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Chapter 8

Dipeptidyl Peptidase-IV and Related Proteases in Brain Tumors

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Additional information is available at the end of the chapter

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1. Introduction

Malignant gliomas rank among the most aggressive human tumors. The hallmarks of these tumors include a highly infiltrative behavior, aberrant cell proliferation and apoptosis, increased angiogenesis and intratumoral as well as systemic immunosuppression [1, 2]. Proteases localized on the cell-surface or released extracellularly may significantly contribute to these characteristics by mediating the breakdown of the components of the extracellular matrix (ECM), liberating growth factors sequestered by binding to the ECM, regulating the activity of paracrine mediators and shedding of cell-surface proteins [3]. There is substantial evidence for the role of matrix metalloproteinases (MMP), the serine protease urokinase-type plasminogen activator (uPA) and the cysteine protease cathepsin B in glioma invasion [4], angiogenesis [5] and proliferation. In addition, expression of proteases such as cathepsin D, uPA or MMP-9 in the clinical material may predict patient prognosis [6-8]. Nevertheless, the role of several proteases including the canonical dipeptidyl peptidase-IV (DPP-IV) and related proteases in glioma progression remains largely unknown with only few studies using synthetic inhibitors or genetic manipulation to determine their function. In this chapter, we review the basic characteristics of DPP-IV and related proteases, focus on their functional role in the transformed as well as stromal cells, and discuss the implications for the biology of human gliomas.

1.1. "Dipeptidyl peptidase-IV activity and/or structure homologous" (DASH) molecules

Historically, dipeptidyl peptidase-IV (DPP-IV, EC 3.4.14.5, identical with the lymphocyte differentiation antigen CD26) was described by Hopsu-Havu and Glenner [9] in liver homogenates on the basis of its unique hydrolytic activity cleaving N-terminal dipeptides from synthetic chromogenic substrates with the proline residue in the penultimate position. The

presence of similar enzymatic activity was observed in body fluids soon after that [10, 11]. At that time, DPP-IV was hypothesized to participate on the turnover of the regulatory as well as structural proteins bearing the consensus cleavage sequence. However, the speculations about its particular biological roles awaited experimental confirmation. Subsequently, multiple authors noted substantial heterogeneity of molecular forms that possessed strikingly similar enzymatic activity but differed in molecular weight, isoelectric point and subcellular localization [12]. It took several years to identify and characterize other "DPP-IV-like" molecules, individual gene products, exhibiting various degree of structural homology with the canonical DPP-IV. These comprise the intracellularly localized DPP8 and DPP9 (both still assigned under the same EC 3.4.14.5) [13, 14], the plasma membrane fibroblast activation protein-alpha/seprase (FAP, EC 3.4.21.B28) [15] as well as the DPP-IV sequentially dissimilar intracellular DPP-II (quiescent cell proline dipeptidase, DPP7, EC 3.4.14.2)[16]. Besides, highly structurally similar but hydrolytically inactive DPP6 and DPP10 were discovered later [17]. Recently, all these molecular species are by some authors referred to as the "Dipeptidyl peptidase-IV activity/and or structure homologous" (DASH) molecules [18-24]. Formerly, Glutamate carboxypeptidase II (GCPII, N-acetyl-L-aspartyl-L-glutamate peptidase I, NAALADase I, prostate specific membrane antigen, EC 3.4.17.21) and Attractin were proposed to belong to this group on the basis of the presumed DPP-IV-like enzymatic activity [24]. However, further research did not confirm the hydrolytic potential of these molecules [25, 26]. Since both of them also lack any significant structural homology with DPP-IV, they are no more included in the DASH group.

1.1.1. Dipeptidyl peptidase-IV

In humans, the canonical DPP-IV is almost ubiquitously expressed as a single-pass type II integral transmembrane glycoprotein in a variety of cell types, tissues and organs (reviewed in [11, 27]). Its soluble counterpart is detectable in body fluids, being either a product of proteolytic shedding from the cell surface or a putative specific secretory form [28]. Upregulation of the plasma membrane DPP-IV is associated with cell differentiation in e.g. T cells [29, 30], hepatocytes [31] and intestinal epithelium [32]. The expression and function of DPP-IV/CD26, a marker of a subset of activated T-cells, was intensively studied in the immune system [33]. Its crosslinking in T cells affects the synthesis and secretion of a number of cytokines and interleukins [34, 35]. DPP-IV is also identical with the adenosine daeminase binding protein and participates on the immunoregulations by influencing the pericellular concentration of free adenosine [36, 37]. The physiological relevance of the interaction of DPP-IV with plasminogen 2 [38] and several proteins of the ECM [39, 40] is still more speculated than proven.

1.1.2. Fibroblast activation protein

Possessing about 52% amino acid sequence identity with DPP-IV, FAP represents its closest homologue within the DASH group. Its gene is located on chromosome 2q23 and is believed to be a product of DPP-IV gene duplication (reviewed in [15]). FAP is typically expressed as a type II transmembrane protein and its soluble counterpart is present in blood plasma and

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is also known as α2-antiplasmin cleaving enzyme [41, 42]. In contrast to DPP-IV, FAP expression is substantially restricted and the majority of normal adult cells are FAP negative [27]. FAP expression is significantly induced in non-malignant states associated with tissue remodeling such as wound healing, embryogenesis, osteoarthritis as well as rheumatoid arthritis [43, 44], in liver cirhosis [45], and in cancer stroma [46]. In addition to the DPP-IV-like exopeptidase activity, FAP also possesses gelatinolytic endopeptidase activity [47, 48], and was thus suggested to participate in the degradation of structural proteins of the extracellular matrix during tissue remodeling and cancer cell invasion (reviewed in [15]). Matrix metalloproteinases (MMP), in particular MMP 2 [49], seem to be important functional partners of FAP in the modification of extracellular matrix [15]. Interestingly, heteromeric DPP-IV/FAP complexes, possessing both the DPP-IV-like exopeptidase and proline-specific endopeptidase enzymatic activity, are suspected to influence the migratory and invasive potential of fibroblasts and endothelial cells [49, 50].

1.1.3. Dipeptidyl peptidase 8 and 9

DPP8 and 9 are cytosolic dimeric proteins that are expressed in the majority of tissues including the human brain [13, 14, 51, 52], for review see [53]. The enzymatic activity of DPP 9 is thought to be important for the degradation of intracellular proline containing proteins with presentation of the peptide fragments on MHC-I molecules [54]. Some reports also suggest the involvement of both DPP8 and 9 in the processes of cell growth, migration and adhesion, probably via an indirect, enzymatic activity independent effect on the cell-extracellular matrix interactions [55]. DPP 9 may also influence the intracellular signaling cascades: DPP9 overexpression reduces the EGF mediated Akt activation by an enzyme activity dependent mechanism, and in addition DPP9 interacts with Ras [56]. Both proteases are expressed in the immune system [52, 57] and some of the effects of non-selective DPP inhibitors on immune cells may be in fact caused by the inhibition of DPP8 and 9 [53].

