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Institute of Biochemistry and Experimental Oncology

Dipeptidyl peptidase-IV Activity and/or Structure Homologues: Their role in gliomagenesis

Jarmila Stremeňová

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Supervised by Professor Aleksi Šedo, MD, PhD, DSc

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STATEMENT

I hereby declare, that presented dissertation thesis was prepared solely using cited literature and under the leadership of my supervisor professor Aleksi Šedo, MD, PhD, DSc and I did not use here presented results to reach another educational degree.

Prague 2009 Jarmila Stremeňová

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SUMMARY

Dipeptidyl peptidase-IV (DPP-IV, EC 3.4.14.5) together with fibroblast activation protein-alpha (FAP), DPP-7, -8 and -9 belong to the functionally defined group of "DPP-IV Activity and/or Structure Homologues" (DASH). They hydrolyse N-terminal X-Pro dipeptides from a number of biologically active peptides like neuropeptide Y, substance P and chemokines such as stromal cell derived factor-1alpha (SDF-1). Limited proteolysis of such mediators by DPP-IV-like enzymatic activity can modify consequent biological responses of the target cells. By that, DASH molecules are supposed to be important for multiple cellular processes, including cell proliferation, malignant transformation, migration and invasion and thus involved in cancer development and progression.

This study was set up to characterise DASH expression pattern and DPP-IV-like enzymatic activity in human astrocytic tumours in comparison with non-tumorous brain tissue, and to assess its context with the expression of receptors of some local mediators- DASH substrates implicated in gliomagenesis. Moreover, the possible functional relevance of DASH molecules in growth properties of transformed astrocytic cells was studied in model of primary cell cultures derived from the glioblastoma *in vitro*.

We observed an increase of DPP-IV-like enzymatic activity in human astrocytomas along with the WHO grade of malignancy. The major part of DPP-IV-like enzymatic activity in non-tumorous brain tissue as well as in astrocytomas is probably derived from the intracellular DPP8/9. However, the substantial part of the DPP-IV-like hydrolytic activity increment observed in the high-grade astrocytomas is an attribute of the plasma membrane DPP-IV and probably FAP. DPP-IV expression and enzymatic activity tightly correlated with the expression of the SDF-1 cognate receptor CXCR4. On the other hand, our *in vitro* studies demonstrated that the primary cell cultures with higher DPP-IV-like enzymatic activity exhibited decreased cell growth.

Hence we speculate that although the upregulated DPP-IV potentially trims down SDF-1 signalling, such effect may be compensated by an increase of the appropriate receptor. This would then favour progression of astrocytoma containing cell population capable of effective tuning of CXCR4-DPP-IV balance within the tumour microenvironment. Taken together, our results suggest, that DASH molecules, namely

DPP-IV might execute an anti-oncogenic effect in transformed cells themselves, while it could still be beneficial to other cell populations within the complex tumour environment, with a resultant net pro-oncogenic effect.

LIST OF ABBREVIATIONS

ADA	Adenosine deaminase
ATB	Antibiotics
CD	Cluster of differentiation
DASH	Dipeptidyl peptidase-IV activity and/or structure homologues
DMEM	Dulbecco's Modified Eagle's Medium
DPP	Dipeptidyl peptidase
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EFGR	Epidermal growth factor receptor
FACS	Fluorescence-activated cell sorting
FAP	Fibroblast activation protein-alpha
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GFAP	Glial fibrillary acidic protein
H-Gly-Pro-AMC	H-7-(L-Glycyl-L-Prolylamido)-4-methylcoumarin
H-Lys-Ala-AMC	H-7-(L-Lysyl-L-Alanylamido)-4-methylcoumarin
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
NAALADase	N-acetylated alpha-linked acidic dipeptidase
NPY	Neuropeptide Y
PBS	Phosphate buffered saline
PDGF	Plateled-derived growth factor
PTEN	Phosphatase and tensin homologue
QPP	Quiescent cell proline dipeptidase
Rb	Retinoblastoma
RT-PCR	Reverse-transcription polymerase chain reaction
SDF-1	Stromal cell-derived factor-1
SFM	Serum free medium
SP	Substance P
TRIC	Tetramethyl rhodamine isothiocyanate
WHO	World Health Organisation

CONTENTS

1. INTRODUCTION

Dipeptidyl peptidase-IV (DPP-IV, EC 3.4.14.5) is a proline specific serine protease belonging to the S9 prolyl-oligopeptidase family. It typically hydrolyses N-terminal dipeptides from substrates with proline at the penultimate position. Such specifity makes DPP-IV a crucial functional regulator of a number of biologically active peptides, which conserve Pro- residue as a sort of proteolytic checkpoint (Tanaka *et al*, 1992; Vanhoof *et al*, 1995). DPP-IV has for years been believed to be the unique plasma membrane post-proline dipeptidyl aminopeptidase, but subsequently further molecules displaying DPP-IV-like enzymatic activity and a varying degree of structural similarity to the canonical DPP-IV have been discovered and grouped as the "Dipeptidyl peptidase-IV activity and/or structure homologues" (DASH) (Sedo & Malik, 2001). The group comprises enzymatically active seprase/fibroblast activation protein-alpha, DPP-II/DPP7/Quiescent cell proline dipeptidase, DPP8, DPP9, and enzymatically inactive DPP6 and DPP10. Attractin, structurally completely unrelated to the canonical DPP-IV, was formerly supposed to belong to the DASH group on the basis of its putative enzymatic activity (Duke-Cohan *et al*, 1998). However, the existence of that hydrolytic activity was later challenged by results of Friedrich et al (Friedrich *et al*, 2007). Likewise DPP-IV-like enzymatic activity of N-acetylated alpha-linked acidic dipeptidase-I, -II and -like (NAALADase) formerly described by Pangalos et al (Pangalos *et al*, 1999) was later disputed by Barinka et al (Barinka *et al*, 2002). Therefore NAALADase as well as attractin were recently withdrawn from the DASH group.

From the functional point of view, most of the DASH molecules are believed to be involved in a broad array of biological processes and thus belong to the so called "moonlighting proteins" (Jeffery, 1999). Among the multiple physiological and pathological functions, DASH play a critical role in immunoregulations and carcinogenesis, due to postsecretory processing of biologically active peptides implicated in the control of cell growth, migration and invasion. Consequently, examination of the DPP-IV family representation and activity has become a major focus of preclinical and clinical studies.

Malignant gliomas, characterised as highly proliferative and locally invasive neoplasias, account for more than 50% of central nervous system tumours. Despite

intensive basic research and efforts in the fields of therapeutic regimens and modalities, the prognosis of patients suffering from these tumours remains dismal.

Gliomagenesis is a complex multifactorial process, which involves the deregulation of signalling of a number of neuropeptides and chemokines, leading to disrupted tumour cell proliferation, migration, invasiveness, and enhanced protection against apoptosis. Dipeptidyl peptidases might represent important regulators of several mediators pathogenetically implicated in gliomagenesis.

1.1. Classification of "Dipeptidyl peptidase-IV activity and/or structure homologues"

Since we reviewed DASH molecules in our recent paper (Enclosure 1), here we provide just concise information about the individual group members.

1.1.1. Dipeptidyl peptidase-IV

Dipeptidyl peptidase-IV is a multifunctional transmembrane type II glycoprotein present on the surface of most cell types, firstly described by Hopsu-Havu and Glener in the human liver in 1966 (Hopsu-Havu & Glenner, 1966). It is identical with the differentiation antigen CD26 of activated T-cells (Aytac *et al*, 2003; Ulmer *et al*, 1990). DPP-IV structure comprises 3 domains: a short intracellular domain consisting of 6 highly conserved amino acids, a 22 amino acid hydrophobic transmembrane segment and a 738 amino acid extracellular domain. The extracellular domain is subdivided into a 20 amino acid flexible stalk region, a cysteine rich region and a 260 amino acid C-terminal catalytic region (Figure 1, (Mentlein, 1999)). The catalytic triade of DPP-IV is composed of residues Ser630, Asp708, and His740, which are located within the last 140 residues of the C-terminal region (Ogata *et al*, 1992).

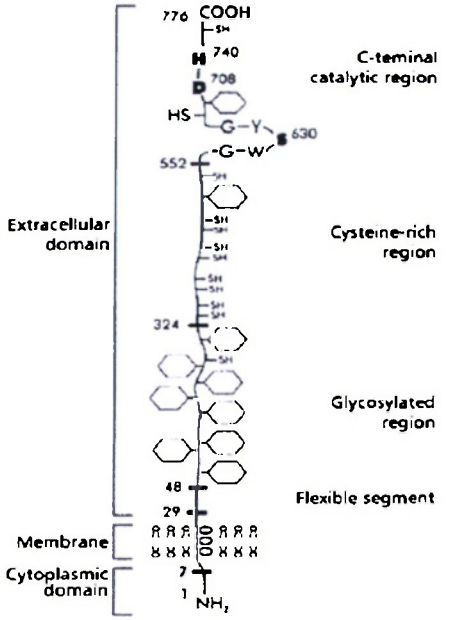

Figure 1. Highly glycosylated structure of DPP-IV comprises short cytoplasmic domain, transmembrane segment and extracellular domain with serine catalytic triade. Taken from Mentlein, 1999.

DPP-IV is enzymatically active as a homodimer. Its folding leads to the formation of a beta-propeller domain and an alpha/beta-hydrolase domain (Figure 2, (Aertgeerts *et al*, 2004b)). Several authors reported that N-linked glycosylation is a prerequisite for correct protein folding and dimer formation, which are a condition for hydrolytic activity and cell-surface expression (Fan *et al*, 1997; Loch *et al*, 1992). However, point mutation of nine Asn residues in glycosylation site of the molecule refuted that assumption (Aertgeerts *et al*, 2004a).

A soluble form of DPP-IV lacking the transmembrane and cytoplasmic domains was found in blood plasma (Durinx *et al*, 2000; Iwaki-Egawa *et al*, 1998), saliva (Elgun *et al*, 2000; Ogawa *et al*, 2008), cerebrospinal fluid, synovial fluid, seminal plasma and urine (Kullertz & Boigk, 1986; Kullertz *et al*, 1986; Narikawa *et al*, 2006; Scherberich *et al*, 1992). The enzymatically active soluble DPP-IV is thought to be either the result of a secretion from different cell types, most probably from the lymphocytes, endothelial and epithelial cells, or the product of proteolytic shedding of the formerly membrane-bound DPP-IV. The protease discharging the soluble form has not been identified yet and it is not even certain whether this proteolysis occurs endosomally during vesicle transfer to the plasma membrane or whether it happens extracellularly.

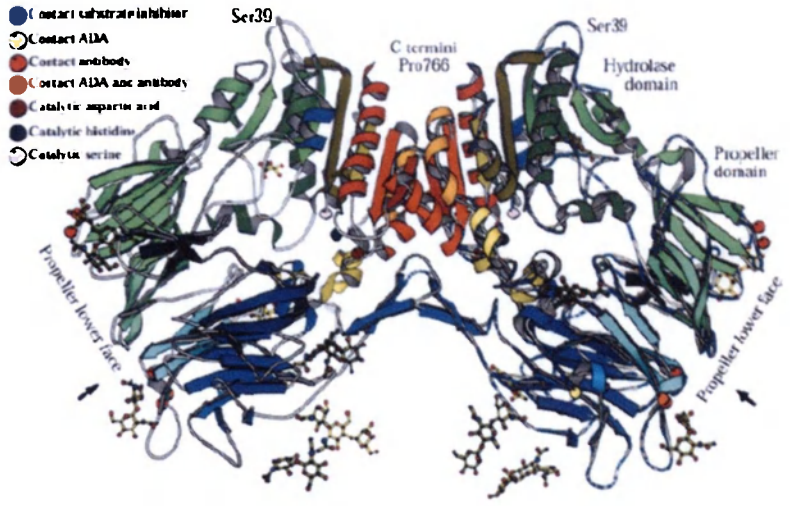

Figure 2. The crystal structure of human DPP-IV homodimer. The orange and yellow residues Leu294 and Val341 are essential for adenosine deaminase (ADA) binding, whereas the red positively charged residues Arg343 and Lys441 are important in epitopes of antibodies that inhibit ADA binding. Glu205 and Glu206, coloured blue, are essential for the enzymatic activity. Both domains contribute to the dimerisation interface, the beta-propeller contributing two beta-strands that protrude from blade 4. Substrate access to the catalytic site occurs via the side openings that face each other and are between the eight-blade beta-propeller (bottom) and alpha/beta hydrolase (top) domains. Taken from Gorrell, 2005.

1.1.2. Fibroblast activation protein-alpha

Fibroblast activation protein-alpha (FAP) also referred to as seprase (surface expressed protease), was formerly known as "F19 Cell Surface Antigen", identified in cultured fibroblasts using the monoclonal antibody F19 (Rettig *et al*, 1993). FAP is structurally highly similar to DPP-IV (Goldstein *et al*, 1997). It bears not only the characteristic dipeptidyl aminopeptidase activity, but also endopeptidase collagenase/gelatinase activity (Levy *et al*, 1999; Park *et al*, 1999). FAP is as an inducible cell surface protease expressed by reactive tumour stromal fibroblasts in epithelial cancers. It is also present in granulation tissue during wound healing, hepatic stellate cells in the cirrhotic liver as well as in certain embryonic tissues, where it is believed to play a pivotal role in proteolytic degradation of extracellular matrix (ECM) components facilitating cell invasion and tissue remodelling (Levy *et al*, 1999; Rettig *et al*, 1994; Scanlan *et al*, 1994). To execute such functions, it is typically localised in the

invadopodial membranes (Chen, 1996; Monsky *et al*, 1994). Moreover, Ghersi et al provided evidence of FAP and DPP-IV heterodimerisation and eventual concerted action of the DPP-IV-FAP complexes with other proteases, e.g. matrix metalloproteinases (MMP) in activated fibroblasts but also in endothelial cells (Ghersi *et al*, 2002; Ghersi *et al*, 2006).

Similarly as for DPP-IV, a soluble form of FAP is present in human blood plasma, where it is identical with formerly described circulating antiplasmin-cleaving enzyme (Lee *et al*, 2006).

1.1.3. Dipeptidyl peptidase-II

Dipeptidyl peptidase-II (DPP-II, EC 3.4.14.2), also referred to as DPP7 and quiescent cell proline dipeptidase (QPP), is an intracellular protease localised to vesicles distinct from lysozymes (Araki *et al*, 2001; Maes *et al*, 2005). DPP-II is enzymatically active preferably in the acidic pH range 5 – 6 (Fukasawa *et al*, 1983) as a homodimer of two identical glycosylated subunits of 50 – 65 kDa. The leucine zipper motif upstream the catalytic triade appears necessary for both homodimerisation and enzymatic activity (Chiravuri *et al*, 2000).

In humans, DPP-II is found in normal as well as in a number of malignant cells, tissues and body fluids, e.g. lymphocytic T- and B-cells, kidney, placenta and seminal fluid (Gossrau *et al*, 1987; Khalaf *et al*, 1986; Maes *et al*, 2005; Sakai *et al*, 1987).

DPP-II is supposed to participate in the turnover of short peptides, preferably tripeptides, in the intracellular vesicular system. Beside that, it could be secreted and act extracellularly. Although some early studies claimed an involvement of DPP-II in the extracellular degradation of collagen (McDonald *et al*, 1985), this functional potential has not been confirmed by contemporary studies (Maes *et al*, 2005).

1.1.4. Dipeptidyl peptidase 8 and 9

Both DPP8 and DPP9 share high degree of sequence identity and similarity with the canonical DPP-IV (Abbott *et al*, 2000). They are catalytically active as 100 kDa non-glycosylated monomers in slightly alkalic pH. DPP8 and DPP9 were identified in 4 and 2 splice variants, respectively, however, their biological relevance remains as yet unexplored. Two of the four splice variants of DPP8 and one full-length 892 amino acid DPP9 variant are catalytically active, but the enzymatic activity of the shorter, 863 amino acid form of DPP9 is still in discussion (Ajami *et al*, 2004; Bjelke *et al*, 2006).

DPP8 and DPP9 are widely expressed. High mRNA levels of DPP8 were found in testis and placenta, activated T-cells and T- and B-cell derived cell lines (Abbott *et al*, 2000). The highest expression of DPP9 mRNA was detected in skeletal muscle, heart, liver and peripheral blood leukocytes, and the lowest in the brain (Olsen & Wagtmann, 2002). Due to their potential to mitigate cell adhesion and migration and to enhance apoptosis, Yu et al proposed their role in processes of tissue remodelling and wound healing. However, these functional activities seem to be independent on their DPP-IV-like enzymatic activity (Yu *et al*, 2006). Till today, physiological substrates of DPP8 and DPP9 *in vivo* are just speculated (Ajami *et al*, 2008).

1.1.5. Dipeptidyl peptidase 6 and 10

Due to the substitution of the catalytic triade serine residue, DPP6 and DPP10, also referred to as DPPX and DPPY, respectively, are enzymatically inactive members of the DASH group with high structural similarity to DPP-IV. DPP6 and DPP10 were identified in 3 and 2 splice variants, respectively. DPP6 and DPP10 have been shown to regulate the expression and gating of K^+ channels of the Kv4 family by tight binding to the pore-forming subunits. They actually associate with A-type K^+ channel subunits, modulating their transport and function in somatodendritic compartments of neurons (Jerng *et al*, 2004; Li *et al*, 2006; Takimoto *et al*, 2006).

1.2. Non-enzymatic functions of DASH molecules

Most of non-enzymatic functions have so far been attributed to the canonical DPP-IV. However, there is emerging evidence of biologically important non-hydrolytic interactions of some other DASH with their molecular partners as well.

1.2.1. DPP-IV/CD26 as a marker of immune cell activation

DPP-IV has been shown to be identical with CD26 on T-cells and is regarded as a non-lineage antigen, whose expression is regulated by the differentiation and activation status of the immune cells (Aytac *et al*, 2003). Although DPP-IV/CD26 is almost absent on resting B and NK cells, its expression on their surface is effectively induced upon stimulation (Bauvois *et al*, 1999). Moreover, the expression of DPP-IV/CD26 is markedly enhanced following T-cell activation, where DPP-IV/CD26 participates as a coreceptor in the signal transduction processes. Despite its short

cytoplasmic domain of only 6 amino acids, signalling is dependent on further interaction with other molecules.

1.2.2. Interaction with CD45

DPP-IV/CD26 interacts with the membrane-linked protein tyrosine phosphatase CD45, where DPP-IV/CD26 binds to the cytoplasmic domain of CD45. This interaction promotes aggregation of lipid rafts and facilitates colocalization of CD45 to the T-cell receptor signalling molecules p56(Lck), ZAP-70, and TCR zeta, thereby enhancing consequent protein tyrosine phosphorylation of various signalling molecules and eventual interleukin-2 production. Hence, this interaction leads to the amplification of immune responses (Ishii *et al*, 2001; Torimoto *et al*, 1991).

Furthermore, within $CD4^+$ T-cell subsets, DPP-IV/CD26 is preferentially expressed on the memory/helper $CD45RO^+CD29^+$ population that has the ability to respond to recall antigens, to induce B-cell immunoglobulin production and provide MHC-restricted help to the cytotoxic T-cells. Moreover, overexpression of DPP-IV/CD26 is associated with an increase in antigen sensitivity (Morimoto & Schlossman, 1998). DPP-IV/CD26 is functionally capable of transmitting signals relating to T-cell activation through the CD3/T-cell receptor complex or the CD2 pathway (Dang *et al*, 1990), which was also observed in thymocyte activation (Dang *et al*, 1991).

1.2.3. Interaction with mannose-6-phosphate/insulin-like growth factor II receptor

Mannose-6-phosphate/insulin-like growth factor II receptor (M6P/IGFIIR) binds DPP-IV/CD26 via mannose-6-phosphate residues in the carbohydrate moiety of DPP-IV/CD26. Upon T-cell activation, mannose-6-phosphorylation increases, leading to the increased binding to the M6P/IGFIIR and resulting in DPP-IV/CD26 internalisation. DPP-IV/CD26-M6P/IGFIIR interaction has an essential role in T-cell activation and migration (Ikushima *et al*, 2000; Ikushima *et al*, 2002).

1.2.4. Interaction with caveolin-1

Another binding partner of DPP-IV is caveolin-1 in antigen presenting cells. The interaction results in caveolin-1 phosphorylation, leading to activation of nuclear factor NF-κB and following upregulation of CD86. The upregulation of CD86 enhances

subsequent interaction of CD86 and CD28 on T-cells, which induces antigen-specific T-cell proliferation and activation (Ohnuma *et al*, 2004).

1.2.5. Interaction with adenosine deaminase

DPP-IV is also known as an adenosine deaminase (ADA, EC 3.5.4.4) binding protein acting as a signalling coreceptor in the immune system (Kameoka *et al*, 1993; Weihofen *et al*, 2004). This interaction can be executed by not only the enzymatically active DPP-IV homodimer but also by the inactive monomeric DPP-IV. Recent study showed similar interaction of DPP-II and ADA, but with one order of magnitude higher dissociation constant (Sharoyan *et al*, 2008).

Localizing cytosolic ADA to the cell surface by its binding to DPP-IV enhances conversion of adenosine to inosine. This reduces extracellular adenosine-mediated inhibition of T-cell proliferation. Moreover, adenosine has been proposed to have a complex, mostly pro-oncogenic, effect on the biology of tumour cells and tumour behaviour: it stimulates motility and proliferation of tumour cells and induces neoangiogenic activity within the tumour while it suppresses the cell-mediated immune response (Linden, 2006; Tan *et al*, 2004).

1.2.6. Interaction with extracellular matrix

The cysteine-rich domain of DPP-IV is believed to be responsible for binding to the collagen type I and III, and fibronectin (Cheng *et al*, 2003; Loster *et al*, 1995; Piazza *et al*, 1989). The ability of DPP-IV to interact with ECM has a projection in immune regulations (Masuyama *et al*, 1992) and cancer progression. For example, DPP-IV mediated cell-cell and cell-ECM adhesion has been demonstrated to participate in the process of tumour invasion (Cheng *et al*, 2003; Kajiyama *et al*, 2002).

1.2.7. Interaction with plasminogen-2

Plasminogen-2 binds non-covalently to DPP-IV through O-linked polysialylated carbohydrate chains. The resulting plasminogen-2-DPP-IV unit may form a ternary complex with ADA (Gonzalez-Gronow *et al*, 2008), which facilitates conversion of plasminogen to plasmin, eventually leading to augmented degradation of ECM, and thus enhancing metastasising (Figure 3, (Dano *et al*, 2005)). Interestingly, soluble FAP antiplasmin-cleaving enzyme may abrogate such pro-metastasising potential of DPP-IV due to hydrolytic activation of alpha2 antiplasmin (Lee *et al*, 2006).

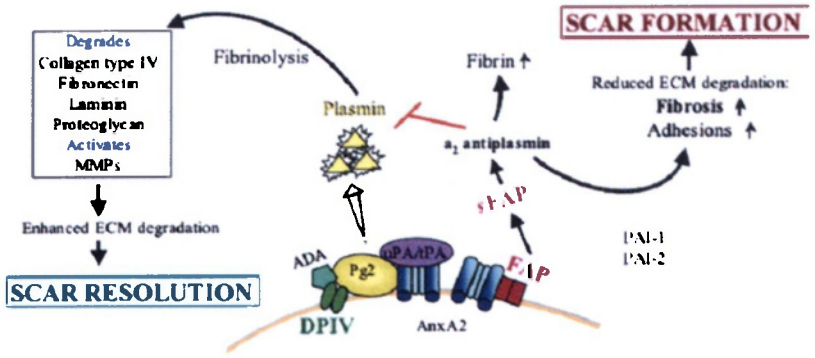

Figure 3. Plasminogen-2 (Pg2) activation to plasmin increases following binding to DPP-IV and ADA. Serum FAP (sFAP) converts alpha2 antiplasmin into a more active form that is better able to inhibit plasmin. FAP associates with the plasminogen receptor annexin 2 (AnxA2), which is also urokinase-type (uPA) and tissue-type (tPA) plasminogen activator receptor. Taken from Gorrell, 2005.

1.2.8. Channel function

As mentioned above, DPP6 and DPP10, both completely devoid of DPP-IV-like enzymatic activity, are involved in the structure and function of voltage-gated K^+ channel of the Kv4 family in neurons (Jerng *et al*, 2004; Takimoto *et al*, 2006).

1.3. Enzymatic functions of DASH molecules

By virtue of their hydrolytic activity "Dipeptidyl peptidase-IV activity and/or structure homologues" process a number of biological mediators and thus cause their inactivation or alter their receptor preference and physiological effect. Examples of proven and hypothetical DASH substrates are listed in Table 1. Thus, regulation of DPP-IV-like enzymatic activity is speculated to be of broad pathogenetic as well as therapeutic potential in many diseases, including cancer. For example, the DPP-IV inhibitors such as Sitagliptin (Januvia, Merck) or Vildagliptin (Galvus, Novartis) that enhance insulin- and reduce glucagon-secretion by preventing the degradation of incretins already represent therapy in type 2 diabetes mellitus (McIntosh, 2008).

Table 1. Examples of biologically active peptides amenable to cleavage by DPP-IV-like enzymatic activity; published in Enclosure 1.

Types of substrates	Examples	Physiological consequence
Incretins and gastrointestinal hormones	Glucagone-like peptide-1, 2 Gastric inhibitory polypeptide Pituitary adenylate cyclase-activating peptide (PACAP)	Inactivation Inactivation Inactivation
Neuropeptides	beta-Casomorphin- 2 Endomorphin- 2 Substance P Neuropeptide Y Peptide YY	Inactivation Inactivation Inactivation, increased susceptibility to proteolytic degradation Changed receptor preference Changed receptor preference
Chemokines	Stromal-cell derived factor-1alpha Eotaxin Monokine induced by gamma interferon (MIG) Interferon-inducible protein-10 Regulated on Activation, Normally T-cell- Expressed and Secreted (RANTES) Macrophage-derived chemokine Macrophage Inflammatory Protein-1beta (MIP-1beta) LD78beta	Inactivation Inactivation Inactivation Inactivation Changed receptor preference Changed receptor preference Changed receptor preference Enhanced activity

1.3.1. DASH in cancer

Deregulation of DASH molecules has been observed in a multitude of tumours (Table 2). Functional studies of the majority of DASH in cancer pathogenesis are still lacking and thus particular biological functions of these molecules as yet remain mostly speculative. However, compared to the most other types of proteases as e,g, urokinase-type plasminogen activator, matrix metallo- and cysteine proteases, participating in the glioma pathogenesis dominantly via cleavage of structural proteins (Binder & Berger, 2002; Levicar *et al*, 2003; Rao, 2003), DASH molecules modify activity of regulatory peptides by limited proteolysis.

Table 2. DASH molecules in neoplasia versus non-tumorous tissue; published in Enclosure 1.

DASH	Expression/ enzymatic activity	Type of cancer
DPP-IV	**Decreased**	Endometrial cancer
		Melanoma
	Increased	Prostate cancer
		Mesothelioma
		Skin basal cell carcinoma Precancerous dermatosis
		Lung papillary adenocarcinoma and squamous cell carcinoma
		Hepatocellular cancer
		Renal cancer
		Astrocytic brain tumors
		Differentiated thyroid papillary and follicular cancer
FAP	**Increased**	Breast cancer
		Melanoma
		Gastric cancer
		Colorectal adenocarcinoma
		Cervical cancer
		Astrocytic brain tumours
		Lung squamous cell carcinoma and adenocarcinoma
DPP-II	**Increased**	Lung squamous cell carcinoma

1.3.2. Mediators-DASH substrates implicated in gliomagenesis

Since the role of DASH in limited proteolysis of biologically active substrates involved in cancerogenesis has been previously extensively reviewed (Busek *et al*, 2004), here we concentrate on mediators specifically important for gliomagenesis.

1.3.2.1. Stromal cell-derived factor-1

Stromal cell-derived factor-1 (SDF-1, CXCL12) is a chemotactic cytokine of the CXC subfamily, existing in 3 splice variants SDF-1alpha, beta and gamma, SDF-1alpha being the most abundant in the brain. SDF-1 is a chemotactic factor for T cell, monocytes, pre-B-cells, and dendritic cells. It induces migration of hematopoietic stem and progenitor cells, and it is thought that they play a crucial role in the homing and mobilization of these cells to/from the bone marrow (Christopherson *et al*, 2002).

