Oncogene dependent regulation of migration and proliferation in human tumor cells

PhD Thesis

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Budapest
2013

INTRODUCTION

Most of the solid tumors have a steadily growing incidence and, in the majority of cases, not primary tumor growth but distant metastases are the main cause of death. Metastasis formation is a complex process that requires a spatiotemporal regulation of cell adhesion, cell proliferation and cell migration. Thus, investigation of these cellular processes and the connection between cell migration and cell proliferation is extensively studied.

Based on experimental data and on the concept that cytoskeletal machinery cannot be used for proliferation and migration concurrently, the "go or grow" hypothesis - formulated for tumor cells - postulates that migration and cell division are mutually exclusive, and tumor cells defer proliferation for cell migration. Most experimental and theoretical approaches addressing the "go or grow" hypothesis have been performed on intracranial tumor cells of neuroectodermal origin and the data is rather conflicting. In the context of glioma the regulation of FAK expression, the mir451/ LKB1/AMPK or mir9/CREB/NF1 signaling and carboxypeptidase E (CPE) expression were proposed as molecular mechanisms that could lead to migration and proliferation dichotomy, respectively.

The "go or grow" hypothesis is particularly important in the development of effective anti-cancer drugs that can also target the survival-prone subpopulation of tumor cells being able to escape from the primary tumor and survive in metastatic tissue microenvironment. If tumor cells defer cell proliferation for cell migration than migrating cells should have a decreased sensitivity to treatment modalities targeting the proliferating tumor cells. Thus, anti-proliferative therapies may unintentionally select for migratory cells or even induce cell migration in surviving cell populations. Furthermore,

inhibition of cell migration might induce the proliferation of disseminating cells and lead to primary or secondary tumor growth. For this reason, understanding better the connection between proliferation and migration is essential for the development of therapies inhibiting both of these cellular processes. Since evaluation of the "go or grow" hypothesis is currently largely based on brain tumor cells, in this thesis, we extended the investigation of this hypothesis on 2D cell cultures of tumor cell lines originating from different embryonic cell layers such as cells of neuroectodermal, mesodermal and entodermal origin, using long-term time-lapse videomicroscopy.

The currently available anticancer treatment modalities often interact with members of the various growth factor (GF) and TGF signaling pathways to influence proliferation and migration. EGF acting on EGFR and FGF2 activating all four types of FGFRs have extensively overlapping downstream signaling cascades, as Ras and Raf play a central role in the downstream activation of both EGF and FGF2 signaling. Impaired EGF and FGF2 signaling is involved in a great variety of malignancies and pro-tumorigenic effect of the signaling of both ligands is proven in malignant melanoma. Furthermore EGF and FGF2 signal transduction is affected by oncogenic driver mutations in BRAF or NRAS, which are present in about 40 to 70% and in 10 to 30% of melanoma cases, respectively. Inhibition of EGF/FGF2 signaling can be exerted on the level receptors (monoclonal antibodies and receptor tyrosine kinase inhibitors) and on the level of the downstream elements. A currently investigated mechanism for the latter is the treatment with zoledronic acid that inhibits prenylation, which is a post-translational modification step of Ras. The facts that both EGF and FGF2 act on extensive overlapping downstream signaling networks and that the most common oncogenic mutations in malignant melanoma are activating mutations of their downstream effectors led us to

investigate the activation and inhibition of EGF and FGF2 signaling on melanoma cells with different NRAS and BRAF mutational status.

Activin is a member of TGFβ signaling family. It is a dimeric protein and interacts with several activin type I and type II receptors. In tumors, activin signaling can be associated with both inhibition and promotion of cell proliferation and tumor progression. In hepatocellular carcinoma, breast cancer and prostate cancer, activin signaling takes part in inhibition of cell proliferation and tumor progression. In contrast, activin can promote cell proliferation in endometrial carcinoma, oral squamous cell carcinoma, testicular and gastric cancer and in a great variety of thoracic tumors like esophageal squamous cell carcinoma, esophageal adenocarcinoma and lung adenocarcinoma. One of the exogenous antagonists against the activin type I is SB-431542 that acts on all three activin type I receptors. Since activin has been shown to be pro-tumorigenic in a variety of thoracic tumors and malignant mesothelioma lacks for targeted therapy our aim was to investigate the potential effect of inhibiting activin signaling in human mesothelioma cells.