1.1.4. Dipeptidyl peptidase-II

DPP-II (DPP7, QPP, EC 3.4.14.2) possesses the unique DPP-IV-like enzymatic activity, but is structurally different from the canonical DPP-IV. It is a widely expressed intracellular enzyme that is typically localized in lysosomes and extralysosomal vesicles [16]. It is the only enzyme from the DASH group that has an acidic pH optimum [16]. Although the physiological function of DPP-II remains largely unknown, it is speculated to participate on the intralysosomal turnover of short peptides [58, 59]. In addition, several reports from the Huber lab argue for its role in the maintenance of quiescence in lymphocytes and fibroblasts [60, 61] and possibly also in glucose homeostasis [62]. DPP-II knockout is embryonic lethal in mice [62, 63], inhibition of DPP-II triggers apoptosis in noncycling G0 lymphocytes [64, 65] probably through deregulation of the cell cycle entry, and its absence in T cells leads to faster proliferation and differentiation into Th17 cells [63].

1.1.5. Dipeptidyl peptidase 6 and 10

DPP6 (dipeptidyl peptidase-IV like protein 1, DPPX) [66] and DPP10 (dipeptidyl peptidase-IV like protein 2)[67] are the enzymatically inactive, DPP-IV structurally related members of the DASH group [17]. Both proteins participate on the regulation of the voltage gated potassium channels [68] and may play a role in the development of the central nervous system and neurodegenerative diseases [69, 70]. There are currently no data on their role in gliomagenesis and only two studies suggested an association of mutations in DPP6 with pancreatic cancer [71, 72].

A substantial leap of interest in the DASH molecules was induced i) by the introduction of DPP-IV inhibitors in the treatment of type II diabetes [19, 244] and ii) observations of marked alterations of their expression and activity in the course of several diseases especially involving the immune system, and in cancer, where a direct pathogenetic role for DPP-IV and FAP seems to be highly probable. A significant proportion of the biologically active, mostly pro-proliferative peptides, systemic as well as local hormones, chemokines, neuropeptides, incretins and growth factors (Figure 1) contains a penultimate N-terminal proline residue as an evolutionary conserved proteolytic regulatory "check-point" [245]. Thus, the DPP-IV enzymatic activity is believed to be a functional regulator of their biological action. Limited proteolysis of these peptides by DPP-IV may lead both to quantitative and, due to the diversification of receptor subset preference, also to qualitative changes of their signaling potential [73-75].

While the systematic description of individual DASH molecules is available, including the cloning and structure resolution, the interpretations of biological studies are often equivocal because of their "moonlighting" character [76]. First, the overlap of enzymatic activities among the DASH molecules implies their sharing of similar sets of substrates (Figure 1) and thus, to some extent, DASH molecules may substitute each other. Second, DASH molecules execute more biological functions, depending on the given cell population and actual context of the biologically active substrates and the relevant receptors within the immediate environment. Third, the functional potential of DASH molecules is broadened by interactions with non-hydrolytic molecular partners (Ramirez-Montagut et al. 2004; Wang et al. 2005).

2. Expression and function of dipeptidyl peptidase-IV and related proteases in the microenvironment of human malignancies

2.1. Expression in transformed cells- tumor type specific and context dependent functions

Altered expression of DPP-IV and FAP is associated with several malignancies including brain tumors [87]. Both molecules may be expressed by the transformed as well as stromal cells and are associated with tumor promotion or suppression depending on the cancer type (for review see [88, 89, 27]). The mechanisms by which these molecules contribute to cancer pathogenesis and progression remain largely unknown, but several re-

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ports indicate that DASH molecules may serve as diagnostic and prognostic markers as well as therapeutic targets.

