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Increased endogenous DNA oxidation correlates to increased iron levels in melanocytes relative to keratinocytes

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Synopsis

The endogenous oxidative state of normal human epidermal melanocytes was investigated and compared to normal human epidermal keratinocytes (NHEKs) in order to gain new insight into melanocyte biology. Previously, we showed that NHEKs contain higher levels of hydrogen peroxide (H_2O_2) than melanocytes and that it can migrate from NHEKs to melanocytes by passive permeation. Nevertheless, despite lower concentrations of H_2O_2, we now report higher levels of oxidative DNA in melanocytes as indicated by increased levels of 8-oxo-2'-deoxyguanosine (8-oxo-dG): 4.49 (±0.55 SEM) 8-oxo-dG/10^6 dG compared to 1.49 (±0.11 SEM) 8-oxo-dG/10^6 dG for NHEKs. An antioxidant biomarker, glutathione (GSH), was also lower in melanocytes (3.14 nmoles (±0.15 SEM)/cell) in comparison to NHEKs (5.98 nmoles (±0.33 SEM)/cell). Intriguingly, cellular bioavailable iron as measured in ferritin was found to be nearly fourfold higher in melanocytes than in NHEKs. Further, ferritin levels in melanocytes were also higher than in hepatocarcinoma cells, an iron-rich cell, and it indicates that higher relative iron levels may be characteristic of melanocytes. To account for the increased oxidative DNA and lower GSH and H_2O_2 levels that we observe, we propose that iron may contribute to higher levels of oxidation by reacting with H_2O_2 through a Fenton reaction leading to the generation of DNA-reactive hydroxyl radicals. In conclusion, our data support the concept of elevated oxidation and high iron levels as normal parameters of melanocytic activity. We present new evidence that may contribute to our understanding of the melanogenic process and lead to the development of new skin care products.

INTRODUCTION

Human melanocytes are specialized, neural crest-derived epidermal cells that synthesize melanin and provide coloration to the skin. In order to synthesize melanin, melanocytes engage in a complex, multifactorial polymerization inside subcellular melanosomal organelles. As these melanosomes mature, they travel through the cell's dendritic projections and, at the completion of melanin synthesis, are transferred to keratinocytes by

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attachment to PAR2 receptors whereupon the melanin then migrates to the top of the nucleus to form a protective perinuclear cap (1).

Although environmental exposure of human skin to ultraviolet (UV) radiation can increase the level of reactive oxygen species (ROS) in melanocytes and initiate melanogenesis, oxidation/reduction mechanisms are, nevertheless, an integral part of the basal activity of melanocytes (2). For example, a critical first step in melanogenesis occurs when the Cu(II)-centered enzyme, tyrosinase, is reduced to Cu(I) leading to DOPA formation. Additionally, reduction of phenylalanine hydroxylase and tyrosine hydroxylase 1 by the electron donor (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (6BH4) is a specific requirement for tyrosinase activation (3).

To date, much biochemical research has been devoted to the etiology of melanin formation and the role that oxidation/reduction mechanisms play in this process (4). However, the endogenous oxidative state of the melanocyte has received less attention, yet it may yield clues to understanding some of the paradoxes encountered in melanocyte biology. In support of this concept, Schallreuter *et al.* (5) reported higher concentrations of hydrogen peroxide in melanocytes of vitiligo patients and, in normal epidermal cells, we observed the transfer of hydrogen peroxide from keratinocytes to melanocytes (6). Further, Wang *et al.* (7) found that reduced levels of oxidative DNA damage repair in melanocytes may also contribute to higher levels of oxidation. Because oxidative stress contributes to mutations, inflammatory processes, and aging (8,9), understanding how oxidative differences in melanocytes affect their normal activity may lead to new insights regarding melanogenesis, melanoma, and solar lentigenes (10). Moreover, a recent study on postmenopausal skin (11) showed higher levels of ferritin and may indicate that postmenopausal women may be more susceptible to UV-induced oxidative damage than other groups due to the presence of more cutaneous bioavailable iron and, thus, iron may also play a critical role in melanocytic function in this group. Although the role of iron in melanocytes has been explored by others (12,13), much of their work was focused on tyrosinase activity. Here, we compare the difference in oxidative state between melanocytes and keratinocytes, two contiguous epidermal cells that cross talk and interact intimately with each other in human skin. We compare oxidative lesions in DNA in the form of 8-oxo-dG, which is a well-characterized indicator of oxidation, ferritin as a biomarker for bioavailable iron, and glutathione (GSH) as a measure of cellular antioxidant status. We also discuss a possible mode of action involving iron in melanocytes in order to account for these differences.

