Alterations of Mitochondria in Peripheral Blood Mononuclear Cells of Vitiligo Patients

MARIA LUCIA DELL'ANNA1, SANDRA URBANELLI2, ARIANNA MASTROFRANCESCO3, EMANUELA CAMERA1, PAOLO IACOVELLI3, GIOVANNI LEONE3, PAOLA MANINI4, MARCO D’ISCHIA4 and MAURO PICARDO1

1Laboratorio di Fisiopatologia Cutanea, Istituto Dermatologico San Gallicano (IRCCS), Rome, Italy; 2Dipartimento di Genetica e Biologia Molecolare, Università ‘La Sapienza’, Rome, Italy; 3Struttura Semplice Organizzativa di Fototerapia, Istituto Dermatologico San Gallicano (IRCCS), Rome, Italy; 4Dipartimento di Chimica Organica e Biochimica, Università Federico II, Napoli, Italy

*Address reprint requests to Mauro Picardo, Laboratorio di Fisiopatologia Cutanea, Istituto Dermatologico San Gallicano (IRCCS), Rome, Italy. E-mail: picardo@ifo.it

Received 31 May 2003; in final form 27 June 2003

The possible role for a defective mitochondrial functionality in the pathogenesis of vitiligo was investigated by measuring intracellular levels of reactive oxygen species and of antioxidants, the activity of Krebs cycle enzymes, as well as the effects of inhibitors of the electron transport chain, in peripheral blood mononuclear cells from patients with active or stable disease vs. normal subjects. Plasma glyoxal levels were also determined in the same groups of subjects as an index of systemic oxidative stress. In patients with vitiligo in active phase, we observed an increased intracellular production of reactive oxygen species with a consequent imbalance of the prooxidant/antioxidant equilibrium, whereas plasma did not show apparent alterations in glyoxal levels, ruling out a systemic oxidative stress. In patients with stable disease, the balance between pro-oxidants and anti-oxidants seems to be maintained. Moreover, a marked increase in the expression of mitochondrial malate dehydrogenase activity and a specific sensitivity to electron transport chain complex I inhibitor were observed. Overall, these data provide further evidence for an altered mitochondrial functionality in vitiligo patients.

Key words: Vitiligo, Complex I, Malate dehydrogenase, Oxidative stress, Mitochondria, Glutathione, Catalase, Superoxide dismutase

INTRODUCTION

Vitiligo is an acquired idiopathic hypomelanosis, characterized by the appearance of depigmented areas on the skin, and affecting 0.5–2% of the world population. The pathogenesis is still to be fully clarified even if several factors have been recognized as possible determinants of the disease. At least three pathogenic mechanisms, such as immunological, neural, and biochemical ones, have been proposed for the occurrence of the manifestations, although none of those can completely account for the disease (1–3). Moreover, epidemiological studies have revealed a high degree of familial segregation (4, 5) supporting a genetic predisposition, although the molecular characterization needs extensive exploration. As the hypothesized mechanisms are not mutually exclusive and are supported all by experimental and/or clinical evidences, a convergent theory has also been formulated (6).

Different authors have reported evidence suggesting that oxidative stress plays a central role in the process of melanocyte degeneration (7–10). Accumulation of hydrogen peroxide and reduced catalase activity have been found in the epidermis of vitiligo subjects as possible consequence of bipterin metabolism alterations, leading to the production of 7-tetrahydrobipterin, an inhibitor of melanin synthesis (7). In cultured vitiligo melanocytes, an imbalance of the antioxidant pattern associated with an increased sensitivity to pro-oxidants (8) has been observed as well as an altered Tyrp-1 expression with an abnormal interaction with calnexin. The subsequent irregular folding of the TRP-1 polypeptide chain

Abbreviations – DCFH-DA, 2’,7’-dichlorofluorescein diacetate; ETC, electron transport chain; PBMC, peripheral blood mononuclear cell; PBS, phosphate buffer saline; PI, propidium iodide; PTP, permeability transition pore; ROS, reactive oxygen species
can produce an augmented release of intermediates metabolites of melanin synthesis (10). Ultimately, clinical data suggest that topical or systemic administration of antioxidants can improve the course of the disease (11–13).

