SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Organizing and modulatory role of plasma membrane microdomains in tumor cell signaling and tumor therapy

by Árpád Szöőr, MD

Supervisor: György Vereb, MD, PhD, DSc

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES

DEBRECEN, 2016

ORGANIZING AND MODULATORY ROLES OF PLASMA MEMBRANE MICRODOMAINS IN CELL SIGNALING AND TUMOR THERAPY

BY **ÁRPÁD SZÖŐR, MD**

SUPERVISOR:. GYÖRGY VEREB, MD, PHD, DSC

DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES, UNIVERSITY OF DEBRECEN

HEAD OF THE EXAMINATION COMMITTEE:	ÁRPÁD TÓSAKI, PD, PHD, DSC
MEMBERS OF THE EXAMINATION COMMITTEE:	TAMÁS BÍRÓ, MD, PHD, DSC
	MIKLÓS KELLERMAYER, MD, PHD, DSC

THE EXAMINATION TAKES PLACE IN THE LIBRARY OF THE DEPARTMENT OF PHARMACOLOGY,
FACULTY OF PHARMACY, UNIVERSITY OF DEBRECEN
APRIL 25TH, 2016., 11 AM

HEAD OF THE DEFENSE COMMITTEE:	ÁRPÁD TÓSAKI, PD, PHD, DSC
REVIEWERS:	ISTVÁN ÁBRAHÁM, MD, PHD, DSC
	PÉTER ANTAL-SZALMÁS, MD, PHD
MEMBERS OF THE DEFENSE COMMITTEE:	TAMÁS BÍRÓ, MD, PHD, DSC
	MIKLÓS KELLERMAYER, MD, PHD, DSC

THE PHD DEFENSE TAKES PLACE AT THE LECTURE HALL OF BLDG. A,
DEPARTMENT OF INTERNAL MEDICINE, FACULTY OF MEDICINE,
UNIVERSITY OF DEBRECEN
APRIL 25TH, 2016., 1 PM

1. Abstract

In the last two decades, several publications dealt with the GM1-ganglioside rich domains of the cell membrane — also termed lipid rafts — as essential modulators of growth factor dependent signaling. These cholesterol- and sphingolipid-enriched submicron sized membrane domains possess not only structural, but also functional roles. Rafts are able to simultaneously isolate or accumulate diverse membrane proteins involved in various signaling pathways; furthermore, these platforms are also able to organize effector and adaptor proteins together with receptors in small membrane compartments, thus evoking more efficient signal transduction.

In my PhD thesis I aim to demonstrate the modulatory and organizing function of GM1 rich microdomains in the membrane of tumor cell lines, and their possible role in targeted therapies. My observations encompass the significance of lipid rafts in serving as a platform for cell confluence dependent regulation of the functional outcome of signaling by the receptor tyrosine kinase PDGFR β, as well as for generating an apoptotic response to combination treatment with platinum complexes and TRAIL. Overall, my results support the view that GM1 rich microdomains are important regulators of various signaling pathways, and can be considered essential platforms for the optimal execution of cellular responses to external signals.

2. Introduction

2.1. Lipid rafts

Until the 1980s, it has been widely accepted that phospholipids and membrane proteins are randomly distributed in cell membranes, according to the Singer-Nicolson fluid mosaic model, published in 1972. In this model, the cell membrane was defined as a bilayer of amphipathic lipid molecules surrounding transmembrane proteins in greater or lesser amounts. Fluidity has been largely owed to horizontal displacement of lipid and protein components, while stability has been attributed to lateral interconnection between various proteins, and between proteins and lipids. Although the fluid mosaic model provided scientists with a new perspective of membrane structure, it was unable to answer a number of function-related questions.

In the mid-eighties, some new studies have pointed out that biological membranes are not only randomly distributed lipid bilayers with uncontrolled diffusion of integral and peripheral proteins, but rather they are hierarchically structured cell compartments, with complex physical nature influenced by lateral heterogeneity. According to some new hypotheses from these years, the cell membrane was described as a lively, dynamic structure with heterogeneous and ever-changing permeability, fluidity and mobility characteristics. Increased organization of lipids and proteins, furthermore their intermolecular communication hinted that unique, submicron motives in cell membranes have an essential role in the regulation of important cell functions.

In 1997, Simons and Ikonen described some new, well organized membrane structures built of sphingomyelin, glycolipids, cholesterol and GPI-anchored proteins, and exhibiting intramembrane mobility. These organized membrane domains were termed lipid rafts, or detergent resistant membrane domains (DRM). Cholesterol is an essential molecular component of rafts, but they also contain exoplasmic sphingolipids and endoplasmic phospholipids linked to membrane proteins by their acyl chain. According to this new model, the plasma membrane is a “sea” of disorganized lipids

with some scattered and well-organized free-cruising “raft” structures composed of cholesterol, sphingolipids and proteins.

In the years following their initial proposition, evidence has accumulated that lipid rafts are not only structural units of the cell membrane, but they might have important functional roles. It was hypothesized that clustered organization of the lipid bilayer has an impact on the function and distribution of GPI-anchored transmembrane proteins, such as the Lck tyrosine-kinase, Src kinases and some G-proteins.

