

Molecular Changes in PDEGF and bFGF in Malignant Melanomas in Relation to the Stromal Microenvironment

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Abstract. *Background: Aberrant expression of either growth factors or growth factor-receptors by stromal cells can be an important factor promoting the growth of solid tumours. It may also affect differentiation of malignant cells and support tumour spread. The aim of the present study was to investigate the hypothesis that basic-fibroblast growth factor (bFGF) and platelet-derived growth factor (PDEGF) may be involved in tumour-stromal microenvironment interactions in primary malignant melanomas. Materials and Methods: PDEGF and bFGF expression in malignant cells and surrounding stromal elements was assessed using indirect immunohistochemistry. Results: It was confirmed that PDEGF can be involved in the reciprocal interactions between tumour cells and stroma, including aberrant angiogenesis. Interestingly, bFGF was present both in malignant melanoma lesions and benign nevi accompanied by different intracellular localisation of the protein, suggesting its implication in regulation of nevus cell proliferation and maturation. Conclusion: The present results suggest that bFGF and PDEGF participate in malignant melanoma progression.*

Malignant melanoma (MM) is a very aggressive type of cancer and the chances of survival often depend on early diagnosis and treatment. It is a highly malignant tumour with rapidly increasing incidence, which originates from pigmented cells derived from the neural crest.

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The prognosis of MM is still based only on histological criteria: tumour size, depth of invasion, ulcerations and mitotic activity. However, these parameters do not permit precise prognosis. A small-sized tumour can metastasise very early, while, on the other hand, tumours of advanced stages may remain localised for many years (1).

The possibility that the tumour microenvironment might play a role in the process of carcinogenesis and metastasis development represents a potential therapeutic target. It has been shown that interactions between tumour cells and the surrounding stromal environment are significant (2). Alterations of the stromal microenvironment, including increased angiogenesis, changes in extracellular matrix composition, increased protease activity and the presence of inflammatory cells are important factors determining tumour growth and invasion. The most recent studies provide evidence for the importance of stromal cells in carcinogenesis (3). This microenvironment can be different in central areas of a tumour where hypoxia and necrosis occur, while more viable areas are found toward the periphery (4). In this regard, tumour cells can regulate development of specific tumour-promoting stroma *via* aberrant expression of either growth factors or growth factor-receptor induction (5), and stromal cells within a stroma may reciprocally affect differentiation of malignant cells (6) and support tumour spread. Invasion of malignant cells into blood or lymphatic vessels is associated with aberrant angiogenesis, which is an important factor determining further dissemination and tumour progression.

The aim of our study was to examine the basic-fibroblast growth factor (bFGF) and platelet-derived growth factor (PDEGF) expression in MM cells and the surrounding stromal environment. PDEGF drives cellular responses, including proliferation, survival, migration and the deposition of extracellular matrix and tissue remodeling factors (7). bFGF stimulates proliferation of mesenchymal,

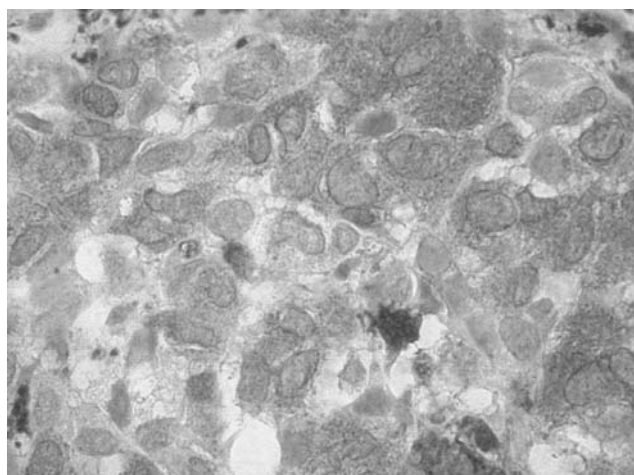


Figure 1. Strong cytoplasmic PDEGF expression in malignant melanoma cells (original magnification 800x).

