Lymphoid elements and apoptosis-related proteins (Fas, Fas ligand, p53 and bcl-2) in lichen sclerosus and carcinoma of the vulva

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Summary

We studied some of the morphological and immunohistochemical parameters of lichen sclerosus (LS) and carcinomas of the vulva in order to verify some characteristics in LS related to neoplasm transformation. Parameters such as proliferating index, rate of proliferation of lymphoid elements into a tumor and types of such elements were studied. In parallel, the number of cells positive to apoptosis-related proteins such as Fas, Fas ligand, p53 and bcl-2 were evaluated. Biopsy material from patients with different vulvar disorders – 22 samples with LS and 23 samples with vulvar squamous cell carcinoma (VSCC) – was studied by the methods of morphometry and immunohistochemistry. In LS, the number of T cells is a few times higher than those of B cells. Among the T cells, the number of killers is significantly higher than the number of helpers. Carcinomas, especially those with lymphoid depletion, are characterized by a further significant increase in some parameters such as the rate of lymphoid proliferation and the number of T helpers and killers. The progression in to tumorigenesis was accompanied with a significant increase in the number of Fas^+ and $FasL^+$ lymphocytes. In tumor epithelial cells the proliferative index increased in carcinomas with lymphoid depletion. The number of $p53^+$ epithelial cells increased whereas the number of bcl-2^+ cells showed a distinct tendency to decrease with progression in to tumorigenesis. Development of a tumor is manifested in deep changes in relationships between different lymphoid components. Only two lymphoid markers are significantly different in VSCC compared to LS: the number of T killers and macrophages. The other parameters studied (rate of proliferative activity, the total number of T cells and T helpers, B cells, IL-2-connective cells) already showed high expression in LS as the first signs of transformation of this inflammation into neoplasia.

Key words: Apoptosis; Carcinoma of vulva; Lichen sclerosus; Proliferation; T and B cells.

Introduction

Among epithelial disorders of the skin and mucosa of external sexual organs in females, lichen sclerosus (LS) and squamous hyperplasia are the main types of disorders which may end as neoplasmic transformations [1]. Vulvar squamous cell carcinoma (VSCC) is an uncommon neoplasm that is associated with granulomatous vulvar diseases, papillomavirus and chronic skin disorders [2, 3].

LS is the most common dermatosis of the vulva of unknown etiology which in many cases plays an important role in the etiology and pathogenesis of VSCC [4-6]. Among patients with VSCC, 40% had LS and 33% had vulvar intraepithelial neoplasia [7]. LS is characterized by progressive thinning of the epithelium, subepithelial edema with fibrin deposition, and an underlying zone of chronic inflammation within the dermis. Squamous cell hyperplasia is also a relatively common skin disorder [8] which often may transform into neoplasia: 85% of vulvar malignant tumors are constituted by squamous cell carcinomas [5]. This epithelial disorder is characterized by acanthosis and variable hyperkeratosis without atypia, is associated with inflammation and sometimes with specific dermatosis.

Diagnosis of both disorders includes gross examination and mainly microscopic analysis [1]. Immunohistochemical findings are restricted by detection of fibrinogenic cytokines within the subepithelial dermis [9], and complement C3 and IgM within edematous areas. But these parameters have no clinical significance because they have also been found within areas of superficial trauma. Although the p53 tumor-suppressor protein expression was significantly higher in VSCC compared to LS or squamous cell hyperplasia [10], the finding has no prognostic value because of the absence of p53 overexpression in precancerous lesions of the vulva [11]. Moreover, p53 overexpression, similar in non-neoplastic vulvar epithelial disorders and vulvar intraepithelial neoplasia [12], is manifested only in late stages of VSCC [13, 14] and does not correlate with the histological grade of a tumor [15]. Despite the fact that p53 antibodies have been detected in the sera of patients with VSCC, no significant association has been found between the p53 serum antibody status in vulvar cancer patients and disease-free women and overall survival [16]. Findings by electron microscope of laminin-positive proteins in the basement membrane zone has more theoretical significance [17]. Expression of proliferating cell nuclear antigen (PCNA) and bcl-2 was found to be almost indentical in lichen

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sclerosis and vulvar intraepithelial neoplasia whereas Ki-67 was a better marker of vulvar dysplasia [18]. The role of immunologic parameters such as CD4 and CD8 T lymphocytes, B lymphocytes, macrophages, IgG, IgM and C3c in the pathogenesis of vulvar lichen sclerosus has been studied very little [19].

