Increased Sensitivity to Peroxidative Agents as a Possible Pathogenic Factor of Melanocyte Damage in Vitiligo

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To examine the sensitivity of vitiligo melanocytes to external oxidative stress, we studied enzymatic and non-enzymatic anti-oxidants in cultured melanocytes of normal subjects (n = 20) and melanocytes from apparently normal skin of vitiligo patients (n = 10). The activity of superoxide dismutase and catalase and the intracellular concentrations of vitamin E and ubiquinone were evaluated in cultures at the fourth or fifth passage. In addition, cells were exposed to various concentrations of a peroxidizing agent, cumene hydroperoxide (CUH, 0.66–20 μM), for 1 and 24 h. Compared to normal melanocytes, vitiligo melanocytes showed normal superoxide dismutase and significantly lower catalase activities and higher vitamin E and lower ubiquinone levels. At the concentration used, CUH did not significantly affect cell number or viability of melanocytes after either period of culture. On the contrary, vitiligo melanocytes were susceptible to the toxic effect of CUH after 24 h of continuous treatment at concentrations greater than 6.6 μM. The degree of CUH toxicity correlated strictly with the anti-oxidant pattern, defined as the ratio between vitamin E concentration and catalase activity, suggesting that the alteration in the anti-oxidants was the basis for sensitivity to the external oxidative stress. Our results demonstrate the presence of an imbalance in the anti-oxidant system in vitiligo melanocytes and provide further support for a free radical-mediated damage as an initial pathogenic event in melanocyte degeneration in vitiligo.
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The pathogenic mechanisms in vitiligo, an acquired depigmenting disorder that affects 0.5–5% of the world population, have not been completely clarified, although important clues have been found in recent years (Nordlund and Ortonne, 1992). Several reports have suggested a specific melanocyte defect, because melanocytes in the depigmented areas can be absent or functionally inactive and in normally appearing skin can present morphologic and biologic alterations and lower expression of the c-Kit membrane receptor (Nordlund and Ortonne, 1992; Norris *et al*, 1996). *In vitro*, vitiligo melanocytes (VMs) grow more slowly than normal melanocytes (NMs), are more dependent on the presence of external catalase (Puri *et al*, 1987; Medrano and Nordlund, 1990; Boissy *et al*, 1991), and present structural aberrations in rough endoplasmic reticulum (Boissy *et al*, 1991). Recently, however, some data have suggested the involvement of the entire epidermis in the disease.

Damaged keratinocytes and functionally altered Langerhans cells have been described in depigmented areas (Nordlund and Ortonne, 1992), and a defect in calcium uptake and in the thioredoxine/thioredoxine reductase system has been reported in keratinocytes (Schallreuter *et al*, 1986; Schallreuter and Pittelkow, 1988). Defective recycling of tetrahydrobiopterin in the phenylalanine hydroxylase reaction in epidermis has been proposed as a possible pathogenic factor of the disease (Schallreuter *et al*, 1994b, 1994c). This metabolic impairment could lead to an accumulation of hydrogen peroxide in melanocytes and the consequent oxidation of (6R)-5,6,7,8-tetrahydrobiopterin, the essential cofactor for phenylalanine hydroxylation, to 6-biopterin, which is toxic for human melanocytes (Schallreuter *et al*, 1994a, 1994b, 1994c). In keratinocytes, increased synthesis of (6R)-5,6,7,8-tetrahydrobiopterin stimulates the catecholamine biosynthetic pathway (Schallreuter *et al*, 1994b, 1994c), which induces monoamine oxidase activity (Schallreuter *et al*, 1996). The generation of hydrogen peroxide as byproducts could be responsible for the alteration of catalase activity in the epidermis and for melanocyte damage (Schallreuter *et al*, 1991, 1996). Moreover, in patients with vitiligo, the plasma level of norepinephrine and the urinary concentrations of catecholamine catabolites homovanillic acid and vanil mandelic acid are significantly increased, independent on the type of the manifestations but related to the onset and active phase of the disease (Morrone *et al*, 1992; Schallreuter *et al*, 1994c), indicating that increased catecholamine release, directly or indirectly, could play a role in depigmentation (Morrone *et al*, 1992). These data suggest

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Abbreviations: SOD, superoxide dismutase; CAT, catalase; CUH, cumene hydroperoxide; VM, vitiligo melanocyte; NM, normal melanocyte.

that the generation of oxidative stress in the skin could be the basis for the disease.