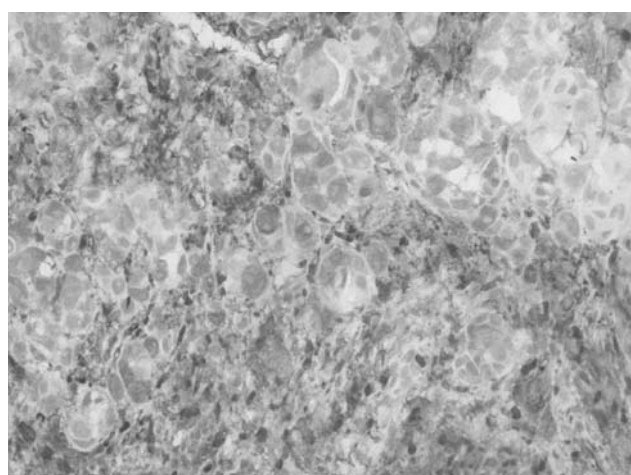


Figure 2. Overexpression of PDEGF in stromal cells surrounding the tumour (original magnification 250x).

epithelial and neuroectodermal cells. Both bFGF and PDEGF are implicated in normal as well as tumour-associated neo-angiogenesis. Tumours with higher expression of these regulatory factors seem to have increased risk for metastases. On the other hand, malignant lesions lacking the receptors and factors probably will not respond to lymphatic and blood cytokines and their malignant potential will be decreased.

Materials and Methods

Samples. The formalin-fixed, paraffin-embedded, routine biopsy specimens were taken from the Institute of Pathology, Faculty of Medicine, Palacky University, Olomouc, Czech Republic. Each sample was assessed histologically by a pathologist before inclusion into the study. Forty-five of the 76 melanomas were classified as nodular melanomas (NM), 33 as superficial spreading melanomas (SSM). Forty-three specimens of common nevi and 10 specimens of dysplastic were used as controls.

Immunohistochemical analysis. Indirect immunohistochemistry using the EnVision visualization system was performed. Briefly, 5- μ m-thick sections were deparaffinised, microwave-oven treated in citrate buffer (pH 6.0) for antigen retrieval, rinsed in Tris buffer (pH 7.6), blocked with 3% hydrogen peroxide solution for 10 min, incubated with 5% dry milk in Tris buffer for 30 min, and incubated overnight at 4°C with the mouse primary antibodies (PDEGF, clone P-GF.44C, 1:100, Novocastra Laboratories Ltd., Newcastle-upon-Tyne, UK; FGF-2, clone 147, 1:100, Santa Cruz Biotechnology, CA, USA). Following washing in Tris buffer, all sections were incubated with anti-mouse EnVision™ + Kit (DakoCytomation, Glostrup, Denmark) for 1 h. All incubations were carried out in a humid chamber. After rinsing in Tris buffer, the antigens were then visualized by incubating with a solution of 3-amino-9-ethyl-carbazole (AEC) (DakoCytomation) for 10 min. Slides were counterstained with haematoxylin and eosin, dehydrated in alcohol, cleared in xylene

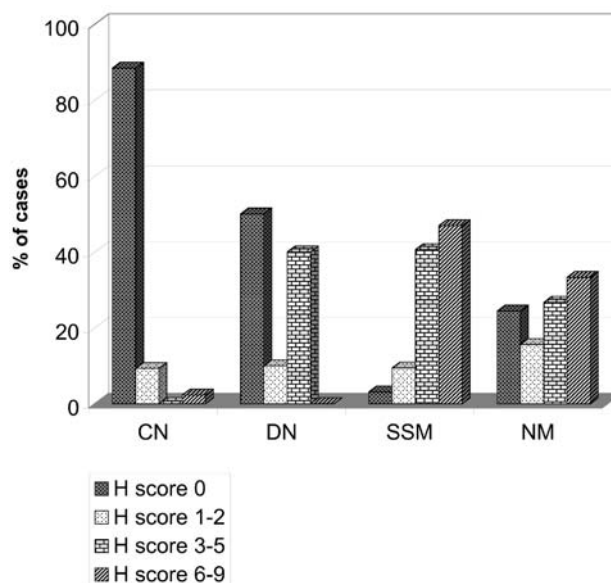


Figure 3. Correlation between diagnoses and amount of the PDEGF expression in melanocytes (CN: common nevi, DN: dysplastic nevi, SSM: superficial spreading melanomas, NM: nodular melanomas). H-score 0 refers to negative staining; 1-2 to weak; 3-5 to moderate; 6-9 to strong staining.

and mounted with Canada balsam. In all experiments, negative controls omitting the primary antibody and positive controls on human colon carcinoma tissue were carried out and evaluated in parallel.