SDF-1 exerts its effects via binding to CXCR4, a G-protein-coupled receptor. A series of studies have identified a CXCR7 as another possible receptor for SDF-1, but activation of CXCR7 does not cause Ca^{2-} mobilization or cell migration (Balabanian *et al*, 2005; Burns *et al*, 2006). Upregulation of SDF-1 and CXCR4 was observed in many cancer types (Bajetto *et al*, 2007; Mizokami *et al*, 2004; Scala *et al*, 2006; Stremenova *et al*, 2007; Su *et al*, 2005), where SDF-1/CXCR4 signalling axis promotes tumour growth, enhances tumour neoangiogenesis, participates in tumour metastasising, avoids tumour cell apoptosis and contributes to immunosuppressive networks within the tumour microenvironment (Kryczek *et al*, 2007). The truncated fragment $SDF\text{-}1_{(3\text{-}68)}$, which results from DPP-IV cleavage, acts as an antagonist of the CXCR4 receptor. Therefore, DPP-IV mediated attenuation of SDF-1-CXCR4 axis seems to be an important anti-tumorigenic event (Sato & Dang, 2003). SDF-1 cleavage and inactivation is speculated to be mediated also by DPP8 and may occur *in vivo* upon cell lysis and release of DPP8 or upon the chemokine/receptor complexes internalisation (Ajami *et al*, 2008).

1.3.2.2. Substance P

The neuropeptide substance P (SP) belongs to the tachykinin family of peptide transmitters. It was discovered as a neurotransmitter or cotransmitter in the central and peripheral nervous system (Harrison & Geppetti, 2001), however, its functional potential is much broader. Substance P and closely related neuropeptides neurokinin A, neuropeptide K and neuropeptide gamma are products of alternative splicing of the preprotachykinin A gene transcript (Carter & Krause, 1990). Tachykinins, such as SP, exerts their function through the activation of G-protein coupled receptors NK1, NK2 and NK3. However, NK1 receptor has the highest binding affinity for SP (Regoli *et al*, 1994).

SP exerts pro-proliferative activity in many cellular systems. In malignant glial cells it induces the release of multiple cytokines increasing proliferation, and thus facilitates tumour progression (Palma *et al*, 2000). In addition, the expression of NK1 receptors in peritumoral and tumoral blood vessels suggests a role of SP in tumour neoangiogenesis and vasodilatation (Hennig *et al*, 1995). Since the C-terminus of SP is responsible for appropriate receptor binding and following signalisation, the truncated fragments $SP_{(3\text{-}11)}$ and $SP_{(5\text{-}11)}$ resulting from the DPP-IV cleavage still retain signalling activity, but with decreased binding affinity to the NK1 receptor. However, SP cleavage

by DPP-IV makes it more susceptible for ultimate scavenging by other peptidases, such as aminopeptidase N (EC 3.4.11.2) or neprilysin (EC 3.4.24.11) (Mentlein, 1999).

1.3.2.3 Neuropeptide Y

Neuropeptide Y (NPY) is a 36 amino acid peptide known mainly as a sympathetic cotransmitter and a vasoconstrictor. NPY is also a potent mitogenic and chemotactic factor for vascular smooth muscle and endothelial cells (Zukowska-Grojec *et al*, 1998). Moreover, it has an important role in appetite control, energy homoeostasis, blood pressure, immune responses and behavioural stress responses. NPY also stimulates angiogenesis, capillary growth and restoration of blood vessels after ischemia. The pleiotropic action of NPY is accomplished by the molecular heterogeneity of its Y1 – 6 G-protein coupled receptors. The predominant vascular NPY receptor Y1 is involved mainly in vasoconstriction and vascular smooth muscle cell proliferation. Y2 and Y5 receptors appear to be the main NPY angiogenic receptors. Interestingly, the Y1 receptor can be stimulated only by the full-length peptide $NPY_{(1-36)}$, whereas the shorter, DPP-IV truncated form $NPY_{(3-36)}$, can activate Y2 and Y5 receptors. This suggests a possible role of proline dipeptidyl peptidases in switching the vasoconstrictive activity of NPY to the angiogenic stimulation (Kitlinska *et al*, 2002). NPY actions are with lower efficiency also regulated by DPP8 and DPP9 *in vitro*, but not by DPP-II (Frerker *et al*, 2007).

1.4. Human brain tumours

1.4.1. Astrocytic tumours

The majority of primary tumours of the central nervous system are of astrocytic origin. Malignant astrocytomas belong to the most aggressive intracranial tumours with a high potential of proliferation and progression. The World Health Organisation (WHO) classifies astrocytomas on the basis of histologic features into the pilocytic astrocytomas WHO grade I, diffuse astrocytomas WHO grade II, anaplastic astrocytomas WHO grade III and the most malignant glioblastoma multiforme WHO grade IV. Glioblastomas are the most frequent astrocytic tumours. The vast majority of them develop *de novo* without previous occurrence of the low-grade tumour and thus represent so called primary glioblastomas. Unfortunately, despite of advances in

diagnosis and treatment, patients suffering from high-grade astrocytomas have dismal prognosis, typically with survival shorter than 1 year after the diagnosis. Astrocytomas locally infiltrate surrounding brain tissue, and rarely metastasise extracranially. Histology together with cytogenetics represent together with clinical diagnostics and imaging techniques the major avenue to diagnose, classify and to monitor the disease course (Khwaja, 2007; Louis & Gusella, 1995; Wen & Kesari, 2008).

Genetic changes occurring during the conversion of low-grade astrocytomas to secondary glioblastomas are different from genetic changes characteristic for primary glioblastomas, diagnosed mostly in elderly patients. Mutation of tumour suppressor gene p53 on chromosome 17p and overexpression of plateled-derived growth factor (PDGF) are seen in low-grade astrocytoma WHO grade II. The transition to anaplastic astrocytoma WHO grade III is associated with loss of tumour suppressor genes located on 9p, 13q and 19q chromosomes, which causes inactivation of the p16/cyclin-dependent kinase-4/retinoblastoma (Rb) pathway, important in cell cycle arrest. Further malignant progression to glioblastoma multiforme WHO grade IV is associated with Rb hypermethylation, PDGF receptor amplification and phosphatase and tensin homology gene (PTEN) mutation located on 10q chromosome. On the other hand, the investigation of primary glioblastomas showed silencing of both 10p and 10q chromosomes and amplification of epidermal growth factor receptor (EGFR) located on 7p chromosome (Khwaja, 2007; Louis & Gusella, 1995; Wen & Kesari, 2008).

1.4.2. Meningiomas

Meningiomas are brain tumours derived from the meninges, the membranes surrounding the brain or spinal cord. They account for 20% of all intracranial tumours. Meningiomas are generally benign (90%) and only rarely undergo malignant transformation. They are categorized as benign (WHO grade I), atypical (WHO grade II) and malignant anaplastic meningiomas (WHO grade III) with high risk of recurrence. Meningiomas display a wide variety of histopathological subtypes. Prognosis for more than 80% patients is estimated for five years, but varies depending on tumour type, its location and size, degree of malignancy and proliferation index (Marosi C, 2008; Sanson *et al*, 1992; Woo *et al*, 2008).

The most common genetic alteration, observed up to 60% of sporadic meningiomas, is the loss of the tumour suppressor nuclear factor-2 gene located on 22q chromosome. Other cytogenetic alterations are chromosomal loss of 1p, 3p, 6q, and

14q. Loss of chromosome 10q, PTEN mutation, is associated with increased tumour grade, shortened time to recurrence, and shortened survival. Progression to anaplastic meningioma has been associated with involvement of chromosomal site 17q (Woo *et al*, 2008).

2. AIMS OF THE THESIS

"Dipeptidyl peptidase-IV activity and/or structure homologues" (DASH) are understood to play a critical role in regulating signalling capacity of chemokines, neuropeptides and other extracellular messengers involved in tumorigenesis. Consequently, examination of DASH representation and activity in gliomagenesis has become a major focus of the thesis.

Aim I. To assess the possible association of DASH and receptors of their biologically active substrates in human brain tumours.

For that purpose, expression pattern and enzymatic activity of DASH and expression of NK1 and CXCR4, receptors of substance P and stromal cell-derived factor-1alpha, were determined in bioptic samples of human non-tumorous, astrocytoma and meningioma tissues and in primary cell cultures.

Aim II. To analyse the possible functional role of DASH molecules in the growth properties of transformed glial cells.

Primary cell cultures derived from human high-grade astrocytic tumours were used for studies of relation of DASH phenotype to the cell growth and migration

3. MATERIAL AND METHODS

3.1. Patients, sample preparation and histological characterisation

Tumour specimens were collected from patients undergoing brain tumour resection at the Department of Neurosurgery at Hospital Na Homolce in Prague. Written informed consent was obtained from the patients before their entry into the study according to the guidelines of the institutional Ethical Committee. Tumours were graded in compliance with current WHO Classification Criteria in cooperation with the Department of Pathology at Hospital Na Homolce in Prague. Non-tumorous brain specimens were obtained from patients in whom brain surgery was performed for drug-resistant temporal lobe epilepsy (Table 3). Tissue samples of weight of about 80 – 150 mg clear of macroscopic vessels and necrosis were frozen on solid CO_2 and then stored at -80 °C. Vascularisation and necrotic areas in the tissue samples were scored semi-quantitatively on a 5-titered scale (0 to 4 crosses) and expressed as the average calculated from the individual values of each visual field determined (Table 4). Proliferation activity was approached by quantification of Ki67 antigen expression using mouse monoclonal anti-human (1:50, clone MIB-1, DAKOCytomation, CZ) as a part of routine histological diagnosis.

Table 3. Characteristics of the experimental cohort. Median values are presented; the ranges of values are shown in brackets.

Experimental group	Diagnosis	Number of patients	Age	Gender Male/Female
Control	Drug-resistant epilepsy	15	40 (22 – 72)	7/8
Grade II	Diffuse astrocytoma	9	36 (19 – 67)	4/5
Grade III	Anaplastic astrocytoma	10	42,5 (25 – 68)	8/2
Grade IV	Glioblastoma multiforme	46	60,5 (27 – 78)	23/23
MG grade I	Microcystic meningioma	1	59	0/1
	Meningothelial meningioma	1	79	0/1
	Fibrous meningioma	1	47	0/1
	Transitional meningioma	7	59 (42 – 66)	0/7
MG grade II	Atypical meningioma	4	51 (37 – 71)	1/3

Table 4. Diagnostic characteristics of bioptic samples. Median values are presented; the ranges of values are shown in brackets.

Experimental group	Ki67 [%]	Vascularisation	Necroses
Control	ND	0 (0 – 1)	0
Grade II	3 (1 – 15)	1 (0,5 – 1)	0
Grade III	9 (3 – 50)	2 (1 – 2,5)	0,5 (0 – 0,5)
Grade IV	20 (2 – 40)	2 (1 – 3)	1 (0 – 4)
MG grade I	3 (2 – 5)	0 (0 –1)	0 (0 – 1)
MG grade II	8 (3 – 12)	1 (0 – 1)	0 (0 – 1)

3.2. Primary cell cultures, preparation and cultivation

Primary cell cultures were derived from tumour tissue samples collected from patients undergoing astrocytic tumour resection. A fresh tissue sample was sectioned into small pieces and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma, CZ) supplemented with 20% fetal bovine serum (FBS, Sigma, CZ), 100 g/ml Streptomycin and 100 U/ml Penicillin G (Sigma, CZ). About 5 – 7 days after explantation when outgrowths were observed, explants were removed and medium was replaced with DMEM supplemented with 10% FBS and antibiotics, and cultured under standard conditions at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After reaching confluence, cultures were harvested by Trypsin-EDTA (Sigma, CZ), and subcultured. Cell culture plastic was obtained from Nunc (USA).

3.3. Isolation and quantification of total RNA, real time RT-PCR

Total RNA was isolated using the TriZol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The concentration of total RNA was determined using the RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, USA).

Gene coding region-specific oligonucleotide primers and fluorogenic TaqMan probes for the real time RT-PCR assays of expression of the investigated transcripts were designed with Primer Express software (Applied Biosystems, USA) and were synthesized at Proligo (France) and Applied Biosystems (USA) (Table 5). The expression of DPP-IV, FAP, DPP8, DPP9, NK1, CXCR4 and beta-actin (an internal reference transcript) mRNA was quantified by coupled real time RT-PCR assays using

ThermoScript One-Step System (Invitrogen, USA). The real time RT-PCR assays were run in duplicate in MicroAmp Optical 96-well Reaction Plates on the ABI PRISM 7700 Sequence Detection System operated by the Sequence Detection System software (Applied Biosystems, USA). The reverse transcription was carried out at 58 °C for 30 min and the subsequent PCR amplification included a hot start at 95 °C for 5 min and 45 cycles of denaturation at 95 °C for 15 s and of annealing/extension at 58 °C for 1 min. The threshold cycle (Ct) values of the amplification reactions, represented by the plots of background-subtracted fluorescence intensity (ΔFI) of the reporter dye (6-FAM or VIC) against PCR cycle number were determined with Sequence Detection System software. Target transcript expression was normalized to beta-actin mRNA expression using the ΔCt method and the linearized ΔCt (i.e. $2^{-\Delta Ct}$) was used for comparative purposes (Livak & Schmittgen, 2001).

3.4. DPP-IV-like enzymatic activity biochemical assay in tissues and cell cultures

Tissue samples were homogenized in ice-cold phosphate buffered saline (PBS), pH 6.0, with an Ultra-Turrax homogeniser fitted with a S8N-5G probe (IKA, Germany) and used for assay immediately. Cell cultures were harvested from the culture dish using 0,02% EDTA in PBS. To determine both cell surface and total DPP-IV-like enzymatic activity from one sample of cell suspension, 0.1% Triton X-100 was added into the reaction mixture to permeabilise cells during the continuous monitoring of the enzymatic reaction. DPP-IV-like enzymatic activity was determined by continuous rate fluorimetric assay at 37 °C with H-7-(L-Glycyl-L-Prolylamido)-4-methylcoumarin (H-Gly-Pro-AMC, Bachem AG, Switzerland) in PBS of pH 7.4, and with H-7-(L-Lysyl-L-Alanylamido)-4-methylcoumarin (H-Lys-Ala-AMC, Bachem AG, Switzerland) in citrate/Na_2HPO_4 buffer of pH 5.5 as substrates at the final concentration of 50 μmol/l (Sedo *et al*, 1989). The release of 4-amino-7-methylcoumarin was monitored at 380 nm excitation and 460 nm emission wavelengths (spectrofluorimeter Perkin Elmer LS50B, USA). Selective DPP-IV and DPP8/9 inhibitors (Jenkins PD, Jones DM, Szelke M: DP-IV-serine protease inhibitors PCT Int. Appl. 1995, WO95/15309, gift from Ferring Pharmaceuticals, UK) were used at the final concentration of 250 nmol/l.

3.5. DPP-IV-like enzymatic activity histochemistry

DPP-IV-like catalytic histochemistry was performed according to Lojda (Lojda, 1981) in 10 μm cryostat sections cut at –20 °C (Bright Instrument Company Ltd., Huntingdon, UK). The sections were fixed in a 1:1 mixture of acetone and chloroform for 2 min at 4 °C and were incubated with Gly-Pro-4-methoxy-betanaphtylamide (0.83 x 10^{-3} mol/l) as a substrate (Bachem, Switzerland) and Fast Blue B in PBS of pH 7.4 at 4 °C. In staining controls, the DPP-IV substrate was omitted from the incubation medium.

3.6. Immunohistochemistry and immunocytochemistry

The detection of DPP-IV/CD26, FAP, DPP8, DPP9, NK1, CXCR4 and GFAP was performed in cryostat sections, and cells grown on coverslips, preincubated in 3 % of heat-inactivated FBS for 20 min, followed by overnight incubation at 4 °C with the respective primary antibodies: mouse monoclonal anti-human CD26 (1:100, clone M-A261, Acris, Germany), mouse monoclonal anti-human FAP (1:200, clone F11-24, Alexis Biochemical, USA), rabbit polyclonal anti-human DPP8 and DPP9 (1:150, Abcam, UK), rabbit polyclonal anti-human NK1 (1:200, Abcam, UK), rabbit polyclonal anti-human CXCR4 (1:200, Acris, Germany), goat polyclonal anti-human CXCR4 (1:200, Abcam, UK), and mouse monoclonal anti-human GFAP (1:100, Exbio, CZ). This was followed by incubation with anti-mouse- (1:200, Sigma, USA) or anti-rabbit- (1:200, Sigma, USA) -IgG-FITC conjugates respectively, anti-goat-IgG-TRITC (1:100, Jackson ImmunoResearch, CZ) or anti-mouse AlexaFluor 488 (1:1000, Invitrogen, USA). In staining controls, the primary antibodies were omitted from the incubation medium. The catalytic enzyme histochemistry, the immunohisto- and immunocytochemistry sections were mounted in antifading Gel/Mount (Biomeda corp., USA), and examined by transmission or fluorescence microscopy (Axiophot, Zeiss-Opton, Germany; Olympus, CZ respectively). Staining intensity was scored semi-quantitatively on a subjectively determined 5-titered scale (0 to 4 crosses) relative to the negative controls, averaged from 10 segments of 2 – 3 non-sequentional histological sections.

3.7. Cell proliferation assay

Cell proliferation assays were performed in 96 well plates. Cells were seeded at a density of 4.000 cells per well in 10% FBS in DMEM, harvested every 2 – 3 days in triplicates and counted on Coulter Counter Z (Beckman, Germany). Population

doubling time was determined from the least square regression fit of the exponential part of the growth curve.

3.8. Cell migration assay

Cells were seeded at a density of 60.000 cells per insert ("transwell migration" inserts for 24 well plates, pore size 8 μm, Becton Dickinson, USA) and cultured for 24 hours in either serum free DMEM or DMEM with 1% FBS used as chemoattractant in the lower compartment. Non-migrated cells were removed using a cotton swab, cells on the lower side of the insert were fixed with 5% glutaraldehyde in PBS and stained with methylene blue (5g/l in 50% v/v ethanol/H_2O). Migration was quantified as a mean of 4 inserts, each counted in 5 microscopic fields at magnification 200x.

Functional studies and phenotypisation were performed in cells of the same passage.

3.9. Flow Cytometry

Immunophenotypisations were performed by a flow cytometer FACS Canto II (BD Biosciences, USA) with Diva software for acquisition and FlowJo (TreeStar Inc., USA) for data evaluation. For this purpose, cells were harvested using 0,02% EDTA in PBS and fixed in 2% paraformaldehyde. Rat monoclonal anti-human CD26 conjugated to phycoerythrin (R&D Systems, UK) was used to detect DPP-IV/CD26.

3.10. Total protein concentration

Total protein concentration was assayed according to Lowry (Lowry *et al*, 1951).

3.11. Statistical analysis

Statistica 8.0 sofware (StatSoft, Inc. Tulse, OK, USA) was used. Differences between groups were evaluated with Kruskal-Wallis or Mann-Whitney tests; correlations were analysed by means of Spearman correlation coefficient.

Table 5. Primers and TaqMan probes used for the real time RT-PCR quantitation of expression of the investigated transcripts

Transcript	GeneBank Accession No.	Sequences and final concentration of primers and TaqMan probes	
DPP-IV	NM_001935	Forward primer:	5'-TGGAAGGTTCTTCTGGGACTG-3', 200 nmol/l
		Reverse primer:	5'-GATAGAATGTCCAAACTCATCAAATGT-3', 200 nmol/l
		TaqMan probe:	5'-(6-FAM)CACCGTGCCCGTGGTTCTGCT(TAMRA)-3', 200 nmol/l
FAP	NM_004460	Forward primer:	5'-TGCCACCTCTGCTGTGC-3', 200 nmol/l
		Reverse primer:	5'-GAAGCATTCACACTTTTCATGGT-3', 200 nmol/l
		TaqMan probe:	5'-(6-FAM)TGCATTGTCTTACGCCCTTCAAGAGTTC(TAMRA)-3',200nmol/l
DPP8	NM_197960	Forward primer:	5'-CCTGTCACCGAGGGCTTAA-3', 400 nmol/l
		Reverse primer:	5'-AACCCTGAAGATATCTGACCTCTG-3', 400 nmol/l
		TaqMan probe:	5'-(6-FAM)CAGGTGGAAGGACTCCAATATCTAGCTTCTCG(TAMRA)-3', 200nmol/l
DPP9	DQ_417928	Forward primer:	5'-GGTGGAGATCGAGGACCAG-3', 400 nmol/l
		Reverse primer:	5'-TGGCCACCTTGAACACCT-3', 400 nmol/l
		TaqMan probe:	5'-(6-FAM)AAGCCATACTTCTCGGCCACGAACTG(TAMRA)-3', 200 nmol/l
NK1	NM_001058	Forward primer:	5'-CAGTGGTGAACTTCACCTATGCT-3', 400 nmol/l
		Reverse primer:	5'-GATGTATGATGGCCATGTACCTATC-3', 400 nmol/l
		TaqMan probe:	5'-(6-FAM)TCCACAACTTCTTTCCCATCGCCG(TAMRA)-3', 200 nmol/l
CXCR4	NM_001008540	Forward primer:	5'-CATGGGTTACCAGAAGAAACTGA-3', 400 nmol/l
		Reverse primer:	5'-GACTGCCTTGCATAGGAAGTTC-3', 400 nmol/l
		TaqMan probe:	5'-(6-FAM)CACCTGTCAGTGGCCGACCTCCT(TAMRA)-3', 200 nmol/l
beta-Actin	NM_001101	Forward primer:	5'-CTGGCACCCAGCACAATG-3', 200 nmol/l
		Reverse primer:	5'-GGGCCGGACTCGTCATAC-3', 200 nmol/l
		TaqMan probe:	5'-(VIC)AGCCGCCGATCCACACGGAGT(TAMRA)-3', 200 nmol/l

4. RESULTS

4.1. DASH molecules and receptors of their substrates NK1 and CXCR4 in human non-tumorous brain, astrocytic tumours and meningiomas

4.1.1. DPP-IV-like enzymatic activity

In astrocytic tumour tissues, DPP-IV-like enzymatic activity correlated with the extent of necrosis ($R = 0.731$, $p < 0.01$), vascularisation ($R = 0.755$, $p < 0.01$) as well as with the proliferation activity characterised by Ki67 expression ($R = 0.314$, $p < 0.05$). In line with that, DPP-IV-like enzymatic activity in bioptic samples increased along with the WHO grade of malignancy in astrocytomas ($R = 0.763$, $p < 0.01$), as well as in meningiomas ($R = 0.549$, $p < 0.05$) (Figure 4). The median activity observed in glioblastomas (WHO grade IV) was significantly higher than that one in non-tumorous brain ($p < 0.01$). DPP-IV-like enzymatic activity in meningiomas was comparable or even higher than that in glioblastoma.

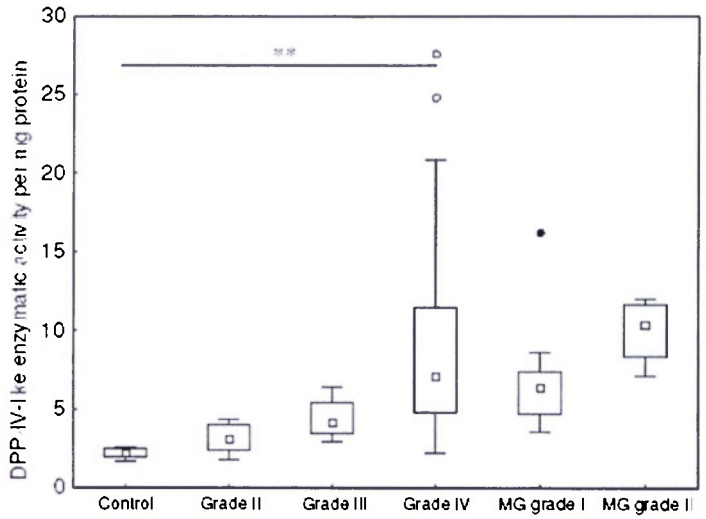

Figure 4. DPP-IV-like enzymatic activity in non-tumorous brain (Control), astrocytic tumours grade II-IV (Grade II-IV) and meningiomas grade I-II (MG grade I-II) measured by fluorimetric assay using H-Gly-Pro-AMC as a substrate. Squares: Medians; Boxes: middle 25-75% of measured values; Bars: Minimal resp Maximal values, o: remote values; •: extreme values; double asterisk: $p < 0.01$, Kruskal-Wallis test.

Only negligible DPP-IV-like enzymatic activity in non-tumorous as well as in tumorous human brain tissues was detected at the acidic pH 5.5 using H-Lys-Ala-AMC (data not shown), which suggests that DPP-II does not significantly contribute to the overall DPP-IV-like enzymatic activity.

To assess the relative participation of individual enzymatically active DASH members on the whole DPP-IV-like enzymatic activity in the tissue samples, inhibition studies using selective inhibitors of DPP-IV and DPP8/9 were performed (Figure 5). Using an inhibitor with 60-fold higher potency for DPP-IV than for DPP8/9, the DPP-IV-like enzymatic activity was more profoundly inhibited in astrocytic tumours of all grades and meningiomas compared to non-malignant brain (Figure 5A). In contrast, no such differential inhibition was seen using an inhibitor possessing 50-fold higher potency for DPP8/9 than for DPP-IV (Figure 5B), suggesting the major proportion of canonical DPP-IV on the DPP-IV-like enzymatic activity increment observed in high-grade gliomas.

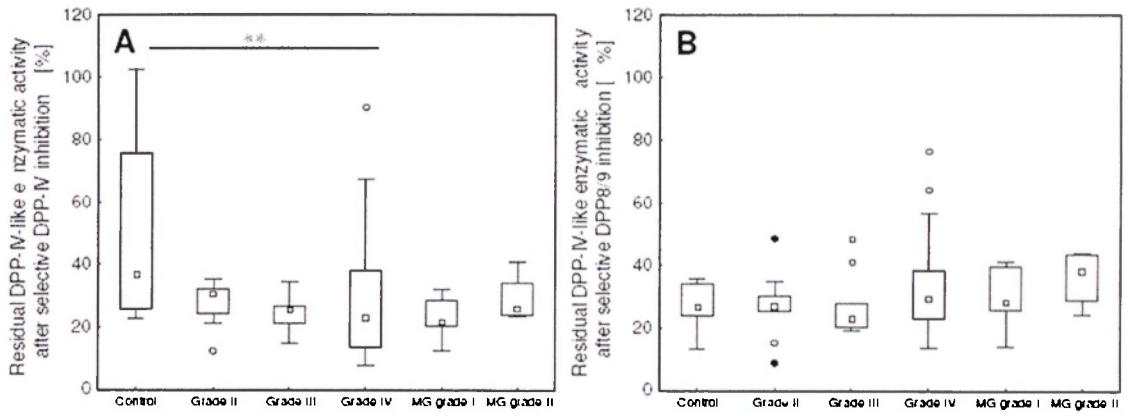

Figure 5. Residual DPP-IV-like enzymatic activity after selective DPP-IV (A) and DPP8/9 (B) inhibition in non-tumorous brain (Control), astrocytic tumours grade II-IV (Grade II-IV) and meningiomas grade I-II (MG grade I-II). Squares: Medians; Boxes: middle 25-75% of measured values; Bars: Minimal resp Maximal values; o remote values; •: extreme values; double asterisk: $p < 0.01$, Kruskal-Wallis test.

4.1.2. Expression of DASH, NK1 and CXCR4 transcripts

Expression of plasma membrane DASH molecules DPP-IV and FAP, and intracellular DPP8 and DPP9, together with receptors of their biologically active substrates, NK1 and CXCR4, were assayed. In contrast with DPP-IV-like enzymatic activity, expression of DPP-IV, FAP, DPP8 and DPP9 transcripts did not display significant differences neither among individual WHO grades of astrocytomas nor meningiomas (Figure 6). Positive correlation between DPP-IV and FAP mRNA expression was found in the whole experimental cohort ($R = 0.497$, $p < 0.01$). Moreover, in glioblastoma tissue, the residual enzymatic activity after DPP-IV inhibition inversely correlated with DPP-IV mRNA expression ($R = -0.468$, $p < 0.01$).

