Examination of different factors influencing the vascularization of human cutaneous melanoma

PhD thesis booklet

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PhD School of Pathological Sciences

Program leader: Prof. László Kopper

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1. Introduction

Despite the immunogenicity of melanoma observed by researchers and clinicians, melanoma belongs to the most aggressive human tumors, with survival of patients with distant metastases still very low. What can be the reason of this contradiction?

Melanoma antigens are processed by official antigen-presenting cells, primarily by dendritic cells (DCs), which then present antigenic peptides to T cells in the neighboring lymph nodes. Activated $CD8^+$ T lymphocytes directly attack tumor cells. $CD8^+$ T lymphocytes, macrophages and eosinophil granulocytes are stimulated by $CD4^+$ T cells through cytokines.

Unfortunately this immune response is not able to prevent tumor progression. Several mechanisms have been described to explain this phenomenon: down-regulation of melanoma-associated antigens or MHC I molecules on the surface of tumor cells; insufficient presentation of antigens; absence of costimulatory signals; immunosuppressive cytokines produced in the microenvironment of tumors; apoptosis of tumor-infiltrating lymphocytes; development of immunotolerance.

The process of angiogenesis is regulated by the balance between the angiogenic (VEGF, bFGF, PDGF) and angiostatic (angiostatin, endostatin, IFNγ) cytokines. Tumor vascularization is probably influenced by an interaction between malignant cells and host cells. Since the majority of tumor-infiltrating cells are derived from the circulation, it is expected that the density of peri- and intratumoral microvessels might be associated with that of the infiltrating cells in melanoma. On the other hand, since tumor-

infiltrating host cells are rich sources of angiogenic factors, their presence might be associated with MVD.

According to our observations, melanoma induces neoangiogenesis peritumorally, and then incorporates part of the microvessels around the tumor. In the tumor microenvironment intratumorally only a proportion of capillaries survive. Melanoma cells are able to mimic endothelial cells, and the process of glomeruloid microvascular proliferation has also been described in melanoma.

Due to its unique mechanism of vascularization, the literature is highly controversial over the association of microvessel density (MVD) and clinicopathological parameters of malignant melanoma. Several investigators have suggested a relationship between the extent of tumor vascularity and prognosis in cutaneous melanomas. However, others did not find tumor vascularity of prognostic value, or even came to the conclusion that high vascularization was associated with a better prognosis.

Most of the clinical trials examining the efficacy of anti-angiogenic agents (thalidomid, semaxanib, sorafenib, marimastat, topotecan, Vitaxin) in melanoma have failed so far. The most effective compounds were tumor necrosis factor (TNF) given in isolated limb perfusion, and the intratumoral injection of plasmid DNA encoding IL-12.

In the second part of my dissertation, I introduce a potential chemotherapeutic and angiostatic agent. Hyperforin is an acilphloroglucinol, lipophilic compound that can be derived from St. John's wort (Hypericum perforatum). In traditional medicine, the lipophilic extracts of St. John's wort are often used to treat burns and skin injuries. Recently, it has gained reputation because of its antidepressive effects. It

also inhibits the proliferation of several gram-positive bacteria, including the meticillin-resistant Staphylococcus aureus. Hyperforin directly inhibits the activity of cyclooxygenase-1 (COX-1), and 5-lipoxygenase (5-LOX). Due to its antibacterial and anti-inflammatory effects, it has been effective in the treatment of atopic dermatitis.

2. Purposes

- Observation of infiltrating cells in melanoma according to tumor progression
- Analysis of the association between microvascular density and tumor-infiltrating cells
- Examination of antitumoral and angiostatic effects of hyperforin

3. Materials and methods

3.1 Patient characteristics

Archival tissue samples were obtained from 52 patients (or 82 patients for the analysis of dendritic cell density) with primary cutaneous malignant melanoma, who underwent surgery between 1980 and 2000 at the Institute of Dermatology, Venerology and Dermatooncology, Semmelweis University, and at the National Institute of Oncology, Budapest. None of the patients received any anticancer treatment prior to surgery. The tumors were grouped into three thickness categories based on the current AJCC (American Joint Committee on Cancer) staging system (Balch et al, 2001) (1.01-2.0 mm, 2.01-4.0 mm, >4.0 mm), and into three categories according

to disease progression during the 5-year follow-up period (nonmetastatic, lymph node metastatic, visceral metastatic).

