

Expression of STATs and their inhibitors SOCS and PIAS in brain tumors. *In vitro* and *in vivo* study.

J. EHRMANN*, N. STRAKOVA, K. VRZALIKOVA, R. HEZOVA, Z. KOLAR

Laboratory of Molecular Pathology & Department of Pathology, Faculty of Medicine and Dentistry, Palacky University Olomouc, Czech Republic, e-mail: jiri.ehrmann@tiscali.cz

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Proteins of STAT family belongs to the transcription factors. Through their binding to the DNA specific sites and consequent regulation of transcription of various genes, these signaling proteins play an important role in many cell functions. Recent studies demonstrated persistent activation of STATs and loss of their natural inhibitors SOCS and PIAS in various human cancers. There is also evidence that experimental pharmacologic or genetic modulation of their function might by a new approach in anticancer treatment. The aim of this study was *in vitro* assesment and analysis of expression of STATs, SOCS and PIAS in glioblastoma cell lines undergoing treatment by PPAR γ agonists/antagonists because PPAR γ and STATs are tightly regulated by an overlapping set of nuclear regulatory proteins. We further analysed immunohistochemical expression of these proteins *in vivo*, with its correlation to grading in various brain tumors. The results of *in vitro* study showed decreased expression of phosphorylated form of STAT3 and increase of its inhibitors SOCS3 and PIAS3 in glioblastoma cell lines after treatment with IC⁵⁰ of PPAR γ agonist ciglitazone. *In vivo* study failed to reveal changes in STAT3 and SOCS3 expression in either low and high grade astrocytomas, however we detect lower expression of STAT2 in low grade astrocytomas when comparing with high grade astrocytomas and lower expression of STAT3 in ependymomas when comparing with anaplastic ones. The results showed existing relationship between STAT and PPAR γ signaling in glial tumors and further support expected important role of STATs in regulation of growth and differentiation in these tumors.

Key words: STAT, SOCS, PIAS, PPARs, brain tumors

Janus kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathway represents main signaling mechanism for cytokines and growth factors influencing regulation of important cellular functions such as cell proliferation, differentiation and apoptosis. STATs are latent signaling cytoplasmic molecules activated by tyrosine-phosphorylation catalysed by JAK upon binding of cytokine/growth factors on its transmembrane cell surface domain. Phosphorylated STAT forms dimeric complex and translocate to the nucleus where it binds to DNA and modulates gene transcription (Fig.1). It has been described 6 classes of STAT (STAT1-6) and 4 classes of JAK (JAK1-4) in mammalian cells. JAK/STAT pathway is negatively regulated by three major classes of proteins: Suppressors Of Cytokine Signaling (SOCS), Proteins that Inhibit Activated STATs (PIAS) and Protein Tyrosine Phosphatases (PTP). SOCS bind phosphorylated JAK and in-

hibit its kinase activity. Moreover, SOCS also facilitate ubiquitination of JAK. PIAS prevent STAT binding to DNA and PTP reverse activity of JAK [1–12].

It has been demonstrated that impaired signaling of JAK/STAT may lead to immune surveillance failure and to development and progression of various tumors. It may be also responsible for resistance to immunomodulation therapy of some tumors and non-tumor diseases such as hepatitis C [13–23].

Loss of function of and more frequently overexpression and persistent activation of STAT family members has been found in many tumors such as leukaeimas and lymphomas [24–26], malignant melanomas [27, 28], squamous cell carcinomas of head and neck [29], lung [30] and breast carcinoma [31–33]. There is experimental evidence that inhibition of activity of STAT proteins and modulation of their regulators may represent a new target for anticancer therapy [34–36]. Interestingly, several studies have shown that some agonists of Peroxisome Proliferator Activated Receptor (PPARs): 15d-

* Corresponding author

PGJ2, clofibrate, rosiglitazone are able to suppress JAK/STAT signaling pathway in lymphocytes [37] but also in glial cells [38, 39]. In our previous study we found significant antiproliferative and proapoptotic effect of PPAR γ agonist ciglitazone on glioblastoma cell lines [40, 41]. Based on these results we hypothesize that eventual changes of JAK/STAT signaling pathway in glioma cell lines influenced by PPAR γ agonists/antagonists may reflect tight relationship between inflammatory and neoplastic processes in glial tumors. Therefore, the aim of this study was *in vitro* assesment and analysis of expression of STATs, SOCS and PIAS in glioblastoma cell lines undergoing treatment by PPAR γ agonists/antagonists with further analysis of immunohistochemical expression of these proteins *in vivo* in various brain tumors, with its correlation to grading.

