PYK2 AND FAK ACTIVATION BY MICROGLIAL CYTOKINES IN INVASION AND PROLIFERATION OF HUMAN GLIOBLASTOMA

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ABSTRACT

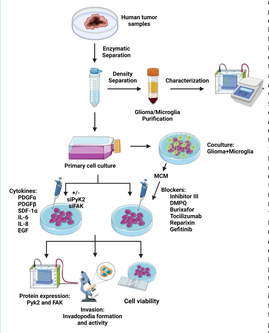
Glioblastoma (GBM) is the most aggressive and highly invasive primary brain tumor in adults. Evidence suggests that microglia create a microenvironment favoring glioma invasion and proliferation. Indeed, previous reports indicate the involvement of focal adhesion kinase (FAK) signaling cascades in glioma cell proliferation. Besides, studies from our laboratory support a critical role of Pyk2, a relative of FAK, in glioma invasion by tumor-infiltrating microglia. However, the microglial-released factors modulating Pyk2 and FAK signaling pathways are unknown. In this study, 20 human GBM specimens were evaluated to identify the cytokine expression patterns in purified microglia and FAK and Pyk2 phosphorylation in glioma cell fraction by RT-PCR and western blot. A Pierson correlation test demonstrated a high correlation (0.6-1.0) between the gene expression levels for PDGFβ, SDF-1α, IL-6, IL-8, and EGF in tumor-purified microglia, and levels of pPyk2(Y579/580) and pFAK(Y925) in glioma cells. The role of cytokines in cell invasion and proliferation by Pyk2/FAK activation was further investigated in primary cell lines from three patients. Thirty percent up-regulation of pPyk2 and pFAK was detected in glioma cells treated (2hrs) with microglia conditioned media (MCM) compared to control cells. siPyk2 or siFAK knockdown identified IL-6 (100 μM), PDGFβ (5 μM) SDF-1α (100 μM), and EGF (1 μM) as key factors of Pyk2- and FAK-dependent activation in all glioma cell lines. Similar results with siPyk2 or siFAK were observed for matrix degradation, invadopodia formation, cell viability, and mitosis. Indeed, Tocilizumab (IL-6R blocker, 100 ng/mL) DMPQ (PDGFβ blocker, 200nM), Burixafor (SDF-1α blocker, 1 μM), and Gefitinib (EGFR blocker, 1 μM) reversed the effect of MCM on glioma cell proliferation and invasion in all cell lines evaluated. These findings support a pivotal role of Pyk2 and FAK in enhancing proliferation and invasion of glioma tumors through IL-6, PDGFβ, SDF-1α, and EGF-dependent pathways. The latter could be of clinical relevance for new therapeutic developments in GBM patients.

INTRODUCTION

Glioblastoma multiforme (GBM, World Health Organization grade IV) is the most common and malignant adult brain tumor. Despite recent advances in health care standards, it has a poor prognosis with median survival rates of approximately 15 months.[1] The ability of glioma cells to restrict absorption of therapeutic chemicals,[2] intense microvascular angiogenesis, uncontrolled proliferation, rapid invasion into the surrounding brain tissue, and the supportive role of tumor microenvironment[3] lays in a basis of glioma resistance to treatment and fast relapse after surgical resection. It was demonstrated that the tumor microenvironment plays a vital role in glioma progression and treatment resistance, with microglia representing a critical component. Under the influence of glioma, microglia are recruited to the tumor site and secrete cytokines such as IL-6 and IL-8, promoting glioma cell proliferation, invasion and migration.

Recent studies from our lab,[4] point to microglia-driven glioma cell invasiveness through the proline-rich tyrosine kinase 2 (Pyk2) mechanism. Pyk2 and its relative Focal Adhesion Kinase (FAK) function as important signaling effectors by stimulating cell proliferation, migration, and survival pathways in astrocytes, fibroblasts, epithelial cells, and including glioma cell.[5] However, specific cytokines or factors released by microglia that modulate Pyk2 and FAK to promote glioma invasiveness and proliferation are poorly understood. In this work, we identified key cytokines and chemokines, released by tumor-infiltrating microglia that induce the activation of Pyk2- and FAK-dependent glioma cell proliferation and invasiveness.