Oxidative stress can be defined as any derangement between pro-oxidants and anti-oxidants in which pro-oxidants prevail (Halliwell and Gutteridge, 1989). We have studied the anti-oxidant enzyme activities superoxide dismutase (SOD) and catalase (CAT), and the intracellular levels of the lipophilic anti-oxidants vitamin E and ubiquinone in cultured NMs (n = 20) and melanocytes from apparently normal skin of vitiligo patients (n = 10). Moreover, we correlated sensitivity to the cytotoxic effects of a peroxidizing agent, CUH, with the anti-oxidant pattern.

MATERIALS AND METHODS

Pyrogallol, hydrogen peroxide, ubiquinone 10, and phorbol esters were from Sigma (St. Louis, MO). Ham's F10 medium, fetal bovine serum (FBS), and antibiotics were provided by GIBCO (Paisley, Scotland, United Kingdom). Buthylated hydroxytoluene, tricosanoic acid (C23:0), N,O-bis-(trimethylsilyl)trifluoroacetamide, trimethylchlorsilane, sodium methoxide, and other reagents and solvents were from Merck AG (Darmstad, Germany) at the highest purity grade.

Patients In 10 subjects affected with vitiligo in the active phase (7 males and 3 female; median age, 34 y; range, 25–48 y; 3 focal, 2 acrofacial, 4 diffuse, 1 generalized), after informed consent, 2-cm biopsies were taken in normal appearing areas (generally lower back). Normal human skin was obtained from 20 subjects undergoing plastic surgery age and were sex-matched with patients (13 males and 7 females; median age, 36 y; range, 27–50 y).

Cell Cultures Epidermis was separated from dermis by incubation of biopsies with trypsin/ethylenediamine tetraacetic acid at 37°C for 1 h. Epidermal cells were isolated after mechanical and enzymatic dissection and cultured in Ham's F10 medium with 10% FBS, 5000 international units penicillin and 5000 μg streptomycin per ml, and phorbol 12-myristate-13-acetate (16 nM). The purity of the cultures was checked by optical and electron microscopic examinations. Subconfluent cultures at the fourth or fifth passage were used (Picardo *et al*, 1996). The batch of FBS was analyzed for the fatty acid pattern and the vitamin E level and was the same for the full experimental period. Cells from three different flasks (25 cm^2) were extensively washed in phosphate buffer, harvested with a rubber policeman, centrifuged at 400 $\times$ *g* for 5 min, and counted with trypan blue before analysis.

Vitamin E Analysis Cells (4 $\times$ 10^6 cells) were extracted three times in hexane:ethanol, 3:1, with 1% sodium dodecyl sulfate and 50 ng of (+)-γ-tocopherol and 50 ng of (+)-δ-tocopherol were added in each sample as internal standards before extraction. The extract was treated with 25 μl of dry pyridine and then directly silylated with 25 μl of N,O-bis-(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorsilane as catalyst. Tocopherols were analyzed by gas chromatography mass spectrometry with an Ultra 2 column (30 m $\times$ 0.20 μm internal diameter, 0.25 mm, Hewlett Packard, Cupertino, CA) by a selected ion(s) monitoring technique. The ions selected were 237, 277, and 502 for α-tocopherol; 223, 263, and 488 for γ-tocopherol; and 209, 249, 474 for δ-tocopherols (Picardo *et al*, 1996). Analyses were repeated twice in each extract with a difference less than 5%. Results are reported as mean of two determinations from two different experiments and expressed as nanograms per 10^6 cells.