One of the important consequences of lipid protein interaction is the flexible, environment-dependent structure and function of the rafts. It was observed that following stimulation, small lipid domains easily melt together and form larger units. It was also proposed that various proteins can be recruited into, or excluded from these domains dynamically, as required by function. This notion, incorporated into the dynamically structured mosaic model, also brought with it the conviction that protein-protein and protein-lipid interactions have similar importance as the forces building structured and less organized domains out of various lipids. The model also proposed that lipid rafts hosting specific protein-lipid interactions are able to increase the lifetime of intermolecular interactions required for efficient signaling, as well as facilitate the binding of membrane surface proteins to cytoplasmic adaptor and regulatory proteins, or cortical actin cytoskeleton.

In the last decade, development in the fields of microscopy and immunofluorescent staining made possible the precise observation of lipid raft structure and function. According to recent publications, the size of detergent resistant membrane domains varies from membrane rings measuring a few nanometers, through small islands measuring below a hundred nanometers to clusters measuring hundreds of nanometers. Although the lipid composition of various sized membrane microdomains also varies, incorporation of glycosphingolipids and cholesterol is common feature. Binding of cholera toxin B-subunit to ganglioside GM1 was one of the earliest markers to identify lipid rafts.

2.2. Receptor models for the investigation of lipid raft function

2.2.1. The PDGF receptor

Platelet-derived growth factors (PDGFs) and their receptors (PDGFRs) have been demonstrated as prototypes for growth factor and receptor tyrosine kinase function for more than 30 years. PDGF stimulates migration and proliferation of connective tissue cells and has an important role during embryonic development and wound healing, but its abnormal expression also contributes to a variety of diseases. PDGF and its receptor are currently under investigation as targets in numerous proliferative disorders, including cancers, fibrosis, and cardiovascular diseases. PDGF was identified from the alpha-granules of human thrombocytes in the 1970s as an essential soluble growth factor for normal fibroblast, smooth muscle cell and glial cell function. According to the original hypothesis, PDGF consisted of two different polypeptide chains — PDGF-A and PDGF-B — linked together as a heterodimer (PDGF-AB). Finding of PDGF-AA and PDGF-BB forms expanded the PDGF family to three proteins encoded by two genes, PDGF-A and PDGF-B. In the first years of the 21st century, characterization of two additional PDGF genes and proteins — PDGF-C and PDGF-D — has further expanded our understanding of this complex signaling system.

The binding of the PDGF dimer formed from the A and/or B polypeptide chains leads the formation of a receptor dimer similarly composed of α and β chains. The β-type receptor executively binds the B subunit, whereas α-type receptors are able to bind both A and B subunits. Expression of PDGFR α was demonstrated on the surface of mesenchymal cells such as chondrocytes, oligodendrocytes and alveolar macrophages, while PDGFR β plays an important role in the regulation of malignant human gliomas. Expression of the PDGF ligand and its receptor correlates with tumor grade and proliferative activity.

PDGF evokes its effect on target cells through activating the intrinsic tyrosine kinase of its receptor upon dimerization, which induces autophosphorylation of the tyrosine 857 residue and is essential for the autoactivation of the receptor.

Consequently generated further phosphotyrosine residues serve as docking sites for proteins possessing a Src homology 2 (SH2) domain or a phosphotyrosine binding (PTB) domain and initiate downstream signaling. On PDGFR, tyrosine 716 (Tyr716) is an important residue which binds the growth factor receptor binding protein-2 (GRB2). This SH2 domain protein activates the Ras-MAPK pathway which plays a central role in cell proliferation. Dephosphorylation of Tyr716 by protein tyrosine phosphatase PTPN1 (PTP1B) inhibits PDGF-induced activation of the ERK1/2 MAP kinases. Negative regulation of this pathway is mediated by the phosphorylation of tyrosine 771 (Tyr771) leading to the binding of RasGAP, the GTPase activator of Ras. Phosphorylation of Tyr771 is regulated by protein tyrosine phosphatase PTPN11 (SHP-2). Decreased recruitment of RasGAP leads to prolonged activation of the Ras-MAP kinase pathway and thus promotes cell proliferation.

Tyrosine 751 (Tyr751) is situated in the kinase insert region and binds the regulatory p85 subunit of phosphatidylinositol 3-kinase (PI3-kinase). The phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)-P3) generated by PI3-kinase enhances the activity of the pro-survival Akt kinase. PTPN1 is also able to dephosphorylate Tyr751 on PDGFR, thus decreasing activation of Akt.

Tyrosine 1021 (Tyr1021) is known as the binding site of phospholipase C-γ1 (PLCγ). PLCγ initiates the inositol 1,4,5-trisphosphate (Ins (1,4,5)-P3) / diacylglycerol pathway, which mediates intracellular calcium mobilization and protein kinase C (PKC) activation and is important in the regulation of cell migration via RhoA.

Our earlier results have shown that cells of confluent A172 glioblastoma cultures, but not of sparse ones, exhibit a sustained two-phase calcium rise upon PDGF stimulus. Furthermore, expression of PDGFR β in these cells increased with confluence of the culture and these receptors were mainly localized to GM1 rich lipid microdomains.

