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RESEARCH ARTICLE

Effects of diphencyprone on expression of Bcl-2 protein in patients with Alopecia areata

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Abstract

Background: Alopecia areata (AA) development is attributed to a T cell involved autoimmune process. Apoptosis is one of the suspected culprits in pathogenesis of this disorder. This disorder can be treated by contact sensitizers like diphencyprone (DPCP). We investigated the effects of treatment with DPCP on the expression of Bcl-2 protein in hair follicle epithelial cells of AA patients and its relation to clinical response to treatment.

Materials and Methods: Patients with chronic and extensive AA who had not received any treatment for at least 6 months were included. Furthermore, 3-mm punch biopsies were obtained from the affected areas before starting the treatment, and, six months after DPCP application, punch biopsies of the same size were taken from the following groups of patients: Group 1: six patients with complete hair regrowth, Group 2: six patients with partial regrowth, and Group 3: six patients with no regrowth. The samples were studied by immunohistochemistry to detect and compare the rate of Bcl-2 expression.

Results: Level of Bcl-2 expression in respondent patients (Group 1) was significantly higher after DPCP treatment (36.50 ± 4.23) compared to pretreatment state (3.67 ± 1.406, $P < 0.001$). Similar finding was observed in second group with partial regrowth (17.67 ± 1.745 versus 5.33 ± 2.076, $P < 0.01$). Such significant change was not observed in third group (4.75 ± 1.315 versus 3.50 ± 0.645, $P > 0.05$).

Conclusion: The results of this study indicate the positive effect of DPCP on regulation (inhibition) of apoptotic process in patients with AA.

Keywords: *Alopecia areata; apoptosis; Bcl-2; diphencyprone; immunohistochemistry*

Introduction

The exact etiology of Alopecia areata (AA) is unknown but it likely is in the category of autoimmune diseases. In pathology, the inflammatory cells infiltration is seen around and inside the hair bulbs in the areas surrounding the hairless area. These cells are predominantly T cells and a mixture of macrophages and Langerhans cells also exist. In this disorder, no inflammation seen around the stem cells thus the hair follicles are not damaged.[1] Different treatments exist for AA such as corticosteroid injection, topical minoxidil, anthralin and immunotherapy.[2] Among these, contact immunotherapy with diphencyprone (DPCP) is choice for widespread hair loss (>50%) and in resistant cases, however in a systematic review no randomized clinical trial was found on the use of DPCP.[3] DPCP can modulate the immune response in skin, thus can treat AA which has its origin in T cells with a change in ratio of CD4/CD8 cells around hair follicle.[4] Apoptosis is regulated by a plenty of proteins produced by number of genes which two pairs of them are Bcl-2 and Bax (Bcl-2 associated x-protein). Molecular

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regulation of keratinocytes apoptosis in hair follicle is of special complexity and is clinically important. Bcl-2 has an important role in hair follicle keratinocyte development during hair follicle cycle. In Bcl-2 lacked mice, skin appears with pigment reduction during next anagen and probably leads to increase in melanocytes death.[4,5] Bcl-2 proteins are found in external surface of mitochondria and are divided to three groups. One group are antiapoptotic like Bcl-2 and Bcl-xL. The second group are proapoptotic like Bax and BAD and the third group have apoptotic activity like Bik. If cell DNA is largely damaged and cannot be repaired it will undergo apoptosis in which activated p53 causes expression of Bax. Bax protein makes an orifice in external membrane of mitochondria from which cytochrome *c* exits which along with some other proteins make a complex named apoptosome.[6–8] Apoptosome has active role in activating the caspases which two-thirds of them are involved in apoptosis.[7,9,10]

This study was designed to determine if immunotherapy with DPCP changes the rate of expression of Bcl-2 protein on the hair follicles of AA patients.

Materials and methods

Patients with chronic widespread AA who had not received treatment for the last six months or had not been treated at all and had been referred to a referral university skin hospital were selected. The study was approved by ethics committee of Center for Research and Training in Skin Diseases and Leprosy and all patients provided written informed consent. Before starting the treatment 3-mm punch biopsies were taken from bald areas of 31 patients with AA (20 males and 11 females) and the same was repeated after 6 months of treatment with DPCP in 18 selected patients who used the treatment regularly including 6 patients who responded with complete hair regrowth (Group 1), six patients who had partial hair regrowth (Group 2) and six patients who did not respond at all (Group 3) consisting of four male and two female patients in each group. Complete response was defined as regrowth in 75% or more of the hairless area, partial and no-response were defined as 25–75% and <25% regrowth, respectively.

Samples were put in phosphate buffered saline 0.01 mol, and stored in –70°C. They were fixed in formalin, soaked in graded ethanol and then xylol. Sections were embedded in paraffin, cast and cut to tissue sections of 2–4 μm by microtome for immunohistochemistry (IHC) staining. All sections were dewaxed in xylene and rehydrated in graded ethanol. Antigen retrieval was performed by treating the sections with 10 mM sodium citrate and 1 mM ethylenediaminetetraacetic acid. The sections were incubated with primary antibody (monoclonal anti-Bcl-2, mouse ascites fluid) and secondary antibody (antimouse immunoglobulin G, whole molecule alkaline phosphatase conjugated, antibody developed in goat, affinity isolated antigen specific antibody, adsorbed human serum proteins). Endogenous alkaline phosphatase was inhibited by levamisole. Activity of alkaline phosphatase was assessed by bromo chloro indole phosphate, nitroblue tetrazolium.

In IHC, Bcl-2 protein takes the dye and this shows the amount of Bcl-2 expressed by cells quantitatively. The number of dots which had taken the dye per unit of area under florescent microscope was used as a quantitative scale for the expression of this protein.

