhodamine123 in the cultured MC and nevus cells in four subsequent experiments. The average increase in fluorescence in the DN cells was 61%. With these experiments we confirmed and extended our earlier finding that DN cells under in vitro conditions produce more ROS than do their corresponding normal MC [13]. The presence of chronic oxidative stress is known to lead to various types of oxidative damage. Oxidative DNA damage can cause increased risk of mutations.

In our previous studies with single-cell gel electrophoresis (comet assay) we demonstrated that cultured DN cells had more DNA damage than normal MC [15]. Figure 4.5 demonstrates that DNA of cultured DN cells was significantly more fragmented than DNA of normal skin MC. In the present study, we also paid attention to oxidative DNA damage. To this end, we performed the comet assay in the presence of FPG. This hydrolytic enzyme recognizes oxidized purines in DNA molecules.

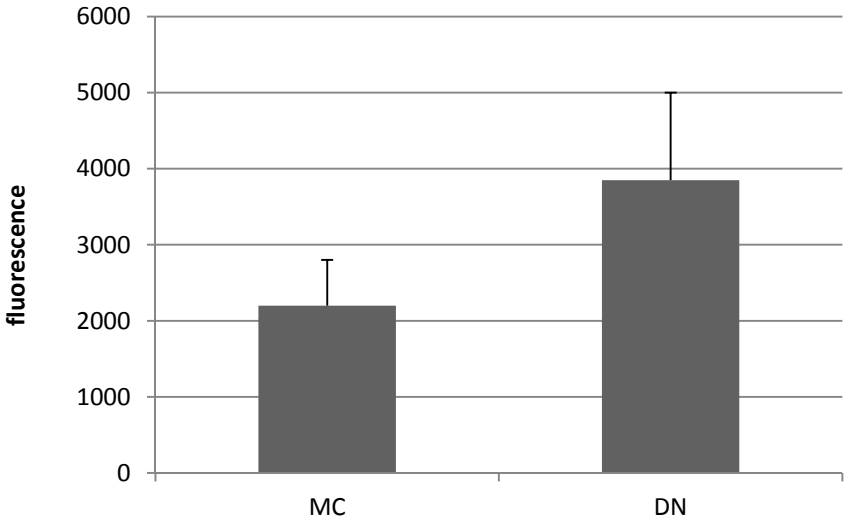

Figure 4.4: FACS analysis of cultured cells incubated overnight with DHR123 shows that dysplastic nevus (DN) cells produce significantly (P = 0.007) more reactive oxygen species than do the corresponding normal melanocytes (MC) of the same patient (culture 4, Table 1). Results from four subsequent experiments with normal MC and DN cells are expressed as mean ± SD.

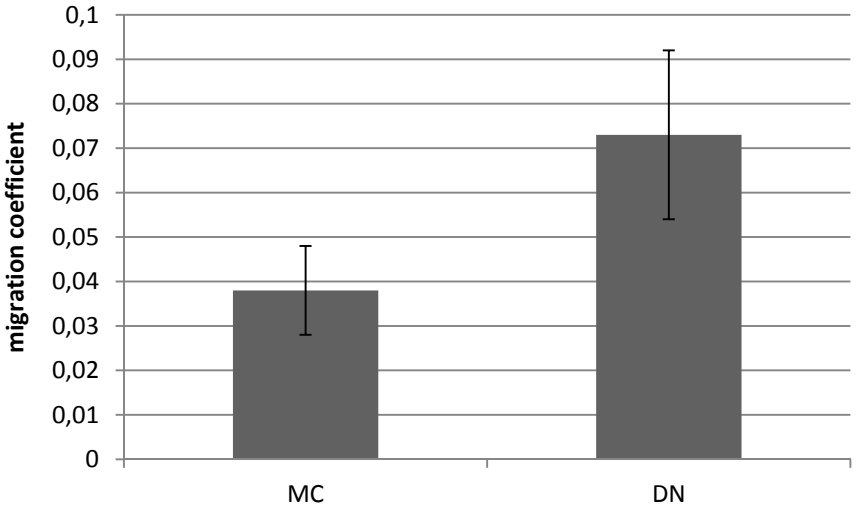

Figure 4.5: Comet assay shows higher fragmentation of the nuclei of cultured dysplastic nevus (DN) cells than those from normal melanocytes (MC) ($P < 0.01$). Results are expressed as mean ± SEM of four cultures of normal MC and four cultures of DN cells. Twenty nuclei per one cell culture were analyzed.