MATERIALS AND METHODS

CELLS

Primary normal human epidermal keratinocytes (NHEKs), melanocytes, and growth media were obtained from Life Technologies (Carlsbad, CA) as primary culture cells isolated from fetal foreskin. NHEKs were cultured in EpiLife (calcium-free) medium containing 1% human keratinocyte growth factors. Eumelanin-producing melanocytes were cultured in Medium 254 and also supplemented to 1% with human melanocyte growth factors. At approximately 50% confluency of the third passage, there were sufficient numbers of cells to perform experiments. Melan a and melan c cells were kind gifts from Drs. S. Orlow and S. Pifko-Hirst (New York University School of Medicine, New York,

NY). Human hepatocarcinoma HepG2 cells and immortalized human mammary epithelial MCF-10A cells were also obtained (ATCC, Manassas, VA) and cultured.

ULTRAVIOLET RADIATION

A bank of four Sylvania Sunlamp fs 40 bulbs, which generate UVB radiation (290–320 nm), was used to irradiate cells. Before irradiation, the culture medium was removed and replaced with 6 ml of Dulbecco's Phosphate Buffered Saline (D-PBS). Fluences were measured with an International Light IL1400A radiometer with a UVB probe attachment.

ISOLATION OF CELLULAR DNA

Precautions were taken to minimize artefactual oxidation (14), and DNA was isolated as previously described (15). Briefly after cell lysis, samples were treated with Ribonuclease A and Proteinase K followed by precipitation with sodium acetate, pH 4.5. Chloroform/isoamyl alcohol (24:1) was then used to remove proteins and lipids and the aqueous phase precipitated in isopropyl alcohol and washed in 70% ethanol. The samples were then resuspended in 10 mM Tris-HCl, pH 7.4 and quantitated.

ISOLATION AND ANALYSIS OF 8-OXO-dG

Purified DNA was sequentially digested to the nucleoside level by DNAse 1, Nuclease P1, and alkaline phosphatase as described previously (15,16). Further, after additional purification by centrifugal filtration, the samples were subjected to high-performance liquid chromatography (HPLC) analysis by injection onto a reverse phase column using a 100-mM lithium acetate, pH 5.2, 0.6 ml per min mobile phase, and interfaced to a UV detector for nucleoside analysis in series with an electrochemical detector for sensitive 8-oxo-dG analysis.

GLUTATHIONE ANALYSIS

GSH was measured by means of the GSH/GSSG-Glo Assay (Promega, Madison, WI), which is a luminescence-based assay that allows detection directly on lysed cells with a luciferin derivative sensitive to glutathione S-transferase. Concentrations of GSH were determined against a standard curve, normalized to cell number, and expressed as nmoles/cell number.

FERRITIN ANALYSIS

To unify the background levels of ferritin, cells were initially grown in their respective media but subsequently changed to DMEM containing 1% FBS for at least 24 h before ferritin analysis. Ferritin was analyzed with an ELISA assay that utilized primary antibodies to human ferritin and secondary antibodies conjugated to peroxidase as described previously (17).

RESULTS

OXIDATIVE DNA BASE DAMAGE

Primary keratinocyte and melanocyte cell cultures at approximately 50% confluency were harvested and subjected to DNA isolation and digestion to the nucleoside level as described in the previous section. HPLC/EC analysis revealed that the endogenous level of 8-oxo-dG in NHEKs was 1.49 (±0.11 SEM)/10^6 dG, whereas melanoyctes were observed to have a higher level at 4.49 (±0.55 SEM) 8-oxo-dG/10^6 dG. These data represent a statistically significant difference ($p < 0.005$, two-tailed t-test) between these cell types and indicate higher oxidative DNA levels in melanocytes (Figure 1A). Further, in order to determine the effect of UVB-induced oxidative stress, melanocytes were then exposed to UVB. Interestingly, when melanocytes were exposed to 125 mJ/cm^2 UVB, 8-oxo-dG levels increased nearly twofold to 8.09 (±1.22 SEM). In comparison, NHEKs increased similarly to 3.82 (±0.3 SEM) when exposed under the same conditions and indicates that