2.2.2. The TRAIL receptor

The main goal of anticancer therapy is to selectively induce apoptosis in cancer cells while sparing untransformed cells and healthy tissues. One promising approach is to trigger the extrinsic apoptotic pathway by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor superfamily, which has been shown to selectively induce apoptosis in various cancer cells in vitro and in vivo. The mechanism of differential sensitivity to TRAIL in normal and cancer cells is still a matter of debates among the experts. Several potential ways of resistance of non-tumorigenic cells to TRAIL have been proposed so far, at the level of both surface and intracellular molecules. Furthermore, many tumor cells have been shown to be resistant to the effects of TRAIL due to deficiencies in apoptotic pathways or over-activated prosurvival signaling. Elucidation of the molecular mechanisms of resistance and designing safe combination therapies using agents capable of sensitizing cancer cells but not normal ones to TRAIL-induced apoptosis are important prerequisites of the successful clinical application of this cytokine.

There are five known types of TRAIL receptors. Death receptors (DRs) DR4 (TRAIL-R1) and DR5 (TRAIL-R2) containing a death domain (DD) are responsible for transmitting the apoptotic signal, whereas decoy receptors (DcRs) DcR1 (TRAIL-R3, lacking DD) or DcR2 (TRAIL-R4, with truncated DD), and a soluble receptor osteoprotegerin (TRAIL-R5) are unable to signal apoptosis. Intriguingly, the cell surface expression of a particular TRAIL receptor may not fully correlate with its functional importance in inducing or inhibiting apoptotic TRAIL signaling.

DR4 and DR5 mediate TRAIL-induced apoptosis by recruiting Fas-associated DD protein and pro-caspase-8 to form a death-inducing signaling complex (DISC). The amount of caspase-8 activated by the DISC is an important factor affecting further progression of apoptotic signaling. In so-called type I cells, abundantly activated caspase-8 directly cleaves and activates effector caspases, which leads to direct implementation of cell death. In type II cells, the amount of caspase-8 activated by the

DISC is not sufficient to trigger adequate effector caspase stimulation; therefore, amplification of the apoptotic signal via mitochondria is required.

The initial steps of TRAIL signaling have been studied intensively, especially the expressional regulation of the TRAIL receptors, their translocation to the cell surface, plasma membrane distribution, lipid raft localization and internalization. Recent studies have suggested the role of lipid rafts as platforms for DR-mediated apoptotic signaling. Relocation of TRAIL DRs into lipid rafts has been shown to facilitate DISC formation and apoptosis mediated by caspase-8 activation, whereas TRAIL-DISC assembly in the non-raft regions of the plasma membrane resulted in inhibition of caspase-8 cleavage and promotion of antiapoptotic signaling.

Combined treatment with chemotherapeutic drugs has been shown to overcome TRAIL resistance in many cancer cell types. Diverse molecular mechanisms have been proposed to be responsible for the synergistic effects of these agents in inducing apoptosis of target cells. Chemotherapy may have a great impact on the crucial steps of the TRAIL signaling pathway, e.g. through increase of TRAIL DR expression, lipid raft localization, decrease of the cellular level of FLICE-like inhibitory proteins, facilitating DISC formation and caspase-8 activation, up/downegulation of pro/antiapoptotic molecules, or stimulation of mitochondria.

Platinum complexes, e.g. cisplatin, carboplatin and oxaliplatin belong to the most widely used chemotherapeutic agents in the treatment of solid cancers. By creating covalent bonds with DNA, they induce DNA damage signaling, which leads either to cell cycle arrest providing time to repair the damage or to immediate activation of apoptotic signaling via the intrinsic mitochondrial pathway and killing of cancer cells. In the past two decades, plenty of newly synthesized analogues of platinum complexes including Pt(IV) complexes were assessed and some of them have entered clinical trials. LA-12 is a novel adamantylamine containing Pt(IV) complex currently in phase I clinical trials. In vivo studies on murine xenografts revealed a higher antitumor activity of LA-12 than that of cisplatin or the Pt(IV) complex satraplatin, as well as enhanced tissue penetration and lower acute systemic toxicity.

Due to its improved hydrophobicity, LA-12 was shown to effectively penetrate tissues and tumors and can be administered orally. These data imply that LA-12 is a promising candidate for cancer therapy, with high efficiency in killing cancer cells in vitro and in vivo and low toxic side effects in vivo.

3. **Aims**

In this work, we have investigated the role of lipid rafts in growth factor receptor and death receptor signaling, as well as the programmed cell death of cancer cells.

We tried to answer the following questions:

- Do lipid rafts and cell confluence have any regulatory significance for the receptor tyrosine kinase PDGF β in glioblastoma cells?

- Do lipid rafts play a role in the apoptotic response induced by the combination of platinum complexes and TRAIL?

4. Materials and methods

4.1. Cell lines

Lipid raft dependent regulation of β-type PDGF receptor was measured on A172 and T98G human glioblastoma cells, maintained in a humidified incubator at 37°C in a 5% CO2 atmosphere in DMEM medium supplemented with 10% fetal calf serum and antibiotics. The cells were passaged three times a week.

We used sparse (low density) and confluent (high density) cell cultures. Initial cell concentration was 15,000/cm2 for sparse and 60,000/cm2 for confluent conditions. Cells were cultured for 2 days before measurements, unless otherwise mentioned.

For Western blot experiments, cells were seeded onto cell culture dishes. For sparse and confluent cultures 570,000 cells were seeded in 100 mm and 35 mm Petri dishes yielding an initial density of 7,250 and 60,000 cells/cm2 for sparse and confluent samples, respectively. Cells were cultured for 2 days before measurements.

