The role of vascular endothelial growth factors and their receptors in malignant melanomas

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Vascular endothelial growth factors (VEGFs) have a leading role among variety of angiogenic factors. Together with their receptors, they play an important role in endothelial cell proliferation and/or elongation, migration and vascular morphogenesis. In order to determine their possible role in malignant melanoma progression, VEGF (representing VEGF-A), VEGF-C and VEGFR-1, -2, -3 immunohistochemical expression on formalin-fixed, paraffin-embedded tissue sections were evaluated. A total of 196 tissue samples consisting of 130 malignant melanomas (MM) with various vertical depth of invasion, 15 metastatic melanomas, and 66 nevi including dysplastic nevi and melanocytic nevi were analysed. Production of both VEGFs were common in benign melanocytic tumors while MM exhibited significant upregulation of VEGF ($p<0.0027$) and VEGF-C ($p<0.0001$). The proteins were also detected within stromal cells surrounding tumors, particularly in fibrocytes/fibroblasts, macrophages and endothelial cells. They also exhibited significant increase in malignant lesions ($p<0.0001$). VEGFRs were localized in tumor, as well in stromal cells. Although expression of VEGF receptors was significantly higher in MM versus nevi ($p<0.002$ for VEGFR-1, $p<0.004$ for VEGFR-2 and $p<0.0001$ for VEGFR-3), a considerable percentage of MM were negative. There were no correlations between sentinel node positivity and all investigated proteins. When clinical outcome was evaluated, progression of the disease positively correlated with VEGF ($p<0,007$) and VEGF-C ($p<0,008$) expression VEGF ($p<0.001$) and VEGF-C ($p<0.0001$) positively correlated with nestin expression in the capillary endothelium, which was used for angiogenesis detection. Our work demonstrated that upregulation of VEGFs is associated with progression of malignant melanomas. The protein expression in the tumor microenvironment highlights their importance in malignant stromal phenotype which may serve as a potential target for the anticancer therapy.

Key words: VEGF, VEGFR, malignant melanoma, stromal microenvironment, immunohistochemistry

It is well-known that the biological behavior of malignant melanomas (MM) is difficult to predict on the basis of histological criteria. Increasing attention is being focused on the interactions of tumor cells with host; stroma/tumor interaction is even considered critical in carcinogenesis, tumor invasion, and metastasis [1]. Angiogenesis is an example of how tumor stroma differs from normal connective tissue. The induction of new blood vessel growth into tumors from a preexisting vascular beds has been reported as a parameter of potential prognostic value in solid tumors, as it may facilitate tumor growth and metastasis [2]. It has been shown that solid tumors require for their growth beyond 1-2 mm in size, constant vascular growth and remodeling [3]. Studies on human breast carcinomas have shown that vascular stroma formation occurs before invasion by tumor cells [1]. In contrast, normal adult vasculature is generally quiescent in nature, with endothelial cells dividing approximately every 10 years [4]. Extensive angiogenesis occurs normally only during the female reproductive cycle and in body repair processes, as in wound healing [5]. Tumor angiogenesis is controlled by a variety of angiogenic factors whose production can change over time [6, 7]. Among these, vascular endothelial growth factors (VEGFs) pose the leading role.

VEGFs and their receptors have been established as distinctive proteins playing a role in endothelial cell proliferation and/or elongation, migration and vascular morphogenesis.

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Table 1

antibody	type	firm	dilution
VEGF	mouse monoclonal (C-1)	Santa Cruz Biotechnology	1:50
VEGF-C	goat polyclonal (N-19)	Santa Cruz Biotechnology	1:50
VEGRF1	rabbit polyclonal	Lab Vision Corporation	1:75
VEGRF2	rabbit polyclonal	Lab Vision Corporation	1:75
VEGRF3	rabbit polyclonal	Chemicon International	1:50
nestin	mouse monoclonal	Chemicon International	1:200

VEGFs are produced by a variety of cell types, including keratinocytes, macrophages, mast cells, smooth muscle cells and fibroblasts [8, 9]. Malignant cells of a spectrum of human solid tumors such as breast, colon, lung and gastric have been proved to express these vascular factors [10, 11].