METHODS



Primary human glioblastoma cell culture: Tissue samples (1.5-2.0 cm³) from human resected glioblastoma tumor (grade IV GBM, WHO) specimens were enzymatically digested with collagenase/hyaluronidase (StemCell Technologies, Vancouver, Canada, cat. # 07912) at 37° C for 1 hour in RPMI medium (Sigma-Aldrich, St. Louis, MO,USA) with rocking. Tumor suspensions were filtered through 70 μm sterile nylon gauze and mixed with 10% FBS Dubelcco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA).

Glioblastoma and microglia purification: Glioblastoma cells were purified from the homogenized tissue using Percoll (Sigma-Aldrich, St. Louis, MO, USA) gradients of 30%, 37% and 70%. Both glioma and microglia fractions were used for protein and gene expression characterization.

Preparation of Microglia Conditioned Medium: HMC3 microglia (AATC, VA, USA) and glioma cells were seeded in co-culture in a ratio 1:1 in DMEM for 24 hours. Co-cultured cells were incubated in serum free medium for 24 hours prior to obtaining microglia conditioned medium (MCM) for the experiments.

Experimental protocol: Glioma cells (+/- siPyK2 or siFAK) were divided in the following groups: Control group, cells incubated in serum-free medium; MCM treatment; cytokines group, cells treated in independent groups with cytokines for 30 min: 5 μM PDGFα, 5μM PDGFβ, 100 μM SDF-1α, 100μM IL-6, 10 μM IL-8, and 1 μM EGF; inhibitors group, cells pre-treated in independent groups for 30 min with their respective receptor inhibitor: 50 nM PDGFR Tyrosine Kinase Inhibitor III, 200 nM DMPQ dihydrochloride, 1 μM Burixafor, 100 ng/mL Tocilizumab, 1 μM Reparixin, and 1 μM Gefinitinib, followed by corresponding treatment in combination with MCM for 120 min.

Western blot: Immunoblotting was performed as described previously.[4] A total of 10μg of proteins from glioma cells were loaded, and the antibodies used were against p-pyk2 Y579/580 (1:500, Invitrogen, Carlsbad, CA, USA), Pyk2 (1:1000), p-FAK Y925 (1:1000), FAK (1:1000) (Cell Signaling Technology, Danvers, MA, USA). Total protein staining with REVERT Total Protein Stain from LI-COR Biotechnology (cat. # 926-11016) was used as loading control. Signals were visualized by Odyssey CLx Quantitative Fluorescent Imaging System. (LI-COR Biotechnology, Lincoln, NE, USA).

Invadopodia assay: Glioma cells were plated on fluorescein-conjugated gelatin-coated glass coverslips (gelatin concentration 0.2 mg/ml, Invitrogen, Carlsbad, CA, USA, cat. #G13187) for 16-48 hours (the optimum incubation time was identified for each cell culture), fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton-X 100. Cells were stained with phalloidin-tetramethyl-rhodamine and DAPI (Sigma-Aldrich, St. Louis, MO, USA). Fluorescent images were visualized using Olympus Fluoview FV1000 confocal microscope (Olympus Corporation) with 40x oil immersion objectives. Images were analyzed using Image J software. Quantification of invadopodia formation: the number of cells forming invadopodia was counted and normalized to the number of total cells in that same image. Quantification of invadopodia activity: black and white images of gelatin degradation were analyzed using ImageJ. The percent of matrix degraded was normalized to the number of nuclei in each image as measured from the DAPI.

Cell viability: Cell viability was determined by the trypan blue exclusion assay. Glioma cells were incubated for 3 days with the treatments mentioned above in serum free medium. Total number of dead cells (trypan blue positive) and live cells (trypan blue negative) were determined by cell counting.

