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

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SOUHRN

Dipeptidylpeptidáza-IV (DPP-IV, EC 3.4.14.5) patří spolu s proteinem aktivovaných fibroblastů-alfa (FAP) a dipeptidylpeptidázami -7, -8 a -9 do funkčně definované skupiny „Dipeptidylpeptidáze-IV aktivitou a/nebo strukturou homologních molekul“ (DASH). Ty odštěpují X-Pro dipeptidy z N-konce řady biologicky aktivních peptidů, například neuropeptidu Y, substance P a některých chemokinů, zejména faktoru stromálních buněk-1alfa (SDF-1). Limitovaná proteolýza těchto mediátorů DPP-IV-podobnou enzymovou aktivitou může významně ovlivňovat biologické odpovědi cílových buněk. Proto je též předpokládána významná role DASH molekul v regulaci buněčné proliferace, maligní transformace, migrace a invaze a tím jejich patogenetický význam v procesu vzniku a rozvoje a nádorů.

Cílem této studie bylo charakterizovat expresní vzorec DASH molekul a DPP-IV-podobnou enzymovou aktivitu v lidských astrocytárních nádorech a porovnat je s nenádorovou mozkovou tkání, s přihlédnutím k jejich kontextu s expresí receptorů některých lokálních mediátorů-DASH substrátů, účastnících se v gliomagenezi. Zároveň byl na modelu primárních kultur odvozených z glioblastomů studován možný funkční význam DASH molekul pro růstové vlastnosti transformovaných astrocytárních buněk *in vitro.*

V lidských astrocytárních nádorech jsme v tumorech vyššího stupně malignity pozorovali významně vyšší DPP-IV-podobnou enzymovou aktivitu. Hlavní podíl na DPP-IV-podobné enzymové aktivitě nenádorové mozkové tkáně i tkáně astrocytomů je pravděpodobně tvořen intracelulární DPP8 a DPP9, zatím co výrazný nárůst u vysoce maligních astrocytomů je atributem membránově vázané DPP-IV a pravděpodobně i FAP. Zároveň exprese a aktivita DPP-IV významně korelovala s expresí CXCR4, receptoru pro SDF-1. Zvýšení aktivity DPP-IV degradující SDF-1 může tedy být v rámci nádorového mikroprostředí kompenzováno zvýšenou expresí příslušného receptoru. Naše *in vitro* studie ukázaly, že primární kultury s vyšší DPP-IV-podobnou enzymovou aktivitou vykazují pomalejší růst.

Proto předpokládáme, že ačkoli DPP-IV na úrovni transformované buňky může představovat anti-onkogenní molekulu, svými dalšími biologickými funkcemi, například v procesech angiogeneze může ve svých důsledcích na úrovni nádorové tkáně působit pro-onkogenně.

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

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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 [1, 2]. 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) [3]. 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 [4]. However, the existence of that hydrolytic activity was later challenged by results of Friedrich et al [5]. Likewise DPP-IV-like enzymatic activity of N-acetylated alpha-linked acidic dipeptidase-I, -II and -like (NAALADase) formerly described by Pangalos et al [6] was later disputed by Barinka et al [7]. 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" [8]. 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 [9], 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 [10]. It is identical with the differentiation antigen CD26 of activated T-cells [11, 12]. 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 [13]. 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 [14].

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 [15]. 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 [16, 17]. However, point mutation of nine Asn residues in glycosylation site of the molecule refuted that assumption [18].

A soluble form of DPP-IV lacking the transmembrane and cytoplasmic domains was found in blood plasma [19, 20], saliva [21, 22], cerebrospinal fluid, synovial fluid, seminal plasma and urine [23-26]. 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.

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 [27]. FAP is structurally highly similar to DPP-IV [28]. It bears not only the characteristic dipeptidyl aminopeptidase activity, but also endopeptidase collagenase/gelatinase activity [29, 30]. 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 [30-32]. To execute such functions, it is typically localised in the invadopodial membranes [33, 34]. 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 [35, 36].

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 [37].

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 [38, 39]. DPP-II is enzymatically active preferably in the acidic pH range 5 – 6 [40] 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 [41].

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 [39, 42-44].

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 [45], this functional potential has not been confirmed by contemporary studies [39].

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 [46]. 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 [47, 48].

