New insight in vitiligo: immunopathology

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EXPRESSION AND MODULATION OF APOPTOSIS REGULATORY MOLECULES IN HUMAN MELANOCYTES: SIGNIFICANCE IN VITILIGO

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Abstract

Although the etiology of the hypopigmentary disorder vitiligo is ill understood it is clear that pigment producing cells are absent from vitiliginous lesional skin. The present study was designed to investigate the possible role of melanocyte expressed apoptosis regulatory molecules in melanocyte disappearance. Flow cytometric evaluation of p53, p21, Bcl-2 and Bax revealed no differences in \textit{in vitro} expression levels between normal control and nonlesional melanocytes. Moreover, no \textit{in situ} immunohistological differences were observed in melanocytes present in control, nonlesional and perilesional skin. However, an enhanced number of p53$^+$ nuclei, in the absence of detectable p21 expression, was detected in involved areas. The observed p53 expression pattern did not involve melanocytes and could be the result of UVA irradiation. Further, we showed that UVB is capable of modulating melanocyte expressed apoptosis regulatory molecules. Consequently, a lethal dose of UVB was given to two groups of cultured normal control and nonlesional melanocytes. No significant differences were found when comparing the percentages and kinetics of UVB induced apoptosis in these groups. In conclusion, our results indicate that the relative apoptosis susceptibility of melanocytes in vitiligo is comparable to that of normal control cells. It is, therefore, unlikely that vitiligo is causally related to disregulation of apoptosis regulatory molecules.

Introduction

Human melanocytes are the pigment (melanin) producing cells of the skin. Melanin protects the host from harmful ultra violet (UV) radiation\textsuperscript{1} and oxidative stress.\textsuperscript{2} Therefore, cells located in epidermis or dermis of depigmented skin are thought to be more susceptible to induction of cell death by environmental stress. Vitiligo is an acquired skin disorder that results in completely depigmented lesions, which may change in size and shape over time.\textsuperscript{3} Melanocytes are absent from these lesions\textsuperscript{4} and their destruction has been suggested to be a key event in the pathogenesis of this disease. Various hypotheses have been put forward to explain the disappearance of melanocytes in vitiligo, including the occurrence of melanocyte-specific autoimmunity.\textsuperscript{3,5} However, so far it's etiology is ill understood and no information is available concerning the mechanisms of melanocyte cell death. Apoptosis is a controlled process of cell death\textsuperscript{6} in which the dying cells are phagocytosed before lysis may occur. In contrast, necrosis is characterized by cell lysis and the subsequent release of potentially harmful cell contents may provoke an acute inflammatory response.\textsuperscript{7} Since acute inflammation of the perilesional area is
rarely seen in vitiligo\(^8\) it is considered for this study that melanocytes in vitiligo die by apoptosis rather than by necrosis.

Induction and execution of apoptosis are complex and well controlled processes. The Bcl-2 family of proteins constitutes one of the most relevant classes of apoptosis modifying gene products.\(^9\) Bcl-2 can protect a cell from a wide range of apoptotic stimuli\(^9\) and its importance in melanocyte survival is emphasized by the accelerated disappearance of melanocytes in Bcl-2 deficient mice.\(^10,11\) Another member of the Bcl-2 family of proteins, i.e. Bax, is an apoptosis agonist that can act via heterodimerization with Bcl-2.\(^9\) The ratio of Bcl-2 and Bax is, therefore, important in determining cell susceptibility to induction of apoptosis. Bcl-2 and bax gene expression can be regulated by the tumour suppressor p53.\(^12\) The cyclin-dependent kinase inhibitor p21 is an important mediator of p53 dependent induction of cell cycle arrest.\(^13\) In addition, p21 has been implicated in the induction of apoptosis.\(^13,14\) Clearly, disregulation of these well balanced mechanisms can lead to inappropriate cell death.