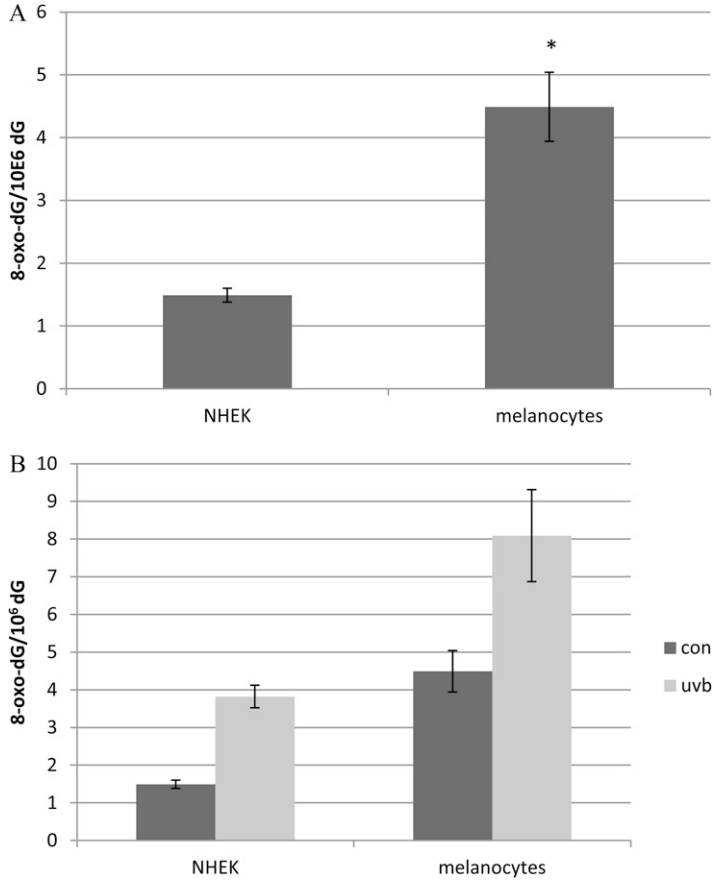

Figure 1. DNA extracted from NHEKs and melanocytes were digested to the nucleoside level and analyzed by HPLC/EC for 8-oxo-dG. (A) Increased levels of 8-oxo-dG were observed in melanocytes compared to NHEKs. Data are expressed as mean ± SEM, $*p < 0.005$. (B) Cells were exposed to 125 mJ/cm^2 UVB and then analyzed. Similar UV-induced increases in 8-oxo-dG were measured for both NHEKs and melanocytes. Data are expressed as mean ± SEM.

although melanocytes have higher endogenous levels of oxidative DNA, they have the same relative susceptibility as NHEKs to UVB-induced oxidative stress (Figure 1B).

One possible explanation for the higher 8-oxo-dG values in melanocytes may have been the difference in growth media. To test this, melanocyte medium was incubated with NHEKs overnight to determine the contribution of medium components to oxidative DNA damage. The next day, cells were harvested and 8-oxo-dG analyzed. In these experiments, the average control value for NHEKs was 1.96 (±0.5 SEM)/10^6 dG while the melanocyte-medium-treated NHEK value was 1.65 (±0.57 SEM)/10^6 dG. Thus, the higher oxidative levels observed in the DNA of melanocytes were not due to differences in medium components. Additionally, NHEK and melanocyte cell cultures were synchronized by serum starvation and their 8-oxo-dG levels assessed for any possible cell cycle effects. The results from this experiment were consistent with our other data, which indicated higher oxidative DNA levels in melanocytes (data not shown).

To further understand the nature of the relative differences in 8-oxo-dG between NHEKs and melanocytes, two murine melanocytic cell lines referred to as melan a and melan c were also evaluated. Although both types of cells are melanocytic in origin, the melan c line has a defective tyrosinase gene and is devoid of melanin. If melanogenesis increases the level of oxyradicals in the cell, the level of 8-oxo-dG should be lower in the melan c cells. However, as shown in Figure 2, only a nonsignificant decrease in 8-oxo-dG was observed in melan c cells as compared to melan a cells. Nevertheless, both melan a and melan c cells were higher in oxidative DNA than in NHEKs and show a greater similarity to human melanocytes, which might be characteristic of these cells.