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 [46]. The highest expression of DPP9 mRNA was detected in skeletal muscle, heart, liver and peripheral blood leukocytes, and the lowest in the brain [49]. 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 [50]. Till today, physiological substrates of DPP8 and DPP9 *in vivo* are just speculated [51].

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 [52-54].

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 [12]. Although DPP-IV/CD26 is almost absent on resting B and NK cells, its expression on their surface is effectively induced upon stimulation [55]. 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 colocalisation 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 [56, 57]. 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 [58]. 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 [59], which was also observed in thymocyte activation [60].

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 [61, 62].

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 [63].

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 [64, 65]. 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 [66].

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 [67, 68].

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 [69-71]. The ability of DPP-IV to interact with ECM has a projection in immune regulations [72] 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 [71, 73].

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 [74], which facilitates conversion of plasminogen to plasmin, eventually leading to augmented degradation of ECM, and thus enhancing metastasising [75]. Interestingly, soluble FAP antiplasmin-cleaving enzyme may abrogate such pro-metastasising potential of DPP-IV due to hydrolytic activation of alpha2 antiplasmin [37].

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 [52, 54].

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 [76].

Table 1. Examples of biologically active peptides amenable to cleavage by DPP-IV-like enzymatic activity; published in [9].

Types of substrates	**Examples**	**Physiological consequence**
Incretins and gastrointestinal hormones	Glucagone-like peptide-1, 2	Inactivation
	Gastric inhibitory polypeptide	Inactivation
	Pituitary adenylate cyclase-activating peptide	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
	Monokine induced by gamma interferon	Inactivation
	Interferon-inducible protein-10	Inactivation
	Regulated on Activation, Normally T-cell-Expressed and Secreted (RANTES)	Changed receptor preference
	Macrophage-derived chemokine	Changed receptor preference
	Macrophage Inflammatory Protein-1beta	Changed receptor preference
	LD78beta	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 [77-79], DASH molecules modify activity of regulatory peptides by limited proteolysis.

Table 2. DASH molecules in neoplasia versus non-tumorous tissue; published in [9].

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 tumours
		Differentiated thyroid papillary and follicular cancer
FAP	**Increased**	Breast cancer
		Melanoma
		Gastric cancer
		Colorectal adenocarcinoma
		Cervical cancer
		Astrocytic brain tumors
		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 [80], 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 [81]. 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 [82, 83]. Upregulation of SDF-1 and CXCR4 was observed in many cancer types [84-88], 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 [89]. The truncated fragment SDF-$1_{(3\text{-}68)}$, 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 [90]. 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 [51].

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 [91], 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 [92]. 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 [93].

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 [94]. In addition, the expression of NK1 receptors in peritumoral and tumoral blood vessels suggests a role of SP in tumour neoangiogenesis and vasodilatation [95]. Since the C-terminus of SP is responsible for appropriate receptor binding and following signalisation, the truncated fragments $SP_{(3-11)}$ and $SP_{(5-11)}$ 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) [13].

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 [96]. 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 [97]. NPY actions are with lower efficiency also regulated by DPP8 and DPP9 *in vitro*, but not by DPP-II [98].

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 [99-101].

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 [99-101].

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 categorised 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 [102-104].

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 [104].

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.

<u>Aim I.</u> 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.

<u>Aim II.</u> 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. Proliferation activity was approached by quantification of Ki67 antigen expression using mouse monoclonal anti-human (1:50, clone MIB-1) 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

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) supplemented with 20% fetal bovine serum (FBS), 100 g/ml Streptomycin and 100 U/ml Penicillin G. 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, and subcultured.

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

Total RNA was isolated using the TriZol Reagent according to the manufacturer's instructions. The concentration of total RNA was determined using the RiboGreen RNA Quantitation Kit.

Real time RT-PCR was performed as described previously [88]. Briefly, total RNA was isolated using the TriZol Reagent according to the manufacturer's instructions. The expression of DPP-IV, FAP, DPP8, DPP9, NK1 receptor, CXCR4 and beta-actin mRNA (an internal reference transcript) was quantified using gene coding region-specific oligonucleotide primers and fluorogenic TaqMan probes by coupled real time RT-PCR assays on the ABI PRISM 7700 Sequence Detection System operated by the Sequence Detection System software. Target transcript expression was normalised to beta-actin mRNA expression using the ΔCt method and the linearised ΔCt (i.e. 2-ΔCt) was used for comparative purposes.

