In Vivo and *In Vitro* Evidence for Hydrogen Peroxide (H₂O₂) Accumulation in the Epidermis of Patients with Vitiligo and its Successful Removal by a UVB-Activated Pseudocatalase

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To date there is compelling *in vitro* and *in vivo* evidence for epidermal H₂O₂ accumulation in vitiligo. This paper reviews the literature and presents new data on oxidative stress in the epidermal compartment of this disorder. Elevated H₂O₂ levels can be demonstrated *in vivo* in patients compared with healthy controls by utilizing Fourier-Transform Raman spectroscopy. H₂O₂ accumulation is associated with low epidermal catalase levels. So far, four potential sources for epidermal H₂O₂ generation in vitiligo have been identified: (i) perturbed (6R)-L-erythro 5,6,7,8 tetrahydrobiopterin (6BH₄) *de novo* synthesis/recycling/regulation; (ii) impaired catecholamine synthesis with increased monoamine oxidase A activities; (iii) low glutathione peroxidase activities; and (iv) "oxygen burst" via NADPH oxidase from a cellular infiltrate. H₂O₂ overproduction can cause inactivation of catalase as well as vacuolation in epidermal melanocytes and keratinocytes. Vacuolation was also observed *in vitro* in melanocytes established from lesional and nonlesional epidermis of patients (n = 10) but was reversible upon addition of catalase. H₂O₂ can directly oxidize 6BH₄ to 6-biopterin, which is cytotoxic to melanocytes *in vitro*. Therefore, we substituted the impaired catalase with a "pseudocatalase". Pseudocatalase is a bis-manganese III-EDTA-(HCO₃⁻)₂ complex activated by UVB or natural sun. This complex has been used in a pilot study on 33 patients, showing remarkable repigmentation even in long lasting disease. Currently this approach is under worldwide clinical investigation in an open trial. In conclusion, there are several lines of evidence that the entire epidermis of patients with vitiligo is involved in the disease process and that correction of the epidermal redox status is mandatory for repigmentation. *Key words: oxidative stress/tetrahydrobiopterin/repigmentation. Journal of Investigative Dermatology Symposium Proceedings 4:91–96, 1999*

Vitiligo is an acquired depigmentation disorder affecting 0.5%–4% of the world population (Ortonne and Bose, 1993). Despite its recognition several thousand years ago, the etiology is still unclear. There are several hypotheses proposed for the development of vitiligo but none of them can satisfy the entire spectrum of this cosmetically disfiguring disorder (LePoole *et al*, 1993a; Ortonne and Bose, 1993). The clinical hallmark is the loss of constitutive pigment. Almost all publications describe decreased numbers of functioning melanocytes or complete absence of these cells in lesional skin (LePoole *et al*, 1993b; Ortonne and Bose, 1993). In addition, altered functionality of keratinocytes and Langerhans cells has been documented (Schallreuter and Pittelkow, 1988; Nordlund and Ortonne, 1992). Numerous studies have been undertaken to identify melanocyte specific cytotoxic antibodies to imply immunologic mechanisms in this disorder (Norris *et al*, 1988a; Bystryn, 1989; Harning *et al*, 1991). An impaired growth of melanocytes derived from "normal" epidermis of patients with

vitiligo was previously observed under *in vitro* conditions showing marked improvement after the addition of exogenous catalase (Medrano and Nordlund, 1990; Boissy *et al*, 1991). Nowadays, there is accumulating evidence for increased oxidative stress of the entire epidermal compartment in this disease (Moellmann *et al*, 1982; Bhawan and Bhutani, 1983; Boissy *et al*, 1991; Schallreuter *et al*, 1991; Maresca *et al*, 1997). This review includes the current evidence to support the concept of oxidative stress in vitiligo.

