Originals

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Expression of Vascular Endothelial Growth Factor (VEGF) in Vulvar Squamous Cancer and VIN

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Angiogenesis plays an important role both in progression of solid tumors and in metastasizing. An invasive growth of a neoplasm is mainly connected with appearing of blood vessels within a tumor. Inhibition of angiogenesis in solid neoplasms may deter both tumor growth and metastases. New treatment strategies based on suppressing of angiogenesis and selective damaging of neoplastic blood vessels may prove to be as efficient as those based on direct destruction of neoplastic cells. One of important angiogenic factors is vascular endothelial growth factor (VEGF), which is produced by neoplastic cells and shows high promitotic activity almost entirely for endothelial cells (paracrine activity). We decided to investigate VEGF expression in precancerous lesions as well as in squamous cancers of vulva. Our material included 31 cases of vulvar squamous cancer, 28 cases of VIN (vulvar intraepithelial neoplasia) III, 10 VIN II cases and 12 VIN I cases. A diagnosis was established according to WHO criteria on the ground of post-operative histopathological examination complemented with proliferation index estimated by the use of MIB-1 antibody. Immunohistochemical examinations were performed on paraffin-embedded material, using MIB-1 antibody (Immunotech), VEGF antibody (Santa Cruz), Goat serum Normal (DAKO), DAKO StreptAB-Complex/HRP Duet, Mouse/Rabbit DAKO DAB Chromogen Tablets, TBS (Sigma). Positive cytoplasmic expression of anti-VEGF polyclonal antibody (diffuse and/or focal and of various intensity) was observed in almost all samples from precancerous and cancerous lesions. The expression was especially strong and diffuse in all cancer cases; in cases of VIN it was mainly focal and weak.

Introduction

Vulvar intraepithelial neoplasia – the term introduced in 1980' – defines the progression of vulvar lesion from mild dysplasia to intraepithelial carcinoma. The process may occur multifocally (70% of cases), involving the entire vulva or may be limited to labia, posterior frenulum and clitoris.

In women between 20 and 40 years of age VIN takes a little risk of progression to invasive cancer but the risk increases significantly in older women. Spontaneous regression of VIN is observed in about 6% of the patients.

In the light of the latest papers about angiogenesis, in particular Folkman's studies on important role of angiogenesis in tumor progression, VEGF (vascular endothelial growth factor) expression in precancerous and cancerous vulvar lesions seems worth of investigating. VEGF is one of more important angiogenic factors and invasiveness of a neoplasm is closely connected with the development of new blood vessels [3–12]. Only two authors reported the angiogenesis in the vulvar lesions [1, 2].

Material and Methods

The investigation was performed on sections from 31 squamous cancers (mainly non-keratinizing, G-2), 12 VIN I, 10 VIN II and 28 VIN III (Table 1). The patients' average age was 58 (range 34–82). Diagnosis was established according to WHO criteria and for each case complemented with proliferation index estimated by the use of MIB-1 antibody. Sections 3–4 μm thick were mounted on poly-L-lysine-coated glass slides (Polylysine TM Microslides; MENZEL-GLASER). A purified rabbit polyclonal antihuman VEGF (A-20) sc antibody (Santa Cruz Biotechnology INC., CA, USA) (1:500), monoclonal mouse MIB-1 antibody (DAKO) (1:50), Goat Serum Normal (DAKO) (1:5) and DAKO StreptABComplex/HRP duet, Mouse/Rabbit (DAKO) DAKO DAB Chromogen Tablets, TBS (Sigma) were used. The staining was performed strictly according to the producer's instruction. A microwave oven was used in order to reveal the antigens. The first reaction was performed overnight at 4^{o}C and other

TABLE 1
Characteristics of the study material

Number of cases	Histological type	Inflammatory infiltrate intensity/pattern	Lymph node (number of metastases)
12	VIN I	+1/ lymphocytes	0
10	VIN II	+1/ lymphocytes	0
28	VIN III	+2/ lymphocytes	0
16	Ca in situ	+2/ lymphocytes	0
31	Squamous cell ca (G1, G2, G3)	+2/lymphocytes and plasma cells	3

reactions at room temperature. Sections from anaplastic gastrointestinal and lung cancers and from tonsils were used as a positive control. An expression of VEGF was estimated in two ways. First, an intensity of the expression was measured on the four-point scale (0 – negative, 1 – slightly positive, 2 – moderately positive, 3 – highly positive); next the pattern of expression – diffuse or focal – was observed [28, 30].

