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TOPIC I

STRUCTURE AND FUNCTION

CHAPTER 1

PEPTIDE SUBSTRATES OF DIPEPTIDYL PEPTIDASES

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1. INTRODUCTION

DPP IV has been attributed a large array of functions, some of which are mediated by its exopeptidase activity. Although it only removes two amino acid residues at the N-terminus of the peptide, this cleavage can inactivate or modify the activity of regulatory peptides, peptide hormones, chemokines and neuropeptides. Several excellent DPP IV substrates with high specificity constants were identified by the *in vitro* kinetic study of the truncation of bioactive peptides by DPP IV. *In vivo* studies e.g. with DPP IV negative animals and *in vivo* inhibition experiments could enlighten us on the physiological relevance of these truncations.

The DPP IV mediated truncation of bioactive peptides has been reviewed on several occasions (Mentlein *et al* 1999, Lambeir *et al* 2003, De Meester *et al* 2000 and 2003). Here we want (1) to present some data on a novel DPP IV substrate, (2) to review some of the recent literature on known substrates and (3) to discuss the possible truncation of known DPP IV substrates by related dipeptidyl peptidases.

2. *IN VITRO* TRUNCATION OF BIOACTIVE PEPTIDES BY DPP IV

2.1. Overview of Specificity Constants

Figure 1 represents the specificity constants k_{cat}/K_m of the cleavage of natural peptides by DPP IV (Lambeir *et al* 2001 and 2002). All data were obtained using the same batch of human natural DPP IV, purified from seminal plasma. The experiments were performed in identical circumstances of incubation and analysis, except for the data on hemorphin-7 which were obtained from Cohen *et al* (2004). The full names of the peptides are given further in this paper.

From the *in vitro* studies several conclusions can be drawn. Not only the amino acid sequence surrounding the scissile bond, but also the specific structural features of the substrate

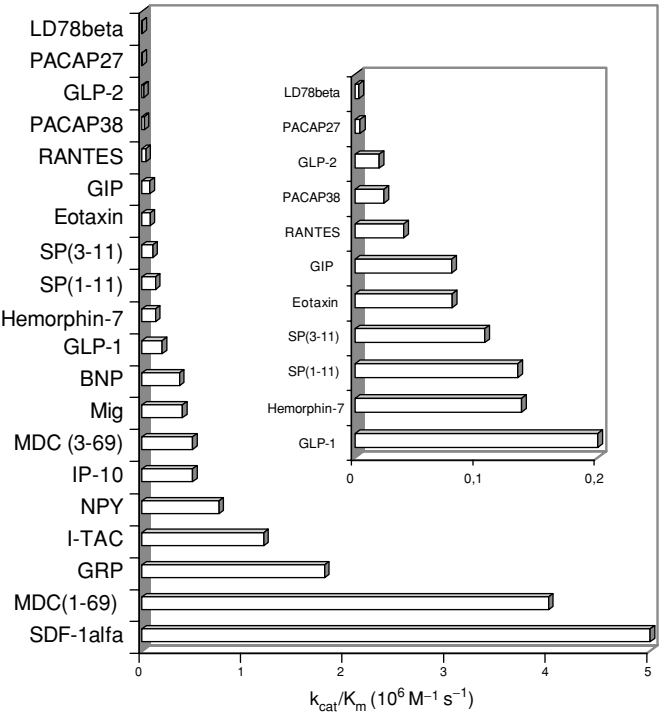

Figure 1. The specificity constants k_{cat}/K_m of the cleavage of natural peptides by DPP IV.

are of importance (Kühn-Wache *et al* 2003). There seems to be no link between peptide length up to 125 amino acids and the kinetic constants. A free and flexible N-terminus is required (Lambeir *et al* 2003).

2.2. Brain Natriuretic Peptide 32 (BNP-32) is Cleaved Efficiently by DPP IV

The natriuretic peptide family consists of four members which all have a characteristic 17 amino acid residue ring formed by an intramolecular disulfide bridge between two cysteine residues. The heart secretes two cardiac natriuretic peptides, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), which are involved in the regulation of extracellular fluid and blood pressure homeostasis.

Before they are released into the circulation they each exist as a high molecular weight prohormone (Vanderheyden *et al* 2004). BNP prohormone, proBNP, consists of 108 amino acids and is produced mainly by the cardiac myocytes. The biologically active BNP-32 is separated from the N-terminal part of the prohormone, called NT-proBNP. The physiological effects of BNP-32 are caused by its binding to the natriuretic peptide receptor type A that is coupled to cGMP. The effects thus produced include diuresis, vasodilatation, inhibition of renin and aldosterone production and of cardiac and vascular myocyte growth (Hall 2004). Measurements of blood BNP-32 proved useful in identifying patients with left ventricular dysfunction. In addition, nesiritide, a recombinant form of human BNP, has been approved for the treatment of acutely decompensated heart failure (Munagala *et al* 2004).