Effect of platinum complexes on the colocalisation of lipid rafts and TRAIL receptors was measured on HCT-116 human colon adenocarcinoma and PC-3 prostate epithelial cancer cell lines maintained in McCoy's 5A modified medium supplemented with 10% heat-inactivated fetal calf serum and antibiotics. Cells were incubated in a humidified incubator at 37°C in a 5% CO2 atmosphere and passaged twice a week. For confocal microscopy, cells were seeded onto 12 mm glass coverslips.

4.2. PDGF ligand stimulation of glioblastoma cells

Before experiments, A172 and T98G cell lines were starved in serum-free HEPES buffer, pH 7.4, for 2h at 37°C. Ligand stimulation of PDGFR β was done at 37°C with recombinant PDGF-BB (the homodimer of the B isoform of platelet derived growth factor) used at a final concentration of 20 ng/ml in 50 μl solution, the lowest dose previously established to cause maximum calcium signals in confluent glioblastoma cell cultures. For microscopy experiments, ligand stimulation was 2min when

measuring receptor phosphorylation, and 2h for assessing migration and proliferation. For Western blot-based analysis of signaling, PDGF-BB stimulation was performed for 1, 2, 5, 15, 30 and 60 min in order to obtain a time-function of events.

4.3. TRAIL ligand stimulation and platinum-complex treatment of colon and prostate carcinoma cells

Before experiments, HCT-116 and PC3 carcinoma cell lines were starved in serum-free HEPES buffer, pH 7.4, for 2h at 37°C. Ligand stimulation of TRAIL receptors and platinum treatment were done at 37°C with recombinant TRAIL used at a final concentration of 5 ng/ml, cisplatin used at a final concentration of 10 μM and LA-12 used at a final concentration of 0,5 μM in 50 μl solution. Cells were incubated for 5-60 minutes at 37°C, then washed with ice cold HEPES buffer.

4.4. Immunofluorescent labeling

Glioblastoma, colon and prostate cancer cell lines on coverslips were washed three times in ice-cold HEPES buffer and incubated with specific monoclonal antibody for 10 min on ice. After three washes, fluorescent dye conjugated secondary antibody was added at 15 μg/ml together with 4 μg/ml Alexa Fluor 488-conjugated cholera toxin B-subunit for 10 min on ice. After three washes cells were fixed with 4% formaldehyde on ice and allowed to warm to room temperature.

Intracellular staining of specific Tyr716, Tyr751, Tyr771 and Tyr1021 phosphotyrosine residues, pRhoA, cortactin and Ki-67 proteins on glioblastoma cells was performed immediately after extracellular labeling, or after fixing cells with 1% formaldehyde for 10 min on ice. The cell membrane was permeabilized with HEPES buffer containing 0.1% Triton-X 100 and 1% BSA for 10 min. Following incubation with the appropriate monoclonal antibodies for 35 min at RT, cells were washed three times with HEPES buffer containing 0.05% Triton-X 100. Primary antibodies were labeled with Cy3, Alexa Fluor 546 or Alexa Fluor 647 conjugated secondary antibodies. After three washes, cells were fixed with 4% freshly depolymerized paraformaldehyde. Finally, the coverslips were mounted with Mowiol.

4.5. Confocal Laser Scanning Microscopy

Membrane distribution of PDGFR β and its phosphotyrosine residues was quantitatively analyzed by a confocal laser scanning microscope (LSM 510, Carl Zeiss GmbH, Jena, Germany). Alexa Fluor 488 was excited at 488 nm, Cy3 and Alexa Fluor 546 at 543 nm, and Alexa Fluor 647 at 633 nm. Their fluorescence emission was detected through 505 to 550-nm, 560 to 615 band-pass and 650-nm long-pass filters, respectively. The images were taken in multitrack mode to exclude channel cross talk. 512 × 512-pixel, 1.5 μm thick optical sections were obtained with a 40× C-Apochromat water immersion objective (NA=1.2).

4.6. Digital image processing

4.6.1. Determining colocalization from image cross-correlation

Colocalization of any two molecules at the few-hundred-nanometer scale was determined from confocal laser scanning microscopy images of double-labeled cells.

The optical section was taken from the top horizontal slice of the membrane of adherent cells. The images were gated on the presence (above-background intensity) of at least one of the fluorophores. A custom written program in LabView was used to analyze the images and calculate the crosscorrelation coefficient C. The theoretical maximum is C = 1 for identical images and a value close to 0 implies independent random localization of the two labeled molecules.

4.6.2. Digital image processing for the quantitative analysis of specific phosphotyrosine distribution inside and outside of lipid rafts

A quantitative digital image processing pipeline, created for this purpose in ImageJ, was used to calculate specific phosphotyrosine density inside and outside of lipid rafts. First, background intensities of phosphotyrosine and GM1 positive channels were subtracted. Two binary masks in inverse relation – raft and non-raft – were created and used to generate inside raft and outside raft phosphotyrosine images.

From these, probability distribution curves of pixel-intensities were generated, and the mean intensities calculated.