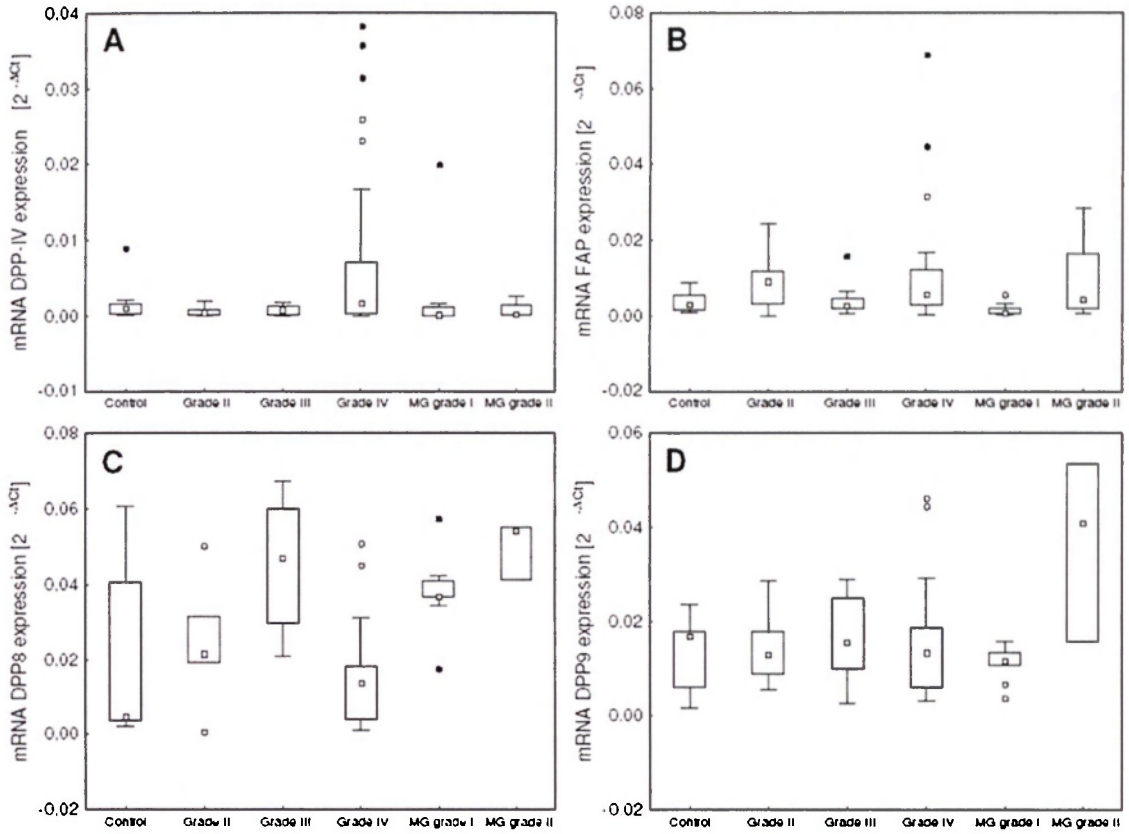

Figure 6. The mRNA expression of DPP-IV (A), FAP (B), DPP8 (C), DPP9 (D) determined by real time RT-PCR normalised to human beta-actin in non-tumorous brain (Control), astrocytic tumours grade II-IV (Grade II-IV) and meningiomas grade I-II (MG grade I-II). Squares: Medians; Boxes: middle 25-75% of measured values; Bars: Minimal resp. Maximal values; o: remote values, •: extreme values.

As to the expression of receptors of DASH catalytic partners, no statistically significant differences suggesting tumour grade dependence were observed in case of NK1 receptor (Figure 7A). Nevertheless, the positive correlations between NK1 and FAP mRNA expression ($R = 0.381$, $p < 0.05$) in glioblastoma, and NK1 and DPP-IV mRNA expression ($R = 0.547$, $p < 0.05$) in meningiomas were observed. In contrast with NK1, transcription of another important receptor involved in gliomagenesis, CXCR4, was significantly higher compared to controls in glioblastomas grade IV ($p < 0.01$, Figure 7B). Interestingly, positive correlations between CXCR4 and DPP-IV transcription in glioblastoma tissue ($R = 0.532$, $p < 0.01$), and between CXCR4 and FAP in meningiomas ($R = 0.609$, $p < 0.05$) were observed. The expression of CXCR4 mRNA positively correlated with DPP-IV-like enzymatic activity in all astrocytomas ($R = 0.417$, $p < 0.01$). Moreover, in glioblastoma tissue, the residual enzymatic activity after DPP-IV inhibition inversely correlated with CXCR4 mRNA expression ($R = -0.309$, $p < 0.05$).

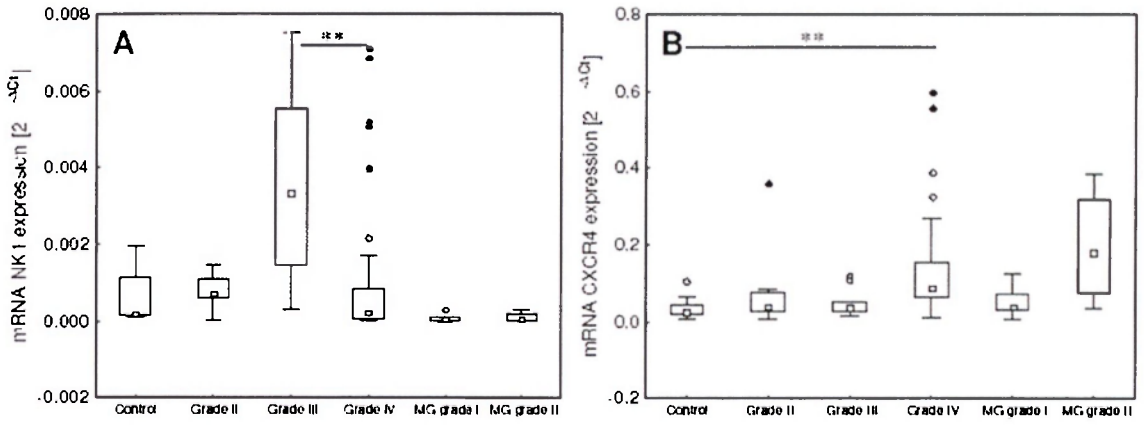

Figure 7. The mRNA expression of NK1 (A) and CXCR4 (B) receptors determined by real time RT-PCR normalised to human beta-actin in non-tumorous brain (Control), astrocytic tumours grade II-IV (Grade II-IV) and meningiomas grade I-II (MG grade I-II). Squares: Medians; Boxes: middle 25-75% of measured values; Bars: Minimal resp Maximal values; o: remote values, •: extreme values; double asterisk: $p < 0.01$, Kruskal-Wallis test.

4.1.3. Expression of DASH, NK1 and CXCR4 protein

To quantify and characterise the protein distribution of DASH molecules and receptors of their substrates within the tissue, immunohistochemical approach was used in context with DPP-IV-like enzymatic activity histochemistry (Table 6, Figure 8). In non-tumorous tissue samples, DPP-IV-like enzymatic activity, as well as expression of DPP-IV and FAP, and CXCR4 receptor was very low. The staining intensity for DPP8 and DPP9 was more intensive. DPP-IV-like enzymatic activity together with expression of other DASH and NK1 and CXCR4 receptors increased along with the rising degree of malignancy. Meningiomas demonstrated higher degree of DPP-IV-like enzymatic activity and DPP8 and DPP9 expression, on the other hand, the expression of DPP-IV and FAP, and CXCR4 was very weak.

Intra-specimen distribution of staining intensity of all markers was highly variable in most tumour samples. Their increased expression was mostly detected in solitary segments of the capillary/vascular bed and also in the perivascular cells with expansion to both vascular and parenchymal tissue compartments in more positive samples.

Table 6. DPP-IV-like enzymatic activity histochemistry (DPP-IV-like) and immunohistochemistry of DPP-IV, FAP, DPP8, DPP9 and receptors NK1 and CXCR4 in non-tumorous brain tissues (Control), astrocytomas grade II-IV (Grade II-IV) and meningiomas. The positivity was scored semi-quantitatively on a 5-titered scale (0 to 4 crosses); ND – not determined.

Group	DPP-IV-like	DPP-IV	FAP	DPP8	DPP9	NK1	CXCR4
Control	0/+	0/+	0/+	++	++	+	0/+
Grade II	++	+++	0/+	ND	ND	+	+++
Grade III	++	++++	+++	ND	ND	++++	++
Grade IV	++++	+++	+++	++++	++++	+++	++++
Meningioma	+++	+	+	++++	+++	ND	+

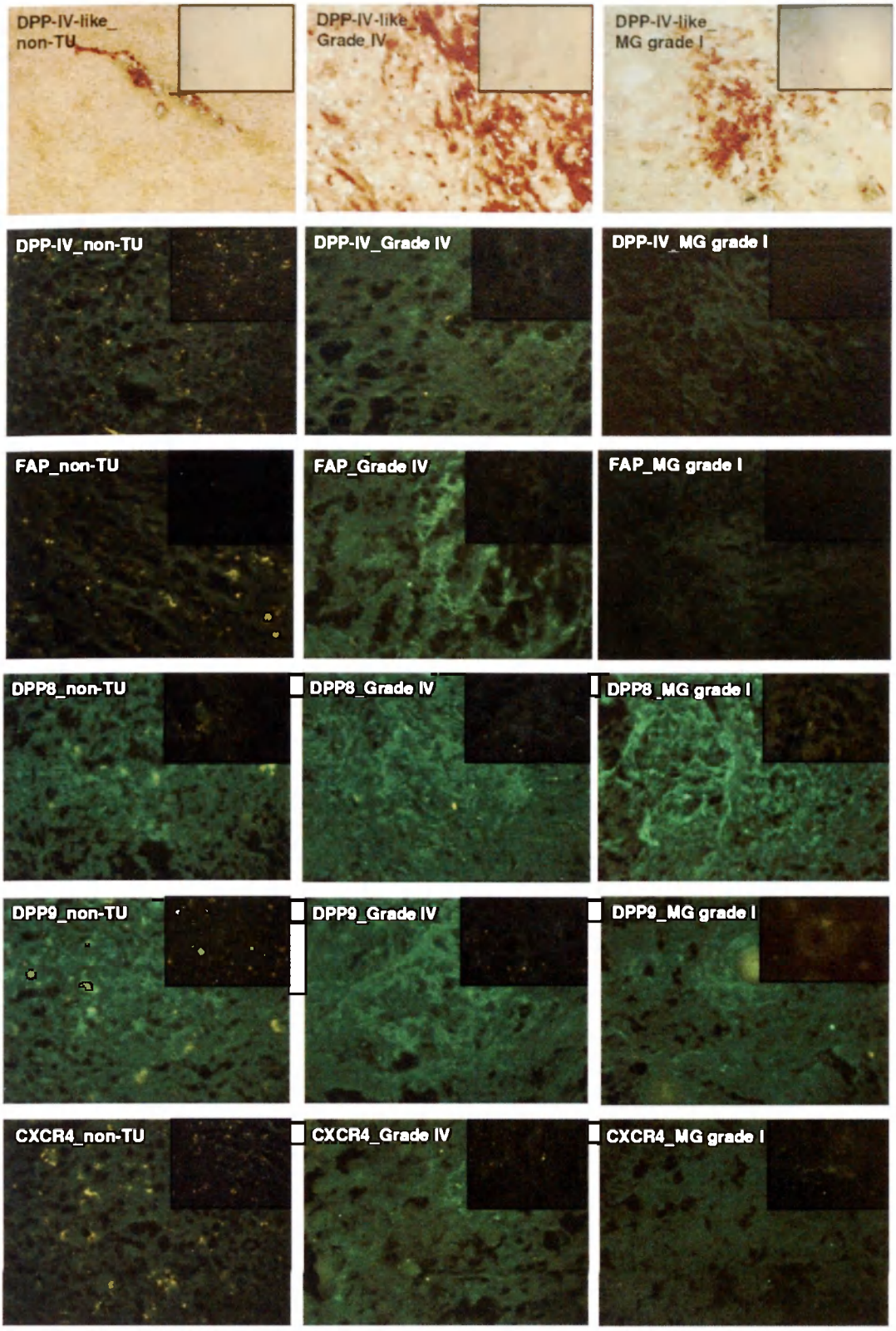

Figure 8. Representative pictures of DPP-IV-like enzymatic activity (DPP-IV-like) determined by enzymatic activity histochemistry, and expression of DPP-IV, FAP, DPP8, DPP9 and CXCR4 detected by immunohistochemistry in non-tumorous brain (non-TU), glioblastomas (Grade IV) and meningiomas grade I (MG grade I) as described in Material and Methods; insets = staining control.

Together, analysis of DASH expression pattern in tissue samples revealed that:

(i) The major part of DPP-IV-like hydrolytic activity in non-malignant brain tissues as well as in astrocytomas and meningiomas irrespective of the WHO grade is carried by intracellular DPP8/9

(ii) The increment of DPP-IV-like enzymatic activity associated with high-grade of malignancy in astrocytomas is mostly an attribute of plasma membrane localised DASH, canonical DPP-IV with possible participation of FAP

(iii) Expression of DPP-IV and CXCR4 receptor seems to be associated within the astrocytic tumour microenvironment

4.2. Expression of DASH, NK1 and CXCR4 and growth properties of glioblastoma primary cell cultures

4.2.1. Expression pattern of DASH, NK1 and CXCR4

Seventeen primary cell cultures were derived from high-grade astrocytoma tissue samples collected from the patients undergoing tumour resection, included in our experimental cohort. After 2 – 5 days, primary explants with star-like shaped cells in outgrowth zone were visible (Figure 9).

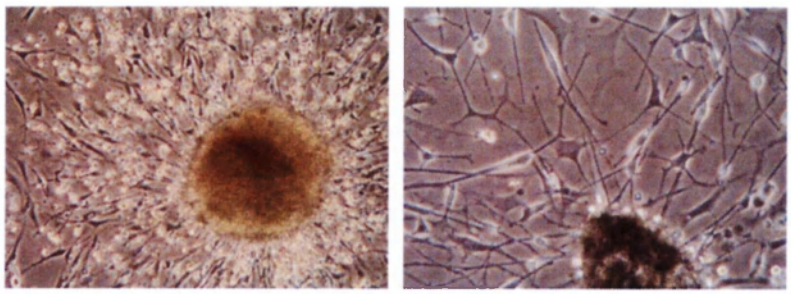

Figure 9. Primary explant culture from glioblastoma multiforme tissue. Explant and early-stage outgrowth after 2 – 5 days of cultivation in 20% FBS in DMEM supplemented with antibiotics. Phase contrast, original magnification 200x.

Individual primary cell cultures displayed diverse morphology. Cultured cells were of polygonal shape growing in cobblestone pattern (Figure 10A), polymorphic (Figure 10B) or spindle-shaped (Figure 10C). Cell cultures were morphologically heterogeneous in early passages (1-6), reaching some degree of morphological uniformity in the later ones (10 and more).

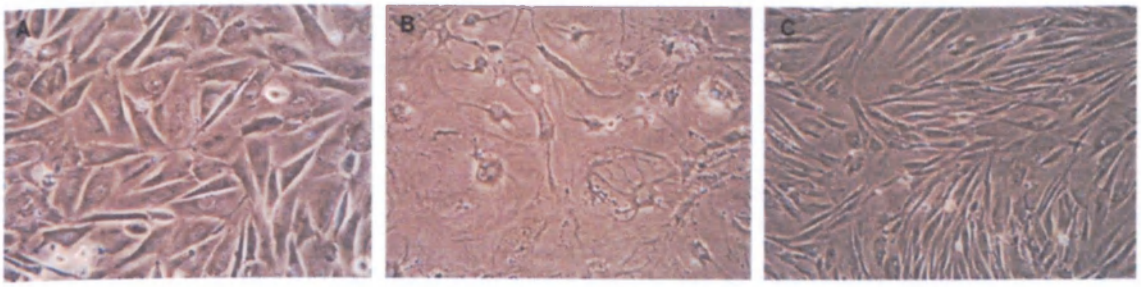

Figure 10. Differing morphology of primary cell cultures derived from glioblastoma multiforme. Polygonal shaped cells (A), polymorphic cells (B) and spindle-shaped cells (C). Phase contrast, original magnification 200x.

To confirm the astrocytic origin of the primary cell cultures, presence of glial fibrillary acidic protein (GFAP), a marker of astrocytic cells, was assayed. Most of the cultures were weakly positive, with varying distribution and staining intensity (Figure 11).

Figure 11. The GFAP expression in primary cell cultures derived from glioblastoma multiforme.

The DPP-IV-like enzymatic activity varied among different primary cell cultures as well as changed among passages during the culture propagation. Indeed, corresponding variation of the DASH expression pattern was observed also on the transcriptional and protein level. Similarly as in the tumour tissue, tight positive correlation between DPP-IV and FAP ($R = 0.670$, $p < 0.01$) transcripts expression was observed. Unlike DPP-IV transcript, FAP mRNA expression correlated ($R = 0.370$, $p < 0.05$) with the cell surface DPP-IV-like enzymatic activity. In contrast to the positive correlation of DPP-IV-like enzymatic activity and CXCR4 mRNA observed in astrocytoma tissues, the inverse correlation of both parameters occurred in primary cell cultures ($R = -0.528$, $p < 0.01$ for the cell surface, and $R = - 0.636$, $p < 0.01$ for the total DPP-IV-like enzymatic activity). Immunocytochemical detection indicated varying expression of canonical DPP-IV and CXCR4 among individual primary cell cultures

with heterogeneous intra-culture distribution with some cells co-expressing both molecules (Figure 12).

NK1 mRNA and protein expression was detected in all primary cell cultures, however, it was not associated with other studied molecules.

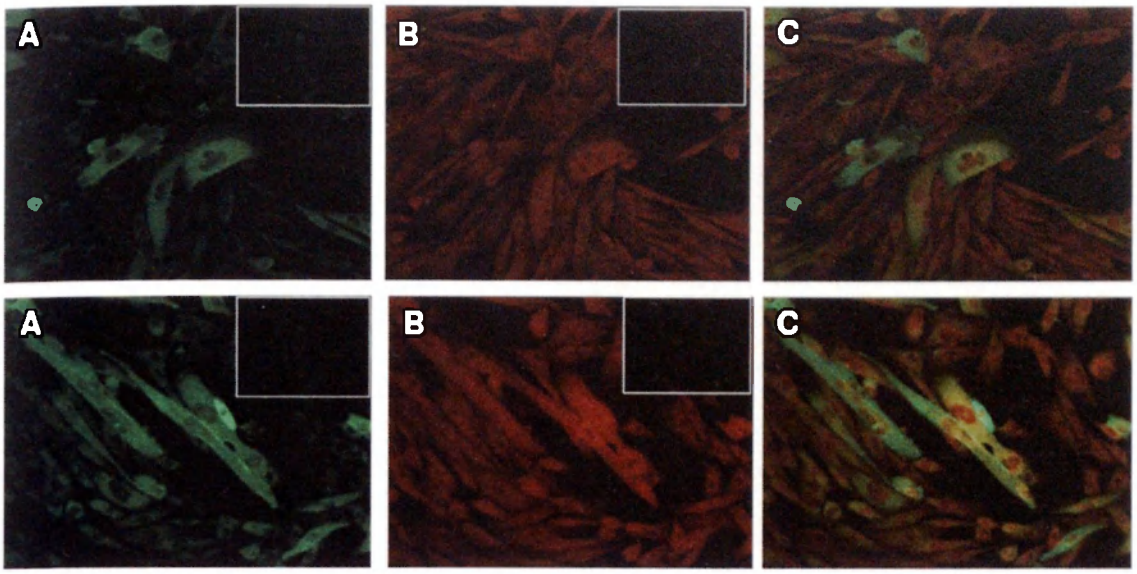

Figure 12. Representative pictures of DPP-IV and CXCR4 expression in primary cell cultures. DPP-IV (A), CXCR4 (B), DPP-IV/CXCR4 (C) expression. Fluorescence microscopy, magnification 200x; insets = staining control.

4.2.2. DASH and growth properties of glioblastoma derived primary cell cultures

Doubling time of primary cell cultures (Figure 13B) positively correlated with the cell surface DPP-IV-like enzymatic activity ($R = 0.452$, $p < 0.05$). Additionally, „slow growing cultures" (doubling time higher than the arbitrary threshold 100 hours) exhibited significantly higher cell surface DPP-IV-like enzymatic activity ($p < 0.05$; Figure 13C) and FAP mRNA expression ($p < 0.05$, data not shown) than the „fast growing cultures" (doubling time lower than the arbitrary threshold 100 hours). Furthermore, the inherent inter-passage variability of primary cell cultures was exploited to examine the association of changes of DASH phenotype with alterations of growth properties in the course of culture propagation. DPP-IV-like enzymatic activity of primary cell cultures differed in early (6th) and late (12th) passages, which was typically accompanied by inverse changes of population doubling time (Figure 13A). Decrease of the DPP-IV-like enzymatic activity during the cell culture propagation was

associated with acceleration of the cell proliferation in 3 out of 6 cultures (P5, P7, P8). In another 2 cultures (P10A, P13), increased DPP-IV-like enzymatic activity was associated with slower proliferation. One of the cultures (P10B) displayed increased proliferation although increased DPP-IV-like enzymatic activity was observed. However, in the 19th passage of this particular culture, steep decrease of its DPP-IV-like enzymatic activity associated with enhanced cell growth was detected (data not shown).

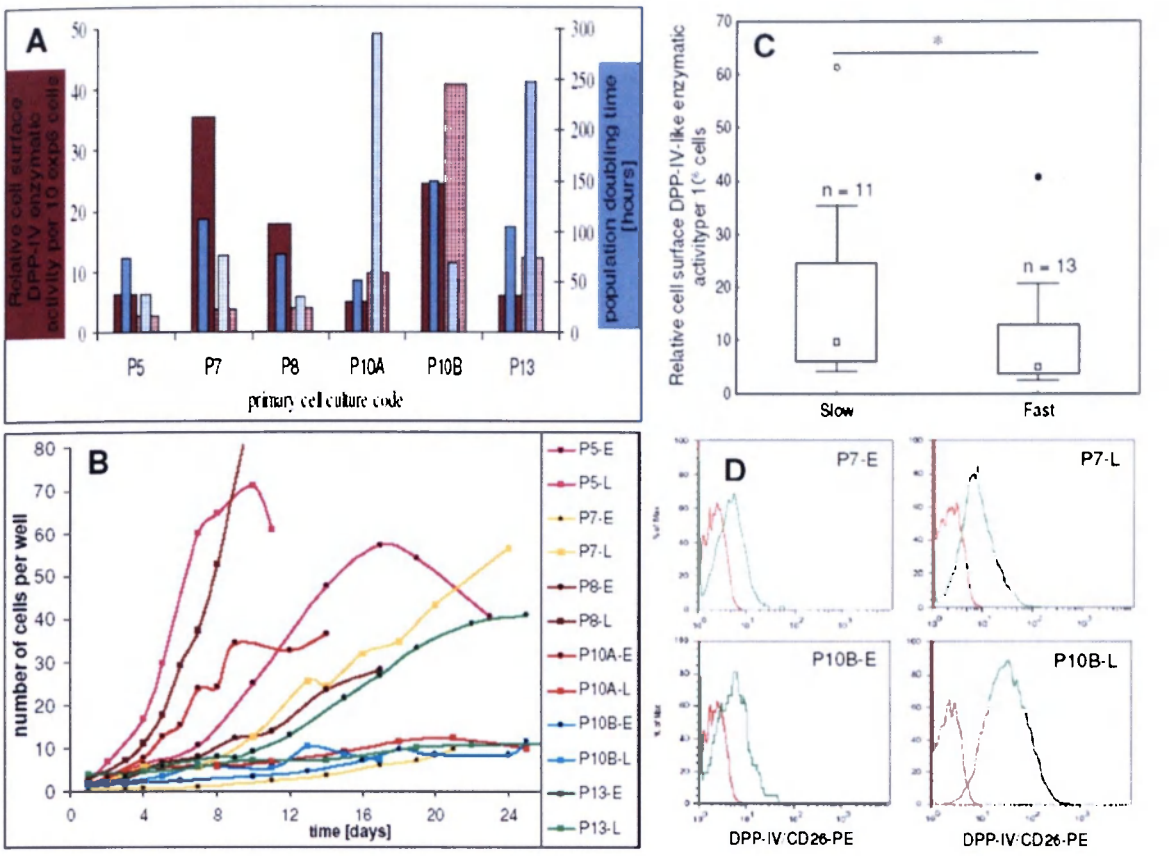

Figure 13. (A) Alteration of cell culture proliferation associated with changes of cell surface DPP-IV-like enzymatic activity. Full boxes: early (6th) passage; shaded boxes: late (12th) passage. In 5 out of 6 primary cell cultures, higher DPP-IV-like enzymatic activity was associated with slower proliferation. **(B) Growth curves** of primary cells cultures. **(C) Cell surface DPP-IV-like enzymatic activity in "slow growing cultures" and "fast growing cultures".** Squares: Medians; Boxes: middle 25-75% of measured values; Bars: Minimal resp Maximal values; o: remote values; •: extreme values; asterisk: $p < 0.05$; Mann-Whitney test. **(D) Representative histograms of changes in DPP-IV/CD26 expression** in P7 and P10B primary cell cultures in early (E) and late (L) passage. Green curves: DPP-IV/CD26 positive cells; Red curves: staining control.

Further, cell migration of the primary cell culture elements was assayed in serum free medium (SFM) and medium supplemented with 1% fetal bovine serum (1% FBS), the later one used as a chemoattractant. Cell migration varied among individual passages during the culture propagation. Surprisingly, some of the cell cultures migrated more readily in SFM conditions than in the medium supplemented with 1% FBS (Figure 14A). Migration of glioma primary cell cultures seemed to be independent on their DPP-IV-like enzymatic activity and the DASH expression pattern (Figure 14B,C).

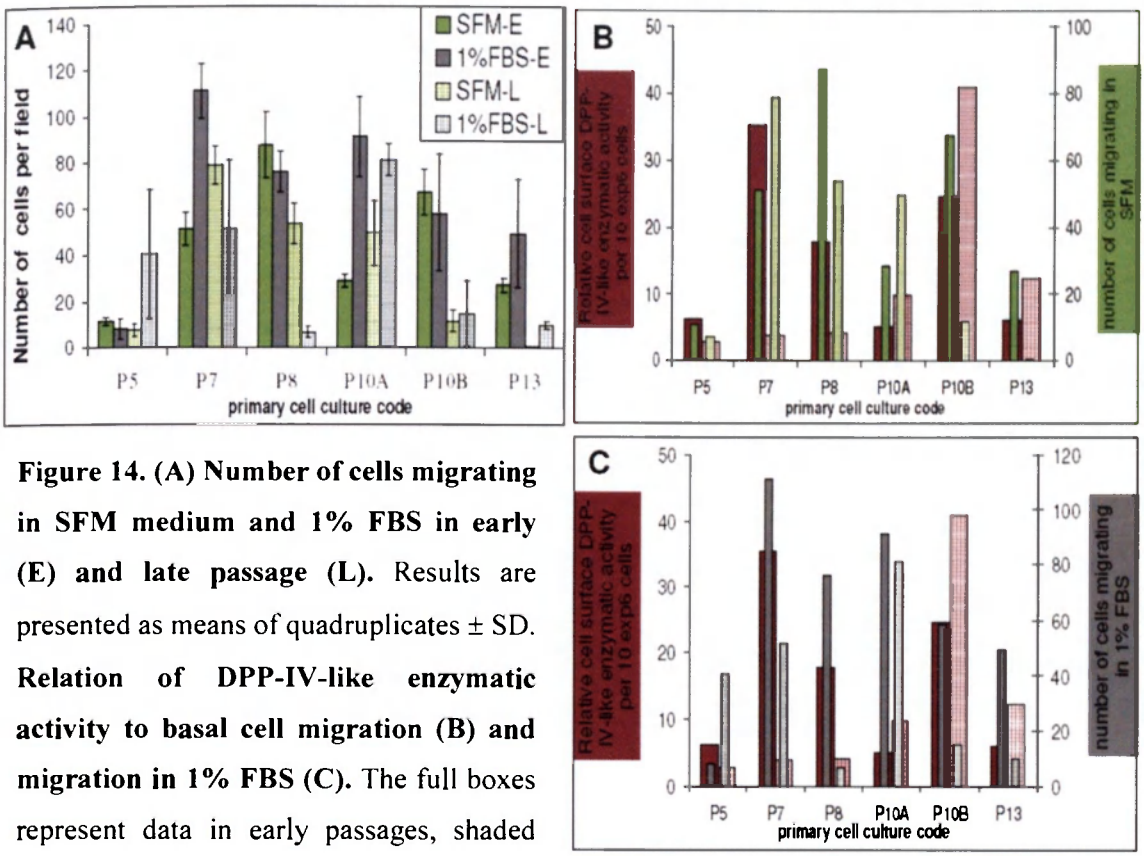

Figure 14. (A) Number of cells migrating in SFM medium and 1% FBS in early (E) and late passage (L). Results are presented as means of quadruplicates ± SD. **Relation of DPP-IV-like enzymatic activity to basal cell migration (B) and migration in 1% FBS (C).** The full boxes represent data in early passages, shaded boxes in later ones.