An imbalance of antioxidants was also demonstrated in peripheral blood mononuclear cells (PBMCs) from patients with active vitiligo, associated with a hyper-production of reactive oxygen species (ROS), and a drop of the mitochondrial transmembrane potential, which was inhibited by Cyclosporin A, an agent interfering with the mitochondrial permeability transition pores (PTP) (9). Therefore, we suggested that the reduced antioxidant capacity is a consequence of the increased ROS generation, possibly of mitochondrial origin, rather than a primary defect in the antioxidant defense system.

To better understand whether a defective function of mitochondria is involved in the pathogenesis of vitiligo, we determined the intracellular ROS levels and the activity of Krebs cycle enzymes, as well as the effects of inhibitors of the electron transport chain (ETC), in PBMC from a further group of patients with active or stable disease and in healthy subjects. Considering that alteration of antioxidants can be consequent to a systemic oxidative stress and/or to an increased catecholamine discharge, as previously reported (14, 15), in the same patients we have measured the plasma levels of the α-oxoaldehydes glyoxal and methylglyoxal, as markers of oxidative degradation of carbohydrates.

Our results confirm that in PBMCs of active vitiligo patients an increased intracellular ROS production and an alteration of the antioxidant system take place, which is not associated with signs of systemic oxidative stress. Moreover, in the same cells we found an increased susceptibility to inhibitors of mitochondrial complex I, and a marked increase in the expression of mitochondrial malate dehydrogenase activity. These data further suggest an altered functionality of mitochondria in vitiligo patients.

**MATERIALS AND METHODS**

**Subjects and Preparation of Blood Samples**

Fifty consecutive patients with non-segmental vitiligo were enrolled into the study. Patients were assigned to the active phase group when a progression of existing lesions or the appearance of new depigmented areas were recorded during the six preceding months, whereas when new lesions or enlargement of depigmented spots were not observed in the previous 6 months, the patients were included into the stable phase group. Accordingly, 35 patients were defined as active, and 15 as stable ones. The women:men ratio was 2:1. Ages of patients ranged from 20 to 60 yr (mean 35 yr). Thirty healthy individuals, age and sex matched, were analysed as the control group. Blood was withdrawn by venipuncture and collected directly into tubes containing LiEDTA. The plasma obtained by centrifugation (300 g for 15 min, room temperature) was stored at −80°C until analysis. The PBMCs were isolated by stratification (400 g for 30 min at room temperature) on a gradient of Ficoll-Hypaque (Uppsala, Sweden). The PBMCs layer was removed and washed twice with NaCl 0.9% (200 g for 15 min at 4°C).

**Determination of Enzymatic Antioxidants and Glutathione**

The PBMCs were lysed in bidistilled water by two consecutive freeze and thaw cycles in liquid N₂, and centrifuged at 800 g for 10 min at 4°C. Protein concentrations were determined, after centrifugation, in the supernatant by Bradford assay and are expressed as mgP/ml.

Total superoxide dismutase (SOD) activity was evaluated in the cell lysates by spectrophotometer based on a competitive inhibition assay of the reaction between superoxide generated by the xanthine-xanthine oxidase and nitroblue tetrazolium, which forms a product absorbing at 560 nm upon reduction (16). Briefly, the supernatant was filtered through a Microcon device (16 000 molecular weight cut off) by centrifugation at 14,000 g and 4°C for 30 min and the retentate, containing the SOD enzyme, was used after dilution in 0.2 M Tris–HCl (pH 7.8). The calculated values are reported as units of SOD per mg of protein (U_{SOD}/mg_p).

Catalase (Cat) activity assay was performed according to the method reported by Claiborne, which measures the rate of disappearance of hydrogen peroxide (10 mM) at 240 nm measured by spectrophotometer (17). One unit of Cat decomposes 1 μmole of H₂O₂ per min at pH 7 and 25°C. The results are reported as units of Cat per mg of protein (U_{Cat}/mg_p).

Reduced glutathione (GSH) levels were determined by a Liquid Chromatography–Mass Spectrometry (LC–MS) assay based on a previously described method (18). Briefly, PBMCs were lysed at 4°C for 30 min in 10 mM aqueous N-ethylmaleimide. After centrifugation, a measured volume of supernatant of thiosalicylic acid (Sigma, St Louis, MO, USA) was added, as an internal standard, at a final concentration of 20 μM. Then, proteins were precipitated by cold CH₃CN and removed by centrifugation. For the HPLC-MS analysis, 10 μl of the supernatant, deprived of proteins, were injected onto a diol column. The results are expressed as nmol GSH/mg_p.

