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Formal abstract by the author

Definice expresního vzorce „DASH systému“ v transformovaných gliálních buňkách, koexprese proteinu aktivovaných fibroblastů a dipeptidylpeptidázy-IV.

Definition of the expression pattern of DASH system in transformed glial cells, the coupled expression of fibroblast activation protein and dipeptidyl peptidase-IV.

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Table of contents

Abstrakt

Dipeptidylpeptidáza-IV (DPP-IV) je multifunkční transmembránový glykoprotein odštěpující X-Pro dipeptid z N-konce peptidového řetězce. Tato evolučně konzervovaná sekvence chrání řadu biologicky aktivních peptidů před nespecifickým štěpením. DPP-IV patří do skupiny "Dipeptidylpeptidáze-IV Aktivitou a/nebo Strukturou Homologních" molekul (DASH), do které mimo ní patří například fibroblastový aktivační protein-α/sepráza (FAP) a několik dalších molekul. I když většina z těchto molekul jsou primárně enzymy, je známo, že alespoň některé své biologické funkce mohou vykonávat nezávisle na své vnitřní enzymové aktivitě. DASH molekuly, spolu s molekulami, s nimiž funkčně interagují, představují „DASH systém", který se patrně významně uplatňuje v řadě patologických procesů, například tumorogeneze. O DPP-IV a jejím strukturálně nejbližším protějšku FAP se předpokládá, že jsou zapojeny do regulace mnoha biologických procesů, významných mimo jiné pro vznik a progresi maligních gliálních nádorů.

V této práci popisujeme expresi a kolokalizaci DPP-IV a FAP v nádorově transformovaných gliálních buňkách *in vitro* a v bioptickém materiálu astrocytárních tumorů. Kromě DPP-IV/FAP dvojitě pozitivních buněk jsme v glioblastomu též nalezli kvantitativně významnou populaci FAP pozitivních mesenchymálních buněk přítomných ve vaskulárním kompartmentu. Dále popisujeme korelativní expresi DPP-IV a FAP v primárních buněčných kulturách odvozených z glioblastomu a asociaci dynamiky změn exprese obou molekul v permanentních astrocytárních buněčných liniích. Absence vztahu exprese endogenního FAP v buňkách exprimujících transgenní, a tudíž v nefyziologickém genomickém kontextu kódovanou DPP-IV, svědčí spíše pro koregulaci exprese obou molekul na transkripční než posttranskripční úrovni. Naše experimenty věnované studiu funkčního významu DPP-IV a FAP v nádorové progresi prokázaly, že exprese obou molekul negativně ovlivňuje adhezi transformovaných gliálních buněk ke komponentám extracelulární matrix, přičemž pro dosažení tohoto efektu je třeba jejich hydrolytická aktivita.

Poznání expresního vzorce DPP-IV a FAP a jejich funkční koordinace v nádorovém mikroprostředí může pomoci pochopit jejich biologickou roli v maligních gliomech.

Abstract

Dipeptidyl peptidase-IV (DPP-IV) is a multifunctional transmembrane glycoprotein removing X-Pro dipeptide from the amino-terminus of the peptide chain. This evolutionary conserved sequence protects a number of biologically active peptides against the unspecific proteolytic cleavage. DPP-IV belongs into the group of "Dipeptidyl peptidase-IV Activity and/or Structure Homologues" (DASH), which, except the canonical DPP-IV, comprises fibroblast activation protein-α/seprase (FAP), and several other molecules. However several of DASH molecules are the enzymes, they execute at least some of their biological functions by non-proteolytic protein-protein interactions. DASH molecules, their substrates and binding partners are parts of "DASH system" which is affected in several pathological process including a cancer. Specifically DPP-IV and its closest structural relative FAP are among others expected to be involved in the development and progression of malignant glioma.

In this study we showed the expression and colocalization of DPP-IV and FAP in glioma cells *in vitro* and in human high grade gliomas. In addition to the DPP-IV/FAP double positive transformed glial cells, we also identified a subpopulation of FAP positive mesenchymal cells located in the perivascular compartment. Moreover we described the correlative expression of DPP-IV and FAP in the glioblastoma-derived primary cell cultures and the associated expression dynamics of both molecules in astrocytoma cell lines. Uncoupled expression of the endogenous FAP and DPP-IV transgene, placed into the non-physiological genomic context argues for the joint control of DPP-IV and FAP genes expression rather than the indirect reciprocal regulation, involving the changes of their mRNA and/or protein. Our experiments focused on the functional relevance of DPP-IV and FAP to cancer progression demonstrate that the overexpression of both molecules impaired the cell adhesion to proteins of extracellular matrix.

Understanding of the DPP-IV and FAP expression pattern and their functional coordination in the tumour microenvironment may help to clarify their biological role and molecular mechanisms in the malignant gliomas.

1. Introduction

Cancer is one of the leading causes of death in the world. Brain tumours belong to devastating neoplasm, causing a significant disability, decreasing quality of life and ultimately leading to death. Gliomas are the most frequent primary brain tumours, with an annual incidence of 5-6 cases per 100,000 populations. Within them, glioblastoma multiforme is the most malignant and the most common tumour. Due to its aggressive growth, less than half of the patients survive more than one year (Louis 2007).

Aberrant response of the malignant cells to humoral growth regulators leads to the tumour progression. Most of the growth regulators are peptides and their turnover is a result of their balanced production and degradation. The amino acid, proline in the penultimate position of the amino-terminal of the peptide chain is evolutionary conserved and protects peptides against unspecific proteolytic cleavage (Vanhoof et al. 1995).

The plasma membrane-bound serine protease dipeptidyl peptidase-IV (DPP-IV; EC 3.4.14.5) was originally described on the basis of its unique substrate specificity processing a chromogenic substrate glycyl-prolyl-beta-naphthylamide (Hopsu-Havu and Glenner 1966). DPP-IV was believed to be the only enzyme cleaving X-Pro dipeptides from the amino-terminus of peptides. Subsequently, several other molecules possessing the DPP-IV-like enzymatic activity and bearing a variable degree of structural similarity to DPP-IV were described. This led to a definition of a group of "Dipeptidyl peptidase-IV Activity and/or Structure Homologues" (DASH). In addition to the canonical DPP-IV, fibroblast activation protein-α/seprase (FAP; EC 3.4.21.B28), dipeptidyl peptidase 8 and 9, dipeptidyl peptidase II, dipeptidyl peptidase-like protein, thymus-specific serine protease, dipeptidyl peptidase IV-β and N-acetylated-α-linked acidic dipeptidase have been included (Sedo and Malik 2001).

Members of the DASH group, their substrates and non-hydrolytic partners were included into functionally defined "DASH system" known to be involved in numerous biological processes, involved also in cancer development and progression. Understanding of DASH expression pattern and its functional coordination with their interacting molecules may help to identify the role of "DASH molecules" in gliomagenesis.

1.1. Dipeptidyl peptidase-IV (DPP-IV)

Human DPP-IV is a 220-240 kDa membrane-associated homodimeric serine protease. In addition to this transmembrane form, a soluble isoform of DPP-IV was detected in human serum and body fluids (Hino et al. 1976; Durinx et al. 2000). DPP-IV preferentially releases X-Pro or X-Ala dipeptides from the amino-terminus of the peptide chain. However, it also acts as a binding molecule for adenosine deaminase, plasminogen 2 and several structural proteins. The DPP-IV gene is localized on the chromosome 2, locus 2q24.3. Several splicing forms were described or hypothesized, however, only some of these proteins seemed to possess hydrolytic activity (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi?db=human&c=Gene&l=DPP4).