LOW EPIDERMAL CATALASE LEVELS IN VITILIGO

The discovery of low epidermal catalase (EC 1.11.1.6) levels in both lesional and nonlesional epidermis of patients with vitiligo suggested for the first time that the entire epidermis may be involved in this disorder (Schallreuter *et al*, 1991). Two possible conclusions stemmed from this observation: (i) the heme active site of this enzyme could be irreversibly inactivated by its own substrate, H₂O₂ (Aronoff, 1965), and (ii) decreased expression of catalase mRNA could lead to lower enzyme levels. Reverse transcriptase–polymerase chain reaction analysis of catalase mRNA extracted from melanocytes and keratinocytes established from healthy controls and patients did not show any differences in expression (**Fig 1**). These earlier data were recently supported by Maresca *et al*, who demonstrated decreased catalase activities in melanocytes established from patients with vitiligo (Maresca *et al*, 1997). In this context, it is interesting that melanocytes from
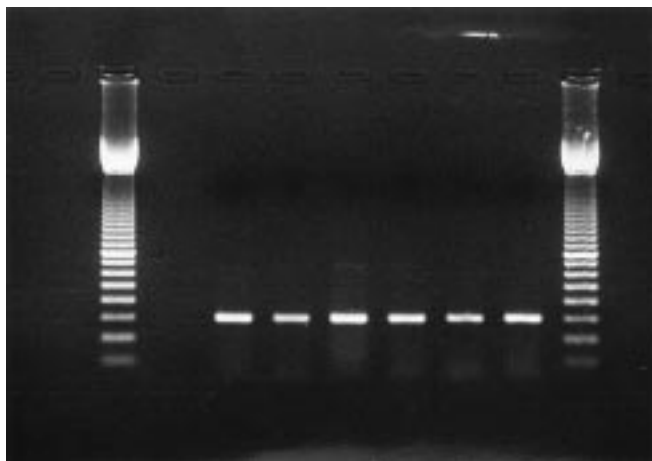
Figure 1. Catalase mRNA expression in melanocytes. Unremarkable mRNA expression of catalase in cell extracts from melanocytes established from patients with vitiligo (n = 5) compared with a healthy control using reverse transcriptase–polymerase chain reaction analysis (55°C, 35 cycles) (from left to right: negative control, 1, 2, 3 (lesional epidermis), 4, 5 (nonlesional epidermis), 6 (healthy control).
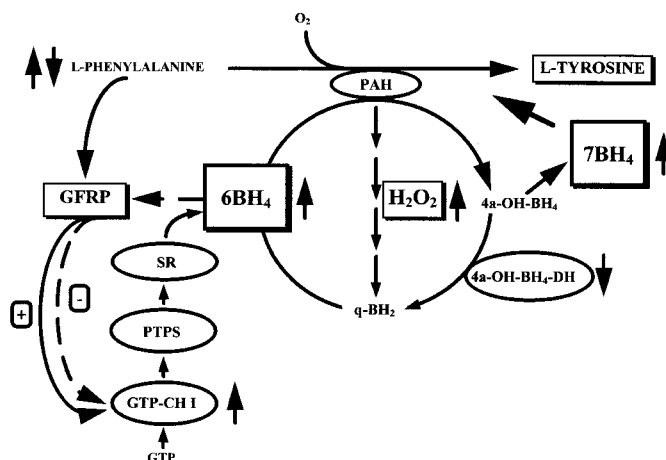

Figure 2. *De novo* synthesis/recycling/regulation of 6BH$_4$ in melanocytes and keratinocytes. 6BH$_4$ is synthesized *de novo* from GTP by the rate-limiting enzyme GTP-cyclohydrolase I (GTP-CH-I) followed by two further enzymatic steps [6-pyruvoyl tetrahydropterin synthase (PTPS), sepiapterin reductase (SR)]. The essential cofactor 6BH$_4$ serves for the conversion of L-phenylalanine to L-tyrosine by phenylalanine hydroxylase (PAH), leading to the formation of 4a-OH-carbinolamine (4a-OH-BH$_4$) and the nonenzymatic production of 7BH$_4$. 4a-OH-BH$_4$ continues through the recycling program to quinonoid dihydropterin via 4a-OH-BH$_4$ dehydratase (4a-OH-BH$_4$-DH), and is then fully reduced to 6BH$_4$ by an NADPH dependent reductase. The *de novo* synthesis/recycling of 6BH$_4$ is tightly controlled by the GTP-cyclohydrolase I feedback regulatory protein (GFRP), where the binding of L-phenylalanine to GFRP upregulates GTP-CH-I and 6BH$_4$ binding to the protein downregulates the *de novo* production of 6BH$_4$ (Harada *et al*, 1993; Milstien *et al*, 1996). In vitiligo 6BH$_4$ is overproduced in association with increased GTP-CH-I and extremely low DH activities. PAH activities are low as a consequence of 7BH$_4$ inhibition concomitant with increased epidermal L-phenylalanine levels. This scenario suggests that the feedback mechanism via GFRP must be impaired because the downregulation of GTP-CH-I via GFRP/6BH$_4$ does not function in vitiligo. At the present time it remains unclear what the primary event is in the perturbed 6BH$_4$ homeostasis in this disorder.

