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Impairment of Angiogenesis in Patients with Granuloma Annulare and Necrobiosis Lipoidica

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Abstract

Necrobiosis lipoidica (NL) and granuloma annulare (GA) belong to the granulomatous skin diseases with unclear pathogenesis. Despite the quite common occurrence of these entities in dermatological practice, research into the subject is limited. Thus, we decided to perform an immunohistochemical analysis of skin biopsies in order to assess the expression of selected proteins involved in angiogenesis. The study group consisted of 21 patients with NL and 23 with GA, selected from the database at the Department of Dermatology, Medical University of Łódź. Six healthy subjects served as the controls. Skin sections were stained with monoclonal antibodies directed against VEGF and CD34. The intensity of expression of epidermal VEGF, and the number of $CD34^+$ dermal blood vessels were assessed. The mean intensity of VEGF immunoexpression in GA patients was 0.91, and was significantly higher than in either the NL patients (0.45; $p<0.01$) or the control group (0.14; $p<0.009$). The mean CD34+ microvessel density per mm^2 in the GA group was 79.04, which was significantly higher than in the NL group (64.84; $p<0.009$) and in the controls (52.03; $p<0.001$). The obtained results confirm the similarity of the histological features of NL and GA. However, in GA, the biopsy changes in angiogenesis were more marked in the GL than in the NL group. In conclusion, based on our results we can assume that imbalance in the process of angiogenesis is one the factors involved in development of both NL and GA.

Keywords: Necrobiosis Lipoidica; Granuloma Annulare; Angiogenesis; $Cd34^+$ Microvessels

Introduction

Necrobiosis Lipoidica (NL) and Granuloma Annulare (GA) belong to the granulomatous skin diseases with unclear pathogenesis. Despite the quite common occurrence of these entities in dermatological practice, research into the subject is limited. Microangiopathy and immunological disturbances, followed by immune vascular deposits, are regarded as potential pathogenic factors in NL [1-4]. Other authors postulate that NL is a connective tissue disorder and that subsequent skin inflammation leads to the development of skin lesions [5]. According to data from the literature, the pathological processes in GA are initiated in the dermis by activation of $CD4^+$ T-lymphocytes and the synthesis of proinflammatory cytokines i.e., of IL-2 and TNF-α- which leads to the release of lysosomal enzymes including metalloproteinases [6-8]. Skin damage results in the enhanced synthesis of collagen I [6]. Many authors have suggested that elastic fiber degeneration is the primary event that subsequently provokes the granulomatous response, and the collagen degeneration is a secondary effect [9-11]. Japanese researchers believe that skin lesion relapses in GA result from uncontrolled inflammation mediated by apoptosis [12]. Because of the limited data on the pathogenesis of these two entities, we decided to perform an immunohistochemical analysis of skin biopsies in order to assess the expression of selected proteins involved in angiogenesis.

Material and Methods

The study group consisted of 21 patients with NL (15 women, 6 men; mean age 61.3 y.o.) and 23 with GA (13 women, 10 men; mean age 53.4 y.o.), selected from the database at the Department of Dermatology, Medical University of Łódź. Six healthy subjects (3 men, 3 women, mean age 50.8 y.o.) served as the controls. Each individual gave written informed consent before entering the study, and the experimental protocol was approved by the local Ethics Committee of the Medical University of Łódź. All subjects underwent a thorough physical examination and the appropriate laboratory investigations. The diagnosis of NL or GA was based on the usual clinical picture and skin histology. Three mm punch biopsies were collected from the lesions of each patient and from the healthy skin of the controls. The biopsies were formalin-fixed and paraffin-embedded. The paraffin-embedded tissue sections were mounted onto SuperFrost slides, deparaffinized, and treated in a microwave oven in a solution of TRS (Target Retrieval Solution, Dako) for 30 minutes (2×5 minutes at 360W, 2×5 minutes at 180W, 2×5 minutes at 90W) and transferred to distilled water. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in distilled water for 30 minutes, and then the sections were rinsed with Tris-buffered saline (TBS, Dako, Denmark). To determine the expression of the proteins being investigated, we used monoclonal antibodies directed against CD34 (clone QBEnd 10; Dako, Denmark) and against VEGF (clone VG1, Dako Denmark). Immunoreactive proteins were visualized using an EnVision horseradish peroxidase kit (Dako, Carpinteria, CA, USA) according to the manufacturer's instructions.

Semiquantitative evaluation

In each specimen, the staining intensity of VEGF was recorded semiquantitatively by two independent observers under 5-7 adjacent high power fields, and was graded as 0 (staining not detectable), 1 (weak immunostaining), 2 (moderate immunostaining intensity), or 3 (strong staining). The mean grade was calculated by averaging the grades assigned by the two observers and approximating the arithmetical mean to the nearest unity.