VEGF is the most well-characterized member of a family of structurally related proteins that act as endothelial cell (EC) mitogens and angiogenic factors [5, 12]. VEGF is a dimeric glycoprotein with structural homology to PDGF [13]. One of the most striking characteristics of VEGF is its ability to induce vascular permeability [12]. This enhanced permeability leads to subsequent fibrin deposition in the extracellular matrix that can then serve as a scaffold for migrating endothelial cells. VEGF-C, a structural homolog with VEGF and PDGF, has been demonstrated to stimulate the growth of lymphatic endothelium and thus induce lymphangiogenesis [14]. The known responses of VEGFs are mediated through their receptors. VEGFRs, including VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 [15]. The activation of VEGFRs by its ligands results in enhanced permeability of the vasculature and increased migration and proliferation of endothelial cells, making them also major targets for therapy [16]. VEGF binds and activates two receptors, VEGFR-1 and VEGFR-2. The binding-affinity of VEGFR-1 for VEGF-A is one order of magnitude higher than that of VEGFR-2, whereas the kinase activity of VEGFR-1 is about 10-fold weaker than that of VEGFR-2 [17]. VEGFR-1 plays a dual role: a negative role in angiogenesis in the embryo most likely by trapping VEGF, and a positive role in adulthood in a tyrosine kinase-dependent manner. VEGFR-2 has strong tyrosine kinase activity and it transduces the major signals for angiogenesis. VEGFR-2 is a direct signal transducer for pathological angiogenesis including cancer [17].

VEGF-C binds and activates VEGFR-2 and VEGFR-3 [18]. VEGFR-3 is essential for the development of the lymphatic vasculature. Experimental tumors that overexpress VEGFR-3 ligands induce lymphatic vessel sprouting and enlargement and show enhanced metastasis to regional lymph nodes [19].

Proliferating endothelial cells have been shown to express nestin. On the other side, reduction or loss of nestin expression is associated with a gradual decrease in the proliferation and an increased differentiation of endothelial cells. According to Sugawara et al., nestin is even considered to be a marker of endothelial proliferation [20].

The study was aimed to investigate the expression of VEGF (representing VEGF-A), VEGF-C and their receptors VEGFR-1, -2, -3 in cutaneous malignant melanomas. The expression of this protein was evaluated in relation to the nestin expression in the surrounding endothelium and the clinicopathological parameters of the disease. These levels were then compared with the expression of this protein in benign nevi.

Materials and methods

A total of 196 tissue samples consisting of 130 malignant melanomas comprising 77 nodular melanomas, 38 superficial spreading melanomas, 15 metastatic melanomas (META), and 66 nevi (N) including dysplastic nevi and melanocytic nevi were analyzed. Nodular and superficial melanomas were divided into two groups according to the depth of invasion (T1< 1mm and T2-T4 >1mm). Indirect immnunohistochemistry on formalin-fixed, paraffin-embedded, 5 μm thick tissue sections, using monoclonal and polyclonal antibodies (Tab. 1), was performed. The paraffin-embedded sections were deparaffinated and treated for 20 minutes in microwave generator (750 W) in 0.01 M citrate buffer (pH 6.0; Lachema, Czech Republic) for antigen retrieval. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 15 minutes. Sections were then incubated overnight with the primary antibodies. The antibody complexes were visualized using the Dako EnVision Plus system (Dako Cytomation, Glostrup, Denmark) for 60 minutes at room temperature with aminoethylcarbazole as a chromogen. Tissue sections of secretory endometrium served as positive controls. For negative controls primary antibodies were omitted. The results were analyzed using a semiquantive scale: (0), negative, (1), weak = up to 25% positive cells, (2), moderate = up to 50% positive cells, (3), strong = more than 50% positive cells. An immunohistochemical scoring system (H-score), where the percentage of stained cells is multiplied by intensity of staining was used for evaluation of the total protein expression. The cases were sorted into four subgroups according to the intensity of the staining: H-score 0 refers to negative expression; H-score 1-2 to weak expression; H-score 3-5 to moderate expression; H-score 6-9 to strong expression. The results were compared with histological diagnosis, tumor grade using the Breslow and TNM classification, endothelial nestin expression within or in the neighborhood of the tumors, sentinel lymph node status and clinical state, estimated as progression or regression of the disease within a 3-year follow-up. Statistical assessment was performed using the Chi-square test and ($p<0.05$) was regarded as statistically significant.