Ubiquinone Analysis Cells (4 $\times$ 10^6 cells) were extracted in hexane: methanol, 3:1, in the presence of tocopherol succinate as an internal standard (50 ng) and the solvents were evaporated to dryness under a nitrogen stream. Ubiquinol 10 and ubiquinone 10 were analyzed by high-performance liquid chromatography (1090 HP) on reversed-phase column (RP18, 5 μm, 25 cm) using a gradient of methanol:isopropanol from 90:10 to 50:50 in 25 min at a rate of 1 ml/min. UV detection was performed at 280 nm and 220 nm. To better quantify the total amount of this anti-oxidant, each sample was analyzed twice, immediately after the extraction and after complete oxidation with benzoquinone (0.1 mM). The difference in the detection of ubiquinone gave the concentration of the reduced form (ubiquinol). Results are reported as mean of two determinations from two different experiments of total ubiquinone (reduced and oxidized) detected in each sample and expressed as nanograms per 10^6 cells.

Enzyme Activities Melanocytes (4 $\times$ 10^6 cells) were sonicated in sodium phosphate buffer (50 mM, pH 7.2) and centrifuged at 10,000 $\times$ *g* for 10 min at 4°C. SOD activity was evaluated by spectrophotometer as inhibition of pyrogallol autooxidation according to Roth and Gilbert (1987), and CAT activity was determined by the disappearance of hydrogen peroxide (Claiborne, 1985). Standard curves were obtained by using purified human SOD and bovine CAT at different concentrations (1, 2, 5, and 10 units per ml). One unit of CAT is defined as the amount that degrades 1 μM H_2O_2 per min at 25°C, and one unit SOD is defined as the amount of enzyme that induces 50% inhibition of pyrogallol oxidation (0.2 mM) at 25°C. At least two determinations were performed on each supernatant and experiments were repeated twice. Results are reported as mean of two different determination performed in each sample and expressed as units per 10^6 cells.

SOD and CAT activities were also evaluated in the same patients' erythrocytes and in a group of 30 controls and expressed as units per g of hemoglobin or units per mg of hemoglobin, respectively.

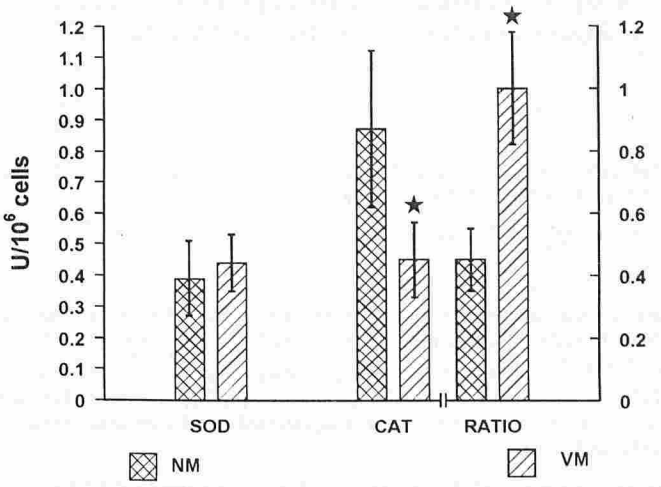

Figure 1. CAT activity is significantly lower in VMs than in NMs. NMs (n = 20) and VMs (n = 10) at the fourth or fifth passage were collected, sonicated in phosphate-buffered saline, and SOD and CAT activities were evaluated by spectrophotometer. *Error bars,* SD. *p < 0.001.

Peroxidative Treatment CUH was dissolved in ethanol at 10 mM, and subsequent dilutions were in culture medium. NMs and VMs were plated in 24-well plates at a density of 10^5 cells per well and treated with concentrations of 0.66 μM, 6.6 μM, or 20 μM for 1 h or 24 h in medium without FBS. Cell number and viability was evaluated by the trypan blue exclusion test immediately afterword and after a subsequent 24-h culture in complete medium. Results are reported as the mean $\pm$ SD of cell viability in three experiments in triplicate.

Statistical Analysis Statistical significance was determined by Student's t test.