Morphometry

Histological morphometry was performed by means of an image analysis system consisting of a PC computer equipped with a Pentagram graphical tablet, an Indeo Fast card (frame grabber, true-color, real-time; produced by Indeo, Taiwan), and a Panasonic color TV camera (Japan) coupled to a Carl Zeiss microscope (Germany). This system was programmed (MultiScan 8.08 software by Computer Scanning Systems, Poland) to calculate the number of objects (automatic function with manual correction, as needed).

The colored microscopic images were saved serially in the memory of a computer, and then quantitative examinations were carried out. The microvessel density ($CD34^+$) was measured in ten monitor fields (0.0205 mm^2 each), and the results were expressed as the number of the microvessels per mm^2.

Statistical methods

All values were expressed as the mean ± SD (standard deviation). The differences between groups were tested using Student's *t*-test for independent samples, preceded by an evaluation of normality and of homogeneity of variances with Levene's test. Additionally the Mann–Whitney *U*-test was used where appropriate. Correlation coefficients were calculated using Spearman's method. The results were considered statistically significant if $p<0.05$.

Results

In the GA and NL groups, the cytoplasmic staining for VEGF protein was detected in epithelial and endothelial cells, and in some cases on inflammatory infiltrates (Figure 1).

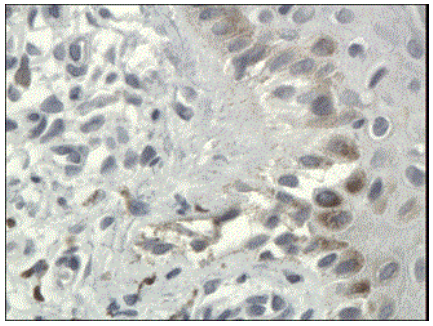

Figure 1: The immunoexpression of VEGF in epithelial cells and inflammatory infiltrates in GA group; magnification 400X

In the controls, the immunoexpression of VEGF protein was found in the keratinocytes of normal epidermis and in endothelial cells (Figure 2).

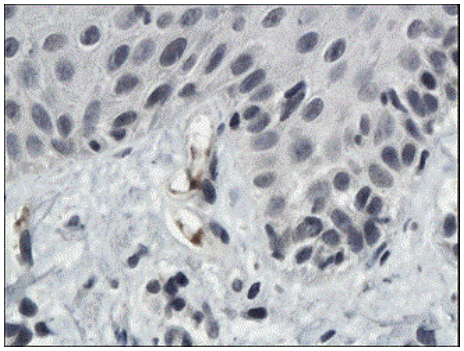

Figure 2: The immunoexpression of VEGF in endothelial cells in NL group; magnification 400X

The mean intensity of VEGF immunoexpression in GA patients was 0.91 ± 0.65, and was significantly higher than in either the NL patients (0.45 ± 0.43; $p<0.01$) or the control group (0.14 ± 0.17; $p<0.009$) (Figure 3).

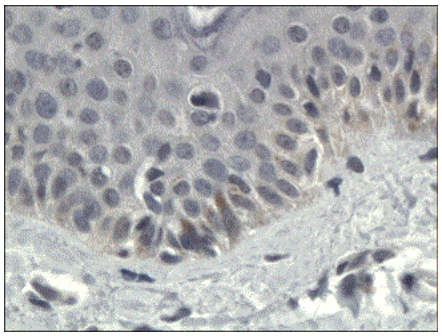

Figure 3: Weak cytoplasmic immunoexpression of VEGF in control group; magnification 400X

Although the relative values of VEGF expression were higher in NL patients than in the controls, there were no significant differences between the measured values ($p=0.10$) (0.45 vs. 0.14) (Figure 4).

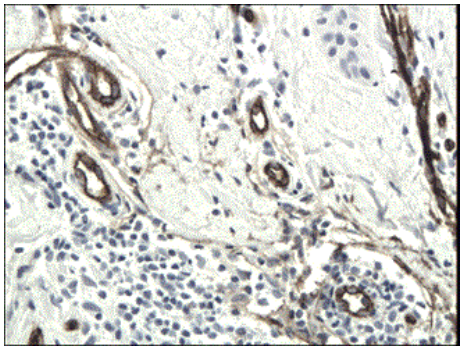

Figure 4: The immunoexpression of CD34 in endothelial cells in GA group; magnification 200X

CD34 immunoexpression was detected in both the GA and NL groups. In the control group, it was found exclusively in endothelial cells (Figure 5).

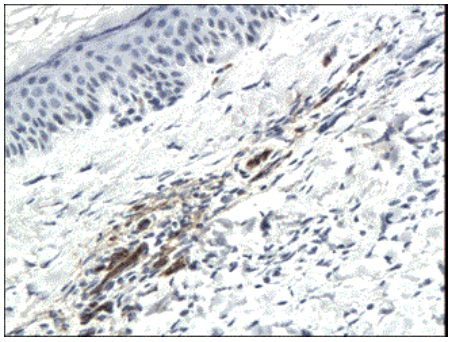

Figure 5: The immunoexpression of CD34 in endothelial cells in NL group; magnification 200X

The mean $CD34^+$ microvessel density per mm^2 in the GA group was 79.04 ± 16.31, which was significantly higher than in the NL group (64.84 ± 18.10; $p<0.009$) and in the controls (52.03 ± 4.91; $p<0.001$) (Figure 6).