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 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) in PBS of pH 7.4, and with H-7-(L-Lysyl-L-Alanylamido)-4-methylcoumarin (H-Lys-Ala-AMC) in citrate/Na_2HPO_4 buffer of pH 5.5 as substrates at the final concentration of 50 μmol/l [105]. The release of 4-amino-7-methylcoumarin was monitored at 380 nm excitation and 460 nm emission wavelengths. Selective DPP-IV and DPP8/9 inhibitors (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 [106] in 10 μm cryostat sections cut at –20 °C. 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 (0.83×10^{-3} mol/l) as a substrate 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), mouse monoclonal anti-human FAP (1:200, clone F11-24), rabbit polyclonal anti-human DPP8 and DPP9 (1:150), rabbit polyclonal anti-human NK1 (1:200), rabbit polyclonal anti-human CXCR4 (1:200), goat polyclonal anti-human CXCR4 (1:200), and mouse monoclonal anti-human GFAP (1:100). This was followed by incubation with anti-mouse- (1:200) or anti-rabbit- (1:200) -IgG-FITC conjugates respectively, anti-goat-IgG-TRITC (1:100) or anti-mouse AlexaFluor 488 (1:1000). In staining controls, the primary antibodies were omitted from the medium. The catalytic enzyme histochemistry, the immunohisto- and immunocytochemistry sections were mounted in antifading Gel/Mount and examined by transmission or fluorescence microscopy. 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. 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) 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. Total protein concentration

Total protein concentration was assayed according to Lowry [107].

3.10. Statistical analysis

Statistica 8.0 sofware was used. Differences between groups were evaluated with Kruskal-Wallis or Mann-Whitney tests; correlations were analysed by means of Spearman correlation coefficient.

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 1). The median activity observed in glioblastomas (WHO grade IV) was significantly higher than the one in a non-tumorous brain ($p < 0.01$). DPP-IV-like enzymatic activity in meningiomas was comparable or even higher than that in glioblastoma.

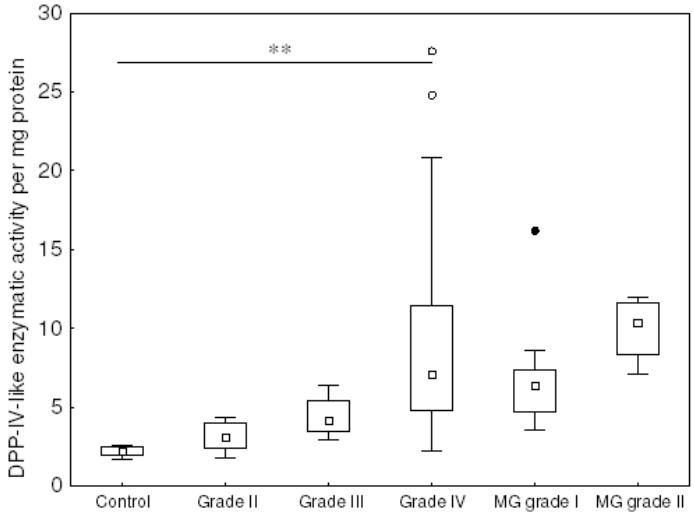

Figure 1. DPP-IV-like enzymatic activity in non-tumorous brain (Control), astrocytic tumours grade II-IV (Grade II-IV) and meningiomas grade I-II (MG grade I-II) measured by fluorimetric assay using H-Gly-Pro-AMC as a substrate. Squares: Medians; Boxes: middle 25-75% of measured values; Bars: Minimal 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 2). 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 a non-malignant brain (Figure 2A). In contrast, no such differential inhibition was seen using an inhibitor possessing 50-fold higher potency for DPP8/9 than for DPP-IV (Figure 2B), suggesting the major proportion of canonical DPP-IV on the DPP-IV-like enzymatic activity increment observed in high-grade gliomas.