Altered expression of Bcl-2, Bax, p53 and p21 might cause an increased susceptibility to induction of apoptosis in melanocytes from vitiligo patients compared to those from healthy individuals. In the present study we therefore investigated the \textit{in situ} as well as \textit{in vitro} expression levels of these molecules in relation to vitiligo pathogenesis. UVB can induce cellular apoptosis by causing direct DNA damage\(^15\) as well as via the induction of reactive oxygen species.\(^16\) Therefore, we studied the UVB induced modulation of apoptosis regulatory molecules in human melanocytes. In parallel, we used the apoptosis inducing capacity of UVB to compare cultured nonlesional vitiligo and normal control melanocytes in their response to a lethal UVB dose.

\textbf{Materials and methods}

\textit{Tissue specimens}

Eight generalized type of vitiligo patients with progressive lesions were included in this study. Perilesional biopsies (3-mm \(\phi\)) were taken after marking carefully the pigmented side bordering the vitiliginous spot with ink. This facilitated the precise cutting of the biopsies, resulting in sections that contain both lesional and nonlesional skin. Nonlesional and lesional biopsies were taken at a distance of at least 5 cm from the perilesional area. Normal, asymptomatic control skin (n=4) was obtained from fresh plastic surgery material. All donors, vitiligo as well as normal controls, were Caucasian. Biopsies were snap frozen in liquid nitrogen and stored in -80 °C. Six \(\mu\)m-thick cryostat sections were cut, air dried and acetone-fixed;
subsequently, sections were stored at -20 °C until use.

**Melanocyte culture**

To investigate whether an imbalance of apoptosis regulatory molecules in nonlesional vitiligo melanocytes exists *in vitro*, cultured cells were not only compared to normal adult cells but also to less differentiated cells of the melanocyte lineage (fetal, neonatal, nevus). Melanocytes were isolated and cultured as described before. In short, fetal (n=4) and nevus (n=5) tissue, as well as nonlesional vitiligo shave biopsy specimens (n=5) were directly incubated in 0.1% trypsin. Alternatively, adult (n=5) and neonatal (n=5) skin tissue specimens were first overnight incubated in 0.5 mg/ml thermolysin (Sigma-Aldrich, Zwijndrecht, the Netherlands), after which the epidermis was removed and the tissue was incubated in trypsin. The resultant cell suspensions were plated in medium that consisted of Ham’s F10 (Gibco Life Technologies, Breda, the Netherlands) with 1% v/v Ultrocer G (Gibco), 10 ng/ml tetradecanoylphorbol 13-acetate (Sigma), 0.1 mmol/L 3-isobutyl-methylxanthine (Sigma), 100 IU/ml penicillin/100 mg/ml streptomycin (Gibco) and 2 mmol/L glutamine (Gibco). The cellular melanin content was determined as reported and was related to the total cellular protein content, measured according to Bradford.

**Antibodies**

| antibody     | specificity          | source            | dilution | dilution 
|--------------|----------------------|-------------------|----------|----------
| NKI-beteb    | melanocytes          | Sanbio            | 1:40     | not applicable |
| mouse MoAb   | (gp100)              |                   |          |           |
| EA10         | p21<sup>WAF1/CIP1</sup> | Oncogene Science | 1:25     | 1:10     |
| mouse MoAb   |                      |                   |          |           |
| 124          | Bcl-2                | Dakopatts         | 1:40     | 1:10     |
| mouse MoAb   |                      |                   |          |           |
| CM1          | p53                  | Novo Castra       | 1:1000   | 1:50     |
| rabbit PoAb  |                      |                   |          |           |
| P-19         | Bax                  | Santa Cruz        | 1:100    | 1:25     |
| rabbit PoAb  |                      |                   |          |           |
Specificity, source and working dilution of monoclonal (MoAb) and polyclonal (PoAb) antibodies used are listed in table 1. Using extracts from cultured melanocytes, Western blotting experiments were carried out according to Plettenberg et al. All antibodies bound specifically to proteins with the expected molecular mass as reported in literature (data not shown).

Immunohistochemistry

Immunohistochemical single and double stainings were performed as described earlier. Endogenous peroxidase was inactivated by preincubating the sections with 0.1% sodium azide/0.3% hydrogen peroxide. Control sections were incubated with isotype-matched control MoAbs (Dako, Glostrup, Denmark), or normal rabbit serum (DAKO).