BNP-32 turned out to be a very good substrate for DPP IV having a higher specificity constant (k_{cat}/K_m $0.37*10^6$ $M^{-1}s^{-1}$) than several known physiological substrates of the enzyme. Shimizu et al reported the release of 2 amino-acids (Ser-Pro) from the aminoterminus of BNP(1-32) upon incubation in whole blood. In their assay sytem, the immunoreactivity of BNP was preserved. No other fragments of BNP were observed after 24-h incubation. The authors did not identify the protease responsible for the observed cleavage. (Shimizu *et al* 2002) Whether the cleavage of BNP-32 by DPP IV has physiological implications remains to be elucidated.

3. *IN VIVO* RELEVANCE OF PEPTIDE TRUNCATION BY DPP IV

In vitro a large number of substrates are cleaved by DPP IV. To date only a few of them have been proven to be endogenous substrates upon the following criteria: (1) cleavage occurs *in vitro* at the penultimate residue, and (2) cleavage products are observed *in vivo* but are absent in the presence of a selective inhibitor or in DPP IV $-/-$ mice (Weber 2004). Even if *in vivo* truncation by DPP IV can be demonstrated, the relevance of the DPP IV cleavage and eventually its inhibition, should be studied thoroughly before conclusions are drawn (Mentlein *et al* 2003). For some known (e.g. substance P (Ahmad *et al* 1992)) or putative (e.g. procalcitonin (Wrenger *et al* 2002)) *in vivo* substrates, no new data became available since the publication of our most recent review (Lambeir *et al* 2003).

3.1. The Pancreatic Polypeptide Family

3.1.1. Neuropeptide Y

Neuropeptide Y (NPY) displays a large array of functions; it is involved in the control of energy homeostasis, blood pressure, angiogenesis, immune responses and behavioural stress responses. It has at least 5 receptors, Y1, Y2, Y4, Y5 and Y6 (for recent review see Groneberg *et al* 2004).

The N-terminally truncated form of NPY(3-36), the NPY fragment created by DPP IV, has considerably reduced activity for the Y1 receptor, but remains an agonist for the Y2 and Y5 receptor subtypes. In vitro stimulation of human umbilical endothelial cells by a cocktail of inflammatory cytokines (LPS, TNF-α and IFN-γ not only increased the NPY and DPP IV synthesis, but also changed the Y2 and Y5 receptor expressions. The NPY system thus seems very flexible during the process of an inflammatory response (Silva *et al* 2003).

The NPY Y1 receptor mediates anxiolytic-like effects of NPY. Karl *et al* (2003) administered intracerebroventricularly different doses of NPY in wild-type F344 rats and mutants lacking endogenous DPP IV-like activity. In the DPP IV-negative substrains NPY administration was more potent in exerting anxiolytic-like and sedative-like effects.

The above mentioned and other recent studies (Li *et al* 2005, chapter 14 in this volume; Kitlinska *et al* 2003) provide evidence that NPY-mediated effects in the periphery as well as in the central nervous system are modulated by DPP IV-like enzymatic activity. Whether long-term inhibition of DPP IV leads to increased NPY activity in treated patients, is to be awaited.

At the moment, no NPY-based molecules are in clinical use, so the human NPY system remains largely elusive (Chronwall and Zukowska, 2004). A number of recent studies analyzing NPY and Y receptor overexpressing and knockout animal models may give some hints on the specific functions of signalling via the different receptors (Lin *et al* 2004).

3.1.2. Peptide YY (PYY)

Another member of the pancreatic polypeptide family is the gut hormone peptide YY for which the L-cells of the gastro-intestinal tract are the major source. PYY is produced by endocrine cells of the small intestine and the colon. Postprandially it is released as a peptide hormone. It circulates in the blood and inhibits several gastro-intestinal functions, for example gastric acid release (Mentlein 1999). There are two endogenous forms of peptide YY, PYY(1-36) and PYY(3-36), the latter is produced by DPP IV. The liberation of the N-terminal dipeptide from peptide YY, converts it from a vasoconstrictive and insulinotrophic factor into a potent secretion inhibitor (Keire *et al* 2002). In humans, peripheral infusion of PYY(3-36) at a dose which produced normal postprandial concentrations significantly decreased appetite and reduced food intake (Batterham and Bloom 2003). These results indicate that the regulation of food intake does not occur via the Y1 receptor.