In humans, DPP-IV is almost ubiquitously expressed in the epithelial cells of the gastrointestinal and biliary tract, in the exocrine pancreas, kidney, uterus, placenta, prostate, endothelial cells of various organs, thymus, lymphatic nodes and activated lymphocyte subpopulation (reviewed in (Gorrell et al. 2001). Altered DPP-IV expression and/or the change of DPP-IV blood plasma concentration are hallmarks of several pathologies including the rheumatoid arthritis, lupus erythematodes, multiple sclerosis, diabetes mellitus as well as cancer. DPP-IV is supposed to orchestrate the processes of cell growth, migration and invasion in a both enzymatic as well as non-enzymatic manner.

1.2. Fibroblast activation protein-α (FAP)

FAP is a 150-170 kDa integral transmembrane serine protease (Rettig et al. 1986). DPP-IV and FAP proteins share 52% amino acid sequence identity (Abbott et al. 1999). Moreover, both genes are localized close to each other on the chromosome 2 (Mathew et al. 1995), thus some authors suggest them to be a product of gene duplication (Havre et al. 2008). Similarly to DPP-IV, not all the proteins encoded by the alternatively spliced forms are enzymatically active (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi?db=human&c=Gene&l=FAP). Homodimerization of FAP is a condition of its proteolytic activity. Except to its DPP-IV-like exopeptidase activity, FAP is also endopeptidase with a gelatinase/collagenase activity (Rettig et al. 1994). FAP was detected in human blood plasma where it was previously known as an antiplasmin cleaving enzyme (Collins et al. 2004; Lee et al. 2006).

FAP is typically expressed in the foetal mesenchymal tissue. In adults, it is transiently expressed on the activated fibroblast during wound healing, and FAP positive cells can be also found in the pancreas and the endometrium (Rettig et al. 1988). Contrary to the restricted physiological distribution, FAP is abundantly expressed in the stromal cells in most of epithelial and mesenchymal tumours as well as in malignant elements of melanoma, bone and soft tissue tumours, breast, lung and ovarian carcinoma (reviewed in (O'Brien and O'Connor 2008). Previously, we described FAP transcripts in glioblastoma (Stremenova et al. 2007); however the particular cell type expressing FAP remains still unknown.

Although FAP is known to cleave collagen type III and the denatured form of the collagen type I, its function in the degradation of the extracellular matrix is not completely understood yet (Christiansen et al. 2007). On the other hand, soluble form of FAP is known to process α2-antiplasmin, a potent inhibitor of plasmin. Thus, via inhibition of plasmin, FAP/antiplasmin cleaving enzyme may modify the plasmatic fibrinolytic system, but also may alter degradation of the extracellular matrix (Lee et al. 2006).

Upregulation of FAP is demonstrated to decrease the adhesion and migration of epithelial cells. Interestingly, enzymatically inactive FAP mutant has not such functional potential and thus it seems that the non-hydrolytic mechanism is involved (Wang et al. 2006).

The results of Ghersi and coworkes suggest the presence of oligomeric complexes formed from both DPP-IV and FAP molecules at the invasive edge of fibroblasts and on the surface of endothelial cells (Ghersi et al. 2006). Such molecular complexes exhibited exopeptidase as well as endopeptidase proline-specific enzymatic activity, the latter was even more potent than that of the homodimeric FAP/seprase. Thus, the authors concluded, that there is a functional cooperation of DPP-IV and FAP, acting as a collagen binding protein and a gelatin cleaving protease respectively, on the matrix degradation. Moreover, the combination of antibodies targeting the DPP-IV collagen binding domain and inhibitors of matrix metalloproteinases (MMP) exhibited stronger inhibition of cell migration than each of the compounds alone. These results demonstrate that both, DPP-IV as well as FAP cooperate with MMPs on the extracellular matrix (ECM) degradation.

1.3. Other DASH molecules

DPP-IV and FAP are the most intensively studied members of the functional group of "Dipeptidyl-peptidase-IV Activity and/or Structure Homologues" so far (Sedo and Malik 2001). Although the involvement of other members of the DASH group in cancer is possible, this thesis focuses on the study of DPP-IV and FAP in pathogenesis of malignant glioma from the following reasons. The plasma membrane localization and extracellular orientation of DPP-IV and FAP is an important prerequisite to their interaction with the components of the tumour microenvironment. Both DPP-IV and FAP have been reported to cleave the

bioactive peptides relevant to the glioma progression such as substance P, Stromal cell-derived factor and neuropeptide Y etc. (Mentlein 1999; Bajetto et al. 2006; Keane et al. 2011). Finally, DPP-IV and FAP directly interact with an extracellular matrix (ECM); DPP-IV as a fibronectin and collagen binding molecule (Hanski et al. 1988), while FAP as an enzyme possessing the gelatinolytic activity.

1.4. DPP-IV and FAP in malignant tissue, "a tale of contradictions"

Expression of DPP-IV and FAP was described in numerous types of cancers so far (as e.g. melanoma, ovarial carcinoma, colorectal and breast cancer) (reviewed in (Kotackova et al. 2009). Recent knowledge argues for possible pro- as well as anti-oncogenic role of DPP-IV and FAP, which may depend on the particular cancer type (Sulda et al. 2006; Kotackova et al. 2009). Such seeming contradiction may be explained by tumour type-specific local microenvironment and by the cell-specific function(s) of DPP-IV and FAP in the given cell population within either the tumour stroma or parenchyma (Cheng et al. 1998; Iwata and Morimoto 1999; Chen and Kelly 2003; Wesley et al. 2005; Sulda et al. 2006).

1.5. DPP-IV, FAP and microenvironment of high grade gliomas

The glioma cells typically possess high invasive potential; they preferentially infiltrate along the blood vessels, the subpial glial space and along the white matter tracts. The brain parenchyma has a unique composition of extracellular matrix; the major ECM component is hyaluronan while the common fibrillar proteins such as collagen, fibronectin or laminin are restricted mainly into the perivascular compartment. The glioma progression is associated with the alteration of ECM composition in both parenchymal as well as perivascular compartment (Delpech et al. 1993; Bellail et al. 2004).

The important prerequisite of high potential of glioma cells to invade is the complex system of ECM processing enzymes. Among them, the MMPs, especially MMP-2 and MMP-9, cathepsin B and uPA/uPAR/plasmin are the most important. These proteases are localized on the invading edge of tumour and in perivascular compartment (Bellail et al. 2004).

In our previous work we demonstrated the expression of DPP-IV and FAP transcripts in tissues of the high grade gliomas (Stremenova et al. 2007). These observations well correspond to the later results of Mentlein et al., describing the major expression of DPP-IV on the endothelial cells, and just the minor one on the glioma cells (Mentlein et al. 2011). Moreover, Mentlein's observations suggest the dominant presence of FAP in GFAP positive glioma cells within the glioblastoma tissue. Our further research demonstrated, that the transgenic overexpression of wild type as well as mutated, enzymatically inactive DPP-IV impairs the glioma cell migration and adhesion to fibronectin (Busek et al. 2012).

1.6. DPP-IV and FAP, "a tale of hidden cooperation"

In order to interpret properly the role of DPP-IV and FAP in processes of tumour progression, several facts have to be considered : (1) DPP-IV and FAP are structurally similar enzymes, thus their identification by using antibodies or specific substrates/inhibitors is difficult; (2) DPP-IV and FAP, due to their similar exopeptidase enzymatic activity and thus sharing similar set of biologically active substrates, can functionally overlap, at least in some of their functions; (3) despite both being enzymes, some of their biological effects are independent on their proteolytic activity; and finally (4) DPP-IV and FAP have been shown to be co-expressed and speculated to functionally cooperate with two major matrix degrading proteolytic systems, MMP (Salamone et al. 2006) and plasminogen/uPAR (Artym et al. 2002).