Numbers of p53+ nuclei were determined for nonlesional, perilesional and lesional areas of the vitiligo patients, as well as for the normal skin specimens from control individuals. For the vitiligo patients, the comparison of p53+ nuclei in the three areas was statistically evaluated by the nonparametric Kruskal-Wallis test and, subsequently, by the Mann-Whitney test. Differences were considered to be significant when p < 0.05.

Flow cytometry

Melanocytes were detached from the culture flask by scraping with a rubber policeman. All cultures studied were between passages 2 and 6. Prior to staining of the cell suspensions for the intracellular antigens Bcl-2, Bax, p21 and p53, cell membranes were permeabilised. Briefly, cells were incubated in 2% paraformaldehyde for 10 minutes, followed by 10 minutes in 0.1% Triton-X-100. Cell suspensions were then incubated with primary antibody (diluted as described in table 1) for one hour, followed by biotinylated rabbit-anti-mouse IgG (Dako, 1:25) in case of mouse monoclonal primary antibodies, or biotinylated swine-anti-rabbit IgG (Dako, 1:200) in case of polyclonal rabbit primary antibodies. After 30 minutes cells were incubated with phycoerythrin-labelled streptavidin (Dako, 1:25). All incubations were carried out on ice and cells were washed after each incubation step, using PBS supplemented with 0.1% sodium azide and 2% Fetal Calf Serum. Negative controls consisted of Ig isotype-matched control MoAb's (Dako) or normal rabbit serum (Dako). Flow cytometric analysis was performed on a FACScan flow cytometer (Becton & Dickinson, San Jose (Ca), USA).
Data were analysed using WinMDI flow cytometry application software (http://facs.scripps.edu/). For data analysis viable melanocytes were gated according to their light scatter characteristics. Specific Mean Fluorescent Intensities (MFI) are after subtraction of control values, and Bcl-2/Bax ratios are obtained by dividing the respective MFIs. Results were tested for statistical significance using the nonparametric Kruskal-Wallis and Mann-Whitney tests and differences between groups are considered significant when p values are less than 0.05.

**UVB exposure**

As a UVB source for experimental induction of apoptosis, we used 4 Philips TL12 tubes with an emission spectrum between 250-400 nm and a peak at 315 nm. Irradiation was quantified using an IL 443 photo therapy radiometer with a SEE 1240 probe (International Light, Newburyport, MA, USA).

Four-mm Ø skin biopsies were taken from surgical material of a normal control donor and were cultured as described before.\(^2^5\) The skin explants were irradiated with a UVB dose of 200 J/m\(^2\) and control explants were not irradiated. At 16 and 24 hours after irradiation, explants were snap frozen in liquid nitrogen and stored at -80 °C. Immunohistochemistry was performed as described above. The experiment was performed in triplicate.

In addition, cultured melanocytes were examined. One million foreskin-derived melanocytes were seeded in a Petri culture dish. Cells were cultured for 4 days, and then irradiated with a UVB dose of 200 J/m\(^2\). During irradiation, medium was replaced with PBS. After 6 hours of additional culture, cells were harvested by trypsin treatment. Expression of Bcl-2, Bax, p21 and p53 was assayed by flow cytometry as described above. Baseline expression levels were determined in non-irradiated cultured melanocytes.

**Quantification of UVB-induced apoptosis of cultured melanocytes**

To avoid bias of results by differences in melanin-related protection to UVB irradiation, all nonlesional vitiligo and normal control cultures used in these experiments were selected to have comparable low melanin content.