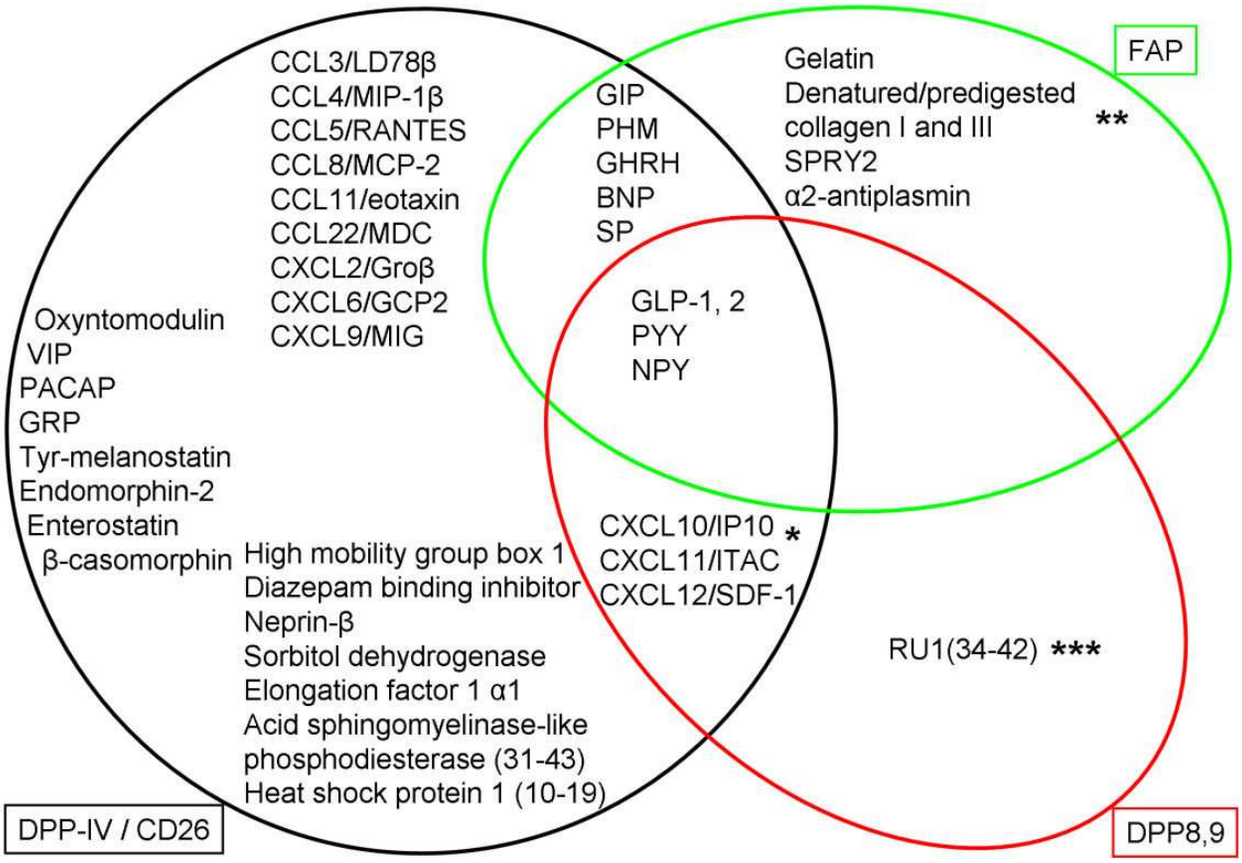

Figure 1. Potential overlaps of DPP-IV and related proteases in the processing of biologically active peptides. The pathophysiological importance of the cleavage is established e.g. for GLP-1, GIP, CXCL12 and NP Y, some of the in vitro cleaved substrates are unlikely to be of significance in vivo (e.g. Heat shock protein 1 for DPP-IV, SPRY2 for FAP). Not all identified DPP-IV substrates were tested with DPP8, 9 and FAP, the cleavage by these proteases is usually slower compared to DPP-IV. * cleavage has only been established for DPP-IV and DPP8; ** substrates of the endopeptidase activity of FAP; *** only tested for DPP8 and DPP9; VIP= Vasoactive intestinal peptide, PACAP= Pituitary adenylate cyclase-activating peptide, GRP= Gastrin-releasing peptide, GIP= Gastric inhibitory polypeptide/ glucose-dependent insulinotropic peptide, PHM= Peptide Histidine-Methionine, GHRH= Growth hormone releasing hormone, BNP= Brain natriuretic peptide, SP= Substance P, GLP-1, 2= Glucagon-like peptide-1, 2, PYY= Peptide YY, NP Y= Neuropeptide Y, SPRY2= sprouty (Drosophila) homolog 2, RU1(34-42)= antigenic peptide VPYGSFKHV. Compiled based on [15, 54, 73, 77-86].

DPP-IV expression is typical for a subset of aggressive T cell malignancies, which may be related to its function in T cell activation [90, 91]. The presence of DPP-IV is also associated with a more malignant behavior in B-cell chronic lymphocytic leukaemia [92], thyroid cancer [93], gastrointestinal stromal tumors [94], and was recently linked to a subpopulation of cancer stem cells responsible for the metastatic spread of colorectal cancer [95].

Recent studies aimed at examining the functional role of DPP-IV in malignant cells. In mesothelioma, DPP-IV is expressed in tumors in situ and in mesothelioma cell lines [96]. By binding fibronectin and collagen I, DPP-IV likely contributes to the interaction of these cells with

the ECM [97]. A different mechanism may operate in Ewing sarcoma: DPP-IV (and likely also the intracellular proteases DPP8 and 9) proteolytically cleaves NP Y1-36 to NP Y3-36 and thus abolishes its cell death inducing activity in the cancer cells and switches it to an angiogenesis promoting mediator [98].

Contrary to the above cited reports, some malignancies exhibit decreased DPP-IV expression. This is typical for melanoma and melanoma derived cell lines [99-102], cancer cell lines derived from neuroblastoma [103] and prostate [104] as well as non-small-cell lung cancer [105]. It should be however noted that on the tissue level, the data on DPP-IV expression are equivocal at least for prostate and lung cancer [106-109]. DPP-IV was shown to act as a tumor suppressor in melanoma [99, 102], neuroblastoma [103], prostate [104] and non-small-lung cancer [105] cells: its reexpression in the transformed cells led do decreased growth, increased apoptosis and sensitivity to growth factor withdrawal, decreased invasiveness and slower xenotransplant growth in immunodeficient animals.

The mechanisms that account for these diverse effects of DPP-IV on tumor cells are rather poorly understood. The currently best characterized physiological function of DPP-IV is proteolytic inactivation of incretins and possibly other biologically active peptides [73, 110]. The biological relevance of this phenomenon is confirmed by the clinically exploited DPP-IV inhibitors resulting in systemic elevation of DPP-IV substrates such as GLP-1 [111]. In addition, a variety of growth factors, chemokines and neuropeptides implicated in the progression of human tumors are potential DPP-IV substrates (reviewed in [74]) and DPP-IV may therefore act as a "gate-keeper" regulating their biological function on the systemic and/or local level. The decreased clearance of biologically active substrates due to the absence of DPP-IV may lead to sustained pro-proliferative signaling and promote tumor growth and metastasis. Masur et al. [112] showed that the growth promoting and promigratory activity of GLP-2 in colon cancer cells in vitro is increased in the presence of a DPP-IV inhibitor. Similarly, the inhibition of the DPP-IV enzymatic activity facilitated metastatic spread of prostate cancer cells by preventing the cleavage of the chemokine CXCL12 (SDF-1, stromal cell derived factor -1) [113].

On the other hand, DPP-IV also triggers changes in signaling cascades and expression of molecules mediating interaction with the ECM that are harder to reconcile with the cleavage of biologically active substrates in the pericellular space. In ovarian carcinoma, DPP-IV expression led to suppression of MAPK signaling, enhanced E-cadherin expression and the downregulation of MMP-2 and MT-MMP-1, which was associated with decreased invasiveness, tumor progression and enhanced chemosensitivity [114-116]. In prostate cancer cells, re-expression of DPP-IV interfered with the signaling of a non-DPP-IV substrate bFGF and inhibited their malignant phenotype in the study by Wesley et al. [104]. Yet, Goznalez-Gronow et al. [117] identified DPP-IV as a receptor for plasminogen 2 epsilon that promoted the invasiveness of the prostate cancer cell line 1-LN.

To test the relevance of the hypothesized non-proteolytic functions of DPP-IV, several groups including ours engineered an enzymatically inactive form of DPP-IV with a single amino acid substitution in the active site ($Ser^{630} \rightarrow Ala^{630}$). Reintroduction of this mutant form

of DPP-IV frequently results in similar tumor suppressing effects as observed with the enzymatically active DPP-IV [99, 102, 105, 118].

The proteolytic and non-proteolytic mechanisms may also combine and thus extend the portfolio of the biological functions of DPP-IV. Arscott et al. [103] showed that DPP-IV reexpression in neuroblastoma cells induced differentiation, increased their sensitivity to serum withdrawal and reduced their migration, invasion and pro-angiogenic capacity in vitro as well as in vivo. This was most likely caused by the downmodulation of the CXCL12-CXCR4 axis and possibly also other chemokine systems. Although not specifically demonstrated by the authors, DPP-IV most likely inactivated CXCL12 proteolytically, resulting in the downregulation of its downstream effectors, but in addition it downregulated the mRNAs of CXCL12 and CXCR4 and several other chemokines including non-DPP-IV substrates [103].

FAP was originally described to be typically expressed in the stromal compartment of tumors (see section 2.2), but several reports, including ours, also show its expression in the transformed elements. A prototypical example is the LOX melanoma cell line, where FAP co-localizes with the urokinase plasminogen activator receptor (uPAR) on the invadopodia and likely contributes to the pericellular proteolysis and invasiveness of these cells [119, 120, 47, 121, 122]. Somewhat surprisingly, Ramirez-Montagut et al. [123] were able to show that in mouse melanoma cells FAP may actually act as a tumor suppressor with the main effects on cell growth and survival independently of its enzymatic activity. These results are in agreement with Rettig et al., who observed loss of FAP during Ras mediated transformation of melanocytes [124] and with the fact that FAP is upregulated upon reintroduction of DPP-IV into melanoma cells with the resulting tumor suppressing effects [99]. Similarly, FAP negative subclones in osteosarcoma were tumorigenic and grew to high densities in contrast to non-tumorigenic FAP positive subclones [124].