Together, analysis of DASH expression pattern and growth properties of primary cell cultures revealed that:

(i) Association of increased cell surface DPP-IV-like enzymatic activity with slower cell growth demonstrated that DPP-IV and FAP might hamper proliferation of transformed glial cells

(ii) DASH expression and DPP-IV-like enzymatic activity of primary cell cultures had no effect on the cell migration

5. DISCUSSION

An imbalance of extracellular proteolysis has been demonstrated to be a general hallmark of malignancy. Changed proteolytic equilibrium, affecting processing of structural and regulatory proteins within the tumour microenvironment, has multiple downstream projections including regulation of neovascularisation, modulation of cancer cell proliferation, migration and invasion. Altered DPP-IV-like enzymatic activity has been observed in numerous tumours and consequently several roles have been proposed for DPP-IV in cancer pathogenesis. However, the overall DPP-IV-like activity frequently encompasses hydrolytic potential of several co-expressed "Dipeptidyl peptidase-IV activity and/or structure homologues" (DASH). It is becoming evident that it is more likely the complex expression pattern of DASH molecules in context with available bioactive substrates and their receptors, which have to be considered to interpret the results of functional studies (Sedo *et al*, 2008).

In the present study, we aimed to approach a possible role of DASH molecules in gliomagenesis. To that point, in the first step of our analyses, we compared DASH phenotype of astrocytic tumours with both non-tumorous human brain tissue and meningiomas, the later as a model of brain tumour of non-astrocytic origin. Then, for the purpose of functional studies, we prepared glioblastoma derived primary cell cultures as a model enabling to study the relation of DASH expression pattern to the cell growth and migration.

Our results suggest that the major part of DPP-IV-like hydrolytic activity in non-malignant human brain but also in astrocytomas and meningiomas is carried by DPP8 and DPP9. Although the rise of DPP-IV-like enzymatic activity associated with the astrocytoma WHO grade of malignancy might be in the same extent caused by the upregulation of DPP8 and DPP9, however, its major fraction seems to be an attribute of DPP-IV, with possible participation of FAP. In contrast with astrocytic tumours, contribution of DPP-IV and FAP on the whole DPP-IV-like hydrolytic activity is very low in meningiomas.

Consistent positive correlation of DPP-IV and FAP was observed in glioblastomas as well as in meningiomas and non-tumorous tissue. This observation fits well with ours and other authors' results, demonstrating co-regulation of both molecules in cell lines of differing origin (Enclosure 2) (Wesley *et al*, 2004). Though in a different

cellular context, the identification of DPP-IV-FAP heterodimers also indirectly points to the possible functional cooperation of both enzymes (Ghersi *et al*, 2002; Ghersi *et al*, 2006).

Increased DPP-IV expression and activity has so far been reported in multiple types of cancer, as e.g. thyroid, skin and prostate and in some white blood cell-derived malignancies. In contrast, decreased DPP-IV expression was observed in melanoma and endometrial adenocarcinoma (reviewed in Enclosure 1). It has been suggested that the balance of proteolytic processing of growth regulators by DPP-IV-like enzymatic activity may have profound effects upon the local tumour microenvironment. To that point, in contrast with the cytosolic DPP8 and DPP9, the transmembrane DPP-IV and FAP, possessing extracellularly exposed active site, are likely actors of such regulations.

Considering our results from tissue biopsies, upregulation of DPP-IV is associated with high degree of malignancy and thus seems to act as a pro-oncogenic molecule. To verify this hypothesis on the level of transformed astrocytic cells, experiments with primary cell cultures derived from glioblastomas were implemented. Surprisingly, increased cell surface DPP-IV-like enzymatic activity was associated with the deceleration of cell growth. It might argue for an anti-proliferative role of DPP-IV and/or FAP; the later one also in virtue of positive correlation of DPP-IV-like enzymatic activity with FAP mRNA expression. Indeed, as we described previously in transfected glioma cell lines, overexpression of DPP-IV leads to inhibition of cell growth and accumulation of cells in the G2/M phase of the cell cycle (Busek *et al*, 2008). One possible explanation is that DPP-IV degrades or inactivates cell growth promoting mediators and thus, on the level of transformed glial cells acts as an anti-oncogenic molecule.

There is seeming contradiction of observations suggesting DPP-IV serves as an "enemy" for transformed cells themselves, while it could still be beneficial to other cell populations within the tumour environment, with a resultant net pro-oncogenic effect (Enclosure 2). Indeed, in a complex tissue microenvironment fine-tuning of protease activity and peptides availability is more tightly regulated than in a test tube or in a cell culture *in vitro*. Hence, *in vitro* results do not adequately predict the potential impact of peptides proteolysis *in vivo* in conditions such as tumour progression. Considering the above mentioned multifunctional nature of DASH molecules, it does not surprise that their impact for the local proteolytic equilibrium, affecting multiple regulatory proteins

functions and the complexity of their mechanisms alerts to the serious risk of adverse effects. The objections for DPP-IV targeting in clinical settings might come from two possible mechanisms: (i) Structurally related targets – DASH molecules - might be involved in pathogenesis of different diseases and/or separate metabolic pathways. Since biological functions have not yet been assigned to all DASH, the undesirable consequences of unselective inhibition have to be expected. (ii) Individual DASH molecule may be involved in multiple physiological processes throughout the body. Thus, „doublespecific" inhibitors, targeting specific DASH molecules in an appropriate cell, could address both abovementioned objections (Enclosure 1).

6. CONCLUSIONS

The aims of the Thesis were accomplished and our results led us to the following conclusions:

- DPP-IV-like enzymatic activity is associated with the decrease of growth of glioblastoma derived primary cell cultures *in vitro*
- DPP-IV-like enzymatic activity in human astrocytoma tissue increases along with the WHO grade of malignancy
- The major part of the DPP-IV-like enzymatic activity in non-tumorous brain as well as in astrocytoma and meningioma biopsies is probably derived from the intracellular DPP8/9
- Unlike to the meningiomas, the increase of the DPP-IV-like hydrolytic activity found in the high-grade astrocytomas is probably an attribute of the plasma membrane DPP-IV and FAP
- Receptors of biologically active mediators of DASH molecules, NK1 and CXCR4, are upregulated in glioblastoma tissue
- Expression of DPP-IV and CXCR4 in glioblastoma significantly correlates and thus DPP-IV mediated degradation of pro-oncogenic chemokine SDF-1 might be compensated by the increase of CXCR4 in the glioblastoma tissue
- Although the biological function of DPP-IV in glioblastoma tissue remains elusive, its parallel involvement in more cellular programs within the tumour is probable. Thus, estimation of its resulting net "pro-" or "anti-oncogenic" effect should respect its contextual role within the tumour microenvironment

7. FURTHER PERSPECTIVES

Our studies suggested link of DASH molecules to the pathogenesis of astrocytomas and may hold promise for further exploitation in the diagnostic and therapeutic arena (Enclosure 1). Nevertheless, our results also raised new questions awaiting further efforts. To that point, we noted striking heterogeneity of DASH expression pattern and activity among individual patients with glioblastoma WHO grade IV. To explain that, we tried to find an association between DASH phenotype and clinical characteristics, such as mitotic index, expression of Ki67, mutation of p53, cytogenetic profile etc. However, to collect representative data essential for definitive assessments, further expansion of experimental cohort is required. Our preliminary observations demonstrate, that the positive correlation of CXCR4 and DPP-IV, observed in glioblastoma tissue, is missing in the patients surviving more than one year (long-term survivors), compared to these who survived less than 6 months after the surgery (short-term survivors). This may suggest that not the absolute values, but effective tuning of CXCR4 and DPP-IV balance within the glioblastoma microenvironment might favour glioblastoma progression. Thus, our further work aims to confirm this hypothesis and to approach molecular mechanisms of the putative CXCR4–DPP-IV co-regulation.

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9. ENCLOSURES

I. **Dipeptidyl peptidase-IV and related molecules: markers of malignancy?**
Sedo A, **Stremenova J**, Busek P, Duke-Cohan. *Expert Opinion on Medical Diagnostics* 2008; 2(6): 677-689. ***IF – newly established journal, invited review***

II. **Dipeptidyl peptidase-IV enzymatic activity bearing molecules in human brain tumors-good or evil?**
Busek P, **Stremenova J**, Sedo A. *Frontiers in Bioscience* 2008; 13: 2319-2326. ***IF 2.99; Times Cited: 1***

III. **Expression and enzymatic activity of dipeptidyl peptidase-IV in human astrocytic tumours are associated with tumour grade.**
Stremenova J, Krepela E, Mares V, Trim J, Dbaly V, Marek J, Vanickova Z, Lisa V, Yea Ch, Sedo A. *International Journal of Oncology* 2007; 31(4): 785-792. ***IF 2.29; Times Cited: 3***

IV. **Dipeptidyl peptidase-IV and related molecules: their role in growth of primary cell cultures derived from human brain tumors.**
Stremenova J, Busek P, Balaziova E, Sromova L, Krepela E, Sedo A. *International Journal of Molecular Medicine* 2008; 22(Suppl 1): S60-60. Conference Report - Poster: 13. World Congress on Advances in Oncology and 11th International Symposium on Molecular Medicine; 9-11.10.2008, Hersonissos, Greece. ***IF 1.85***

V. **Modulation of substance P signaling by dipeptidyl peptidase-IV enzymatic activity in human glioma cell lines.**
Busek P, **Stremenova J**, Krepela E, Sedo A. *Physiological Research* 2008; 57(3): 443-449. ***IF 1.51***

I.

Dipeptidyl peptidase-IV and related molecules: markers of malignancy?

Sedo A, Stremenova J, Busek P, Duke-Cohan.

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Expert Opinion

Dipeptidyl peptidase-IV and related molecules: markers of malignancy?

Aleksi Šedo[†], Jarmila Stremenová, Petr Bušek & Jonathan S Duke-Cohan
[†]*Charles University in Prague, Laboratory of Cancer Cell Biology of the 1st Faculty of Medicine, U Nemocnice 5, 128 53, Prague 2, Czech Republic*

Background: The dipeptidyl peptidase-IV (DPP-IV) family has outgrown its humble origins as a simple enzymatic activity cleaving dipeptides from peptides with an accessible N-terminal penultimate proline with no clear role in metabolism. It is now understood to play a critical role in regulating signaling capacity of chemokines, neuropeptides and other extracellular messengers in addition to playing direct roles by means of non-enzymatic interactions to regulate the local extracellular proliferative environment. Consequently, examination of DPP-IV family representation and activity in immune and oncogenic processes has become a major focus. *Objectives*: To review the evidence for DPP-IV family members as markers of malignancy. *Methods*: Overview of published data. *Results/conclusion*: The DPP-IV family, which is probably linked directly to the pathogenesis of cancer, holds significant promise for exploitation in the diagnostic arena.

Keywords: cancer, DASH, dipeptidyl peptidase, fibroblast activation protein, marker

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1. Introduction

Dipeptidyl peptidase-IV (DPP-IV, EC 3.4.14.5, CD26) was first described by Hopsu-Havu and Glenner in the human liver in 1966 [1]. Subsequently, its activity and expression were detected in almost all tissues and cell types, with particularly strong expression in the kidney and on the surface of endothelial cells [2]. The development of monoclonal antibodies quickly resolved that the molecule previously identified as the T-cell activation antigen CD26 was the source of the DPP-IV activity [3], and confirmed that expression was constitutive in expressing cells, with the special exception of T cells, where expression is very low in the resting state and is rapidly upregulated upon activation [4]. This led early on to the possibility that CD26 may be a marker of leukaemic T cells. This serine-type protease is enzymatically active as a homodimer composed of two 110 kDa subunits, typically expressed as a glycosylated transmembrane type II protein [2]. It exhibits a rare enzymatic activity that cleaves two N-terminal amino acids from peptides and small proteins with Pro or Ala in the penultimate position (**Figure 1**) that are otherwise relatively resistant to proteolytic attack by most proteases [2,5]. Interestingly, the proline residue at the penultimate position seems to be an important proteolytic checkpoint conserved in multiple biologically active peptides [6]. Many chemokines/cytokines and lymphokines are susceptible to DPP-IV cleavage (**Table 1**, for a review, cf. [2,7]), which led to the view that the enzyme serves a dual function: changing the functional activity of its substrates and also acting as a gatekeeper to general proteolytic degradation.

In addition to the 'canonical' DPP-IV/CD26, several molecules that exhibit similar enzymatic activity and/or varying degree of structural homology have been discovered and are sometimes referred to as 'dipeptidyl peptidase-IV activity

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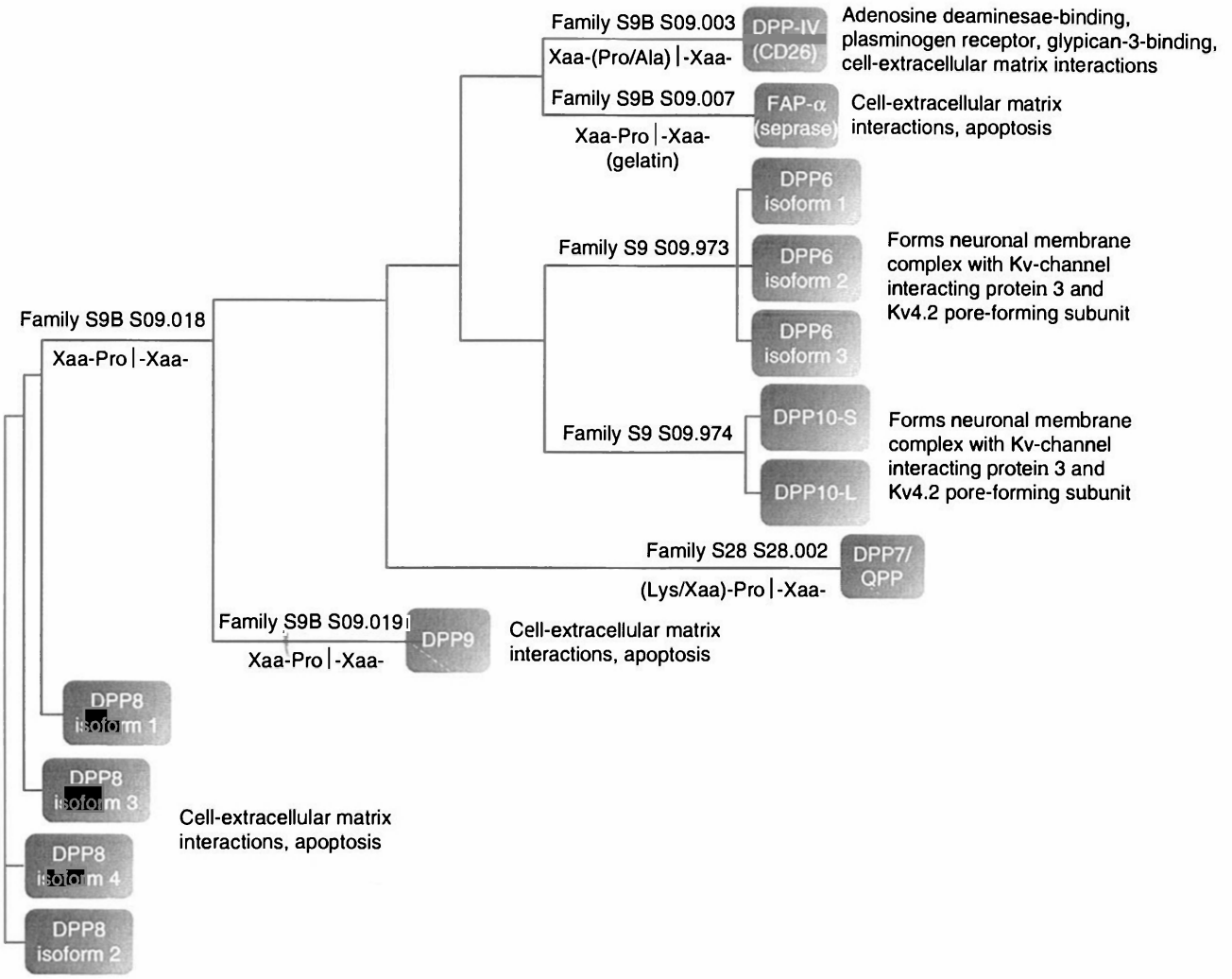

Figure 1. A phylogenetic tree of DPP-IV family protein sequence relationships. Distances represent relationship predicted after alignment using the CLUSTAL2 algorithm followed by PHYLIP analysis (Genbank accession numbers: DPP4: NP_001926; DPP6.1: NP_570629; DPP6.2: NP_001927; DPP7/DPPII/QPP: NP_037511; DPP8.1: NP_569118; DPP8.2: NP_060213; DPP8.3: NP_932064; DPP8.4: NP_932065; DPP9: NP_631898; DPP10-S: NP_001004360; DPP10-L: NP_065919; FAP-alpha/seprase: NP_004451). The blue boxes indicate enzymatically active proteins and the brown boxes indicate members with no dipeptidyl peptidase-IV enzymatic activity. All proteins are members of the SC clan representing enzymes with an α/β-hydrolase fold; family S9B represents the dipeptidyl peptidase-IV family, whereas peptidase family S28 contains the DPP-IV-sequence divergent DPP7/DPPII/QPP. DPP6 and DPP10 are classified as S9 homologs with no peptidase activity. The Family descriptor is followed by the unique family identifier under which the general substrate specificity is indicated [120]. To the right of each colored box are listed known interactions/functionalities believed to be *independent* of enzymatic activity.

and/or structure homologs' (DASH) [8]. These comprise enzymatically active fibroblast activation protein-alpha (FAP, also referred to as seprase), DPP-II (also referred to as DPP7 or quiescent cell proline dipeptidase [QPP]), DPP8 and DPP9. Figure 1 provides an overview of the family relationships. Given the striking overlap of substrate specificity, and to some degree catalytic specific activity, it is clear that tissue-specific regulation and expression of DASH members and their substrates is critical. In addition, through examination of the interactions of the ectodomains of the various DASH, it is clear that the ability to influence tumor development may reside in domains unrelated to the peptidase activity. The critical role of the domains outside the catalytic site is revealed by the high conservation of primary structure between various DPP-IV-related molecules within mammalian species in addition to the very short cytoplasmic domains, which are too short either to interact with intracellular signaling adapters or to signal directly themselves [9]. These properties imply that the transmembrane and non-catalytic extracellular domains have evolved to interact with a variety of other functional moieties. This latter aspect is confirmed by the recruitment of DPP-IV-related molecules to complex

Table 1. Examples of biologically active peptides amenable to cleavage by dipeptidyl peptidase-IV enzymatic activity.

Types of substrate	Examples	Physiological consequence
Incretins and gastrointestinal hormones	Glucagone-like peptide-1, 2	Inactivation
	GIP	Inactivation
	PACAP	Inactivation
Neuropeptides	Beta-casomorphin – 2	Inactivation
	Endomorphin – 2	Inactivation
	Substance P	Inactivation, increased susceptibility to proteolytic degradation
	Neuropeptide Y	Changed receptor preference
	Peptide YY	Changed receptor preference
Chemokines	Stromal cell-derived factor-1alpha	Inactivation
	Eotaxin	Inactivation
	MIG	Inactivation
	IP-10	Inactivation
	RANTES	Changed receptor preference
	MDC	Changed receptor preference
	MIP-1beta	Changed receptor preference
	LD78beta	Enhanced activity

*For more comprehensive information, see [2].
GIP: Gastric inhibitory polypeptide; IP-10: Interferon-inducible protein-10; MDC: Macrophage-derived chemokine; MIG: Monokine induced by gamma interferon; MIP-1beta: Macrophage inflammatory protein-1beta; PACAP: Pituitary adenylyl cyclase-activating peptide; RANTES: Regulated on activation, normally T-cell-expressed and secreted.

protein assemblies in both lymphocytes [10] and neurons [11] and to the invadopodia of metastatic cells [12].

1.1 Dipeptidyl peptidase-IV

Owing in large part to the co-identity of DPP-IV with CD26, defined initially as a human T lymphocyte activation antigen, there has been a focus over the years on a possible role for CD26 in aberrant immunoregulation potentiating the development of multiple sclerosis, tissue graft rejection, inflammatory rheumatic diseases and inflammatory bowel disease, among others [13-15]. This interest has extended to tumorigenesis as DPP-IV enzymatic activity is often significantly altered not only in the tumor microenvironment (**Table 2**), but also in the systemic circulation (**Table 3**). In contrast to proteases involved in cancer development and progression as the executors of tissue degradation leading to increased metastatic capability, DPP-IV operates mostly as a regulatory molecule, modifying biological activity of local peptide growth factors and chemokines. In addition to its cell surface localization, DPP-IV lacking the transmembrane domain is present in significant amount in body fluids such as serum, cerebrospinal fluid, synovial fluid, seminal plasma and urine, making the greatest contribution to the total DPP-IV-like activity in these materials [16]. The soluble DPP-IV is thought to originate predominantly from lymphocytes, endothelial and epithelial cells by the proteolytic cleavage of the membrane-bound DPP-IV/CD26. The protease liberating this soluble form has not been characterized and it is not even certain yet whether this proteolysis occurs endosomally during vesicle transfer to the plasma membrane or whether it happens extracellularly.

The enzymatic activity of DPP-IV with regard to tumor development may well turn out to be quite complex. First, there exists the possibility that for tumors abnormally secreting high levels of peptide messengers, subclones may develop that are selected for high or low levels of DPP-IV activity depending on whether those peptides are inhibitory or stimulatory for the developing transformed cells. A further layer may then be added if the clipping of the N-terminal dipeptide of a substrate alters its receptor preference [17]. This scenario may be considered cell autonomous, but DPP-IV may also exert effects upon the environment around the tumor cell, either through modulating the chemokine/cytokine environment with subtle alterations on a tumor stem cell niche [18], or even aiding immune evasion by modifying local chemokine gradients resulting from recognition of the transformed cells by the immune system.

The non-enzymatic interactions of DPP-IV also offer up some intriguing possibilities regarding development of transformed cells. Proteolysis by fibrinolysin of extracellular matrix is a critical aspect of metastatic invasion [19]. It has been shown recently that cell-surface DPP-IV can recruit plasminogen to

Table 2. Dipeptidyl peptidase (DPP)-IV and related molecules in neoplasia versus non-tumorous tissue.

DASH	Expression/enzymatic activity	Type of cancer	Method of detection	Ref.
DPP-IV	Decreased	Endometrial cancer	Immunodetection	[61,62]
		Melanoma	Enzymatic assay, immunodetection	[49,60]
	Increased	Prostate cancer	Enzymatic assay, immunodetection	[68,69]
		Mesothelioma	Immunodetection	[66]
		Skin basal cell carcinoma	Immunodetection	[74]
		Precancerous dermatosis		
		Lung papillary adenocarcinoma and squamous cell carcinoma	Enzymatic assay, immunodetection	[71,72]
		Hepatocellular cancer	Enzymatic assay	[73]
		Renal cancer	Immunodetection	[67]
		Astrocytic brain tumors	RT-PCR, enzymatic assay, immunodetection	[65]
		Differentiated thyroid papillary and follicular cancer	RT-PCR, enzymatic assay, immunodetection	[63,64,104-109]
FAP	Increased	Breast cancer	Enzymatic assay, immunodetection	[34,110-112]
		Melanoma	Enzymatic assay, immunodetection	[86]
		Gastric cancer	RT-PCR, enzymatic assay, immunodetection	[87,88]
		Colorectal adenocarcinoma	Enzymatic assay, immunodetection	[34,90,91]
		Cervical cancer	Immunodetection	[89]
		Astrocytic brain tumors	RT-PCR, immunodetection	[65[
		Lung squamous cell carcinoma and adenocarcinoma	Enzymatic assay, immunodetection	[34]
DPP-II	Increased	Lung squamous cell carcinoma	Enzymatic assay	[82]

DASH: Dipeptidyl peptidase-IV activity and/or structure homologs; FAP: Fibroblast activation protein-alpha; RT-PCR: Reverse transcriptase polymerase chain reaction.

form a complex involving a sialylated O-linked glycosyl chain on the plasminogen [20]. For tissue-invading tumors, the surface expression of CD26 may thus be quite important, whereas for the leukemias, DPP-IV expression may be an equivocal event. Even more intriguingly, the plasminogen may form a ternary complex with adenosine deaminase [20], already known to interact with the ectodomain of CD26 [21]. The membrane proximal presence of adenosine deaminase may be critical in at least two respects. First, proliferating cells tend to accumulate high extracellular concentrations of adenosine, which may be toxic, particularly to lymphoid cells, depending on the relative expression of the A2A and A2B adenosine receptors that stimulate intracellular adenylyl cyclase activity generating cyclic adenosine monophosphate, and the A1 and A3 receptors that inhibit adenylyl cyclase activity [22]. Adenosine deaminase catalyzes the breakdown of adenosine to inosine, which can then participate in the nucleotide salvage pathway. Accordingly, the presence of adenosine deaminase complexed to CD26 may have a subtle influence on the proliferative potential of a cell. The relative expression of adenosine receptors on a tumor cell may well direct the outgrowth of tumor subclones with differing levels of cell-surface DPP-IV.

More recently, glypican-3 has been identified as a CD26-interacting protein [23]. The most intriguing aspect of this report is that glypican-3 is essentially a form of heparan sulfate proteoglycan GPI (glycosyl phosphatidylinositol)-linked to the cell membrane as opposed to being an integral part of the basement membrane. It is normally expressed in embryonic developing tissues and is significantly downregulated in adult tissue – there is evidence that through an undefined regulatory event it can modulate the activity of a large number

Table 3. Plasma/serum dipeptidyl peptidase (DPP)-IV-like enzymatic activity in cancer patients.

DASH	Enzymatic activity	Type of cancer	Method of detection	Ref.
DPP-IV	Decreased	Gastric cancer	Enzymatic assay	[94,113,114]
		Pancreatic cancer	Enzymatic assay	[94]
		Acute lymphocytic leukaemia	Enzymatic assay	[114]
		Lymphosarcoma		
		Hodgkin's disease		
		Oral squamous cell cancer	Enzymatic assay	[98,115-117]
			Immunodetection	
		Colorectal cancer	Immunodetection	[96]
		Differentiated thyroid cancer	Enzymatic assay	[118]
	Increased	Colorectal cancer	Enzymatic assay	[97]
		Hepatic cancer	Enzymatic assay	[73,95,119]
		Bile duct cancer	Enzymatic assay	[94]
DPP-II	Increased	Hepatic cancer	Enzymatic assay	[73]
		Oral cancer	Enzymatic assay	[98]

DASH: Dipeptidyl peptidase-IV activity and/or structure homologs.

of growth-signaling peptides, including insulin-like growth factor 2 (IGF2). The expression of glypican-3 is strongly associated with induction of apoptosis. It appears that the interaction of glypican-3 with CD26 is a critical step in the induction of apoptosis, and that this may be mediated in part by a direct inhibition of the DPP-IV enzymatic activity of CD26. If this is confirmed by further reports, then the heterogeneity of DPP-IV expression in many tumor types (described further below) may reflect the state of glypican-3 expression, where low levels of glypican-3 would not influence DPP-IV expression but high levels would select for tumor subclones with low DPP-IV/CD26 levels. This report also defines glypican-3 as the first natural regulator of DPP-IV enzymatic activity, and points to this as a fruitful area of investigation.