2. Hypothesis and aims

Our previous data confirmed significant correlation of DPP-IV and FAP transcripts and protein expression in astrocytic tumours (Stremenova et al. 2007). However the particular cell type or cell population expressing DPP-IV and/or FAP remains unknown. Moreover, although the co-expression of DPP-IV and FAP was observed in several biological systems and experimental models (Monsky et al. 1994; Wesley et al. 2004; Stremenova et al. 2007; Goscinski et al. 2008), their putative co-regulation was not noted so far. Thus, on the basis of ours and others results, suggesting functional association of both molecules in the biological processes, relevant to the glioma progression, we aimed to test the hypothesis of co-regulation of DPP-IV and FAP in transformed glial cells and to test their effect on the cell adhesion.

Aim 1: To characterise expression and staining pattern of DPP-IV and FAP in human glioblastoma multiforme.
Aim 2: To verify co-expression of DPP-IV and FAP in permanent glioma cell lines and in the primary cell cultures derived from human glioblastoma multiforme.
Aim 3: To approach mechanism of putative coupling of DPP-IV and FAP expression in glioma cell lines.
Aim 4: To determine the relevance of DPP-IV, FAP and their enzymatic activity for adhesion of glioma cells to the components of extracellular matrix *in vitro*.

3. Material and methods

3.1. Cell lines, primary cell cultures and sample preparation

Cell lines U138MG, U87MG (both derived from human glioma WHO grade IV; ATCC, Teddington, UK), U373 (derived from glioma WHO grade III; ATCC), stable DPP-IV-transfected U87MG and stable DPP-IV or FAP -transfected U373 clones (see below) were cultured in the Dulbecco's modified Eagle's medium (DMEM; Sigma, Prague, Czech Republic) supplemented with 10% foetal calf serum (FCS; Sigma). In some experiments, a serum-free DMEM was used to model growth factors deficiency conditions, inducing adaptive cell differentiation (Sedo et al. 1998). Primary cell cultures were derived from biopsies of human high grade astrocytic tumours. A fresh tissue sample was cut into small pieces and cultured in DMEM, supplemented with 20% FCS (Sigma), and streptomycin in f.c. 100 μg/ml and penicillin G in f.c. 100 U/ml (Sigma). Between the 5^{th} - 7^{th} day of explantation, the medium was replaced with fresh DMEM supplemented with 10% FCS and the antibiotics. Total cell lysates were prepared on ice in a lysis buffer supplemented with a mixture of protease inhibitors (pepstatin A 25 μM, AEBSF 200 μM, E-64 50 μM) and cleared by centrifugation. For separation of the soluble and membrane fraction, the differential centrifugation was used.

3.2. Fluorescent in situ hybridisation (FISH)

FISH analyses were carried out on the suspensions of fixed cells. To identify chromosome 2, the commercially available centromeric DNA chromosome enumeration probe 2 (CEP 2; D2Z1) Spectrum Orange and a differently labelled DNA probe CEP 18 (D18Z1) Spectrum Green, serving as a control, were used according to the manufacturer's recommendations (Abbott Molecular, Des Plaines, USA). To localize the DPP-IV and FAP DNA sequences, respectively, the commercially available bacterial artificial chromosome probes were used (FAP - RP11-576I16 and DPP-IV - RP11-178A14, Pentagen, Kolin, Czech Republic).

3.3. Construction of DPP-IV and FAP cDNA vector and cell transfection

U87MG cells were transfected with human DPP-IV using the Mifepristone inducible Gene Switch System (Invitrogen, Life Technologies, Prague, Czech Republic) as described previously (Busek et al. 2008). U373 cell line was transfected with human DPP-IV or FAP using the Tetracycline inducible pTet-On-Advanced system (Clontech, Saint-Germain-en-Laye, France) as described (Busek et al. 2012).

3.4. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

To determine the expression levels of DPP-IV and FAP-α mRNAs, a coupled RT-PCR was used as described previously (Busek et al. 2008). Total RNA was isolated by TriZol Reagent (Invitrogen) according to the manufacturer's instructions. The expression of DPP-IV, FAP and β-actin trasncripts was quantified by using gene coding region-specific oligonucleotide primers carrying flourogenic TaqMan probes by real time RT-PCR assay; ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Carlsbad, USA). The transcript expression was normalized to β-actin mRNA using ΔCt method (Livak and Schmittgen 2001).

3.5. Enzymatic activity assays and gel filtration chromatography

The cell surface and the total cellular enzymatic activities were determined using the continuous rate fluorimetric assay as described in detail previously (Sedo et al. 1998; Sedo et al. 2003; Lee et al. 2006). To describe the elution profile of DPP-IV-like exopeptidase and endopeptidase enzymatic activities of soluble and solubilized membrane cell fraction, gel filtration chromatography on a column of Sephacryl S-300 (Pharmacia, Uppsala, Sweden) was used (Sedo et al. 2003).

3.6. Western blot and zymography assay

The cell lysates were diluted in the Tris-HCl sample buffer, pH 6.8, containing 1% sodium dodecyl sulphate (SDS), 10% glycerol and 0,005% bromphenol blue and 25 μg of total protein without boiling were loaded per well onto SDS 7,5% polyacrylamid gel. The electrophoresis run in the conditions of 25 mM Tris buffer containing 1.92 M glycine and 0.1% SDS. Proteins were then transferred onto the PVDF membrane (Biorad, Prague, Czech republic) by semidry blotting, the membrane was blocked for 3 hours with 5% non-fat milk in 0,05% Tween 20 Tris-buffered saline and then incubated overnight at 4°C with primary monoclonal antibodies targeting DPP-IV (clone E19, dilution 1:1000) and FAP (clone D28, dilution 1:3000) diluted in 5% non-fat milk. The blots were subsequently developed with horseradish peroxidase conjugated secondary antibody (R1371HRP, Acris, Herford, Germany, 1:10 000 in 5% non-fat milk) and enhanced chemiluminiscence. The blots were exposed to the photographic film (Hyperfilm^{TM} ECL, Amersham).

The zymography was run on SDS 7.5% polyacrylamid gel containing 0.1% of gelatine. The gels were incubated under the condition of 2 mM EDTA or 2 mM $CaCl_2$ Tris-buffered saline. The gels were stained with Coomassie Blue.

3.7. Enzyme-Linked ImmunoSorbent Assay (ELISA)

The DPP-IV and FAP proteins were assayed by DuoSet DPP-IV and DuoSet FAP ELISA kits (DY1180 and DY3715, R&D Systems, Abingdon, UK) according to the manufacturer's recommendations.

3.8. Immunocytochemistry and immunohistochemistry

All procedures of the immunocytochemistry and immunohistochemistry were held in a humidified chamber. In the staining controls, the primary antibodies were omitted. The cells, cultured on the glass coverslips, were fixed by 4% paraformaldehyde and permeabilised by 0.1% Triton X-100. The coverslips were pretreated in 3% heat-inactivated FCS. The samples were incubated with the monoclonal primary antibodies, anti DPP-IV (clone MA 261) and anti FAP (clone D8). This step was followed by the incubation with the Alexa Fluor 488-labelled goat anti-mouse IgG and the Alexa Fluor 546-labelled goat anti-rat IgG, respectively. The coverslips were finally mounted. Frozen specimens of tumour and non-tumorous brain were cut into 10 μm-thick sections. The slides were fixed with 4% paraformaldehyde and permeabilised by 0,1% Triton X-100. After the preincubation in 10% FCS 0.1% BSA TBS, the immunodetection of antigens was performed. Double immunostainings were performed to identify the cell type expressing DPP-IV and FAP. The sequential protocol was introduced; the slides were incubated with first primary antibody (anti DPP-IV or anti FAP) and followed by the incubation with the appropriate secondary antibodies. Subsequently, the second staining for the markers of differentiation was done (vW, GFAP, CD105, TEM-1, SOX-2, TE-7). The nuclei were stained by Hoescht (bisbenzimide, Sigma).