Statistical analysis: Data are presented as mean ± standard deviation (SD). Statistical comparisons were performed with One-way analysis of variance (ANOVA). A p value of <0.05 was considered significant. When a significant overall effect was present, intergroup comparisons were performed using a Tukey-Kramer correction for multiple comparisons, using GraphPad Prism statistical software 9.1.0 (San Diego, CA, USA).

RESULTS

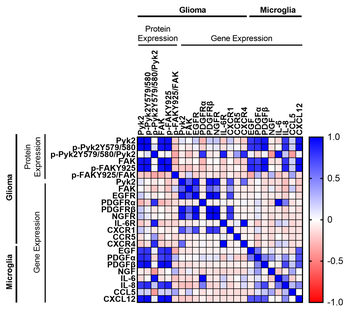


Figure 1. Heat map of Pearson correlation matrix for the gene expression of main microglia cytokines/chemokines and Pyk2/FAK gene and protein expression in human glioma cells. Cytokines with a large strength of correlation (0.6-1.0) were selected for further studies. (n=20)

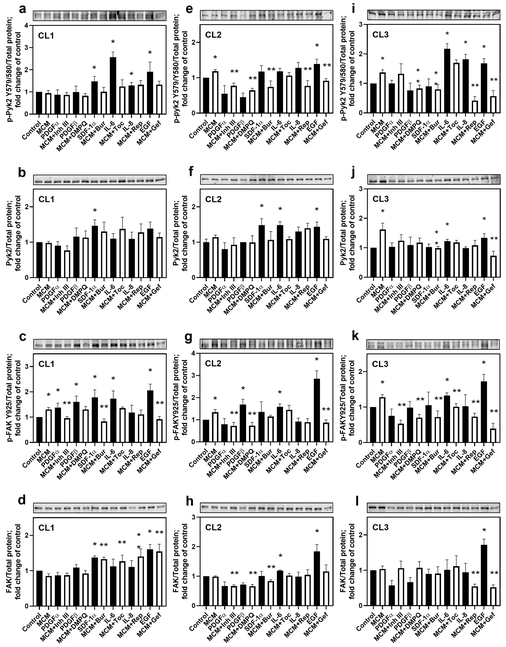


Figure 2. Cytokines/chemokines released by microglia upregulate Pyk2 and FAK protein phosphorylation in human glioma cells. Representative Western blots and quantitative data of total and phosphorylated Pyk2 (Y579/580) and FAK (Y925) are presented for (a,-d), CL1; (e-h), CL2; and (i-l) CL3. Proteins were calculated as the ratio of total and phosphorylated protein to total protein and normalized to control for each kinase. The values are shown as means ± SD of three to six experiments per group. *p ≤ 0.05 vs Control; **p ≤ 0.05 vs MCM. Inh III = Inhibitor III, Bur = Burixafor, Toc = Tocilizumab, Rep = Reparixin, and Gef = Gefitinib.

RESULTS (Cont.)

