9. Appendix

Part of the data of this work was presented as a poster on the congress Proteolytic Enzymes & Their Inhibitors, June 2012, Barga, Italy.

Expression and enzymatic activity of fibroblast activation protein in human glioma cells

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Introduction

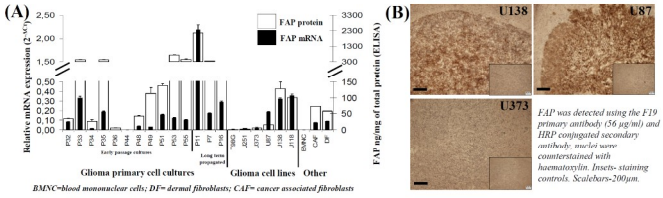
Human gliomas are highly invasive tumors and the mechanisms that influence their interaction with the surrounding tissue are still incompletely understood. Fibroblast activation protein (EC 3.4.21.B28; FAP) is a dual specificity serine dipeptidyl peptidase and gelatinase that is expressed in stromal as well as transformed cells in several tumors. FAP is strongly associated with the development, progression and outcome of human carcinomas, and also represents a potential therapeutic target.

The pathophysiological role of FAP in tumor microenvironment is nevertheless poorly understood. Its gelatinolytic activity may participate in the modification and degradation of extracellular matrix and thus facilitate tumor invasion and metastasis. However, several reports demonstrate that some functions of FAP are independent of its enzymatic activity and that in certain tumor cells FAP may act as a tumor suppressor.

Previous data suggested that FAP is expressed in human gliomas (1, 2). In this report we analyze the expression and enzymatic activity of FAP in glioma cells and the role of FAP in the interaction of glioma cells with the surrounding extracellular matrix.

Results

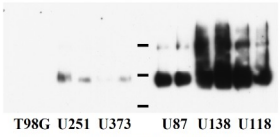
I. FAP is expressed to a variable extent in glioma cell lines, primary cell cultures derived from high grade gliomas (A) and orthotopic xenotransplants (B).



BMNC=blood mononuclear cells; DF= dermal fibroblasts; CAF= cancer associated fibroblasts

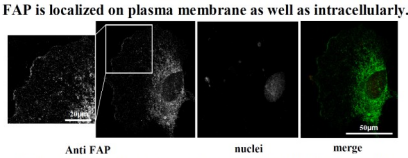
FAP was detected using the F19 primary antibody (56 μg/ml) and HRP conjugated secondary antibody; nuclei were counterstained with haematoxylin. Insets- staining controls. Scalebars-200μm.

WB detection of FAP



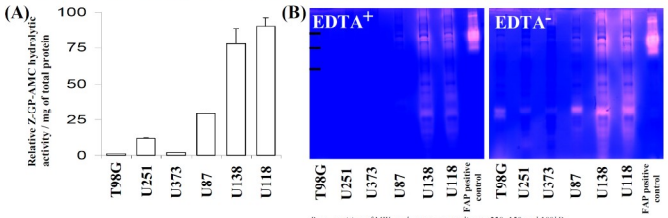
75μg of total protein per well, non denaturing SDS PAGE. FAP was detected with the F19 monoclonal antibody (1,4 μg/ml). Bars- position of MW markers corresponding to 250, 150 and 100kDa.

FAP is localized on plasma membrane as well as intracellularly.



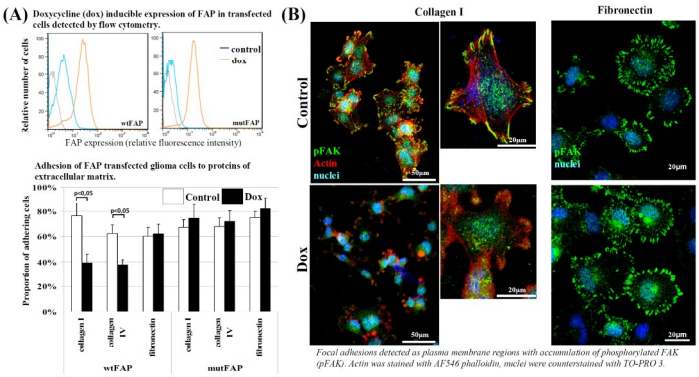
Confocal microscopy images of U118 cells fixed with 4% paraformaldehyde and stained using the F19 monoclonal antibody (56μg/ml). Nuclei were counterstained with TO-PRO 3.