normal healthy individuals are more sensitive to H$_2$O$_2$ than keratinocytes, which could be due to their decreased catalase and glutathione peroxidase activities (Norris *et al*, 1988b; Yohn *et al*, 1991). The cardinal question arises, however, of where the H$_2$O$_2$ in vitiligo originates from. To date there are at least four potential sources of epidermal H$_2$O$_2$ generation identified in this disorder, in addition to low epidermal catalase levels in lesional and nonlesional skin of these patients.

Defective de novo *synthesis/recycling/regulation of the cofactor (6R)-L-erythro 5,6,7,8 tetrahydrobiopterin (6BH$_4$) in vitiligo*   In vitiligo, a defective *de novo* synthesis/recycling/regulation of the essential cofactor 6BH$_4$, with concomitant micromolar accumulation of the isomer (7R)-L-erythro 5,6,7,8 tetrahydrobiopterin (7BH$_4$), has been shown in cell extracts from epidermal suction blister tissue, and from melanocyte and keratinocyte cell cultures established from the same material of these patients (Schallreuter *et al*, 1994a, b). This impaired 6BH$_4$ *de novo* synthesis/recycling/regulation heralds several potential pitfalls for the healthy survival of the epidermal unit.

1 The micromolar accumulation of the 7-isomer is sufficient to inhibit phenylalanine hydroxylase (PAH; EC 1.14.16.1), and therefore high epidermal levels of 7BH$_4$ in vitiligo are in agreement with low enzyme activities as observed in this patient group (Davis *et al*, 1992; Schallreuter *et al*, 1994a, b). One consequence of inhibited PAH is the accumulation of L-phenylalanine. Recently Fourier-Transform Raman spectroscopy has been utilized *in vivo* to identify this accumulation of epidermal L-phenylalanine, which further supports the inhibition of PAH in vitiligo (Schallreuter *et al*, 1998a).
2 The inhibited enzyme together with the decreased 4a-hydroxy tetrahydrobiopterin dehydratase (EC 3.4.5.10) in the recycling process leads to a nonenzymic over-production of 7BH$_4$ with a concomitant accumulation of H$_2$O$_2$ due to an enhanced short circuit to quininoid dihydropterin (Davis *et al*, 1992; Schallreuter *et al*, 1994b). **Figure 2** summarizes our current understanding of the pathologic events in vitiligo with regard to *de novo* synthesis/recycling/regulation of 6BH$_4$ in association with accumulation of the 7-isomer.

Increased epidermal monoamine oxidase A (MAO-A) activities in vitiligo   The increased production of 6BH$_4$ in epidermal keratinocytes established from patients with vitiligo coincides with an enhanced norepinephrine biosynthesis in these cells (Schallreuter *et al*, 1994b). These findings were associated with increased MAO-A (EC 1.4.3.4) activities in the entire epidermis, unveiling additional H$_2$O$_2$ stress as this metabolite is a product of the catecholamine degrading enzymic reaction (Schallreuter *et al*, 1996). Furthermore, it has been shown that norepinephrine plasma levels as well as urinary levels of catecholamine metabolites are

significantly increased in these patients, particularly in active vitiligo (Morrone *et al*, 1992; Schallreuter *et al*, 1994b). A perturbed catecholamine degradation in this disorder is also supported by increased catecholamine-O-methyl transferase (COMT; EC 2.1.1.6) activities (LePoole *et al*, 1994).