The cited data show that till now immunohistochemical studies have not been helpful diagnostically for early diagnosis and prognosis of VSCC and its treatment [20]. The management of LS poses a challenge for clinicians. Early diagnosis and treatment may minimize the risk of the development of VSCC. However, the true history of the disease is not clear. It is still uncertain which factors determine transformation of an inflammation into cancer or which mechanism is responsible for recurrence of disease after successful surgery [21].

The present study is devoted to of some of the morphological and immunohistochemical parameters which usually accompany the very beginning of the neoplastic process in order to verify which of them is related to neoplasmas of the vulva and whether it is possible to use them for clinical diagnosis and oncological prognosis. Parameters such as the proliferating index, rate of proliferation of lymphoid elements into a tumor and their subtypes as well as expression of apoptosis-related proteins (ARP) (Fas and Fas ligand (FasL), p53 and bcl-2) were used as criteria for inclusion.

Materials and Methods

Patients. Biopsy material from patients with different vulvar disorders (22 samples with LS and 23 samples with VSCC at different stages of malignant transformation) was studied by the methods of morphometry and immunohistochemistry. The following five groups of patients were analyzed: the 1st group contained 12 patients with LS, the 2nd group included eight patients with LS and focal squamous hyperplasia (vulvar intraepithelial neoplasia I and II - VIN-2), the 3rd group contained five patients with VIN-3 and carcinoma in situ, the 4th group was comprised of 13 patients with VSCC and distinct lymphoid infiltration, and the 5th group contained five patients with VSCC and lymphoid depletion.

Immunohistochemical and Morphometric Studies. The total number of immunocompetent cells (T and B lymphocytes, macrophages) in different parts of the biopsy, and the average number of different types of cells per 50,000 μm^2 of a slide were measured with an ocular grid at a magnification of x400. In each case, the number of 1,000 to 3,000 cells was evaluated. Areas of lymphoid-macrophageal infiltrates in tissues studied were determined by the method of point evaluation in all slides at a magnification of x100. The proliferating index was calculated as the number of mitotic cells per 50,000 μm^2 using a marker for Ki-67 protein (Novocastra Labs, Newcastle, England). For evaluation of different types of cells, the immunoperoxidase technique was used with commercial markers CD3 (mature T lymphocytes), CD4 (T helpers), CD8 (T cell killers and suppressors), CD20 (B lymphocytes), CD68 (macrophages) (Novocastra Labs, Newcastle, England), CD25 (IL-2Ralpha - DBS Co., Pleasanton, USA). The number of cells reacting to ARP such as Fas and Fas ligand (FasL), p53 and bcl-2 was determined using commercial markers (Santa Cruz Biotechnology, CA).

Statistical analysis was performed using the one way ANOVA-test and determination of coefficients of correlation (r) between different parameters was studied. Pairs of means were compared using the Tukey HSD test.

Results

Lichen sclerosus and vulvar squamous cell carcinoma are characterized by different relationships among the parameters studied. In patients with LS (1st group), areas of lymphoid infiltrates in the stroma were small and the total number of the immunocompetent cells was not high (Table 1). The number of intraepithelial immunoreactive cells are shown in Table 2. The number of T cells was high with a high percentage of T cell killers and suppressors among them (Figures 1, 2).

The beginning stages of tumorigenesis (VIN group 2) were characterized by significant increases in the areas of lymphoid infiltrates (2.7 times compared to LS) and an increase 1.5 times the content of cells contained in them (Table 1). The number of IL-2-positive cells increased whereas the total number of lymphocytes and especially T helpers decreased compared to LS (Figure 1).

In carcinoma *in situ* (VIN group 3), further progress in the described changes was seen (Table 1, Figure 1). The number of T lymphocytes decreased while the number of macrophages and B lymphocytes increased (Table 2, Figure 2).