Altogether, growth factor receptor signaling and oncogenic mutations affecting this pathway are major regulators of the spatiotemporal interplay and activation of cell migration and proliferation in human tumor cells. In the PhD dissertation we investigated the following research question.

OBJECTIVES

1. *Do human tumor cells defer proliferation to cell migration in adherent cultures as it had been postulated in the "go or grow" hypothesis?* Accordingly, we measured proliferation, migration and length of cytokinesis in 35 lung cancer, melanoma and mesothelioma cell lines by videomicroscopy and performed correlation analysis between these cellular processes at both single cell and population level.
2. *Does invasion of the ECM from multicellular spheroids require concurrent cell proliferation?* We raised this question because a number of mathematical models of 3D matrix invasion of tumor cells incorporate the assumption that proliferation is a prerequisite to invasive behavior, however, there was no experimental evidence available for this. In order to evaluate this hypothesis we characterized the invasion pattern of proliferating and proliferation-inhibited cells from multicellular spheroids into collagen type I gel.
3. *Do BRAF and NRAS oncogenic mutations determine the migratory and proliferative response of melanoma cells to activation and inhibition of EGFR and FGFR?* First, BRAF and NRAS oncogenic mutations as well as EGFR and FGFR expression of melanoma cells were determined. Then, migration and proliferation as well as the activation of downstream signaling were explored under baseline conditions and after treatment with EGF and/or FGF or with the inhibitors of these receptors.
4. *Does prenylation inhibition interfere with migration, proliferation and the activity of Ras signaling pathway in human melanoma cells in vitro and in vivo?* BRAF and NRAS mutation dependent effect of ZA treatment on cell migration, proliferation and apoptosis induction

was determined in melanoma cells *in vitro*. Furthermore, the effect of ZA on primary tumor growth and metastasis formation was assessed using animal models of melanoma cells with different mutational status.

5. *Does activin signaling support or interfere with migration and proliferation of human mesothelioma cells?* Accordingly, we assessed the effect of activin and activin-receptor inhibitor SB431542 treatment on cell proliferation, cytokinesis and migration in human mesothelioma cells via videomicroscopy.

METHODS

Cell lines

Thirteen melanoma, twelve mesothelioma, ten lung cancer and two brain tumor derived human cell lines were used in the studies.

Compounds/drugs

The following compounds were used in the experiments: EGF, FGF2, the FGFR inhibitors ponatinib, BGJ-398, BIBF-1120, AZD-4547, the EGFR inhibitors gefitinib, erlotinib, CI-1033, pelitinib, the prenylation inhibiting zoledronic acid (ZA), activin and the activin inhibitor SB-431542.

Videomicroscopy

Duration of cytokinesis, cell proliferation and migration was assessed in adherent 2D cell cultures by the evaluation of long term phase-contrast microscopy time-lapse recordings.

Collagen invasion assay

Glioma cell pellets were drawn into pipette tips and cylindrical aggregates were embedded into collagen gel and photographed to evaluate 3D migration pattern.

Mutation analysis

Genomic DNA was isolated from pellets of melanoma cells and oncogenic mutations in BRAF and NRAS were investigated via microcapillary restriction fragment length analysis and by direct DNA sequencing.

Quantitative PCR

Total RNA was isolated and reverse transcription was performed. Next, quantitative real-time PCR was used to measure expression of EGFR and FGFRs in melanoma cells.

Cell viability assay
Adherent cells cultures were fixed using trichloroacetic acid after treatment and total protein amount was measured colorimetrically after SRB staining to evaluate cell viability.

Apoptosis assay
Apoptosis induced by zoledronic acid treatment was assessed using TUNEL staining on PFA fixed cell cultures and direct counting of the TUNEL-positive cells.

Activation of cell signaling
Actiavtion of Erk1/2, S6, FAK and Src proteins was quantified by immunoblot analysis phosphorylation of these proteins in human melanoma cells.