Age-dependent reduced glutathione peroxidase activities in vitiligo   Determination of glutathione peroxidase (EC 1.11.1.9) activities in total blood samples demonstrated significantly lower values in patients (n = 36) compared with controls (n = 14) (Beazley *et al*, 1999). In this context, it has also been demonstrated that no patients (n = 61) revealed selenium deficiency when compared with normal controls (n = 5932, normal range 0.80–1.20 μmol per liter); however, 56% of the group had higher levels (range 1.20–2.00 μmol per liter). Because glutathione peroxidase can provide a back-up system for catalase (Stryer, 1988), these new findings suggest that the selenium-dependent enzyme is very likely not compensated for by the selenium-independent glutathione peroxidase. Hence, this important H$_2$O$_2$ degrading system also appears to be impaired in vitiligo. Our results contradict earlier reports from De Luca *et al* who found no significant difference in enzyme activities between patients and controls (De Luca *et al*, 1997). Based on our results, we conclude that oral supplementation with selenium in vitiligo is unwarranted, as recommended by others.[1]

Possible production of H$_2$O$_2$ via the cellular infiltrate in active vitiligo   Several investigators have shown the presence of a cellular infiltrate in the peri-lesional skin of patients with vitiligo (Ortonne and Bose, 1993). Due to this infiltrate the biologic oxygen burst of these cells via

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[1]Picardo M, Camera E, Maresca V, Pittarello A, Leonetti F, Passi S: Antioxidant treatment in vitiligo. *Pigment Cell Res* 10:360, 1997 (abstr.)
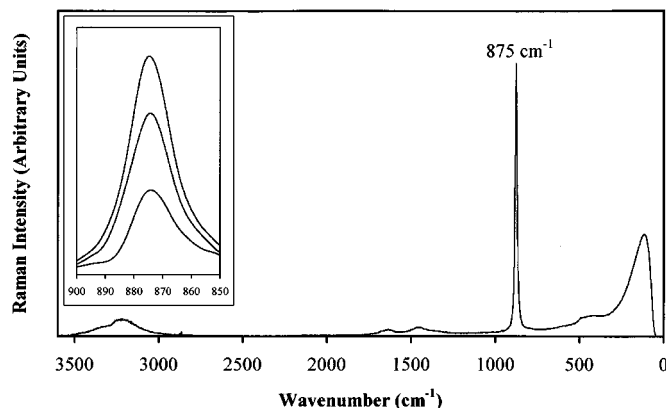
Figure 3. Identification of H_2O_2 using Fourier-Transform Raman spectroscopy. The H_2O_2 peak (8.8 M) could be assigned *in vitro* to 875 cm^{-1} based on the O–O stretch.
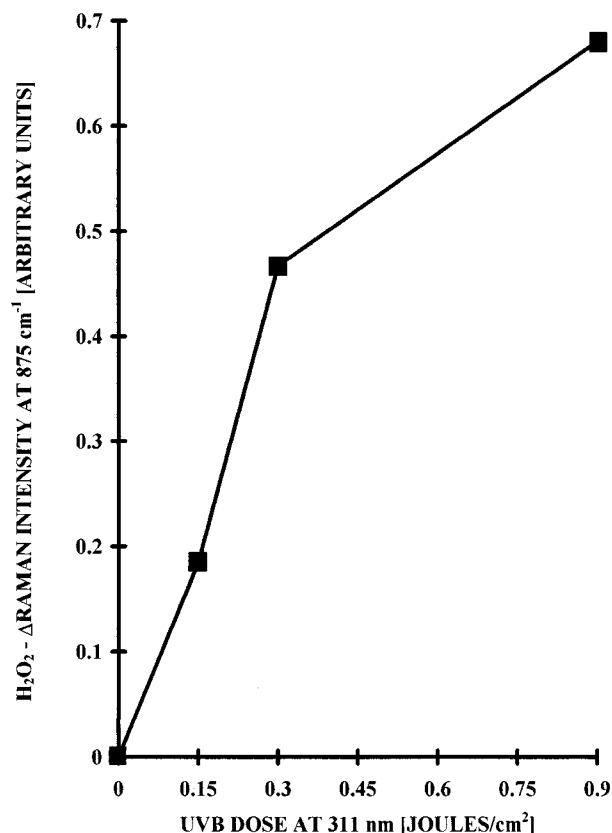
Figure 4. *In vivo* epidermal H_2O_2 generation using Fourier-Transform Raman spectroscopy. *In vivo* H_2O_2 production by UVB (311 nm) is dose dependent (0.15–0.9 J per cm^2). Each measurement was taken directly after UV exposure in healthy skin of photo skin type V [each data point presents the mean of three measurements $\pm$ SEM].

