ORIGINAL ARTICLE

Effectiveness of photodynamic therapy in the treatment of Lichen sclerosus: cell changes in immunohistochemistry

Anita Olejek [1], Iwona Kozak-Darmas [1], Sylwia Kellas-Sleczka [1,2], Katarzyna Steplewska [3], Tomasz Biniszkiewicz [4], Beata Birkner [4], Anna Jarek [1], Leszek Nowak [1], Krystyna Stencel-Gabriel [5], Aleksander Sieron [4]

1 Department of Gynecology, Obstetrics and Oncological Gynecology, Bytom, Medical University of Silesia;
2 Department of Brachytherapy, Maria Skłodowska-Curie Memorial Cancer Centre – Institute Oncology, Gliwice;
3 Department of Histopathology, Zabrze, Medical University of Silesia;
4 Department and Clinic of Internal Diseases, Angiology and Physical Medicine, Bytom, Medical University of Silesia;
5 Department of Pediatrics, Bytom, Medical University of Silesia; Poland.

Correspondence to: Professor Anita Olejek, MD, PhD
Department of Gynecology, Obstetrics and Oncological Gynecology, Bytom, Medical University of Silesia SUM, ul.Batorego 15, 41- 902 Bytom, Poland.
PHONE +48 032 7861540; FAX +48 032 7861647;
E-MAIL: bytomobstgyn@sum.edu.pl

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Abstract

BACKGROUND: Vulvar lichen sclerosus (LS) affects primarily women at postmenopausal age and its background remains unknown. One of the treatment modalities is photodynamic therapy (PDT). The aim was to investigate the efficacy of PDT in women with LS and the analysis of protein expression before and after PDT. **MATERIAL AND METHODS**: From 04.2006-01.2008 28 women, with LS underwent photodynamic diagnosis and next PDT: six-courses every second week with using 5-aminolevulinic acid (ALA) as a photosensitizer. Punch biopsies were taken before and after treatment and immunohistochemy was done with Ki67,CD44,CD34 and CD3. **RESULTS**: Before PDT all patients suffered from pruritus and after in 89.3% the relief was noted. The histological examination showed that 35.7% patients hadn't LS after therapy completion. Anti-CD44 staining intensities was scored qualitatively - there were no statistical difference at the expression of protein CD44 in the epidermis ($p>0.05$) before and after therapy. Microvessel density was assessed at the hot spots, marked with anti-CD34. Statistical difference in AVD before and after therapy: ($p<0.05$). The staining intensity of Ki-67 didn't differ before and after PDT ($p>0.05$). The expression of CD3 on T lymphocytes showed statistical difference of the lymphocytic infiltration before and after PDT ($p<0.05$). **CONCLUSION**: The immunohistochemical staining in vulvar LS showed increasing microvessel density and decreasing lymphocytic infiltration. There were a clinical, and less histological improvement in patients with LS. We suggest that the photodynamic therapy is an effective, alternative treatment in some but not all patients with LS. Therefore, further studies are needed.

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INTRODUCTION

Lichen sclerosus (LS) is a chronic disease of unknown pathogenesis, which is mostly found in women at postmenopausal age. Its main symptoms are pruritus and discomfort (Rolfe *et al.* 2002) that appear independently of an inflammation or other external factors. Lichen sclerosus affects both the epidermis and the dermis (Farell *et al.* 1999, Lukowsky *et al.* 2000).

Many recent studies focus on the pathomechanism of LS development (Farell *et al.* 1999, Li *et al.* 2004, Rolfe et al.2002, Rolfe et al. 2001). Some researches study the role of the immune system in the LS pathogenesis. e.g. the role of HLA, Ki67, MVD or T lymphocytes, in LS development (Rasspollini *et al.* 2007, Rolfe *et al.* 2002, Tchórzewski *et al.* 2005). A connection between autoimmune diseases and autoantibody occurrence was reported in LS patients and their families. A special interest was focused on the importance of HLA-DQ7, HLA-DQ8 and HLA-DQ9 in patients with LS (Lukowsky *et al.* 2000).

