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Cell confluence induces switching from proliferation to migratory signaling by site-selective phosphorylation of PDGF receptors on lipid raft platforms

Árpád Szöőr [a], László Ujlaky-Nagy [b], Gábor Tóth [a], János Szöllősi [a,b], György Vereb [a,b,*]

[a] Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary
[b] MTA-DE Cell Biology and Signaling Research Group, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

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ABSTRACT

Platelet derived growth factor receptors (PDGFR) play an important role in tumor pathogenesis and are frequently overexpressed in glioblastoma. Earlier we have shown that only confluent glioblastoma cell cultures exhibit a biphasic calcium transient upon PDGF stimulation. Here, we examined how the change in cell density leads to differential cellular responses to the same PDGF stimulus.

PDGF beta receptors and their specific phosphotyrosine residues were fluorescently co-labeled on A172 and T98G glioblastoma cells. The distribution in cell membrane microdomains (lipid rafts) and the phosphorylation state of PDGFR was measured by confocal microscopy and quantitated by digital image processing. Corresponding bulk data were obtained by Western blotting. Activation of relevant downstream signaling pathways was assessed by immunofluorescence in confocal microscopy and by Western blot analysis. Functional outcomes were confirmed with bulk and single cell proliferation assays and motility measurements.

In non-confluent (sparse) cultures PDGF-BB stimulation significantly increased phosphorylation of Tyr716 specific for the Ras/MAPK pathway and Tyr751 specific for the phosphoinositide 3-kinase/Akt pathway. As cell monolayers reached confluence, Tyr771 and Tyr1021 were the prominently phosphorylated residues. Tyr771 serves as adaptor for Ras-GAP, which inactivates the MAPK pathway, and Tyr1021 feeds into the phospholipase C-gamma/PKC pathway. Coherent with this, MAPK phosphorylation, Ki-67 positivity and proliferation dominated in dispersed cells, and could be abolished with inhibitors of the MAPK pathway. At the same time, RhoA activation, redistribution of cortactin to leading edges, and increased motility were the prominent output features in confluent cultures. Importantly, the stimulus-evoked confluence-specific changes in the phosphorylation of tyrosine residues occurred mainly in GM1-rich lipid microdomains (rafts).

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 and survival in sparse cultures and inhibition of proliferation and promotion of migration in confluent monolayers. In our model, the ability to switch the final output of the same stimulus as a function of cell density could be a key to the balance of proliferation and invasion in malignant glioblastoma.

1. Introduction

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 [1], but its abnormal expression also contributes to a variety of diseases [2]. PDGF and its receptor are currently under investigation as targets in numerous proliferative disorders, including cancers, fibrosis, and cardiovascular diseases [2]. PDGFR-β plays an important role in the regulation of human malignant gliomas [3,4]. PDGF and PDGFR expression correlates with tumor grade and proliferative activity [5–7],

Abbreviations: DAG, diacylglycerol; GM1, monosialotetrahexosylganglioside; GRB2, growth factor receptor binding protein-2; IP3, inositol 1,4,5-trisphosphate; PDGF, platelet-derived growth factor; PDGF-BB, homodimer of the B isoform of platelet derived growth factor); PDGFR, platelet-derived growth factor receptor; PI3-kinase, phosphatidylinositide 3′-kinase; PKC, protein kinase C; PLCγ, phospholipase C-γ1; PtdIns(3,4,5)-P3, phosphatidylinositol (3,4,5)-trisphosphate; PTPN1, protein tyrosine phosphatase, non-receptor, type 1 (PTP1B); Ras-GAP, GTPase activator of Ras; Ras-MAPK, Ras mediated mitogen-activated protein kinase; SH2 domain, Src Homology 2 domain; Tyr716, tyrosine residue 716; Tyr751, tyrosine residue 751; Tyr771, tyrosine residue 771; Tyr1021, tyrosine residue 1021.

* Corresponding author at: Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen, Egyetem tér 1, H-4032 Debrecen, Hungary.
E-mail address: vereb@med.unideb.hu (G. Vereb).

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and inhibition of PDGFR-β drastically reduces the proliferation and migration of glioblastoma cells [8].

Phosphotyrosine residues on the activated PDGFR initiate downstream signaling. On PDGFR-β, 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 [9] which plays a central role in cell proliferation. Dephosphorylation of Tyr716 by protein tyrosine phosphatase PTPN1 (PTP1B) inhibits PDGF-induced ERK1/2 MAPK activation [10]. The negative regulation of this pathway is mediated by the phosphorylation of tyrosine 771 (Tyr771) leading to the binding of Ras-GAP, the GTPase activator of Ras [11]. Phosphorylation of Tyr771 is regulated by protein tyrosine phosphatase PTPN11 (SHP-2) [12]. Dephosphorylation of Tyr771 decreases the recruitment of Ras-GAP leading to prolonged activation of the Ras/MAP kinase pathway and thus promoting cell proliferation.

Tyrosine 751 (Tyr751) is situated in the kinase insert region and binds the regulatory p85 subunit of phosphatidylinositide 3′-kinase (PI3-kinase) [13]. The phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)-P3) generated by PI3-kinase enhances the activity of the pro-survival Akt kinase [14,15]. PTPN1 is also able to dephosphorylate Tyr751 on PDGFR, thus decreasing activation of Akt [10].

Tyrosine 1021 (Tyr1021) is known as the binding site of phospholipase C-γ1 (PLCγ) [16]. PLCγ initiates the inositol 1,4,5-trisphosphate (Ins (1,4,5)-P3)/diacylglycerol (DAG) pathway, which mediates intracellular calcium mobilization and protein kinase C (PKC) activation [17] and is important in the regulation of cell migration [16,18].

