The role of the motogenic signal in human melanoma cells

PhD thesis booklet

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INTRODUCTION

The incidence of cutaneous malignant melanoma is increasing worldwide, which tendency is typical in the Hungarian epidemiology. Malignant melanoma is resistant to common cytotoxic therapies, improvement in survival can only be achieved with early detection and complete surgical removal. However, malignant melanomas have a potential to form organ metastases in the very early phase of primary growth. For this reason, a better understanding of the mechanisms involved in their progression is urgently needed.

In the multifactorial process of the metastatic cascade, every step is associated with the triple potency for adhesion, degradation and migration. The components of the extracellular matrix serve as adhesion sites for the tumor cells. Heparins interfere with different function of heparan sulfates in the matrix, the most known example for the clinical application is the synthetic heparin and its derivatives as anticoagulant agents. Clinical observations showed that the use of heparin treatments in cancer patients delayed tumor progression and prolonged survival. In numerous experimental tumor models data supported the positive effect of heparin derivatives, such as low-molecular-weight heparins (LMWHs). According to our
previous results, LMWHs and unfractionated heparin inhibited microvascular arrest and metastasis formation of human melanoma cells in preclinical spleen-liver and lung colonization models. An important element of the antimetastatic effect was that LMWH as well as heparins inhibited melanoma cell migration and invasion. Other works proved that shorter heparin derivatives, including non-anticoagulant fragments, inhibited the tumor induced angiogenesis, however, their direct effect on the tumor cells (e.g. in the case of migration) has not been studied previously.

CD44 is a cell surface molecule involved in cell-matrix and cell-cell interactions. The prognosis of melanomas expressing CD44 isoforms containing the v3 exon (CD44v3) proved significantly poorer than that of CD44v3-negative cases. CD44v3 is glycated by HS, therefore it could be a potential target for modulation by heparin derivatives in melanoma.

The components of the extracellular matrix are more than just adhesion sites for the migrating tumor cells: following enzymatic degradation of the ECM, the release of the sequestrated growth factors increases, and thus they become available for the cells. The receptor tyrosine kinase pathway directly regulates cell proliferation, survival and motility. In a
number of cancers (e.g. glioblastoma, non-small-cell cancer, head and neck cancers) dysfunction of the epidermal growth factor receptor (EGFR) or the hepatocyte growth factor receptor (c-Met) contributes to the malignant transformation. c-Met is present on normal epithelial cells and melanocytes as well, and its ligand is expressed by mesenchymal cells of the skin. Moreover, melanoma cells produce HGF themselves, and the correlation of c-Met overexpression with the growth of tumor cells suggests that the c-Met/HGF pathway has a pivotal role in melanoma progression in autocrine and paracrine manners as well, which suggests that c-Met is a potential target for molecular therapy in human malignant melanoma. The mutation of c-Met was described in human melanoma cells in the juxtamembrane domain, but not at the TK domain. In the case of our own melanoma cell lines, the members of our workgroup found no genetic alterations in c-Met either in the extracellular or in the intracellular domains.

Unlike c-Met, according to previous studies, the expression and function of EGFR was contradictory in malignant melanoma. EGFR gene copy number alterations in surgical samples of primary cutaneous malignant melanomas are associated with poor prognosis. According to the analysis of our colleagues, wild-type sequences were found in 2 of the
studied 8 cell lines, while in two cell lines they did not detect extracellular domain at all. They found total deletion in two cell lines and a new splice variant in two other melanoma cell lines.

Different elements of the tyrosine kinase pathway are impacted by the level of intracellular calcium, which could result in the induction of apoptosis. The intracellular level of Ca$^{2+}$ is critical in the processes of survival and cell migration as well. The source of calcium intracellularly is the endoplasmic reticulum, while extracellularly the channels of the plasma membrane, including the ligand-dependent and the voltage-dependent forms. Based on our microarray study, our group demonstrated a novel finding that human melanoma cell lines overexpressed a Ca$^{2+}$-release channel, ryanodine receptor 2 (RyR2), and two of its regulators. Meanwhile, RyR2 does not function as a release channel in melanoma. The other important element of Ca$^{2+}$ homeostasis, the purinergic Ca$^{2+}$ channel P2X$_7$ was overexpressed in both malignant melanoma cells and melanocytes, but it functioned as a calcium entry pathway in melanoma cells but not in melanocytes.
AIMS

1. Could short heparin oligosaccharides interfere with the heparan sulfate group of the extracellular matrix, and could they affect the migration of human malignant melanoma cells?
2. Which forms of c-Met are expressed in human melanoma, and how could they impact cell proliferation, apoptosis, migration and invasion in vitro and in vivo?
3. Which forms of EGFR are expressed in human melanoma, and what could be their role in regulation of cell proliferation, apoptosis, migration and invasion?
4. How could the intracellular Ca$^{2+}$ signal regulate survival of human melanoma cells?

MATERIALS AND METHODS

For detecting the expression of EGFR and c-Met in human melanoma cell lines, we used immunocytochemistry, flow cytometry, Western blot, nested PCR and quantitative PCR. Applying specific inhibitors, siRNA and oligosaccharides, we determined the modulatory effect on
cellular processes (proliferation – MTT-assay; migration, invasion – modified Boyden-chamber).

Staining with propidium-iodide or double staining with FITC-Annexin V and propidium-iodode was performed for detecting apoptosis.

The effect of the treatments on the in vivo colony formation of melanoma cells was determined in spleen-liver metastasis model and lung coloniziation assay in SCID mice.