One million cells were seeded, and four days later irradiated with a UVB dose of 40 J/m\(^2\). Two days after irradiation already detached as well as adherent cells were harvested from the cultures. Cells were resuspended in 100 µl of culture medium and incubated with 10 µg/ml of the cell-membrane permeable DNA binding dye Hoechst 33342 (Molecular Probes, Leiden, The Netherlands) for 15 minutes at 37 °C. Subsequently, cell-membrane impermeable propidium iodide (PI, Molecular Probes) was added for one minute at a concentration of 20 µg/ml. Cells
were then analysed using an epifluorescence microscope (Leitz, Wetzlar, Germany), and scored as apoptotic when chromatin was condensed or when nuclei were fragmented. In early stages of condensation cells were Hoechst+/PI-, and, due to secondary necrosis, Hoechst+/PI+ in late stages of condensation. Cells were scored necrotic when Hoechst+/PI- without chromatin condensation. For each culture at least one thousand cells were evaluated per time point. Statistical analysis was performed using the nonparametric Mann-Whitney test and differences are considered significant when p values are less than 0.05.

Results

**In vitro expression levels of p53, p21, Bcl-2 and Bax**

Cultured melanocytes were assayed for expression levels of p53, p21, Bcl-2 and Bax by flow cytometry. No significant differences in p53 and p21 expression were observed when comparing the average expression levels of cells in different stages of differentiation (data not shown). Interestingly, nevus cells seemed to have lower Bcl-2/Bax ratios when compared to the other differentiation stages (figure 1). However, these differences were not statistically significant.

\[ \begin{align*}
\text{fetal} & \quad \text{natal} & \quad \text{normal} & \quad \text{NL} & \quad \text{nevus} \\
(n=4) & \quad (n=5) & \quad (n=5) & \quad (n=5) & \quad (n=5)
\end{align*} \]

![Figure 1](image_url)

Figure 1. Bcl-2/Bax protein expression ratios in various melanocyte cell cultures, as determined from the respective mean fluorescence intensities by flow cytometry. Ratios are expressed as the mean +/- standard deviation.
In situ epidermal expression of gp100, p53, p21, Bcl-2 and Bax

Single staining with the anti-gp100 monoclonal antibody (NKI-Beteb) indicated the presence of gp100+ cells, that is, melanocytes, in the basal layer of the epidermis in normal control and in nonlesional skin and in the pigmented part of perilesional vitiligo skin. As expected, no melanocytes were observed in the lesional area of vitiligo skin.

Normal control and nonlesional vitiligo skin sections were mostly p53 negative, except for occasional cells with positive nuclei that were detected throughout the epidermis. Interestingly, higher numbers of p53+ nuclei were observed in lesional and/or perilesional vitiligo skin sections in 5 out of 8 patients (Fig 2).

![Figure 2](image-url)  
Figure 2. Number of p53+ nuclei/mm epidermis in skin biopsies from vitiligo patients and from control individuals. Numbers 1 to 8 correspond with different individuals.

Differences between lesional and nonlesional areas in this respect, were found to be statistically significant (p < 0.015). P53 staining patterns are illustrated for nonlesional (Fig 3a), perilesional (Fig 3b) and lesional (Fig 3c) areas of vitiligo skin from one patient (patient number 8). The highest number of lesional p53+ cells was observed in patient 8, who was the only patient receiving UVA therapy when biopsies were taken. P53/gp100 double-positive melanocytes were never observed in any of the investigated skin sections. Epidermal immunoreactivity to p21 was absent from all investigated biopsies.
The epidermal basal layer of all sections was weakly reactive to anti Bcl-2 antibody. In addition, cells with high Bcl-2 expression levels were detected at regular intervals in the basal layer of normal control and nonlesional skin and in the pigmented part of perilesional skin. These strongly Bcl-2 positive cells could all be double-stained for gp100 (Fig 4). Bcl-2 staining intensity of gp100+ cells in perilesional areas was comparable to that of melanocytes in normal control and nonlesional epidermis.

Figure 3. P53 expression in nonlesional (A), perilesional (B) and lesional (C) skin of patient 8 (original magnifications 100x).
Immunostaining by the anti Bax monoclonal antibody did not vary between normal control, nonlesional, perilesional and lesional vitiligo skin sections. Cells located in the basal and supra-basal epidermal layers expressed low cytoplasmic level of Bax. Staining for Bax expression in the upper epidermal layers was more intense and was detected in the cell nuclei (Fig 5).