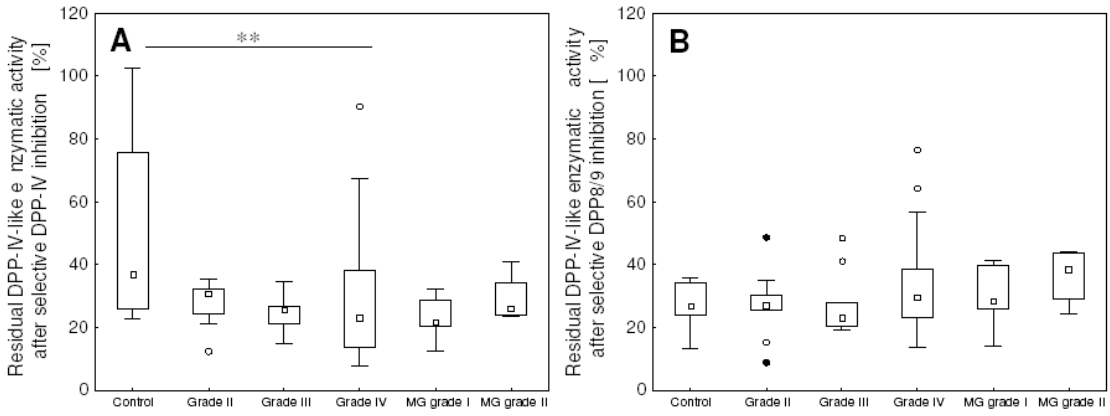

Figure 2. Residual DPP-IV-like enzymatic activity after selective DPP-IV (A) and DPP8/9 (B) inhibition in non-tumorous brain (Control), astrocytic tumours grade II-IV (Grade II-IV) and meningiomas grade I-II (MG grade I-II). Squares: Medians; Boxes: middle 25-75% of measured values; Bars: Minimal resp 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 3). 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 ($R = -0.468$, $p < 0.01$).

As to the expression of receptors of DASH catalytic partners, no statistically significant differences suggesting tumour grade dependence were observed in the case of NK1 receptor (Figure 4A). 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 4B). 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 ($R = -0.309$, $p < 0.05$).