3.9. Adhesion and spreading assays

Cell adhesion was assessed in collagen type I or type IV coated 96-well plates. Cells were grown in 10% FCS supplemented DMEM with or without Doxycycline in final concentration 10^{-6} mg/ml. After 72 hours of DPP-IV or FAP induction, cells were harvested and resuspended in 0.1% BSA (Sigma) in DMEM. 50 000 cells were added to the wells and attached. Non-adherent cells were gently removed. The adherent cells were fixed and stained with methylene blue. Cells were lysed with 1% SDS and the absorbance was read at 630 nm.

Cells were prepared by the same way as for adhesion assay. Cell spreading was assessed in the collagen type I or type IV coated 12-well plates. 50 000 cells were added to the wells that have been blocked with 0.1% BSA, and allowed to attach for indicated time. The adherent cells were fixed by 5% glutaraldehyde and stained with methylene blue. The cells were photographed using inverse fluorescent microscope Olympus IX70.

3.10. Statistical analysis

The Mann-Whitney test was used to evaluate the differences between the investigated groups. Correlation of quantitative variables was assessed by computing the Spearman's correlation coefficient.

4. Results

4.1. In situ DPP-IV and FAP expression in human glioblastoma multiforme

To characterize the distribution of DPP-IV and FAP in the brain tumour microenvironment, immunohistochemistry analyses were performed by using mAb clone E19 or MA 261 (anti DPP-IV) and mAb clone D8 and D28 (anti FAP). DPP-IV and FAP were detected mainly in tumorous tissue and were either absent or present only in traces in non-tumorous brain. In tumours, DPP-IV was detected mainly in the parenchyma, it was also sporadically observed in the perivascular compartment. FAP expression was evident in both parenchymal as well as in the perivascular compartments.

Double staining analysis revealed that the intraparenchymal FAP positive cell population co-expressed also DPP-IV, glial fibrillary acidic protein (GFAP) and the

transcription factor SOX-2, a marker of the multipotent neural and glioma stem cell. These cells were negative for the markers of the endothelial and mesenchymal elements. Thus, these cells apparently represent the transformed elements of glial origin. On the other hand, perivascular FAP positive subpopulation was vW, GFAP, CD105 and SOX-2 negative, it sporadically expressed SMA, TEM-1 and the fibroblast antigen detected by TE-7 antibody. Interestingly, they typically surrounded dysplastic and hypertrophic vessels. Since the FAP positive perivascular cells co-expressed several mesenchymal but lacked the glial markers, they highly probably belong among to the mesenchymal elements.

4.2. DPP-IV and FAP co-expression in primary astrocytic cell cultures

We observed a positive correlation between the expression of DPP-IV and FAP on the mRNA and protein level in the primary cell cultures (Figure 1A). Immunoblotting using anti FAP and anti DPP-IV identified single band at about 150 kDa in non-denaturating conditions (Figure 1B). Membrane overlay assay using H-Gly-Pro-AMC as a substrate targeting post-proline-specific exopeptidase activity confirmed that the band contains the active enzyme possessing DPP-IV-like enzymatic activity (Figure 1C). Using the zymography we demonstrated the band of MW 150 kDa contained the gelatinolytic activity (Figure 1D).

A

		Transcript		Protein	
		DPP-IV	FAP	DPP-IV	FAP
Transcript	DPP-IV	x	R=0,58; p<0.0001 N=47	R=0,67; p<0.004 N=16	n.s.
	FAP		x	n.s.	n.s.
Protein	DPP-IV			x	R=0,497; p<0.016 N=23
	FAP				x

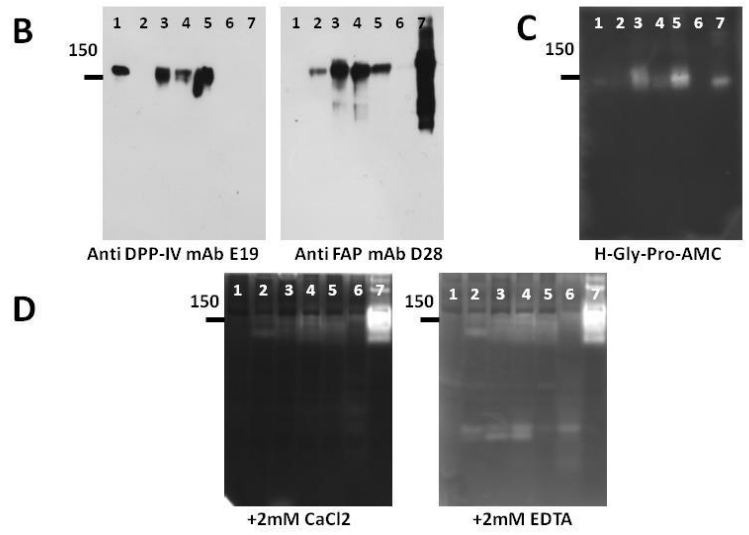

Figure 1. **DPP-IV and FAP in primary cell cultures derived from human glioblastoma multiforme.**

(**A**) correlation of DPP-IV and FAP transcripts and proteins; (**B**) immunodetection of DPP-IV (mAb E19) and FAP (mAb D28); (**C**) membrane overlay assay (H-Gly-Pro-AMC) of DPP-IV-like enzymatic activity; (**D**) zymography assay of gelatinolytic activity. Zymograms incubated in the buffers activating (+2mM $CaCl_2$) and inhibiting (+2mM EDTA) matrix metalloproteinases.

Line 1: DPP-IV positive control; Line 2: primary cell culture 7; Line 3: primary cell culture 9; Line 4: primary cell culture 10-II; Line 5: primary cell culture 16-II; Line 6: primary cell culture 20; Line 7: FAP positive control

4.3. DPP-IV and FAP co-expression during experimentally induced changes of DPP-IV-like enzymatic activity

U87MG and U138MG cell line co-express DPP-IV and FAP and exhibit the respective gene loci in the proper localization on the chromosome 2 (Figure 2A). Cell culture under the conditions of growth factor deficiency in serum-free medium substantially increases the DPP-IV-like enzymatic activity in these cell lines (Sedo et al. 1998). The upregulation of biochemically assayed DPP-IV-like enzymatic activity was associated with the increase of the expression of both DPP-IV and FAP mRNAs and also with the increase of DPP-IV and FAP proteins as determined by ELISA, immunoblotting and immunocytochemisty (Table 1, Figure 2B, 2C, 2D). Similarly to the primary cell cultures, we observed single band of about 145 kDa detected by the anti DPP-IV as well as by anti FAP antibody. This band corresponded to the DPP-IV-like exopeptidase enzymatic activity visualized by the membrane overlay assay (Figure 2C). Thus, similarly to the primary cell cultures, we identified active 150kDa homodimers of DPP-IV and FAP and moreover we demonstrated the co-expression of both the DPP-IV and FAP in permanent human glioma cell lines U87MG a U138MG.