**UVB modulated expression levels of p53, p21, Bcl-2 and Bax**

In cultured explants of non-irradiated skin, single stainings indicated few p21+ cells in the epidermis. Upon exposure to UVB, expression of p53 was also observed, and p21 expression had further increased, at both 16 and 24 hours p21 and p53 immuno reactive cells were detected throughout the epidermis. Double staining for gp100 also indicated the presence of a small number of p53 (Fig 6a) and p21 (Fig 6b) double positive melanocytes both at 16 and at 24 hours after UVB treatment; double-positive cells were not observed in non irradiated cultures.
Figure 6a. UVB-induced expression of p53 (red) in a fraction of gp100 positive (blue) melanocytes 24 hours after irradiation. Double-positive cells are indicated with arrows (original magnification 160x).

Figure 6b. UVB-induced expression of p21 (blue) in a gp100 positive (red) melanocyte, 24 hours after irradiation indicated with arrow, (original magnification 160x).

Melanocytes with high Bcl-2 expression were detected by immunodouble staining for gp100 in UVB-irradiated as well as in control cultures. Single staining experiments indicated that at sixteen hours after irradiation the expression level of melanocytic Bcl-2 was not changed in response to UVB (Fig 6c). However, at 24 hours after UVB irradiation, melanocytic Bcl-2 expression was markedly decreased in comparison to the expression in the non irradiated skin (Fig 6d). UVB treatment did not induce marked differences in Bax expression levels (not shown).
Exposure of cultured normal melanocytes to UVB *in vitro* did enhance the expression levels of p53, p21 and Bax, as observed by flow cytometry at 6 hours after irradiation. In contrast, the expression of melanocytic Bcl-2 was lowered (data not shown).

**UVB induced in vitro melanocyte apoptosis**

UVB irradiation of melanocytes *in vitro* at a dose of 40 J/m² does induce apoptosis. At 48 hours after irradiation chromatin condensation was observed as a characteristic morphological feature of late stage apoptotic cells (Fig 7). Dependent on the integrity of the cell membrane, condensed chromatin was observed at relatively early stages (Hoechst⁺, blue) as well as at relatively late stages (Hoechst⁺ / PI⁺, red).
The kinetics of UVB (40 J/m²)-induced apoptosis in a melanocyte culture were studied (Fig 8a). Chromatin condensation started to occur at approximately 24 hours after irradiation. Based on these kinetics, it was decided to compare susceptibility to induction of apoptosis of nonlesional vitiligo and normal control cells at 40 hours after UVB irradiation. No significant differences in cell death kinetics between nonlesional vitiligo and normal control cultures were detected, as indicated by the percentages of cells with early and late chromatin condensation as well as the total number of cells with chromatin condensation (Fig 8b).
Necrotic cells were observed in irradiated as well as nonirradiated cultures. The fraction of necrotic cells did not vary between nonlesional vitiligo and normal control cells and never exceeded 3%.

![Figure 8b. Percentages of apoptotic cells 40 hours after UVB (40 J/m²) irradiation, comparing normal adult cells (white bars) with nonlesional vitiligo derived cells (black bars). Cells with “early” stage chromatin condensation were Hoechst positive, cells with “late” stage chromatin condensation were Hoechst and Propidium Iodide positive. Data represent the mean +/- standard deviation.](image)

**Discussion**

The key feature of vitiligo is the absence of pigment producing cells in lesional skin, which is thought to be due to their destruction in situ. Since an acute inflammatory response is not a general finding in vitiligo, we hypothesized that melanocytes die in a controlled manner via apoptosis. Because melanocytes in patients with vitiligo may intrinsically be more susceptible to apoptotic stimuli we compared melanocytes from control individuals and from vitiligo patients both in situ and in vitro.

Bcl-2 and Bax were investigated because of their opposing important roles in regulating susceptibility to induction of apoptosis. Cultured nevus cells showed a tendency of lower ratios of Bcl-2/Bax expression levels than melanocyte cultures from control individuals. However, no significant differences were observed between nonlesional vitiligo and normal adult melanocytes in vitro. In accordance with earlier reports, in situ analysis revealed high melanocytic Bcl-2 expression in normal skin. Equally high Bcl-2 expression was observed in nonlesional and, most interestingly, also in perilesional melanocytes of vitiligo skin. In conjunction, the Bax expression...
apoptosis-related molecules in vitiligo

pattern in vitiligo skin specimens was also unchanged. Our observations do not completely exclude the possibility that melanocytes might change their Bcl-2/Bax ratio just prior to cell death. However, these results clearly indicate that melanocytes in vitiligo do not have intrinsically abnormal Bcl-2/Bax ratios.