**Measurement of Intracellular Reactive Oxygen Species**

The PBMCs (0.5–1 × 10⁶) were stained with 2.5 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Fluka AG, Basel, Switzerland), a compound that is oxidized to fluorescent 2′,7′-dichlorofluorescein by H₂O₂, other ROS and low molecular weight peroxides (19). The incubation was performed at 37°C, 5% CO₂ in phosphate buffer saline (PBS) containing calcium, magnesium, and 5 mM glucose. After 30 min, stained PBMCs were analysed using a flow cytometer (Cytoron Absolute, Ortho Diagnostic Systems; excitation at 488 nm; 256 resolution channels). The median of the FL-1 channel (530 nm), corresponding to the maximal number of cells with the highest fluorescence, was the parameter utilized to evaluate the ROS levels.

**Assessment of Cell Viability**

To determine the extent of cell death PBMCs were stained with 1 μg/ml propidium iodide (PI) (Sigma) (λem 630 nm). Immediately after staining, PBMCs were analysed by means of a flow cytometer. Viable (region A) and apoptotic (region B)
cells were distinguished also on the basis of cell morphology changes, detected through Differential Light Scatter (DLS), and were independently evaluated for the positive staining with PI (20). PI was compatible with a DCFH-DA co-staining, based on the different emission wavelengths, thus allowing the simultaneous identification of viable cells and measurement of intracellular ROS levels.

**Determination of Mitochondrial Trans Membrane Potential**

Mitochondrial transmembrane potential ($\Delta \Psi_m$) was evaluated by means of JC-1 (5,5′,6′,6′-tetrachloro-1,1′,3′,3′-tetrathyizolium, inner filter probe; Molecular Probes, Eugene-Oregon, USA). The stock solution (2.5 mg/ml) was prepared in N,N-dimethylformamide (Merck AG, Darmstadt, Germany). PBMCs were stained with 2.5 µg/ml JC-1 for 15 min at 37°C and 5% CO₂, washed in PBS containing 5 mM glucose, and immediately analysed by flow cytometry for green (530 nm) and for orange (570 nm) fluorescence (21).

**Inhibition of Mitochondrial Respiratory Chain**

Rotenone (Sigma), an inhibitor of complex I, was dissolved in DMSO/ethanol mixture, whereas oligomycin (Sigma), a specific inhibitor of complex V, was dissolved in ethanol. The stock solutions were stored at 4°C before use, and applied to PBMCs at different doses (0.6, 1.2, 2.5 and 5 µg/ml) before DCFH-DA addition. The final concentration of the solvents was less than 1%.

**Evaluation of Krebs Cycle Enzymes**

The zymografic analysis of the Kreb’s cycle enzymes was performed on 300 ng of protein from PBMCs lysates and carried on horizontal starch gel at 12% (w/v). The gel was performed with hydrolysed potato starch powder (Connaught Medical Research Laboratory, Toronto, Canada) dissolved in the appropriate buffer. Standard horizontal electrophoresis was carried out at 7–8 V/cm for 3–7 h at 5°C. We analysed five enzymes in each sample, encoded by eight putative loci: malate dehydrogenase (Mdh-1, Mdh-2, EC 1.1.1.37), isocitrate dehydrogenase (Idh-1, Idh-2, EC 1.1.1.42), aspartate aminotransferase (Aat-1, Aat-2, EC 2.6.1.1), nucleoside phosphorylase (Np, EC 2.4.2.1), and fumarase (Fh, EC 4.2.1.2). Previously published data performed on the total and mitochondrial homogenates of cells showed that the Mdh-1, Aat-1, Idh-1 loci synthesized cytoplasmic enzymes whereas the other synthesized mitochondrial enzymes (22). The buffer systems utilized were: Tris-versene-borate, for malate dehydrogenase (23) and fumarase (22) enzymes, and phosphate-citrate, for isocitrate dehydrogenase (23), aspartate aminotransferase (24) and nucleoside phosphorylase (22). A semi-quantitative evaluation of the enzymatic activities was performed by densitometric measurements of gel electrophoresis by an IBAS 2000 system (Kontron, Germany).