Culture conditions		DPP-IV	FAP
		Mean ± S.D. [pg/μg protein]	
U87MG	**SFM**	56.1 ± 0.5	90.5 ±12.3
	FCS	13.5 ± 6.7	23.3 ± 1.2
U138MG	**SFM**	66.4 ± 1.2	108.7 ± 0.7
	FCS	36.6 ± 3.3	88.4 ± 1.8
DPP-IV transfected U87MG	**Mifepristone**	105.4 ± 1.7	5.4 ± 1.7
	Controls	3.8 ± 0.7	8.4 ± 1.8

Table 1. **DPP-IV and FAP in permanent glioma cell lines, U87MG, U138MG and DPP-IV transfected U87MG.**

U87MG and U138MG cells cultured in serum free media (SFM) compared to the cells grown in 10% foetal calf serum supplemented medium (FCS). DPP-IV transfected U87MG cells stimulated to express transgenic DPP-IV by 0,25 nM Mifepristone (Mifepristone), untreated DPP-IV transfected U87MG cells (Controls).

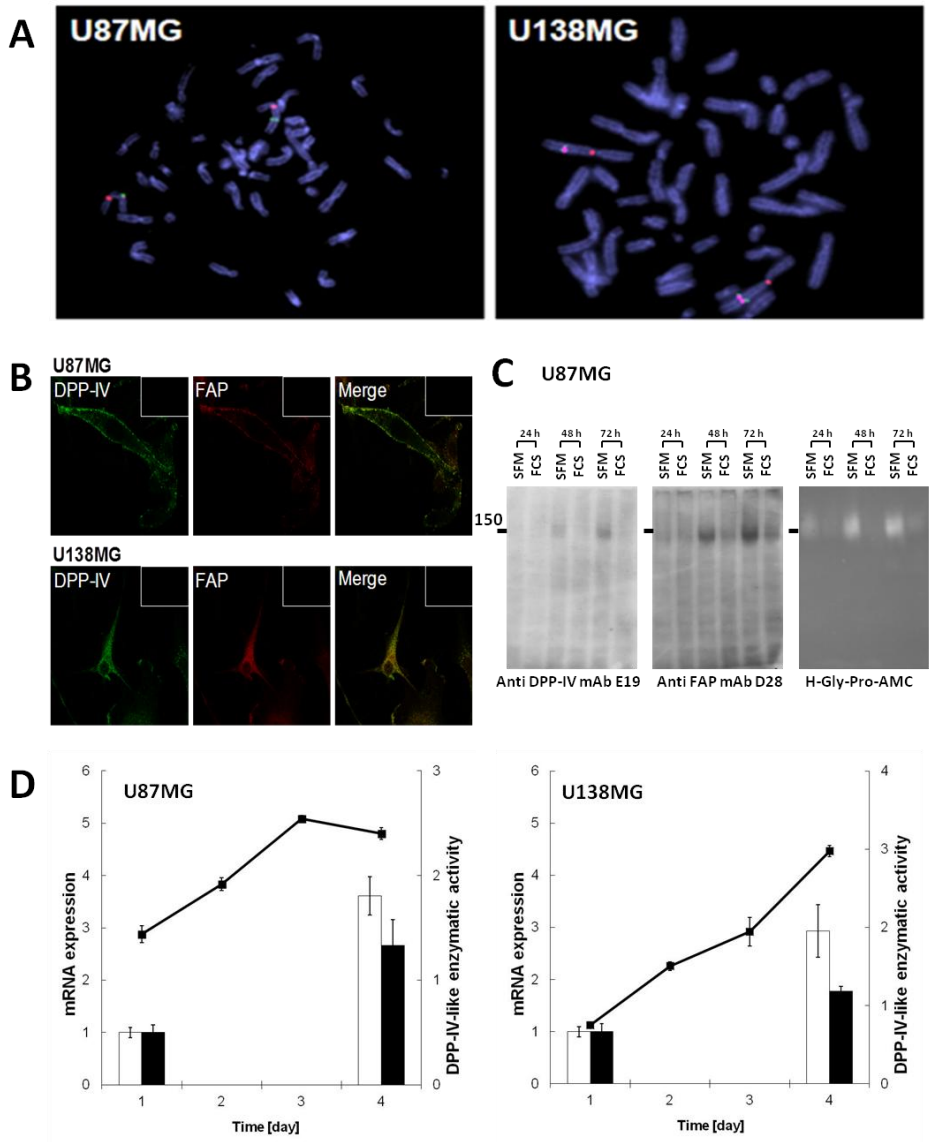

Figure 2. **DPP-IV and FAP in permanent glioma cell lines U87MG and U138MG.**

(**A**) genomic localization of DPP-IV (green) and FAP (rouge) loci on the chromosome 2 detected by FISH. Centromeric probe for chromosome 2 in red; (**B**) immunodetection of DPP-IV (mAb MA 261, green) and FAP (mAb D8, red); (**C**) immunobloting and membrane overlay assay (H-Gly-Pro-AMC) of DPP-IV and FAP (150 kDa) derived from the U87MG cells cultured in serum free media (SFM) compared to the ones grown in 10% foetal calf serum supplemented medium (FCS) for 24, 48 and 72 hours (h); (**D**) relative increment of DPP-IV (white bars) and FAP (black bars) mRNA expression and plasma membrane DPP-IV-like enzymatic activity (■). U87MG and U138MG cells cultured in serum free media compared to the cells grown in 10% foetal calf serum supplemented medium.

Elution profile of the exopeptidase DPP-IV-like enzymatic activity, assayed by the gel filtration, demonstrated two molecular weight (MW) forms of about 410 - 610 kDa and 130 - 230 kDa MW respectively. Using Z-Gly-Pro-AMC as a substrate to detect the proline-specific endopeptidase enzymatic activity, two peaks corresponding to 410 - 610 kDa and 60 – 90 kDa were detected. In U87MG cells cultured in serum free media, the DPP-IV-like exopeptidase as well as the proline-specific endopeptidase enzymatic activity increased in the MW-region of about 410 - 610 kDa (Figure 3A, 3B). The changes in DPP-IV-like exopeptidase and the proline-specific endopeptidase enzymatic activities were fully reverted by the addition of 10% foetal calf serum into the culture media (Figure 3C).

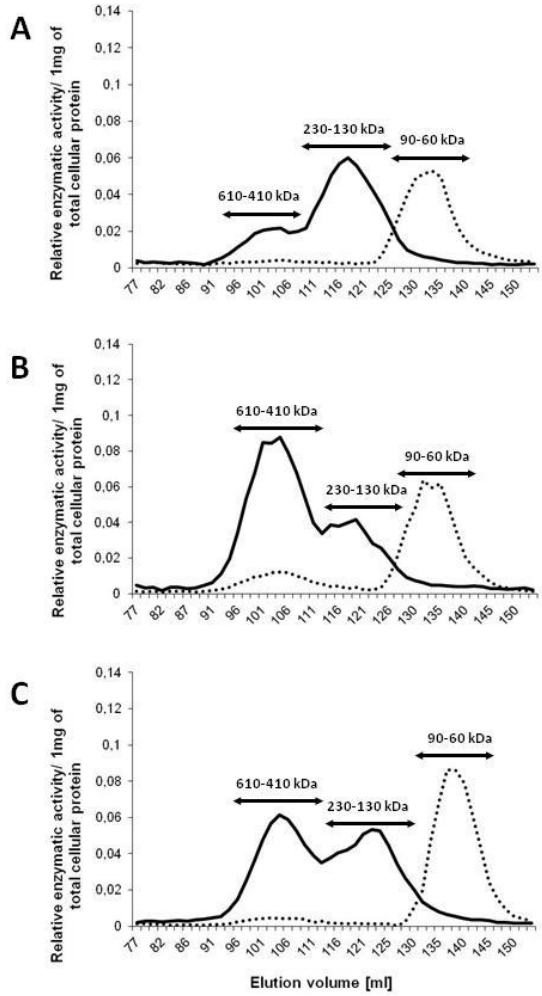

Figure 3. **Elution profile of the DPP-IV-like exopeptidase and proline-specific endopeptidase activity in the U87MG cell line.**

U87MG cells were cultured for (**A**) 72 hours in 10% foetal calf serum supplemented medium; (**B**) 72 hours in serum free medium; (**C**) 72 hours in serum free medium followed by 48 hours in 10% foetal calf serum supplemented medium. DPP-IV-like exopeptidase activity (full line); proline-specific endopeptidase activity (dotted line).