Ratios of Bcl-2/Bax expression levels can be regulated by the tumour suppressor p53. An additional p53 target gene that may be involved in apoptosis is p21. Nonlesional vitiligo and normal adult melanocytes had comparable baseline in vitro expression levels for both p53 and p21. Likewise, nonlesional, perilesional and control skin specimens did not differ in the absence of p53+ or p21+ melanocytes in vivo. As a positive control, a fraction of the gp100+ melanocytes could be double-stained with anti-p53 or anti-p21 antibodies in normal skin biopsies exposed to UVB. In biopsies from 5 out of 8 vitiligo patients, we observed increased numbers of p53 nuclei predominantly in the epidermal basal layer in both lesional and perilesional areas. A similar p53 expression pattern has been reported to occur specifically in UVA-irradiated skin. Since all vitiligo biopsies, including those from the nonlesional area, were taken from sun-exposed areas of the body, our finding may reflect the absence of pigment-mediated UVA protection in both lesional skin and in the lesional side of perilesional skin biopsies. Despite the enhanced p53 expressions, p21+ cells were not detected in vitiligo skin biopsies. Because UV irradiation in vivo has been reported to induce expression of both p53 and p21 in normal skin, our observations may be vitiligo specific.

Subsequently, we investigated the possibility that the functional capacity of melanocytes to respond to an apoptotic stimulus in vitiligo patients, is different from that in control individuals. Both ex vivo and in vitro it was observed that UVB irradiation induces enhanced expression of p53 and p21 and down modulation of Bcl-2 in normal melanocytes. Correspondingly, UVB was found to induce in vitro melanocyte apoptosis, confirming earlier observations. In this model system, the kinetics of UVB-induced cell death revealed a broad time frame in which apoptosis occurred, indicating heterogeneity of the investigated melanocyte cell population. Since it is known that apoptotic stimuli often arrest growth before inducing cell death, this may be due to absence of cell cycle synchronization prior to irradiation. Importantly, susceptibility to induction of apoptosis did not appear to be different for nonlesional and normal control melanocytes. Our observations are compatible with a report from Im et al, in which they mention an equal decrease of viable melanocytes upon UVB exposure when comparing nonlesional vitiligo and normal control cells.

Melanocyte cell death in generalized vitiligo occurs in the absence of neutrophil influx and erythema. Therefore, we hypothesized that this form of single cell death might be due to an insufficient protective response to pro-apoptotic stimuli. Indeed, dysregulation of cellular
apoptotic pathways is thought to be involved in the pathogenesis of a wide variety of diseases. However, data obtained in the present investigation do not indicate that the apoptotic machinery of melanocytes in vitiligo patients differs from that in control individuals. Importantly, earlier observations from our group demonstrated that infiltrating T cells and macrophages can be observed adjacent to remaining perilesional melanocytes in generalized vitiligo (van den Wijngaard et al, submitted for publication). Additionally, it was shown that cytotoxic T cells (manuscript in preparation) and the macrophage mediator nitric oxide (Ivanova, personal communication) are both capable of inducing melanocyte apoptosis in vitro. Therefore, it is feasible that in vivo melanocyte apoptosis in vitiligo is caused by autoreactive T cells and/or macrophages. The present communication indicates, that such an autoimmune process is probably not causally related to changed apoptotic defences in melanocytes.

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References


CHAPTER 4

ABERRANT EXPRESSION OF COMPLEMENT REGULATORY PROTEINS, MEMBRANE COFACTOR PROTEIN (MCP) AND DECAY ACCELERATING FACTOR (DAF), IN THE INVOLVED EPIDERMIS OF PATIENTS WITH VITILIGO

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