**Glyoxal and Methylglyoxal Determination in Plasma Samples**

Glyoxal and methylglyoxal determination was carried out in plasma from 20 vitiligo patients (10 in stable and 10 in active phase) and 10 normal subjects, according to the procedure of Espinosa Mansilla (25). Briefly, 200 µl of plasma were placed in a 3 ml vial and treated with 300 µl of a solution of 6-hydroxy-2,4,5-triaminopipridine (7.02 × 10^{-3} M) and 200 µl 0.02 M sodium acetate/acetic acid (pH 4.0) buffer solution. Deionized water was added up to 2 ml. The samples were heated at 60°C for 45 min. Aliquots of 100 µl were withdrawn and subjected to HPLC with fluorimetric detection (HPLC-FD). A 250 × 4.60 mm Sphereclone ODS(2) 5 μm column was eluted with 0.02 M sodium acetate/acetic buffer (pH 4.0)/acetonitrile (99:1 v/v), at a flow rate of 1.3 ml per min. Under these conditions, pterin and 6-methylpterin eluted at 9.2 and 21.1 min, respectively. A Jasco FP-110 fluorescence detector with excitation set at 365 nm and emission at 447 nm was used for detection. Quantitative analysis of glyoxal and methylglyoxal was carried out by comparing integrated peak areas with calibration curves obtained by submitting authentic samples to the derivatization procedure described above. All determinations were run at least in triplicate.

Statistical significance was evaluated using Student’s test.

### RESULTS

**Antioxidant/Pro-Oxidant Imbalance in PBMCs from Patients with Active Vitiligo**

The values of antioxidant enzymes activities, the levels of GSH and ROS, in the two groups of vitiligo subjects and in normal individuals are reported in Table 1. Confirming previous data, patients in the active phase of the disease showed a considerably lower Cat activity and reduced GSH levels as well as a significantly higher activity of total SOD in lymphocytes compared with the groups of stable vitilgo and of normal subjects ($P < 0.05$). Consequently, the SOD/Cat activity ratio was significantly altered in active phase group with respect to the stable and normal ones, being 0.55, 0.34, and 0.33, respectively. Even ROS levels were significantly higher in PBMCs of patients with active disease whereas in the stable group the increase was not statistically significant.

<table>
<thead>
<tr>
<th></th>
<th>Cat ($U_{cat}/mg_p$)</th>
<th>SOD ($U_{sod}/mg_p$)</th>
<th>SOD/Cat</th>
<th>GSH (nmol GSH/mgp)</th>
<th>DCFH-DA (MF)</th>
<th>JC-aggregates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active vitilgo</td>
<td>104 ± 40*</td>
<td>58 ± 19*</td>
<td>0.55 ± 0.01</td>
<td>33 ± 4*</td>
<td>177 ± 11*</td>
<td>63 ± 5*</td>
</tr>
<tr>
<td>Stable vitilgo</td>
<td>149 ± 35</td>
<td>51 ± 18</td>
<td>0.34 ± 0.05</td>
<td>65 ± 8</td>
<td>164 ± 20</td>
<td>70 ± 7**</td>
</tr>
<tr>
<td>Normal</td>
<td>146 ± 48</td>
<td>48 ± 19</td>
<td>0.33 ± 0.03</td>
<td>64 ± 9</td>
<td>137 ± 15</td>
<td>97 ± 2</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. normal group values. **P < 0.01 vs. normal group.
in comparison with those in normal subjects. As previously reported (9), neither significant difference among lymphocyte subpopulations (CD4, CD8, CD45RA, CD45RO), nor a significant correlation with CLA+ cells (data not shown) were observed. ROS levels correlated with the SOD/Cat ratio in either normal subjects or stable vitiligo groups (R = 0.75), whereas, in the active vitiligo group, DCFH-DA fluorescence intensity was inversely correlated with the Cat activity (R = −0.99). These data suggest that in vitiligo there is an established hyper-production of ROS that, during the stable phase of the disease, is properly balanced by the cellular antioxidant defence system and, when an additional oxidative stimulus occurs, cells increase the ROS production without oppose further antioxidant defence possibly by exhaustion of the stock.