Further analysis of the elution profiles revealed that both DPP-IV-like exopeptidase and prolyl-endopeptidase enzymatic activities in the MW-region of about 410 - 610 kDa were detectable in the solubilized membrane fraction, but not in the soluble fraction. On the contrary, the 130-230 kDa DPP-IV-like exopeptidase and 90-60 kDa prolyl-endopeptidase activities were characteristic for the soluble fractions (Figure 4).

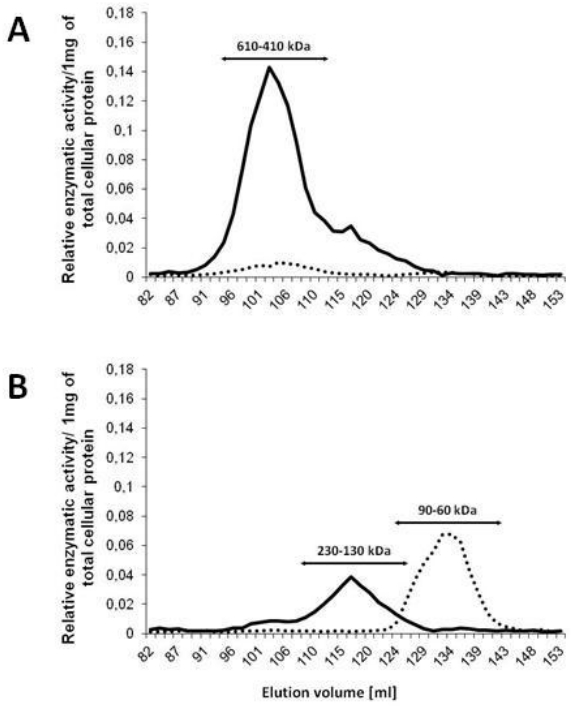

Figure 4. **Elution profile of the DPP-IV-like exopeptidase and proline-specific endopeptidase activity in U87MG cell fractions.**

(**A**) solubilized membrane fractions; (**B**) soluble fractions. U87MG cells were cultured in serum free medium for 72 hours. DPP-IV-like exopeptidase enzymatic activity (full line) and proline-specific endopeptidase enzymatic activity (dotted line).

4.4. Expression of FAP in DPP-IV transfected U87MG cell line

In model of DPP-IV transfected U87MG, the DPP-IV transgene is highly probably localized in the aberrant genomic context and thus operated by different transcriptional factors. In these experiments, neither the upregulation of FAP mRNA and protein expression (Figure 5A, Table 1) nor the increment of the relevant proline-specific endopeptidase enzymatic activity (Figure 5B) were observed after the induction of DPP-IV overexpression.

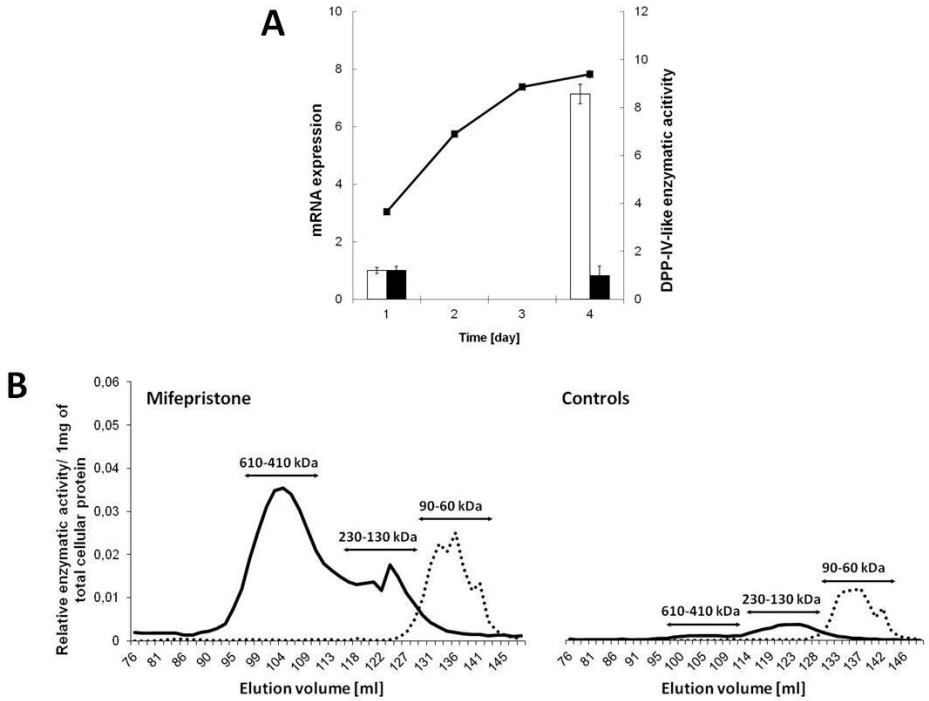

Figure 5. **DPP-IV and FAP in the DPP-IV transfected U87MG cell line.**

(**A**) relative increment of DPP-IV (white bars) and FAP (black bars) mRNA expression and plasma membrane DPP-IV-like enzymatic activity (■); (**B**) elution profile of the DPP-IV-like exopeptidase (full line) and proline-specific endopeptidase (dotted line) activity. DPP-IV transfected U87MG cells were stimulated to express the DPP-IV transgene by 0.25 nM Mifepristone (Mifepristone) treatment. Controls = untreated DPP-IV transfected U87MG cells.

4.5. The effect of DPP-IV and FAP expression on cell adhesion and spreading

To characterize the effect of DPP-IV and FAP on cell adhesion and spreading, we used the transgenic system of DPP-IV or FAP expressing U373 cell line. Upregulation of transgenic wild type DPP-IV impaired adhesion of U373 glioma cells on a plastic coated with collagen type I. The overexpression of mutated enzymatically inactive transgenic DPP-IV did not alter adhesion of U373 at all (Figure 6A). Similarly to the cells expressing wild type DPP-IV, transgenic expression of the wild type FAP negatively influenced adhesion and spreading of U373 glioma cells on collagen type I and IV (Figure 6B, 6C, 6D). The expression of mutated enzymatically inactive FAP did not induce the impairment of cell adhesion (Figure 6B, 6D).