RESULTS

Vitiligo Melanocytes Show an Alteration of the Anti-Oxidant Pattern. The cell growth rate was different in the cell cultures analyzed. To avoid interference due to the experimental procedures, we studied subconfluent cultures at the fourth or fifth passage grown in a medium containing the same batch of FBS, so that the supplies of external fatty acids, vitamin E (1.2 μg per ml), and essential elements were the same in all the cultures.

In cultures of NMs, the mean SOD activity was 0.39 $\pm$ 0.20 unit per 10^6 cells (range, 0.25–0.55), the mean catalase activity was 0.87 $\pm$ 0.25 unit per 10^6 cells (range, 0.45–1.4), and the ratio of units of SOD to units of CAT was 0.45 **(Fig 1)**. These results are not significantly different from those previously reported (Yohn *et al*, 1991; Picardo *et al*, 1996). In contrast, in VMs, the mean SOD activity was 0.44 $\pm$ 0.09 unit per 10^6 cells (range, 0.38–0.54), whereas mean catalase activity was significantly lower to 0.45 $\pm$ 0.26 unit per 10^6 cells (range, 0.15–0.5; $p < 0.001$). Consequently, the ratio of units of SOD to units of CAT was 1 **(Fig 1)**. The evaluation of the anti-oxidant enzyme activity in the erythrocytes of the same patients showed values comparable to those of a control group **(Table I)**.

In measurement of lipophilic anti-oxidants, the vitamin E concentration in NMs was 1.69 $\pm$ 0.33 ng per 10^6 cells and the total

Table I. Erythrocyte SOD and CAT Activities Are Comparable in Control Subjects and Vitiligo Patients

	Controls (n = 20)	Vitiligo patients (n = 10)
SOD (units per g of hemoglobulin)	560 ± 35[a]	580 ± 25
CAT (units per mg of hemoglobin)	302 ± 35	327 ± 65

[a] Values represent the mean ± SD.

ubiquinone was 3.9 ± 0.12 ng per 10^6 cells **(Fig 2)**. In contrast, VMs showed a higher vitamin E level (3.75 ± 0.8 ng per 10^6 cells; $p < 0.005$), whereas the ubiquinone concentration was lower (1.78 ± 0.8 ng per 10^6 cells; $p < 0.005$) **(Fig 2)**. No differences were detected in the ratio of ubiquinolol to ubiquinone between NMs and VMs.

Increased Sensitivity to Peroxidative Agents of VMs Culture for 24 h in medium without FBS did not significantly alter cell viability, even though cell growth was reduced with respect to controls in both NMs and VMs **(Fig 3)**. At the concentrations of CUH used (0.66–20 μM), the cell number and viability of NMs were comparable to those of non-treated cells after both 1 and 24 h **(Fig 3)**. Even in VMs, cell viability was comparable to that of control cultures after a 1-h treatment at the lowest concentration used. At 6.6 μM and 20 μM of CUH, however, a cytotoxic effect was observed in 3 of 10 cell cultures, and in these cells, CAT activity was the lowest detected. Correlated with the concentration of CUH, treatment for 24 h induced a reduction of cell viability in all the VM cultures that was statistically significant at 6.6 μM and 20 μM **(Fig 3)**. The sensitivity to the external peroxidative agent seemed to be dependent on the imbalance of the anti-oxidants, because the correlation between the ratio ng of vitamin E to units of CAT and cell viability after a 24-h treatment with CUH at 20 μM was highly significant **(Fig 4**; $p < 0.0001$; $r = -0.954$).