### Alteration of Mitochondria Function in PBMCs from Active Vitiligo Patients

As the main intracellular source of ROS is the mitochondrion, several functional parameters of this organelle were evaluated. A significant reduction of membrane potential (ΔΨm), as indicated by the decreased level of JC-1 aggregates, was observed in cells from vitiligo patients in the active and in the stable phase, although at a different extent (63 ± 5 and 70 ± 7%, respectively, vs. 97 ± 2% in normal subjects P < 0.005) (Table 1). Even the response to the ETC complex I inhibitor rotenone was different in the three groups. In PBMCs of normal subjects, cell viability and percentage of JC-1 aggregates were reduced only at the highest doses used (2.5–5 μg/ml); whereas in the active vitiligo group, a significant modification was observed at 0.6 μg/ml and in PBMCs from the stable vitiligo group at 2.5 μg/ml of the inhibitor (Figs 1 and 2). On the contrary, no significant differences were observed between PBMCs from vitiligo and from normal subjects in the response to the ETC complex V inhibitor oligomycin (Table 2). These results suggest that PBMCs of vitiligo patients are particularly susceptible to the inhibitor of the complex I ETC.

In order to evaluate the efficiency of mitochondrial metabolism, some Krebs cycle enzymes were analysed. No differences in the electrophoretic mobility of any of the enzymes evaluated were seen among any of the groups (data not shown). However, the mitochondrial isoform of malate dehydrogenase showed a higher activity in vitiligo patients with respect to unaffected subjects, whereas the cytoplasmic isoform was normally expressed. Consequently, the ratio of cyt/mit malate dehydrogenase activity, as determined by densitometry, significantly decreased with respect to those of normal subjects (P < 0.005) (Fig. 3).

### Glyoxal Levels in Vitiligo Patients

Glyoxal and methylglyoxal, converted to the highly fluorescent pterin and 6-methylpterin, were determined by flow cytometry (Fig. 4). The glyoxal production by vitiligo patients was significantly higher than normal subjects (P < 0.05) (Table 3). These results suggest that in vitiligo there is an increase in the production of glyoxal and methylglyoxal.

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**Table 2. Effect of oligomycin treatment. Cell viability, ROS production and mitochondrial membrane potential in PBMCs of vitiligo and normal subjects**

<table>
<thead>
<tr>
<th>Oligomycin (µg/ml)</th>
<th>0</th>
<th>0.6</th>
<th>1.2</th>
<th>2.5</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active vitiligo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A %</td>
<td>67 ± 4</td>
<td>56 ± 4</td>
<td>55 ± 5</td>
<td>46 ± 5</td>
<td>34 ± 4</td>
</tr>
<tr>
<td>ROS</td>
<td>177 ± 11</td>
<td>168 ± 9</td>
<td>165 ± 10</td>
<td>150 ± 6</td>
<td>131 ± 9</td>
</tr>
<tr>
<td>ΔΨm</td>
<td>63 ± 3</td>
<td>61 ± 4</td>
<td>59 ± 5</td>
<td>54 ± 4</td>
<td>48 ± 6</td>
</tr>
<tr>
<td>Stable vitiligo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A %</td>
<td>67 ± 6</td>
<td>63 ± 5</td>
<td>59 ± 4</td>
<td>53 ± 3</td>
<td>50 ± 5</td>
</tr>
<tr>
<td>ROS</td>
<td>164 ± 20</td>
<td>152 ± 12</td>
<td>148 ± 11</td>
<td>148 ± 7</td>
<td>141 ± 4</td>
</tr>
<tr>
<td>ΔΨm</td>
<td>70 ± 2</td>
<td>68 ± 5</td>
<td>63 ± 4</td>
<td>57 ± 5</td>
<td>55 ± 2</td>
</tr>
<tr>
<td>Normal</td>
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<tr>
<td>A %</td>
<td>70 ± 2</td>
<td>65 ± 4</td>
<td>60 ± 7</td>
<td>50 ± 6</td>
<td>45 ± 8</td>
</tr>
<tr>
<td>ROS</td>
<td>137 ± 15</td>
<td>132 ± 6</td>
<td>133 ± 6</td>
<td>130 ± 4</td>
<td>129 ± 7</td>
</tr>
<tr>
<td>ΔΨm</td>
<td>97 ± 4</td>
<td>95 ± 4</td>
<td>88 ± 6</td>
<td>88 ± 5</td>
<td>70 ± 5</td>
</tr>
</tbody>
</table>

All data come from flow cytometric analysis. A % represents the percentage of viable cells. ROS correspond to DCFH-DA FL1 median intensity fluorescence. ΔΨm was expressed as percentage of aggregates within the viable region.