Breast cancer cells also express FAP in vivo [15, 125]. FAP is associated with their decreased dependence on growth factors in vitro and formation of more rapidly growing and more vascularized tumors in a xenotransplantation model [125-127]. Interestingly and somewhat in contrast to the previously published data in other cancer types, the tumor promoting activities in breast cancer cells may be independent of the intrinsic enzymatic activity of FAP [128]. FAP is also expressed in the tumor cells of mesenchymal origin in malignant and benign tumors, but is probably rather linked to their myofibroblastic differentiation than to their malignant potential [129]. Epithelial cancer cells e.g. in gastric [130], esophageal [131], colorectal [132] and cervical cancer [133] were also demonstrated to be FAP positive. The function of FAP in these cells is unclear but in analogy to other cancers it is presumed to be linked to their invasiveness.

There is limited data on the expression and in particular the function of DPP8, DPP9 and DPP-II in cancer cells. Yu et al. described increased DPP9 mRNA in testicular cancers on a small patient sample [52], both DPP8 and DPP9 are expressed in human breast, ovarian and hepatic cancer cells as well as in lymphoma cells lines [52, 134] and chronic B cell leukemia cells [135]. Interestingly, transgenic DPP9 was shown to induce apoptosis in hepatoma cells and decrease the EGF mediated activation of Akt [56]. These effects were dependent on the enzymatic activity of DPP9, but in addition to that, both DPP9 and DPP8 were demonstrat-

ed to interact with Ras [56]. In Ewing sarcoma cells, DPP8 and DPP9 seem to exert similar effects as DPP-IV (see above) due to their similar enzymatic activity [98] and efficient cleavage of NP Y [77](Figure 1.). Whether DPP8 and DPP9 regulate the adhesion and migration [55] of malignant cells remains to be established. Lower DPP-II catalytic histochemistry staining was suggested as a favorable prognostic marker in chronic lymphocytic leukemia (CLL) [136]. In addition, DPP-II inhibition leads to induction of apoptosis in CLL cells in approximately 60% of patients, which is associated with the presence of other established positive prognostic markers and a clinically more benign disease course [137]. Whether DPP-II is functionally involved in the pathogenesis in CLL and/or other cancers is currently not known.

2.2. Expression and role of dipeptidyl peptidase-IV and related proteases in the stromal compartment of tumors

Tumor stroma is composed of an extracellular matrix and a diverse set of cell types that significantly contribute to tumor progression [138]. Among others, the stroma is an important source of tumor associated proteases including the DASH molecules.

Vascular and lymphatic endothelial cells express DPP-IV in cell culture as well as in situ [139, 140], but the expression is variable and several reports show no DPP-IV staining of cell vessels [141]. Similarly ambiguous are the data regarding the function of DPP-IV in endothelial cells: it is speculated to contribute to their interaction with the extracellular matrix proteins, convert NP Y to its pro-angiogenic form and promote their migration and invasion [50, 142-144]. Contrarily, a recent report showed that DPP-IV ablation using either genetic or pharmacologic approaches may increase endothelial cell proliferation and migration induced by the inflammatory cytokines TNF-α or IL-1β [139]. These somewhat conflicting results may be due to regional differences in the proteolytic makeup of endothelial cells as well as differing functions of DPP-IV depending on the presence of its "molecular partners" and microenvironmental stimuli. FAP mRNA was also detected in endothelial cells cultured in vitro [145]. Interestingly, Ghilardi et al. [146] observed higher FAP expression in endothelial cells derived from ovarian and renal carcinoma compared to cells derived from normal tissues. The functions of FAP in endothelial cells are mostly speculative but it may (probably together with DPP-IV) contribute to the degradation of extracellular matrix [50]. FAP may be also expressed by pericytes [138], although in some cancer models its expression was restricted to isolated infiltrating stromal cells rather than pericytes [147].

Expression of DPP-IV in the normal and cancer associated fibroblasts is rather variable [148-151], but cultured fibroblasts and myofibroblasts in the majority of epithelial cancers strongly express FAP [15, 152, 153]. Stimuli leading to the upregulation of FAP may involve inflammatory mediators such as TGF beta, Il1 and oncostatin M [43, 154], factors secreted by tumor cells (i.e. PDGF-BB, TGF-beta1 and Wnt5a in melanoma cells [155]) and the transcription factor EGR1 [156]. Pathophysiologically, FAP likely participates in the turnover and modification of the extracellular matrix [157]. Lee et al. [158] found that fibroblasts engineered to express FAP seeded on gelatin produced matrices with changed composition and structure, which promoted the migratory behavior of pancreatic carcinoma cells. These

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changes were mediated by the enzymatic activity of FAP as demonstrated by using a FAP inhibitor naphthalenecarboxy-Gly-boroPro [158]. FAP inhibition was also shown to block the growth of lung and colon cancer in a mouse model by increased accumulation of collagen, decreased myofibroblast content and vessel density [159] suggesting its crucial role for the effective establishment of tumor stroma. Other mechanisms may also contribute to the important role of FAP in tumor microenvironment. By selectively depleting the FAP positive stromal cells, Kraman et al. demonstrated that they are crucial for the suppression of antitumor immune response [160]. Whether FAP is just an "innocent by-stander" marker of these cells, or plays a direct role in this process remains to be established. In multiple myeloma, the stromal FAP expression is important in promoting the survival of myeloma cells [18, 161], but the mechanisms are unknown. The expression of FAP in tumors is in general associated with a more aggressive disease course and shorter patient survival [153, 162, 163]. Surprisingly, one study in breast cancer [164] described longer overall survival and the disease free interval in patients with higher stromal FAP expression.

Immune cells are another important constituent of the tumor microenvironment that may express DPP-IV, DPP8 and DPP9, but do not express FAP [135, 165, 166]. In cancer patients, changes in the DPP-IV levels are frequently seen in serum and in lymphocytes [80] and cytokines such as TGF-β may contribute to these changes in peripheral blood lymphocytes as documented in patients with oral cancer [167]. Despite the well established role of DPP-IV in human lymphocyte proliferation and activation [33], its function, as well as the possible significance of DPP8 and DPP9 in mediating or suppressing effective antitumor responses is unknown. Talabostat (PT-100), an inhibitor of DPP-IV and FAP, was demonstrated to stimulate the immune response to several experimental tumors, but the mechanisms were not dependent on the inhibition of DPP-IV [168].

In conclusion, the expression of DPP-IV and related proteases is frequently deregulated in the parenchymal and/or stromal compartment in human malignancies. The molecules may promote or suppress tumor progression depending on the tumor type and the presence of their substrates and/or interactors in the microenvironment, which are characteristic for individual tumors. This highly context dependent role is the likely explanation for the conflicting data reported on their role in cancer [27, 74, 169]. The mechanisms seem to involve proteolytic processing as well as non-proteolytic protein-protein interactions and modification of intracellular signaling pathways. Similar mechanisms likely operate for the intracellular proteases DPP8 and DPP9, but the evidence for their role in human cancers is currently limited.