3.2 Immunohistochemical detection of infiltrating cells and microvessels in melanoma samples

Three-µm sections cut from formalin-fixed, paraffin-embedded cutaneous melanoma samples were used in the study. Immunohistochemistry was performed on deparaffinized serial sections after microwave antigen retrieval. Endogenous peroxidase activity was blocked with incubation of the slides in 3% H_2O_2 in methanol, and non-specific binding sites were blocked with 3% bovine serum albumin. Polyclonal anti-CD3 (diluted 1:100), anti-CD8 (diluted 1:100), anti-CD20 (diluted 1:100), anti-CD34 (diluted 1:50) and anti-CD68 (diluted 1:100) (all from Dako, Glostrup, Denmark), and monoclonal anti-CD1a and anti-DC-LAMP (Immunotech, Marseilles, France) were used as primary antibodies, and mouse IgG1 (Sigma, St. Louis, MO) for negative control. Biotinylated anti-mouse/anti-rabbit Ig was used as secondary reagent, followed by streptavidin-peroxidase treatment (LSAB2 System, HRP; Dako). Antibody binding was visualized with 3-amino-9-ethylcarbazole (AEC; Vector Laboratories, Inc., Burlingame, CA), then the slides were counterstained with hematoxylin.

Microvessels were highlighted by staining endothelial cells with anti-CD34 antibody. We selected three vascular hot spots both intra- and peritumorally, and counted the $CD34^+$ microvessels. The number of $CD3^+$, $CD8^+$ or $CD20^+$ lymphocytes, $CD1a^+$ DCs, and $CD68^+$ macrophages was

registered separately in intratumoral and peritumoral areas at five randomly selected sites, and the density of positive cells/mm^2 was calculated.

3.3 Statistical analysis

Statistical comparison between cell densities in different tumor groups was made by using the Mann-Whitney U test and Kruskal-Wallis test. The correlation between $CD3^+$, $CD8^+$, $CD20^+$, $CD68^+$, $CD1a^+$ infiltrating cells and microvessels was evaluated by using Spearman rank correlation test. All statistics were calculated using the BMDP Statistical Software Pack.

3.4 Hyperforin and cytotoxic drugs

Hyperforin was extracted from Hypericum perforatum (Flavex, Rehlingen, Germany). Paclitaxel was obtained from Tocris, camptothecin and vincristine from Sigma. All drugs were dissolved in dimethyl-sulphoxide (DMSO), and further diluted with phosphate-buffered saline (PBS). Further dilutions were made with medium to obtain final concentrations. The maximal final DMSO concentration was 1%.

3.5 Cell lines

The following cell lines were used: human dermal microvascular cells, A-431 human epidermoid carcinoma, HT-144, MV3, 1F6, SB1 and SB3 human melanomas, Jurkat human acute T-cell leukemia, MCF-7 and MDA-MB-468 human breast adenocarcinomas, SK-OV-3 human ovarian carcinoma, MAT-Lu and AT-2.1 rat prostate carcinomas, AR42J and ARIP rat pancreatic carcinomas, RG2 rat glioblastoma**,** BDX2 rat fibrosarcoma and MT-450 rat mammary adenocarcinoma**.**

3.6 Proliferation test

Adherent cells were collected with trypsin/EDTA. All cells were washed twice with PBS, resuspended in medium, and further cultured in 96-well microtiter plates ($1x10^5$ cells/ml). Following addition of hyperforin (2.5-90 µg/ml), camptothecin, vincristine and paclitaxel (0.0001-1.0 µg/ml), cells were incubated for 24 hours. The incorporation of 5-bromo-2'-deoxyuridine (BrdU) into the DNA of proliferating cells was measured by using a colorimetric BrdU cell proliferation ELISA.

3.7 Photometric determination of apoptosis

Cells were treated with hyperforin for 24 hours as described above. Cells were then evaluated for apoptosis using a cell death detection ELISA (Cell Death Detection ELISA^{PLUS}, Roche Molecular Biochemicals). The principle of this test is the detection of mono- and oligonucleosomes in the cytoplasmic fractions of cell lysates using biotinylated anti-histone and peroxidase-coupled anti-DNA antibodies. The amount of nucleosomes is photometrically quantified at 405 nm by the peroxidase activity retained in the immunocomplexes.