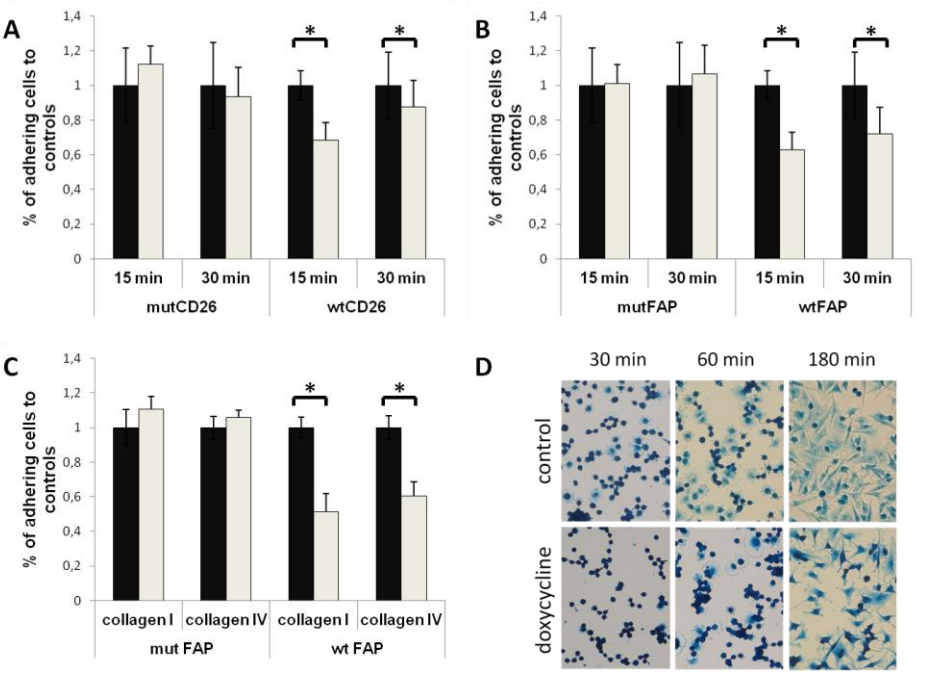

Figure 6. **Effect of DPP-IV and FAP on glioma cell adhesion and spreading.**

(A) % of adhering DPP-IV overexpressing cells (white bars) to the controls (black bars) to the collagen I in 15 and 30 min (**B**) % of adhering FAP overexpressing cells (white bars) to the controls (black bars) to the collagen I in 15 and 30 min (**C**) % of adhering FAP overexpressing cells (empty bars) to controls (full bars) to the collagen I and IV in 15 min. (**D**) Spreading of wild type FAP transfected U373 cells on collagen type I. DPP-IV or FAP transfected U373 cells were stimulated to express the given wild type (wt) or mutated (mut) transgene by 10^{-6} M Doxycycline treatment. Controls = untreated transfected U373 cells.

5. Discussion

The group of "Dipeptidyl peptidase-IV activity and/or structure homologous" molecules was first defined in 2001. Strong evidence about their contribution on multiple physiological and pathological processes, including the growth, migration and invasion of transformed cells has been bought in the last decade. In this study, we describe DPP-IV and FAP expression pattern within the human glioblastoma microenvironment and demonstrate for the first time coupled DPP-IV and FAP expression in transformed glial cells. Finally, we analyze the possible implication of DPP-IV and FAP in the transformed cell adhesion.

5.1. Expression pattern and localisation of DPP-IV and FAP in human glioblastoma multiforme (Aim 1)

In our study, we demonstrate the parenchymal and perivascular localisation of both DPP-IV and FAP. In the tumour parenchyma, but not in the perivascular compartment, we found dispersed double positive DPP-IV/FAP and FAP/GFAP glial cells. The sporadic expression of SOX-2 within these cells suggests that, at least part of this population, represents cells of glial origin possessing characteristics of malignant transformation (Fang et al. 2011). In addition, we found FAP positive but DPP-IV and GFAP negative cell population in the perivascular localization. Such cells were tightly associated with the dysplastic and hyperproliferative tumour vessels. Because such FAP positive cells sporadically express the markers of mesenchymal cells, such as SMA, TEM-1, TE-7, but not antigens attributed to the endothelia, microglial and astrocytic elements (vW, CD105, Iba, GFAP), their mesenchymal origin seems probable. The pericytes, vascular smooth muscle cells and myofibroblast/fibroblast belong to the group of mural mesenchymal cells, surrounding

endothelium and basal membrane (Kurz et al. 2004). They have a potential to transdifferentiate (Rajkumar et al. 2005), thus their strict identification remains partially equivocal. FAP, a marker of activated fibroblasts, was reported to be expressed on the peripheral myofibroblast/pericytes in a rodent experimental model (Wang and Shi 2009)**.** On the basis of our results and published observations, we conclude that the perivascular FAP positive cells in glioblastoma tissue are eight the pericytes or activated fibroblast/myofibroblasts.

5.2. Co-expression of DPP-IV and FAP in glioma (Aim 2)

The significant correlation of DPP-IV and FAP mRNA templates and the respective proteins is found in the glioblastoma-derived primary cell cultures (Table 1A). Further analysis of the putative molecular forms possessing DPP-IV enzymatic activity using immunoblotting revealed single band of about 150 kDa, stained by both anti DPP-IV and anti FAP antibody respectively. This band was colocalizing with the one detected by fluorogenic substrate H-Gly-Pro-AMC membrane overlay assay as well as by gelatinolytic enzymatic activity visualized by the zymography assay. Similarly to that, DPP-IV and FAP immunopositive band of about 150 kDa comigrating with the DPP-IV-like enzymatic activity was found also in both tested permanent glioma cell lines, U87MG and U138MG. Molecular weight of DPP-IV and FAP, both naturally occurring as homodimers, varies depending on the source, typically around 220kDa and 170-150 kDa, respectively. However, Gorrell et al observed 140 – 160 kDa homodimers of DPP-IV in cirrhotic liver and lymphocytes (Gorrell et al. 2001) and group of Chen described 37 kDa enzymatically active FAP isoform in melanoma cells (Chen et al. 2006). The authors suggested that the lower MW of DPP-IV and FAP can either be product of an alternative splicing or can represent differentially glycosylated form, specific for the given cellular source. Together, we presume that the glioma cells most probably express enzymatically active DPP-IV and FAP, both of 145-150 kDa MW. However, existence of DPP-IV/FAP heterodimers, hypothesized by Ghersi et al. (Ghersi et al. 2003; Ghersi et al. 2006) might also partially explain conjunction of results of our immunodetection and enzyme activity assays. However, the existence of such "chimeric" heterodimers still awaits to be confirmed or denied.

5.3. Coupled expression of DPP-IV and FAP in glioma cells (Aim 3)

To confirm the dynamic association of the DPP-IV and FAP expression, hypothesized on the basis of previous correlative observations, we choose U87MG and U138MG cell lines, both spontaneously expressing DPP-IV and FAP. The respective DPP-IV and FAP gene loci in the proper localization on the chromosome 2, a necessary prerequisite for a proper biological regulation, were verified (Figure 1A). The upregulation of DPP-IV-like enzymatic activity is associated with the coupled upregulation of both DPP-IV and FAP transcripts and protein in both glioma cell lines (Figure 1; Table 1). Due to their similar hydrolytic properties, both molecules are probably participating on the overall assayed DPP-IV-like enzymatic activity. To assess the share of FAP on the total DPP-IV-like enzymatic activity, the proline-specific endopeptidase enzymatic activity exhibited by FAP, but not by the canonical DPP-IV, was measured (Barelli et al. 1999). Since the prolyl endopeptidase (PEP; EC 3.4.21.26), differing from FAP by the MW, is in general responsible for the substantial part of cellular proline-specific endopeptidase activity, gel filtration chromatography preceded the biochemical enzyme activity assay in order to take apart FAP from PEP.

In U87MG cells cultured in the conditions of growth factors deficiency, the increment of both the DPP-IV-like exopeptidase and the proline-specific endopeptidase enzymatic activities are observed in the MW-region of about 410 - 610 kDa (Figure 2). Similar

oligomeric complexes possessing DPP-IV-like enzymatic activity (480 - 900 kDa) are known to occur in the other cell types and are suggested to be a result of DPP-IV and FAP homodimers aggregation (Scanlan et al. 1994; Ghersi et al. 2002). 80 kDa proline-endopeptidase activity peak (Figure 4) present in the elution profiles of the soluble, but not solubilized membrane cell fraction corresponds well with the cytosolic PEP with expected molecular weight (Polgar 2002).