***In vivo* subcutaneous xenograft model**
To examine the effect of ZA on primary tumor growth, human melanoma cells were injected subcutaneously into the flank region of NSG mice, treated intraperitoneally on a weekly basis and tumor volumes were evaluated.

***In vivo* spleen to liver colonization assay**
In order to investigate the effect of ZA on metastatic potential, colonization experiment from spleen to liver was performed in NSG mice. Animals were treated intraperitoneally for three weeks before the spleen and liver were removed and weighed.

Statistical analysis
All statistical analyses were computed in GraphPad Prism 5.

RESULTS

Migration/proliferation dichotomy in 2D cell cultures

Since migration, proliferation and the interplay of these cellular processes, postulated by the "go or grow" hypothesis, are crucial in terms of tumor progression and investigated currently in central nervous system tumors we evaluated this hypothesis on tumor cell lines with neuroectodermal, mesodermal and entodermal origin. Videomicroscopy recording and assessment of migration, proliferation and cytokinesis-length were performed on both individual cells and on population levels in 2D cell cultures of thirty-five (12 mesothelioma, 13 melanoma and 10 lung) cancer cell lines.

Significantly higher averaged 24-hour migration distance was found in mesothelioma cells when compared to melanoma and lung cancer cells. Average expected number of cell divisions within 24hs showed a range of one order of magnitude and the highest averaged proliferation was found in mesothelioma cells followed by proliferation of melanoma and lung cancer cells. The average duration of one division showed no significant differences between the three tumor subtypes.

The statistical correlations between the probability of cell division, the average migrated distance and the duration of cytokinesis has been established in all three tumor types. Interestingly, a strong positive correlation was found between cell proliferation and cell migration in melanoma cells and in lung cancer cells, as well; whereas, no correlation was observed in the examined mesothelioma cell lines

Correlation between average duration of cytokinesis and cell migration was calculated for each tumor type. Interestingly, significant negative correlation was found between duration of cytokinesis and cell migration in melanoma cell lines. There was no significant correlation between cytokinesis and migration in mesothelioma and lung cancer cells. Correlation

between cell proliferation and duration of cytokinesis was significant in mesothelioma cells but failed to show significance in melanoma and lung cancer cells.
Since the FAK/Src signaling is an important regulatory pathway in 2D migration, activation of FAK and Src kinases were explored by examining total and phosphorylated amount of proteins via immunoblot assay. Next, we divided melanoma cell lines into two groups at the median migratory potential and averaged the activation for the six slowest and six fastest migrating melanoma cells. Interestingly, activation of FAK tended to be higher in fast migrating melanoma cells. In contrast, activation of Src was essentially equal in fast and slow migrating cells.

Proliferation and migration in 3D cell cultures
Interplay between proliferation and migration plays an important role in 3D tumor growth and invasion. Since proliferation is thought to be a prerequisite for the 3D extracellular matrix invasion of tumor cells, the interdependence of proliferation and migration was studied in glioblastoma cells. Our evaluation was also included in a novel mathematical model that describes the invasion patterns of tumor cells into the surrounding matrix. Accordingly, cell invasion from an aggregate into a surrounding ECM was studied in the presence/absence of a cell proliferation inhibitor. Nevertheless, the invasion patterns and migrated distances in the first 24 hours after treatment were essentially the same in division inhibited and control cells.

Oncogenic mutations in melanoma cells
In order to evaluate whether the most important oncogenic mutations indeed influence migration and cell division in a distinct manner in melanoma, mutational status of the investigated cell lines were determined. Altogether four cell lines with BRAF (V600E), two cell lines with NRAS (Q61K

and Q61R) mutations and two cell lines wild-type for these genes were used for the experiments.

Ligand dependent activation of EGFR and FGFR in melanoma

Prior to testing the activation of EGFR and FGFR receptors on melanoma cells with known mutational status, expression of EGFR and FGFR relative to GAPDH was investigated by qPCR. High expression of EGFR, FGFR1 and FGFR4 was confirmed in each of the investigated cell lines. Interestingly, FGFR2 and FGFR3 were not expressed in the two NRAS-mutant cell lines. In general, the lowest expression of growth factor receptors was found in the double wild-type cells.