3.2. Opioid Peptides

3.2.1. Hemorphins

The hemorphins are a family of small bioactive peptides derived from hemoglobin hydrolysis through a yet unknown mechanism. In addition to their opioid like activity a number of other biological activities of hemorphins were observed, including interaction with the renin-angiotensin system. Although the presence in physiological or pathological conditions as well as potential activities of hemorphins have been extensively studied, only few reports are related to their metabolism. Cathepsin D has been hypothesized to be involved in the first step of their generation, with the release of LVV-Hemorphin-7 (LVVH7; sequence: LVVYPWTQRF) and VVH7 (Fruitier-Arnaudin *et al* 2003a). LVVH7 incubated with microsomal fraction gave rise to Hemorphin-7, which could be degraded by the DPP IV present in this fraction. A subsequent kinetic study with purified DPP IV permitted to confirm that H7 is very good substrate for the enzyme (Cohen *et al* 2004).

In vivo relevance for a role for DPP IV in the hemorphin-7 metabolism has not been reported yet. Intriguingly, significantly lower levels of serum hemorphin-7 peptides are found in diabetic patients. It is hypothesized that the *in vivo* formation of hemorphins resulting from hemoglobin hydrolysis is altered by glycosylation (Fruitier-Arnaudin *et al* 2003b). Given the many effects of hemorphins *in vivo*, one can wonder 1) whether the significantly altered levels in diabetics contribute to the symptomatology and 2) whether inhibition of DPP IV 'rescues' part of the hemorphin activities.

3.2.2. Endomorphins

Endomorphin-1 (EN1) is an endogenous mammalian opioid agonist with high affinity and selectivity for μ-opioid receptors. During tests in a buccal delivery system, EN1 was shown to be very unstable. Diprotin A, a dipeptidyl peptidase inhibitor provided significant inhibition of its degradation (Bird *et al* 2001).

Another endogenous ligand for the μ-opioid receptor, endomorphin-2, was incubated with mouse brain synaptic membranes in order to study its metabolism. The degradation of the substrate was inhibited by the use of DPP IV inhibitors, such as diprotin A and B. Furthermore, purified DPP IV hydrolyzed endomorphin-2. These findings add further evidence for the

proposed role of DPP IV in the metabolism of endomorphin-2. The intracerebroventricularly injection of diprotin A together with endomorphin-2 in mice enhanced endomorphin-2-induced antinociception (Sakurada *et al* 2003), suggesting a role for diprotin A-sensitive DPP activity. Repetition of this kind of experiments with highly selective, potent inhibitors of the different members of the dipeptidyl peptidase family would greatly enhance our understanding of the central metabolism of the endomorphins.

3.3. PACAP/Glucagon Family of Peptides

3.3.1. Pituitary Adenylate Cyclase Activating Polypeptide (PACAP)

PACAP has been attributed a number of functions, such as glucose control, lipid metabolism and adaptive thermogenesis. An intact N-terminus is required for its bioactivity. PACAP is a 38 amino acid peptide with a C-terminally truncated 27-residue splicing variant PACAP27. *In vitro* a differential processing of PACAP27 and PACAP38 has been observed. PACAP38 has a higher affinity for DPP IV and a higher turnover rate (Lambeir *et al* 2001). Using tandem mass spectrometry Zhu *et al* (2003) measured the intact PACAP(1-38) and the metabolite PACAP(3-38) in plasma of wild type and DPP IV deficient C57Bl/6 mice after intravenous dosing with supraphysiological levels of the neuropeptide. They found that in wild type mice PACAP38 was rapidly degraded with concomitant formation of the inactive PACAP(3-38). This metabolite was virtually absent in DPP IV$-/-$ mice and the clearance of intact PACAP38 from the circulation was significantly lower in these animals. In a similar experiment monitoring the catabolism of exogenous PACAP27 in wild type mice, there was no evidence for the formation of the corresponding PACAP(3-27) metabolite. Recently Ahrén and Hughes (2005) were able to show that in mice DPP IV inhibition by Valine-pyrrolidide augmented the insulin responses to intravenous administration of PACAP38. From these results they concluded that prevention of inactivation of this peptide augments islet function.

3.3.2. Vasoactive Intestinal Peptide (VIP)

VIP is a prominent neuropeptide with a wide spectrum of biological activities in mammals, such as suppression of inflammation and perception of pain. It is released after stimulation of the parasympathetic nerves in the pancreas and increases insulin and glucagon secretion in a glucose-dependent manner. As residues 1 and 5 are involved in VIP binding and residue 3 is involved in VIP binding and VIP structure (Nicole *et al* 2000), the sequential truncation of this peptide by DPP IV is likely to have an impact on its bioactivity. So far there is no *in vivo* evidence for relevance of DPP IV mediated truncation of VIP.