MIB-1 expression was seen in cell nucleus as homogeneous brown pigmentation. For each section a ratio was counted as proportion of MIB-1 positive cells to a number of counted cells (not less than 500 counted cells), using an image analysis system consisting of a IBM-compatible computer equipped with an optical mouse, Indeo Fast card (frame grabber, true-color, real-time), produced by Indeco (Taiwan), and color TV camera Panasonic (Japan) linked to a Carl Zeiss Jenaval microscope (Germany). This system was programmed (program MultiScan 8.08, produced by the Computer Scanning System, Poland) to calculate the number of objects in the whole specimen. This method of estimation is admitted to be the most objective and reproducible [23]. Number and kind of inflammatory cells in the stroma of the lesions examined were studied, too.

Results

All the lesions examined revealed positive nuclear expression of MIB-1 (Figs. 1. a, b, c). In normal, healthy squamous epithelium less than 10% cells of parabasal layer were MIB-1 positive. Along the progression of VIN grade MIB-1 positive cells appeared in higher and higher layers of the epithelium. Only keratinizing cells were the exception. In VIN III lesions MIB-1 expression was strong in all cells except of keratinizing cells and in carcinomas it was highly positive in all cells except of "cancer pearls". Proliferation index was lowest in VIN I and VIN II lesions (less than 10% of

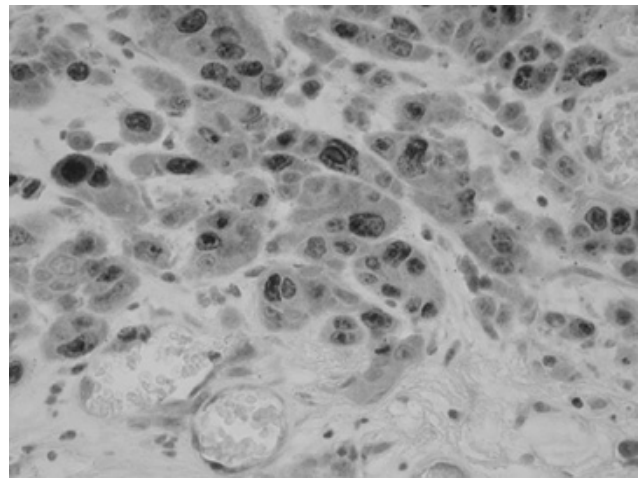

Fig. 1a. Positive nuclear expression of MIB-1 in vulvar squamous cancer. Magn. 250×.

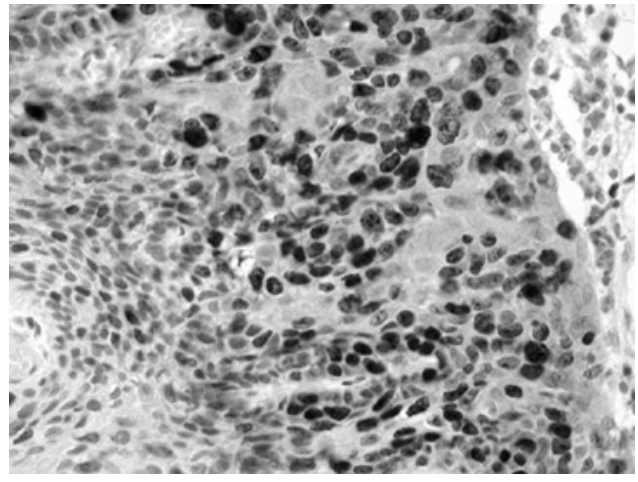

Fig. 1b. Positive nuclear expression of MIB-1 in vulvar intraepithelial neoplasia. Magn. 250×.