3.8 In vitro "angiogenesis" assay

To investigate the effect of hyperforin on endothelial cells in an *in vitro* model of angiogenesis, we used ECMatrix™ (Chemicon), a solid gel of basement proteins, prepared from the EHS mouse tumor. The gel was placed in a heated (37°C) incubation chamber of a TILL-Photonics™ time-lapse video microscope equipped with a video imaging system. Human

dermal microvascular endothelial cells (HDMEC, $2x10^5$) suspended in medium were placed on top of the gel with or without addition of 5 µg/ml hyperforin. Transmitted light images were obtained every 10 sec for 12 h.

3.9 Determination of VEGF in cell culture supernatants

MT-450 cells ($1x10^5$/ ml) were cultured for 24 h with hyperforin at the indicated concentrations. Supernatants were tested for secreted vascular endothelial growth factor (VEGF) using a specific ELISA (human VEGF Quantikine ELISA, R&D Systems, Wiesbaden, Germany).

4. Results and discussion

4.1 Variation of macrophage, T-cell and dendritic cell infiltration with clinical parameters

Intratumoral and peritumoral density of $CD68^+$ macrophages was significantly higher in melanomas thicker than 2.0 mm, compared to thinner ones. There was no significant difference in macrophage infiltration between the 2-4 mm and >4 mm thickness categories. Similarly to MVD, the density of intratumoral macrophage infiltration was lower in females than in males ($p=0.0527$), while no difference was observed in peritumoral cell density. This finding might be explained by gender-specific differences in the recruitment of macrophages in the tumor, supported by the demonstration of the inhibitory effect of estrogen on the expression of monocyte chemotactic protein-1 (MCP-1) and on macrophage infiltration in various tissues. Localization, histological type, ulceration and clinical outcome did not significantly influence macrophage infiltration.

Distant organ metastasis was associated with lower intra- and peritumoral density of $CD3^+$ T lymphocytes, compared to the non-metastatic group (p=0.0903 and p=0.0659, respectively, borderline significance). Similar association was observed in the case of peritumoral $CD8^+$ T cells (p=0.0947, borderline significance). Intra- and peritumoral T-cell infiltration was not affected by tumor thickness, localization, histological type, and patients' sex, while ulcerated tumors showed a trend to be associated by lower intratumoral density of $CD3^+$ T lymphocytes (p=0.0972) and lower peritumoral density of $CD8^+$ T cells (p=0.0729).

The degree of infiltration by $CD1a^+$ and DC-$LAMP^+$ DCs showed strong inverse correlation with the thickness of melanomas ($p<0.001$). Compared to tumors that developed distant visceral metastases during the 5-year follow-up period, nonmetastatic ones were characterized with more intense peritumoral DC infiltration, however, these associations were lost when only melanomas thicker than 1.0 mm were included in the analysis. On the other hand, high peritumoral density of mature DCs was associated with longer survival, both in univariate (p=0.0195) and multivariate analysis (p=0.008).

4.2 Microvessel density of primary melanoma

According to our observations, peritumoral microvessel count showed a statistically not significant tendency to increase parallel with tumor thickness. On the other hand, intratumoral MVD in medium-thick (2.01-4.0 mm) melanomas exceeded that of the other two thickness categories, although the differences were not statistically significant. We could not detect significant differences in MVD when comparing the patients

according to the localization, histological type (SSM vs. NM), metastasis formation, or ulceration of the primary tumors. However, in the male patient group intratumoral MVD was significantly higher ($p=0.0303$) than in females.

4.3 Correlation between microvessel densities and immune infiltrates

We have analyzed the correlations of infiltrating cell densities with MVD in samples grouped according to tumor thickness and metastasis formation. Intratumoral infiltration did not show significant association with MVD in any of these groups for any of the cell types studied. In the case of peritumoral cell densities, when analyzing the whole patient population, the only statistically significant correlation was found between MVD and the density of $CD3^+$ T cells ($p<0.05$). This association was stronger in the highest thickness category (>4.0 mm), as well as in the group of visceral metastatic tumors ($p<0.001$ and $p<0.01$, respectively). In these subgroups of patients similar phenomenon was observed in the case of $CD8^+$ cells.

The association of MVD with T-cell density suggests that it could influence the intensity of peritumoral T-cell infiltration, therefore, a higher MVD might even have a positive role in contributing to an increased local immune response. On the other hand, by the production of angiogenic cytokines T-cells can promote the vascularization of melanoma.

In melanomas thicker than 4.0 mm MVD positively correlated with macrophage density as well, while weak associations were found in the case of B-cell and DC infiltration in visceral metastatic tumors. In contrast, no correlations could be seen for thinner (1.01-2.0 mm) or nonmetastatic melanomas in the case of the majority of markers.