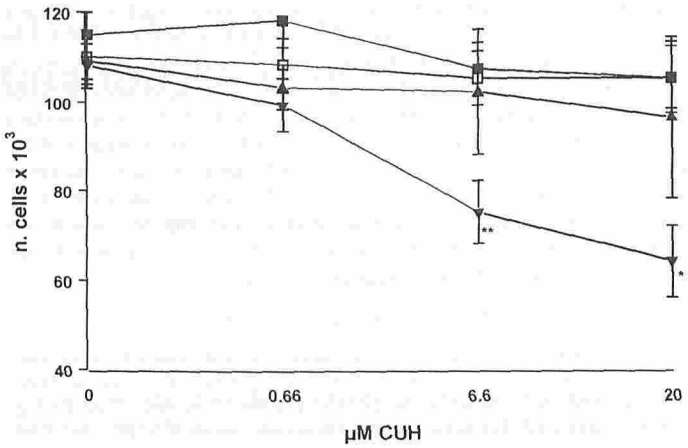

Figure 3. VMs are susceptible to the toxic effect of CUH. Cells (100×10^3 cells) were plated in 24-well plate and incubated with 0.6 μM, 6.6 μM, or 20 μM CUH. Viability was evaluated by trypan blue exclusion. ■, NMs treated for 24 h in medium without FBS; □, NMs treated for 1 h and then cultured for 24 h in complete medium; ▲, VMs treated for 1 h and then cultured for 24 h in complete medium; ▼, VMs treated for 24 h in medium without FBS. *Error bars,* SD (n = 3 experiments in triplicate). $^{**}p < 0.005$; $^{*}p < 0.001$ as compared to NMs values.

DISCUSSION

The pathogenic events underlying melanocyte damage in vitiligo have not been completely clarified; however, *in vitro* and *in vivo* data suggest a free radical-mediated mechanism. Previous reports have shown reduced catalase activity in the epidermis of vitiligo patients, even though the values have been significantly influenced by the presence of keratinocytes, as the measurements were performed on the whole epidermis obtained from a suction blister (Schallreuter *et al*, 1991). Our data show that melanocytes from vitiligo patients, as compared with NMs, possess an imbalance of both enzymatic and non-enzymatic anti-oxidants detectable as normal SOD and reduced CAT activities, decreased ubiquinone, and increased vitamin E concentrations.

The imbalance of anti-oxidants detected in VMs could be either the source or the consequence of peroxidative damage of the cells. Generation of reactive oxygen species and lipoperoxides is associated with a decrease in the anti-oxidant levels in the skin (Applegate and Frenk, 1995), and products of lipoperoxidation could be possible pathogenic factors in hypopigmentary disorders other than vitiligo (Nordlund and Abdel-Malek, 1988; Picardo *et al*, 1991; De Luca *et al*, 1996). CAT is a heme-containing enzyme and is

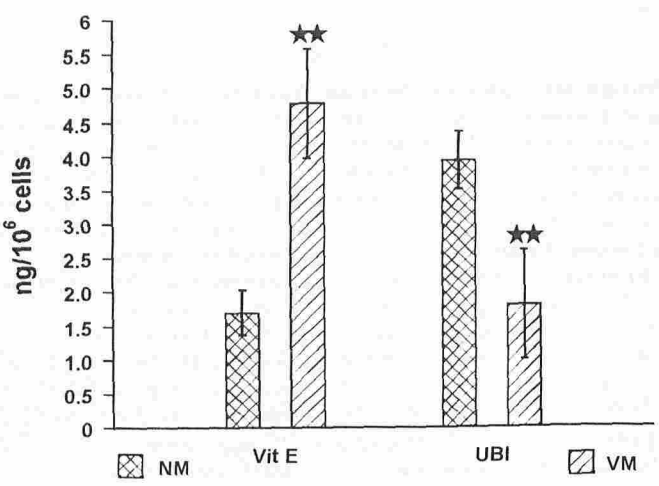

Figure 2. Lipophilic anti-oxidants are significantly altered in VMs. NMs (n = 20) and VMs (n = 10) at the fourth or fifth passages were extracted, vitamin E (Vit E) was analyzed by gas chromatography mass spectrometry, and ubiquinone (UBI) was analyzed by high-performance liquid chromatography as reported in *Materials and Methods*. *Error bars,* SD. $^{**}p < 0.001$.