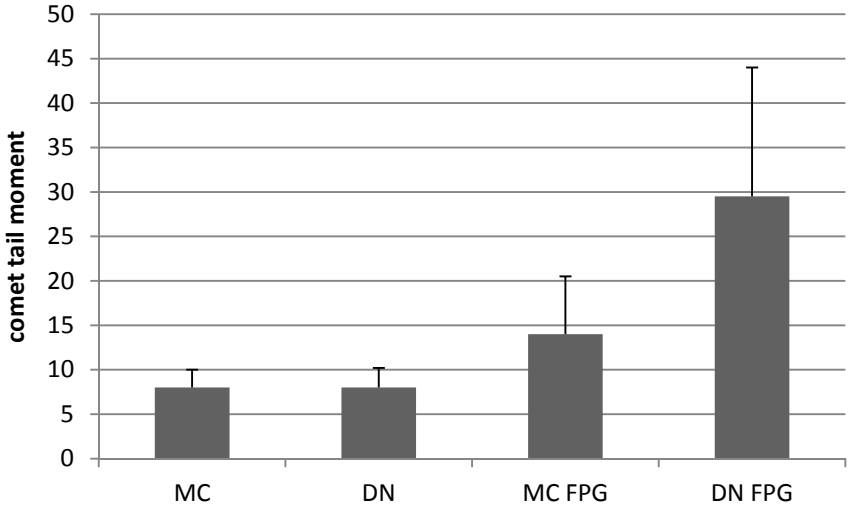

Figure 4.6: Nuclei from dysplastic nevus (DN) cells conceal more oxidative DNA damage than do normal melanocytes (MC) from the same donor. A demonstration of the results of comet assays performed with MC and DN cells in the absence and presence of formamidopyrimidine-DNA glycosylase (FPG) that recognizes oxidized nuclear bases. Even when in this particular case no difference in comet assay between normal melanocytes and DN cells was found, the utilization of FPG demonstrated a significantly higher number of FPG-sensitive sites in the nuclei of the DN cells. Similar results were obtained in three other experiments with the same cultures (no. 4, Table 1). The measurements including 50 nuclei were performed at least twice.

A special example in Figure 4.6 illustrates that even if the comet tail moments of normal MC and DN cells were not different, a significantly higher comet tail moment of DN cells was obtained when the examined cells were preincubated with the glycosylase. From these experiments it can be concluded that DN cells harbor more oxidative DNA damage than do normal MC. As under our experimental conditions UV radiation cannot be blamed for this damage, an internal cause must be involved. The sustained overproduction of ROS in DN cells could be a logical cause.

DISCUSSION

Melanin production is an oxygen-consuming process that generates reactive indolic and phenolic compounds. These substances undergo redox reactions and polymerize to form the melanin. Some of these melanin precursors are also present in the cytoplasm. The redox reactions (named redox-cycling) of phenols are known sources of hydrogen peroxide. Glutathione serves as the first line of cellular defense against the (geno)toxic effect of hydrogen peroxide. We show that the stimulation of melanin production by increased concentration of tyrosine in culture medium is accompanied by significantly decreased concentration of (total) glutathione. There are different possibilities how to explain this phenomenon. The most probable explanation is a relative shortage of cysteine, the building stone of glutathione, that is consumed during the synthesis of (pheo)melanin. However, other options, like a nucleophilic addition of glutathione to the quinone products of melanogenesis [25] or binding to the thiol groups of proteins (forming GSH-protein mixed disulfides) [26] should be considered as well. A combination of these possibilities is very likely to occur. Regardless of the underlying mechanism, reduced glutathione concentration

increases the risk of oxidative damage in these cells. Pigment cells with stimulated production of pheomelanin [27] are the best candidates for such increased risk.

Dysplastic nevus cells commonly produce more melanin.

The first report on the higher production of pheomelanin in DN was published by Salopek et al. [14]. We have recently confirmed this observation by finding notably elevated melanosomal sulfur (indicator of pheomelanin) in various histologic preparations of DN [13].

In the present study, we show for the first time that in cultured DN cells the pigmentation differs from the normal MC and is often increased without the stimulation by tyrosine. In these cases the pheomelanin／eumelanin ratio is also clearly elevated.

Cutaneous MC can partly lessen the risk of oxidative stress by transferring melanin-synthesizing melanosomes to surrounding keratinocytes. However, DN cells have no regular contact with keratinocytes but still synthesize melanin.

We recently found that DN cells contained an increased cytoplasmic concentration of iron [13]. The existence of free transition metals, such as iron, presents a significant risk of structural intracellular damage due to their interaction with hydrogen peroxide, yielding extremely toxic hydroxyl radicals [28]. With the use of three different molecular probes designed for visualizing ROS generation in living cells, we observed increased intracellular ROS production in DN cells.

Experimental oxidative imbalance is probably the most often used inducer of stress-induced premature senescence [29]. During this process, a rapid telomere shortening can occur. In fibroblasts subjected to a rather mild oxidative stress, telomeres were lost five to ten times faster than under nonstress normal situation [30]. A comparison of telomere shortening rates in different human diploid fibroblast lines under different conditions revealed that the ratio between oxidative damage and antioxidative defense was quantitatively the most important determinant of telomere shortening [31].

The acquired knowledge of the existence of sustained oxidative stress in DN cells is in agreement with the proposition that these cells exhibit senescent behavior [32]. Indeed, we and others [33] have regularly observed that DN cells in culture stop growing after reaching only a few passages (Fig. 3). However, the production of pigment continues and the generation of ROS can be visualized. Using different redox probes we showed at least 60% increase of ROS generation in DN cells when compared with normal MC cultured under the same conditions. As mentioned before, such a situation can cause telomere attrition, which is only a special indicator of DNA damage. Under normal culture conditions, the nuclei of DN cells contain significantly more strand breaks (characterized as alkali labile sites) than do normal MC [15]. Single strand breaks, double strand breaks, cross-links and incomplete excision repair sites are responsible for the fragmentation pattern of the nuclei. By introducing lesion-specific glycosylase that recognizes oxidatively damaged DNA bases, we were able to demonstrate that even if the ‘‘normal’’ comet assay does not show any differences in DNA fragmentation between MC and DN cells, FPG digestion causes significantly increased numbers of DNA fragments in DN cells.

Taken together, our work suggests that elevated melanin production is connected with the increased risk of oxidative imbalance that is associated with oxidative DNA damage. Such a situation may lead to higher numbers of mutations and provide a basis for malignant transformation of pigment cells.

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