Table 1. — *The number of different subsets of lymphocytes in the stroma of tumors obtained from different groups of patients (Mean ± SE).*

Patient groups	no.	Areas of lymphoid infiltrates	Number of lymphocytes	Number of $CD3^+$ cells	Number of $CD4^+$ cells	Number of $CD8^+$ cells	Number of $CD20^+$ cells	Number of macrophages	Number of $IL\text{-}2^+$ cells
1	12	4.6±0.4[a]	138.7±10.2[b]	96.8±8.7[b]	34.7±4.2[b]	62.0±6.7[b]	10.4±2.3[b]	30.0±2.3[b]	6.1±0.7[b]
2	8	12.4±1.2[c]	206.7±27.4[c]	123.2±11.7	30.2±2.8	92.3±10.2[c]	45.4±4.8[c]	38.2±3.3	23.4±4.1[c]
3	5	20.4±2.1[c]	248.2±25.1	150.2±12.5	13.6±1.5[c]	136.0±11.4[c]	50.1±5.2	48.1±5.4	85.1±10.2[c]
4	13	25.8±1.4[c]	241.0±47.2	131.1±13.1	14.9±1.6	117.1±9.6	65.1±5.9	45.1±4.2	63.1±5.2
5	5	5.8±0.8[c]	86.9±18.7[c]	35.4±2.8[c]	5.0±0.8[c]	30.2±4.2[c]	12.7±2.9[c]	38.4±3.9	5.3±0.4[c]

Groups of patients with 1, LS and atrophicus; 2, LS with VIN 1 and 2; 3, carcinoma in situ, VIN-3; 4, VSCC with lymphoid infiltration; 5, VSCC with lymphoid depletion.

[a]In % of the area of a slide.

[b]The number of lymphocytes/50,000 μm^2.

[c]Significantly different from the previous group, $p < 0.05\text{-}0.01$.

Table 2. — *Number of different subsets of immunocompetent cells in the parenchyma of tumors obtained from different groups of patients (Mean ± SE).*

Groups	No.	Number of					
		lymphocytes	CD3⁺ cells	CD4⁺ cells	CD8⁺ cells	CD20⁺ cells	macrophages
1	12	13.7±1.1[b]	12.1±0.5[b]	2.7±0.5[b]	9.4±0.9[b]	0.2±0.2[b]	1.4±0.6[b]
2	8	17.5±1.4[c]	16.4±1.5[c]	1.4±0.3[c]	14.2±1.1[c]	0.3±0.2	1.1±0.4
3	5	16.5±1.6	13.1±0.7	1.5±0.5	11.6±0.6	1.8±0.5[c]	1.6±0.7
4	13	41.9±4.7[c]	22.7±2.6[c]	1.3±0.3	20.9±2.4[c]	1.5±0.3	17.7±4.5[c]
5	5	22.6±2.2[c]	3.5±0.8[c]	0.3±0.2[c]	3.1±1.1[c]	0.7±0.2[c]	18.4±4.4

Table 3. — *Proliferative index and the number of lymphocytes positive to Fas, FasL and bcl-2 in the stroma of tumors obtained from different groups of patients (% of the total number of cells/ 50,000 μm²) (Mean ± SE).*

Groups	No.	Proliferative index	Number of Fas⁺ cells	Number of FasL⁺ cells	Number of bcl-2⁺ cells
1	12	3.1±0.5	18.6±1.3	34.2±3.4	18.5±1.1
2	8	5.8±0.7	20.6±1.9	22.2±2.6	48.5±5.1[a]
3	5	4.2±0.5	70.0±6.9[a]	35.6±3.4	35.1±2.8
4	13	4.8±0.4	15.2±1.8[a]	23.7±2.1	36.2±4.1
5	5	3.6±0.4[a]	9.3±0.2[a]	14.9±1.7[a]	29.3±3.1

[a]Significantly different from the previous group, $p < 0.05$-0.01.

Table 4. — *Proliferative index and the number of epithelial cells positive to p53 and bcl-2 in the parenchyma of tumors obtained from different groups of patients (% of the total number of cells/50,000 μm²) (Mean ± SE).*

Groups	No.	Proliferative index	Number of p53⁺ cells	Number of bcl-2⁺ cells
1	12	26.2±2.6	44.7±3.9	53.1±6.7
2	8	27.2±1.5	59.5±5.4[a]	48.5±4.1
3	5	27.8±1.6	56.0±4.8	35.1±2.6
4	13	27.2±2.7	41.6±4.3[a]	19.3±1.8[a]
5	5	36.2±3.0[a]	68.1±5.4[a]	17.8±2.1

[a]Significantly different from the previous group, $p < 0.05$-0.01.