Materials and methods

***In vitro* study. Cell lines.** Four human glioblastoma cell lines, T98G, U-87 MG, U-118 MG and A172 were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1.7 mM L-glutamine and 50 mg/100 ml gentamycine. U-87 MG and T98G cells were cultured in humidified atmosphere with 5% CO₂ at 37°C and U-118 MG and A172 with 10% CO₂ at 37°C.

PPAR agonists/antagonists. The cell lines were treated by the following agonists and antagonists of PPARs: Ciglitazone (agonist) was obtained from Alexis Biochemicals (Läufelfingen, Switzerland) and dissolved in DMSO (see ref. 41). Antagonists mifepristone (RU486), MK-886, BADGE, diclofenac, GW 9662, nimesulid and T0070907 were purchased from Cayman Chemical (USA) and dissolved in DMSO, too. The final concentration of DMSO was lower than 0.3%. The effect of the solvent on cell viability assay was checked in each experiment and we did not recorded any changes in cell viability.

Cell viability assay. Cell survival was determined using a colorimetric MTT assay. Cells were plated out in 96-well cell culture plates at a density of 2 800 – 5 000 cells per well. Following attachment overnight, the cells were treated from 12 to 72 hours by the ligands of PPAR. Concentrations of PPAR ligands leading to 50% inhibition of growth (IC₅₀) was determined by measuring MTT reductase activity (3,[4,4-dimethylthiazol-2-yl] 2,5 diphenyltetrazolium bromide; Serva Electrophoresis, Heidelberg, Germany).

10 ml of 0.5% MTT was added to each well, and the cells were incubated with substrate for 4 hours at 37°C. After incubation, blue formazan crystals were solubilized in 100 μ l 10% SDS. The absorbance was read at 540 nm using a microplate reader and this directly correlated with the cell viability. IC₅₀ were determined as 50% decrease of absorbance compare with the absorbance of the cells treated only by DMEM (control cells). In addition, cell viability of the treated cells were compared with both cells in DMEM and cells in DMEM with

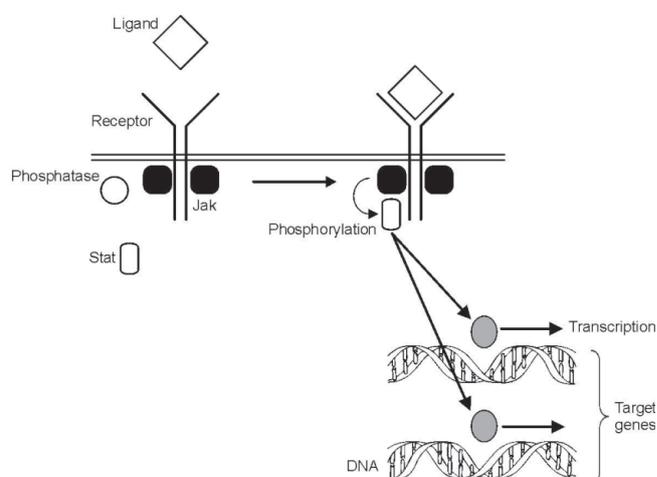


Figure 1. JAK/STAT signaling pathway

maximum used concentration of vehicle. All experiment were done in quadruple.

Western blotting Standard immunoblotting techniques were used. The proteins from whole cell lysate were run on 10 – 12% polyacrylamide gels. Electrophoresed proteins were transferred onto nitrocellulose membrane by semi-dry electrophoretic transfer. Non-specific binding sites were blocked with blocking buffer (5% fat-free skimmed milk with 0.1% Tween 20 in PBS). Subsequently, the membrane was incubated with the primary antibody (Table 1) diluted in the blocking buffer overnight at 4°C. Then the membrane was washed with the washing buffer (PBS – 0.1% Tween 20) for 1 hour at room temperature. After that the membrane was incubated with diluted goat anti-mouse IgG-horseradish peroxidase conjugated antibody (dilution 1 : 6 000, Santa Cruz, California) or goat anti-rabbit IgG-horseradish peroxidase conjugated antibody (dilution 1 : 2 000, DakoCytomation, Carpinteria, California) for 30 minutes at 4°C. The proteins were detected by chemiluminescence reagent – ECL plus (Amersham Biosciences, Vienna, Austria).