Results

VEGF (representing VEGF-A) and VEGF-C were localized cytoplasmatically. VEGF was present in most benign and malignant lesions, however in different intensities (Fig.1A, 2A and 2B). Statistically significant increase in the protein

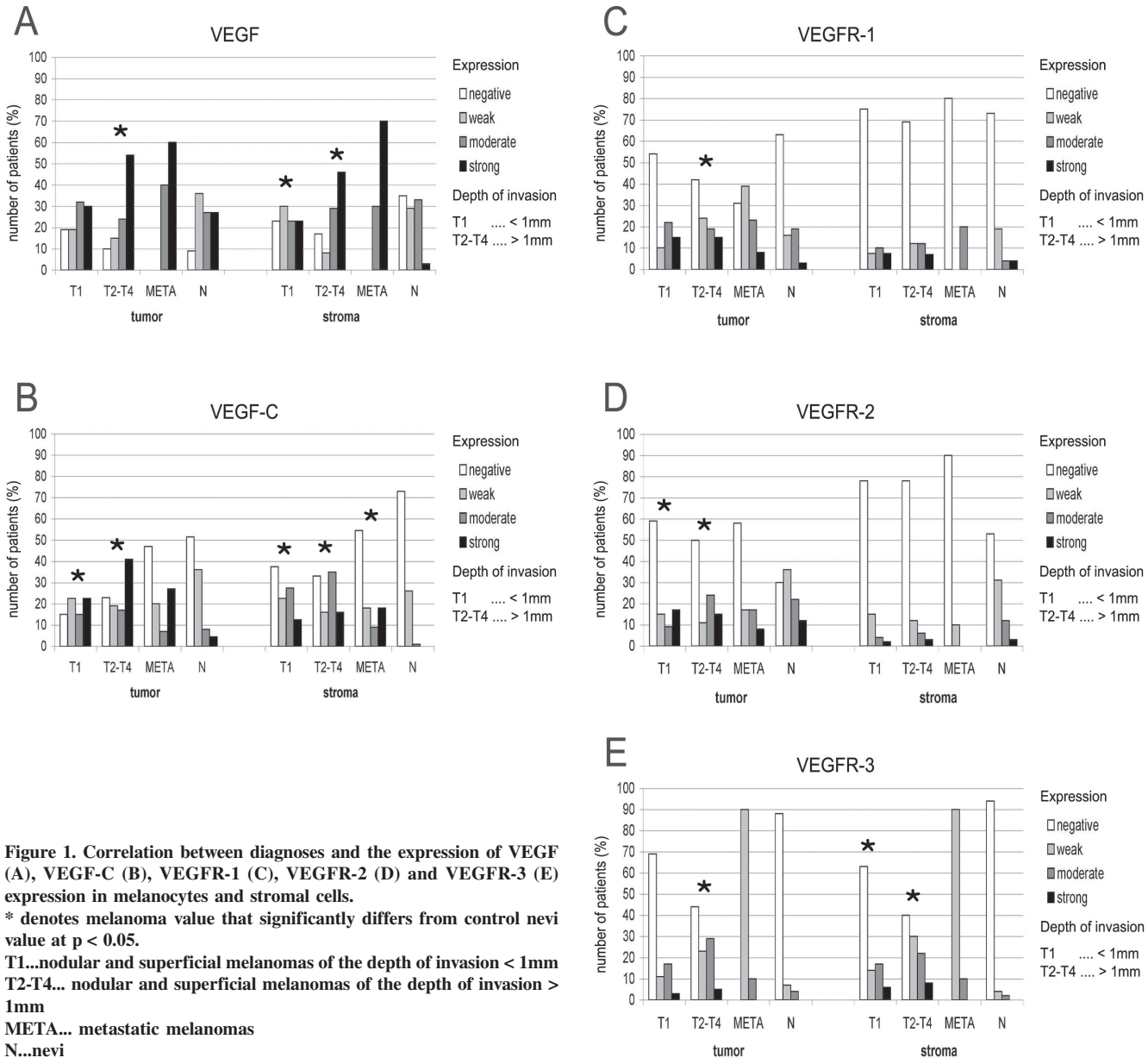

Figure 1. Correlation between diagnoses and the expression of VEGF (A), VEGF-C (B), VEGFR-1 (C), VEGFR-2 (D) and VEGFR-3 (E) expression in melanocytes and stromal cells.
*** denotes melanoma value that significantly differs from control nevi value at p < 0.05.**
T1...nodular and superficial melanomas of the depth of invasion < 1mm
T2-T4... nodular and superficial melanomas of the depth of invasion > 1mm
META... metastatic melanomas
N...nevi

expression was found in advanced MM exceeding vertical invasion 1 mm if compared to nevi (p<0.0027). No differences were observed comparing early melanomas and nevi.On the other hand, as for VEGF-C, nevi were mostly negative or weakly positive, while malignant lesions showed significant higher protein levels, where moderate to strong expression predominated (p<0.0001; Fig 1B). There were no differences between early (T1), and advanced melanoma stages (T2-T4). VEGFs were also detected within stromal cells surrounding tumors, particularly in fibrocytes/fibroblasts, macrophages and endothelial cells. The microenvironment of MM exhibited significantly higher expression of both proteins in comparison with nevi (p<0.0001). Moreover, the stroma of advanced melanomas showed higher proteins levels than early lesions (p<0.0055).

The results of VEGFR-1, -2, -3 in tumors and stromal microenvironment are summarized in Fig. 1C, 1D, 1E. All these proteins were found predominantly cytoplasmatically, less frequently nuclear or combined nuclear/cytoplasmic staining were seen. Although VEGFR-1 was exprimed in higher levels in melanoblasts mostly of advanced MM when compared with nevi (p<0.002) (Fig. 2C), a considerable part of these tumors were negative or weakly positive. The protein was also detected in scattered stromal elements, mostly in the capillary endothelium, lymphocytes, macrophages and fibrocytes/fibroblasts, but the differences of its levels were not signifi-