Glutathione. As a gauge on the overall oxidative state of the cells, total GSH levels were also measured and found to be lower in melanocytes than in NHEKs. GSH in NHEKs was calculated to contain 5.98 nmoles (±0.33 SEM)/cell while in eumelanin-producing melanocytes this level was 3.14 nmoles (±0.15 SEM)/cell and would appear to be consistent with an oxidatively challenged cellular environment. However, the oxidized dimer of GSH, GSSG, was 12.2% for both cell types and these results are summarized in Figure 3. Although lower total GSH levels in melanocytes should foster a more oxidatively

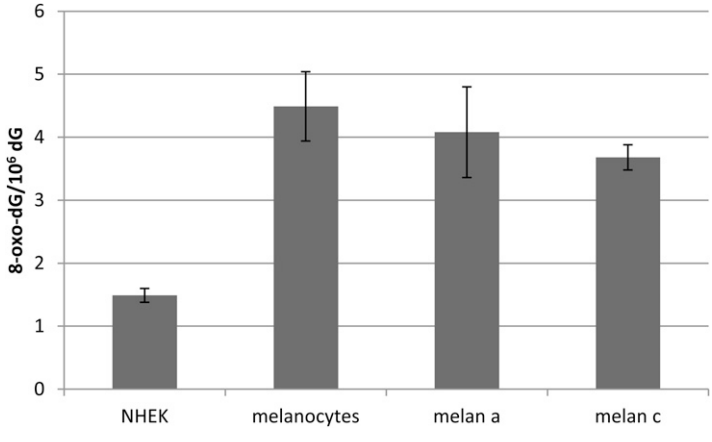

Figure 2. DNA extracted from NHEKs and melanocytes were digested to the nucleoside level and analyzed by HPLC/EC for 8-oxo-dG. Oxidative DNA levels in two melanocyte-derived cell lines: melan a and melan c were more similar to melanocytes than to NHEKs. Data are expressed as mean ± SEM.

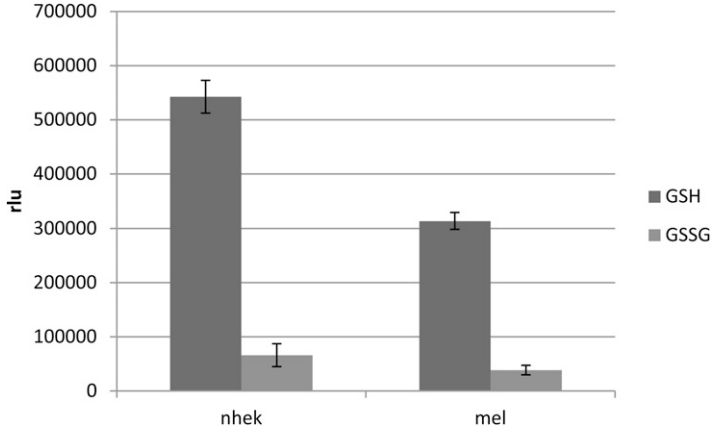

Figure 3. GSH and GSSG were measured in NHEKs and melanocytes using a chemiluminescence-based assay. GSH levels were lower in melanocytes than in NHEKs but GSSG levels were both 12.2% of the total GSH. A linear regression curve was also developed with GSH standards to determine cell molar concentrations and then normalized to cell number as reported in the text. Data are expressed as mean ± SEM.

challenged cellular environment, the finding that GSSG is the same in both cell types raises the possibility that eumelanin-producing melanocytes may normally utilize less GSH for their cellular functions.

Ferritin. As iron plays a significant role in the generation of ROS, we next measured the levels of ferritin, a storage protein of bioavailable iron, in both types of cells. Because serum is an exogenous source of iron in cell culture, we were careful to standardize both cell lines in 1% serum. As shown in Figure 4, ferritin levels in melanocytes were nearly fourfold higher than in NHEKs. Thus, NHEKs contained 23.3 ng (±5 SEM) ferritin/mg protein, whereas melanocytes contained 97.1 ng (±6 SEM) ferritin/mg protein. To further understand this difference in the context of other cells, we compared ferritin levels in other cell types. MCF-10a cells, immortalized mammary epithelial cells, had 13.0 ng