4.6.3. Digital image processing for the quantitative analysis of specific relative receptor phosphorylation inside and outside of lipid rafts

The ImageJ algorithm developed for this purpose starts by subtracting the background in the PDGFR, phosphotyrosine and GM1 channels, and then normalizes the intensity of the specific phosphotyrosine label to the total PDGFR on a pixel by pixel basis. The image thereby generated represents the relative specific PDGFR phosphorylation on the cell surface. Raft and non-raft binary masks are then created from the GM1 channel and applied to calculate the mean relative receptor phosphorylation inside and outside of lipid rafts. Finally, the inside raft value is normalized by the outside raft value, so that acquisition parameters that could change across experiments are canceled to allow the pooled evaluation of results from many independent experiments. Values of the final quotient above and below 1 indicate preferential phosphorylation and dephosphorylation, respectively, of a given tyrosine residue inside rafts.

4.7. Western blotting

Cell lysates were prepared from sparse and confluent A172 and T98G cell lines. Cells were washed with ice cold HEPES buffer and lysed with lysis buffer. Protein concentration was measured according to Bradford, samples were diluted with SDS-PAGE sample buffer to contain the same concentration of total protein and finally boiled for 10 min. Proteins (20 µg per well) were separated by 6-12% SDS-PAGE gels depending on the targeted molar mass, and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were blocked for 60 min with TBS buffer supplemented with 5% dry milk, then incubated with specific monoclonal antibodies. After overnight incubation on a rocking table at 4°C, membranes were washed with TBS-T for 30 min at room temperature, and then peroxidase-conjugated secondary antibody was added for 2 hours. After a 30 min wash with TBS-T,

membranes were developed with Super Signal West Pico Chemiluminescent Substrate and imaged with a FluorChem Q system.

4.8. Assessment of cell proliferation

Proliferation in confluent and sparse cultures was measured using an MTT based colorimetric assay. Cells plated at various densities were grown in 96-well plates for 2 days. After incubation with EZ4U, a Synergy HT Multi-Detection microplate reader was used to measure 488 nm absorption, which, corrected with the 620 nm absorption was converted to cell numbers using a calibration curve from freshly plated and adhered cells seeded at various densities.

To follow the growth rate in real time, impedance based real time cell adherence assay (RTCA) was performed using an ECIS ZΘ analyzer. T98G cells seeded at low and high densities were grown in 8W10E PET 8 well arrays with gold electrodes at the bottom. Weak alternating current was applied at frequencies from 1 to 100,000Hz to continuously measure the complex impedance spectrum of cells adhering to each electrode in the well. The measured impedance at any time point is proportional to the area of the electrode covered by cells. Impedance values from 4 wells were averaged, normalized to initial cell number, and plotted as a function of time.

4.9. Statistical analysis

The data from lipid raft and cell confluence dependent regulation of PDGFRs was statistically analyzed by two-way analysis of variance followed by Tukey's test.

Lipid raft dependent synergism of platinum complexes and TRAIL receptors was analyzed by ANOVA followed by Tukey's test, or by a nonparametric Mann–Whitney U-test.

All statistical analyses were performed by the XLSSTAT module supplemented MS Office Excel 2010 software. A P value of <0.05 was considered significant.

5. Results and discussion

5.1. Cell confluence and lipid raft dependent regulation of PDGFR β on glioblastoma cell lines

5.1.1. PDGFR β clusters in the cell membrane co-localize with lipid rafts

Preliminary experiments have shown that expression of PDGFR β on A172 and T98G glioblastoma cells increases with the confluence of cells in culture and that these receptors are mainly localized to GM1 rich lipid microdomains. We have employed confocal microscopy yielding 1.5 micron thick optical slices of the upper cell membrane to quantitate the degree of overlap between clusters of PDGFR β and lipid rafts. Colocalization was quantified by calculating the crosscorrelation coefficient. Overlap of PDGFR with lipid rafts was directly proportional to the confluence of the monolayer, regardless of whether cells were seeded at varying densities and measured after having adhered to the substrate, or seeded at the same density, and left to grow continuously over several days to reach increasing levels of confluence. This indicates that cell culture confluence as a state, and not the process of reaching it regulates receptor expression and raft localization.

5.1.2. Lipid raft and cell confluence dependent regulation of PDGFR β

To investigate whether the confluence dependent raft localization of the receptors has any functional consequence, we have quantitated by immunofluorescence the amount of specific phosphotyrosine residues inside and outside GM1 rich membrane domains both in resting and PDGF-BB stimulated cells. The pixel by pixel distribution of specific phosphotyrosine residues both inside and outside lipid rafts was calculated.

Sparse cultures exhibited basal phosphorylation of Tyr716 inside rafts slightly higher than confluent cultures, and showed a greatly increased activation upon stimulation with PDGF-BB, whereas p-Tyr716 hardly increased in rafts of confluent cells upon stimulus.

Basal phosphorylation of Tyr751 was 1.6x higher in sparse than in confluent cultures. Following PDGF-BB stimulus, sparse cells showed increased phosphorylation inside lipid rafts.

Cell confluence had no prominent effect on the distribution of Tyr771 residue. Following PDGF-BB stimulation Tyr771 was more phosphorylated in confluent cultures, with a dominance of lipid raft-localized receptors

Tyr1021 phosphorylation was globally higher in GM1 rich membrane areas, and exhibited considerable increase upon ligand stimulation in confluent cells.

5.1.3. Relative specific phosphorylation of PDGFR β is a function of both cell confluence and lipid raft localization

Stimulation induced effects on the cell surface distribution, raft localization and activation of PDGF receptors was simultaneously observed by triple immunofluorescent staining. We measured confluence dependent expression, specific tyrosine phosphorylation and raft localization of PDGFR in membrane before and after PDGF-BB stimulation. While Tyr716 and Tyr751 residues were primarily phosphorylated in GM1 rich domains of sparse cultures, Tyr771 and Tyr1021 activation was increased in rafts of confluent cells.