To further approach the mechanism of observed association of DPP-IV and FAP expressions, a model of DPP-IV transfected glioma cells was used, but the coupled expression of transgenic DPP-IV and endogenous FAP was not observed there (Balaziova et al. 2011).

Together, we demonstrated for the first time the coupled expression of DPP-IV and FAP transcripts and proteins in the transformed glial cells. Uncoupled expression of transgenic (exogenous) DPP-IV and the intrinsic (endogenous) FAP in the DPP-IV transfected glioma cells suggests that the coupling of expression of both molecules is more likely a result of a joint control of their expression, rather than a consequence of an indirect reciprocal posttranscriptional regulation involving changes of their mRNA and/or protein expression (Balaziova et al. 2011).

5.4. DPP-IV and FAP in adhesion of glioma cells to the components of extracellular matrix (Aim 4)

Demonstrated co-regulation of DPP-IV and FAP in glioma elements implies their functional cooperation in the glioma cells biology.

Overexpression of transgenic FAP significantly decreased the cell adhesion to collagen type I and IV. Moreover, this effect was dependent on the FAP enzymatic activity. Preliminary results of our laboratory also suggest participation of FAP in the processes of glioma cell migration and invasion (not shown). In view of these observations, we hypothesize that in our model of the glioma cells, FAP alone or in cooperation with other matrix degrading system processes collagen, which then serves as worse adhesion substrate for the cells. Interestingly, the FAP role in cellular adhesion and migration might be cell type specific. Similarly to our results, Wang et al observed decrease of cell adhesion and migration in a model of HEK 293 epithelial cell line. In contrast to that, the same authors noted an opposite effect of FAP in LX-2 human stellate cell line (Wang et al. 2006). Although FAP is known to cooperate on the cleavage of extracellular matrix components with the matrix metalloproteinases and plasmin (Chen and Kelly 2003; Christiansen et al. 2007), Wang et al. demonstrated that the FAP effect in their experiments was independent on its hydrolytic activity at all. Based on these results, Wang concluded that the impairment of cell adhesion might be caused by the FAP-associated downregulation of integrin-β1 and upregulation of matrix metalloproteinase 2 and CD44.

Similarly to FAP, overexpression of enzymatically active, but not mutant, transgenic DPP-IV impaired the adhesion of the glioma cell to the collagen type I. These results fit well with previously observed potential of DPP-IV to hinder glioma cell migration and adhesion to fibronectin (Busek et al. 2012). The collagen binding domain of DPP-IV is presumed to be localized in its cystein-rich region (Loster et al. 1995). Wang demonstrated positive effect of overexpression of both enzymatically active and inactive DPP-IV on the cell adhesion to fibronectin, but not to collagen, and the negative effect on migration of HEK 293 epithelial cells. Similarly, transgenic DPP-IV expression increased the adhesion of ovarian carcinoma cells to fibronectin. Considering all of that, here we report for the first time observations suggesting a DPP-IV enzymatic activity may hinder cell adhesion to collagen. The presence of collagen binging domain within the DPP-IV sequence primarily implies its adhesive function. However, it might also be speculated that the hydrolytic effect of the enzyme on the

locally present bioactive peptides, controlling processes of cell adhesion and migration, may, cell-system specifically, weaken or even revert the assumed straightforward pro-adhesive potential of DPP-IV molecule.

Together, we suggest that DPP-IV and FAP might represent important players in complex molecular machinery moderating cell adhesion and migration. Demonstrated coupled expression of DPP-IV and FAP, their proven potential to affect balances of bioactive substrates as well as structural proteins of extracellular matrix, together with their non-hydrolytic functions argue for contextual role of the "DASH system" in biological processes.

6. Conclusion

In this study, we demonstrated:

- In human glioblastoma multiforme, DPP-IV and FAP are expressed on the GFAP and SOX-2 positive cells, which represent probably the transformed glioma elements.
- In human glioblastoma multiforme, FAP is also expressed on the mesenchymal cell population, which is tightly associated with dysplastic and hyperproliferative vessels.
- In human glioblastoma multiforme, DPP-IV and FAP are co-expressed, at least, at some of glioma cell populations. Using *in vitro* model of the permanent glioma cell lines, we demonstrate that the coupled of their expression is likely a result of a joint DPP-IV and FAP transcriptional control.
- Individual expression of both transgenic, enzymatically active DPP-IV and FAP has the negative effect on cell adhesion.

These observations argue for putative involvement of DPP-IV and FAP into the malignant glioma progression and suggest the role in glioma vascularisation.

7. Literature

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. "The AceView Database; http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi?db=human&c=Gene&l=FAP."

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8. List of publication related to the thesis

8.1. Original papers and review

8.1.1. **ENCLOSURE 1:** Busek, P., Stremenova, J., Sromova, L., et al. (2012) Dipeptidyl peptidase-IV inhibits glioma cell growth independent of its enzymatic activity. International journal of biochemistry and cell biology. 2012, 44: 738-747. IF 4.7

8.1.2. **ENCLOSURE 2:** Balaziova, E., Busek, P., Stremenova, J., et al.(2011) Coupled expression of dipeptidyl peptidase-IV and fibroblast activation protein-alpha in transformed astrocytic cells. Molecular and cellular biochemistry. 354: 283-289. IF 2.0

8.1.3. **ENCLOSURE 3:** Kotackova, L., Balaziova, E., Sedo, A. (2009) Expression Pattern of Dipeptidyl Peptidase IV Activity and/or Structure Homologues in Cancer. Folia biologica. 55: 77-84. IF 1.1

8.2. Posters

8.1.4. **ENCLOSURE 4:** Balaziova, E., Busek, P., Fejfarova, E., et al. Expression of fibroblast activation protein-α in human glioblastoma and its effect on glioma cell adhesion and invasion. (Poster) Podosomes, invadopodia and focal adhesion in phyiology and pathology, 2011, Madrid, Spain.

8.1.5. **ENCLOSURE 5:** Balaziova, E., Kotackova L., Sedo A. Regulation of co-expression of Dipeptidyl peptidase-IV and Fibroblast activation protein in glioma cell lines. (Poster) 14th World Congress on Advances in Oncology and 12th International Symposium on Molecular Medicine, 2009, Loutraki, Greece.

9. Abbreviations

AEBSF – 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; **BSA** – Bovine serum albumin; **CEP** – Chromosome enumeration probe; **DASH** – Dipeptidyl peptidase-IV activity and/or structure homologues; **DPP** – Dipeptidyl peptidase; **DMEM** – Dulbecco's modified Eagle's medium; **ECM** – Extracellular matrix; **ELISA** – Enzyme-linked immunosorbent assay; **FAP** – Fibroblast activation protein α; **f.c**. – final concentration; **FCS** – Foetal calf serum; **FISH** – Fluorescence in situ hybridization; **GFAP** – Glial fibrillary acidic protein; **h** – Hour; **M** – mol/l; **mAb** – Monoclonal antibody; **min** – Minute; **MMP** – Matrix metalloproteinase; **MW** – Molecular weight; **PBS** – Phosphate buffered saline; **RT-PCR** – Reverse transcriptase polymerase chain reaction; **SMA** – α-smooth muscle actin; **TEM-1** – Tumor endothelial marker 1, endosialin; **vW** – von Willebrand factor; u**PA/uPAR** – Urokinase-type plasminogen activator/urokinase-type plasminogen activator receptor; **WHO** – World health organisation