3. Dipeptidyl peptidase-IV and related proteases in the pathogenesis of brain tumors

3.1. Expression of DPP-IV and related proteases in glioma cell lines

In astrocytoma cells, DPP-IV was first detected using immunohistochemistry by Medeiros [170]. Subsequent work in our laboratory revealed the presence of DPP-IV-like enzymatic

activity together with the expression of mRNA for DPP-IV, FAP and DPP-8 and 9 in permanent glioma cell lines C6, U373, T98G, U251, U87, U138, U118 and in human glioma primary cell cultures as well as in glioma stem-like cells ([118, 171-175] and unpublished data). The DPP-IV-like enzymatic activity in the permanent glioma cell lines is only partially inhibited by a highly specific DPP-IV inhibitor sitagliptin. In U87, U138 and U118 lines 30, 60 and 85% respectively of the total enzymatic activity is inhibited and can therefore be attributed to the canonical DPP-IV. In contrast, only 12-15% of the DPP-IV-like enzymatic activity in U373, T98G, U251 cells is inhibited by sitagliptin (Busek et al. unpublished data). These results correspond well with the relatively high DPP-IV mRNA expression in the U87, U138 and U118 cell lines [175, 176].

FAP is also expressed in glioma cells. The early work by Rettig et al. [177] detected FAP in 19 out of 22 of astrocytoma cell lines by immunohistochemistry using the F19 monoclonal antibody. Similarly Mentlein et al. [176] showed high FAP mRNA expression in 6 out of 7 glioma cell lines. According to our data, the expression of FAP may be more variable [118] with substantial variation in individual primary cell cultures as well as permanent cell lines. In the panel of glioma cell lines examined in our studies, FAP expression mirrored the expression of DPP-IV and was substantially higher in U87, U138 and U118 cell lines than in U373, T98G, U251 lines [175]. Therefore, although FAP is significantly less efficient in cleaving the H-Gly-Pro-AMC substrate compared to DPP-IV [48], it may partly contribute to the sitagliptin resistant DPP-IV-like enzymatic activity in some of the glioma cells lines. Interestingly, we have consistently observed a positive correlation between the mRNA expression of the two transmembrane proteases DPP-IV and FAP in glioma cell lines as well as in glioma primary cell cultures [178]. Moreover, the expression of both DPP-IV and FAP increased concomitantly in glioma cells cultured in serum free media and decreased after the addition of serum to the starved cells [178] suggesting that similar pathways regulate their expression in this model, most likely on the transcription and/or mRNA stability levels [178]. The mRNAs for the ubiquitous intracellular enzymes DPP8 and DPP9 are expressed in similar quantities in the glioma cell lines tested in our laboratory and probably make the largest contribution to the residual DPP-IV-like enzymatic activity after inhibition with sitagliptin (Busek et al. unpublished data).

It is currently unclear to what extent the in vitro cell culture conditions and the standard process of glioma cell line establishment may influence the observed expression of DPP-IV and especially FAP, a known phenotypic marker of mesenchymal cells such as activated fibroblasts [15]. The typical media supplemented with 10% fetal calf serum are known to promote the mesenchymal phenotype in cultured cells ("mesenchymal drift"; [179]) which could lead to the upregulation of FAP. Xenotransplants generated by orthotopic implantation of the glioma cells into immunodeficient mice exhibited higher DPP-IV-like enzymatic activity compared to the contralateral hemisphere (Figure 2) and the enzymatic activity was in part sensitive to a specific DPP-IV inhibitor. DPP-IV as well as FAP could be detected on the mRNA level as well as by immunohistochemistry in the xenotransplants from the U87 and U138 cells (data not shown), which suggests that their expression in glioma cells is retained under the conditions closely mimicking the microenvironment of human gliomas.

Similarly, Li et al. [180] were able to demonstrate the enzymatic activity of FAP in a U87 tumor model, although the subcutaneous implantation used in their study exposed the glioma cells to somewhat unnatural microenvironment. Orthotopic xenotransplantation of freshly isolated glioma cells or expanded glioma stem-like cells are necessary to determine whether the expression of FAP in particular is maintained in glioma cells in situ. Such experimental approach would be suitable for the preclinical tests of therapies targeting this protease.

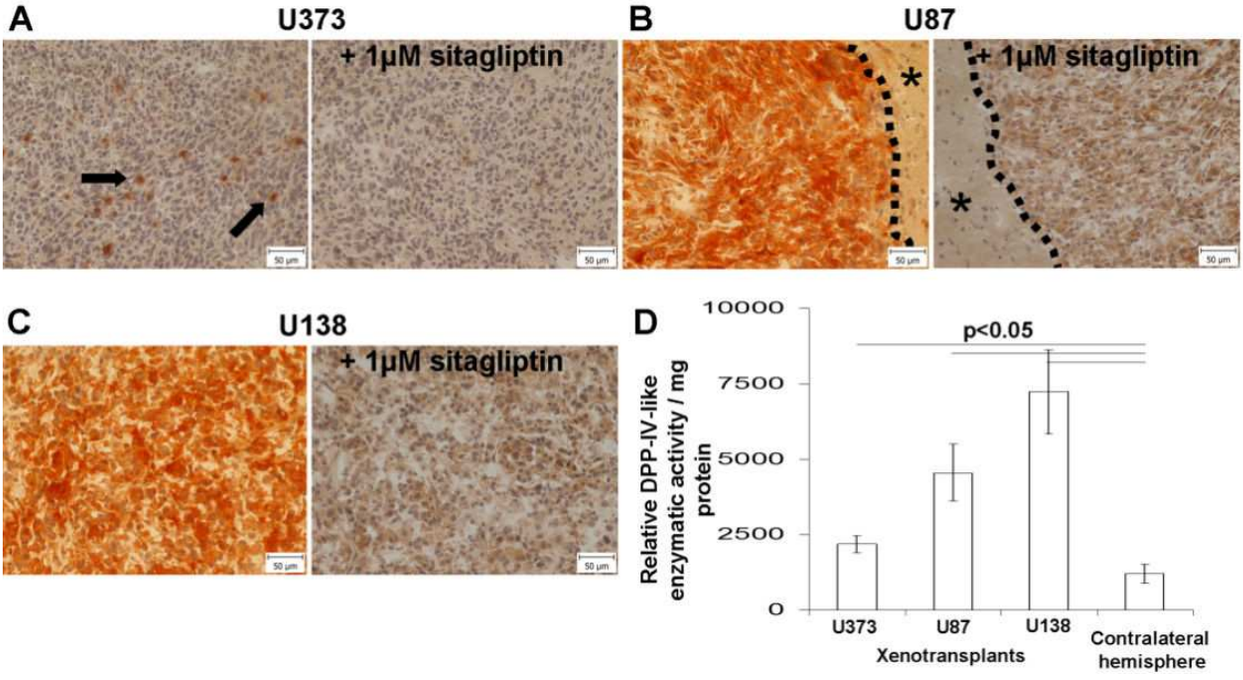

Figure 2. DPP-IV-like enzymatic activity detected by catalytic histochemistry in orthotopic glioma xenotransplants (A-C). 10^6 cells of the indicated cell lines were used for intracerebral implantation into immunodeficient mice [118]. DPP-IV-like enzymatic activity (red precipitate) was detected by incubating 10µm frozen sections with 7-(glycyl-l-prolylamido)-4-methoxy-β-naphthylamide hydrochloride as a substrate and Fast Blue B in PBS (pH 7.4) at 4°C overnight [181]. 1µM sitagliptin was used to inhibit the enzymatic activity of canonical DPP-IV (CD26), nuclei were counterstained with haematoxylin. Only small areas of DPP-IV-like enzymatic activity (arrows) can be detected in U373 tumors (A). In (B), the dashed line marks the interface between the diffusely stained tumor tissue and surrounding normal brain (asterisk). (D) DPP-IV-like enzymatic activity in homogenates from the xenotransplants compared to the contralateral hemisphere. H-Gly-Pro-7-amino-4-methylcoumarin was used as a substrate at pH 7.5 and 37°C [118].

