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Original article

Reduced immunohistochemical expression of adhesion molecules in vitiligo skin biopsies

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ABSTRACT

Because defects in adhesion impairment seem to be involved in the etiopathogenesis of vitiligo, this study aimed to compare the immunohistochemical expression of several adhesion molecules in the epidermis of vitiligo and non lesional vitiligo skin.

Sixty-six specimens of lesional and non lesional skin from 33 volunteers with vitiligo were evaluated by immunohistochemistry using anti-beta-catenin, anti-E-cadherin, anti-laminin, anti-beta1 integrin, anti-collagen IV, anti-ICAM-1 and anti-VCAM-1 antibodies. Biopsies of vitiligo skin demonstrated a significant reduction in the expression of laminin and integrin. The average value of the immunohistochemically positive reaction area of the vitiligo specimens was 3053.2 μm^2, compared with the observed value of 3431.8 μm^2 in non vitiligo skin ($p = 0.003$) for laminin. The immuno-positive area was 7174.6 μm^2 (vitiligo) and 8966.7 μm^2 (non lesional skin) for integrin ($p = 0.042$). A reduction in ICAM-1 and VCAM-1 expression in the basal layer of the epidermis in vitiligo samples was also observed ($p = 0.001$ and $p < 0.001$, respectively). However, no significant differences were observed with respect to the expression of beta-catenin, E-cadherin, and collagen IV between vitiligo and non lesional skin.

Our results suggest that an impairment in adhesion exists in vitiligo skin, which is supported by the diminished immunohistochemical expression of laminin, beta1 integrin, ICAM-1 and VCAM-1.

1. Introduction

Vitiligo is an acquired depigmenting disorder that is characterized by the loss of functional epidermal melanocytes. Multifactorial and overlapping pathogenic mechanisms seem to be involved in its etiology [1]. A recent theory termed melanocytorrhagy has suggested that a primary epidermal defective cellular adhesion function may be involved in the loss of melanocytes in vitiligo [2,3].

Cell adhesion, which is critical for the regulation of tissue development and maintenance of tissue architecture, is regulated by various epidermal adhesion molecules (proteins) on the cell surface [4,5].

Interactions between melanocytes and keratinocytes are mediated by cadherins [6], which connect basal and suprabasal cells, and by catenins, which, in association with cadherins, form cell-cell adherence junctions [5,7].

Interactions between melanocytes and the basement membrane are mediated by integrins, which are adhesive proteins that are constitutively found on basal cells. Integrins mediate cell-cell and cell-matrix communication through a connection with collagen and laminin, among others [5,6,8,9].

Although they are present in smaller amounts, the expression of adhesion molecules from the immunoglobulin superfamily, such as intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), is also observed in the epidermis [5]. Both normal and vitiligo melanocytes have shown similarly low levels of constitutive expression of ICAM-1 [10]. However, it is unclear whether and how skin injuries can induce the inappropriate expression of ICAM-1 and other proinflammatory genes in melanocytes, as ICAM-1 expression has also been detected in melanocytes around active vitiligo patches as well as in surgically

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transplanted melanocytes [4,11]. Al-Badri et al. and van denWijngaard et al. also demonstrated the presence of ICAM-1 expression in perilesional melanocytes around active vitiligo patches. The expression of ICAM-1 by melanocytes may contribute to the abnormal immune response in vitiligo [12,13,14].

In normal skin, the expression of VCAM-1 has been observed on perivascular dendritic cells and some follicular keratinocytes [5,15]. In view of this, we decided to study the immunohistochemical expression of several adhesion molecules (beta-catenin, E-cadherin, lamninin, beta1 integrin, collagen IV, ICAM-1 and VCAM-1) found in the skin and to compare vitiligo and non lesional skin specimens.

2. Materials and methods

This study was approved by the Research Ethics Committee of Pontifical Catholic University of Paraná and was conducted according the principles of the Declaration of Helsinki.

Thirty-three patients who were evaluated by a single dermatologist (CCSC) were enrolled in this study. The main inclusion criteria were as follows: absence of topical or systemic treatment for vitiligo in the last 30 days, area for a biopsy of non lesional vitiligo skin with a 15-cm radius from any vitiliginous macules, as skin biopsies up to 15-cm that had already shown alterations that were not found in non lesional skin [11]. After signing an informed consent, two skin specimens (vitiligo and non lesional skin) were obtained with a 3 mm punch. The vitiligo specimen was obtained from a well-defined achromic area, without any signs of clinical inflammation (erythema).

Formalin-fixed paraffin-embedded skin samples were prepared using the tissue microarray (TMA) technique, mounted from the original paraffin blocks containing vitiligo and non lesional skin. The TMA blocks were sectioned to originate multisamples slides that were analyzed by using immunohistochemistry.