The concept of natural regulators of DPP-IV function has wider implications. In fact, several reports had attributed DPP-IV activity to the proteins attractin and NAALADase (*N*-acetylated alpha-linked acidic dipeptidase), which are now known not to have any intrinsic activity [24,25]. Despite being purified to apparent homogeneity, attractin preparations were actually contaminated with a small amount of CD26. This confusion arose in part because of the high DPP-IV enzymatic activity, which was out of all proportion to the apparent contamination by CD26. Although variations in DPP-V activity have been reported depending upon glycosylation, one further possible explanation for this observation is that attractin may be a natural potentiator of DPP-IV activity. Several recent reports have identified the CUB ('for complement C1r/C1s, Uegf, Bmp1') domain of procollagen C-protease enhancers as being critical for the enzymatic activity of tolloid proteases. The CUB structural motif of ~ 110 amino acid residues is present in multiple developmentally regulated plasma membrane-associated proteins and executes diverse biological roles, including complement activation, developmental patterning, tissue repair, axon guidance and angiogenesis, cell signaling, fertilization, hemostasis, inflammation, neurotransmission, receptor-mediated endocytosis and tumor suppression. On the basis of structural commonalities, protease activity enhancement is likely to be a function of all proteins containing a complete CUB domain [26,27]. It is then reasonable to examine whether the CUB domain of attractin regulates DPP-IV enzymatic activity. As an interesting aside, the DPP-IV activity of plasma is ~ 200-fold greater than that of cerebrospinal fluid (CSF) where attractin is circulating at high levels in the plasma, but is essentially absent in normal CSF [28-30].

1.2 Dipeptidyl peptidase-II

In contrast to DPP-IV, which was initially discovered as an ectopeptidase activity, DPP-II was initially identified as an intracellular activity. Despite the passing of more than 40 years since its discovery, the physiological role of DPP-II has still not been elucidated [31]. Its ubiquitous distribution argues for a general role for DPP-II as a housekeeping protein. Its presumed lysosomal localization and the limited substrate range (tripeptides) suggest it may be involved in the final steps of peptide degradation. Moreover, DPP-II is the only lysosomal DASH molecule cleaving postproline peptide bonds and probably plays an important role in intracellular protein metabolism. In addition, DPP-II contains a leucine zipper motif, which may endow it with capacity to execute some of its biological functions through non-hydrolytic protein–protein binding interactions. Despite a report of an

antiapoptotic role for DPP-II in leukocytes, the likely interactions and mechanism remain unclear [32].

1.3 Fibroblast activation protein-alpha

Fibroblast activation protein-alpha, the closest structural relative of DPP-IV, has proved to be an important player in oncogenic processes and represents a promising therapeutic target [33]. The expression of FAP is more restricted than that of DPP-IV and DPP-II and has been identified in activated fibroblasts, during wound healing in tumor stroma, in hepatic stellate cells in the cirrhotic liver, in some embryonic tissues as well as in certain epithelial cancer cells, but not in most normal human tissues [34,35]. Moreover, a secreted form of FAP is present in human plasma and is identical to the circulating antiplasmin-cleaving enzyme [36]. In addition to its possible role in the processing of biologically active peptides by means of its DPP-IV-like enzymatic activity, FAP has also been demonstrated to degrade endoproteolytically components of the extracellular matrix [34]. Probably owing to the relatively high degree of sequence similarity, in cells co-expressing DPP-IV and FAP these two chains may form a heterodimer [35]. It is not known whether the enzymatic activity of the heterodimer and its effect upon extracellular matrix is substantially altered on comparison with that of co-expressed homodimers of DPP-IV/CD26 and FAP, respectively.

1.4 DPP8, DPP9

Although there is evidence that DPP8 and DPP9 are involved in the processes of cell growth and migration, cancer-related functional as well as clinical studies are still absent. Nevertheless, studies so far suggest that the roles of DPP8 and DPP9 in the regulation of cell-extracellular matrix interactions are independent of the DPP-IV activity [37].

1.5 Enzymatically inactive DASH molecules

Two DPP-IV homologs, DPP6 and DPP10, are enzymatically inactive owing to the substitution of the active site Ser residue. Both DPP6 and DPP10 have been shown to be an integral component of a complex assembly in neuronal somatodendrite membranes, which acts as a K voltage-gated channel [38,39]. Perhaps reflecting the limited tissue-specific distribution, there is no evidence that these proteins are involved in tumorigenesis.

1.6 DASH peptidase activity is the pre-eminent functionality

As discussed above, a wide array of biological functions independent of the hydrolytic activity have been proposed for DASH molecules. The main and best-understood function, however, is the hydrolytic processing of local and endocrine mediators that modifies their signaling potential. In this way, DPP-IV participates in the regulation of glycemia by means of the cleavage of incretins [40,41] and influences mobilization, homing and engraftment of hematopoietic stem cells by modulating the stromal cell-derived factor (SDF)-1alpha-CXCR4 axis [42]. In clinical practice, DPP-IV represents a validated therapeutic target and its inhibitors (sitagliptin, vildagliptin) have gained extensive use for the treatment of diabetes [40,41,43]. DPP-IV inhibition also holds promise for increasing the efficacy of hematopoietic stem cell transplantation [42,44]. Similarly, DPP-IV enzymatic activity processes several biologically active peptides implicated in the control of cell growth, migration and invasion. Limited proteolysis of DASH substrates such as substance P, neuropeptide Y, as well as the chemokines RANTES (regulated on activation, normally T-cell-expressed and secreted) and SDF-1alpha (Table 1) appears to be critical for quantitative and also qualitative changes of their signaling potential [45]. The pro- or anti-oncogenic effect of DASH molecules might be a result of different composition of humoral signals within a particular tumor environment. Consequently, modulation of DPP-IV enzymatic activity is also speculated to be of therapeutic potential in oncology. Although concerns exist about the use of DPP-IV inhibitors stemming from the almost ubiquitous expression of DASH molecules and heterogeneity of the gene products possessing DPP-IV enzymatic activity, clinical trials in cancer patients are continuing [46].

2. DASH alterations as markers of tumorigenesis, prognosis and therapy

The search for new cancer markers represents a rapidly growing area of biomedical research. In general, the ideal tumor marker should be easily measurable in plasma or other body fluids and tissue specimens, have satisfactory sensitivity, specificity and reproducibility, and should be cost-effective. It should be informative for diagnosis, screening, prediction of prognosis, indication and monitoring of the therapy and design of the follow-up. There have not been any systematic cohort studies of DASH representation in cancer development. Although the evidence is strong, for example, that FAP in non-small cell lung carcinoma and other epithelial-type cancers may have an essential role in metastatic activity [35], these are predicted associations and cannot be compared with the quantitative assays measuring carcinoembryonic antigen or prostate-specific antigen as markers of cancer development and therapy. As we approach the era of personalized genomics and medicine in general, where the individual tailoring of diagnosis and therapy are approaching the realm of increased benefit/risk ratios together with cost-effectiveness, it may be beneficial to add DPP-IV activity to the list of neoplastic markers where a systemic/normal tissue set of values is established for an individual, and variations from this baseline can then be monitored. The implication, borne out by almost 40 years of measuring serum DPP-IV, in normal and many different pathological states, is that the range of baseline activities is wide. This has to be contemplated further in the light that

modifying molecules such as glypican-3 may modify activity independent of the actual amount of DPP-IV present [23].

2.1 Neoplastic expression of DPP-IV

In vitro, an anti-oncogenic effect of DPP-IV was demonstrated in cell lines derived from melanoma [47-49], ovarian carcinoma [50,51], non-small cell lung carcinoma [52], prostatic carcinoma [53] and, lately, glioblastoma [54]. This is speculated to be the result of locally increased degradation of soluble pro-proliferative mediators – DPP-IV substrates such as SDF-1alpha, DPP-IV-mediated interaction with extracellular matrix [55,56] or upregulation of other molecules such as E-cadherin [57]. However, the situation in solid tumors is complicated by the cell heterogeneity representing a majority of more differentiated cells limited in tumorigenic capacity, tumor stem cells in niches that, if equivalent to stem cells in other arenas, are probably maintained by a complex arrangement of stromal cell and chemokine interactions [18], as well as necrotic tissue.

There are conflicting reports on the expression of DPP-IV in melanocytic skin lesions [58]. Van den Oord [59] did not observe expression of DPP-IV in normal melanocytes as had previously been reported by Houghton *et al.* [49]. However, a decline in DPP-IV expression accompanied the progression from radial to vertical phase and DPP-IV was absent in metastases [59]. Recently, Roesch *et al.* [60] reported that DPP-IV might be useful in distinguishing deep penetrating nevi from malignant melanoma as it is absent in the latter. Decreased DPP-IV expression inversely correlating with tumor grade was also observed in endometrial cancer [61,62]. On the contrary, an increase of DPP-IV enzymatic activity occurs in several types of cancer compared with the noncancerous tissue (Table 2). DPP-IV has been proposed as an important diagnostic and prognostic marker of differentiated thyroid follicular and papillary cancer [63,64]. Grade-dependent upregulation of DPP-IV expression and its enzymatic activity are documented in high-grade astrocytic tumors [65]. The results of Inamoto *et al.* [66,67] argue for the possibility of using DPP-IV as a diagnostic tool as well as a therapeutic target in mesothelial and renal cancers. In addition, the increased DPP-IV activity in prostatic tumors and in urine obtained after prostatic massage was put forward as a potential diagnostic marker [68,69]. Interestingly, compared with the primary prostate cancer, a significantly lower expression was detected immunohistochemically in its metastases [70]. Compared with non-cancerous matched tissue, an augmented DPP-IV was determined in lung adenocarcinoma [71,72]. However, there are conflicting results in lung squamous cell carcinoma; biochemical assays demonstrated significantly increased DPP-IV-like activity, namely in lung tumor grade I [71], whereas enzyme activity histochemistry revealed just sporadic DPP-IV positive elements [72]. Apart from the different methodological approach used in both studies, absence of grade specification in the experimental cohort in the latter study should be considered. Higher DPP-IV activity was detected in human hepatocellular carcinoma compared with the normal liver tissue [73]. Similarly, high DPP-IV activity was observed in basal cell carcinoma and precancerous dermatosis compared with normal skin, whereas poorly differentiated malignant squamous cell carcinomas showed no histochemically detectable DPP-IV activity at all [74].

Given that CD26 in humans was originally described as a T-cell activation antigen, it was perhaps not unexpected to observe that increased DPP-IV/CD26 expression was a characteristic of some T-cell-derived neoplasias [75], including T-large granular lymphoproliferarive disorder, T-lymphoblastic lymphoma, acute T-lymphoblastic leukemia and T-cell CD30$^+$ anaplastic large cell lymphoma, where it was suggested as a marker of aggressiveness and worse prognosis [76,77]. Conversely, the underexpression of DPP-IV/CD26 was described in elements of Sezary syndrome/mycosis fungoides by cDNA microarray/flow cytometry [78-80]. Active DPP-IV/CD26 downregulation by promoter hypermethylation has also been demonstrated in cells from adult T-cell leukemia/lymphoma [81].

2.2 DPP-II expression in neoplasias

So far, investigation of the relationship of DPP-II to neoplastic development has been limited. Krepela *et al.* [82] observed increased DPP-II activity in grade I squamous cell lung carcinoma. Peripheral blood lymphocytes of patients with B-cell chronic lymphocytic leukemia (CLL) were reported to express DPP-II. In patients with stable disease, a significantly lower number of DPP-II positive lymphocytes was found. On the other hand, a high number of DPP-II positive lymphocytes indicated a progressive disease [83]. Interestingly, a more recent study suggested that CLL patients with B lymphocytes resistant to DPP-II inhibitor-induced apoptosis had worse prognosis [84]. Studies based, however, on biochemical detection of DPP-II using preferential but not selective substrates and discriminating DPP-II from DPP-IV on the basis of different pH optima cannot avoid some level of overlap of DPP-IV and DPP-II enzymatic activities.

2.3 FAP and tumor development

Although FAP was claimed to have anti-oncogenic roles in melanoma cells *in vitro* [85], it is evidently pro-oncogenic in most of the transformed cells and tumors studied so far (Table 2), including melanoma [86], possibly because of its collagenolytic activity. FAP expression has been determined in stromal fibroblasts of various epithelial tumors, such as breast, lung, gastric and colon cancers [34]. Also, upregulation of FAP in stromal and transformed cells has been demonstrated in gastric cancer [87,88]. FAP has been proposed to be an early marker of tumor invasion in squamous lesions of the uterine cervix [89]. In colon cancer, correlation of FAP expression and clinical stage, invasive potential and a negative correlation with patients' prognosis has

been affirmed [90,91]. In human astrocytic tumors, a grade-dependent rise of FAP expression has been shown [65]. Immunopositivity of FAP in fluids and cells of abdominal and pleural effusions accompanying ovarian cancer indicated poor patient prognosis and thus it was proposed to represent a diagnostic and prognostic marker in this disease [92]. Expression of a soluble, shortened form of FAP, probably derived from alternative splicing, has been described in colon, stomach, breast and ovarian tumors and in melanoma [93]. The preferential expression of FAP in tumor stroma and cancer cells made FAP also a candidate therapeutic target [33].

It is readily apparent that there is often a conflict between the predominantly anti-oncogenic effect of DPP-IV and in some cases also FAP in transformed cell lines *in vitro* and their frequent upregulation in tumor tissue representing the origin of the cell lines. However, the local signaling milieu, endowing tumors with their growth and progression potential, is determined by both transformed and tumor stromal cells, whose individual requirements for proteolytic processing of auto- and paracrine signals may vary. Thus, although DPP-IV and FAP molecules might be a burden for transformed cells themselves, they could still be beneficial for other cell populations within the tumor, with a resultant net pro-oncogenic effect [54]. This effect, essentially on tumorigenic niches in the context of the non-enzymatic interactions involving plasminogen, glypican-3 and extracellular adenosine, may render cell lines grown *in vitro* a poor model of tumorigenesis *in vivo*.

2.4 Circulating DPP-IV activities

Many diseases, including neoplasias, have been associated with altered DPP-IV-like enzymatic activity in blood plasma/serum. Increased blood plasma DPP-IV has been proved in patients with bile duct and hepatocellular carcinoma and with metastases into the liver [73,94,95]. In most cancers, however, enzymatic activity of DPP-IV as well as the level of the enzyme protein in plasma or serum tend to decrease (**Table 3**). Using immunodetection of serum DPP-IV in colorectal cancer, Cordero *et al.* [96] claimed a decreased level of DPP-IV protein. In a separate study using an enzyme activity assay, however, increased DPP-IV activity in the plasma of colorectal cancer patients was observed [97]. In patients with oral squamous cancer, DPP-IV levels tended to reflect the clinical status and therapeutic response. The serum DPP-IV activity in patients with a fair prognosis was significantly elevated towards the normal range, whereas the activity in patients with a poor prognosis was significantly decreased. Conversely, DPP-II serum activity in these patients was significantly increased compared with that of healthy subjects, and was inversely related to DPP-IV activity. The authors concluded that levels of these serum enzymes might become an aid for the diagnosis of malignant tumors and for estimating patients' prognosis [98]. Both serum DPP-II and DPP-IV activities were increased in patients with hepatic cancer but the increase in DPP-II was greater than that of DPP-IV, thus the ratio of the two activities increased significantly. These reports suggest that the increase of the serum DPP-II:DPP-IV rario might serve as a biochemical index of some blood and solid tumors, closely correlating with the tumor size and degree of progression [31,73].

Although canonical DPP-IV represents the paramount source of DPP-IV-like activity in plasma/serum, CD26 knockouts still retain ~ 10% of blood plasma DPP-IV-like hydrolytic activity. This supports the notion that other DASH molecules, most probably DPP-II and FAP, are present in the systemic circulation as well [31,36]. There are at present no reports about FAP in the plasma of cancer patients, although the recent purification of a soluble seprase from bovine plasma implies that such a form might exist in humans [99].

3. Expert opinion

3.1 Does DPP-IV activity or expression have diagnostic potential?

Most clinically useful diagnostic and prognostic markers are likely to be molecules causally involved in cancer progression [100]. In this respect, DASH molecules, which are probably linked directly to the pathogenesis of cancer, hold significant promise for exploitation in the diagnostic arena. In assessing the eventual clinical potential of selected DASH for inclusion in the current portfolio of cancer markers, several matters should be taken into consideration:

- DPP-IV and FAP may represent promising markers for cancer diagnostics, grading and prognosis in several rypes of solid as well as hematological malignancies. Also, DPP-IV/CD26 may represent a lead to tailor suitable therapeutic modalities owing to the current availability of FDA-approved inhibitors. Furthermore, the availability of several crystal structures both as apoenzyme and complexed to substrate/inhibitor will allow development of agents that may interfere with the non-enzymatic interactions of DPP-IV/CD26 and may be of great significance in regulating tumor progression [101-103].
- Changes of DPP-IV-like activity in plasma/serum in cancer patients are not specific for a particular tumor type and also occur in non-malignant diseases. Moreover, although the differences between control groups and patient cohorts are statistically significant, there is a substantial overlap, which does not provide a strong enough discrimination needed for clinical decision-making. Some reports, however, suggest that monitoring of plasma/serum DPP-IV on an individual basis may be valuable for following disease course and/or response to therapy.
- DPP-IV-like enzymatic activity in tissue specimens as well as in body fluids may be derived from several co-expressed molecules with overlapping enzymatic activity. Moreover,

other gene products may modify the whole DPP-IV activity of the given biological material. The complex interplay in an *in vivo* environment may open up new therapeutic modalities, which target the transcription activity of particular DASH, presence and subcellular distribution of its splice variants, analysis of post-translational processing in addition to its enzymatic activity.

- In the end it is the protein activity that is important in pathogenesis. Upregulation of transcripts detected by array hybridization techniques cannot represent the potential at the translational level. The differential catalytic activity following post-translational modification and the relative expression of interacting proteins and substrates in the larger tissue environmental context will prove to be much more informative.
- Finally, there is simply not enough information published at present to make definitive assessments. The individual reports published so far suggest definite trends, but larger prospective studies using rigidly defined patient cohorts representing a screening population, with external validation and comparable protocols, will provide the required statistical power for verification of the potential of DASH molecules in clinical diagnostics.

Declaration of interest

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Affiliation

Aleksi Šedo[†1,2] MD PhD, Professor,
University Teacher, Scientist,
Jarmila Stremenová[1,2] MSc, University Teacher,
PhD Student, Petr Bušek[1,2] MD,
University Teacher, PhD Student &
Jonathan S Duke-Cohan[3] PhD,
University Teacher, Scientist
[†]Author for correspondence
[†1]Charles University in Prague,
Laboratory of Cancer Cell Biology of the 1st
Faculty of Medicine,
U Nemocnice 5, 128 53,
Prague 2, Czech Republic,
Tel: +420 728 748 692; Fax: +420 224 965 826;
E-mail: aleksi@cesnet.cz
[2]The Institute of Physiology,
Academy of Sciences,
Prague, Czech Republic
[3]Dana-Farber Cancer Institute and
Department of Medicine,
Harvard Medical School,
Department of Medical Oncology,
Boston, USA

II.

Dipeptidyl peptidase-IV enzymatic activity bearing molecules in human brain tumors-good or evil?

Busek P, Stremenova J, Sedo A.

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Dipeptidyl peptidase-IV enzymatic activity bearing molecules in human brain tumors - good or evil?

Petr Busek, Jarmila Stremenova, Aleksi Sedo

Laboratory of Cancer Cell Biology, Department of Biochemistry and Experimental Oncology, 1st Faculty of Medicine, Charles University in Prague, Institute of Physiology, Academy of Sciences, Czech Republic

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1. ABSTRACT

Dipeptidyl peptidase-IV (DPP-IV) represents a unique proteolytic activity cleaving N-terminal X-Pro dipeptides. In addition to canonical DPP-IV/CD26, a number of other molecules have been discovered which exhibit DPP-IV-like enzymatic activity and various degree of structural similarity. These comprise enzymatically active fibroblast activation protein-alpha, DPP-II, DPP8, DPP9 and enzymatically inactive DPP6 and DPP10 that have been grouped as "DPP-IV activity and/or structure homologues" (DASH). Because the enzymatically active DASH can share similar sets of biologically active substrates and are frequently coexpressed within single cell or on tissue level, it is tempting to consider their participation on biological function(s) previously attributed to DPP-IV/CD26. It is speculated that disrupted expression and enzymatic activity of some DASH might corrupt the message carried by their substrates, with consequent promotion of abnormal cell behavior. Thus, modulation of activity of a particular enzyme using e.g. inhibitors, specific antibodies or modifying its expression may be an attractive therapeutic concept in cancer treatment. This review summarizes current knowledge of the expression and possible function of DPP-IV enzymatic activity bearing molecules in human brain tumors.

2. INTRODUCTION

Dipeptidyl peptidase-IV (DPP-IV, EC 3.4.14.5) is a serine protease originally described by Hopsu-Havu and Glenner (1) in liver homogenates as an activity cleaving glycyl-prolyl-beta-naphthylamide. This protease is unique as it is able to remove two N-terminal amino acids from peptides and small proteins with Pro or Ala in the penultimate position that are otherwise rather resistant to proteolytic degradation (2). In the course of time, further molecules exhibiting DPP-IV enzymatic activity and varying degree of structural homology to canonical DPP-IV have been discovered and grouped as Dipeptidyl peptidase-IV activity and/or structure homologues (DASH, (3)). The group comprises enzymatically active DPP-II (also referred to as DPP7 or quiescent cell proline dipeptidase), DPP8, DPP9 and fibroblast activation protein alpha (FAP, also referred to as seprasc) and enzymatically inactive DPP6 and DPP10. Attractin, which is structurally unrelated to DPP-IV (4), was formerly supposed to belong to DASH on the basis of its putative enzymatic activity, but a recent report by Friedrich *et al* (5) strongly argues against its intrinsic hydrolytic potential.

DPP-IV and some other DASH exhibit biological functions independent of their hydrolytic activity. For

example, DPP-IV has been shown to be identical to CD26 expressed on the T lymphocyte surface and to act as a signaling coreceptor in the immune system (6), to bind adenosine deaminase, plasminogen (7, 8) and some components of the extracellular matrix (9, 10). DPP8 and 9 are speculated to influence cell migration and adhesion independently of their intrinsic enzymatic activity (11). DPP6 and DPP10, both structurally related to DPP-IV but enzymatically inactive, were shown to be part of the neuronal voltage gated K^+ channels (12, 13). However, the DPP-IV-like hydrolytic activity driven cleavage of a number of biologically active peptides including chemokines and various neuropeptides is considered to be the main mechanism, by which DASH molecules can execute their biological functions. Proteolytic nicking of N-terminal dipeptides of DASH biologically active substrates is considered to be an important regulator of both their half-lives as well as receptor preference, fine-tuning their signaling capacity prior to receptor binding (14, 15). For example, cleavage of the chemokine RANTES 1-68 (regulated on activation normal T-cell expressed and secreted, CCL5) produces RANTES 3-68 that is inactive at receptors CCR1 and CCR3, but retains the ability of full-length molecule to activate CCR5. This conversion abrogates the ability of RANTES to induce migration of monocyte (16-18). Similarly, DPP-IV-like enzymatic activity cleaves neuropeptide Y 1-36 (NP Y) yielding NP Y 3-36 that has decreased affinity for the Y1 receptor subtype, but retains binding capacity for the Y2 and Y5 receptors (19, 20). As a physiological consequence, NP Y loses the vasoconstrictive potential while retaining the ability to promote angiogenesis via Y2 receptors (21). Moreover, expression of NP Y during angiogenesis seems to be orchestrated with expression of its cognate receptors Y1-Y5 and DPP-IV, which increases its proangiogenic potential (22, 23).

Due to the frequently observed coexpression of multiple DASH at the cellular as well as tissue level, their participation on the overall DPP-IV- like hydrolytic activity is evident and mutual functional overlap possible. However, plasma membrane localization, presence in body fluids and the ability to cleave larger substrates make DPP-IV and FAP most feasible as regulators of biologically active peptides. Thus, the cleavage of some of the "classical" DPP-IV substrates demonstrated for intracellular DPP8, 9 (24, 25) is likely of limited physiological impact.

Changes in expression of DPP-IV enzymatically active molecules have been described in a number of pathological states, including cancer. However, the functional consequences of such alterations are not straightforward (26) and may depend on the tissue specific presence of relevant biologically active substrates and respective receptors.

Here, we review data on the expression and possible function of DPP-IV enzymatic activity bearing molecules in the central nervous system focusing on human brain tumors.

3. DPP-IV ENZYMATIC ACTIVITY IN GLIOMA CELL LINES – POSSIBLE ASSOCIATION WITH CELL DIFFERENTIATION AND GROWTH

DPP-IV was detected in D384 astrocytoma cells by Medeiros *et al* (27) and subsequently by Sedo in human neuroblastoma SK-N-SH, rat C6 as well as human U373 and U87 glioma cell lines (28, 29). Moreover, in the C6 cell line DPP-IV enzymatic activity was upregulated during chemically induced differentiation (30). In subsequent studies with rat C6 (31) as well as human glioma cell lines U373, T98G, Hs 683, U138 and U87, substantial heterogeneity of molecules bearing DPP-IV-like enzymatic activity was demonstrated by gel chromatography, enzyme inhibition studies and native electrophoresis (32). These observations implied either the presence of multiple molecular forms of DPP-IV or expression of other enzymatically active DASH. Indeed, RT PCR confirmed expression of DPP-IV, FAP, DPP8 and DPP-II transcripts (32). Furthermore, subcellular localization and inhibition studies suggest that the majority of DPP-IV like enzymatic activity in all glioma cell lines studied may in fact be attributed to intracellular soluble DPP8/9 (unpublished data). However, cell differentiation and growth arrest induced by starvation were accompanied by rise of DPP-IV-like enzymatic activity localized predominantly in the plasma membrane. Indeed, we observed upregulation of both DPP-IV and FAP mRNA upon starvation induced growth arrest in U87 cells ((33) and unpublished data). Our results also revealed positive correlation ($r = 0.9$, $p < 0.05$) between the expression of DPP-IV and FAP mRNA in different glioma cell lines (Figure 1). This is analogous to the results demonstrating that DPP-IV upregulation is accompanied by growth arrest and restoration of non-malignant phenotype in melanoma and lung cancer cells (34, 35). Moreover, reexpression of DPP-IV in these cells was associated with upregulation of FAP. In our experimental setting, overexpression of DPP-IV in T98G human glioma cell line lcad to growth inhibition and accumulation of the cells in G2/M phase of the cell cycle (Figure 2). The mechanisms of growth inhibition and antioncogenic activity, which has been attributed to DPP-IV by several authors, may involve proteolytic processing of its biologically active substrates (for review cf. (36)). In gliomas, substance P (SP), RANTES and stromal cell derived factor-1alpha (SDF-1alpha or CXCL12), can promote malignant behavior of the tumor cells (37-39). We have demonstrated the ability of glioma cells highly expressing DPP-IV to prevent signaling of SP by its proteolytic degradation (40). This cleavage however could not explain the growth arrest observed in T98G cells, which are devoid of functional SP receptor (40). In this case, other substrate(s) or non-enzymatic functions of DPP-IV are likely involved.

Taken together, so far available data suggests possible coregulation of both plasma membrane localized DASH, DPP-IV and FAP, and their link to cell growth and differentiation, although the underlying molecular mechanisms remain to be elucidated.