3.2. Expression of DPP-IV and related proteases in normal brain and human astrocytic tumors

The data on the expression of DPP-IV, DPP-II, FAP and DPP8 and 9 in the human brain is limited. Using immunohistochemistry, Bernstein et al. detected abundant expression of DPP-IV in the immature central nervous system with much lower expression in adults [246]. In rats, mice and ginea-pigs, the expression of DPP-IV was studied in more detail and DPP-IV was detected in the capillaries, meninges as well as certain neuronal structures (see [175] and references therein). By means of its enzymatic activity, DPP-IV is speculated to participate on nociception and behavior regulation most likely by inactivating biologically active

peptides such as SP, endomorphin-2 and NP Y [182-184]. DPP-IV may also be involved in the pathogenesis of ischemia-reperfusion injury. Roehnert et al. [185] described the appearance of DPP-IV immunoreactivity in rat microglia, astrocytes and neurons following unilateral transient occlusion of the medial carotid artery. Interestingly, intracranial administration of a specific DPP-IV inhibitor sitagliptin led to a 21.1±5.8% decrease in infarct size suggesting neuroprotection in this model [185].

Enzymatic activity attributed to DP-II was detected in brain homogenates [186, 187] and histochemically in specific neuronal populations in rat brain by Gorenstein et al. [188]. Later studies demonstrated its presence in glial cells [189] and speculated its association with astrocyte differentiation [190].

DPP8 and 9 are also expressed in the brain tissue [13, 52] and are probably responsible for a significant part of the DPP-IV-like enzymatic activity detected in human brain tissue homogenates [87]. In rats, DPP8 was detected in neurons, but not astroglial cells and microglia by immunohistochemistry [185].

On the contrary, FAP protein is most likely absent in non-tumorous human brain: Rettig et al. [177] failed to detect its expression in human autopsy material using the F19 antibody; similarly, samples obtained from patients with pharmacoresistant epilepsy show no staining using several anti FAP antibodies ([87] and unpublished data). Although FAP mRNA can be detected by sensitive RT-qPCR assays [87, 176], it is probably not being translated or the protein levels are bellow the detection limit of the methods used.

In gliomas the DPP-IV-like enzymatic activity is substantially higher compared to the non-tumorous brain [87, 191] and DPP8 and 9 represent the major part in both cases [87]. In contrast to DPP8 and 9, the expression of DPP-IV and FAP is significantly increased in gliomas compared to the non-tumorous brain. According to The Cancer Genome Atlas ([192], http://cancergenome.nih.gov/) DPP-IV and FAP mRNA are upregulated more than two times compared to controls in 200 of 424 (47%) and in 162 of 424 (38%) glioblastoma patients respectively. In our patient cohort ([87] and unpublished data) DPP-IV and FAP mRNA were upregulated 9.9 and 4.6 fold respectively in newly diagnosed glioblastoma (N=28) compared to controls (pharmacoresistant epilepsy, N=15). Using RT-qPCR, Mentlein et al. also observed upregulation of DPP-IV and FAP in a small cohort of glioblastoma patients compared to the autopsy material [176]. Similarly, increased FAP expression in grade IV tumors and especially in gliosarcomas was observed by Mikheeva et al. [193].

Using catalytic histochemistry, Mares et al. could show that the DPP-IV-like enzymatic activity in grade II astrocytomas was mainly localized perivascularly and in mononuclear-like cells in the parenchyma [191]. In grade IV tumors (glioblastomas), the proportion of these stained cells was markedly increased and in addition, the DPP-IV-like enzymatic activity was present in spindle shaped, smooth muscle- or pericyte-like cells around hyperplastic vessels, and in tumor parenchyma [191]. Interestingly, the overall DPP-IV-like enzymatic activity determined by catalytic histochemistry correlated negatively ($r= -0.30$, $p= 0.04$) with the proliferation marker Ki67 [191]. Immunohistochemistry staining with DPP-IV and FAP specific antibodies revealed minimal positivity in non-tumorous brain with frequent fiber-

like positivity in glioblastomas, occasionally close to the capillaries [191]. Mentlein et al. showed that at least part of the FAP expressing cells also coexpressed GFAP and Ki67 [176]. Collectively, these data demonstrate that the expression of DPP-IV and FAP as well as the DPP-IV-like enzymatic activity is increased in a substantial part of glioblastomas. Both the transformed as well as stromal cells such as reactive astrocytes, cells in the vessel wall and infiltrating immune cells may contribute to this increased expression.

3.3. Possible functions of DPP-IV and FAP in the glioma microenvironment

Glioblastomas are highly heterogenous both histologically and on the molecular level [194, 195]. The transformed cells themselves represent a mixture of cell types that may originate from the stochastic clonal expansion or from a more hierarchical organization of gliomas as postulated by the cancer stem cell hypothesis [196, 197]. In addition to the transformed cells, a variety of host cells contributes to the glioma microenvironment. This stromal compartment is an important contributor to the malignant phenotype of glioma cells and comprises of microglia/macrophages, lymphocytes, neural precursor cells, neurons, pericytes/vascular smooth muscle cells, reactive astrocytes and endothelial cells [198].

Given the marked increase of the expression of DPP-IV and FAP mRNA in glioblastoma tissue homogenates and the increase of the DPP-IV-like enzymatic activity in the microvasculature and parenchyma, the proteases seem to be functionally important for the transformed as well as nontransformed cells.

3.3.1. DPP-IV as a possible regulator of glioma cell growth

The DPP-IV-like enzymatic activity may influence signaling of various soluble mediators involved in the pathogenesis of gliomas (Table 1).

DPP-IV substrate	Role in gliomagenesis
CCL3L1 (LD78beta)	Enhances glioma cell proliferation[199].
CCL5	Possible role in the recruitment of microglia/macrophages [200], promotion of glioma invasion and angiogenesis [201].
CCL22	Recruitment of immunosuppressive Treg cells [202, 203].
CXCL9	Increased expression in glioblastoma [204], promotes glioma cell growth [205].
CXCL10	Pro-proliferative signaling through ERK1/2 in glioma cells [205, 206].
CXCL11	A ligand for CXCR7, which mediates prosurvival signaling in glioma cells [207, 208].
CXCL12	Promotion of glioma invasion, growth and angiogenesis (for review see [209])
SP	Promotion of glioma growth and secretion of cytokines (reviewed in [210, 211]).
PACAP, VIP	Stimulation of glioma cell growth [212, 213].