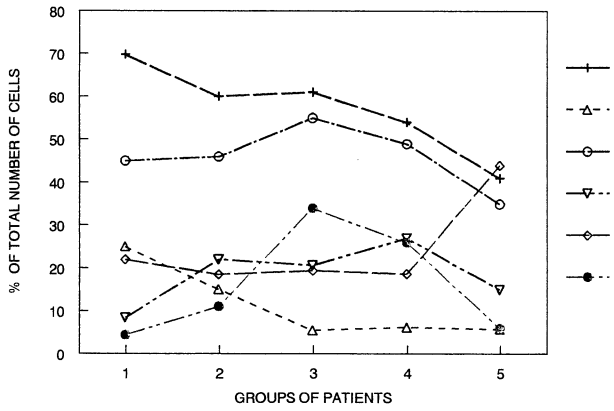

Figure 1. — Relationship between the number of different immunocompetent cells in the stroma of tumors obtained from different groups of patients (%). Groups of patients with: 1, LS and atrophicus; 2, LS with VIN 1 and 2; 3, carcinoma in situ, VIN 3; 4, VSCC with lymphoid infiltration; 5, VSCC with lymphoid depletion. Parameters studied: A, CD3⁺ lymphocytes; B, CD4⁺ lymphocytes; C, CD8⁺ lymphocytes; D, CD20⁺ lymphocytes; E, macrophages; F, IL-2⁺ cells.

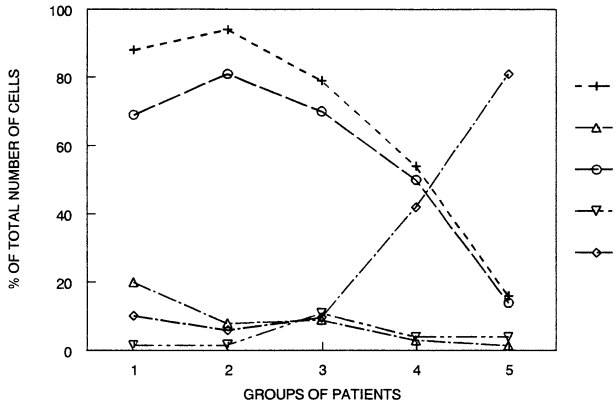

Figure 2. — Relationship between the number of different immunocompetent cells in the parenchyma of tumors obtained from different groups of patients (%). Groups of patients with: 1, LS and atrophicus; 2, LS with VIN 1 and 2; 3, carcinoma in situ, VIN 3; 4, VSCC with lymphoid infiltration; 5, VSCC with lymphoid depletion. Parameters studied: A, CD3⁺ lymphocytes; B, CD4⁺ lymphocytes; C, CD8⁺ lymphocytes; D, CD20⁺ lymphocytes; E, macrophages.

In carcinoma with infiltrates (group 4), a further significant decrease in the number of T lymphocytes including T cell killers was seen whereas the number of macrophages increased sharply (Figure 2).

Carcinoma with lymphoid depletion (group 5) was characterized by a sharp decrease compared to the previous stage in almost all parameters studied except the number of macrophages (Table 2, Figure 2). All these changes reflect deep inhibition of the lymph system.

Progression into tumorigenesis was accompanied by a significant increase in the number of Fas⁺ and FasL⁺ lymphocytes (Table 3). In patients with LS (1st and 2nd groups), Fas and FasL were found mainly in the epithelial cells of the granular and corneal layers (62-73% of cells) whereas in patients with carcinomas, these ARP were found mainly in the central parts of cancer lobules, including cancer "pearls" (30-45%). In tumor epithelial cells, the proliferative index increased in carcinomas with lymphoid depletion (Table 4). The number of p53⁺ epithelial cells increased in patients with VIN-2 compared to those with LS and also in patients with carcinomas with lymphoid depletion compared to those with carcinomas and lymphoid infiltration (Table 4). The number of bcl-2⁺ epithelial cells showed a distinct tendency to decrease with progression into tumorigenesis (Table 4).

A significant difference in the rate of lymphoid infiltration in lichen sclerosus was found between "old" (older than 60 years) and "young" (younger than 60 years) patients. No correlation was seen between the age of patients and the rate of other parameters studied.

Development of a tumor is manifested in deep changes in relationships between different lymphoid components.