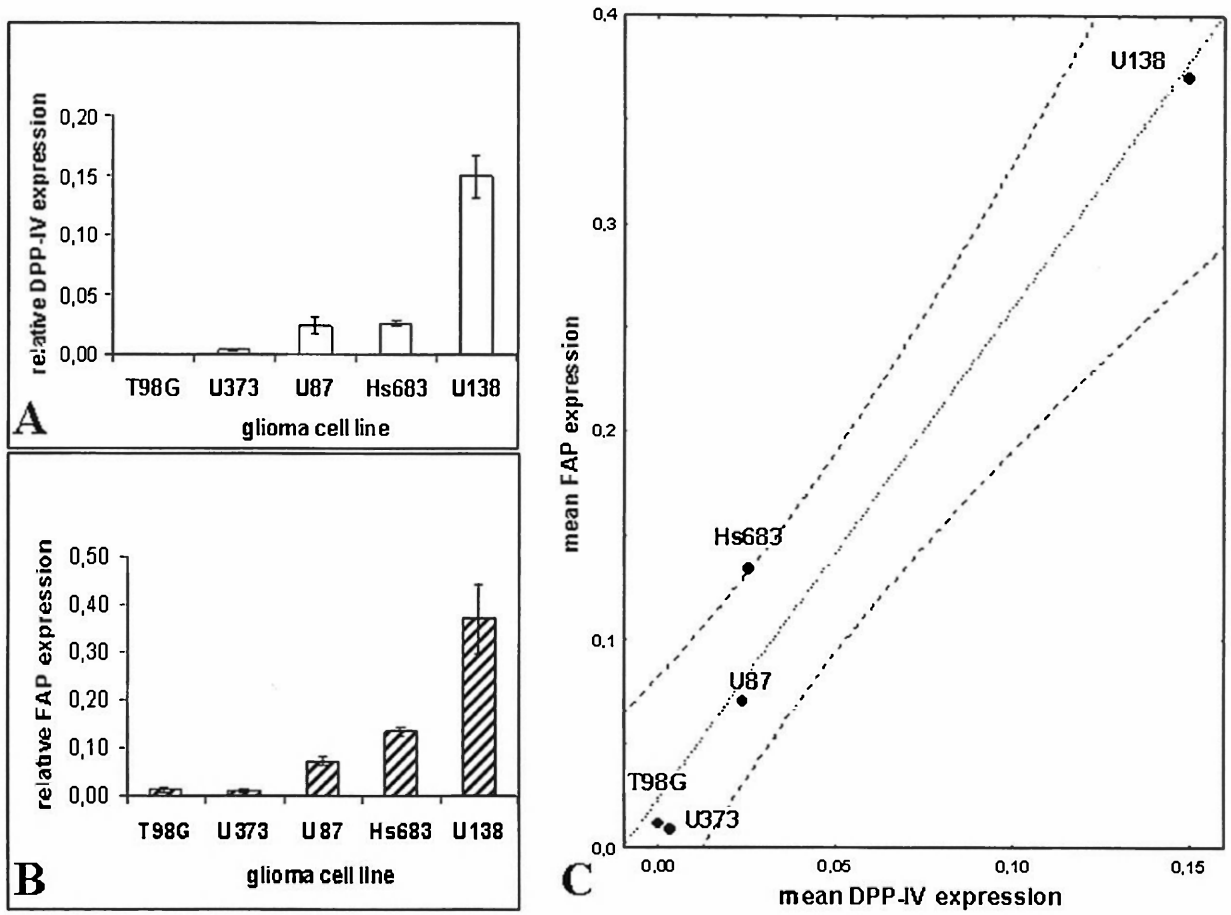

Figure 1. Expression of DPP-IV and FAP transcripts positively correlates in human glioma cell lines. Expression of DPP-IV (panel A) and FAP (panel B) mRNA was assessed using real time RT-PCR with normalization of the values to human beta-actin mRNA. Data are presented as means ± SEM of four measurements. Correlation between the expressions in different glioma cell lines ($r = 0.9$, $p < 0.05$) was assessed by Spearman coefficient using Statistica 7.0 software, regression curve and 95% confidence intervals are shown (panel C).

4. DPP-IV ENZYMATICALLY ACTIVE MOLECULES IN HUMAN BRAIN AND BRAIN TUMORS

Reports on the expression and possible function of DASH in the central nervous system are scarce and mostly concentrate on DPP-IV and DPP-II. DPP-IV as well as its activity has been detected in the capillaries and meninges in rat (41-44) and on certain neuronal structures in rat and mouse (45). Biochemical studies also suggest its presence in various brain regions in rat and guinea-pig (46, 47). A number of studies with DPP-IV inhibitors and DPP-IV deficient animals implicate DPP-IV in the regulation of nociception and behavior possibly via metabolism of biologically active peptides such as SP, endomorphin-2 and NP Y (48-50). To our knowledge only a report by Bernstein *et al* (51) described the presence of DPP-IV in the human brain with abundant expression in the developing central nervous system and a decrease in adults.

DP-II (for review cf. 52) was detected in brain homogenates (53) and histochemically in specific neuronal populations in rat brain by Gorenstein *et al* (54) with no staining over glia. However, later studies demonstrated presence of DPP-II in glial cells (55), linked it to astrocyte differentiation and described the decrease of its activity during maturation of the rat brain (56). DPP-II was also described in neurons, pericytes and ependymal cells in the spinal cord in rat (57). According to Frerker *et al* (24), DPP-II with a significant contribution of DPP8/9 may in fact constitute the majority of DPP-IV-like enzymatic activity in rat brain.

We detected DPP-IV-like enzymatic activity in homogenates of non-tumorous human brain, astrocytic and non-astrocytic tumors (58). Using real time RT-PCR and immunochemistry we observed very low expression of DPP-IV and FAP on mRNA and protein levels in non-tumorous brain. Selective inhibitors showed that the majority of DPP-IV-like enzymatic activity in non-tumorous brain could be attributed to DPP8/9, which are thought to be localized intracellularly. However, DPP-IV and FAP mRNA and protein were upregulated and the DPP-IV-like enzymatic activity was increased in high-

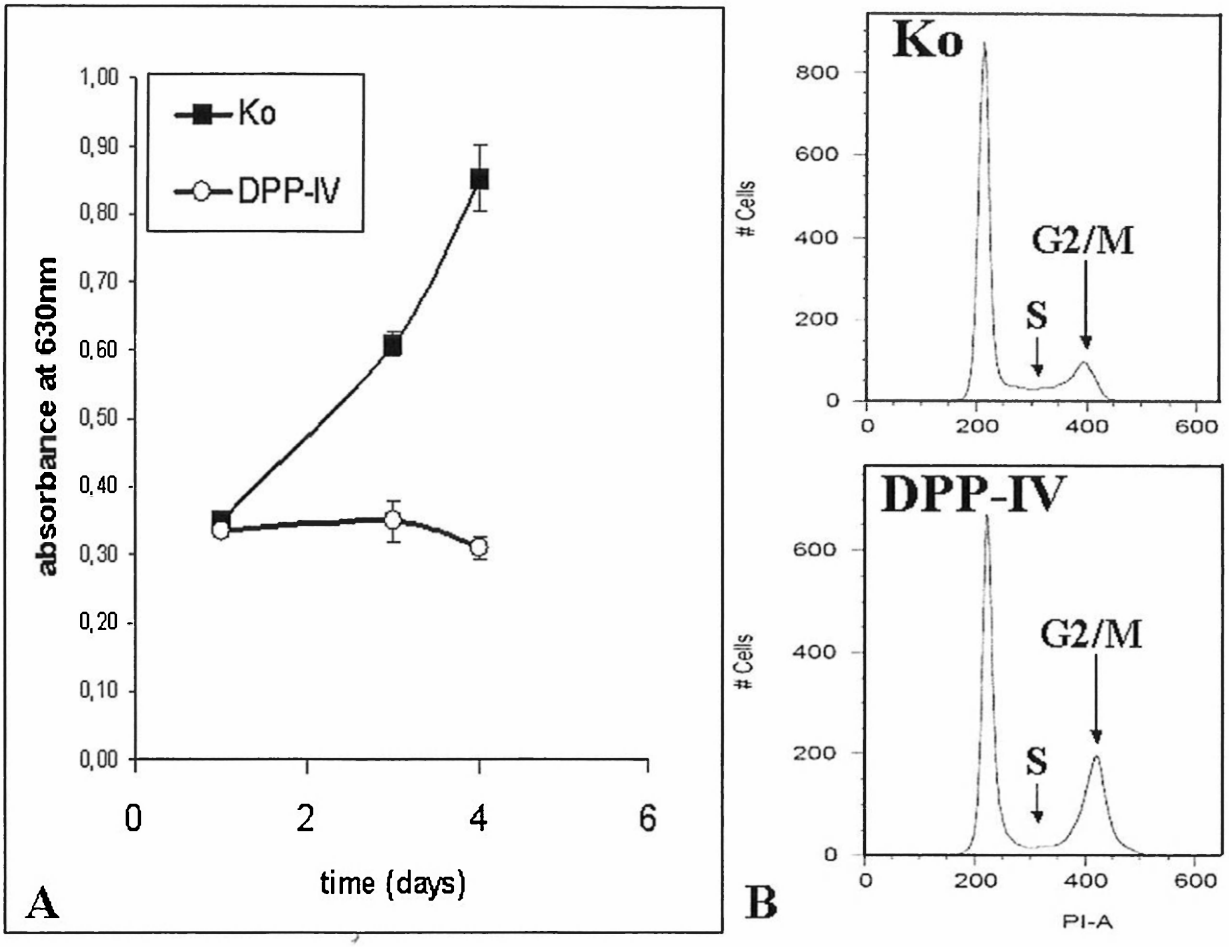

Figure 2. Effect of DPP-IV overexpression on the proliferation of T98G cells. Growth curves (A) and cell cycle analysis (B) of DPP-IV transfected T98G cells without (Ko) and after induction of DPP-IV expression (DPP-IV). T98G cells were transfected with DPP-IV using a mifepristone-inducible expression system (GeneSwitch, Invitrogene). For growth curve construction, cells were fixed and stained with methylene blue followed by colorimetric quantification. Flow cytometric cell cycle analysis was performed 48h after induction of DPP-IV expression. The expression inducing agent mifepristone had no effect on the proliferation of non-transfected cells. Results of a typical experiment are shown. S, G2/M- cells in the S and G2/M phase of the cell cycle, respectively.

grade gliomas compared to non-tumorous brain. Similarly to glioma cell lines (see above), the expression of DPP-IV and FAP positively correlated in tumor tissues. We did not observe a significant difference in contribution of DPP8/9 to the DPP-IV enzymatic activity between non-tumorous and tumorous tissue based on biochemical studies with specific DPP8/9 inhibitors. Our data suggest that a substantial part of the DPP-IV-like enzymatic activity increase in gliomas was due to an upregulation of DPP-IV and possibly FAP (58). This may be seen as contradictory to the antioncogenic properties of DPP-IV observed in several transformed cell lines as mentioned above (section 3.). However, the cellular source of upregulated DPP-IV and FAP in gliomas remains unclear.

Only marginal enzymatic activity in the non-tumorous as well as in the tumorous human brain was detected at the acidic pH 5.5, which suggests that DPP-II does not significantly contribute to the overall DPP-IV-like enzymatic activity in human brain and astrocytic tumors (58).

5. CONCEIVABLE INTERACTION OF DPP-IV WTH THE SDF-1ALPHA- CXCR4 AXIS IN GLIOMAS

Several soluble mediators susceptible to DPP-IV cleavage have been deseribed to promote the malignant behavior of glioma cells (37, 38). SDF-1alpha has been implicated in glioma cell growth, survival, migration and invasion, as well as angiogenesis. Its receptor CXCR4 is abundantly and grade-dependently expressed in gliomas *in vivo* (39, 59-64). DPP-IV is known to effectively cleave SDF-1alpha (65). The functional consequences of SDF-1alpha cleavage by DPP-IV have mostly been documented in hematopoetic system (66-68). In addition, Mizokami *et al* (69) demonstrated that DPP-IV might hamper the growth

of endometrial carcinoma cells probably due to locally decreased availability of SDF-1alpha.

Similarly to Rempel *et al* (60) we observed a tumor grade-related rise in expression of CXCR4 mRNA and protein in human astrocytic tumors (58). We speculate that in high-grade gliomas, upregulated DPP-IV might trim down SDF-1alpha signaling, which may be compensated by the increase in CXCR4. This would favor proliferation of glioma cell populations capable to effectively raise their CXCR4 expression. Indeed, our preliminary data show tight positive correlation between CXCR4 and DPP-IV expression ($r = 0.89$, $p < 0.01$, $N = 8$) in patients with survival under 6 months while no significant correlation ($r = 0.55$, $p > 0.1$, $N = 9$) is present in patients surviving more than one year after surgery (unpublished results). Interestingly, there is not a significant difference in CXCR4 or DPP-IV expression between both groups. This suggests that not the absolute value, but rather a relative ratio of CXCR4 and DPP-IV expression may influence glioblastoma progression.

6. CONCLUSIONS AND PERSPECTIVES

An imbalance of extracellular proteolysis has been demonstrated to be a general hallmark of malignancy (70). Altered proteolytic equilibrium, affecting processing of structural and regulatory proteins within the tumor microenvironment, has multiple downstream projections including regulation of neovascularization, modulation of cancer cell proliferation, migration and invasion.

Altered DPP-IV-like enzymatic activity has been observed in numerous tumors and consequently several roles have been proposed for DPP-IV in cancer pathogenesis. However, the overall DPP-IV-like activity frequently encompasses hydrolytic potential of several coexpressed DASH molecules. It is becoming evident that it is more likely the complex expression pattern of DASH molecules in context with available bioactive substrates and their receptors, which have to be considered to interpret the results of functional studies (36). This broader view may help explain the seemingly contradictory roles of DPP-IV and FAP, which can both act as either tumor suppressors or promoters depending on the tumor type (26).

Plasma membrane localization and slightly alkaline pH optima make the canonical DPP-IV and FAP the most serious candidates among other DASH molecules for proteolytic processing of humoral mediators and for interaction with extracellular matrix. Similarly to several other cancer cell types (34, 35, 71), DPP-IV seems to have a rather antiproliferative effect on glioma cells *in vitro*. This might be due to the locally increased degradation of soluble mediators such as SDF-1alpha or SP. Surprisingly, we observe an increase of DPP-IV like enzymatic activity attributable to DPP-IV or FAP in high-grade gliomas compared to non-tumorous human brain. However, it should be considered that the local proteolytic milieu, endowing tumors with their growth and progression potential, is determined both by transformed and stromal cells. Thus, although DPP-IV might represent "bad guy" for transformed cells themselves, it could still be beneficial to other cell populations within the tumor with a resultant net pro-oncogenic effect.

DPP-IV enzymatic activity is a promising therapeutic target as has been demonstrated by the recent FDA approval of DPP-IV inhibitors for the treatment of diabetes (72) and a number of patents claiming the use of these inhibitors in autoimmune diseases, cancer or stimulation of hematopoiesis. Although few side effects have been reported for the clinically tested DPP-IV inhibitors so far, the severe toxicity of DPP-8 and 9 inhibitors in preclinical studies (73) remains a concern. The objections for DPP-IV targeting in clinical settings might come from two possible mechanisms of adverse effects: (i) Structurally related targets – DASH molecules - might been involved in pathogenesis of different diseases and/or separate metabolic pathways. Since biological functions have not yet been assigned to all DASH, the undesirable consequences of unselective inhibition have to be expected. (ii) Individual DASH molecule may be involved in multiple physiological processes throughout the body. For example, DPP-IV inhibitors used for the treatment of diabetes could increase the risk of promoting an already existing intestinal tumor due to sustained stimulation of tumor cells by glucagon like peptide-2, another DPP-IV substrate (74). „Doublespecific" inhibitors, targeting specific DASH molecules in an appropriate cell, could address both above-mentioned objections.

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Abbreviations: DASH: dipeptidyl peptidase-IV activity and/or structure homologues, DPP: dipeptidyl peptidase, FAP: fibroblast activation protein alpha,, NP Y: neuropeptide Y, RANTES: regulated on activation normal T-cell expressed and secreted, RT-PCR: reverse-transcription polymerase chain reaction, SDF-1alpha: stromal cell derived factor-1alpha, SP: substance P

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Send correspondence to: Dr Aleksi Sedo, Laboratory of Cancer Cell Biology, 1st Faculty of Medicine, Charles University in Prague, U Nemocnice 5, 128 53, Prague 2, Czech Republic, Tel: 420-728748692, Fax: 420-224965826, E-mail: Aleksi@cesnet.cz

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Expression and enzymatic activity of dipeptidyl peptidase-IV in human astrocytic tumours are associated with tumour grade.

Stremenova J, Krepela E, Mares V, Trim J, Dbaly V, Marek J, Vanickova Z, Lisa V, Yea Ch, Sedo A.

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Expression and enzymatic activity of dipeptidyl peptidase-IV in human astrocytic tumours are associated with tumour grade

JARMILA STREMENOVA[1], EVZEN KREPELA[1], VLADISLAV MARES[2], JULIE TRIM[3], VLADIMIR DBALY[4], JOSEF MAREK[4], ZDISLAVA VANICKOVA[1], VERA LISA[2], CHRIS YEA[3] and ALEKSI SEDO[1]

[1]Joint Laboratory of Cancer Cell Biology of the 1st Faculty of Medicine, Charles University in Prague, and the [2]Institute of Physiology, Academy of Sciences, Prague, Czech Republic; [3]Ferring Pharmaceuticals, Southampton, UK; [4]Departments of Neurosurgery and Pathology, Hospital Na Homolce, Prague, Czech Republic

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Abstract. Alterations in dipeptidyl peptidase-IV (DPP-IV) enzymatic activity are characteristic of malignant transformation. Through its well-characterized functionality in regulating the activity of bioactive peptides by removal of the N-terminal dipeptide, DPP-IV activity may have profound effects upon metastatic potential and cell growth. Although DPP-IV/CD26 (EC 3.4.14.5) is the canonical representative of the group, a number of other proteins including DPP-7, 8, 9, and seprase/fibroblast activation protein-α (FAP-α) have been shown to have similar enzymatic activity. This study was set up to address the relative representation and enzymatic activity of plasma membrane localized DPP-IV/CD26 and FAP-α in human brain and astrocytic tumours. In parallel, expression of CXCR4, receptor for glioma cell growth stimulator chemokine SDF-1α known to be a DPP-IV substrate, was investigated. This is the first report showing that non-malignant brain tissue contains a DPP-IV-like enzymatic activity attributable mostly to DPP-8/9, while the substantial part of the activity in glioma is due to increased DPP-IV/CD26, localized in both the vascular and parenchymal compartments. DPP-IV enzymatic activity increased dramatically with tumour grade severity. A grade-related increase in CXCR4 receptor paralleled the rise in DPP-IV expression and activity. These data might support a role for DPP-IV regulation of the CXCR4-SDF-1α axis in glioma development.

Introduction

Malignant gliomas, characterized as highly proliferative and locally invasive neoplasias, account for more than 50% of central nervous system tumours. Despite intensive basic research and efforts in the fields of therapeutic regimens and modalities, prognosis of patients suffering from these tumours remains dismal. A major area of current investigation is identification of alterations in mRNA and protein expression in glioma cells that might contribute to gliomagenesis and also be amenable as therapeutic targets.

Dipeptidyl peptidase-IV (DPP-IV/CD26) is a multifunctional, typically plasma membrane localized protein, widely distributed in various cell systems. It has been for years believed to be a unique protease cleaving peptides and proteins with a proline residue on the penultimate position from their N-terminal. Subsequently, other 'DPP-IV activity and/or structure-homologues' (DASH), were discovered (1). Together with CD26, the canonical DPP-IV, enzymatically active DASH include plasma membrane fibroblast activation protein-α (FAP-α) and the intracellular DPPs-7, 8, 9. Because of their similar enzymatic activity, it is tempting to speculate on their participation in or interference with biological function(s) previously attributed solely to DPP-IV/CD26 although relative activity will be a function of local DASH involvement. Among the multiple physiological and pathological functions so far described, DPP-IV may play a critical role in immunoregulation as well as in carcinogenesis (2-5). In contrast to the proteases involved in structural protein degradation during malignant progression, plasma membrane associated DPP-IV/CD26 probably function dominantly as the regulators of humoral signaling. As such, they participate in postsecretory processing of biologically active peptides implicated in control of cell growth, migration and invasion. Thus, although some biological functions of DPP-IV seem to be independent of its intrinsic hydrolytic activity (6), limited proteolysis of DASH substrates such as substance P, neuropeptide Y, and RANTES and SDF-1α chemokines appears to be critical for quantitative and also qualitative changes of their signaling potential (5).

Chemokine SDF-1α together with its cognate receptor CXCR4 is functionally involved in several cancer progression-related events including chemotaxis, invasion, adhesion and vascularization (7,8) in a variety of malignant tumours (9-11). In glioma cells, CXCR4 represents the major chemokine receptor mediating their survival (12,13). DPP-IV enzymatic

Correspondence to: Dr Aleksi Sedo, 1st Faculty of Medicine, Charles University in Prague, U Nemocnice 5, 128 53, Prague 2, Czech Republic
E-mail: aleksi@cesnet.cz

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activity is responsible for cleavage of SDF-1α at its amino-terminal consensus sequence. Truncation of SDF-1α by DPP-IV-like activity inhibits chemotactic activity, while the resulting fragment may even act as an antagonist of CXCR4 (14). Co-distribution of DPP-IV with CXCR4 and their SDF-1α induced internalization has been observed in human lymphocytes and in several cell lines (15). Such orchestrated regulation of DPP-IV hydrolytic potential within the CXCR4-SDF-1α regulatory axis might serve as an example of the putative regulatory 'DASH system' (5), consisting of plasma membrane localized DPP-IV enzymatic activity-bearing molecules, their biologically active autocrine/paracrine substrates and relevant receptors.

While DPP-IV has been studied in numerous cancer types, there are no reports of DPP-IV activity in human brain and astrocytic tumours, despite the importance of SDF-1α CXCR4 in glioma development and DPP-IV regulation of SDF-1α activity. In this study, we ascertain the relative representation and enzymatic activities of DPP-IV-like proteases in normal brain and astrocytomas of varying grades, and relate this to the presence of SDF-1α receptor CXCR4.

Materials and methods

Patients, sample preparation and characterization. Tumour specimens were collected from 31 patients undergoing astrocytic tumour resection. Tumours were graded in compliance with the current WHO Classification Criteria. Non-tumorous brain specimens were obtained from eleven patients in whom brain surgery was performed for drug-resistant temporal lobe epilepsy (Table I). Tissue samples, clear of macroscopic vessels and necrosis, were frozen on solid CO_2 and then stored at -80°C. Vascularization and necrótic areas in paraffin sections were scored semi-quantitatively on a 5-titered scale (0-4 crosses) and expressed as the average calculated from the individual values of each optical field determined. Written informed consent was obtained from the patients before their entry into the study, according to the guidelines of institutional Ethics Committee.

Isolation and quantification of total RNA. Total RNA was isolated using the TRIzol Reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Spectrophotometric analysis, carried out in 10 mM Tris-HCl buffer, pH 7.5, confirmed that the samples of total RNA had an A_{260nm}/A_{280nm} ratio >1.8. The concentration of total RNA was determined using the RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, OR, USA).

Real-time RT-PCR. Gene coding region-specific oligonucleotide primers and fluorogenic TaqMan probes for the real-time RT-PCR assays of expression of the investigated transcripts were designed with the program Primer Express (Applied Biosystems, Foster City, USA) and were synthesized at Proligo (Paris, France) and Applied Biosystems (Warrington, UK), respectively. The sequences and final concentrations of the primers and probes that were used in the real-time RT-PCR assays are indicated in Table II. The expression of DPPIV, FAP-α, and CXCR4 mRNAs and ß-actin mRNA (internal reference transcript) was quantified by coupled

Table I. Characterization of the experimental cohort.

Diagnosis	Patient code	Age	Sex	Vascularisation	Necrosis
Control	26	46	F	1	0
	27	29	F	1	0
	43	44	M	0	0
	45	43	M	0	0
	46	54	F	0	0
	48	34	F	0	0
	49	30	M	1	0
	50	50	M	0	0
	53	40	F	0	0
	7	72	M	ND	ND
	5A	34	M	0	0
Grade II	5	34	M	1	0
	11	34	F	1	0
	19	34	F	1	0
Grade III	2	25	M	1	0
	15	68	M	1	1
	16	52	M	2	1
	55	33	M	2	0
Grade IV	1	70	M	2	2
	3	53	F	2	0
	4	52	F	2	1
	6	54	F	2	1
	9	59	F	1	0
	10	48	F	3	2
	12	57	F	3	2
	13	32	M	3	1
	14	70	M	3	1
	22	49	M	3	3
	25	67	F	2	2
	28	72	F	2	1
	29	67	F	2	1
	31	46	M	3	3
	32	65	F	3	1
	33	62	M	2	4
	35	73	F	2	1
	36	32	F	2	4
	37	32	F	ND	ND
	39	33	F	2	0
	40	55	M	1	3
	42	57	F	2	0
	44	61	M	2	1
	47	66	F	2	2

ND, not determined.

Table II. Primers and TaqMan probes used for real-time RT-PCR quantitation of expression of the investigated transcripts.

Transcript	GeneBank accession no.	Sequences and final concentration of primers and TaqMan probes
DPP-IV	NM_001935	Forward primer: 5'-TGGAAGGTTCTTCTGGGACTG-3', 200 nM
		Reverse primer: 5'-GATAGAATGTCCAAACTCATCAAATGT-3', 200 nM
		TaqMan probe: 5'-(6-FAM)CACCGTGCCCGTGGTTCTGCT(TAMRA)-3', 200 nM
FAP-α	NM_004460	Forward primer: 5'-TGCCACCTCTGCTGTGC-3', 200 nM
		Reverse primer: 5'-GAAGCATTCACACTTTTCATGGT-3', 200 nM
		TaqMan probe: 5'-(6-FAM)TGCATTGTCTTACGCCCTTCAAGAGTTC(TAMRA)-3', 200 nM
CXCR4	NM_001008540	Forward primer: 5'-CATGGGTTACCAGAAGAAACTGA-3', 400 nM
		Reverse primer: 5'-GACTGCCTTGCATAGGAAGTTC-3', 400 nM
		TaqMan probe: 5'-(6-FAM)CACCTGTCAGTGGCCGACCTCCT(TAMRA)-3', 200 nM
ß-actin	NM_001101	Forward primer: 5'-CTGGCACCCAGCACAATG-3', 200 nM
		Reverse primer: 5'-GGGCCGGACTCGTCATAC-3', 200 nM
		TaqMan probe: 5'-(VIC)AGCCGCCGATCCACACGGAGT(TAMRA)-3', 200 nM

real-time RT-PCR assay. The RT-PCR reaction mixtures of a total volume of 50 μl contained 25 μl of ThermoScript Reaction Mix (a buffer with 3 mM $MgSO_4$ and 0.2 mM of each dGTP, dCTP, dATP and dTTP) and 1 μl of ThermoScript Plus Reverse Transcriptase/Platinum *Taq* DNA polymerase Mix (Platinum Quantitative RT-PCR ThermoScript One-Step System, Invitrogen), the respective gene-specific primers and TaqMan probe at the indicated final concentrations (Table II), 40 U of RNasc inhibitor RNaseOUT (Invitrogen), and 200 ng of total RNA. The final concentrations of the primers and TaqMan probe adopted for real-time RT-PCR quantification of each indicated transcript were determined in optimization experiments. The real-time RT-PCR assays were run in duplicate in MicroAmp Optical 96-well Reaction Plates on the ABI PRISM 7700 Sequence Detection System using Sequence Detection System software (Applied Biosystems). The reverse transcription was carried out at 58°C for 30 min and the subsequent PCR amplification included a hot start at 95°C for 5 min, 45 cycles of denaturation at 95°C for 15 sec and of annealing/extension at 58°C for 1 min. The threshold cycle (C_T) values of the amplification reactions, represented by the plots of background-subtracted fluorescence intensity (ΔFI) of the reporter dye (6-FAM or VIC) against PCR cycle number, were determined with the Sequence Detection System software. The statistical difference of the ß-actin mRNA-normalized target transcript expression in tumour and normal tissues was calculated from the linearized ΔC_T data (i.e. $2^{-\Delta C_T}$) and the tumour/normal ratio of the ß-actin mRNA-normalized target transcript expression was calculated by means of the $2^{-\Delta\Delta C_T}$ method (16).

DPP-IV enzymatic activity biochemical assay. Tissue samples were homogenized in ice-cold phosphate buffered saline (PBS), pH 6.0, with an Ultra-Turrax homogenizer fitted with an S8N-5G probe (IKA, Staufen, Germany) and used for assay immediately. DPP-IV-like enzymatic activities were determined by continuous rate fluorimetric assay (17) with 7-(Glycyl-L-Prolylamido)-4-methylcoumarin (Gly-Pro-NHMec) and with H-7-(L-Lysyl-L-alanylamido)-4-methylcoumarin (Lys-Ala-NHMec) (Bachem AG, Switzerland) as substrates respectively at a final concentration of 50 μM. The assays were performed in PBS of pH 7.4 and in citrate/Na_2HPO_4 buffer of pH 5.5. The release of 4-Amino-7-methylcoumarin was monitored at 380-nm excitation and 460-nm emission wavelengths (spectrofluorimeter Perkin-Elmer LS50B, USA). Selective DPP-IV, DPP-II and DPP8/9 inhibitors (Ferring Pharmaceuticals, UK) were used at the final concentration of 250 nM. Total protein concentration in samples was assayed according to Lowry (18).