Table 1. DPP-IV substrates implicated in the pathogenesis of gliomas

Although DPP-IV and FAP exhibit similar dipeptidyl peptidase activity on small fluorogenic substrates, a recent study found substantial differences in their ability to cleave peptide substrates [79]. All peptides (with the exception of VIP) listed in Table 1 are cleaved rapidly by recombinant DPP-IV; in contrast FAP can only cleave SP effectively and does not cleave chemokines, PACAP or VIP [79](Figure 1). Given that, DPP-IV is the main candidate for influencing the functions of these mediators in gliomas.

By removing the N-terminal dipeptide from the biologically active peptides, DPP-IV in general diminishes their activity and/or increases their susceptibility to cleavage by other proteases [73]. Given that the majority of the substrates listed in Table 1 are thought to promote the malignant phenotype of glioma cells, DPP-IV would be somewhat paradoxically expected to suppress it. Indeed, we have previously shown the ability of DPP-IV to abrogate the calcium mediated signaling of SP in glioma cells [214]. We also observed that higher DPP-IV-like enzymatic activity in primary glioma cell cultures correlated with their slower growth [118]. In addition, overexpression of the transgenic DPP-IV in several glioma cell lines decreased their proliferation, led to a cell cycle block and a 50% decrease of the size of xenotransplanted tumors in immunodeficient mice [118]. Interestingly, our microarray data suggested that expression of several molecules linked to glioma pathogenesis was perturbed in glioma cells with forced expression of DPP-IV [118]. This included e.g. downregulated expression of transcripts encoding membrane growth factor receptors (PDGFRA, CALCRL, GRPR) and adhesion molecules (CD97, COL8A1, COL13A1, NLGN1, NLGN4X, PCDH20, SCARF2, NrCAM) as well as molecules typically overexpressed in gliomas (e.g. CALCRL, COL8A1, HAS2, NES, RRM2 [192], http://cancergenome.nih.gov). On the contrary, several tumor suppressors e.g. BEX2, RAP1GAP, DUSP26, SYT13, TSPYL2 were upregulated ([118] and references therein). In order to determine whether the observed in vitro and in vivo growth inhibitory effects were mediated by the enzymatic activity, the experiments were done in parallel with cells transfected with an enzymatically inactive mutant DPP-IV due to a single amino acid ($Ser^{630} \rightarrow Ala^{630}$) substitution in the active site [118]. These studies revealed similarly decreased growth of glioma cells overexpressing the enzymatically inactive DPP-IV providing evidence that these effects were independent of the enzymatic activity [118]. In summary, our studies demonstrate that DPP-IV may modify the function of its substrates through proteolysis, but likely has also an enzymatic activity independent growth inhibitory effect in glioma cells. The detailed molecular mechanism(s) for these effects however remain to be identified.

These data strongly argue that DPP-IV in glioma cells in vivo is unlikely to directly promote the malignant potential of the expressing cells. However, DPP-IV did not suppress the malignant phenotype of glioma cells completely in our studies – albeit the tumors were smaller with lower percentage of Ki67 positive nuclei, they exhibited an infiltrative growth pattern similar to controls [118]. We also observed a highly infiltrative growth of the xenotransplanted glioma stem-like cells expressing DPP-IV (Busek et al. unpublished). Several possibilities exist to explain these seeming contradictions: i) DPP-IV expression / enzymatic activity may reflect a mechanism striving to prevent the inappropriate proliferation of malignant cells. In support of this possibility, Mares et al. [191] observed an inverse correlation

between the DPP-IV-like enzymatic activity and Ki67 in glioblastoma tissues. On the other hand, these less proliferative cells may be more resistant to conventional adjuvant therapies and therefore contribute to tumor recurrence. ii) Glioblastomas are highly heterogeneous and likely composed of several interacting subpopulations establishing a complex "ecosystem" [197]. DPP-IV may contribute to the interaction with other tumor clones and/or stromal compartment by local proteolytic processing of biologically active peptides with an overall increased tumor growth despite its growth inhibitory effects in the expressing subpopulation. Such a role of DPP-IV could not have been identified using conventional cell-line based xenotransplantation models in immunodeficient animals utilized in our studies. iii) DPP-IV expression may also be linked to the microenvironment typical of glioblastomas. The grade IV tumors characteristically contain necrotic areas and exhibit enormous stimulation of angiogenesis caused by hypoxia. Hypoxia also promotes the aggressiveness of glioma cells through the transcription factor HIF-1α (hypoxia inducible factor-1α) [215]. DPP-IV was demonstrated to be regulated by hypoxia in several systems although with variable outcomes. In extravillous trophoblast cells, the hypoxia induced increase of DPP-IV was associated with their decreased invasiveness [216]. In colon and gastric cancer cell lines, DPP-IV was increased in a HIF-1α dependent manner in response to hypoxia in vitro and in xenotransplants depleted of VEGF [217]. The purpose of this induction of DPP-IV in the response to hypoxia is not clear. The data from other experimental systems nevertheless suggest that by promoting the expression of DPP-IV together with the angiogenic receptor Y2, ischemia may enhance the angiogenic response to NPY [218, 219].

In addition to the transformed glioma cells, DPP-IV is also increased in the microvasculature [191]. Here, DPP-IV may contribute to neoangiogenesis by promoting the proliferation and invasiveness of endothelial cells.

In summary, higher expression of DPP-IV is typical for glioblastomas. Although the function of the protease cannot be currently ascribed with certainty, it may negatively affect glioma cell proliferation even independent of its enzymatic activity [118] and participate on the pericellular proteolysis with possible paracrine effects on other tumor subpopulations including stromal cells. The functional significance of DPP-IV upregulation in the glioma stromal compartment remains to be established.