GM1 rich microdomains (lipid rafts) of the cell membrane play crucial role in growth factor induced signal transduction [19,20]. These plasma membrane microdomains enriched in cholesterol and sphingolipids are able to segregate cellular processes during signal transduction by promoting isolated assembly of membrane protein superstructures [21] and to focus signaling input by accumulation of specific receptors [22,23].

Under normal conditions, cell growth and motility are regulated not only by the availability of growth factors but also by a number of positive and negative co-stimuli delivered by the extracellular matrix and the neighboring cells. Increasing number of cell–cell contacts leads to an arrest of proliferation known as contact inhibition [24]. When normal cells undergo malignant transformation, they can lose contact inhibition causing abnormal cell proliferation and migration [25]. In the case of PDGFR mediated cell proliferation, contact inhibition correlates with low molecular weight phosphotyrosine phosphatase mediated dephosphorylation of the receptor [26].

Previously we have shown that PDGF stimulated glioblastoma cells respond with prolonged biphasic rises of intracellular free calcium concentration when cultures were confluent, however, in sparse cultures this response largely disappears [27]. The distinct behavior was independent of cell cycle [27], however, preliminary experiments hinted that PDGFR expression and lipid raft localization did change with cell culture confluence. Consequently, in the present study we examined how segregation of PDGFR by GM1 rich microdomains could regulate a differential cellular response as a function of cell confluence in glioblastoma cells. We have found that even though the same receptors are stimulated by the same ligand, tyrosine residues that initiate distinct pathways are selectively phosphorylated in lipid rafts and stimulate functions inherently appropriate for the status of tumor cells: sparse cells enter the mitotic cycle, whereas confluent cells start to migrate.

2. Materials and methods

All materials were from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

2.1. Cell culture

A172 (CRL-1620) and T98G (CRL-1690) human glioblastoma cells obtained from American Type Culture Collection were maintained in a humidified incubator at 37 °C in a 5% CO_2 atmosphere in Dulbecco's Minimal Essential Medium supplemented with 10% fetal calf serum and antibiotics. Cells were passaged three times a week.

For confocal microscopy, cells were seeded onto 12 mm glass coverslips (0.17 mm, Menzel-Glaser, Braunschweig, Germany) at various densities. We used sparse/low density and confluent/high density cell cultures. Initial cell concentration was 15,000/cm^2 for sparse and 60,000/cm^2 for confluent conditions. Cells were cultured for 2 days before measurements, unless otherwise stated.

For Western blot experiments, cells were seeded onto cell culture dishes (Corning, via Sigma-Aldrich). Sparse/low density and confluent/high density cultures were used. 570,000 cells were seeded in 100 mm and 35 mm petri dishes which meant 7250 and 60,000 cells/cm^2 for sparse and confluent samples, respectively. Cells were cultured for 2 days before measurements.

Before experiments, cells were starved in serum-free HEPES buffer, pH: 7.4, for 2 h at 37 °C. Ligand stimulation of PDGFR-β was done with the recombinant homodimer of the B isoform of platelet derived growth factor (PDGF-BB) at 37 °C. PDGF-BB was used at a final concentration of 20 ng/ml, the lowest dose previously established to cause maximum calcium signals in confluent glioblastoma cell cultures [28]. For microscopy experiments, ligand stimulation was 2 min long when measuring receptor phosphorylation, and 2 h when assessing migration and proliferation. In order to determine the time-dependence of the events, PDGF-BB stimulation was performed for 1, 2, 5, 15, 30 and 60 min for Western blot-based analysis of signaling.

2.2. Immunofluorescent labeling

Cells on coverslips were washed three times in ice-cold HEPES buffer and incubated with anti-PDGFR-β mAb (10 μg/ml, RD Systems PR7212) for 10 min on ice. After three washes, Cy3-conjugated secondary goat anti-mouse Fab antibody (Jackson ImmunoResearch Europe Ltd., Suffolk, UK) was added at 15 μg/ml together with 4 μg/ml Alexa Fluor 488-conjugated cholera toxin B-subunit (Invitrogene/Life Technologies, Carlsbad. CA) for 10 min on ice. The cholera toxin B-subunit binds to monosialotetrahexosylganglioside (GM1) glycosphingolipid rich domains and serves as one of the most widely used markers of lipid rafts. After three washes (1, 3 and 5 min), cells were prefixed with 1% paraformaldehyde for 10 min on ice, then the cell membrane was permeabilized with HEPES buffer containing 0.1% Triton-X 100 and 1% BSA for 10 min. Phosphotyrosine 716, 751, 771 and 1021 residues of PDGFR were labeled with indirect immunofluorescence. Following incubation with the appropriate mAb (sc-16569-R, sc-21902-R, sc-17174-R, sc-12909-R respectively, Santa Cruz Biotechnologies, Dallas, TX, 12 μg/ml final concentration) for 35 min at RT, cells were washed three times (for 2, 4 and 5 min) with HEPES buffer containing 0.05% Triton-X 100. Primary antibodies were labeled with Alexa Fluor 647 conjugated secondary goat-anti-rabbit antibody (20 min, RT, 10 μg/ml, Invitrogen/Life Technologies, Carlsbad. CA). After three washes, cells were fixed with 4% freshly depolymerized paraformaldehyde. Finally, the coverslips were mounted with Mowiol. Negative controls with one of the primary antibodies omitted, as well as with single secondary antibodies and with no labeling at all were also prepared and confirmed to lack signal in the appropriate fluorescence channel.

For microscopic assessment of cell proliferation and cell migration, intracellular indirect labeling was performed using the same protocol as above, starting with washing, fixation and permeabilization. Primary antibodies were used at 4 μg/ml (M7240 against Ki-67 from Dako, Glostrup, Denmark, and 05-180 antibody against cortactin from Merck