It is crucial to understand the background of LS because this disease may become a precursor of vulvar cancer in a small percentage of women (Bamberger *et al.* 2002 , Rolfe *et al.* 2001). Therefore, it is important to early diagnose and treat possible premalignant lesions. One of the treatment modalities includes photodynamic diagnostics (PDD) and then photodynamic therapy (PDT). PDT enables the finding of a lesion with an inappropriate fluorescence. Then, the idea of the photodynamic therapy is to perform a selective excision of a diseased tissue, in which an oxygen-dependent reaction between photosensitizing dye and light leads to the destruction of tissue (Campbell *et al.* 2004 , Hillemanns *et al.* 2000, Olejek *et al.* 2004). LS is affecting both the epidermis and the dermis. Thus, there are several markers that influence changes in the skin with lichen sclerosus. Firstly, CD44 was suggested as a regulator of keratinocyte proliferation (Kaya *et al.* 2000), secondly CD34 allow to mark the intraepithelial cells of the blood vessels and differentiate the microcirculation dysfunction.

THE AIM

The aim of this study was to investigate the efficacy of PDT in women with lichen sclerosus and to analyze protein expression before and after PDT application. In addition, we provided a subjective assessment of symptoms in patients before and after therapy.

MATERIAL AND METHODS

From April 2006 to January 2008, all 28 women treated at the Clinic of Vulvar Diseases, Department of Gynecology, Obstetrics and Gynecological Oncology with histological diagnosis of LS underwent PDT after PDD. All patients primarily underwent pharmacological treatment without clinical or histological effects.

In the diagnostics protocol Xillix Oncolife System laser was used, and then a six-course of photodynamic therapy using Diomed 630 laser was applied. The study protocol contained a course of PDT every second week. 5-aminolevulinic acid (ALA), which is preferentially absorbed and induces protoporphirin IX accumulation in changed tissue, was used as a photosensitizer. Multiple punch biopsies were taken before and after treatment and tested with monoclonal antibodies Ki67 (N1633), CD44 (M7082), CD34 Class II (N1632), CD3 (RTU-CD3-PSI) (DAKO). Markers were determined by immunohistomorphometric analysis using a three-point analysis ABC (Avidyn-Biotyn-Complex). Immunohistochemical staining was performed according to manufacturer instructions. The expression of markers was compared with a control tissue: Ki67 and CD3 with tonsil tissue, CD34 with hemangioma tissue and CD44 with skin tissue. For tissue evaluation light microscope BX51 was used and the immune reaction had been estimated by 2 independent people at double blind trial. Anti-CD44 staining intensities of skin sections (membrane reaction) was scored qualitatively on a four- point scale of: 0- no staining, 1- weak staining, 2- moderate staining, 3-strong stainig as described by Kaya *et al.* (2000).

Microvessel density (MVD) was analyzed at the neovascularization areas in the dermis (hot spots) (marked with anti-CD34 monoclonal antibody) (Saravanamuthu *et al.* 2003). With a 100x objective, the hot spots were detected and then with a 200x objective, the microvessels were numbered at three different field-of-view (area= 0,950 mm^2). The highest (HVD) and the average (AVD) MVDs were quantified for each sample under high magnification (x200) using an image analysis system.

Inflammatory infiltrations in the dermis were assessed semi-quantitatively using a four- point scale: 0- no inflammation, 1- small-grade infiltration, 2-medium-grade infiltration, 3-high-grade infiltration.

The expression of CD3+ T lymphocytes in the inflammatory infiltration of the dermis was obtained semi-quantitatively on a six- point scale: 0 – lack of CD3+ cells, 1- single CD3+ cells, 2- under 10 % of CD3+ cells, 3 – from 11 to 30 % of CD3+ cells, 4 – from 31 to 60 % of CD3+ cells, 5 – from 61 to 100 % of CD3+ cells (Gross *et al.* 2001).

Epidermis cell proliferation was detected using Ki67- antibody. At 400x objective, the number of positive reactions (dark- brownish cell nucleus)/ 1000 epidermis cells was analyzed and the results are shown as percentage of nucleus/ 1000 cells.

All women completed PDT and had immunohistological examinations performed before and after PDT. All women had vulvar bacterial samples taken. In case of abnormal results, they were treated according to sensitivity to antibiotics.

The study was approved by the Local Ethical Committee of the Medical University of Silesia, and written informed consent was obtained from all participants.