Both the staining intensity and quality were scored using a semi-quantitative scale: (0), negligible, (1), weak = up to 25% positive cells, (2), moderate = up to 50% positive cells, (3), strong = more than 50% positive cells. The staining was assessed in tumour as well as in stromal cells. We used the H-score (intensity x quantity) for evaluation of total protein expression. H-score 0 refers to negative

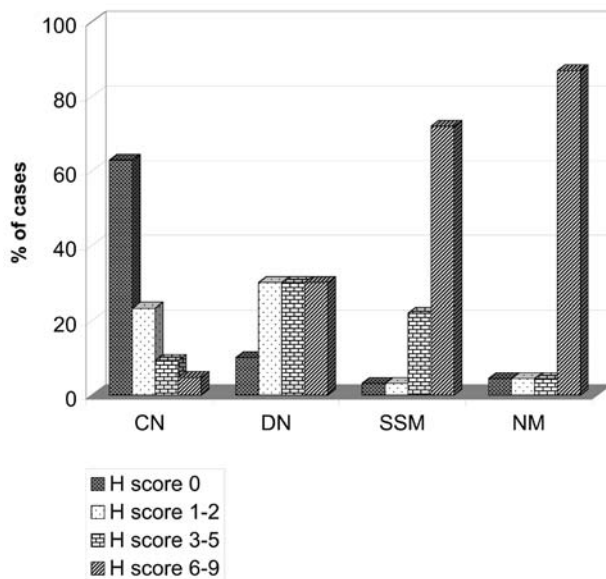


Figure 4. Correlation between diagnoses and amount of the PDEGF expression in stromal cells mainly in fibrocytes (CN: common nevi, DN: dysplastic nevi, SSM: superficial spreading melanomas, NM: nodular melanomas). H-score 0 refers to negative staining; 1-2 to weak; 3-5 to moderate; 6-9 to strong staining.

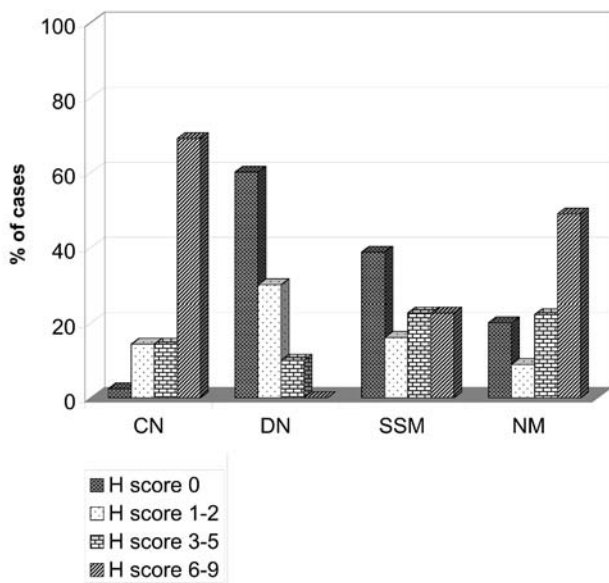


Figure 5. Correlation between diagnoses and amount of the bFGF expression in melanocytes (CN: common nevi, DN: dysplastic nevi, SSM: superficial spreading melanomas, NM: nodular melanomas). H-score 0 refers to negative staining; 1-2 to weak; 3-5 to moderate; 6-9 to strong staining.

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The results were statistically analysed using a Chi-square test ($p < 0.05$).