The data were entered in SPSS software version 16.0 and were compared by paired student's *t*-test, Turkey, and ANOVA tests. $P < 0.05$ was accepted as significant.

Results

There was no significant difference in Bcl-2 expression between groups before treatment (Table 1). Level of Bcl-2 expression in respondent patients (Group 1) was significantly higher after DPCP treatment (36.50 ± 4.23) compared to pretreatment state (3.67 ± 1.406, $P < 0.001$). Similar finding was observed in second group with partial regrowth (17.67 ± 1.745 versus 5.33 ± 2.076, $P < 0.01$). Such significant change was not observed in third group (4.75 ± 1.315 versus 3.50 ± 0.645, $P > 0.05$, Table 1).

Change in Bcl-2 expression after treatment was calculated by subtracting baseline values from after treatment values for each patient and this difference was used for group by group comparison with Tukey test, which showed that in Group 1 increase in expression of Bcl-2 protein was significantly higher than Group 2 ($P < 0.05$) and Group 3 ($P < 0.01$, Figure 1). Moreover, the increase in expression of Bcl-2 protein in Group 2 was significantly more than Group 3 ($P < 0.05$, Figure 1).

Discussion

The effect of DPCP on expression of a plenty of biologic determinants involved in AA have been investigated by

Table 1. Bcl-2 expression rate before and after treatment with DPCP in patients with AA (mean ± SEM).

Group	Bcl-2 expression rate before DPCP treatment	Bcl-2 expression rate after DPCP treatment	*P* value (paired *t*- test)
1 (Complete regrowth)	3.67 ± 1.406	36.50 ± 4.233	0.001
2 (Partial regrowth)	5.33 ± 2.076	17.67 ± 1.745	0.011
3 (No regrowth)	3.50 ± 0.645	4.75 ± 1.315	0.431

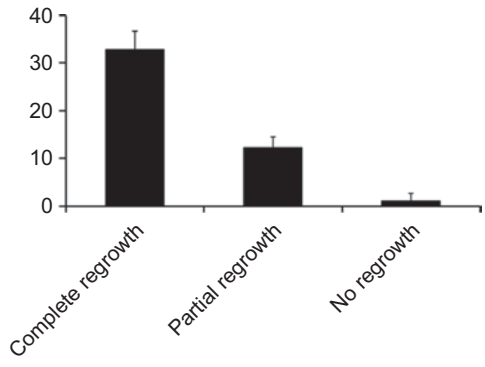

Figure 1. Increase in Bcl-2 expression (mean ± SEM) after 6 months of treatment with DPCP. (Change was calculated subtracting Bcl-2 values before treatment from values after treatment.)

immunohistochemistry staining of affected tissue before and after DPCP application. These include VEGF (vascular endothelial growth factor), factor VIII, survivin, p16, CD4, CD8, and skin-associated chemokine CCL27. After DPCP therapy there were increases in VEGF immunosensitivity, number of factor VIII expressing capillary vessels, p16 in hair follicle outer sheath, CCL27 in outer and inner root sheath along with basal interfollicular keratinocytes and CD8/CD4 ratio.[11] Also the expression of CD44 and CD49d, which are important factors in leukocyte extravasation was increased after DPCP treatment.[12]

To our knowledge, this is the first study showing that immunotherapy with DPCP can affect apoptosis pathway by affecting Bcl-2 expression, and this effect is correlated with the clinical response. In late anagen hair follicles, dermal papilla is strongly positive for Bcl-2 immunostaining. Bcl-2 is expressed in the dermal papilla throughout mature human hair cycle. Apparently, dermal papilla cells are resistant to the apoptotic forces in the hair cycling process by dominant Bcl-2 expression. Also the intense Bcl-2 immunoreactivity in the outermost cell layer of the follicular epithelium at the bulge level in all three stages has been detected. Bcl-2 expression is important for the maintenance of the follicular epithelial stem cells, and the formation of the secondary hair germ. Recently, significant difference was observed in Bcl-2 expression between the areas of the scalp that were clinically affected and unaffected in androgenetic alopecia. Bcl-2 expression was almost lost in the dermal papilla and the bulge area of AA.[13] Bcl-2 gene which causes Bcl-2 protein production is an apoptosis inhibitor. Considering the high rate of Bcl-2 expression in Group 1 after DPCP treatment, it can be concluded that increase in Bcl-2 expression leads to regulation (inhibition) of apoptosis which can be the cause of hair regrowth in AA patients.

The mechanism of action of DPCP in AA is not completely understood. DPCP can induce apoptosis in perifollicular lymphocytes and hair regrowth in AA induced in mice.[12] After treatment with DPCP, the number of CD8 lymphocytes increased in biopsy samples of AA patients.[14,15] In one of these studies a 600% increase in the number of CD8 lymphocytes under the epithelium and about a 250% increase in CD8 around hair bulbs and other epidermal appendages were shown after treatment with DPCP. These results were more significant in a group, which had good clinical response to the treatment.[14] It has been shown that after treatment with DPCP, anti-panCD44 inhibits the immigration of T cells toward lymph nodes and causes disease improvement.[16]

This study showed increased level of Bcl-2 expression in patients with AA who had improvement in hair growth after treatment with DPCP. Whether this increase in Bcl-2 expression is due to DPCP effect, or is it something that occurs with improvement in AA regardless of any particular treatment should be addressed in future studies including AA patients with spontaneous hair regrowth. On the other hand, only Bcl-2 expression was evaluated in this study due to budget restrictions. It is recommended to study other markers involved in cell cycle regulation and apoptosis in future studies.

Declaration of interest

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