Measurements of the response rate of PDT were analyzed with the Wilcoxon test. For statistical analysis the median was used to evaluate the change of Ki67, CD3, CD44, CD34 before and after treatment. The level of significance (*p* value) was set at .05 for all tests.

RESULTS

The mean age of women was 57.8 years (min.32–max. 71). A histological examination confirmed lichen sclerosus of vulva in all 28 patients.

Before PDT, all patients suffered from pruritus at the external genital area and 2 of 28 suffered from discomfort; however, none of the women claimed to have pain.

After PDT, in 25 patients the relief of pruritus was noted, but 3 patients still had discomfort at the external genital area. The histological examination showed that 18 of 28 patients still had lichen sclerosus after therapy completion. 7 women were diagnosed with vulvitis chronica with focal hyperkeratosis and three others had vulvitis chronica only.

Anti-CD44 staining intensities of skin sections (membrane reaction) was scored qualitatively on a four- point scale. We have not found any statistical difference at the expression of protein CD44 in the epidermis. CD44 expression before and after PDT is shown in Table I.

Microvessel density was assessed at the hot spots (region of the most intensive neovascularization at the dermis) and we found a statistical difference in AVD before and after therapy (Fig. 1). Results of AVD measurement are shown in Table II ($p<0.05$).

The staining intensity of Ki-67 did not differ before and after PDT ($p>0.05$) and Table III shows the results of the staining.

Finally, we assessed the expression of CD3+ on T lymphocytes using the semi-quantitative 6-grade scale. Mean epidermal cell proliferation rate before therapy was 22,3% and after PDT 19,5% (Fig. 2). The detailed results of CD3+ T cells infiltration in the skin sections is presented at Table IV. The lymphocytic infiltration at the lesions was acquired using a six-point semi-qualitative scale. There was a statistical difference of the lymphocytic infiltration before and after PDT ($p<0.05$).

Table I. CD44 expression in the skin sections of patients with LS ($p>0.05$).

Reaction intensity	Before PDT (n=28)	After PDT (n=28)
0 – no staining	0	0
1 – weak staining	6	8
2 – moderate staining	8	9
3 – strong staining	14	11

Table II. The average microvessel density (AVD) in the skin sections of patients with LS

AVD	Before PDT (n=28)	After PDT (n=28)
Median	53.66	68.66
Mean	54.16	65.58
CI 95%	47.15–61.17	59.62–71–54
Minimum	27.33	40.33
Maximum	78.00	86.33
Standard deviation	18.07	14.11

Table III. presents Ki-67 expression in sections with lichen sclerosus.

Ki67	Before PDT (n=28)	After PDT (n=28)
Median	22.55	19.70
Mean	22.34	19.54
CI 95%	17.91–26.76	15.84–23.24
Minimum	1.30	7.10
Maximum	39.60	35.40
Standard deviation	11.41	8.76

Table IV. The expression of CD3 on T cells in the skin infiltrate of patients with LS.

Reaction intensification	Before PDT (n=23)	After PDT (n=23)
0 – lack of CD3+ cells	–	3
1 – single CD3+ cells	–	–
2 – under 10 % of CD3+ cells	–	–
3 – from 11 to 30 % of CD3+ cells	–	6
4 – from 31 to 60 % of CD3+ cells	9	6
5 - from 61 to 100 % of CD3+ cells	14	8

DISCUSSION

Lichen sclerosus is not recognized as a premalignant lesion, but it may lead to vulvar cancer (Rolfe *et al.* 2001). It was reported that 40% of patients with vulvar planoepithelial cancer had co-existing lichen sclerosus (Zaki *et al.* 1996). The disease is more frequent in women, often localizing at vulva (Meffert *et al.* 1995) and its development is linked with genetic, environmental, infectious and autoimmune factors (Chil *et al.* 2005).