II. Endopeptidase enzymatic activity of FAP in glioma cell lines. (A) Z-Gly-Pro-AMC cleavage by immunocaptured FAP; (B) Gelatin zymography in the presence, or absence of the metalloproteinase inhibitor (EDTA+ / EDTA-).



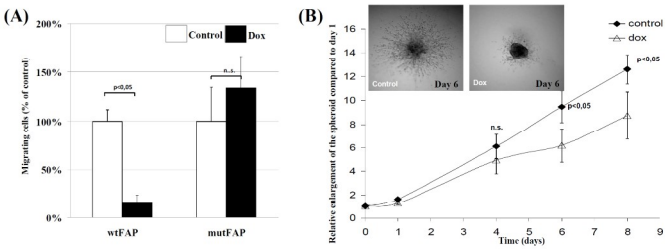
Bars- position of MW markers corresponding to 250, 150 and 100kDa.

III. Expression of transgenic, wild type (wtFAP), but not enzymatically inactive mutant FAP (mutFAP) in U373 glioma cells negatively influences cell adhesion (A). wtFAP impaires spreading and focal adhesion formation on collagen I, but not fibronectin (B).



Focal adhesions detected as plasma membrane regions with accumulation of phosphorylated FAK (pFAK). Actin was stained with AF546 phalloidin, nuclei were counterstained with TO-PRO 3.

IV. Transgenic wild type FAP in U373 cells reduces cell migration (A) and invasion in 3D-collagen I gels in a spheroid invasion model (B).



Conclusions:

- **FAP is expressed by glioma cells *in vitro* and in orthotopic xenotransplants.**
- **In glioma cells, FAP is localized on plasma membrane as well as intracellularly.**
- **U373 cells expressing enzymatically active FAP exhibit decreased adhesion, migration and focal adhesion formation *in vitro*.**
- **FAP may influence the interaction of glioma cells with specific components of the extracellular matrix via its enzymatic activity.**

Matherial and Methods:

Glioma cell lines were from ATCC (U373, T98) and CLS (U251, U87, U118, U138), cells were cultured under standard conditions. U373 cells were transfected with the wild type and mutant (active site Ser^{624}→Ala) FAP using the doxycycline inducible TetOn system (Clontech). Xenotransplants were generated by orthotopic implantation of glioma cells into immunodeficient mice. DuoSet ELISA (RnD Systems) was used to quantify FAP in cell lysates, real-time RT-PCR data were normalized to beta actin. Immunodetection of FAP was performed using the mouse monoclonal anti FAP antibody produced by the F19 hybridoma cell line (ATCC) or by rat monoclonal anti FAP antibodies (clones D8 and D28, Vitatex). An immunocapture assay with the F19 antibody was used to detect the Z-GP-AMC cleaving activity of FAP.

Zymography assays were performed using 0.1% gelatin in 7.5% polyacrylamide gels, gels were incubated in the presence or absence of EDTA at 37°C and stained with Coomasie blue. Adhesion assays were performed in 96WP coated with collagen I, IV or fibronectin (Sigma). Cell migration was evaluated using the modified Boyden chamber assay using cell culture inserts with 8 μm pores. Spheroids were formed by the „hanging drop" method and embeded in collagen I. Cell invasion was assessed by measuring the area containing the invading glioma cells. An antibody against the Tyr397 phosphorylated FAK (Invitrogen) was used to detect focal adhesions.

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References:

1) Stremenova et al. (2007) Int. J. Oncol. 31, 785-792.
2) Mentlein et al. (2011) Biol Chem. 392, 199-207.

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