NADPH oxidase activities must also be taken into consideration for additional H_2O_2 generation (Marks *et al*, 1996).

***In vivo* detection of H_2O_2 in the skin using Fourier-Transform Raman spectroscopy**   Fourier-Transform Raman spectra were produced with a BRUKER RFS 100/S spectrometer equipped with a liquid nitrogen cooled germanium detector. Sample excitation was accomplished using a Nd^{3+}:YAG laser operating at 1064 nm with a laser power of 400 mW. Each spectrum was accumulated over 5 min with 300 scans and a resolution of 4 cm^{-1}. Total H_2O_2 was assigned as a well-defined peak at 875 cm^{-1} based on the O–O stretch (8.8 M) (**Fig 3**). Using this method it is possible to identify *in vivo* directly
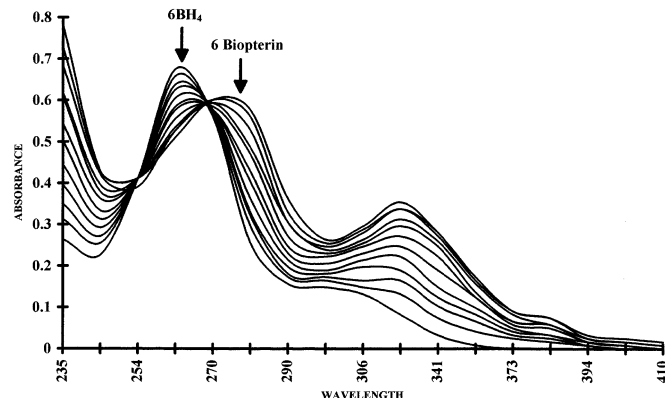

Figure 5. Sequential oxidation of $6BH_4$ to 6-biopterin. $6BH_4$ (0.2 $\times$ 10^{-6} M) was oxidized by H_2O_2 (0.6 $\times$ 10^{-6} M) directly to 6-biopterin over 20 min. The spectra were scanned every 2 min, showing a shift of the peak from $6BH_4$ to 6-biopterin. The isosbestic points prove the full direct 4-electron oxidation to 6-biopterin.

dose-dependent H_2O_2 generation in normal healthy skin following UVB exposure using TL01 bulbs with a narrow band output (maximum at 311 nm, range 310–315) (**Fig 4**). Furthermore, we were able to confirm *in vivo* increased epidermal H_2O_2 production in vitiligo using this noninvasive method in 10 patients with active disease compared with healthy controls. There were no significant H_2O_2 levels detectable in normal healthy skin with different photo skin types (Fitzpatrick classification) under the same experimental conditions.