Ligand activation of EGFR and FGFR was investigated by treating cells with 50 ng/ml EGF, FGF2 or both during videomicroscopy rmeasuerments. Proliferation and migration of untreated cells with different oncogenic mutations was compared first. Importantly, both BRAF and NRAS activating mutations resulted in elevated levels of migration and proliferation compared to double wild type cells. The difference inthe average migrated distance of BRAF-mutant and double wild type cells was statistically significant.

Mutational status dependent effect of EGF and/or FGF2 treatment on proliferation and cell viability of melanoma cells was tested both via videomicroscopy and SRB-assay. There was only a modest increase of cell proliferation in double wild-type cells, but not in cells with BRAF or NRAS oncogenic mutations upon treatment.

Videomicroscopy recordings were also used to define the mutation-dependent effect of EGF and/or FGF2 treatment on migration. The migratory effect was more profound compared to the effect seen in proliferation and convincing effect on migration was only seen in double wild type cells. Migration in both double wild type cell lines increased upon treatment with GFs but EGF caused more elevated migratory activity

than FGF2 in both cell lines. Importantly, the combined treatment resulted in a further increase in migration when compared to treatment with EGF or FGF2 alone. Although significantly elevated migration was observed in the NRAS-mutant cell line M24met after FGF2 treatment, this increase was considerably smaller than those observed in double wild type cells. When taking the average migration distance in cell lines having the same mutation status, BRAF-mutant cells failed to show altered migration after treatment with GFs and NRAS-mutant cells showed only a modest increase in migration in response to FGF2 or combined growth factor treatment.

Phosphorylation of two major downstream effectors, Erk1/2 and S6, were explored using immunoblot measurement to assess the activation of growth factor receptor pathway. Under baseline conditions, BRAF and NRAS oncogenic mutations resulted in a higher phosphorylation of Erk1/2 and S6 as compared to wild type cells.

Treatment with GFs elevated the level of phosphorylation of Erk1/2 and S6 in double wild type cells in a much higher proportion as compared to either BRAF or NRAS mutant cells. Generally, the alteration of Erk1/2 and S6 phosphorylation measured in cells harboring oncogenic mutations was rather modest when compared to the double wild type response.

Inhibition of EGFR and FGFR in melanoma

Since activation of the EGF/FGF signal transduction was found to be dependent on the oncogenic mutation in melanoma cells, the pharmacological inhibition of EGFR and FGFR was also compared in the melanoma cells with different oncogenic mutations.

The oncogenic mutation dependent inhibition of EGF and FGF signaling was performed by treating the cells for 72 h with EGFR (gefitinib, erlotinib CI-1033 and pelitinib) and FGFR

(ponatinib, BGJ-389, BIBF-1120 and AZD-4745) inhibitors and viability was measured via SRB assay. Independent of their mutational status, cell lines were largely insensitive to gefitinib and erlotinib treatment. Although CI-1033 and pelitinib treatment were somewhat effective, there was no difference between the sensitivity of cells having different mutations. Similarly, the effect of FGFR inhibition on cell viability was also independent of cells being BRAF- or NRAS-mutant or wild type for these genes.

Oncogenic mutation-dependent prenylation inhibition response in melanoma

Posttranslational modification – including prenylation among others – of Ras is one of the major regulators of its activity and oncogenic RAS mutations play a major role in malignant melanoma. Thus, the effect of prenylation inhibition was examined by zoledronic acid (ZA) treated human melanoma cell lines carrying either mutant BRAF or NRAS or none of them. After the 24-hour-treatment, the BRAF-mutant cells displayed a profound change in morphology. These cells obtained a rather elongated form, whereas only modest or no change was found in NRAS-mutant or in double wild-type cells.

Effect of the treatment with different concentrations of ZA on cell viability of melanoma cells was measured by SRB-assay. ZA treatment clearly decreased cell viability in NRAS mutant cells even in smaller doses. Cell viability of BRAF mutant and double wild type cells were decreased only to a smaller extent and at higher doses.