A quantitative digital image processing pipeline was used to determine the relative specific phosphorylation inside and outside of lipid rafts. We concluded that in unstimulated cells the dephosphorylated state of specific tyrosine residues dominated in rafts with the exception of Tyr1021.

Following PDGF-BB ligand stimulation, distinct activation profiles of sparse and confluent cells were observed for specific tyrosine residues, with major changes from resting to stimulated state occurring in lipid rafts. Tyr716 involved in the Ras-MAPK pathway was phosphorylated in sparse, while Tyr771 inhibiting the same pathway was activated in confluent cells.

Activation of Tyr751 regulates cell survival by initiating the PI3-Kinase pathway. PLCγ responsible for calcium transients and cell migration is activated on Tyr1021. We found that activation of Tyr751 was dominant in the membrane of sparse cells,

while Tyr1021 phosphorylation was increased in the GM1 rich domains of confluent cells.

5.1.4. Activation of the Ras-MAPK pathway and consequential cell proliferation dominates in sparse cultures

To confirm confluence dependent selective tyrosine phosphorylation and test the activation of relevant downstream signaling pathways, Western blot analysis was performed. Also, to reveal whether the finding can be generalized, a second PDGFR expressing glioblastoma cell line, T98G, was examined in addition to A172 cells.

In accordance with microscopic data, levels of p-Tyr716 which activates the Ras-MAPK pathway showed a transient increase in sparse cultures of both cell lines following PDGF-BB ligand stimulation, and no change in confluent cells. Conversely, increased phosphorylation of the Tyr771 residue, which activates Ras-GAP, an inhibitor of the pathway, was more prominent in confluent cultures, although some transient phosphorylation was also seen in sparse cultures. Next, we measured the phosphorylation of two effector MAPK isoforms, p38 MAPK, and p42/p44 MAPK. Coherent with the specific phosphorylation of tyrosine residues activating and inhibiting these MAP kinases, both p38 and p42/p44 were activated mainly in sparse cultures. We have observed a time delay between the activation of receptors and effector proteins: while Tyr716 and Tyr771 were maximally phosphorylated within 1-2 minutes of ligand stimulus, and by 15 min were already back to baseline, activation of effector MAP kinases peaked in the range of 5 – 30 minutes, land lasted through the 60 min observation period

As logically expected, phosphorylated p38 MAPK, and also p42/44 MAPK were increased in sparse cultures upon PDGF stimulus, in coherence with increased Tyr716 phosphorylation. Meanwhile, in confluent cells, MAPK phosphorylation was low, partly owed to the lower levels of p-Tyr716, and partly to the strong phosphorylation of Tyr771, which inhibits the MAPK pathway by activating RasGAP.

Since activation of the MAP kinase pathway is expected to influence cell proliferation, accumulation of the Ki-67 proliferation marker in cell nuclei was also

assessed by fluorescence microscopy. In line with increased MAPK activation in sparse cultures, we found greatly increased Ki-67 protein levels in the nuclear region of ~ 85% of the cells in sparse cultures two hours after PDGF stimulus. Coherent with low MAPK activation, the proportion of highly Ki-67 positive cells was only 1% in confluent cultures, and a further 12% exhibited a slight increase. Both MTT based and real time proliferation assays confirmed that the rate of proliferation is at least twice as high in sparse cultures as in confluent ones. Without stimulus, no signs of proliferation were detected in either sparse or confluent serum starved cell cultures.

5.1.5. Activation of the PLCγ pathway and consequential cell migration dominates in confluent cultures

Phosphorylation of Tyr1021 occurred both in sparse and confluent cells, started by the first minute of stimulation, but was over within half an hour. Confluent cells showed markedly higher p-Tyr1021 than sparse cells of both cell lines. The phosphorylation of RhoA, an important effector activated along the PLCγ / PKC pathway was correspondingly also greatly increased in confluent cells, while in sparse cells it remained at baseline with only a slight increase at 2-15 minutes. We also observed that by two hours RhoA phosphorylation can even be below the baseline seen in unstimulated cells, while in confluent cells it is still maintained at a very high level.

Based on this observation, we hypothesized that activation of the PLCγ pathway in confluent cells has a functional consequence on cell migration. Cortactin is an important protein facilitating polymerization and rearrangement of the actin cytoskeleton, promoting the formation of lamellipodia, invadopodia, and cell migration. In our confocal experiments, cellular localization and rearrangement of this protein to the leading edges were oppositely regulated in sparse and confluent cultures. In resting sparse cells, cortactin was mostly localized to the polarized cell front protrusion but decreased in quantity and appeared perinuclearly two hours after stimulus. In unstimulated confluent cultures, cortactin dominantly showed

cytoplasmic localization, but following PDGF-BB stimulation, it cumulated in the polarized leading cell front.

5.2. Platinum complex based chemotherapeutic agents and TRAIL induce DR4 and DR5 mediated, caspase-8 regulated apoptosis in a lipid raft dependent manner

5.2.1. Trail receptors form clusters on the cell surface and colocalize with lipid rafts

In our experiments we examined the sensitizing effect of LA-12, a novel platimun-complex on the TRAIL mediated apoptosis of colon and prostate cell lines. We also compared its efficiency and its raft-related effects with the strong and widely used chemotherapy drug, cisplatin.