Analysis of the correlations found in the visceral metastatic group after subdivision of the cases according to tumor thickness indicated that all these associations were present only in the subgroup of melanomas >4.0 mm (n=9), while no significant correlations were seen in the case of tumors ≤4.0 mm (n=16).

4.4 Antiproliferative effect of hyperforin on melanoma- and human dermal microvascular endothelial cells

Another purpose of my research work was the examination of the antiproliferative effect of hyperforin on melanoma- and human dermal microvascular endothelial cells (HDMEC). As melanoma cells are resistant to most chemotherapeutic agents and it is difficult to induce apoptosis of these cells, the development of new drugs is highly important.

With the exception of the fibrosarcoma cell line, hyperforin effectively inhibited the proliferation of 10 human and 7 rat tumor cell lines. Compared to the other cell lines, hyperforin showed an intermediate inhibitory activity on the proliferation of melanoma cell lines.

As the majority of cytotoxic drugs cause apoptosis of tumor cells, we investigated whether hyperforin is able to induce apoptosis in the targeted cells. Apoptotic oligonucleosomes appeared dose-dependently in hyperforin-treated cells.

Since melanoma cells express COX-1 and -2, and there is epidemiological evidence of the anti-melanoma effect of non-steroid inflammatory drugs, the COX inhibitory effect of hyperforin also deserves attention. It is possible that it also contributes to the antiproliferative effect of hyperforin.

We examined the effect of hyperforin on human dermal microvascular endothelial cells. Hyperforin inhibited the proliferation of HDMECs in a dose-dependent manner without inducing apoptosis.

We observed the net-formation of endothelial cells on an extracellular matrix gel of basement membrane proteins under video-microscope. The formation of microvascular tubes was completely inhibited by the addition of 5 µg/ml hyperforin without disturbing cellular adhesion to the matrix. Evaluation of the films in the time-lapse mode revealed that the inhibition of tube formation by hyperforin was not associated with a reduced motility of the endothelial cells.

According to the literature, inhibitors of COX-1 and 5-LOX interfere with angiogenesis, so it is possible that this property of hyperforin contributes to the anti-angiogenic property of this agent.

We asked whether the anti-angiogenic activity of hyperforin is associated with the inhibition of VEGF secretion by tumor cells. To address this issue, MT-450 carcinoma cells were incubated in vitro with hyperforin, using the same concentrations that have been shown to inhibit proliferation of these cells. We observed a dose-dependent inhibition by hyperforin of VEGF secretion by MT-450 cells. It is possible that the decrease in VEGF secretion is in part the result of the cytotoxic effect of hyperforin.

Our results allow us to conclude that hyperforin directly inhibits the proliferation and net formation of endothelial cells and indirectly interferes with angiogenesis by inhibiting the secretion of VEGF by tumor cells.

Due to the cytotoxic and anti-angiogenic properties of hyperforin, it is a candidate for being a new antitumoral agent that deserves further investigations. The anti-melanoma effect is also encouraging, as hyperforin

is able to disrupt apoptosis resistance of melanoma cells. The anti-angiogenic properties of hyperforin could also be useful in the treatment of melanoma, though the anti-angiogenic agents that have been tried until now proved to be ineffective. It is remarkable that hyperforin has been used with minimal toxicity as an antidepressant for years. The most important side effect is that it activates the cytochrome P-450 system of the liver, and it can interact with several medicaments and cytotoxic drugs. As St. John's wort can be found in abundance in Europe, North-America, Asia and North-Africa, the supply of hyperforin is not limited.

5. Conclusions

- Intratumoral and peritumoral density of $CD68^+$ macrophages was significantly higher in melanomas thicker than 2.0 mm, compared to thinner ones. This finding suggests that macrophages might promote tumor progression. Interestingly, macrophage infiltration was significantly higher in the male paient group.

- The intense infiltration by DC-$LAMP^+$ mature dendritic cells appears to be a positive prognostic marker in cutaneous melanoma.

- We found an association between the tumor microvasculature and the density of infiltrating cells (T-, B-, dendritic cells and macrophages) only peritumorally, and primarily in thicker and metastatizing melanomas. This finding is in harmony with the observation that neoangiogenesis in melanoma occurs primarily peritumorally.

- As hyperforin effectively inhibited the proliferation of melanoma cells and dermal microvascular endothelial cells, it appears a promising anti-melanoma and anti-angiogenic agent.

6. List of own publications

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[1] Authors contributed equally to this work

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