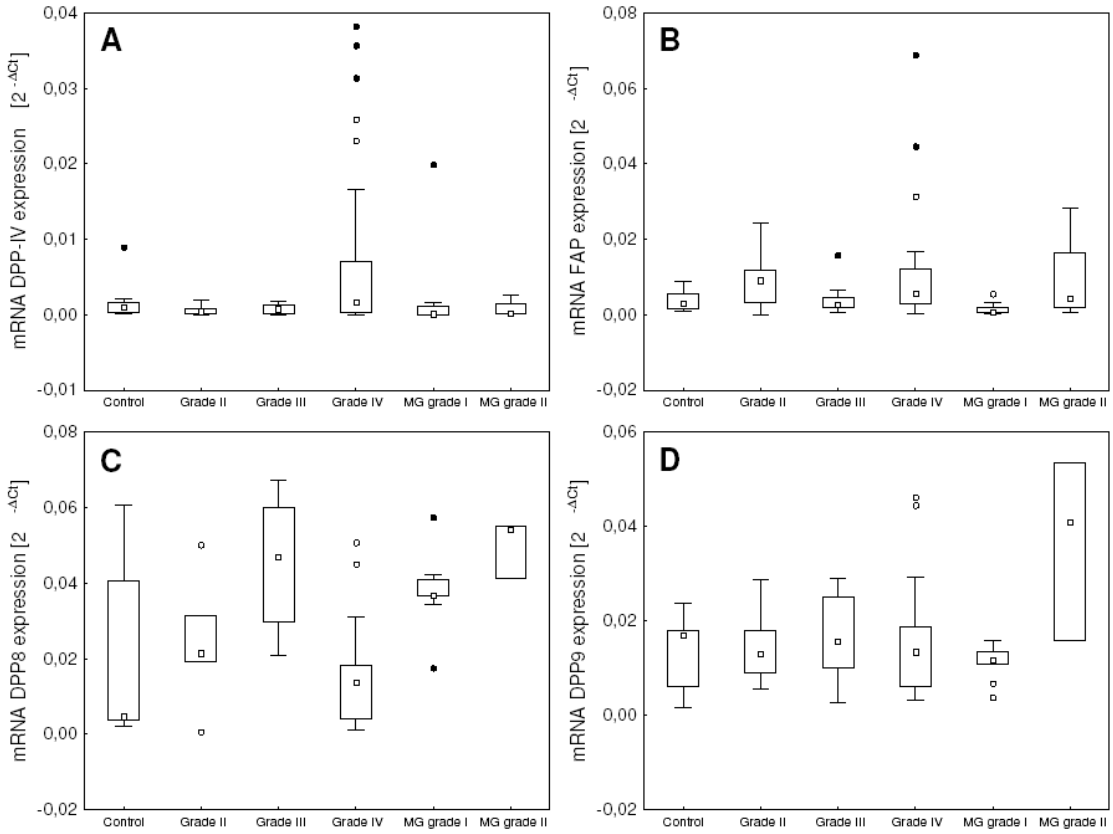

Figure 3. The mRNA expression of DPP-IV (A), FAP (B), DPP8 (C), DPP9 (D) determined by real time RT-PCR normalised to human beta-actin in non-tumorous brain (Control), astrocytic tumours grade II-IV (Grade II-IV) and meningiomas grade I-II (MG grade I-II). Squares: Medians; Boxes: middle 25-75% of measured values; Bars: Minimal resp Maximal values; o: remote values, ●: extreme values.

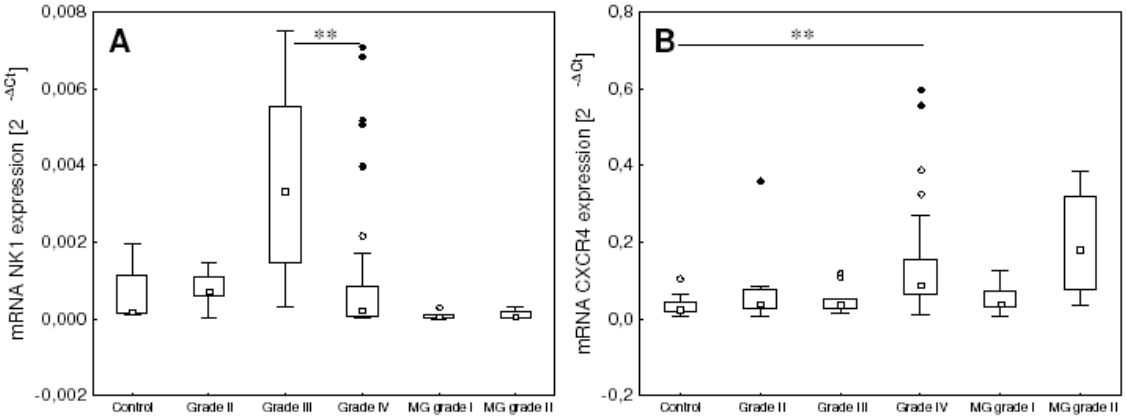

Figure 4. The mRNA expression of NK1 (A) and CXCR4 (B) receptors determined by real time RT-PCR normalised to human beta-actin in non-tumorous brain (Control), astrocytic tumours grade II-IV (Grade II-IV) and meningiomas grade I-II (MG grade I-II). Squares: Medians; Boxes: middle 25-75% of measured values; 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, an immunohistochemical approach was used in context with DPP-IV-like enzymatic activity histochemistry (Table 4). 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 a 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 4. 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	+

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 5).

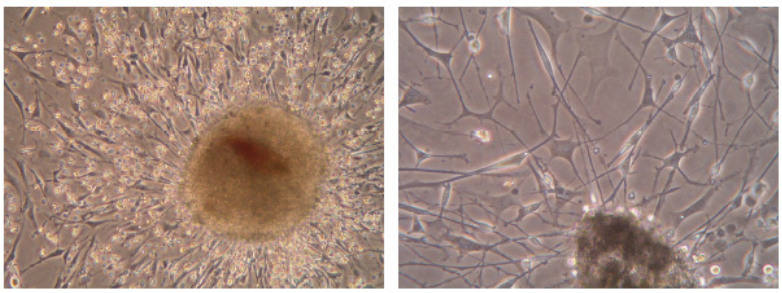

Figure 5. Primary explant culture from glioblastoma multiforme tissue. Explant and early-stage outgrowth after 2-5 days of cultivation in 20% FBS in DMEM supplemented with antibiotics. Phase contrast, original magnification 200x.