3.3.3. Glucagon-Like Peptide 1 (GLP-1) and Glucose-Dependent Insulinotropic Peptide (Gastric Inhibitory Peptide, GIP)

The incretins GIP and GLP-1 are gut-derived factors that potentiate postprandial insulin secretion and glucose clearance; they are released in response to the presence of food in the intestinal lumen. Both are subjected to DPP IV mediated N-terminal cleavage which causes a complete loss of insulinotropic action of these hormones as these residues are important in receptor activation. GLP-1 and GIP are the best studied *in vivo* substrates of DPP IV and they are subject of a number of excellent recent reviews (Ahren 2003; Drucker 2003; Kieffer 2004).

The *in vivo* stabilization of these incretins is now widely accepted as a new approach in antidiabetic treatment. In addition to the use of DPP IV inhibitors, stable incretin analogues have been developed to treat diabetes. Lee *et al* (2005) developed a specific DPP IV resistant mono-polyethylene glycol-conjugated GLP-1 preparation with a greatly improved pharmacological profile. In a recent study by Green *et al* (2004a) alanine residues in both GLP-1 (Ala^8) and GIP (Ala^2) were substituted for a larger amino acid structure, 2-aminobutyric acid (Abu). (Abu^8)GLP-1 turned out to be profoundly resistant to DPP IV degradation, but surprisingly (Abu^2)GIP only had a moderately prolonged half-life compared to native GIP. The same group of researchers also combined this GLP-1 modification with the attachment of palmitoyl moieties to its epsilon-amino group in the side chain of the Lys^{26} residu. They indicate a potential usefulness of fatty acids linked analogues of GLP-1, but indicate the importance of the chain length for peptide kinetics and bioavailability (Green *et al* 2004b). The N-terminal modification of His^7 in GLP-1 also generates DPP IV stable analogues (Green *et al* 2004c). Islam *et al* (2005) transfected rat insuloma cells with a plasmid encoding a mutated form of GLP-1 (GLP-1-Gly^8). The cells produced and stored GLP-1-Gly^8, moreover they displayed an enhanced glucose responsiveness. The results obtained with stabilized GLP derivatives as well as with DPP IV inhibitors (Holst and Deacon 2004; Ahren *et al* 2004) all support a crucial role for DPP IV in the *in vivo* metabolism of this incretin.

3.3.4. Glucagon-Like Peptide 2 (GLP-2)

Next to its important role in intestinal mucosa regeneration and permeability, GLP-2 augments intestinal hexose transport and reduces gastric acid secretion and gastric emptying. In *in vitro* experiments DPP IV-mediated truncation of GLP-2 turned out to be almost ten times slower than that of GLP-1 (Lambeir *et al* 2002). In an elegant study on GLP-2 degradation in humans, Hartmann *et al*. 2000 identified GLP-2(3-33) as the only metabolite formed. However, 1 hr after subcutaneous injection, still 69% of the GLP-2 was intact, confirming a significantly slower cleavage than GLP-1. Nothwithstanding this relatively slow truncation, preclinical *in vivo* studies on the therapeutic potential of GLP-2, use DPP IV-resistant analogues of the peptide e.g. Teduglutide, (Gly2)GLP-2 (Booth et al 2004).Several *in vivo* experiments have been conducted in order to test the hypothesis that the degrading activity of DPP IV would be limiting for the effects of GLP-2. Hartmann *et al* (2000) found that the GLP-2 degradation in Wistar rats could be reduced by Valine-Pyrrolidide and that DPP IV inhibition enhanced the insulinotropic effect of GLP-2 in both rats and and C57BL mice. Administration of rat GLP-2 to DPP IV deficient rats was found to be associated with markedly increased bioactivity of rat GLP-2 resulting in an increase in small bowl weight (Drucker *et al* 1997). Tavares *et al* (2000) determined the importance of DPP IV in the regulation of circulating GLP-2 levels *in vivo* by injecting GLP-2 or the DPP IV-resistant analogue [Gly^2]GLP-2 in normal and in DPP IV-negative rats. They found that normal rats showed a steady degradation of GLP-2(1-33) to GLP-2(3-33) over time. In contrast, little or no conversion of GLP-2(1-33) was observed in DPP IV negative rats. [Gly^2]GLP-2 also remained intact in wild type rats. GLP-2 is known to enhance the repair of damaged intestinal tissue, however Geier *et al* (2005) demonstrated that DPP IV(−/−) mice did not display an increased resistance to or an enhanced recovery from dextran sulphate sodium-induced colitis compared to DPP IV(+/+). As they found that DPP IV(−/−) mice still possessed significant plasma levels of DPP IV-like activity, they hypothesized that other DPP IV family members might also be involved in the cleavage of GLP-2.