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**Fig. 1.** Effect of rotenone treatment on viability of peripheral blood mononuclear cells (PBMCs) from normal and vitiligo subjects. The viable cells were determined on the basis of Differential Light Scatter (DLS) and propidium iodide (PI) staining. The results represent the mean ± SD of all the sample analysed. *P < 0.05 and **P < 0.001 vs. not treated cells.

**Fig. 2.** Effect of rotenone treatment on mitochondrial membrane potential (ΔΨm). The modification of the percentage of JC-1 aggregates was assumed as index of ΔΨm reduction. *P < 0.05 and **P < 0.001 vs. not treated cells.

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**Fig. 3.** Alteration of the mitochondrial metabolism. As the main intracellular source of ROS is the mitochondrion, several functional parameters of this organelle were evaluated (Table 2). These results suggest that PBMCs of vitiligo patients are particularly susceptible to the inhibitor of the complex I ETC.

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**Table 3.** Glyoxal and methylglyoxal levels in PBMCs of normal and vitiligo subjects.

<table>
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<th>1.2</th>
<th>2.5</th>
<th>5</th>
</tr>
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<td>Active vitiligo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glyoxal (nM)</td>
<td>67 ± 4</td>
<td>56 ± 4</td>
<td>55 ± 5</td>
<td>46 ± 5</td>
<td>34 ± 4</td>
</tr>
<tr>
<td>Methylglyoxal (nM)</td>
<td>177 ± 11</td>
<td>168 ± 9</td>
<td>165 ± 10</td>
<td>150 ± 6</td>
<td>131 ± 9</td>
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<td>59 ± 4</td>
<td>53 ± 3</td>
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<td>129 ± 7</td>
</tr>
</tbody>
</table>

All data come from flow cytometric analysis. Glyoxal and methylglyoxal were determined by enzymatic colorimetric assay.
HPLC-FD. By this method, glyoxal and methylglyoxal levels of 1.72 ± 0.06 μM and 514 ± 16.1 nM were determined, respectively, for vitiligo patients vs. control values of 2.03 ± 0.10 mM for glyoxal and 566.7 ± 42.3 nM for methylglyoxal.

**DISCUSSION**

Although there is to date compelling evidence for an oxidative stress condition in vitiligo, there remains considerable uncertainty as to whether elevated free radical production and/or impairment of the major antioxidant defence system is an initial pathogenic event of the disease, or is merely a contributory factor.

In the present study, we have confirmed and extended our previous data on a possible impairment of mitochondrial function in patients with vitiligo. We have indeed detected an imbalance of antioxidants correlated with an increased production of ROS and associated with an alteration of mitochondrial trans-membrane potential in PBMCs from an additional group of active vitiligo patients. The abnormal intracellular ROS generation seems to be an event that occurs upstream the antioxidant alteration and both the increased SOD activity and the reduction of the catalase activity, as well as the depletion of GSH, could be regarded as a consequence of the ROS hyper-production. The levels of ROS, in fact, were correlated with the SOD/Cat ratio in cells from normal subjects as well as in cells from patients with stable vitiligo whereas, in the active phase, ROS levels correlate inversely with Cat activity.

Mitochondria are generally recognized as one of the main sources of ROS and the primary site of superoxide generation in the skin (26). Previously, we have demonstrated that intracellular ROS in PBMCs from vitiligo patients are generated mainly by mitochondria as cyclosporin A, an inhibitor of PTP, significantly reduces their levels (9). Several reports indicate that about 5% of the oxygen at the complexes I and III sites escapes the proper way and gives rise to radical species (27), and that some subunits of the complex I are particularly vulnerable to oxidative injury because of the presence of several sulphhydryl residues (28). In active vitiligo patients, PBMCs are specifically susceptible to the ETC complex I inhibition, as indicated by the increased cell death and decreased mitochondrial membrane potential following rotenone treatment, suggesting that components of the mitochondrial respiratory chain can be altered. The increased susceptibility to rotenone has been correlated with altered functionality of the complex I in different systems (29), and inhibition of complex I activity as well as alteration of PTP have been associated to apoptotic pathway via caspase 3 activation (30).