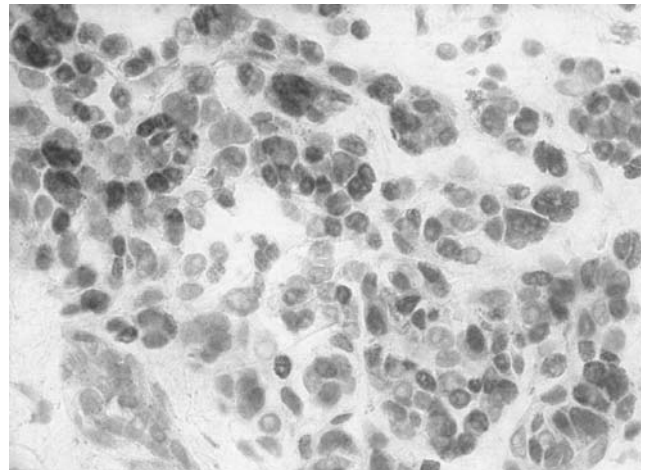


Figure 6. Nuclear bFGF staining in nevi cells (original magnification 640x).

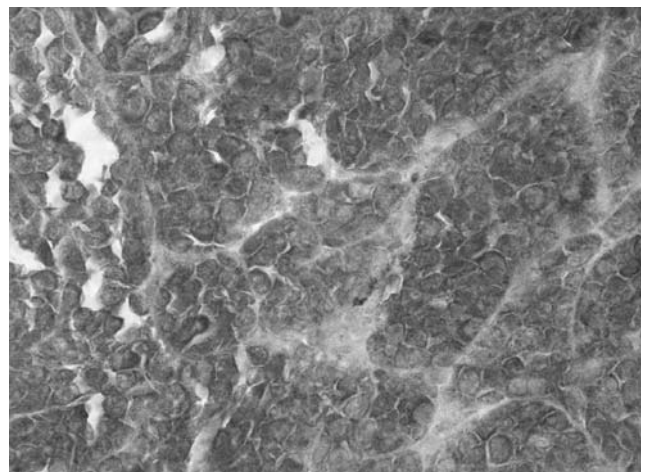


Figure 7. Cytoplasmic bFGF staining predominated in malignant melanomas (original magnification 500x).

Results

PDEGF. PDEGF expression was found in tumour cells (Figure 1) as well as in stromal cells (Figure 2), particularly in fibroblasts surrounding malignant lesions. Moreover, PDEGF staining was occasionally seen in endothelial cells and in epidermal and follicular keratinocytes. PDEGF was localised mainly in the cytoplasm of cells, the nuclear positivity was found infrequently. Melanocytes of all nevi displayed PDEGF protein very sporadically. Statistical analysis confirmed a significantly higher expression of PDEGF in malignant lesions compared to benign ones ($p = 0.0002$ for SSM and $p = 0.033$ for NM). The same phenomenon was observed for PDEGF in stromal cells ($p < 0.0001$ for SSM and $p < 0.0001$ for NM). The distribution of PDEGF expression in differential diagnoses is shown in Figures 3 and 4.

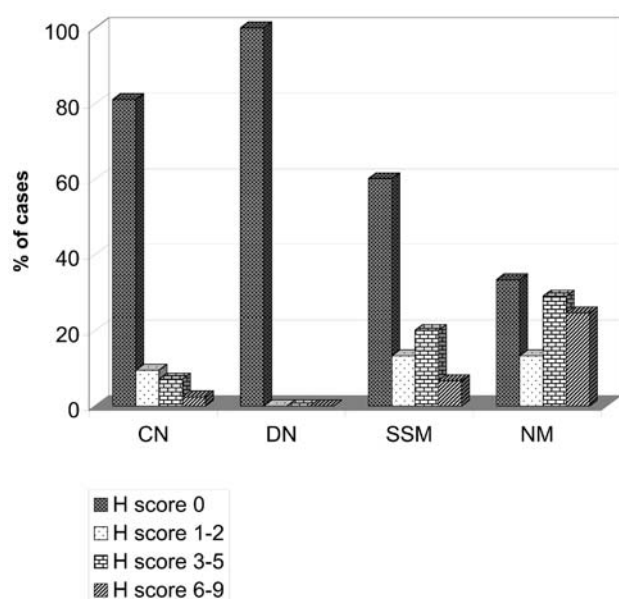


Figure 8. Correlation between diagnoses and amount of the bFGF expression in stromal cells mainly in fibrocytes (CN: common nevi, DN: dysplastic nevi, SSM: superficial spreading melanomas, NM: nodular melanomas). H-score 0 refers to negative staining; 1-2 to weak; 3-5 to moderate; 6-9 to strong staining.