The pharmacological treatment of lichen sclerosus is often inefficient. Therefore alternative form of therapies are suggested including photodynamic therapy. Photodynamic diagnostics (PDD) preludes photodynamic therapy (PDT). PDD is based on analysis of fluorescence in the diseased tissue. Photosensitizer (5-ALA)

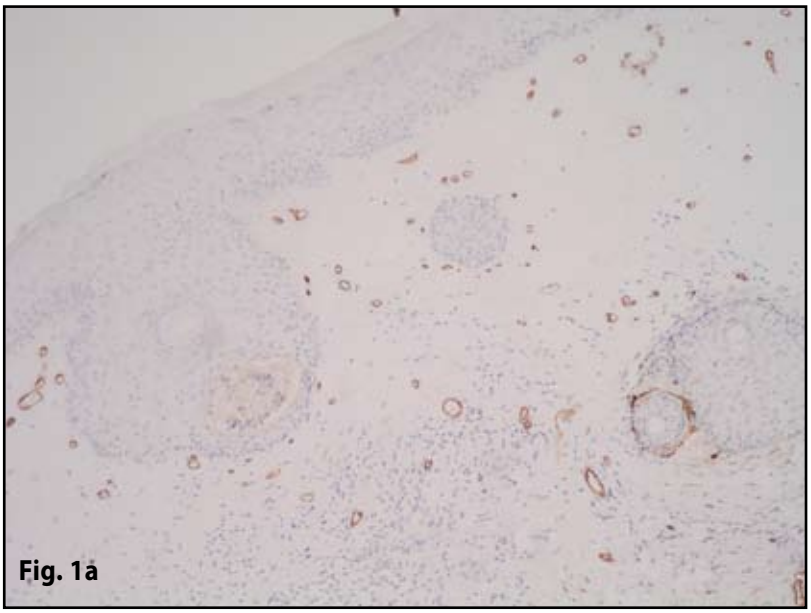

Fig. 1a

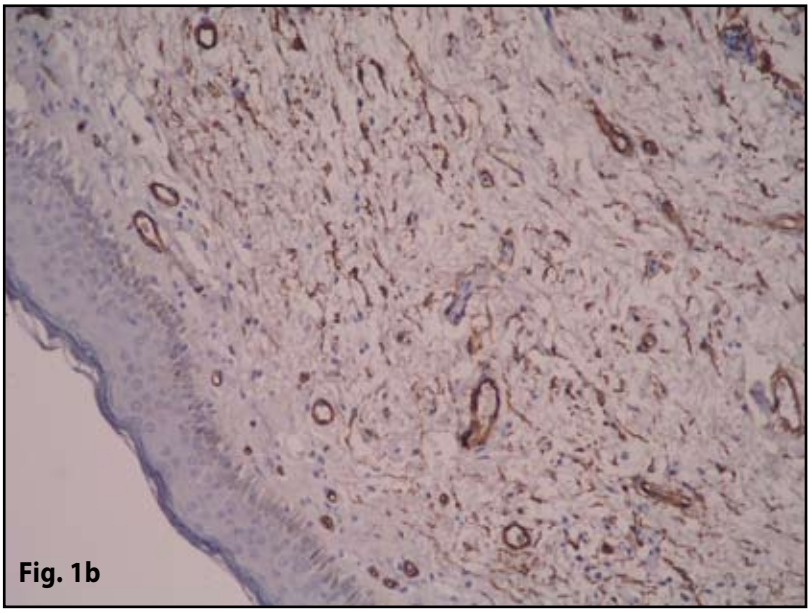

Fig. 1b

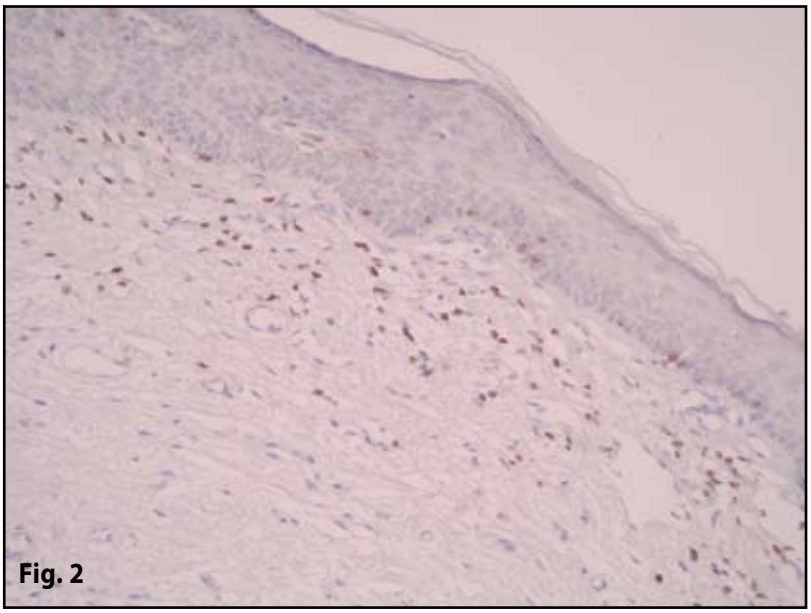

Fig. 2

Fig. 1. CD34 immunohistochemical analysis in vulvar samples. 1a Results before PDT. 1b Results after PDT.

Fig. 2. CD3 immunohistochemical analysis in vulvar samples. 2a Results before PDT. 2b Results after PDT.

locating at the pathological tissue presents with a greater intensity than ALA locating at the healthy tissue. ALA, that marks the skin, enables to target the changed tissue and set a proper histological diagnosis. In addition, during the photodynamic therapy ALA enables to reach the tissue and destroy it precisely. The initial interaction of light with protoporphirin IX results in the generation of singlet oxygen and oxygen radicals which are highly reactive. This leads to tissue damage due to cell necrosis or apoptosis. The results of that interaction depend on the biochemical proprieties of the photosensitizer and the duration of laser application on the tissue. Apart from the lethal effects on cells, PDT has bacteriocidal activity, inducing several immunologic reactions, photoclusion in tumor vessels or modification of angiogenesis. The selective damage of tissue is the main advantage of PDT.

We assessed the expression of Ki67, CD44, CD34 and CD3 of the T lymphocytes before and after PDT with the additional evaluation of MDV at the tissue. Apart from the immunohistochemical studies, we evaluated the clinical improvement after PDT. It was noted that most of the patients had no symptoms after PDT, but only 7 of 28 women had a complete disease regression with histological confirmation. It is probable that the relatively low rate of a complete disease disappearance is due to the small number of PDT courses (6x) or too low photosensitizer concentration (5%).