The immunoperoxidase procedure was used in for immunohistochemistry, as described by Chong [16]. Each immunostaining reaction included positive controls (skin from adults without vitiligo) and negative controls (without incubation with the primary antibody). To observe the immunohistochemical expression of beta-catenin, E-cadherin, ICAM-1, VCAM-1, laminin, beta1 integrin and collagen IV in vitiligo and non lesional skin using immunohistochemistry, the following primary monoclonal antibodies at their respective dilutions were used: from Novocastra® (New Castle upon Tyne, United Kingdom): anti-beta-catenin (1:800), anti-E-cadherin (1:100), anti-ICAM-1 (1:100), and anti-VCAM-1 (1:200). Polyclonal anti-laminin (1:800) from DakoCytomation® (Glostrup, Denmark) anti-beta1 integrin (CD29) (1:400) from Epitomics (Burlingame, California), and anti-collagen IV (1:100) from Santa Cruz Biotechnology, Inc (Europe).

All TMA staining procedures included both a negative control (which was missing primary antibody) and a positive control (normal skin, from a non vitiligo patient). An immunoperoxidase assay with modifications was part of the immunohistochemistry, as reported by Chong et al. [16]. Antigen retrieval was performed using a BioSBTMImmunoRetriever. Tissue samples were incubated with the primary antibodies in a moist chamber at room temperature for one hour. Incubations with the secondary antibody (Dako AdvanceTMHRP System,DakoCytomation, Inc., CA, USA) were carried out for 30 min. Incubations with 3,3-diaminobenzidine and hydrogen peroxide substrate (DakoCytomation, Inc., CA, USA) were performed for 3 min to visualize positive staining.

All specimens underwent optical microscopic analysis using a BX50 Olympus® (Tokyo,Japan), coupled to a Dinoeye video camera enhanced by image analysis software Image Pro Plus™ (Maryland, USA). For each sample, three fields of each skin specimen (200x) underwent the analysis. The observer (ARF) was unaware of the skin type and was only informed of the antibody that was used. Samples that demonstrated an inappropriate reaction were excluded.

One high-quality section of immunohistochemical staining (control and skin control) for anti-beta-catenin, anti-E-cadherin, anti-laminin, anti-beta1 integrin and anti-collagen IV was chosen to serve as a "mask", which contained adequate levels of positive tissue immunoexpression signal. The mask was then superimposed to the samples photomicrographs. Based on the ideal positive tissue immunoexpression signal obtained from the mask the image analysis software Image Pro Plus™ identified the positive areas in the samples and is able to transform these results into a positive tissue immunoexpression area per square micron (μm^2). For each case, an average of positive area was determined in three images.

In this field, the observer manually selected the amount of dark brown color to be considered positive antibody expression, establishing a pattern to be followed in the morphometric analysis, performed by the Image Pro Plus™ software to minimize analysis errors.

This pattern was followed using three high-power fields (HPF) of each sample of beta-catenin, E-cadherin, laminin, beta1 integrin and collagen IV staining were selected (Olympus BX50–HPF = 400X) and evaluated using Image Pro Plus® for morphometric analysis. The "mask" was used to compare the expression of such molecules between vitiligo and non lesional skin through the total area/HPF of the positive immunohistochemical reaction obtained (the results are presented as μm^2).

For ICAM-1 and VCAM-1 staining (Olympus BX40–HPF = 400X), a qualitative evaluation was performed, and the epidermal localization of positive immunohistochemical reactions was specified (the presence or absence of basal cell predominance).

The resultant data were analyzed using the IBM SPSS Statistica v.20.0 software, and paired Student's *t*-test (vitiligo and non lesional skin of the same patient) was used for quantitative variables. The variables normality condition was evaluated by the Kolmogorov-Smirnov test. A p value < 0.05 indicated statistical significance.

3. Results

The median age of the individuals studied was 42.8 years, and the most common type of vitiligo was vitiligo vulgaris (75.8%). The clinical characteristics of all included patients and the biopsy location sites are described in Table 1. The numeric results for the immunochemistry of adhesion molecules and the comparison of the thickness between vitiligo and non lesional skin are described in Tables 2 and 3. The averages of the immunohistochemical positive areas for laminin ($p = 0.003$), beta1 integrin ($p = 0.042$), ICAM-1 ($p = 0.001$) and VCAM-1 ($p < 0.001$) in vitiligo samples were lower than those obtained for non lesional skin ($p < 0.001$) (Fig. 1).

There was no pathological sign of inflammation in all samples (vitiligo and non lesional skin). The slides immunostained with anti-beta-1 integrin and anti-laminin antibodies revealed immunopositivity between epidermal keratinocytes. This immunopositivity was marked in the basal portions of non lesional epidermis with a decrease in the intermediate portions and it was virtually absent in the upper layers (Fig. 1B and D). This aspect presented attenuated in vitiligo skin as shown in Fig. 1A and C. Immunostained with anti-beta catenin and anti-E cadherin antibodies revealed immunopositivity in the full extent of the epidermis with a chicken wire pattern (Fig. 1F and H), the same pattern seen in vitiligo skin, but with slight attenuation as shown in Fig. 1E and G. The samples immunostained with anti-ICAM-1 and anti-VCAM-1 antibodies show light immunopositivity only in basal and