3.4. Gastrin-Releasing Peptide (GRP)

GRP is a 27 amino acid member of the bombesin family of peptide hormones. Its functions are the stimulation of the secretion of various gastrointestinal hormones and the regulation of the endocrine and exocrine pancreas. In *in vitro* experiments GRP is rapidly truncated by DPP IV in two consecutive steps, with a k_{cat}/K_m being significantly higher than the one for GLP-1 and GIP. For the latter two the intact peptide levels are regulated by DPP IV *in vivo*, it is however not known whether DPP IV degrades GRP *in vivo* (Lambeir *et al* 2001). Furthermore, even if DPP IV removes the N-terminal amino acids *in vivo*, it is not clear how this would augment insulin secretion, since the insulin-releasing property of GRP resides in the C-terminal end of the peptide. Recent experiments by Ahrén and Hughes (2005) offer new indications that GRP is not a substrate for DPP IV under *in vivo* conditions. In their mouse model it was surprising to find an augmentation by valine-pyrrolidide of GRP-induced insulin secretion. This augmentation however was prevented by a GLP-1 receptor antagonist, suggesting the GRP induced insulin secretion was, at least partially, dependent on GLP-1. As a possible explanation the authors suggested that GRP causes a release of GLP-1, which in turn contributes to the insulin response to GRP. Hence, although Valine-pyrrolidide augments GRP-induced insulin secretion, it is most likely dependent on prevention of inactivation of GLP-1.

3.5. Chemokines

Chemokines constitute of a family of small cytokines, which exert their effect locally in a paracrine or autocrine fashion. They play fundamental roles in the development, homeostasis and function of the immune system and act as regulatory molecules in leukocyte maturation and traffic and in homing of lymphocytes and the development of lymphoid tissues (for a recent review: Laing *et al* 2004). The target cell specificity of chemokines depends on the cellular expression of the different chemokine receptors (CCRs or CXCRs). The N-terminal domain of chemokines is involved in receptor binding and its processing can either activate chemokines or render them completely inactive (Proost *et al* 1998).

3.5.1. Regulated on Activation, Normal T cell Expressed and Secreted (RANTES, CCL5)

RANTES has been shown to be a substrate of soluble DPP IV. Moreover in the experiments of Oravecz *et al* (1997) the truncated RANTES(3-68) in contrast with the intact form, was not able to increase the cytosolic calcium concentration in human monocytes, but still induced this response in macrophages activated with macrophage colony-stimulating factor. Further analysis learned that the truncated molecule displayed altered receptor specificity. Proost *et al* (1998a) described the physiological occurrence of RANTES missing the first two amino acids. Furthermore this truncated form inhibited infection of mononuclear cells by an M-tropic HIV-strain 5-fold more efficiently than intact RANTES. The authors concluded that the proteolytic processing of RANTES by DPP IV may be an important regulatory mechanism during anti-inflammatory and antiviral responses.

In 2003 Khin *et al* found that DPP IV is expressed in normal endometrial glandular cells and that its expression in endometrial adenocarcinoma is down-regulated with increasing grade. RANTES was shown to be highly expressed in all grades of endometrial adenocarcinoma cells and addition of the peptide to the cells increased the proliferation in a concentration dependent manner. As RANTES is also secreted by other tumors and as an association with enhanced

tumor growth is seen, a prolonged activity of this chemokine may be disadvantageous. Apart from this finding, DPP IV expression is also known to be downregulated on a number of other tumor cells. Further investigations are necessary to clarify whether there is a link between changes in DPP IV activity and RANTES influence on tumor growth (cfr. SDF-1).

3.5.2. Eotaxin (CCL11)

Because of its selective attraction of eosinophils, basophils and TH2 lymphocytes, eotaxin has been characterized as an important mediator in allergic reactions. Its receptor therefore has emerged as a target for the treatment of allergic asthma (Forssmann et al 2004). Eotaxin is efficiently cleaved by DPP IV (Struyf *et al* 1999; Oravecz *et al* 1997) and this cleavage has an effect on its biological activity. There seems to be a difference in the regulation by DPP IV of the chemotactic as opposed to the antiviral properties of eotaxin, since HIV-2 infection of CC chemokine receptor 3-transfected cells was inhibited to a similar extend by eotaxin and eotaxin(3-74) (Struyf *et al* 1999). In contrast the truncated form showed reduced chemotactic activity for eosinophils and impaired binding and signalling properties through the CC chemokine receptor 3. Furthermore, it desensitized calcium signalling and inhibited chemotaxis toward intact eotaxin.