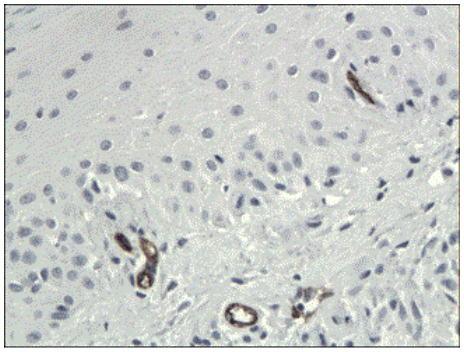

Figure 6: The immunoexpression of CD34 in endothelial cells in control group; magnification 200X

No statistical difference was found between $CD34^+$ microvessel density per mm^2 in NL patients (64.84) and in controls (52.03) ($p=0.10$). The detailed results are presented in Table 1.

	GA (n=23)	NL (n=21)	Controls (n=6)	P values
VEGF (mean score)	0.91 ± 0.65	0.45 ± 0.43	0.14 ± 0.17	$p<0.01^a$, $p<0.009^b$, $p=0.10$ (NS)^c
Microvessel density (per mm^2)	79.04 ± 16.31	64.82 ± 18.10	52.03 ± 4.91	$p<0.009^a$, $p<0.001^b$, $p=0.10$ (NS)^c

Table 1: A comparison of semiquantitative and quantitative data on VEGF immunoexpression and microvessel density in GA, NL, and control groups a) between GA and NL b) between GA and controls c) between NL and controls

We found positive correlations between VEGF expression and $CD34^+$ microvessel density in both NL and GA groups (Table 2).

Pair of variables	GA (n=23)	NL (n=21)	Control group (n=6)
VEGF vs. CD34	r=0.52, p<0.01	r=0.46, p<0.04	r=0.69 p=0.12 (NS)

Table 2: Correlation between immunoexpression of VEGF and CD34 in GA, NL, and controls

Discussion

Angiogenesis is the formation of new blood vessels from the existing vascular bed. Increased vascular growth has also been demonstrated in inflammatory skin diseases, including psoriasis [13,14]. The migration of endothelial cells introduced by vascular endothelial growth factor (VEGF) is a critical step in angiogenesis. VEGF is a multifunctional peptide that is capable of inducing endothelial cell proliferation and angiogenesis both in vivo and in vitro. VEGF induces microvascular hyperpermeability, and its angiogenic effect plays a role in a variety of physiological and pathological conditions-including wound healing, blister formation, and skin neoplasia [15-18]. The raised plasma levels of VEGF have been noted not only in vascular tumors of the skin (such as Kaposi's sarcoma) but also in chronic inflammatory and autoimmune diseases. It is also known that VEGF-induced angiogenesis and increased vascular permeability may promote chronic inflammation [19]. CD34 antigen is presented on the surface of vascular endothelial cells and hematopoietic cells, and its expression plays a significant role in angiogenesis, not only in embryos, but also in adults. It is supposed that endothelial cell injury is an essential pathogenic factor in the development of inflammatory and autoimmune diseases [20].

Data on the pathogenesis of NL and GA is scarce. Their common occurrence and therapeutic problems were the reasons that we decided to perform the present study. The characteristic features of both dermatoses include collagen and elastic fiber degeneration, as well as immune disturbances [21]. According to some authors, GA consists of a delayed-type hypersensitivity reaction with increased synthesis of IL-2, favoring Th1 response [22,23]. The fact of the coexistence of necrobiosis lipoidica and granuloma annulare with diabetes mellitus may testify that microangiopathy is one of the pathological

phenomena leading to their development. This was the motivation for our decision to perform an immunohistochemical study to assess the expression of CD34 and VEGF in skin lesions. Any imbalance in the expression of proteins might demonstrate enhanced angiogenesis in NL and GA. The lack of data on the subject in the literature makes our results original. In one of the studies, biopsies taken from GA patients were analyzed by direct immunofluorescence. The analysis showed bound in vivo IgM and C3 deposits around vessels, which might suggest the presence of vasculitis of autoimmune origin [24].

In our study, we showed a higher expression of VEGF in GA patients than in NL subjects and controls. The enhanced expression of this protein in the patient group (in spite of the lack of statistical difference between the NL group and the controls) points to the role of imbalance in angiogenesis in the pathogenesis of both diseases. Similar observations concern the CD34 (+) cells, a larger number of which was found in GA biopsies. The results obtained confirm the similarity of the histological features of NL and GA; however, in GA, the biopsy changes in angiogenesis were more marked in the GA than in the NL group. In conclusion, based on our results we can assume that imbalance in the process of angiogenesis is one the factors involved in development of both NL and GA.

Funding source:

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