CONSEQUENCES OF EPIDERMAL H_2O_2 ACCUMULATION IN VITILIGO

The importance of the epidermal $6BH_4$/6-biopterin redox status   One consequence of $6BH_4$ and $7BH_4$ overproduction in vitiligo in association with H_2O_2 accumulation is the full oxidation of these reduced pterins to 6- and 7-biopterin, respectively. **Figure 5** presents the kinetics for the sequential H_2O_2 oxidation of $6BH_4$ by scanning every 2 min over a period of 20 min. It is noteworthy, however, that $6BH_4$ can also be partially photo-oxidized specifically by low-dose UVB exposure yielding 7,8-dihydroxanthopterin (Armarego *et al*, 1983; Armarego, 1984; Schallreuter *et al*, 1998b). Moreover, it is interesting that UVA cannot promote this reaction at any wavelength under the same experimental conditions (Schallreuter *et al*, 1998b). Furthermore, under *in vitro* conditions 6-biopterin is cytotoxic to normal healthy melanocytes in the 10^{-7} M range, meanwhile 7-biopterin has no effect under the same conditions (Schallreuter *et al*, 1994c). At present the physiologic role of the nonenzymatically produced $7BH_4$ remains obscure. The micromolar production of $7BH_4$ in vitiligo has significant consequences leading to inhibition of PAH activities in close association with the H_2O_2-producing short circuit in the recycling process of $6BH_4$ (Davis *et al*, 1992; Schallreuter *et al*, 1994a, b). $6BH_4$ is an essential cofactor for the aromatic hydroxylases and for the nitric oxide synthases (Kaufman and Fisher, 1974; Kwon *et al*, 1989). The presence of a complete autocrine *de novo* synthesis/ recycling/regulation of $6BH_4$ has been identified in both epidermal keratinocytes and melanocytes (Schallreuter *et al*, 1994a, b). Clearly, the redox homeostasis of $6BH_4$/6-biopterin in the epidermal unit is crucial to all the above metabolic steps, as well as to melanocyte survival, because only the reduced cofactor is functional. One system efficiently controlling pterin redox status in the epidermis is thioredoxin/thioredoxin reductase (EC 1.6.4.5) (Wood *et al*, 1995). Calcium tightly controls this system via a single EF-hands binding site on thioredoxin reductase (Schallreuter *et al*, 1989). Moreover, it has been shown that melanocytes as well as keratinocytes established from lesional epidermis express a perturbed cellular calcium homeostasis under *in vitro* conditions together with impaired thioredoxin/thioredoxin reductase (Schallreuter *et al*, 1986; Schallreuter and Pittelkow, 1988; Schallreuter-Wood *et al*, 1996).
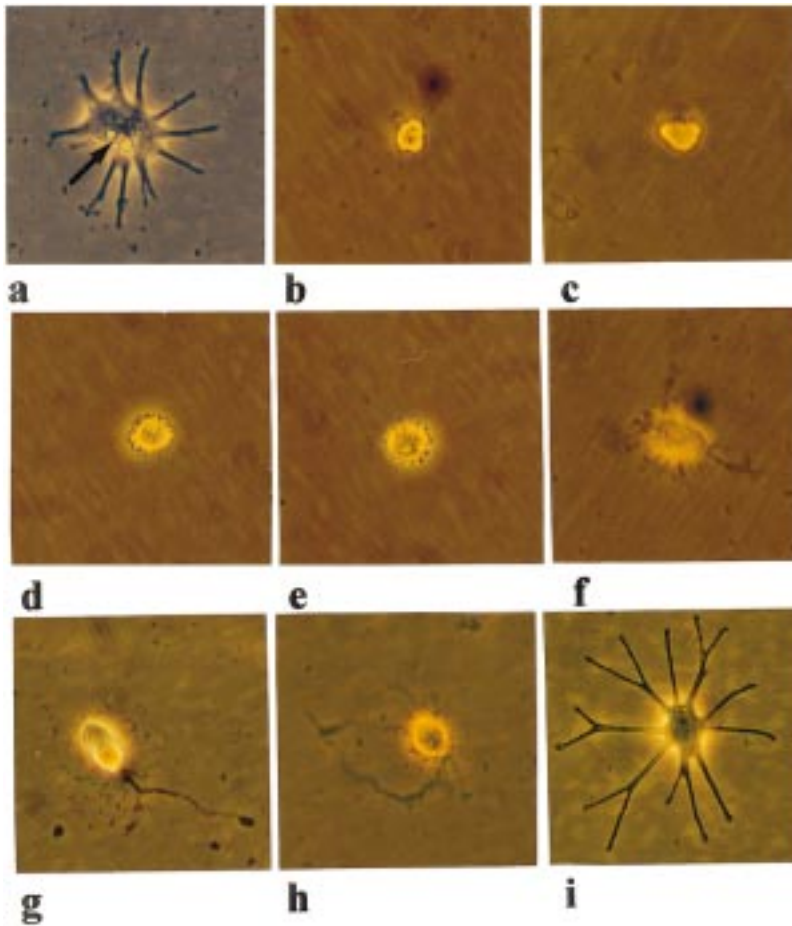
Figure 6. Kinetics of melanocyte vacuolation, loss of dendrites, and subsequent recovery after the addition of exogenous catalase (30 mg per ml). (*a*) Vacuolation in the melanocyte (↑), day 19 in culture; (*b*) subsequent dendrite loss on day 25 in culture; (*c*) addition of bovine catalase to the culture medium, day 27 in culture; (*d*) the same cell after 4 d; (*e*) after 6 d; (*f*) after 7 d; (*g*) after 8 d; (*h*) after 9 d; (*i*) complete recovery on day 13. This experiment has been reproduced in five different cell lines.