Table 1. Primary antibodies used

Antibody (anti-)	Clone	Poly/Monoclonal	Origin	Producer
PPAR α		polyclonal	rabbit	Santa Cruz
PPAR γ	E-8	monoclonal	mouse	Santa Cruz
SOCS1		polyclonal	rabbit	Santa Cruz
SOCS2		polyclonal	rabbit	Santa Cruz
SOCS3		polyclonal	rabbit	Santa Cruz
PIAS1		polyclonal	rabbit	Santa Cruz
PIAS2		polyclonal	rabbit	Santa Cruz
STAT2		polyclonal	rabbit	Santa Cruz
STAT3	124H6	monoclonal	mouse	Cell Signaling
STAT3 phospho		polyclonal	rabbit	Cell Signaling
STAT5		polyclonal	rabbit	Cell Signaling

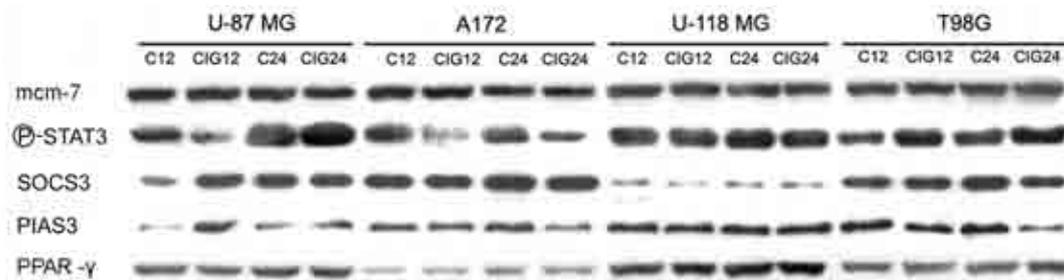


Figure 2. Decreased expression of phosphorylated form of STAT3 and increase of its inhibitors SOCS3 and PIAS3 in U-87 and A172 glioblastoma cell lines after treatment with IC^{50} of PPAR γ agonist ciglitazone.

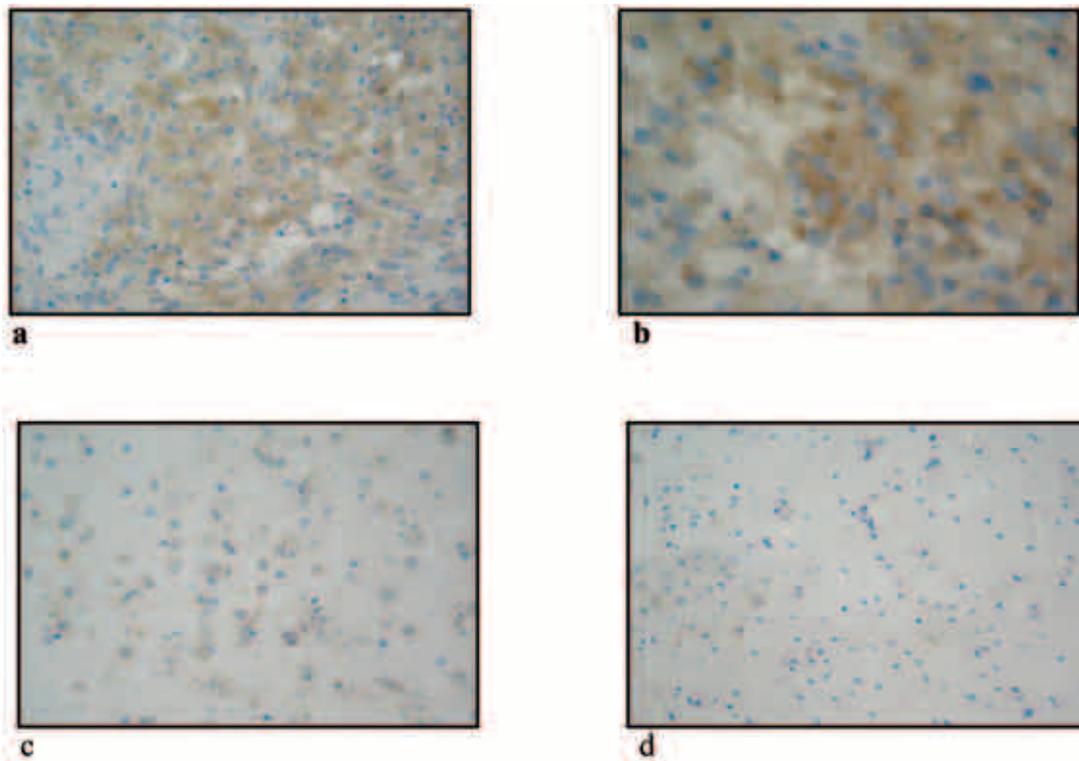


Figure 3. High positivity of expression of STAT 2 in HGA (a,b) compared with low positivity or negativity of STAT2 in LGA (c,d). Magnification 200x

The protein expressions (after 12 hours or 24 hours) of cell lysates of treated cells were compared to suitable controls (12 hours and 24 hours).