Videomicroscopy measurements were used to evaluate the effect of ZA treatment on migration of melanoma cells. Despite the significant increase in the migration of one of the NRAS-mutant cell lines and one of the double wild type cells, ZA treatment increased the migratory activity of BRAF-

mutant cells to a much higher extent compared to NRAS mutant and double wild type cells.

In order to characterize the pro-apoptotic effect of ZA, TUNEL staining was performed on melanoma cell lines treated with 25μM ZA. Though both NRAS-mutant cell lines were sensitive to ZA treatment, there was a huge difference in their sensitivity level. In average, there was a more profound pro-apoptotic effect of ZA treatment in NRAS-mutant cells compared to BRAF mutant and double wild type cells. Of note, amount of apoptotic cells found in the TUNEL assay was in line with the measured reduction in cell viability.

Since a robust apoptotic effect in NRAS mutant melanoma was found the activation of the ribosomal protein S6 a downstream target of RAS involved in the regulation of survival was investigated via immunoblot assay. Interestingly, the treatment with ZA resulted in the increased activation of ribosomal protein S6 in M24met NRAS mutant cells and in a decreased activation in the other NRAS mutant cell line. This differential reduction of S6 activation was in accordance with the results seen in the TUNEL assay.

To examine the effect of ZA on primary tumor growth, human melanoma cells were injected subcutaneously into the flank region of NSG (NOD scid gamma) mice. ZA treatment failed to show an inhibitory effect on subcutaneous tumor growth of injected melanoma cells. In order to investigate the metastasis related effects of ZA, colonization experiment of spleen to liver was performed. As a result, ZA failed to inhibit the metastatic growth of NRAS-mutant or double wild-type melanoma cells and it exerted only modest inhibition on metastatic growth of BRAF-mutant melanoma cells. Importantly, the lower dose (50μg/kg) of ZA resulted in a significantly higher metastasis formation in double wild-type cells.

Modulation of the activin signaling in mesothelioma

Activin signaling can be associated with both inhibition and promotion of tumor progression in various types of tumors. However, the role of this signaling pathway in mesothelioma has not been investigated yet. Videomicroscopy measurements were performed to investigate the effect of activin A (20 ng/ml), the inhibitor SB431542 (20 μM) or the combination of these two agents on cell proliferation and cell migration. Treatment with the activin receptor inhibitor SB-431542 alone or in combination decreased the proliferation of M38K mesothelioma cells significantly compared to control. In contrast, there were no differences in the proliferation of P31 cells upon inhibition or activation of the activin receptors. Unlike cell proliferation, migration of M38K cells was increased after activin treatment, whereas migration of P31 cells remained unaffected.

Videomicroscopy provides a unique opportunity to identify aberrant cytokinesis, which was observed in P31 mesothelioma cell line even under control conditions. Almost 2 percent of mitoses were multipolar even in untreated cell populations. Interestingly, treatment with activin receptor inhibitor SB-431542 alone or combined with activin resulted in a significantly higher proportion of multipolar (in majority tripolar) cytokineses as compared to control.

CONCLUSIONS

1. Revisiting the "go or grow" hypothesis, no negative correlation between proliferation and migration and thus no supporting evidence for the hypothesis was found. In contrast, positive correlation was found between migration and proliferation in melanoma and lung cancer cells.
2. Our experiments using collagen embedded multicellular brain tumor cell spheroids demonstrated that the invasion process in 3D matrices did not require concurrent cell proliferation.
3. Investigation of the mutational status dependence of EGF and FGF response revealed a lower baseline activity and higher inducibility of proliferation and migration in double wild-type melanoma cells compared to cells with oncogenic BRAF or NRAS mutation. In contrast, response to GF receptor tyrosine kinase inhibitors was oncogenic mutation independent.
4. Investigation of the mutation dependence of prenylation inhibition resulted in a decrease of proliferation in NRAS mutant melanoma cells but in the increase of migration *in vitro* and increased metastatic potential *in vivo* in BRAF mutant and double wild type melanoma cells, respectively. The apoptosis induction in NRAS mutant melanoma suggests that prenylation targeting treatment modalities may be effective in this molecular subgroup of melanoma.
5. In certain mesothelioma cells, migratory and proliferative response to the induction and inhibition of activin signaling conferred a pro-tumorigenic effect of activin activation. The presented results suggest that activin may be a valuable candidate for therapeutic interference.