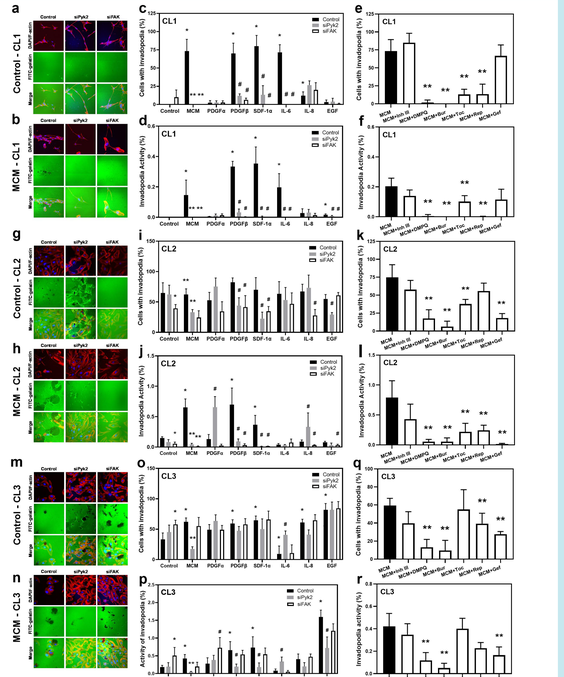


Figure 3. Functional invadopodia formation is enhanced by microglia-derived cytokines/chemokines through Pyk2 and FAK signaling. Fluorescent matrix degradation experiments were performed to observe the invasiveness of glioma cells. (a-b, g-h, m-n) Representative fluorescence images of F-actin (rhodamine-phalloidin, red), FITC-gelatin (green) and nuclei (DAPI, blue) in control (a,g,m) and MCM (b,h,n) conditions in CL1, CL2, and CL3, respectively. Degraded areas of FITC-labeled gelatin are evident as black areas devoid of FITC-labeled gelatin. (c,e,I,k,o,q) Percentage of cells with invadopodia and (d,f,j,l,p,r) invadopodia activity were calculated after treatments with cytokines in presence or absence of siPYk2 and siFAK, and MCM with or without inhibitors. The values are shown as means ± SD of three to six experiments per group. *p ≤ 0.05 vs Control, ** p ≤ 0.05 vs MCM, #p ≤ 0.05 vs corresponding cytokine. Inh III = Inhibitor III, Bur = Burixafor, Toc = Tocilizumab, Rep = Reparixin, and Gef = Gefitinib.

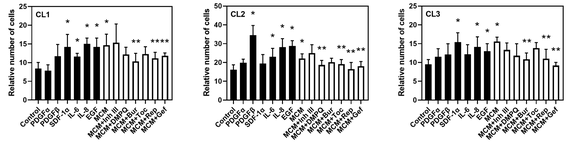
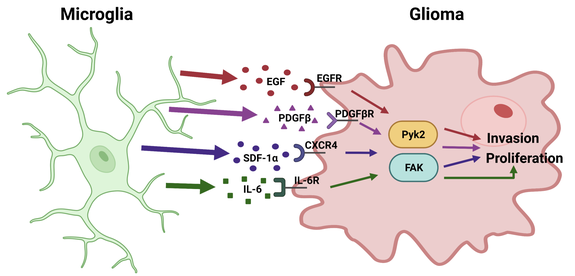


Figure 4. Cytokines/chemokines released by microglia stimulate glioma cell viability. Trypan blue exclusion test was performed to evaluate glioma cell proliferation. Number of viable cells were calculated after treatments with cytokines and MCM with or without inhibitors in (a) CL1, (b) CL2, and (c) CL3. The values are shown as means ± SD of three to six experiments per group. *p ≤ 0.05 vs Control, **p ≤ 0.05 vs MCM. Inh III = Inhibitor III, Bur = Burixafor, Toc = Tocilizumab, Rep = Reparixin, and Gef = Gefitinib.

CONCLUSIONS

- Our findings demonstrate that cytokines released by microglia activate Pyk2 and FAK kinases to promote glioma cell proliferation and dispersal.
- Microglia-derived EGF, PDGFβ, SDF-1α, and IL-6 are the primary activators of Pyk2 and FAK in glioma. Specifically, EGF, PDGFβ, and SDF-1α promote glioma cell invasion by inducing the formation of functional invadopodia.
- EGF and IL-6 play a role in enhancing glioma cell mitosis and viability in all cell lines evaluated, while the effect of PDGFβ and SDF-1α is patient-dependent.
- Glioma cell invasion and proliferation are Pyk2- and FAK-dependent events, although the relevance of each of these kinases is cytokine- and patient-dependent.
- These findings could be of clinical relevance for new therapeutic developments in GBM patients.

WORKING MODEL



FUTURE DIRECTIONS

Future studies involve determinations of the effect of Pyk2 and FAK inhibitors in combination with current GBM treatments in human primary glioma cell lines.

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