Intracellular vacuolation in full skin biopsies and in melanocytes/keratinocytes established from lesional and nonlesional epidermis of patients with vitiligo – evidence for epidermal H_2O_2 stress? Varying degrees of vacuolation and degeneration in melanocytes and keratinocytes have been reported by several investigators (Moellmann *et al*, 1982; Bhawan and Bhutani, 1983). These findings could implicate lipid peroxidation based on H_2O_2 generation. In order to follow directly the fate of melanocytes and keratinocytes *vis-à-vis* H_2O_2 stress, we successfully established these cells from suction blister tissue of lesional and nonlesional epidermis of 10 randomly selected patients with different disease duration (1–25 y) according to the method of Pittelkow and Shipley (1989). All patients had active vitiligo at the time point of the study. Six patients were male and four patients were female, with a mean age of 30.7 y (range 15–55) and photo skin types (Fitzpatrick classification) (III, n = 5; IV, n = 1; V, n = 4). To our surprise, we were able to grow and passage melanocytes from both lesional and nonlesional skin in all patients. These cells developed various degrees of vacuolation after day 16 in cell culture, whereas this phenomenon was absent in cells of healthy controls (n = 8) cultured under the same *in vitro* experimental conditions. Further to the observed vacuolation, we also noted a loss of dendrites in the majority of melanocytes. Upon the addition of bovine catalase to the culture medium, all cells began to re-establish dendrites and there was no further evidence for cellular vacuolation. **Figure 6** demonstrates a time course of one representative melanocyte established from lesional white skin showing first vacuolation, followed by a complete loss of dendrites and subsequent sequential recovery of the cell with regrowth of the dendrite after addition of exogenous bovine catalase to the culture medium.

In summary, we conclude that there is *in vivo* evidence for epidermal H_2O_2 accumulation within the entire skin of patients with vitiligo. These results are strongly supported by the *in vitro* observations using epidermal cell extracts, as well as melanocyte and keratinocyte cell cultures established from lesional and nonlesional epidermis of patients with this disorder. Furthermore, the *in vitro* results strongly support that the entire homeostasis of the epidermis is affected in vitiligo, despite the presence of clinically normal pigmented and white skin. All *in vivo* and *in vitro* data implicate the involvement of the entire epidermal compartment in this disease.

Taking all current *in vivo* and *in vitro* observations into consideration, it appeared logical to substitute the impaired H_2O_2 removing systems (i.e., catalase and glutathione peroxidase).

The development of a UVB-activated pseudocatalase complex in association with successful repigmentation of vitiligo What is a pseudocatalase? Pseudocatalase is not an enzyme. It is a nonpolar bis manganese III-EDTA-$(HCO^-_3)_2$ complex capable of degradation of H_2O_2 to O_2 and H_2O after photo-activation with UVB or solar irradiation (Pseudocatalase, Patent No. EPO58417 1A). The reduction of the H_2O_2 peak can be followed *in vivo* over time after topical application of this pseudocatalase preparation. **Figure 7(*a*)** demonstrates *in vitro* the rapid decrease of the H_2O_2 signal upon the addition of pseudocatalase (12×10^{-6} M) over 6 min. **Figure 7(*b*)** shows an *in vivo* Fourier-Transform Raman spectrum in a healthy control, where under normal circumstances no significant H_2O_2 was detected; however, after exogenous exposure of the skin to 13.2 mM per mm^2 H_2O_2 for 5 min, the peak at 875 cm^{-1} was well resolved. There was a rapid decrease of this signal with the application of pseudocatalase after 5 min. **Figure 7(*c*)** presents the H_2O_2 peak of one patient's involved skin *in vivo* before and after treatment with pseudocatalase, yielding a clearly resolved peak at 875 cm^{-1}, which was effectively reduced by pseudocatalase as in the control experiment. Because the addition of exogenous calcium could correct the impaired calcium homeostasis under *in vitro* conditions, the pseudocatalase complex was incorporated into a base cream together with calcium chloride (Schallreuter *et al*, 1993). Patients are required to apply this topical
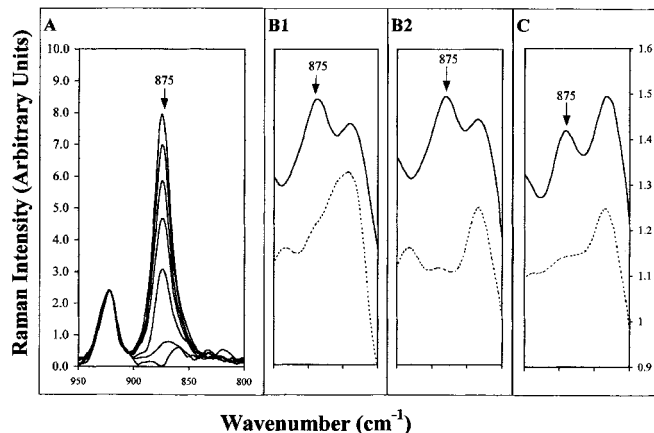
Figure 7. *In vitro* and *in vivo* H_2O_2 removal with pseudocatalase. (*A*) *In vitro* Fourier-Transform Raman spectrum of H_2O_2 (4.4 M) at 875 cm^{-1} and its decrease over time upon the addition of pseudocatalase (the time interval between each spectrum is 1 min). (*B*) *In vivo* Fourier-Transform Raman spectra of (*B1*) normal healthy skin (- - - -) and its exposure to H_2O_2 (13.2 mM per mm^2) (––––) and (*B2*) its removal by pseudocatalase (- - - -) after 5 min. (*C*) *In vivo* results of involved skin of a patient with vitiligo showing a native well-defined peak at 875 cm^{-1} (––––) before treatment and its successful reduction after 5 min by pseudocatalase (- - - -).