PUBLICATIONS

Publications related to the thesis

1. Garay T, Juhász E, Molnár E, Eisenbauer M, Czirók A, Dekan B, László V, Hoda MA, Döme B, Tímár J, Klepetko W, Berger W, Hegedűs B.: Cell migration or cytokinesis and proliferation? - Revisiting the "go or grow" hypothesis in cancer cells in vitro. Exp Cell Res. (impact factor 2012: 3.557), 2013
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ACKNOWLEDGEMENTS

During my PhD studies I was fortunate to meet and work with many helpful people in a number of different laboratories. First of all, I am grateful to my home Institute the 2nd Department of Pathology at the Semmelweis University. It has been a great privilege to conduct research at the Institute of Cancer Research of the Medical University of Vienna, Vienna, Austria and at the Department of Tumor Biology, Institute for Cancer Research, Oslo University Hospital, Oslo, Norway. It was a source of joy of well-done work to cooperate with researchers from the National Korányi Institute of TB and Pulmonology, from the Department of Experimental Pharmacology at the National Institute of Oncology, from the Department of Biological Physics at the Eötvös University, from the Department of Anatomy & Cell Biology at the University of Kansas Medical Center, and from the Department of Thoracic Surgery at Medical University of Vienna, Vienna, Austria.

My first debt of gratitude goes to my advisor, Balázs Hegedűs who was guiding my steps from the first day on and managed - despite my always critical attitude - to serve as a role model to me in science, career and life. My special thanks also to József Tímár, the Head of the Institute who supported my projects and scientific career, gave criticism and wise advice.

My special thanks are extended to the staff of the 2nd Department of Pathology, Semmelweis University, Budapest, Hungary. I am particularly grateful for the assistance given by Eszter Molnár, Éva Juhász, Andrea Réti, Anikó Gaál, Ágnes Biletz, Zita Hegedüs, Erzsébet Rásó, Tamás Barbai, Benedek Gyöngyösi, István Kenessey, Marcell Szász, Gábor Lendvai, Csilla Horváth, Violetta Piurkó, Viktória Gregor, Tibor

Schönfeld. Further, my special thanks to Lenke Balogh and Jánosné Seres, as the Institute would not be working so smoothly without their work. I am also grateful to all collaborators from other institutions in Budapest. I wish especially to acknowledge the help provided by József Tóvári, András Czirók, Balázs Döme, Szilvia Török, Mihály T. Cserepes, Magdolna Keszthelyi, Judit Dobos and Natália Bogdán.

I am grateful to Walter Berger for being always open for inspiring discussions and hosting my visits in Vienna. I also would like to thank for help and support of his co-workers at the Institute of Cancer Research: Karin Schelch, Christine Pirker, Michael Grusch, Maria Eisenbauer, Julia Münzker, Claudia Engelmaier, Nikolaus Floimayr, Daniela Lötsch, Rosa Weiss. Likewise, I would like to thank the members of the Translational Thoracic Oncology Laboratory of the Department of Thoracic Surgery in Vienna: Barbara Dekan, Viktória László, Anita Rózsás, Andreas Wagner, Mir Alireza Hoda and Bahil Ghanim.

I also would like to thank to Øystein Fodstad, the host supervisor of my fellowship to Oslo, and Karianne Risberg from the Institute for Cancer Research at the Oslo University Hospital and Marco Donia from the Center for Cancer Immune Therapy at Copenhagen University Hospital for the opportunity to collaborate in a very exciting project that will only be finished in my post-doc time.

And finally I would like to thank to the people who provided the foundation to my PhD. I am grateful to my family, especially to my wife and my parents who let me travel far and work early and late and encouraged me if something did not go ideally. And special thanks to my twins, without whom this thesis would not have been written so fast.