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

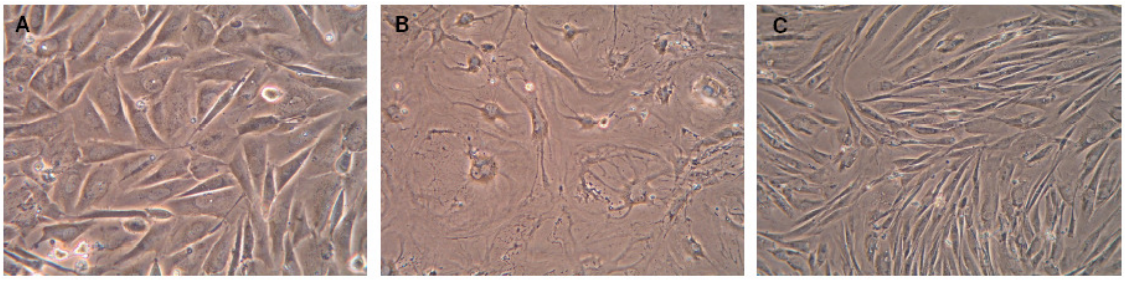

Figure 6. Differing morphology of primary cell cultures derived from glioblastoma multiforme. Polygonal shaped cells (A), polymorphic cells (B) and spindle-shaped cells (C). Phase contrast, original magnification 200x.

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 on the transcriptional and protein level as well. 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 the DPP-IV transcript, FAP mRNA expression correlated with the cell surface DPP-IV-like enzymatic activity ($R = 0.370$, $p < 0.05$). 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 (data not shown).

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

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

Doubling time of primary cell cultures 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 7B) 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 the early (6th) and late (12th) passages, which was typically accompanied by inverse changes of population doubling time (Figure 7A). 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, a steep decrease of its DPP-IV-like enzymatic activity associated with enhanced cell growth was detected.

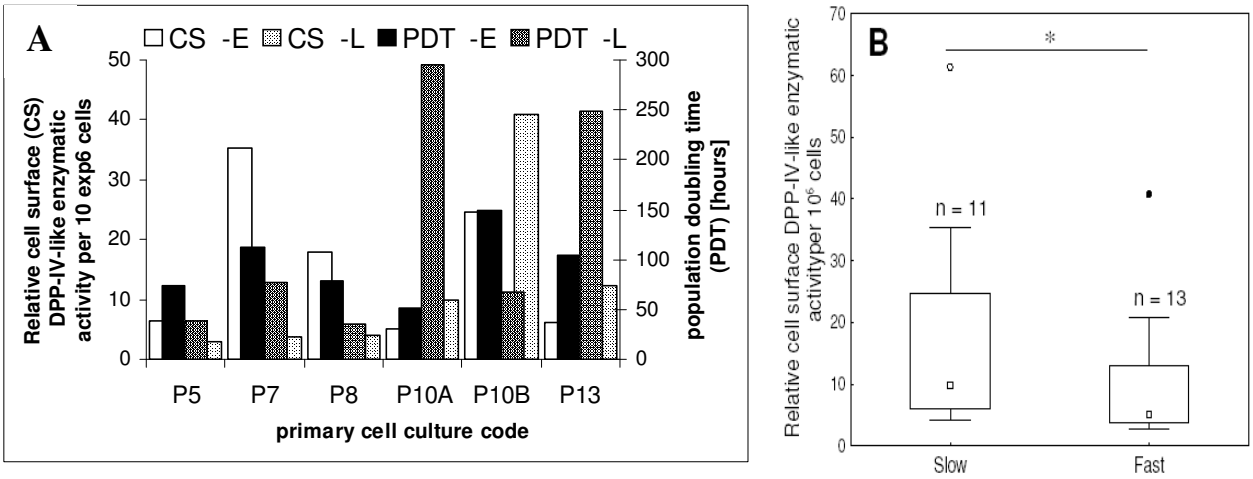

Figure 7. (A) Alteration of cell culture proliferation associated with changes of cell surface DPP-IV-like enzymatic activity. CS: cell surface DPP-IV-like enzymatic activity; PDT: population doubling time; E: early (6th) passage; L: late (12th) passage. In 5 out of 6 primary cell cultures, higher DPP-IV-like enzymatic activity was associated with slower proliferation. **(B) 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.

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 8). Migration of glioma primary cell cultures seemed to be independent on their DPP-IV-like enzymatic activity and the DASH expression pattern.