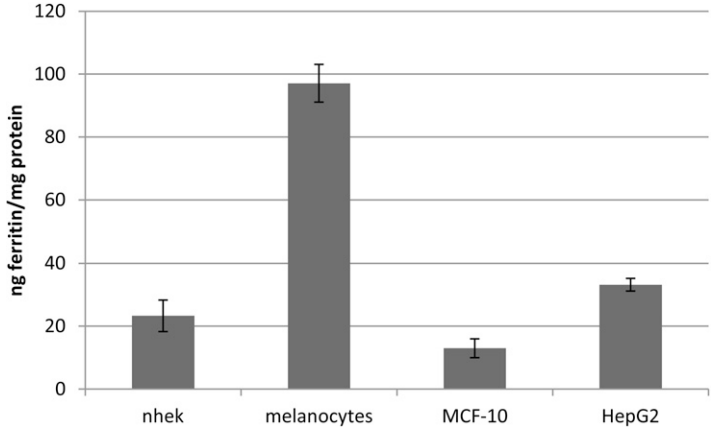

Figure 4. Ferritin concentrations were assayed using an ELISA technique and showed increased levels of ferritin in melanocytes in comparison to NHEKs, MCF-10, as well as in HepG2, an iron-rich hepatocarcinoma cell. Data are expressed as mean ± SEM.

(±3 SEM) ferritin/mg protein. Intriguingly, even HepG2 cells, hepatocarcinoma cells derived from iron-enriched liver tissue, were significantly lower than melanocytes at 33.2 ng (±2 SEM) ferritin/mg protein. These results demonstrate that melanocytes appear to have higher levels of iron in comparison to other cell types and support the concept of a higher oxidative environment in melanocytes.

DISCUSSION

Previously, we reported a fourfold higher level of H_2O_2 in NHEKs than in melanocytes (6), which should indicate less oxidative stress in melanocytes. We further showed that H_2O_2 could permeate by passive diffusion from NHEKs to melanocytes. Nevertheless, despite these previous findings, we now report higher levels of oxidative DNA in melanocytes as compared to NHEKs, as well as lower concentrations of GSH, both of which are well-established biomarkers of oxidative status. Because NHEKs and melanocytes had similar UVB-induced 8-oxo-dG potential and because higher levels of 8-oxo-dG were also found in mouse-derived melanocytes, our data suggest that elevated states of oxidation may be characteristic for melanocytes. To account for these differences, we propose that the higher levels of iron that we measured in melanocytes in this study contribute to higher levels of 8-oxo-dG. A possible reaction sequence might involve a Fenton reaction between iron and H_2O_2 leading to the production of hydroxyl radicals and oxidative DNA. Because H_2O_2 is able to penetrate through membranes and because ferritin has been found in the nucleus (18), the possibility that H_2O_2 will react with ferrous ions inside the nucleus is suggested as a possible mechanism for increased melanocytic oxidative DNA. This reaction sequence might also explain, at least in part, the reduced amount of hydrogen peroxide that we previously determined in melanocytes.

In this report, we show that melanocytes are significantly higher in iron, compared not only to NHEKs but also to iron-rich hepatocarcinoma cells. As melanocytes are specialized epidermal cells dedicated to the synthesis and secretion of melanin in order to protect skin against UV-induced photo damage, the increased presence of iron raises the question as to why a melanocyte would tolerate such a high risk/benefit iron ratio. Although ferritin sequesters approximately 4500 ferric ions per molecule, it also releases iron as reactive ferrous ions (19). Iron ions have been shown to induce DNA base modifications in cellular DNA (20), and understanding this reaction dynamic in melanocytes may be an important factor in understanding the melanogenic process. Intriguingly, 8-oxoguanine has also recently been described as a signaling molecule (21), and perhaps future research will link it to melanocyte gene activation, as well. Further, recent work by Gruber and Holtz (22) demonstrated increased levels of ferritin gene expression after treatment with conventional skin lighteners and their data also support the concept of iron flux in relation to melanocyte function.

As postmenopausal women appear to have higher levels of cutaneous iron (11), our data suggest a possible correlation between this group and the development of age-related melanopathologies, such as lentigines. If future clinical studies support this hypothesis, then research should be targeted toward producing iron-reduction treatments (23). Moreover, increased levels of melanocytic oxidative DNA due to increased iron may be a common denominator that may help to explain the occurrence of melanomagenesis even in sun-protected areas of skin. In conclusion, higher levels of oxidative DNA appear to be present in normal human epidermal melanocytes and correspond to increased amounts of reactive bioavailable iron.

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