To date it is not known whether treatment with DPP IV inhibitors results in enhanced eotaxin activity *in vivo*.

3.5.3. Macrophage Derived Chemokine (MDC, CCL22)

MDC attracts monocytes, dendritic cells, activated lymphocytes and NK cells. DPP IV mediates its NH_2-terminal processing, cleaving off Gly^1-Pro^2 and Tyr^3-Gly^4. Both truncated products display reduced chemotactic activity on lymphocytes and monocyte-derived dendritic cells. They showed impaired binding to and calcium mobilization through the CCR4 receptor. However they retained their ability to attract and to bind monocytes (Proost *et al* 1999). The truncated products are also not recognized by the promiscuous D6 receptor. This receptor has a high structural similarity with chemokine receptors and binds most inflammatory CC chemokines. However, because of the lack of a known signalling function, it was classified as a silent chemokine receptor or chemokine scavenging decoy receptor. The selective recognition of intact MDC versus MDC(3-69) and MDC(5-69) was suggested to represent a strategy to focus the decoy function on the CCR4 agonists, without interference from inactive processed forms (Bonecchi *et al* 2004).

3.5.4. LD78β (CCL3L1)

By interaction with and signalling through CCR1 and CCR5 LD78α and LD78β activate and chemoattract mononuclear cells. Although these two chemokines only differ by 3 out of 70 amino acids, they interact differently with CCR5, for which LD78β shows the highest affinity. LD78α is not cleaved by DPP IV, while LD78β is. *In vivo* produced LD78β(3-70) was recovered from peripheral blood mononuclear cell preparations after *in vitro* stimulation. The truncated chemokine LD78β(3-70) showed increased chemotactic activity in comparison to intact LD78β, which is in contrast to all other chemokines processed by DPP IV. Also the truncated form retained its capacity to induce an intracellular calcium increase in CCR5-transfected cells. On CCR1 transfectants it was far more potent than the intact form (Proost *et al* 2000).

3.5.5. Interferon-Inducible T Cell α Chemoattractant (I-TAC, CXCL11)

Ludwig *et al* (2002) used the DPP IV inhibitor Lys-pyrrolidide to demonstrate that DPP IV was responsible for the cleavage of I-TAC by PHA/IL-2 activated T cells. The truncated form had an eightfold reduced potential to bind and regulate CXCR3, but was completely inactive in calcium flux and chemotaxis assays. Moreover, I-TAC(3-73) desensitized T cell chemotaxis in response to intact chemokine.

However, using the same inhibitor it was also shown that DPP IV cleavage was not rapid enough to disturb the *in vitro* chemotactic activity of I-TAC. Based on this observation and the knowledge that there is a high degree of redundancy in chemokine biology, Mentlein et al (2003) hypothesized that DPP IV does not affect immediate chemotactic effects and that DPP IV inhibitors will have little or no side effects on chemokines. Further *in vivo* observations are needed to see whether this statement holds true for all chemokines.

3.5.6. Stromal-Cell-Derived Factor 1 (SDF-1, CXCL12)

SDF-1 is constitutively expressed in most tissues as SDF-1α(1-68) and SDF-1β (1-72); these forms result from alternative gene splicing. SDF-1 regulates hematopoiesis, lymphocyte homing, B-cell growth and angiogenesis. Moreover, there is increasing evidence that SDF-1 and its receptor CXCR4 are involved in a number of inflammatory processes. SDF-1 is the only physiological ligand for CXCR4. *In vitro*, SDF-1 is the best DPP IV substrate known so far. It was demonstrated that DPP IV mediated cleavage of SDF-1α resulted in a loss of lymphocyte chemotactic activity and signalling properties through CXCR4, although SDF-1α (3-68) still desensitized the SDF-1α(1-68) induced Ca^{2+} response (Proost *et al* 1998b). SDF-1α truncation by DP IV also resulted in a loss of inhibition of HIV cell entry with SDF-1α (Ohtsuki *et al* 1998; Proost *et al* 1998b).

In a recent article by Busso *et al* (2005) it was shown that DPP IV-deficient mice exhibited increased levels of circulating intact, active SDF-1. In an antigen-induced arthritis model this was associated with increased numbers of SDF-1 receptor-positive cells infiltrating in the arthritic joints of the DPP IV-deficient versus wild type mice. Mizokami *et al* (2004) demonstrated that exogenous SDF-1α significantly stimulated proliferation of human endometrial carcinoma cells in a concentration dependent manner. In contrast, upon transfection of DPP IV in these cells, there was no apparent effect on tumor cell proliferation by the addition of exogenous SDF-1α. These examples support that in different species and different *in vitro* settings, DPP IV is involved in the biology of SDF-1.