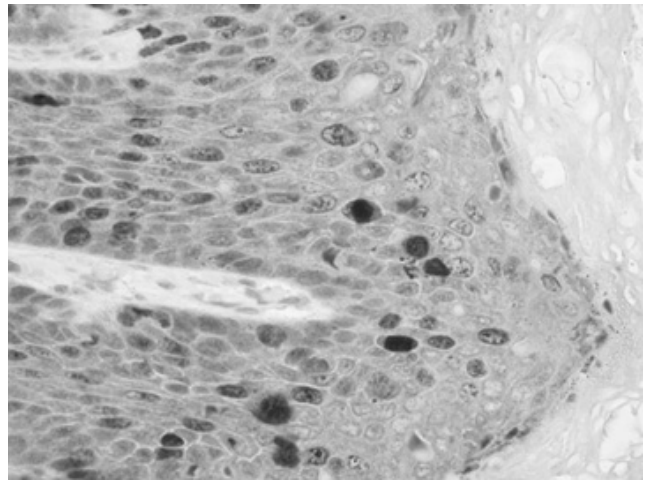

Fig. 1c. Positive nuclear expression of MIB-1 in vulvar intraepithelial neoplasia. Magn. 250×.

TABLE 2
MIB-1 expression in the study material (proliferation index)

Number of cases	Histopathological type	MIB-1 expression % of positive cells
12	VIN I	<10%
10	VIN II	<10%
28	VIN III	10% - 50%
16	Ca in situ	50%
31	Squamous cell ca (G1, G2, G3)	>50 %

TABLE 3
VEGF expression in the study material

Number of cases	Histopathological type	VEGF – intensity of the expression	VEGF – pattern of the expression
12	VIN I	+1	focal
10	VIN II	+1	focal
28	VIN III	+2	diffuse
16	Ca in situ	+2	diffuse
31	Squamous cell ca (G1, G2, G3)	+3	diffuse

MIB-1 positive cells), moderate in VIN III (between 10% and 50% of MIB-1 positive cells) and highest for invasive carcinoma (more than 50% of MIB-1 positive cells) (Table 2). The estimation of proliferation index helped us to classify VIN lesions to I, II, III-grade groups more objectively.

Slight to moderate (+1 to +2) and focal positive cytoplasmic VEGF expression was observed in VIN I and II lesions. Moderate to high (+2 to +3) and diffuse expression was seen in VIN IIII and decidedly high (+3) and diffuse in invasive carcinomas (Table 3 and Figs. 2. a, b). Stromal inflammatory infiltrate consisting of lymphocytes and plasma cells was present.

Discussion

The formation of new blood vessels from pre-existing vasculature is *sine qua non* requirement for the progression of neoplasms [5–12, 20]. Thus, determination of tumor angiogenic profile seems to be helpful in choosing a method of effective therapy [3, 9, 11]. All the more, angiogenesis and its intensity may become a new prognostic factor [14, 15, 19, 21]. It should be remembered that solitary VIN lesions in older women take a great risk of progressing to

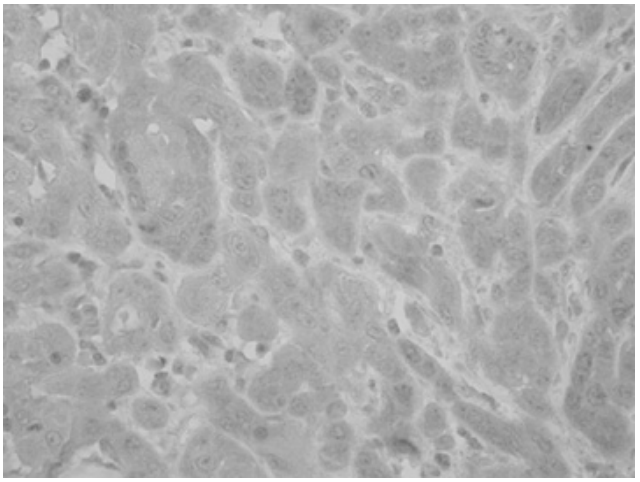

Fig. 2a. Positive cytoplasmic expression of anti-VEGF polyclonal antibody in vulvar squamous cancer. Magn. 250×.