The first hypothesis may soon be confirmed because our laser center is now recruiting a group of 60 women in whom a period of therapy was lengthened until 10 courses of PDT but the final results are still not known. It should be mentioned that the treatment efficacy may also be affected by the time frame between ALA

and laser application. Previous studies applied higher concentration of the photosensitizer and the time in between application was from 2 to 5 hours (Bamberger *et al.* 2002, Fehr *et al.* 2001, Hillemanns *et al.* 2000 , Hillemanns *et al.* 1999, Kurwa *et al.* 2000). In our study, we applied ALA in the form of a paste 2 hours before lasering. Our finding that the average vessel density increase in the skin sections after PDT may suggest the occurrence of the skin regeneration. However, some studies related the increase of microvessel density with the development of planoepithelial cancer in the skin to LS (Rasspollini *et al.* 2007). In general, MVD in lichen sclerosus is decreased comparing to the healthy tissue and the most intensive density is found in VIN or vulvar cancer (Saravanamuthu *et al.* 2003). The increased angiogenesis was reported to influence the growth of tumor and its metastatic activity (Hillemanns *et al.* 1999).

Lesions affected with LS have an atrophic epidermis with a subepidermal vacualization, and the dermis is edematous and hyalinized. It is often accompanied with the infiltration of the lymphocytes (Chil *et al.* 2005). Thus, our observation that lymphocytic infiltration decreases after PDT is noteworthy but both decrease and increase of inflammatory infiltration was previously described (Carlson *et al.* 2002, Fehr *et al.* 2001, Gross *et al.* 2001).

Studies report that photodynamic therapy is a noninvasive treatment with sufficient cosmetic results, but its weakness of PDT is poor efficiency, especially due to resistance to PDT and the recurrence of lichen sclerosus with a short remission period (Gross *et al.* 2001, Tchórzewski *et al.* 2005, Zaki *et al.* 1996). Nevertheless we believe that a better application of photosensitizer, shortening of the time between photosensitizer application and the start of lasering and finally, the lengthening of the treatment protocol may improve the efficacy of PDT in patients with lichen sclerosus.

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