We determined that DR4 and DR5 type TRAIL receptors form clusters on the surface of PC-3 prostate and HCT-116 colon carcinoma cells and colocalize with GM1 rich microdomains of the membrane. Based on this observation we hypothesized that the plasma membrane is an important factor in regulating the LA-12 or cisplatin induced extrinsic apoptotic pathway.

5.2.2. Surface expression of TRAIL receptors is increased upon treatment with platinum complexes

We also observed that treatment for 1h with either 10 μM cisplatin or 0.5 μM LA-12 increased the expression of TRAIL receptors by 20-200% in both examined cell lines. Cisplatin treatment of the HCT-116 cell line especially increased the expression level of the DR5 TRAIL receptor, while LA-12 treatment of the PC-3 cell line affected most prominently that of the DR4 TRAIL receptor.

5.2.3. Lipid raft localization of TRAIL receptors was increased upon treatment with platinum complexes

After 1 h of cisplatin or LA-12 treatment, the colocalization between lipid rafts and TRAIL receptors DR4 and DR5 increased in both HCT-116 and PC-3 cells. Compared to controls, DR4 raft localization increased significantly in PC-3 cells after LA-12 treatment, while DR5 showed a significant increase in HCT-116 owed to cisplatin, and in PC-3 owed to both platinum complexes.

5.2.4. Short term TRAIL treatment increases lipid raft localisation of DR4 and DR5 rceptors

We examined the effects of TRAIL ligand stimulation on raft localization of DR4 and DR5 receptors. We found that short term (5-20 min) TRAIL stimulation at 5ng/ml increased the raft localization of both DR4 and DR5 receptors on both cell lines.

Following 1 hour treatment, raft localization of the TRAIL receptors fell below the baseline on HCT-116 and PC-3 cell lines, most likely owed to internalization.

5.2.5. LA-12- or cisplatin-mediated potentiation of TRAIL-induced cytotoxicity is associated with activation of the apoptotic caspase cascade

In order to examine whether cytotoxicity induced by the combination of platinum complexes and TRAIL occurred via activation of the caspase cascade and apoptotic signaling, analysis of the cleavage of caspase-8, and caspase-3 was performed by Western blotting. Pretreatment with cisplatin or LA-12, followed by TRAIL, resulted in a substantial potentiation of the cleavage of pro-caspase-8, and pro-caspase-3 in both cell lines.

6. Conclusions

6.1. Lipid raft and confluence dependent regulation of PDGF receptor β on glioblastoma cell lines

Receptor tyrosine kinases of the PDGFR family play important roles in the proliferation and survival of cells, including those in glial tumors of the central nervous system. In this current study we described that PDGF receptors are mostly localized in submicron sized clusters on the cell surface. We also determined that the number, receptor density and lipid raft colocalization of these submicron sized clusters increases directly with cell confluence. Furthermore, we observed that receptor-raft interaction and cell confluence has high impact on the activation of various signaling pathways initiated on specific tyrosine residues of PDGFR β. Following PDGF-BB stimulation the Ras-MAPK pathway specific Tyr716 and the PI3-Kinase / Akt specific Tyr751 are phosphorylated primarily in sparse cultures, while Tyr771 phosphorylation which inhibits MAPK signaling decreases in these cells. In accordance with this observation, PDGF stimulus causes significantly higher MAPK phosphorylation and higher rate of Ki-67 antigen positivity, as well as at least twice higher proliferation rate in sparse cultures than in confluent ones.

At the same time, Tyr1021 activating phospholipase-C-γ and consequently the PKC pathway was highly activated in confluent cultures. We could observe the increased phosphorylation of downstream RhoA, and the redistribution of cortactin into leading edges, indicating increased mobility in confluent cultures.

Tyrosine dephosphorylation and phosphorylation was dominantly performed in GM1 positive lipid domains.

These observations suggest that the same stimulus is able to promote distinctly relevant signaling outputs through a confluence dependent, lipid raft-based regulatory mechanism. In particular, cell division in sparse cultures and inhibition of proliferation and promotion of migration in confluent monolayers.

6.2. Platinum complex based chemotherapeutic agents and TRAIL induce DR4 and DR5 mediated, caspase-8 regulated apoptosis in a lipid raft dependent manner

TRAIL, a member of the tumor necrosis factor superfamily has been shown to selectively induce apoptosis in various cancer cells and to modify their resistance against other chemotherapy agents. Death receptors DR4 and DR5, containing a death domain, are responsible for transmitting the apoptotic signal of TRAIL. In this present study, we observed that both of examined platinum complexes increased the expression of TRAIL receptors in prostate and colon carcinoma cell lines. Cisplatin treatment had the greatest effect on DR5 receptors of colon cancer cell lines, while LA-12 treatment increased most eminently DR4 expression on prostate cancer cells. Both complexes increased the raft localization of TRAIL receptors on both cell lines, but LA-12 proved as effective as cisplatin in a concentration twenty times lower. TRAIL stimulation had a time dependent effect on receptor expression and raft localization. 5-20 minutes of TRAIL stimulation increased the expression and raft localization of receptors, but after one hour, raft localization decreased below the baseline, presumably owed to receptor internalization. The joint effect of platinum compounds and TRAIL on TRAIL receptor expression and raft localization can be exploited to induce apoptosis of cancer cell more effectively, as evidenced by the activation of caspases 8 and 3 in our model cell lines. This may offer potential clinical benefits when combining the two treatments.