In vivo study. Material. Archived, formalin fixed, paraffin embedded samples of 14 high-grade astrocytomas (anaplastic astrocytoma, glioblastoma; average age 58 year; median survival 37 months) and 11 low-grade astrocytomas (fibrillary astrocytoma, average age 38 year; median survival 134 months) classified according to the WHO were used. Following cases of other CNS and PNS tumors were also used (up to three for each category): oligodendroglioma,

ependymoma, ganglioneuroma, pinealocytoma, pituitary adenoma, kraniopharyngeoma, neurofibroma and meningioma.

Method. Detection of proteins was done with using indirect immunohistochemistry and analysis of expression was assessed semiquantitatively by „histoscore“ as a result of multiplication of positivity (0 = up to 10% of positive cells, 1 = 11-29% of positive cells, 2 = 30-59% of positive cells, 3 = 60% and more positive cells) and intensity of staining (0 = negative, 1 = weak, 2 = moderate, 3 = strong). Maximal histoscore is 9.

Statistical analysis. The data of the MTT experiments are expressed as means \pm SE of four independent experiments ($p \leq 0.05$).

The data from in vivo study was analysed by t-test ($p \leq 0.05$).

Results

Cell viability – MTT assay – IC₅₀. In our previous studies [40,41] we found that IC₅₀ of substituted synthetic PPAR γ agonist ciglitazone after 24 hour was $1.8 \cdot 10^{-4}$ mol/l in U-118 MG; $2.3 \cdot 10^{-4}$ mol/l in T98G; $2.2 \cdot 10^{-4}$ mol/l in U-87 MG and $2.1 \cdot 10^{-4}$ mol/l in A172. Therefore, we used these concentrations in forthcoming experiments. From studied PPAR γ antagonists, only mifepristone affected A172 line with IC₅₀ after 24 hour $6 \cdot 10^{-5}$ mol/l.

Western blotting. As seen in Figure 2, after treatment with IC₅₀ of PPAR γ agonist ciglitazone we detected decreased expression of phosphorylated form of STAT3 and increased expression of its inhibitors SOCS3 and PIAS3 in U-87MG and A172 glioblastoma cell lines. Other cell lines (U-118 MG and T98G) were unaffected. Mifepristone caused some changes in protein expression after 24 hour, however, these changes were invariable and were reached by concentrations exceeding IC₅₀.

In vivo study. We failed to reveal changes in STAT3 and SOCS3 expression in either low and high grade astrocytomas, however we detect lower expression of STAT2 in low grade astrocytomas when comparing with high grade astrocytomas (Figure 3) and lower expression of STAT3 in ependymomas when comparing with anaplastic ones.

Discussion

It was found that normally latent JAK/STAT signaling proteins are abundantly present in developing human brain [42]. In our study we found higher positivity of STAT2 protein expression in high-grade astroglomas compared to low-grade tumors and all studied high-grade glioma cell lines expressed significant amount of STAT3. Because most tumor cell in high-grade gliomas exists in undifferentiated or less differentiated forms, increased levels of STATs may represent particular form of cell signaling, similar to developing brain. Another possible explanation of increased STATs expression in high grade gliomas may be suggested key role of STAT3 in autocrine VEGF activation [43] and therefore in increased angiogenesis which is characteristic for these high-grade tumors. Moreover, as suggested in study of Yao et al. [44] STAT signaling in high-grade gliomas seems to be a key mediator of interferon γ induced upregulation of thymidine phosphorylase which enhances cytotoxicity of 5-fluorouracil. Interestingly, several studies revealed that JAK/STAT signaling is also inhibited by some PPARs ligands such as 15-deoxy-delta12, 14 prostaglandin J2 (15d-PGJ2) [45] or GW501516 [46] and that this inhibition may have therapeutic

benefit in some malignancies [47]. Study of Park et al. [48] demonstrated that STAT signaling was inhibited by both 15d-PGJ2 and another PPAR γ agonist rosiglitazone by induction of SOCS1 and SOCS3 in glia. This is in accordance with our finding of decreased expression of STAT3 and increased expression of SOCS3 and PIAS3 together with induction of CDK inhibitors p21 and p27 and inhibition of cell cyclin D1 and pRB-P in glioma cell lines after ciglitazone treatment [41]. The fact that not all cell lines are affected by ciglitazone and almost all PPAR γ antagonists can be explained by demonstration of widespread chromosomal instability of glioma cell lines [49] leading to necessity of individual phenotyping of particular glial tumor. Moreover, it should be taken into consideration that very recent study of Coras [50] showed PPAR independent effect of PPAR γ agonist troglitazone, a potent inhibitor of glioma cell migration and brain invasion.

Nevertheless, the results of our study support general concept of existing cross-talk of JAK/STAT signaling pathway and PPAR γ in gliomas which may reflect tight relationship between inflammatory and neoplastic processes in glial tumors and which further support expected important role of STATs and PPARs in regulation of growth and differentiation in these tumors.

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