3.3.2. Implications of FAP for glioma migration, ECM remodeling and angiogenesis

Several reports suggest a possible role of FAP in glioma cell migration not only because of its role in the extracranial malignancies (section 2.1). Lal et al. [220] studied the phenotypic and molecular changes caused by the introduction of the activating mutant form of EGFR (EGFRvIII) into glioma cells with low EGFR expression. They observed increased invasiveness of the EGFRvIII transduced cells, which was accompanied by upregulation of several transcripts encoding proteins of the extracellular matrix and proteases, including FAP [220]. A similarly designed study tested the effects of the introduction of IGFBP2, a molecule with pleiotrophic roles in glioblastoma [221], into the SNB19 glioma cell line. Here, increased invasion was also observed and FAP was among the 28 significantly induced genes with a 4.5 to 16 fold increase in different clones according to the microarray data [222]. Likewise, intro-

duction of TWIST1, a basic helix-loop-helix domain-containing transcription factor implicated in EMT (epithelial mesenchymal transition) and cancer metastasis [223], into glioma cells promoted their invasion and among other genes activated the expression of FAP [193]. Tatenhorst et al. [247] took a different approach and compared the expression profile in two subpopulations isolated from the U373 glioma cell line based on their differing migratory rates on Matrigel. FAP was the top upregulated gene (11.7 fold) in the clone with high migration in vitro. Although these studies do not provide direct evidence that FAP contributes to the high migration and invasiveness characteristic for glioma cells, they strongly suggest an association of FAP expression with the glioma migratory phenotype and its activation in response to molecular abnormalities frequently occurring in glioblastomas. Mentlein et al. [176] addressed the role of FAP in glioma migration directly by siRNA mediated downregulation in the A746 glioma cell line. No effect on cell migration was noted in the transwell assay when uncoated or Matrigel coated inserts were used [176]. However, the cells invaded slightly less efficiently through the gelatin coated inserts and their invasion through brevican, a chondroitin sulfate proteoglycan abundantly present in the adult human brain, was reduced by almost 50% [176]. The underlying mechanisms remain to be established. The extracellular matrix of gliomas is substantially different from the extracranial malignancies: the fibrillary proteins (e.g. collagens, fibronectin, laminin) are much less abundant and mostly present in the perivascular space. Instead, hyaluronic acid and associated proteins such as versican and brevican prevail [224, 225]. Although Mentlein et al. [176] demonstrated that FAP cleaved brevican, the cleavage by the recombinant protease was inefficient and required prolonged incubations. Thus, the siRNA mediated downregulation of FAP in glioma cells could instead have effects on other ECM degrading systems. FAP is known to be part of multiprotein complexes in invadopodia [153, 226] and it was demonstrated to physically interact with uPAR in a β1-integrin dependent manner [121]. Interestingly, simultaneous downregulation of uPAR and cathepsin B was shown to downregulate FAP in glioma cells [227]. FAP therefore seems to act in cooperation with other proteolytic systems and its presence may influence the remodeling of glioma ECM not only by its intrinsic gelatinolytic activity but also by its possible role in the formation and/or stabilization of invadopodia.

Another interesting but unexplored aspect is the possible role of FAP in the promotion of angiogenesis of glioblastomas. The expression of FAP in endothelial cells [145, 146], higher microvessel densities in breast cancer xenotransplants engineered to express FAP [127] and the decreased microvessel density in response to FAP ablation in a lung cancer model consistently imply its role in angiogenesis. This may be –similarly to DPP-IV– via the processing of NP Y 1-36 to an angiogenic NP Y 3-36 [228]. In addition, FAP expressing fibroblasts are able to modify collagen type I matrices in a way that promotes the invasion of tumor cells (see section 2.2, [158]). Possibly, FAP may participate on the transformation of the glioma extracellular matrix into an environment that would be more supportive for the migration of endothelial cells [15].

Glioblastomas typically contain necrotic areas surrounded by pseudopalisades. A model for the pathogenesis of this typical morphological feature of glioblastoma has been proposed [229, 230] and postulates that thrombotic occlusion of the central vessel results in hypoxia,

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which than drives the migration of the surrounding glioma cells and robustly stimulates angiogenesis. Several mechanisms likely contribute to the vaso-occlusive process including the leakage of plasma clotting factors through the damaged vessels and their contact with a procoagulant tumor environment (see [231] for review). FAP was previously demonstrated to be identical with α2-antiplasmin cleaving enzyme [42]. Upon conversion by FAP, α2-antiplasmin is more effectively incorporated into fibrin and protects the fibrin clot from plasmin degradation [232]. By this mechanism, FAP may contribute to the prothrombogenic state in glioblastoma with resulting development of necrosis and stimulation of angiogenesis. The speculated mechanisms listed above are mediated by the DPP-IV-like or prolyl- endopeptidase enzymatic activities of FAP. In addition, FAP has probably other, enzymatic activity independent pro-angiogenic effects as recently demonstrated in breast cancer using catalytically inactive mutant FAP [15, 128].

4. DPP-IV and FAP as possible markers and treatment targets in gliomas?

Glioblastomas have dismal prognosis and despite the multimodality treatment the majority of patients die within 10-14 months [248, 249]. Regardless of ongoing efforts, the pathogenesis of glioblastoma remains unknown and therefore specific targeted therapies are currently not available. Despite their rather peculiar role in cancer pathogenesis, both DPP-IV and FAP were suggested as diagnostic and prognostic markers and therapeutic targets for tumors outside of the central nervous system (reviewed in [15, 88]). DPP-IV staining was suggested as a useful adjunct marker for the differentiation of malignant melanomas from deep penetrating nevi [101] and benign and malignant diseases of the thyroid gland [93, 233]. To the best of our knowledge, there is only one study suggesting a possible prognostic relevance of DASH molecules in the brain tumors. Shaw et al. [234] studied the expression signature that was related to the chemosensitivity of oligodendroglial tumors and observed that FAP was downregulated several fold in tumors that were chemosensitive and/or exhibited the prognostically favorable 1p/19q loss [234].

Preclinical studies with DPP-IV targeting antibodies suggest that DPP-IV may be a new therapeutic target in malignant mesothelioma [96, 97], renal carcinoma [235] and some hematologic malignancies [236]. The highly selective expression of FAP in the tumor microenvironment and its expected direct pathogenetic participation on tumor progression has also raised interest in its possible therapeutic exploitation with a simultaneous impact not only on the transformed cells, but also on the stromal elements („stroma targeted therapies“) [138]. Experimentally, FAP specific antibodies were utilized for the targeting of TNF alpha carrying nanoparticles [237] or in the form of a chimeric protein with the extracellular domain of the ligand of the TNF receptor 4-1BB to enhance the local T cell-mediated antitumor responses [238]. Further, induction of immune response against FAP leads to a decreased tumor growth and an enhanced effect of cytostatic therapy in several experimental models [239-242]. FAP activated cytotoxic prodrugs have also been designed [243] and the inhibition of FAP enzymatic activity by specific low molecular weight inhibitors was tested in experimental myeloma treatment [18].

Several important issues arise when designing treatment modalities targeting the DASH molecules by enzyme inhibitors or antibodies. In the case of DPP-IV, its almost ubiquitous presence, combined with multiple tissue specific biological roles, increases the risk of on-target side effects. In this respect, the restricted expression of FAP seems to be a substantial advantage. Further, the similar enzymatic properties of DASH proteases represent a possible source of off-target side effects [19]. Such a problem may be avoided by using highly specific inhibitors as documented by the successful introduction of DPP-IV inhibitors into clinical practice for the treatment of diabetes mellitus [244]. On the other hand, the moonlighting nature of the DASH molecules (combination of enzymatic and non-enzymatic functions), might hypothetically represent an advantage for their targeting as the non-enzymatic functions would remain untouched when using low molecular weight inhibitors to block the enzymatic functions.

In conclusion, given the emerging role of DPP-IV and FAP in the processes of gliomagenesis and the precedent evidence for their possible therapeutic exploitation in extracranial malignancies, they seem to be promising candidates for the targeting of gliomas.

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