RESULTS

Only oligosaccharide containing 18 units (dp18) had significant inhibitory effect on cell proliferation. In contrast, in vitro cell migration and invasion were inhibited by all oligosaccharides studied except dp8 and dp22. Antibody against CD44v3 stimulated cell migration and invasion, and this effect could be attenuated by oligosaccharides dp4 and dp18. Moreover, oligosaccharides dp4 and dp18 reduced the number of lung colonies formed in SCID mice intravenously injected with human melanoma cells, while dp22 proved to be ineffective in this respect. Analyzing histological sections of tumor colonies under light microscope, dp18 significantly reduced the microscopic colony size compared to the control.
group, which could be explained by the in vitro antiproliferative effect of dp18. Oligosaccharides dp4 and dp22 had no significant effect on the size of tumor colonies. There was no treatment-dependent alteration in the histology of tumor colonies. According to previous studies, shorter length oligosaccharides have minimal anticoagulant potency, these fragments could be promising new tools in the therapy of metastatic malignancies including malignant melanoma, without significant modulatory effect on the hemostatic system.

Based on the PCR analyses of our group, we determined the expression of EGFR and c-Met proteins by flow cytometry and immunocytochemistry. All human melanoma cell lines used were positive for both the extra- and intracellular domains of c-Met. The extracellular domain of EGFR proved negative (except one melanoma cell line), while in permeabilized cells the intracellular domain was present. Using phosphospecific antibody, without exogenous stimulation, EGFR and c-Met showed constitutive activity. Monospecific tyrosine kinase inhibitors caused in vitro decreased proliferation, migration and elevated apoptosis. Administration of a selective c-Met tyrosine kinase inhibitor (TKI) significantly decreased primary tumor growth as well as the capacity for liver colony formation of human melanoma
cells. Selective EGFR-TKI was less effective in the inhibition of metastasis formation, and had no effect on the primary tumor. The inhibitory effect of the dual-specific EKB-569 suggests the necessity of rational drug design against EGFR and c-Met in the therapy of malignant melanoma.

In the tyrosine kinase pathway numerous elements (mainly small GTPases) are regulated by Ca\(^{2+}\). Administration of ATP, the physiological ligand of P2X\(_7\) purinoreceptor Ca\(^{2+}\) channel, did not induce significant apoptosis of melanoma cells, moreover, parallel administration of ATP with the strong apoptosis inducer 2-methoxy-estradiol significantly inhibited this process. However, when we induced apoptosis by 2-methoxy-estradiol treatment, P2X\(_7\)-knocked down cells became significantly more sensitive to the effect of the drug.

Our data on the aberrant function of purinoreceptor P2X\(_7\) may help to elucidate the underlying molecular mechanism of apoptosis resistance of melanoma cells and to explore novel targets for a more efficient therapy.
CONCLUSIONS

1. We identified LMWH-derived 4–18 unit length oligosaccharides, which inhibited *in vitro* proliferation and/or migration of human melanoma cells, and *in vivo* resulted in a decrease of lung colonization. We defined the sequence required for their biological effect.

2. Without exogenous stimulation wild-type c-Met showed constitutive activity in human melanoma cells. *In vitro* treatment with a specific c-Met tyrosine kinase inhibitor resulted in reduced proliferation, migration, and elevated apoptosis, while *in vivo* treatment significantly decreased primary tumor growth as well as the capacity for liver colony formation of melanoma cells.

3. We confirmed that in the majority of our human melanoma cells EGFR showed constitutive activity. *In vitro* treatment with specific EGFR tyrosine kinase inhibitor had antiproliferative, antimigratory activity and induced apoptosis. Administration of monospecific EGFR tyrosine kinase inhibitor inhibited metastasis formation, but had no effect on the primary tumor. Our results suggest the necessity of dual-specific inhibitors
against EGFR and c-Met in the therapy of malignant melanoma.

4. We confirmed that purinoreceptor proved to be an antiapoptotic device in human melanoma cells.

PUBLICATIONS

Papers connected to thesis:


Conference abstracts connected to thesis:

1. **Kenessey I**, Tóvári J, Tímár J. Blocking of EGF receptor tyrosine kinase by ZD1839 (gefitinib) causes inhibition of cell proliferation and migration in human melanoma cell lines. 18th Meeting of the European Association for Cancer Research (EACR18), July 3-6, 2004, Innsbruck, Austria

2. **Kenessey I**, Tóvári J, Rásó E, Kramer Z, Tímár J. Blocking of EGF receptor tyrosine kinase causes inhibition of cell proliferation and migration in human melanoma cell lines. FEBS-EMBO advanced lecture course “Molecular Mechanisms in Signal Transduction and Cancer”, August 15-26, 2005, Spetses, Greece


4. **Kenessey I**, Tóvári J, Rásó E, Kramer Z, Tímár J. The modulatory effect of EGF receptor on human melanoma cells. Semmelweis University, PhD Scientific meeting, April 14-15, 2005, Budapest, Hungary
5. Ádám A, **Kenessey I**. Functional genomics of c-Met in human melanoma cell lines. Semmelweis University, PhD Scientific meeting, April 14-15, 2005, Budapest, Hungary


7. **Kenessey I**, Tóvári J, Rásó E, Mészáros L, Kramer Z, Tímár J. The modulatory effect of EGF receptor on human melanoma cells. 66th Hungarian Pathology Congress, October 4-6, 2007, Balatonfüred, Hungary

Papers not connected to thesis:


2. Rényi-Vámos F, Tóvári J, Fillinger J, Tímár J, Paku S, **Kenessey I**, Ostoros G, Agócs L, Soltész I, Dőme B.