bFGF. The percentages of melanocyte cells stained with bFGF depended on the character of the lesion (Figure 5). Interestingly, we found strong expression of bFGF in nevi. We noticed the nuclear staining of benign melanocytes (Figure 6), whereas in malignant melanomas the cytoplasmic localisation of the protein predominated (Figure 7). A significant difference was found between SSM and benign nevi ($p < 0.0001$) and between NM and benign nevi ($p < 0.0001$). bFGF was also localised in stromal fibroblasts (Figure 8). The immunoreactivity of stromal cells in nodular melanomas was significantly more frequent than in nevi ($p < 0.0001$). There was no correlation between SSM and benign nevi.

Discussion

The molecular events in melanoma development and progression have been described by Saida (8). Transformation of normal human melanocytes to melanoma cells is a multistep process accompanied by loss of growth-restraining functions (such as tumour suppressors) and gain of tumour-promoting factors (such as oncogenes) (9). Malignant melanoma cells express different growth factors and cytokines and their receptors in the various stages of tumour progression, which, by means of autocrine and paracrine effects, support their growth and acquire the ability to metastasise (reviewed in 10). The clue to this

biological role of growth factors in malignant melanoma may lie in the microenvironment character of the lesions. Activated fibroblasts can form a fetal-like environment. These produce a number of factors including bFGF, PDEGF, epidermal growth factor (EGF), transforming growth factor alpha (TGF α) and a family of vascular growth factors (VEGFs) A,B,C. Thus, tumours with increased expression of these regulating factors and their receptors seem to have increased risk of metastatic potential. Higher expression of growth factors, not only in malignant melanoma cells, but in cells of the microenvironment points to the reciprocal influence of these two compartments (11).

In the present study, we investigated PDEGF and bFGF expression using immunohistochemistry to study their potential for malignant cells and the surrounding stromal environment. Higher expression of both PDEGF and bFGF was found, not only in advanced stages of the disease with poor prognosis, but also in stages of early invasion. Therefore, their involvement in early stages of tumour disease is suggested. bFGF is known as a mitogen for human melanocytes. The aberrant expression of bFGF occurs at an early stage of melanocyte lesions, affecting growth and cell dedifferentiation (12). Increased expression of bFGF is connected with induction of malignant phenotype and may be evoked by ultraviolet radiation (13). Halaban *et al.* (14) found bFGF only in malignant melanoma cells and characterised it as a significant autocrine factor influencing melanoma growth. However, these results have not been confirmed by other studies (15, 16), where bFGF has also been described in cells of conventional benign nevi, although significantly less often (17). In our study, we detected bFGF in malignant melanoma lesions as well as in nevi. We can conclude that bFGF cannot be considered to be a marker of melanocyte transformation. We identified nuclear localisation of the protein in nevi, while in malignant melanomas cytoplasmic localisation of bFGF predominated. This finding may reflect an aberrant form of the bFGF molecule, which is either incapable of nuclear transport, or a defect in the bFGF-driven cascade may exist. It is possible that the different localisation may be a key to another effect of bFGF, for example on the growth of malignant lesions and/or stimulation of fibrotisation and maturation of benign nevi.

PDEGF mediates the development of a tumour stroma according to the metabolic requirements of the growing tumour (18). PDEGF is assumed to be an important autocrine growth factor in melanoma, showing significant mitogenic activity (19). Its higher expression is documented, not only in cells of primary skin melanomas, but also in their metastases, emphasising its importance as a growth factor in melanoma progression (20). It has been found that PDEGF has its own transformative potential and anti-apoptotic effect (21). Apart from tumour cells,

PDEGF is produced by activated macrophages and fibroblasts, endothelial cells, smooth muscle vessels and epidermal keratinocytes. Our results are in accord with these previous studies. We found a strong PDEGF expression in stromal cells, indicating its role in the stimulation of tumour stroma development and formation of new blood and lymphatic vessels.

Acknowledgements

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