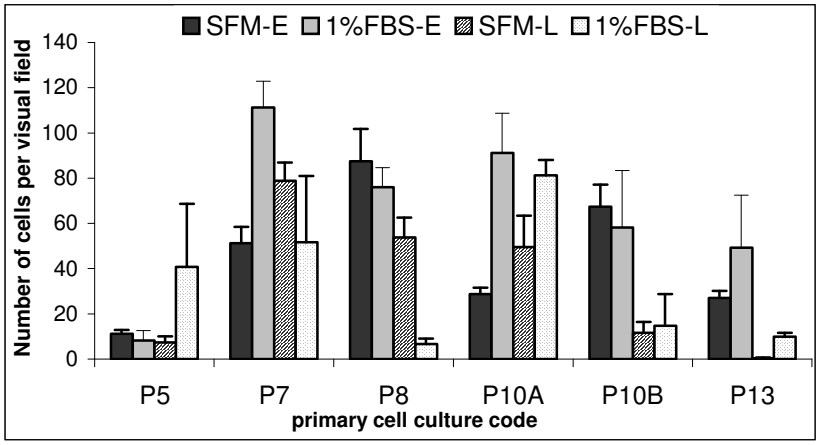

Figure 8. Number of cells migrating in SFM medium and 1% FBS in early (E) and late passage (L). Results are presented as means of quadruplicates ± SD.

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

(i) The 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) The DASH expression and DPP-IV-like enzymatic activity of primary cell cultures had no effect on 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 [9].

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 [108, 109]. Though in a different cellular context, the identification of DPP-IV-FAP heterodimers also indirectly points to the possible functional cooperation of both enzymes [35, 36].

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 [9]). 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 [109].

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 [109]. 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 within the tumour microenvironment, has numerous downstream projections, including regulation of neovascularisation, modulation of cancer cell proliferation, migration and invasion. To that end, recent results support the importance of DASH molecules, namely DPP-IV and FAP in angiogenesis [110]. On the other hand, DPP-IV mediated degradation of local pro-oncogenic mediators of gliomagenesis, as e.g. neuropeptide SP and chemokine SDF-1, might be disadvantageous for transformed cells. Truncation of SDF-1 by DPP-IV-like enzymatic activity inhibits its chemotactic activity, while the resulting fragment may even act as an antagonist of CXCR4. In light of that, upregulation of CXCR4 tightly correlating with DPP-IV expression and activity in glioblastoma [88], but not in meningioma might be interpreted as a compensation of DPP-IV activity-mediated degradation of SDF-1 within the microenvironment of highly malignant tumours. Analogical co-expression of DPP-IV with CXCR4 has been later observed in more cancer types [111, 112]. Suggested orchestrated regulation of DPP-IV hydrolytic potential with the SDF-1-CXCR4 axis led us to the definition of putative "DASH system", consisting of plasma membrane localised DPP-IV-like enzymatic activity-bearing molecules, their biologically active autocrine/paracrine substrates and relevant receptors [88]. Together, it might be speculated that the effective tuning of CXCR4 and DPP-IV represent important pathogenetic momentum of the glioblastoma progression.

Similarly, another substrate of DPP-IV-like enzymatic activity, SP, has been implicated in the regulation of astrocytoma cell lines [113, 114]. NK1 receptor triggers a number of signalling cascades including elevation of $[Ca^{2+}]_i$ and activation of mitogen-activated protein kinases [115]. In our previous work, we described negative effect of DPP-IV upregulation on the SP mediated intracellular calcium mobilization in glioblastoma cell lines [116]. Observed upregulation of NK1 receptor in glioblastoma tissue, correlating with FAP mRNA expression may represent another lead for future studies although there is so far absent precedent knowledge of SP and FAP functional association.

As we mentioned previously, DPP-IV inhibitors are actually used in the treatment of diabetes mellitus. However, in broader sense, "Dipeptidyl peptidase-IV activity and/or structure homologues" are speculated to be possible therapeutic targets in several other diseases, including cancer [117]. Our recent observations of upregulated DASH in glioblastoma partially support that concept as well. However, a result of functional studies indicating DASH multifaceted cellular 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 [9].

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 [9]. 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.

8. REFERENCES

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9. LIST OF PUBLICATIONS RELATED TO THE THESIS

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