DPP-IV enzymatic activity histochemistry. DPP-IV-like catalytic histochemistry was performed according to Lojda (19) in 10-μm cryostat sections cut at -20°C (Bright Instrument Company Ltd., Huntingdon, UK). The sections were fixed in a 1:1 mixture of acetone and chloroform for 2 min at 4°C and were incubated with Gly-Pro-4-methoxy-ß-naphtylamide (0.83×10^{-3} M) as a substrate (Bachem, Bubendorf, Switzerland) and Fast Blue B in PBS of pH 7.4 at 4°C. In controls, the DPP-IV substrate was omitted from the incubation medium.

Immunohistochemistry. Detection of DPP-IV/CD26, FAP-α and CXCR4 was performed in cryostat sections preincubated in 3% of heat-inactivated bovine foetal serum for 20 min, followed by overnight incubation at 4°C with the respective primary antibodies: mouse monoclonal anti-human CD26 (1:100, clone M-A261), rabbit polyclonal anti-human CXCR4 (1:200) from Acris, Germany and mouse monoclonal anti-human FAP-α (1:200, clone F11-24, Alexis Biochemical, USA). This was followed by incubation with anti-mouse- (1:200, Sigma, USA) or anti-rabbit (1:200, Sigma, USA)

Table III. Correlations of clinical, morphological and biochemical charactcristics of human astrocytic tumours and non-tumorous brain tissues.

	mRNA FAP-α	mRNA CXCR4	Total DPP-IV-like activity	Activity after DPP-IV inhibition	Activity after DPP8/9 inhibition	Vascularization	Nccroses
mRNA DPP-IV	**0.471**	**0.617**	**0.590**	**-0.621**	**0.458**	**0.531**	**0.648**
mRNA FAP-α		**0.522**	0.319	-0.327	ns	0.403	0.357
mRNA CXCR4			**0.639**	-0.387	ns	**0.647**	**0.657**
Total DPP-IV-like activity				**-0.452**	ns	**0.769**	**0.758**
Activity after DPP-IV inhibition					**-0.565**	**-0.452**	-0.356
Activity after DPP8/9 inhibition						ns	ns
Vascularization							**0.712**

Spearman's correlation coefficients; probability of correlations: ns, p>0.05; normal font, p<0.05; bold font, p<0.01.

IgG-FITC conjugates respectively. In controls, the primary antibodies were omitted from the medium.

The enzyme histochemistry and the immunohistochemistry sections were mounted in antifading Gel/Mount (Biomeda Corp., USA) and examined by transmission or fluorescence microscopy (Axiophot, Zeiss-Opton, Germany). Staining intensity was scored semi-quantitatively on a subjectively determined 5-titered scale (0-4 crosses) relative to the negative staining controls, averaged from 10 segments of 2-3 non-sequentional histological sections.

Statistical analysis. Statistica 6.0 sofware (StatSoft, Inc. Tulse, OK, USA) was used to perform statistical calculations. Differences between groups were evaluated with Mann-Whittney test; correlations were analyzed by means of Spearman's correlation coefficient (Table III).

Results

Co-expression of both plasma membrane-bound DPP-IV and FAP-α, together with the robust expression of chemokine receptor CXCR4 mRNA was observed in all tissue samples analyzed. Although there were significant relative variations between tumours a significant upregulation of both DPP-IV and FAP-α mRNA expression was found in WHO grade IV glioma (Fig. 1). Furthermorc, expression of DPP-IV and FAP-α transcripts was significantly correlated (Table III). Expression level of CXCR4 mRNA tightly correlated with the WHO glioma grade as well and with both DPP-IV and FAP-α mRNA expression (Table III).

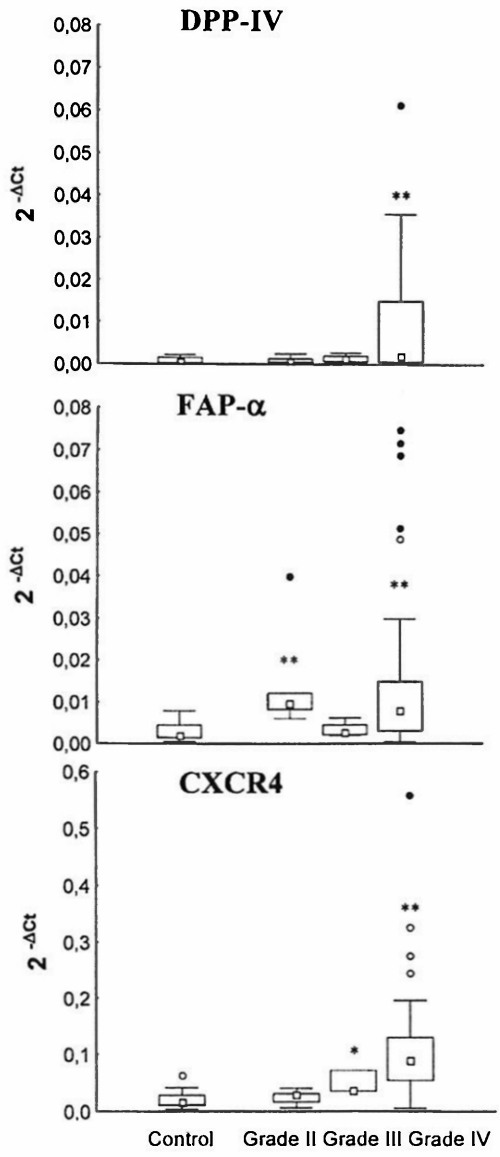

Figure 1. Expression of DPP-IV, FAP-α and CXCR4 mRNA in glioma. Squares, medians; boxes, middle 25-75% of measured values; bars, minimal resp maximal values; ◇, remote values; •, extreme values; p, compared to controls; *p<0.05, **p<0.01; Mann-Whitney U test.

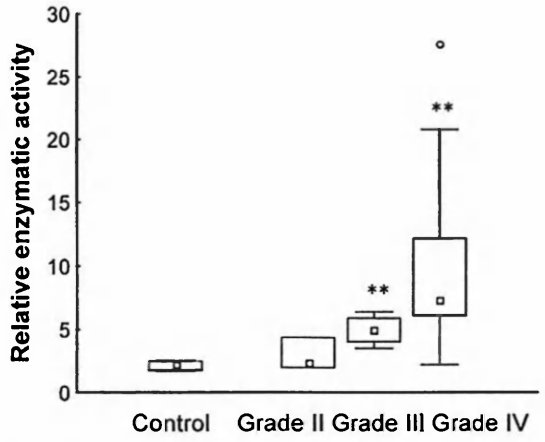

Figure 2. DPP-IV-like enzymatic activity per mg of protein in glioma. Squares, medians; boxes, middle 25-75% of measured values; bars, minimal resp maximal values; o, remote values; p, compared to controls; **p<0.01; Mann-Whitney U test.

Although DPP-IV-like enzymatic activity varied within the individual tumours, it was significantly higher in high-grade tumours compared to the non-malignant brain tissues (Fig. 2). The entire DPP-IV-like enzymatic activity correlated with FAP-α and even more significantly with DPP-IV/CD26 mRNA expression. In addition, DPP-IV-like enzymatic activity tightly correlated with CXCR4 mRNA expression (Table III).

To assess the relative participation of plasma membrane associated DASH molecules to the whole DPP-IV-like enzymatic activity present in the tissue samples, inhibition studies using selective inhibitors were performed. Using an inhibitor with 60-fold higher potency for DPP-IV/CD26 than for DPP8/9 (20), the DPP-IV-like enzymatic activity was more profoundly inhibited in astrocytic tumours of all WHO grades than that in non-malignant brain (Fig. 3). In contrast, no such differential inhibition was seen using an inhibitor possessing 50-fold higher potency for DPP8/9 than for DPP-IV (Fig. 3). Residual enzymatic activity after DPP8/9 inhibition correlated positively with mRNA expression of DPP-IV/CD26 (Table III). In line with that, the residual enzymatic activity after DPP-IV inhibition correlated inversely with both total DPP-IV-like enzymatic activity and with DPP-IV/CD26 mRNA expression (Table III). Together, results of expression and inhibition studies suggest the major part of DPP-IV like hydrolytic activity is carried by intracellular DPP8/9 in non-malignant tissues as well as in gliomas irrespective of their WHO grade. In contrast with that, enzymatic activity of plasma membrane associated DPP-IV and FAP-α seem to grow gradually along with the tumour WHO grade.

Inhibitor of DPP-II, which is intracellular and prefers an acidic pH, was devoid of virtually any effect on the enzymatic assays with Gly-Pro-NHMec as a substrate at pH 7.5, suggesting the lack of DPP-II contribution to the overall DPP-IV-like enzymatic activity. In addition, the DPP-II inhibitor negligibly affected enzymatic reactions with Lys-Ala-NHMec run at pH 5.5, i.e. in conditions typically preferred by DPP-II (not shown).

To characterize the tissue distribution of DPP-IV/CD26, FAP-α and CXCR4, enzymatic activity histochemistry and immunohistochemistry analyses were carried out. DPP-IV-like activity as well as DPP-IV/CD26, FAP-α and CXCR4 proteins in non-tumorous brain are either absent or present at trace levels except some solitary segments of the capillary/vascular bed, as best evident for DPP-IV enzymatic activity histochemistry, also in the surrounding pericyte-like cells. In contrast, significant up-regulation of DPP-IV enzymatic activity (Fig. 4) and expression of DPP-IV/CD26, FAP-α and CXCR4 proteins (Table IV) is evident in both vascular and parenchymal tissue compartments of WHO grade IV tumours (Fig. 5). In most tumour samples substantial intra-specimen variation in staining density was observed for all molecules studied.

Extent of neovascularization and necrosis represent morphological indicators used for astrocytoma tumour grading. Expected positive correlation between the tumour

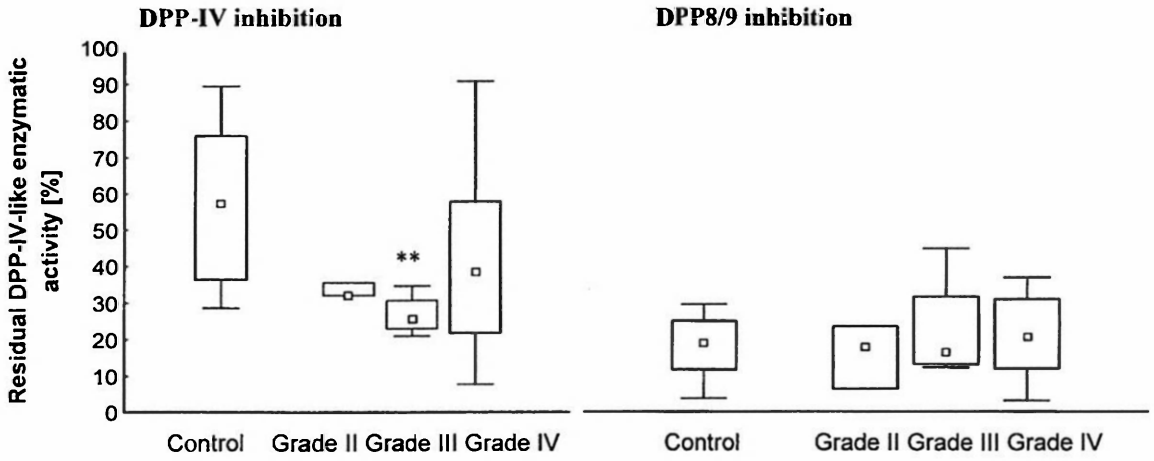

Figure 3. Residual DPP-IV-like enzymatic activity after DPP-IV and DPP8/9 inhibition. Squares, medians; boxes, middle 25-75% of measured values; bars, minimal resp maximal values; p, compared to controls; *p<0.05; Mann-Whitney U test.

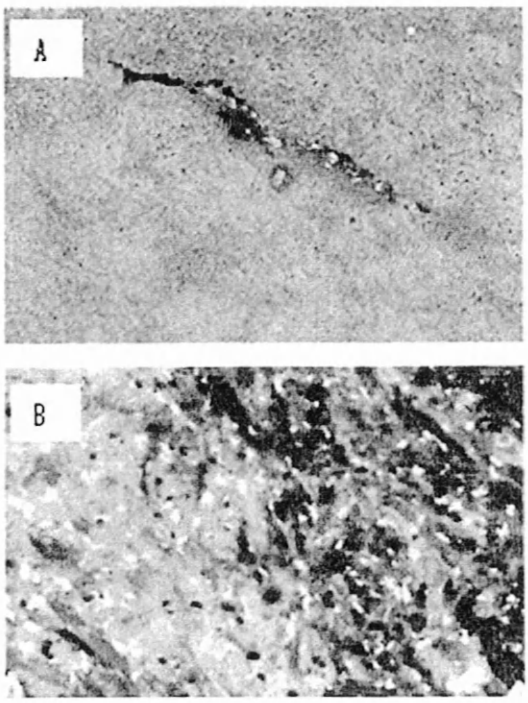

Figure 4. Localization of DPP-IV in non-tumorous (A) and glioblastoma WHO grade IV (B) tissues by enzymatic activity histochemistry. Original magnification x200.

Table IV. Enzymatic activity histochemistry[a] and immunohistochemistry[b] of DPP-IV, FAP-α, and CXCR4 in human astrocytic tumours and non-tumorous brain tissues.

	DPP-IV-like[a]	DPP-IV/CD26[b]	FAP-α[b]	CXCR4[b]
Non-malignant	Traces	Traces	Traces	Traces
Grade II	++	+++	Traces	+++
Grade III	++	++++	+++	++
Grade IV	++++	+++	+++	++++

Values represent modus values of individual samples. Staining intensities were scored as shown in Materials and methods.

grade and both these parameters was indeed observed in our experimental cohort (Table III). There was a substantial degree of correlation of DPP-IV enzymatic activity reached with both vascularization and with relative occurrence of necrosis (Table III). A similar trend was observed for DPP-IV, FAP-α and CXCR4 mRNA expression.

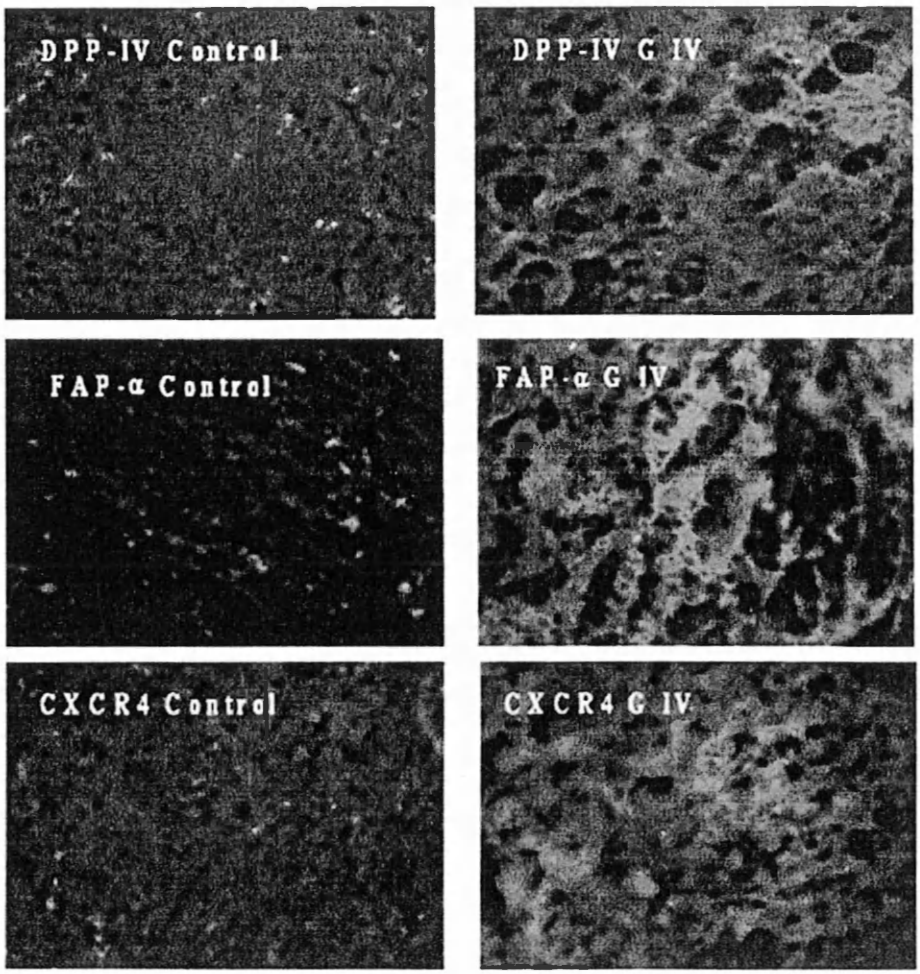

Figure 5. Detection of DPP-IV, FAP-α, and CXCR4 in non-tumorous and glioblastoma Grade IV tissues by immunohistochemistry. Original magnification x400.

Discussion

Increased DPP-IV expression and activity has so far been reported in multiple types of cancer, including thyroid, skin and prostate and in some white blood cell-derived malignancies (5). In contrast, decreased DPP-IV expression was observed in melanoma, endometrial adenocarcinoma and non-small cell lung cancer cell lines (21-23). It has been suggested that the balance of inhibitory and stimulatory protcolytic regulation by DPP-IV can have profound effects upon the available soluble growth stimulatory and growth inhibitory factors within the normal microenvironment with drastic consequences if a tumourigenic event occurs (21). Altering the balance of DPP-IV enzymatic activity therapeutically may have significant beneficial effects, as has already been demonstrated in diabetes (24) and suggested for rheumatoid arthritis (4).

In this report, we established that DPP-IV activity represented by several 'Dipeptidyl peptidase-IV activity and/or structure homologues' may be an important therapeutic target for manipulating glioma growth. The observed correlation of DPP-IV and FAP-α mRNA expression fits well with proposed co-regulation of both gene products, where FAP-α upregulation is associated with transfection-induced DPP-IV expression in some cell lines (23). Though in a different cellular context, the identification of DPP-IV-FAP-α heterodimers (25) also indirectly points to the possible functional cooperation of both enzymes. Compared to the non-malignant brain tissue, substantial upregulation of DPP-IV/CD26 and FAP-α expression associated with significantly higher DPP-IV-like enzymatic activity was observed in grade IV gliomas. Moreover, results of the inhibition studies strongly suggest that plasma membrane DPP-IV/CD26 and FAP-α are responsible for the DPP-IV-like enzymatic activity identified in high-grade tumours. Since there is no specific inhibitor, FAP-α participation can not be precisely quantified, but the weaker correlation of FAP-α rather than DPP-IV/CD26 mRNA expression with the whole DPP-IV-like activity argues for a relatively minor contribution of FAP-α. Thus, plasma membrane DPP-IV/CD26 probably represents most of the DASH cleavage activity for susceptible local growth mediators in glioblastoma. Considering the marked effect of the DPP8/9 selective inhibitor, the participation of intracellularly localized DPPs 8 and 9 in astrocytic tumours is apparent. Even in non-malignant tissues the DPP8/9 activity probably represents the dominant component of all cell-associated DASH hydrolytic activity. Nonetheless, as mentioned before, the functional targets of the intracellular and extracellular DASH molecules presumably differ.

The presence of CXCR4 and SDF-1α were observed across a range of tumours. The pro-malignant potential of the CXCR4-SDF1 axis was noted in the results of Luker and Luker (9), who documented decreased breast cancer growth as a consequence of reduced CXCR4 expression. CXCR4 tumour grade-associated expression has already been reported in gliomas (26,27). The first evidence of a possible functional link of the two so far separately studied molecular groups, chemokine system and DASH molecules, was given by Herrera *et al* (15) who found both structural association as well as a functional relationship of CXCR4 and DPP-IV in T-cells, where both molecules are internalized together upon the SDF-1α-mediated signaling. The functional relation of DPP-IV and the CXCR4-SDF-1α axis in transformed cells was later suggested by several authors (28). In this study, we identified a significant corrclation between the chemokine receptor CXCR4 and DPP-IV expression and even tighter correlation between the CXCR4 expression and DPP-IV enzymatic activity in glioblastoma. The marked upregulation of CXCR4 tightly correlating with DPP-IV might be interpreted as a compensation of DPP-IV activity-caused degradation and thus decreased availability of SDF-1α within the tumour microenvironment. Indeed, inverse correlation of CXCR4 and SDF-1α expression with the tumour grade previously observed in human endometrial adenocarcinoma was associated with DPP-IV downregulation (22).

Our findings indicate that the DPP-IV-like enzymatic activity in human brain and glioma tissues represents conjunction of hydrolytic action of several DASH members. Moreover, the increase of the hydrolytic activity in glioblastoma compared to non-malignant tissue is probably mostly derived from upregulation of canonical DPP-IV. However, the modification of DPP-IV biologically active substrate turnover at the local level might be counterbalanced by consequent regulation of their receptors. Nevertheless, our observations open up the possibility that pharmacological regulation of DPP-IV-mediated modulation of SDF-1α function may alter glioma cell migration, providing insight into downstream therapeutic modalities.

Acknowledgements

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IV.

Dipeptidyl peptidase-IV and related molecules: their role in growth of primary cell cultures derived from human brain tumors.

Stremenova J, Busek P, Balaziova E, Sromova L, Krepela E, Sedo A.

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Conference Report – Poster

Dipeptidyl peptidase-IV and related molecules: their role in growth of primary cell cultures derived from human brain tumors

Stremeňová J., Bušek P., Balažiová E., Šromová L., Křepela E., Šedo A.

Laboratory of Cancer Cell Biology, Department of Biochemistry and Experimental Oncology, 1st Faculty of Medicine, Charles University in Prague, Czech Republic

Abstract

Dipeptidyl peptidase-IV (DPP-IV, EC 3.4.14.5) is a transmembrane serine protease cleaving X-Pro dipeptides from multiple biologically active peptides such as substance P, neuropeptide Y, glucagon-like peptides or chemokines such as stromal cell derived factor-1 (SDF-1), RANTES and many others. However, DPP-IV-like enzymatic activity is also attributed to some other proteins including fibroblast activation protein-alpha (FAP), DPP-7, 8, 9, which have been grouped as "DPP-IV Activity and/or Structure Homologues" (DASH). Their proper regulation is supposed to be important for multiple cellular processes, including cell proliferation, migration, malignant transformation and invasion. We studied the expression and enzymatic activity of DPP-IV and related molecules in primary cell cultures derived from human astrocytic tumors. Using real time RT-PCR, immunocytochemistry and flow cytometry, we assessed the expression of DPP-IV, FAP, DPP-8, DPP-9 and also of receptors of some of their substrates known to be important in gliomagenesis. Primary cell cultures displayed varying morphology and differed in expression of DASH molecules and DPP-IV-like enzymatic activity. We detected the expression of the SDF-1 receptor, CXCR4, but very low expression of the substance P receptor NK1. Higher expression of DPP-IV was associated with slower cell growth. Our data support possible role of DPP-IV in the regulation of growth of primary cell cultures derived from human astrocytic tumors.

Dipeptidyl peptidase-IV and related molecules: Their role in growth of primary cell cultures derived from human brain tumors

Stremeňová Jarmila, Bušek Petr, Balážiová Eva, Šromová Lucie, Křepela Evžen, Šedo Aleksi

Laboratory of Cancer Cell Biology, Department of Biochemistry and Experimental Oncology, 1st Faculty of Medicine, Charles University in Prague, Czech Republic

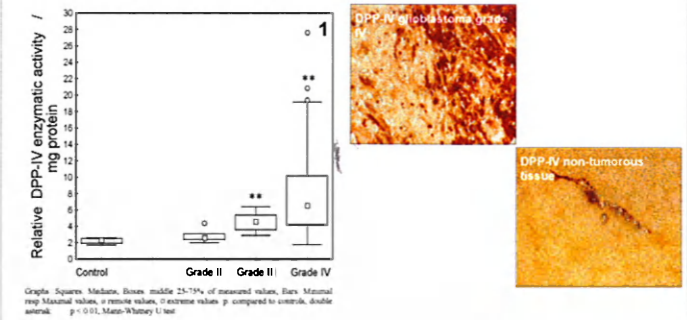
Introduction

Dipeptidyl peptidase-IV (DPP-IV, EC 3.4.14.5) is a transmembrane serine protease cleaving X-Pro dipeptides from multiple biologically active peptides such as substance P, neuropeptide Y, glucagon-like peptides or chemokines, e.g stromal cell derived factor-1 (SDF-1), RANTES and many others. DPP-IV enzymatic activity has recently also been attributed to several other proteins including fibroblast activation protein-alpha (FAP) and intracellularly localized DPP-7, 8, 9, which have been grouped as "DPP-IV Activity and/or Structure Homologues" (DASH). Their proper regulation is supposed to be important for multiple cellular processes, including cell proliferation, migration and invasion [Ref. 1.]. However, there is contradictory evidence with respect to the role of DPP-IV in oncogenesis. While our previous data using glioma cell lines argue for the antioncogenic role of DPP-IV, we observed marked DPP-IV upregulation in glioma tumor tissue [Ref. 2]. To shed light into the role of DPP-IV in gliomas we used the model of primary cell cultures derived from human astrocytic tumors.

The aim of this study was to analyze the expression and enzymatic activity of dipeptidyl peptidase-IV and related molecules and their role in cell growth in primary cell cultures derived from human astrocytic tumors.

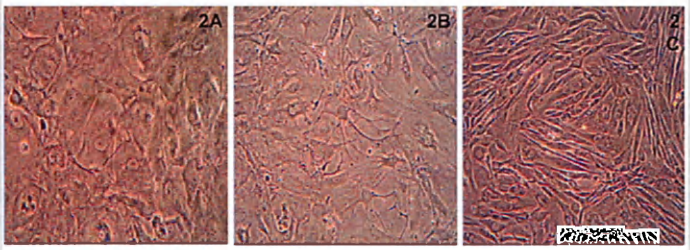
Results

I. Grade dependent increase of DPP-IV enzymatic activity in human gliomas (Fig. 1). Catalytic histochemistry demonstrates heterogeneous distribution of DPP-IV enzymatic activity in the tumor tissue.



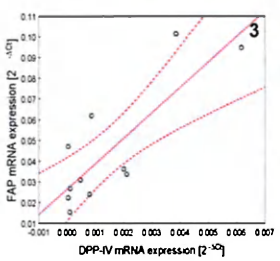
Graphs: Squares: Medians; Boxes: middle 25-75% of measured values; Bars: Minimal resp Maximal values; o remote values; * extreme values; p: compared to controls; double asterisk: p < 0.01, Mann-Whitney U test

II. Primary cell cultures (14 in total) derived from high grade gliomas displayed diverse morphology. The cultured cells were of polygonal shape and cobblestone appearance (Fig. 2A), star-like (Fig. 2B) or spindle-shaped (Fig. 2C). The cell cultures were morphologically heterogenous in early passages (1-6), reaching some degree of morphological uniformity in the later ones (12 and more).

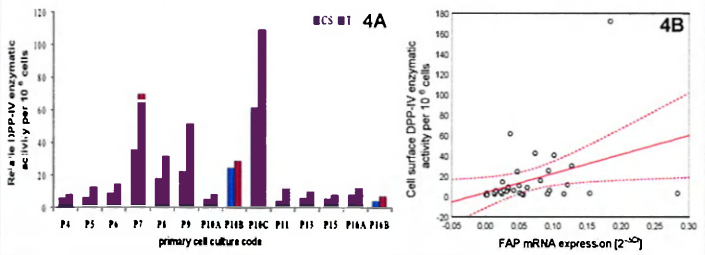


Staining for GFAP, the marker of glial cells, was weakly positive in most primary cultures (Fig. 2D, 2E, 2F).

III. Transcription of several DASH members, DPP-IV, DPP8, DPP9 and FAP was detected and quantified by real time RT-PCR. Interestingly, significant positive correlation of both plasma membrane bound DPP-IV and FAP mRNA (R = 0.673, p = 0.001) was observed (Fig. 3).