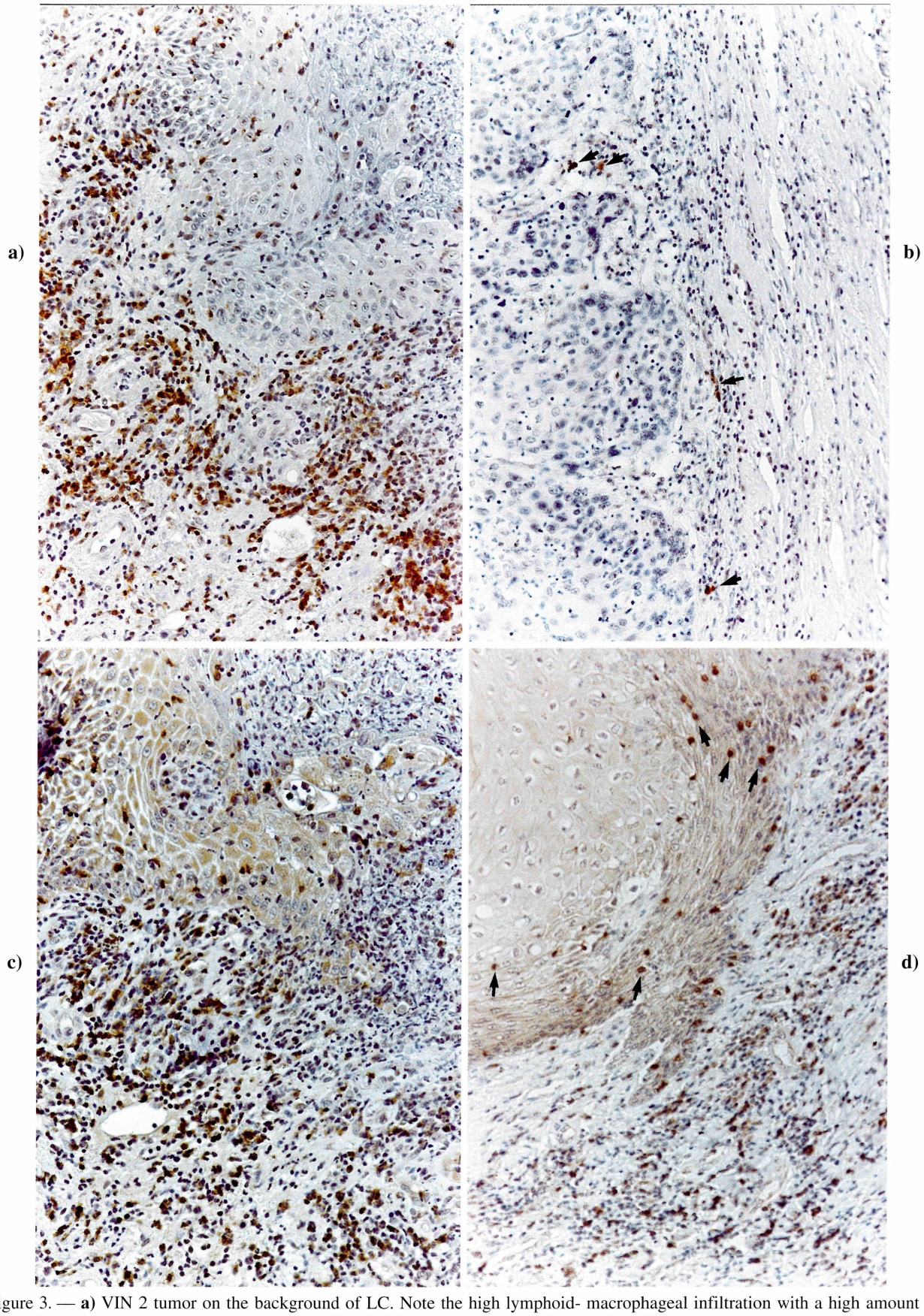

Figure 3. — **a)** VIN 2 tumor on the background of LC. Note the high lymphoid- macrophageal infiltration with a high amount of CD4+ lymphocytes (brown staining) x200. **b)** VSCC: Note lymphoid depletion, weak infiltration and very seldom $CD4^+$ lymphocytes (arrows) in the stroma of tumor x200. **c)** The same place as in Figure **a)**. Note the high number of $CD8^+$ lymphocytes (brown staining) in tumoral stroma and parenchyma. x200. **d)** The same case as in Figure **b)**. Note the small number of $CD8^+$ lymphocytes in tumoral stroma (brown staining) and parenchyma (arrows) x200.