Furthermore, in human serum, SDF-1α as well as SDF-1β are processed at the NH_2 terminus by CD26/DPP IV. The truncated forms are unable to bind heparin and to activate cells (De La Luz *et al* 2004). Although Sadir *et al* (2004) demonstrated *in vitro* that heparin as well as heparan sulphate protected SDF-1 against processing by DPP IV, it remains unknown whether *in vivo* heparan sulphate protects SDF against DPP IV.

Inhibition of the endogenous DPP IV activity on human cord CD34+ progenitor cells enhances the migratory response of these cells to SDF-1α DPP IV mediated cleavage of SDF-1 may therefore represent an additional regulatory mechanism in the migration, homing and mobilization of stem and progenitor cells. DPP IV inhibition may favor homing and engraftment (e.g. after cord blood transplantation), while increasing the DPP IV activity may contribute to the mobilization of hematopoetic cells (Christopherson *et al* 2002).

4. POSSIBLE TRUNCATION OF KNOWN DPP IV SUBSTRATES BY OTHER DIPEPTIDYL PEPTIDASES

Besides DPP IV, only a few proteases are able to cleave the post-proline bond two positions from the N-terminus. The latter small subset of post-proline dipeptidyl aminopeptidases consists of FAPα, DPP8 and DPP9 and DPP II (reviewed recently in Busek *et al* 2004 and Gorrell 2005). The involvement of the DPP IV related peptidases in the processing of known DPP IV substrates is a subject of current research in several groups.

4.1. Fibroblast Activation Protein α (FAPα)

FAPα, as a matrix-degrading enzyme, is involved in tissue remodelling and cancer cell invasion and is not found in healthy adult tissue. It shares the highest homology with DPP IV. *In vitro* FAPα has been found to exhibit dipeptidyl peptidase activity; it showed a preference for Ala-Pro-AFC as compared with Lys-Pro-AFC or Gly-Pro-AFC (Park *et al* 1999). FAPα also displays gelatinase activity *in vitro*, with both gelatin and collagen type I (Sun *et al* 2002). However little is known about its natural substrates and the significance of its peptidase activity *in vivo*. Cheng *et al* (2005) pointed out the importance of this activity. They performed a site-directed mutagenesis at the catalytic site of FAPα, $Ser^{624} \rightarrow Ala^{624}$, which resulted in a severe loss of FAPα ectopeptidase activity. They transfected HEK293 cells with wild type or Ala^{624} mutant FAPα and inoculated them into immunodeficient mice. Cells transfected with wild type FAPα how the characteristic tumor growth rate and tumorigenic potential attributed to the presence of FAPα. The cells with mutant FAPα showed similar behaviour to vector-only transfected cells. Lee *et al* (2004) discovered and purified a proteinase from human plasma and named it antiplasmin-cleaving enzyme, as it cleaved the Pro^{12}-Asn^{13} bond of alpha2-antiplasmin to generate a more active form. This enzyme is similar in primary structure and catalytic properties to membrane-bound fibroblast activating protein α.

4.2. DPP8 and DPP9

The biological substrates of DPP8 and DPP9 have not yet been reported in the scientific literature.

The substrate specificity of DPP8 has been determined by measuring its activity against several chromogenic substrates. Abbott *et al* (2000) have demonstrated that DPP8 is a dipeptidyl peptidase that lacks tripeptidyl peptidase activity, using H-Ala-Ala-Phe-*p*NA and H-Ala-Phe-Pro-*p*NA as possible substrates. It also did not display any endopeptidase activities, using Z-Ala-Pro-*p*NA and succinyl-Ala-Pro-*p*NA. They found that recombinant DPP8 preferred H-Gly-Pro-*p*NA over H-Ala-Pro-*p*NA. Chen *et al* (2004) also report these substrates as the best substrates for DPP8, with a lower K_m however for H-Gly-Pro-*p*NA as opposed to H-Ala-Pro-*p*NA.

When DPP9 was identified and characterized, the presence of a serine-protease GWSYG motif and a catalytic triad identical to that found in DPP IV, FAP and DPP8 suggested that DPP9 might have DPP IV-like activity (Olsen *et al* 2002). Ajami *et al* (2004) were able to show that DPP9-transfected 293T cells hydrolyzed Ala-Pro-AFC and Gly-Pro-*p*NA, whereas untransfeceted and vector-only-transfected 293T cells did not.

The availability of sufficient amounts of pure DPP8 and DPP9 should allow a thorough *in vitro* kinetic investigation of peptide cleavage by these recently discovered enzymes.