preparation twice a day on the entire integument followed by total body low dose narrow band UVB (TL01, 310–315 nm) exposure two to three times a week with a fixed single dose of 0.15–0.3 J per cm^2 per session. It was a surprising observation that the application of broad band UVB (280–320 nm) in association with pseudocatalase yielded in many cases a progression of the disease. The mechanism of this action, however, is still unclear. On the other hand, the pseudocatalase complex can also be activated sufficiently by solar irradiation. It is remarkable that UVB-activated pseudocatalase is 15 times more active in the oxidation of H_2O_2 than natural catalase. The results of a recent pilot study on 33 patients with vitiligo were very promising, providing some important observations: (i) pseudocatalase treatment stopped the progression of active vitiligo in 95% of cases; (ii) the repigmentation process was independent of disease duration, and even patients with long lasting disease presented an excellent recovery (Schallreuter *et al*, 1995).

It is noteworthy that there must be a reservoir of a small population of quiescent melanocytes or possible precursors even after many years of disease, because it was possible to establish melanocyte cell cultures from lesional and nonlesional epidermis using suction blister material of all 10 randomly selected patients with vitiligo of different disease duration. Here, it is interesting to note that the repigmentation process can be followed by WOOD's light examination due to the disappearance of the characteristic fluorescence from the oxidized pterins in association with a light, even repigmentation, followed by a more pronounced and faster peri-follicular activation of melanocytes. Histologic examination of full skin biopsies revealed a complete recovery from epidermal vacuolation and the presence of functioning melanocytes in all patients (n = 18), observed by both light and electron microscopy, as well as immunohistochemical analyses, i.e., staining with characteristic melanocyte markers (NK 1 beteb, HMB 45, dopa-oxidase and Fontana Masson). After complete repigmentation the treatment was stopped and there was no new onset of vitiligo observed. So far, these fully recovered patients have remained stable for up to 5 y. Conversely, if the treatment was terminated before complete repigmentation, there was a rebound phenomenon observed in all cases. The treatment with pseudocatalase is extremely slow or even unsuccessful in the repigmentation of fingertips, toes, and in the medial wrist area. Currently a worldwide open trial with a stable formulation of the pseudocatalase complex is underway. In our own hands, the earlier observations of the pilot study have been confirmed in 73% of the patients (n = 146) (KUS; personal clinical observation).

In conclusion, there is increasing evidence that epidermal H_2O_2

accumulation fosters vitiligo. Whether H_2O_2 is the cause or the consequence of the disease remains to be identified. The substitution with a narrow band UVB-activated pseudocatalase complex in combination with calcium can successfully remove epidermal H_2O_2 in association with a healthy recovery of the entire epidermis and repopulation with functioning melanocytes.

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