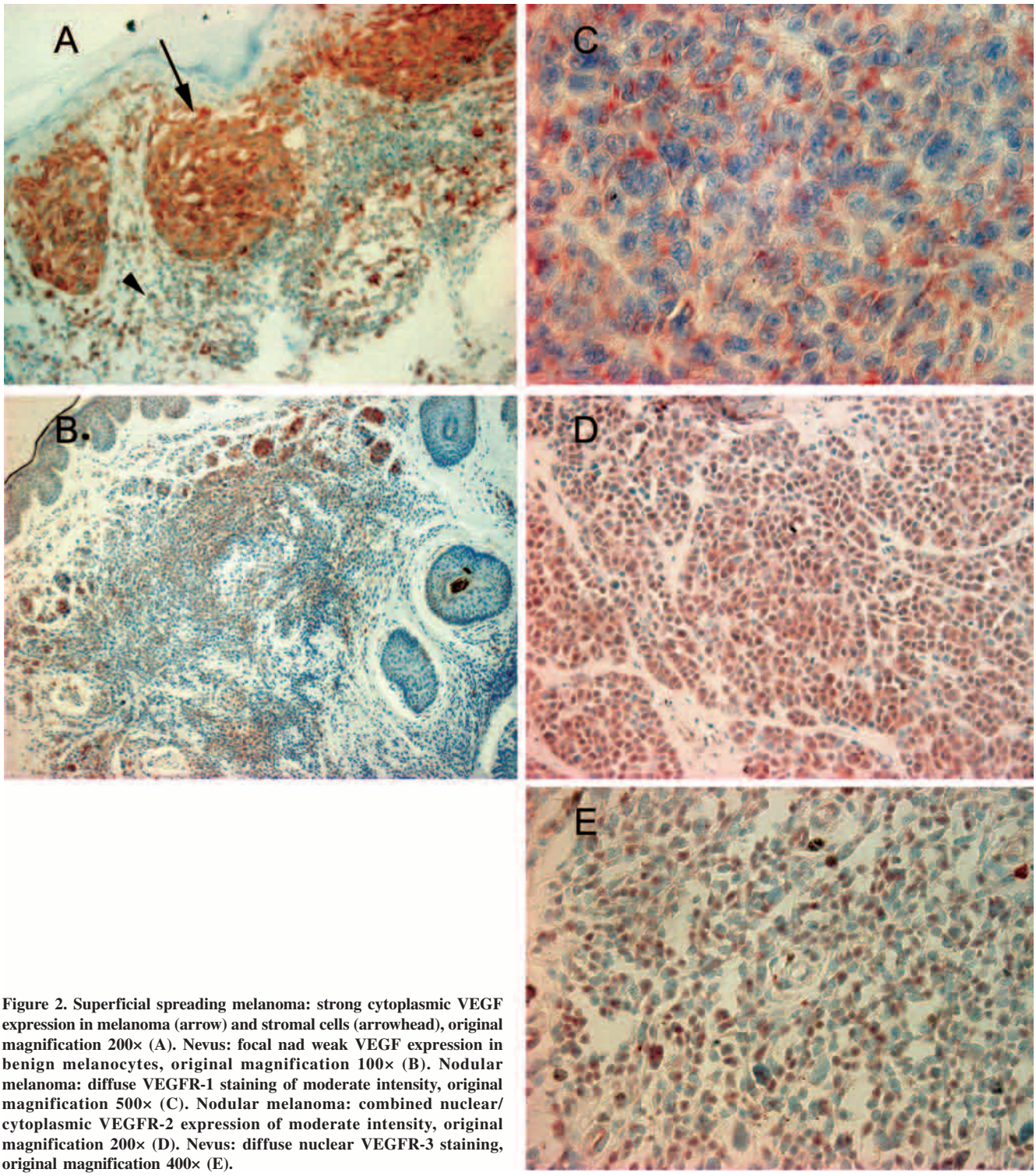

Figure 2. Superficial spreading melanoma: strong cytoplasmic VEGF expression in melanoma (arrow) and stromal cells (arrowhead), original magnification 200× (A). Nevus: focal nad weak VEGF expression in benign melanocytes, original magnification 100× (B). Nodular melanoma: diffuse VEGFR-1 staining of moderate intensity, original magnification 500× (C). Nodular melanoma: combined nuclear/ cytoplasmic VEGFR-2 expression of moderate intensity, original magnification 200× (D). Nevus: diffuse nuclear VEGFR-3 staining, original magnification 400× (E).

cant. VEGFR-2 exhibited, increasing expression in MM versus nevi ($p<0.004$) for both early and advanced MM; (Fig. 2D), even though half melanomas were negative, and furthermore, in a part of nevi lesions the protein expression was moderate or strong in intensity. No differences among lesions were found when stromal expression was evaluated. VEGFR-3 also positively correlated with the depth of invasion, significant increase were found in advanced MM in compari-