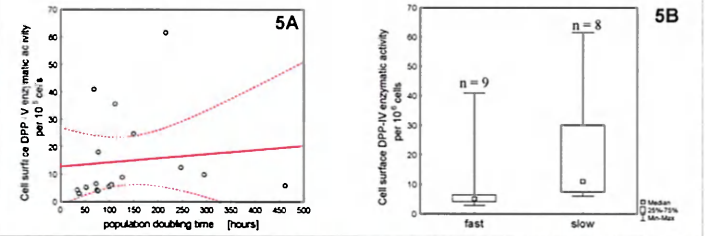


IV. Both cell surface (CS) and total (T) DPP-IV enzymatic activity varied among individual primary cell cultures (Fig. 4A). FAP mRNA correlated with the cell surface enzymatic activity (R = 0.415, p = 0.015; Fig. 4B), while no correlation between DPP-IV enzymatic activity and DPP-IV, DPP8 and DPP9 trancripts was observed.

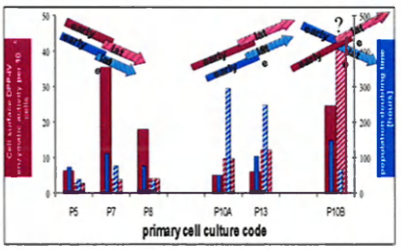


V. Cell surface DPP-IV enzymatic activity positively correlated with population doubling time (R = 0.510, p = 0.037; Fig 5A).

Moreover, when the primary cell cultures were divided into „slow growing" and „fast growing" (population doubling time higher or lower than arbitrary treshold 100 hours, respectively), „slow growing" cultures displayed significantly higher cell surface DPP-IV enzymatic activity (p = 0.029; Fig. 5B).



VI. The DPP-IV enzymatic activity of primary cell cultures differed in early and late passages, which was accompanied by changes of population doubling time (Fig. 6). In 5 out of 6 primary cell cultures, higher DPP-IV enzymatic activity was associated with slower proliferation.



Conclusions

- Expression of DPP-IV, FAP, DPP8 and DPP9 transcripts has been demonstrated in primary cell cultures derived from high grade gliomas, thus the observed DPP-IV enzymatic activity may be derived from more DASH molecules.
- The correlation of cell surface DPP-IV enzymatic activity with slower growth of primary cultures suggests that DPP-IV and FAP may participate on the regulation of transformed glial cell proliferation.
- Alhough DPP-IV and FAP in glioma tissue are associated with higher degree of malignancy, these molecules seem to hamper proliferation of transformed cells.

Methods

Primary cell cultures were derived from tumor tissue samples collected from patients undergoing astrocytic tumor resection. Fresh tissue sample was chopped to small pieces and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% of fetal bovine serum (FBS) with 100 ug/ml Streptomycin and 100 U/ml Penicillin G (Sigma, CZ). The cultivation medium was later replaced with DMEM with 10% of FBS and antibiotics.

Real time RT-PCR - expression of DPP-IV, FAP, DPP8 and DPP9 was normalized to human beta-actin mRNA ($2^{-\Delta CT}$ method) Sequence Detection System ABI PRISM 7700 was used (Applied Biosystems)

DPP-IV enzymatic activity and enzyme catalytic histochemistry was assessed in the cell suspension and cryostat sections using Gly-Pro-NHMec and Gly-Pro-NA (Bachem AG, Switzerland), respectively.

Detection of Glial fibrillary acidic protein (GFAP) was performed using monoclonal mouse anti human GFAP antibody (Exbio, CZ)

Growth curves: 4 000 cells per well were seeded in 10% FBS in DMEM in 96-well plates. Cells were counted using cell counter, population doubling time was determined from a least square regression fit of the exponential part of the growth curve

References: 1 Busek P. et al. (2004) *Int J Biochem Cell Biol* 36 406-21 2. Stremenova J. et al. (2007) *Int J Oncol* 31 785-92

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Contact: aleks@cesnet.cz, http://lbnb.lf1.cuni.cz

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Modulation of substance P signaling by dipeptidyl peptidase-IV enzymatic activity in human glioma cell lines.

Busek P, Stremenova J, Krepela E, Sedo A.

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Modulation of Substance P Signaling by Dipeptidyl Peptidase-IV Enzymatic Activity in Human Glioma Cell Lines

P. BUŠEK, J. STREMEŇOVÁ, E. KŘEPELA, A. ŠEDO

Joint Laboratory of Cancer Cell Biology of the First Faculty of Medicine, Charles University in Prague, and the Institute of Physiology, Academy of Sciences, Prague, Czech Republic

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Summary

Dipeptidyl peptidase-IV (DPP-IV, CD26) is a serine protease almost ubiquitously expressed on cell surface and present in body fluids. DPP-IV has been suggested to proteolytically modify a number of biologically active peptides including substance P (SP) and the chemokine stromal cell derived factor-1α (SDF-1α, CXCL12). SP and SDF-1α have been implicated in the regulation of multiple biological processes and also induce responses that may be relevant for glioma progression. Both SP and SDF-1α are signaling through cell surface receptors and use intracellular calcium as a second messenger. The effect of DPP-IV on intracellular calcium mobilization mediated by SP and SDF-1α was monitored in suspension of wild type U373 and DPP-IV transfected U373DPPIV glioma cells using indicator FURA-2. Nanomolar concentrations of SP triggered a transient dose dependent increase in intracellular calcium rendering the cells refractory to repeated stimulation, while SDF-1α had no measurable effect. SP signaling in DPP-IV overexpressing U373DPPIV cells was not substantially different from that in wild type cells. However, preincubation of SP with the DPP-IV overexpressing cells lead to the loss of its signaling potential, which could be prevented with DPP-IV inhibitors. Taken together, DPP-IV may proteolytically inactivate local mediators involved in gliomagenesis.

Key words

Dipeptidyl peptidase • Substance P • Glioma • NK1 receptor • Calcium signaling

Corresponding author

Aleksi Šedo, Institute of Biochemistry and Experimental Oncology, 1st Faculty of Medicine, Charles University, U Nemocnice 5, 128 53 Prague 2, Czech Republic, Fax: +420 224 965 826. E-mail: aleksi@cesnet.cz

Introduction

Dipeptidyl peptidase-IV (DPP-IV, CD26) is a widely expressed 240 kDa serine protease with a multitude of functions under both physiological and pathological conditions (for review see Lambeir *et al.* 2003). Its relatively restricted substrate specifity for proline or alanine in the P1 position directs its action on a number of biologically active peptides such as neuropeptide Y, substance P (SP), glucagon-like peptide-1 and -2 and a number of chemokines including stromal cell-derived factor-1α (SDF-1α, CXCL12) (Mentlein 1999, de Meester *et al.* 2000). Proteolytic removal of the two N-terminal amino acid residues by DPP-IV mostly decreases the biological activity of the corresponding peptide, while in some cases it can activate the peptide substrate or influence its binding to specific receptor subtypes (Mentlein 1999). DPP-IV is mostly expressed on cell surface and a soluble form is also present in the serum (Durinx *et al.* 2000). DPP-IV enzymatic activity can therefore affect auto-, para- as well as endocrine signaling of biologically active substances.

DPP-IV is frequently dysregulated in cancer, being significantly down-regulated or lost in some tumors and upregulated in others (Sulda *et al.* 2006). A number of DPP-IV substrates promote the malignant phenotype of cancer cells that express appropriate receptors. Thus, DPP-IV hydrolytic activity can interfere with some pro-oncogenic signaling pathways (Bušek *et al.* 2004, 2006). Indeed, Masur *et al.* (2006) have recently shown that a DPP-IV inhibitor can promote the growth stimulating and migratory effect of glucagon-like peptide-2 in DPP-IV

positive colon cancer cell lines. Interestingly, *in vitro* studies have mostly demonstrated that artificial upregulation of DPP-IV has an antioncogenic effect (Wesley *et al.* 1999, 2004, 2005), although the mechanism remains elusive.

In brain tumors, DPP-IV substrates SP and SDF-1α trigger a number of intracellular signaling cascades that affect cell proliferation, survival, migration and invasion (Barbero *et al.* 2003, Palma and Maggi 2000, Sharif 1998). Indeed, antagonists of the corresponding receptors NK1 and CXCR4 exhibit significant antitumor activity in gliomas (Palma *et al.* 2000, Rubin *et al.* 2003). We have previously detected expression and activity of DPP-IV and possibly other molecules bearing similar enzymatic activity, e.g. fibroblast activation protein-α, dipeptidyl peptidases 8 and 9 in human glioma tumors and cell lines (Šedo *et al.* 2004, Stremeňová *et al.* 2006). Here we explore, whether DPP-IV enzymatic activity can influence signaling of selected biologically active DPP-IV substrates in glioma cell lines.

Methods

Chemicals and cell lines

Human SDF-1α was purchased from PeproTech (UK), SP, Diprotin A and Lys[Z(NO2)]-pyrrolidide were from Bachem (Switzerland). Glioma cell lines U373 and T98G (ATCC, USA) and their transfectants (U373DPPIV, T98GDPPIV) were cultured in Dulbecco's Modified Eagle's Medium with 10 % fetal bovine serum (Sigma, Czech Republic) under standard conditions. DPP-IV was transfected into U373 and T98G cells using the mifepristone inducible Gene Switch System (Invitrogen, USA). The transfectants inducibly expressed DPP-IV upon treatment with 5 nM Mifepristone (mife). Maximum DPP-IV upregulation was achieved after 24 hours in U373DPPIV and after 48 hours in T98GDPPIV cells.

Real time RT-PCR

Total RNA was isolated using the TriZol Reagent (Invitrogen, UK) according to the manufacturer's instructions. Spectrophotometric analysis, carried out in 10 mmol/l Tris/HCl buffer, pH 7.5, revealed that the samples of total RNA had an A260 nm/A280 nm ratio greater than 1.8. The concentration of total RNA was determined using the RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, USA).

Gene coding region-specific oligonucleotide primers and fluorogenic TaqMan probes for the real time RT-PCR assays of expression of the investigated transcripts were designed with the program Primer Express (Applied Biosystems, USA) and were synthesized at Proligo (France) and Applied Biosystems (UK), respectively (Table 1). The expression of DPP-IV, NK1 receptor, CXCR4 and β-actin mRNA (an internal reference transcript) was quantified by coupled real time RT-PCR assays. The RT-PCR reaction mixtures of a total volume of 50 µl contained 25 µl of ThermoScript Reaction Mix (a buffer with 3 mmol/l $MgSO_4$ and 0.2 mmol/l of each dGTP, dCTP, dATP and dTTP) and 1 µl of ThermoScript Plus Reverse Transcriptase/ Platinum Taq DNA polymerase Mix (both mixes were from Platinum Quantitative RT-PCR ThermoScript One-Step System, Invitrogen), the respective gene-specific primers and TaqMan probe, 40 units of RNase inhibitor RNaseOUT (Invitrogen), and an input of 200 ng of total RNA. The real time RT-PCR assays were run in duplicate in MicroAmp Optical 96-well Reaction Plates on the ABI PRISM 7700 Sequence Detection System operated by the Sequence Detection System software (Applied Biosystems). The reverse transcription was carried out at 58 °C for 30 min and the subsequent PCR amplification included a hot start at 95 °C for 5 min and 45 cycles of denaturation at 95 °C for 15 s and of annealing/extension at 58 °C for 1 min. The threshold cycle (Ct) values of the amplification reactions, represented by the plots of background-subtracted fluorescence intensity (ΔFI) of the reporter dye (6-FAM or VIC) against PCR cycle number, were determined with the Sequence Detection System software. Target transcript expression was normalized to β-actin mRNA expression using the ΔCt method and the linearized ΔCt (i.e. $2^{-\Delta Ct}$) was used for comparative purposes (Livak and Schmittgen 2001).

DPP-IV enzymatic activity assay

Cell surface DPP-IV enzymatic activity was assessed in suspensions of viable cells by a continuous rate fluorimetric assay using 7-(glycyl-L-prolylamido)-4-methylcoumarin (Gly-Pro-AMC; Bachem, Switzerland) as substrate at pH 7.5 and 37 °C (Šedo *et al.* 1989).

Measurement of intracellular calcium

Mobilization of intracellular Ca^{2+} was monitored in cell suspensions at 37 °C after loading cells with the ratiometric indicator FURA-2 (final concentration 4 µmol/l) for 30 min and scraping the cells gently into a Krebs buffer (120 mmol/l NaCl, 4.75 mmol/l KCl,

Table 1. Primers and TaqMan probes used for real time RT-PCR quantitation of expression of the investigated transcripts

Transcript	GeneBank Accession No.	Sequences and final concentration of primers and TaqMan probes
DPP-IV	NM_001935	Forward primer: 5'-TGGAAGGTTCTTCTGGGACTG-3', 200 nmol/l Reverse primer: 5'-GATAGAATGTCCAAACTCATCAAATGT-3', 200 nmol/l TaqMan probe: 5'-(6-FAM)CACCGTGCCCGTGGTTCTGCT(TAMRA)-3', 200 nmol/l
NK1	NM_001058	Forward primer: 5'-CAGTGGTGAACTTCACCTATGCT-3', 400 nmol/l Reverse primer: 5'-GATGTATGATGGCCATGTACCTATC-3', 400 nmol/l TaqMan probe: 5'-(6-FAM)TCCACAACTTCTTTCCCATCGCCG(TAMRA)-3', 200 nmol/l
CXCR4	NM_001008540	Forward primer: 5'-CATGGGTTACCAGAAGAAACTGA-3', 400 nmol/l Reverse primer: 5'-GACTGCCTTGCATAGGAAGTTC-3', 400 nmol/l TaqMan probe: 5'-(6-FAM)CACCTGTCAGTGGCCGACCTCCT(TAMRA)-3', 200 nmol/l
β-Actin	NM_001101	Forward primer: 5'-CTGGCACCCAGCACAATG-3', 200 nmol/l Reverse primer: 5'-GGGCCGGACTCGTCATAC-3', 200 nmol/l TaqMan probe: 5'-(VIC)AGCCGCCGATCCACACGGAGT(TAMRA)-3', 200 nmol/l

Table 2. Expression of DPP-IV and receptors of its biologically active substrates in wild type glioma cell lines (U373 and T98G) and transfected cells (U373DPPIV and T98GDPPIV) stimulated (mife +) or not stimulated (mife -) to express DPP-IV.

	Relative cell surface DPP-IV enzymatic activity/10^6 cells	Relative mRNA expression ($2^{-\Delta Ct}$) x10^{-3} DPP-IV	NK1	CXCR4
Wild cells				
U373	1.0 ± 0.1	3.148 ± 1.138	1.129 ± 0.338	0.627 ± 0.055
T98G	0.7 ± 0.1	0.004 ± 0.001	0.011 ± 0.003	0.286± 0.067
Transfected cells				
U373DPPIVmife -	2.4 ± 0.1	89.630 ± 17.872	2.314 ± 0.628	0.195 ± 0.067
U373DPPIV mife +	27.4 ± 0.5	663.675 ± 63.162	5.097 ± 1.062	0.665 ± 0.095
T98GDPPIV mife -	1.3 ± 0.1	36.818 ± 10.271	0.143 ± 0.083	0.078 ± 0.042
T98GDPPIV mife +	543.7 ± 90.0	2444.720 ± 326.319	0.005 ± 0.001	0.074 ± 0.026

The expression of DPP-IV, NK1 receptor and CXCR4 mRNAs was normalized to the expression of human β-actin mRNA. Data are presented as means ± SEM of at least three measurements.

1 mmol/l KH_2PO_4, 5 mmol/l $NaHCO_3$, 1.44 mmol/l $MgSO_4$, 1.1 mmol/l $CaCl_2$, 0.1 mmol/l EGTA, 11 mmol/l glucose, 25 mmol/l NaHEPES, 0.1 % bovine serum albumin fraction V, pH 7.4). Fluorescence was measured at 340 nm/380 nm (excitation) and 510 nm (emission) on a Perkin Elmer spectrofluorometer. Stock solution of SP was added to the cuvette with tested cells either directly or after 10-30 min of preincubation at 37 °C with T98GDPPIV cells induced or not induced to upregulate DPP-IV. Concentration of intracellular calcium was calculated using the equation $[Ca^{2+}]_i = K_d \times (R-R_{min})/(R_{max}-R) \times SFB$, where R is the emission ratio value (340 nm/380 nm). R_{max}, the maximum 340 nm/380 nm ratio, was determined by lysing the cells with 0.1 % Triton X-100 in the presence of 1 mmol/l $CaCl_2$. The R_{min} 340 nm/380 nm ratio was obtained by adding 40 mmol/l EGTA. K_d is the dissociation constant of the Fura-2/Ca^{2+} complex (225 nmol/l) and SFB is the ratio of 380 nm fluorescences under Ca^{2+}-free/Ca^{2+} saturated conditions. Trypsin, which is known to induce calcium oscillations in glioma cells (Ubl *et al.* 1998), was used at a fmal concentration of 10^{-4} g/l as a positive control for the above described $[Ca^{2+}]_i$ rise measuring method.

Results

To quantify the potential of our model cell lines to proteolytically process biologically active DPP-IV substrates, cell surface DPP-IV enzymatic activity was determined in wild type U373 and T98G cells and in their transfected counterparts U373DPPIV and T98GDPPIV. Cell surface DPP-IV enzymatic activity was upregulated

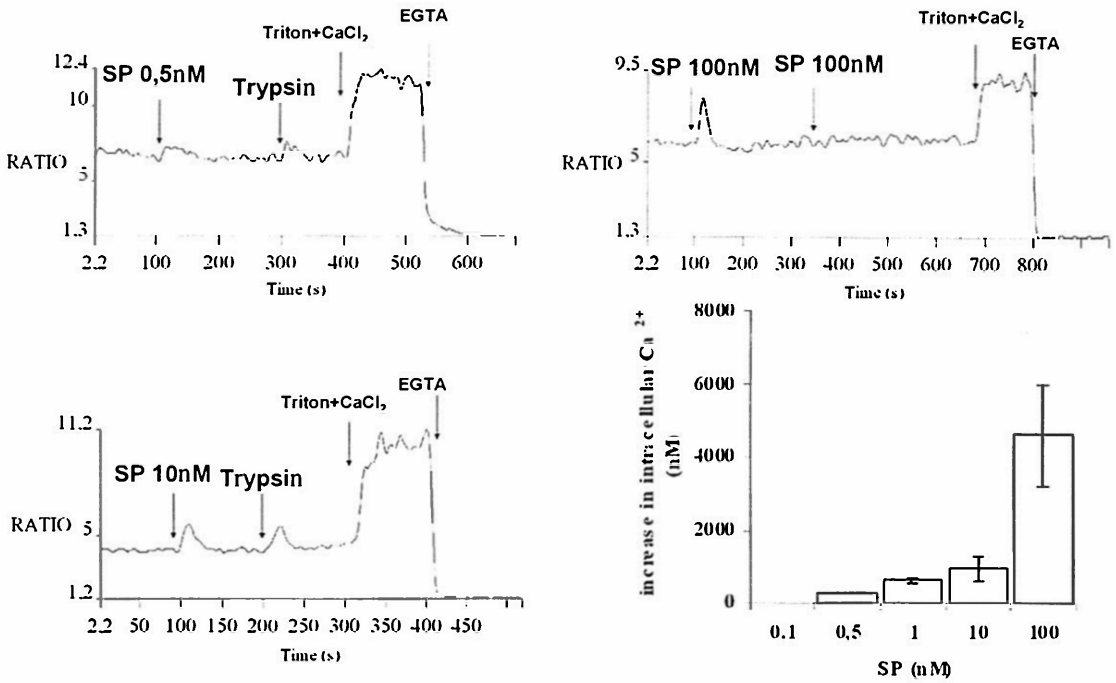

Fig. 1. Substance P triggers concentration-dependent mobilization of intracellular calcium in U373 cell line. Intracellular Ca^{2+} release was measured using the fluorescent probe Fura-2 AM. The fluorescence ratio at the two excitation wavelengths (vertical axis) is presented as an indicator of intracellular Ca^{2+} mobilization. Intracellular Ca^{2+} concentration was calculated as described in Materials and Methods; bars represent means ± SEM. SP: substance P, concentration in nmol/l [nM]

upon mifepristone stimulation 10 times in U373DPPIV and several hundred times in T98GDPPIV in comparison with the corresponding mifepristone unstimulated transfectants and wild type cells. Upregulation of DPP-IV was also confirmed at the transcriptional level (Table 2). We also determined the expression of transcripts of NK1 receptor and CXCR4, the receptors of SP and SDF-1α, respectively (Table 2).

Thereafter we tested the ability of DPP-IV substrates SP and SDF-1α to trigger calcium signaling in our model cell lines. SP caused a concentration dependent transient rise of $[Ca^{2+}]_i$ in U373 rendering the cells refractory to repeated stimulation (Fig. 1). This rise of $[Ca^{2+}]_i$ induced by SP was, however, not significantly affected by upregulation of DPP-IV in U373DPPIV cells (data not shown). To assess the possible effect of prolonged exposure of SP to high DPP-IV activity, U373 cells were exposed to SP preincubated with DPP-IV upregulating T98GDPPIV glioma cells. Such pre-treatment of SP abrogated its signaling potential (Fig 2b). SP exposed to DPP-IV upregulating T98GDPPIV cells also lost its capacity to induce the abovementioned resistance of U373 to repeated stimulation by SP not exposed to DPP-IV enzymatic cleavage (Fig. 2). On the contrary, preincubation of SP with T98GDPPIV cells not upregulating DPP-IV did not affect its potential to trigger calcium signaling (Fig. 2a). DPP-IV inhibitors Diprotin A and Lys[Z(NO2)]-pyrrolidide preserved the signaling potential of SP, which confirms that SP was inactivated specifically by DPP-IV enzymatic activity (Fig. 2d-f).

SDF-1α did not cause measurable changes of $[Ca^{2+}]_i$ in any cell line tested (data not shown).

Discussion

DPP-IV is a widely expressed serine protease that can proteolytically modify a number of biologically active peptides. It is considered to be an important regulator of SP plasma half-life (Ahmad *et al.* 1992, Wang *et al.* 1991) and has been shown to affect some SP regulated physiological processes (Grouzmann *et al.* 2002, Guieu *et al.* 2006). Functional SP receptors NK1 are widely expressed in astrocytic brain tumors and have been implicated in the regulation of their growth (Palma *et al.* 2000, Sharif 1998). NK1 receptor triggers a number of signaling cascades including elevation of $[Ca^{2+}]_i$ and activation of mitogen-activated protein kinases that can mediate the growth promoting effect of SP (Luo *et al.* 1996, Palma *et al.* 1999).

We previously detected DPP-IV enzymatic

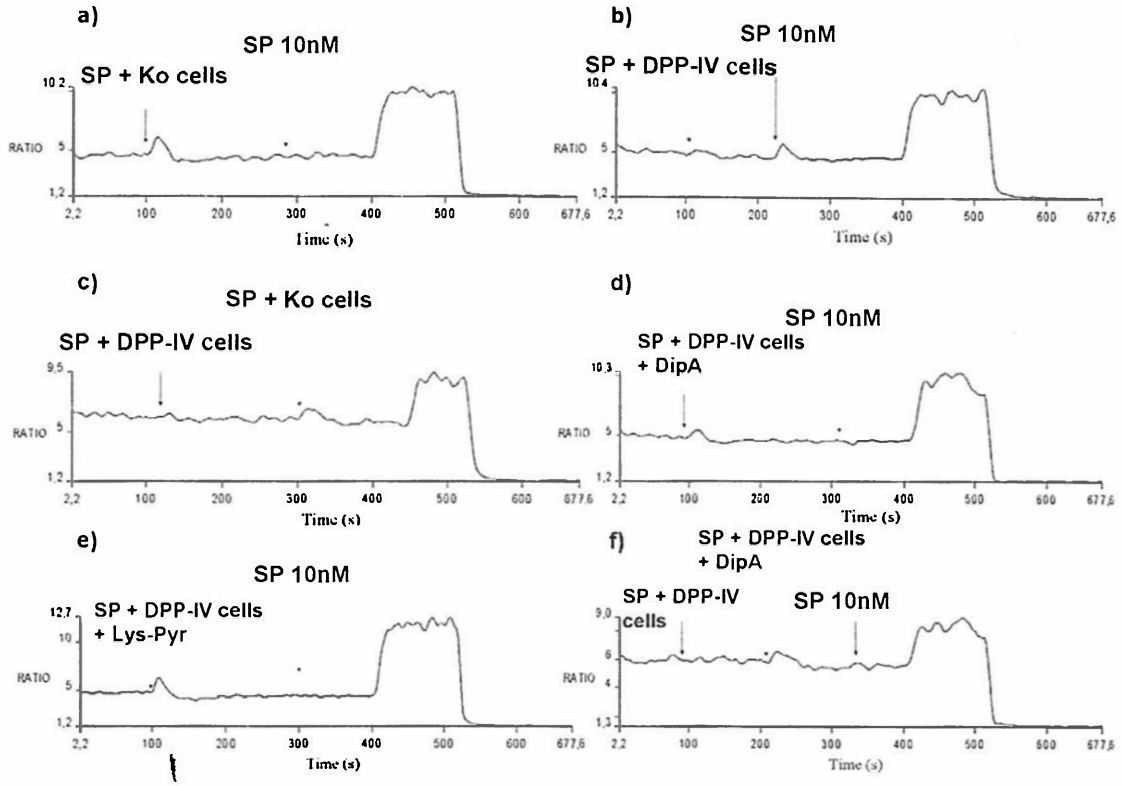

Fig. 2. Substance P loses its ability to trigger calcium signaling in U373 cells after preincubation with DPP-IV overexpressing cells. Intracellular Ca^{2+} release was monitored as described in Fig.1. Substance P (SP, 10 nmol/l [nM]) was preincubated in the suspension of T98GDPPIV cells that (a) had not been (Ko cells) or (b) had been (DPP-IV cells) induced to overexpress DPP-IV. Plots (d), (e), (f): indicated DPP-IV inhibitors DipA (200 μmol/l) and Lys-Pyr (200 μmol/l) were present in the preincubation mixture. Neither T98GDPPIV cells nor inhibitors alone induced calcium signaling in U373 cell line (data not shown). DipA: Diprotin A; Lys-Pyr: Lys[Z(NO2)]-pyrrolidide.

activity in human astrocytic tumor tissues and glioma cell lines (Šedo *et al.* 2004, Stremeňová *et al.* 2006) and observed decreased growth of DPP-IV upregulating transfectants (Bušek *et al.* 2006). Here we demonstrate that DPP-IV enzymatic activity can influence the signaling potential of SP in glioma cell lines. In agreement with the literature (Sharif 1998, Palma *et al.* 1999), SP induced Ca^{2+} signaling in U373 but not in T98G cell line, which well corresponds to the observed substantially lower expression of NK1 receptor mRNA in T98G cells (Table 2). On the contrary, SDF-1α did not cause measurable changes of $[Ca^{2+}]_i$ in any cell line tested, although we detected mRNA expression of its receptor CXCR4. However, Oh *et al.* (2001) also observed SDF-1α induced changes of $[Ca^{2+}]_i$ in glioma cells only after enhancement of CXCR4 expression by cytokines.

Calcium signaling triggered by SP in U373DPPIV cells upregulating DPP-IV was similar in duration and magnitude to that in wild type U373. This is most likely because the second messenger calcium signaling occurs within seconds while degradation of SP by cell surface DPP-IV into the less active SP5-11 fragment may require longer periods of time. The upregulation of DPP-IV was also much lower in U373DPPIV compared to T98GDPPIV, which did not express NK1 receptors (Table 2). However, it should be considered that i) complex cellular programs such as cell proliferation frequently require prolonged exposure to the particular ligand, and ii) the ligand may be subject to functionally relevant proteolytic cleavage by surrounding cells that neither secrete nor respond to it. To simulate functional interaction of two cell types in SP signaling, U373 cells were exposed to SP that had been preincubated with T98GDPPIV glioma cells overexpressing DPP-IV but lacking NK1 receptor. These experiments demonstrated functional inactivation of SP, which was prevented with specific DPP-IV inhibitors.

Cleavage of SP and other susceptible regulatory molecules may contribute to the growth inhibitory effect of DPP-IV that has been observed in several cell lines (Wesley *et al.* 1999, 2004, 2005), including glioma cells (Bušek *et al.* 2006). Our results also suggest that prereceptor modification of signaling peptides by DPP-IV

enzymatic activity may be physiologically relevant even for DPP-IV negative cells. Thus, DPP-IV present in the tissue microenvironment may represent an important regulator of local humoral signaling.

Conflict of Interest

There is no conflict of interest.

Acknowledgement

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