7. **Summary**

Cumulatively, our observations suggest that cholesterol and ganglioside enriched lipid microdomains of the cell membrane, also termed lipid rafts, are important regulators of various signaling processes.

We have shown that PDGF and its receptor, using the organizing ability of lipid raft platforms, can initiate divergent signaling pathways as required by the sparse or confluent state of the cell population. Proliferation is activated is sparse, while migration in confluent cultures. Cell survival is regulated in a less divergent manner.

We observed that platinum complexes widely used for cytostatic therapy increased the cell surface expression of TRAIL receptors and also their raft localization. Both of these effects result in a more effective signaling and increased apoptotic cell death. This newly observed lipid-raft dependent regulation has potential consequences in cancer therapy.

Our results give an overview extending to clinical aspects about the diverse regulatory and organizing function of GM1 ganglioside rich membrane microdomains.

Registry number: DEENK/255/2015.PL
Subject: Ph.D. List of Publications

Candidate: Árpád Szöőr
Neptun ID: ALEKKW
Doctoral School: Doctoral School of Pharmaceutical Sciences
MTMT ID: 10034414

List of publications related to the dissertation

1. **Szöőr, Á.**, Ujlaky-Nagy, L., Tóth, G., Szöllősi, J., Vereb, G.: Cell confluence induces switching from proliferation to migratory signaling by site-selective phosphorylation of PDGF receptors on lipid raft platforms.
Cell. Signal. "Accepted by Publisher" (2015)
DOI: http://dx.doi.org/10.1016/j.cellsig.2015.11.012
IF:4.315 (2014)

2. Vondalova, B.O., Jelínková, I., **Szöőr, Á.**, Skender, B., Soucek, K., Horváth, V., Vaculova, A., Andera, L., Sova, P., Szöllősi, J., Hofmanova, J., Vereb, G., Kozubik, A.: Cisplatin and a potent platinum(IV) complex-mediated enhancement of TRAIL-induced cancer cells killing is associated with modulation of upstream events in the extrinsic apoptotic pathway.
Carcinogenesis. 32 (1), 42-51, 2011.
DOI: http://dx.doi.org/10.1093/carcin/bgq220
IF:5.702

List of other publications

3. Govers, C., Sebestyén, Z., Roszik, J., van Brakel, M., Berrevoets, C., **Szöőr, Á.**, Panoutsopoulou, K., Broertjes, M., Van, T., Vereb, G., Szöllősi, J., Debets, R.: TCR's genetically linked to CD28 and CD3[epszilon] do not mispair with endogenous TCR chains and mediate enhanced t cell persistence and anti-melanoma activity.
J. Immunol. 193 (10), 5315-5326, 2014.
DOI: http://dx.doi.org/10.4049/jimmunol.1302074
IF:4.922

4. Nagy, D., Gönczi, M., Dienes, B., **Szöőr, Á.**, Fodor, J., Nagy, Z., Tóth, A., Fodor, T., Bai, P., Szűcs, G., Rusznák, Z., Csernoch, L.: Silencing the KCNK9 potassium channel (TASK-3) gene disturbs mitochondrial function, causes mitochondrial depolarization, and induces apoptosis of human melanoma cells.
Arch. Dermatol. Res. 306 (10), 885-902, 2014.
DOI: http://dx.doi.org/10.1007/s00403-014-1511-5.
IF:1.902

5. Pázmándi, K., Kumar, B.V., Szabó, K., Boldogh, I., **Szöőr, Á.**, Vereb, G., Veres, Á., Lányi, Á., Rajnavölgyi, É., Bácsi, A.: Ragweed subpollen particles of respirable size activate human dendritic cells.
PLoS One. 7 (12), e52085, 2012.
DOI: http://dx.doi.org/10.1371/journal.pone.0052085
IF:3.73

6. Roszik, J., Sebestyén, Z., Govers, C., Guri, Y., **Szöőr, Á.**, Pályi-Krekk, Z., Vereb, G., Nagy, P., Szöllősi, J., Debets, R.: T-cell synapse formation depends on antigen recognition but not CD3 interaction: Studies with TCR : zeta, a candidate transgene for TCR gene therapy.
Eur. J. Immunol. 41 (5), 1288-1297, 2011.
DOI: http://dx.doi.org/10.1002/eji.200940233
IF:5.103

7. **Szöőr, Á.**, Szöllősi, J., Vereb, G.: Rafts and the battleships of defense: The multifaceted microdomains for positive and negative signals in immune cells.
Immunol. Lett. 130 (1-2), 2-12, 2010.
DOI: http://dx.doi.org/10.1016/j.imlet.2009.12.016
IF:2.511

Total IF of journals (all publications): 28,185
Total IF of journals (publications related to the dissertation): 10,017

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

08 December, 2015

Address: 1 Egyetem tér, Debrecen 4032, Hungary Postal address: Pf. 39. Debrecen 4010, Hungary
Tel.: +36 52 410 443 Fax: +36 52 512 900/63847 E-mail: publikaciok@lib.unideb.hu, ▫ Web: www.lib.unideb.hu