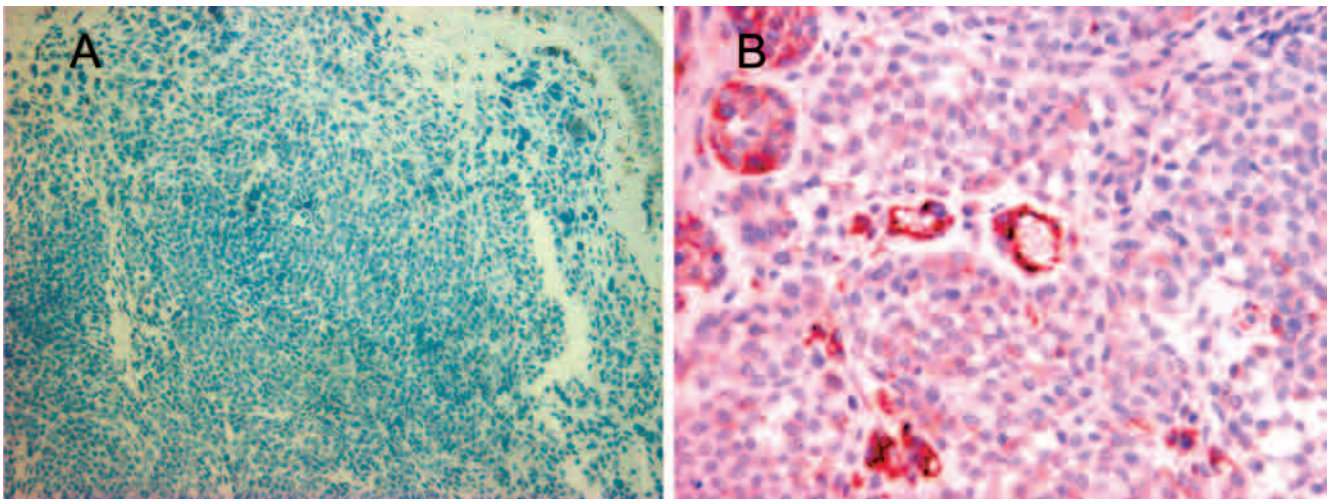

Figure 3. Intradermal nevus: Nestin-negative staining of nevi and endothelial cells, original magnification 200×(A). Nodular melanoma:diffuse nestin expression of moderate intensity in melanoma cells and strong overexpression of the protein within the endothelium, original magnification 400× (B).

son with moles, in which it was detected only sporadically ($p<0.0001$). Similarly, the stromal elements of melanomas revealed higher protein expression, when compared to nevi ($p<0.0001$), which were over 94% cases negative (Fig.2E).

The nestin staining was increased in the capillary endothelium adjacent to the MM (Fig.3A, 3B), although the nestin-positive endothelial cells were also observed in the vessels surrounding melanocytic nevi, especially of larger nevi. The number of nestin-positive cells in the capillary endothelium positively correlated with VEGF ($p<0.001$) and VEGF-C ($p<0.0001$) protein expression.

Positive sentinel lymph nodes were detected in only 33% of cases. There were no correlations between sentinel node positivity and all investigated parameters. When clinical outcome was evaluated, progression of the disease positively correlated with VEGF ($p<0,007$) and VEGF-C ($p<0,008$) expression.

Discussion

In this study, we show that VEGF-A and VEGF-C are commonly present in both benign and malignant tumors. Both proteins were distributed rather evenly in all tumor cells. This contrasts with the data published by Dvorak et al (1995) who found highly elevated protein levels in hypoxic areas of tumors, particularly in areas surrounding tumor necrosis [21]. Although a major stimulus for VEGF expression is hypoxia leading to stimulation of HIF (hypoxia induced factor) gene transcription, other regulatory mechanisms, mainly cytokines and growth factors also contribute to the expression [22, 23]. As we detected no necrosis in our sections and VEGFs were present even in small-sized MM, we stress the role of cytokines in the protein overexpression. Differing data has been published on VEGFs expression in benign tumors; whilst some investigators describe the factors being normally produced by benign tumor cells [5, 24], others associate their upregulation only with melanoma progression and dissemination, and claim that naevocellular nevi contain no detectable VEGFs [25, 26]. Our observed VEGFs staining in benign melanocytes, albeit in lower levels, confirms that the factors regulate angiogenesis under both benign and malignant conditions, and their detection cannot have been used for differentiation of biological character of a lesion. Nestin detection, as a marker of angiogenesis, affirms increased amount of newly formed vessels by the moles, especially of larger ones. We suggest that benign tumor cells influence the composition of the surrounding stroma, tumor growth and/or mechanical irritation induce changes identical to wound healing. The crucial event distinguishing malignant cells seems to be the extended overexpression of the proteins [27]. Whereas in healing wounds and benign tumors VEGFs expression ceases, in malignancies are expressed indefinitely. Proteins levels might be already increased in an early tumor stage; as we demonstrate in our study, where deregulated expression, mainly of VEGF-C in early T1stage melanomas was seen. Local tissue protein changes, thus considerably precede their serological levels: significant increase in plasma VEGF levels was found in patients with melanoma patients in stages I, II and III vs. those in stage IV [28].