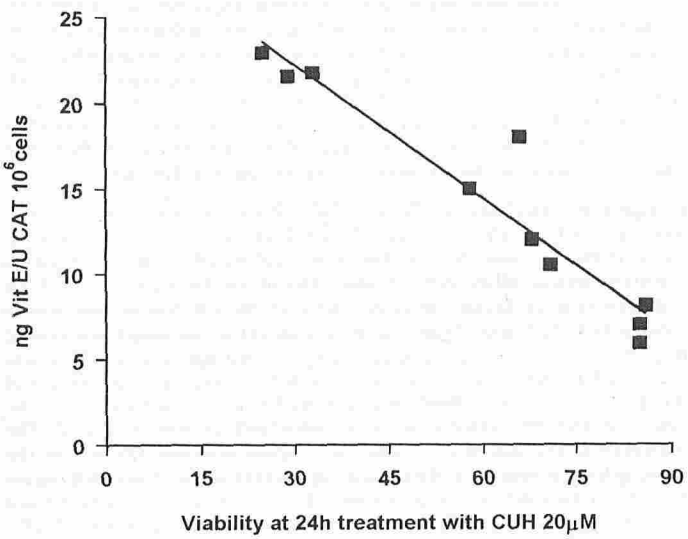

Figure 4. In VMs the sensitivity to CUH is correlated to the imbalance of anti-oxidants. The linear regression curve between the ratio of ng of vitamin E to units of CAT and viability after a 24-h treatment with 20 μM CUH in VMs is shown. $r = -0.954$; $p < 0.0001$.

susceptible to peroxidizing substances such as hydrogen peroxide itself, organic hydroperoxides, and UV light (Halliwell and Gutteridge, 1989). Both biochemical and histologic findings suggest the presence of toxic levels of peroxides in the epidermis of vitiligo patients (Moellman *et al*, 1982; Schallreuter *et al*, 1991), and the production of a localized burst of hydrogen peroxide has been suggested as the consequence of alterations in tetrahydrobiopterin and catecholamine biosynthesis (Schallreuter *et al*, 1996).

We were not able to establish whether the decrease of catalase was due to an alteration in protein synthesis or in enzyme activity; however, the measurements performed in erythrocytes from the same patients showed values comparable to those of controls **(Table I)**, suggesting that the low CAT activity in melanocytes represents the end point of a specific metabolic impairment rather than a systemic alteration.

The correlation between the imbalance of anti-oxidants and the sensitivity of VMs to CUH **(Fig 4)** suggests that the imbalance was the basis for the toxic effect of the peroxidizing agent. Anti-oxidant enzymes operate in combination: SOD dismutates superoxide anion radicals, generating hydrogen peroxide and oxygen, and CAT has been reported to be the main enzyme involved in removing H_2O_2 in melanocytes (Halliwell and Gutteridge, 1989; Yohn *et al*, 1991). When the ratio of activity of these enzymes is altered, hydrogen peroxide can accumulate inside the cells, generating hydroxyl radicals via the Haber-Weiss reaction, leading to the peroxidation of cell membranes (Halliwell and Gutteridge, 1989). In VMs, the decrease in CAT activity was associated with higher vitamin E concentrations that could represent a compensatory mechanism adopted by the cells to prevent or reduce the damage due to the generation of reactive oxygen species. The low concentrations of ubiquinone detected, however, suggest an impairment in the recycling of tocopherol. Vitamin E, in fact, is a chain-breaking anti-oxidant that terminates free radical-mediated peroxidation, producing tocopheryl radicals, and is regenerated by the oxidation of ubiquinol (Mukai *et al*, 1990; Ernster and Beyer, 1991). Therefore, we can speculate that in VMs, after exposure to an external oxidative stress, the concentrations of reactive oxygen species increase and the lipoperoxidative process can go on to produce cell membrane damage.

Our results provide further support for the presence of a metabolic abnormality in VMs and for free radical-mediated damage as the main pathogenic event in melanocyte degeneration. Moreover, it is possible that VMs may reach a different level of equilibrium of anti-oxidants because of a continuous peroxidative process. In line with these results, we have recently reported the therapeutic effect of systemic administration of a group of anti-oxidants in patients with progressive disease, and the topical application of pseudo-CAT and calcium has been proved to be effective in vitiligo subjects (Schallreuter *et al*, 1995).[1]

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