The results achieved indicate that, in the stable phase of vitiligo, the balance between pro-oxidants and antioxidants is maintained and that stimuli capable of leading to a further increase of ROS production and interfering with mitochondrial functions could produce an alteration of the antioxidant system associated with the active phase of the disease. ROS, in particular $\text{H}_2\text{O}_2$, in fact are capable of inducing the opening of PTP and their intracellular generation could amplify the mitochondrial dysfunction (31).

Hydrogen peroxide accumulation is an established biochemical change in vitiligo, and can cause extensive oxidation of biological molecules, including carbohydrates, largely via Fenton-type reactions. Carbohydrates degradation may give rise to a range of genotoxic and cytotoxic $\alpha$-oxoaldehydes and related species which may amplify the primary damage caused by hydrogen peroxide-derived ROS, leading eventually to apoptosis. These $\alpha$-oxoaldehydes are usually reflective of systemic oxidative stress states associated with aberrant carbohydrate metabolism, e.g. diabetes. The failure to demonstrate an alteration of glyoxal and methylglyoxal levels in the plasma of vitiligo patients would apparently argue against a systemic, generalized oxidative stress condition.

Possible mitochondrial impairment in vitiligo is also supported by studies of enzymes involved in the Krebs cycle. The expression of mitochondrial isoform of MDH was increased in PBMCs from vitiligo subjects. MDH acts as a shuttle for the NAD+/NADH across the mitochondrial membrane and favours elevation of NADH in mitochondria, providing the substrate to complex I. The increased activity of the MDH could account for an augmented intra-mitochondrial NADH level intended to produce physiological levels of ATP in a condition of impaired complex I activity (32).

The results described herein concur to delineate a pathogenetic scenario in which a mitochondrial defect may play an important role possibly correlated with a genetic background.

Previous reports have shown that in vitro, vitiligo melanocytes present an imbalance of antioxidants and are more susceptible to the toxic effects of cumene hydroperoxide and of UVB, both agents being capable of inducing intracellular ROS generation and alteration of mitochondrial function (8, 10). Moreover, an increased $\text{H}_2\text{O}_2$ level has been reported in vivo in the epidermis of active vitiligo patients (11). In addition, morphological or metabolic alterations in epidermal cells other than melanocytes have been previously reported in vitiligo, including keratinocytes and Langerhans cells (1). Pathological conditions associated with an oxidative
stress state that share a mitochondrial complex I dysfunction/deficiency include notably Parkinson’s disease, in which the defect of both complex I activity and the oxidative stress has been detected even in cells different from the pigmented dopaminergic neurons of the substantia nigra pars compacta (33, 34). In in vitro models with an inherited complex I defect, the exposure to rotenone precipitates cell degeneration (33). Whilst it is too early to draw any conclusion about the actual significance of this defect in vitiligo, the observation of alterations even in PBMCs, suggest a generalized metabolic impairment that occurs in vitiligo patients compatible with the genetic background of the disease.

Mitochondria, however, possess different threshold sensitivity to pro-oxidant agents depending on the cell type, and intracellular concentration of antioxidants and the level of polynsaturated fatty acids of the membranes – particularly polyunsaturated fatty acids of the membranes – seem to be favouring factors (28, 29).

In this context, exposure to different stimuli such as UV, mechanical trauma and/or release of pro-inflammatory cytokines or H$_2$O$_2$ in the epidermis, could further impair mitochondria with an increased intracellular ROS generation and alteration of the cellular metabolic activities.

According to the convergent theory, mitochondria could represent the target of the stressor stimuli generated by a variety of pathways and provide the biochemical basis for the disappearance of melanocytes. Consequently, as reported by various groups (11–13), supplementation with antioxidants could be advantageous in the treatment of the disease.

As recently proposed, stressor events can participate in the loss of dendricity and the detachment of melanocytes (melanocytorrhagy) possibly linked to a defect in the adhesion process (35). In this connection however, decreases in intracellular ATP content as well as in mitochondrial function have been reported to affect the intracellular routes of adhesion molecules which finally lead to the loss of intercellular and matrix adhesion (36, 37).

Acknowledgements – The study was partly supported by the grant from Italian Minister of Health 02/01/G/7.

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