Upregulated proteins were associated with marked angiogenesis, when increased density of nestin-positive endothelium within or nearby melanomas, especially of advanced stages, was found. Another important point regarded as critical in tumor progression has currently been attracting attention – stromal microenvironment and stromal/tumor interaction [29]. In our study, both protein staining in fibrocytes/fibroblasts, macrophages and endothelial cells exhibited a significant increase compared MM vs. nevi. Moreover, there were differences be-

tween T1 and more advanced lesions. Thus, we found that phenotypic alteration of the microenvironment already accompanies early phases of malignancies. Differing data explaining VEGF expression in the stromal cells has been published. Apart from the possibility that the proteins diffuse from the tumors to the stromal compartment and there subsequently accumulate [5], their direct expression by stromal cells has been shown to be important as a potential therapeutic target [29].

One assumed way that VEGFs may act is via targeting their receptors – VEGFRs. Whereas the role of the receptors has been extensively described during embryogenesis [30], their contribution to other pathological conditions, especially carcinogenesis is still a topic of interest. While malignant lesions were associated with upregulation of the proteins, inhibition of their overexpression resulted in significant reduction in blood and lymphatic vessel density and tumor necrosis [19]. However, in this study, despite the significant increase of the proteins in expression in MM, mainly advanced cases, the majority of malignant lesions were VEGFRs negative. Furthermore, we observed no correlations between the proteins expression and microvessel density. In our study, the receptors were detected both in tumors cells and the endothelial cells, macrophages and fibrocytes/fibroblasts, as well. We thus confirm recent findings that VEGFRs are not exclusively localized in vascular endothelium; they are also found in cancer and various stromal cells [31, 32]. Interestingly, the proteins are expressed not only by malignant melanoblasts but also, albeit in lower amounts, by nevi cells. So we suggest that VEGFRs may then also regulate the growth of benign tumors. The proteins exhibited mostly a cytoplasmatic pattern of staining. As monoclonal antibodies to the cytoplasmic tail of the receptors were used for the detection, we did not observe typical membraneous location of these proteins. However, some published data stress the implication of subcellular re-location of the protein resulting in their funcional changes. Nuclear localization of VEGFRs is decribed to be typical for malignant lesions [24], where associated with a transctiptional activity of the proteins as a consequence of their kinase activity and an ability to positively regulate DNA synthesis, cell proliferation and migration [33] stimulating angiogenesis and/or tumor growth [34]. But as only non-significant minority of our cases exhibited such nuclear VEGFRs re-localization or cytoplasmic/nuclear co-location, we did not confirm the findings. Based on our results we suggest that angiogenic or autocrine pathway of VEGFs action might be either independent of their receptors, or their link may be indirect. Some published investigations affirm that the situation is more complicated than was previously thought. So, in vitro study on the melanoma B16 cells found no VEGFR-1 or -2 expression. Pisacane and Risio described no association between microvascular density and VEGFR-2 [24]. Similarly, Straume and Akslen stress that the development of a tumor-related capillary network in malignant melanomas may be independent on VEGFR expression [35]. Moreover, inhibition of VEGFR-1 or 2 achieved no significant influence on melanoma growth [32]; and the observed inhibition of VEGFR signaling was primarily assigned to strong reduction of inflammatory infiltrate resulting in decreased cytokines levels. But some proteins, e.g. Notch, directly regulating VEGFR expression and altering VEGF function have been recently described [36] and must be considered. Likewise, proangiogenic co-receptors, such as neuropilins, which are known to posses an ability to affect VEGF function and/or VEGFR expression should be regarded [37]. On the other hand, the overexpression of VEGFR-1 was shown to be associated with favorable prognosis of colorectal cancers [38]. One explanation might be that VEGFRs trap VEGFs and limit them to an appropriate range [39]. From this point of view we may also explain negative VEGFR cases in advanced melanoma with clinical progression.

In summary, our work has demonstrated that strong upregulation of VEGF is associated with progression of malignant melanomas. The protein expression in the tumor microenvironment highlights the importance of malignant stromal phenotype which may serve as a potential target for the development of anticancer agents. We hypothesise that VEGF action might not be directly dependent on their receptors. Discovery of the secondary factors responsible for VEGF mediated angiogenesis and/or direct tumor cell stimulation may be an important future goal with clinical utility.

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