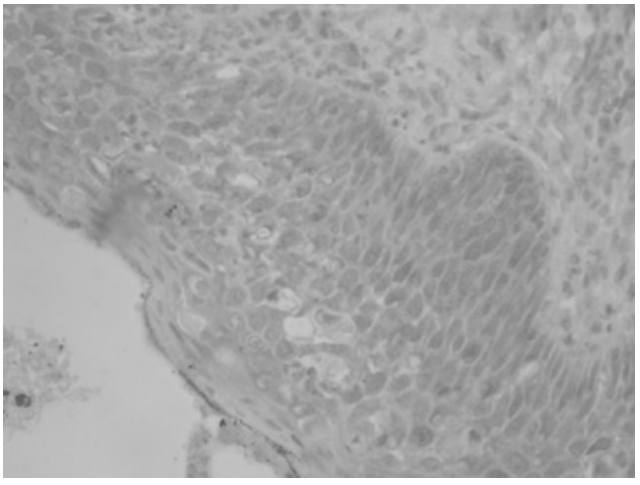

Fig. 2b. Positive cytoplasmic expression of acti-VEGF polyclonal antibody in vulvar intraepithelial neoplasia. Magn. 250×.

a cancer. Studies on angiogenesis may help to find, which VIN lesions are at higher risk of carcinoma development. Our statement, that in more advanced lesions (VIN III) more cells produce VEGF more intensively (slight and focal expression of VEGF in VIN I versus high and diffuse in VIN III) may prove that angiogenesis will start quicker. In invasive cancer samples VEGF immunoreactivity was very strong and diffuse, what reflects a great angiogenic activity of this neoplasm. It correlates directly with increasing number of blood vessels in this cancer and its progression. Bracher-Todesca et al. observed similar results. They have reported also, that VEGF expression in VIN III lesions was much stronger than in VIN I and II. Understanding of neoplastic angiogenesis and explaining the role of pro- and anti-angiogenic factors may give us new therapeutic possibilities [16–18, 27, 29]. According to Folkman following substances are now clinically tested: suppressors of endo-

thelium proliferation – angiostatin, endostatin, plate factor 4, TNP-470, AGN-1470, tamoxifen; suppressor of TNF synthesis – Limomid; inhibitor of bFGF and VEGF production – interferon; interleukin 12, which increases secretion of induced chromosome 10 protein, which has angiogenesis inhibiting activity [11, 17, 22, 25]. Many investigators observed that attempts on destruction of blood vessels within a neoplasm and inhibition of angiogenesis may be of special importance. According to Folkman's observation prolonged anti-angiogenic therapy in experimental animals causes diminishing of tumor size or its total eradication [13]. Inhibition of VEGF production at VIN I or VIN II level could, maybe, stop a progress of dysplasia and thus development of vulvar cancer. On the other hand, if cancer was already developed inhibiting of angiogenesis may cause, as in animal model, diminishing of tumor size and stop metastasizing. VEGF is a very important pro-angiogenic factor and thus it is conducive to development of cancer from precancerous lesions and its further progression. We found VEGF expression in all vulvar intraepithelial changes and also in vulvar cancers. The VEGF immunoreactivity differs only in intensity and extent. It is obvious that this question needs further studies, which may give promising results.

Conclusions

1. Focal or diffuse VEGF expression was observed in all the lesions examined (VIN I–III and vulvar cancer).

2. VEGF expression is strongest in squamous carcinoma cells.

3. In VIN I and II lesions VEGF expression is weak.

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