In LS, a highly significant correlation was seen between only the rate of hyalinization and the number of $CD8^+$ lymphocytes (r = 0.47) and also between different subtypes of T lymphocytes: r = 0.53 and 0.54 between the number of $CD3^+$ and $CD4^+$ cells, or $CD4^+$ and $CD8^+$ cells, respectively. In carcinomas, a high correlation was found between rate of lymphoid proliferation and the number of $Ki\text{-}67^+$ cells (r = 0.56), the total number of T and B cells (r = 0.55), the number of $CD4^+$ and $CD8^+$ lymphocytes (r = 0.52), between the number of $Ki\text{-}67^+$ cells and the number of T helpers (r = 0.57) and macrophages (r = 0.61). High parallelism was found in the secretion of Fas and FasL by immunocompetent cells in patients with VSCC (Table 3).

Discussion

Lichen sclerosus is an inflammatory dermatosis characterized by clinicopathologic persistence and hypocellular fibrosis (sclerosis) [1]. Vulvar squamous cell carcinoma (VSCC) is significantly associated with the presence of LS and diffusely expresses the p53 gene product [1]. Keratinocytes affected by LS show a proliferative phenotype and can exhibit markers of neoplastic progression such as increased p53 expression and DNA aneuploidy. As a chronic scarring inflammatory dermatosis, vulvar LS could act as both "initiator and promoter" of carcinogenesis, thus explaining the frequent coexistence of these diseases. Because keratinocytes of LS significantly express tumor suppressor gene p53 protein, the p53 gene may be involved early on in this proposed pathway of carcinogenesis [1]. Similar data were obtained in our study where a significant increase in the number of p53-positive tumor cells was found in VIN-2-patients compared to those with LS and also in patients with carcinomas and lymphoid depletion compared to those with carcinomas and lymphoid infiltration.

Very little is known about intracellular changes which are characteristic of LS and VSCC. Alterations in cytokine (CD44) and cell adhesion receptor status variably occur in VSCC and may affect tumor morphology and host response with the development of a prominent fibromyxoid stromal tumor and extensive lymph node metastases [22]. Ki-67 and PCNA nuclear staining were largely restricted to basal and parabasal cells in normal tissue and LS [18]. Focal mid-epithelial staining with PCNA and Ki-67 was seen in one case of LS. Both antibodies stained dysplastic cells at higher epithelial levels in vulvar intraepithelial neoplasia (VIN), but Ki-67 was more consistently reactive and showed a sharper distinction from adjacent histologically uninvolved epithelium compared to PCNA. The pattern of bcl-2 staining was identical in normal vulvas, LS and VIN, but bcl-2 coloured occasional mitotic figures in VIN [18]. We found that progression of tumorigenesis of the vulva is accompanied by a tendency to a decreased number of bcl-2-positive tumor cells.

Different immunocompetent cells do not react equally in the progression of tumorigenesis. In the stroma of a tumor, the number of T lymphocytes – among them helpers and killers/suppressors – decreased from LS to VSCC whereas the number of B lymphocytes increased till stage of cancer with lymphoid infiltration (Figure 1). The number of IL-2-positive cells also increased till this stage but then sharply decreased. The number of macrophages did not change during all stages and sharply increased at stage of cancer with lymphoid depletion.

In the epithelium of a tumor, the total number of T lymphocytes and killers decreased in all stages compared to a VIN-2 stage to cancer with lymphoid depletion while the number of macrophages increased sharply in both stages of cancer studied (Figure 2). It seems that T helpers are less resistant because the number has already decreased at the stage of VIN-2, and at the stage of cancer with lymphoid depletion the number is less than 7 times in the stroma and 9 times in the parenchyma.

Tumorigenesis progression is not accompanied by significant changes in the number of most of the lymphoid cell-types studied. We found wide areas of lymphoid infiltrates in LS despite a significant difference from VSCC. This parameter reflects the relatively quiet character of transformation of LS into cancer. The number of proliferative active epithelial cells in LS was high and this parameter did not change in VSCC. The finding is in accordance with the observations of others [18]. We suggest that high proliferative activity, characteristic of dysplasia, has already begun in stages of LS.

A similar conclusion can be made regarding most of the other lymphoid parameters studied. As was found in our study, only two lymphoid markers were significantly different in VSCC compared to LS: the number of T killers and macrophages. The other parameters studied (the total number of T cells and T helpers, B cells, IL-2-positive cells) already showed high expression in LS as the first signs of transformation of this inflammatory neoplasia. A high correlation between different subtypes of T cells suggests that determining one or two of them is enough in the prognosis of the possible development of cancer.

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