Table 1. Bioactive peptides tested for *in vitro* truncation by DPP II.

Peptide	N-terminus	# Residues
Pancreatic polypeptide family		
Neuropeptide Y	YPSKPDNPG-	36
PACAP/Glucagon family		
GLP-1	HAEGTFTSD-	30/31
GLP-2	HADGSFSDE-	33
GIP	YAEGTFISD-	34
VIP	HSDAVFTDN-	28
PACAP27	HSDGIFTDS-	27
PACAP38	HSDGIFTDS-	38
Chemokines		
I-TAC	FPMFKRGRC-	73
SDF1α	KPVSLSYRC-	68
MDC	GPYGANMED-	69
Natriuretic peptides		
BNP32	SPKMVQGSG-	32
Others		
GRP	VPLPAGGG-	27
Substance P	RPKPQEFFG-	11
Bradykinin	RPPGFSPFR-	9

4.3. DPP II does not Cleave Large DPP IV Substrates

Early DPP II articles indicate that DPP II only cleaves very short peptides. McDonald *et al* (1968) showed that DPP II hydrolyses several tripeptides, while tetrapeptides, tripeptide esters and dipeptides were not attacked. Moreover the hydrolysis of otherwise susceptible tripeptides did not occur if either the carboxyl group or the amino group was blocked. Later, Eisenhauer and McDonald (1986) reported that the action of porcine DPP II was limited to tripeptides, although they saw a trace of activity on the tetrapeptide Phe-Pro-Ala-Ala. Mentlein and Struckhoff (1989) stated that the rat brain DPP II preferentially acts on tri- and tetrapeptides, although they also detected activity with peptides up to 11 residues. With the 'rediscovery' of DPP II as 'Quiescent cell postproline dipeptidyl peptidase' (Araki *et al* 2001, Leiting *et al* 2003, Maes *et al* 2005) larger peptides (like chemokines and other cytokines) were suggested for this vesicular enzyme (Chiravuri *et al* 2000). To look into this controversy, we incubated purified human DPP II with several known DPP IV substrates. Under its optimal assay conditions DPP II did not cleave any of the substrates, when using the same incubation times and enzyme activities as for the DPP IV cleavage studies (Table 1).

To study the decay curves of peptide substrates 10 µl of 50 U/l DPP II was mixed with 10 µl of 10 µM substrate solution and incubated at 37 °C in presence of 50 mM cacodylic acid buffer, pH 5.5, 10 mM EDTA. After 1 hour the mixtures were quenched in 0.1% trifluoroacetic acid. C18 ZipTips (Millipore Corp., Bedford, MA) were used to desalt the samples. Elution was performed step-wise with 10 µl of 30 and 50% acetonitrile in 0.1% acetic acid. The composition of the mixture was determined with an Esquire ESI Ion Trap mass spectrometer (Bruker, Bremen, Germany). The instrument was used in a normal range, normal resolution setting, optimized on an m/z value near the most abundant ion of the intact peptide.

5. CONCLUDING REMARKS

At this very moment with advanced clinical trials evaluating the first generation of dipeptidyl peptidase inhibitors for therapeutic use, some dual feelings may appear upon meeting again with the complexity of dipeptidyl peptidase biology. Nevertheless, never before were the conditions so favourable to further unravel the puzzle:

- A number of structurally different DPP IV inhibitors reached the status of investigational new drugs with up to now very promising safety profiles.
- Selective inhibitors for related dipeptidyl peptidases are being developed and will add to a better understanding of their role in physiology and pathology (Lankas *et al* 2004; Jiaang *et al* 2005; Senten *et al* 2004)
- Technology platforms-functional genomics, peptidomics and proteomics-became available that allow a more detailed molecular view of what happens upon *in vivo* administration of this type of drugs (Schulz-Knappe *et al* 2001; Gevaert *et al* 2003).

These and other tools together with an open, critical mind certainly will increase our insight in DPPs. More studies are needed to determine the *in vivo* relevance of DPP mediated cleavage of the various proposed substrates like e.g. brain natriuretic peptide (BNP). Let's hope that future research will reveal the value of DPPs as therapeutic targets in other diseases, rather than toxic side effects that discourage further research in this intriguing area.

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CHAPTER 2

PHOSPHORUS-CONTAINING INHIBITORS OF PROTEOLYTIC ENZYMES

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1. INTRODUCTION

The dipeptidyl peptidase IV (DP IV, EC 3.4.14.5) is a proline specific serine protease, which cleaves Xaa-Pro dipeptides from the N-terminus of oligo- and polypeptides (for review Lambeir *et al* 2003).

During the last years our attention was directed to the development of DP IV inhibitors and their specificity. It is well-known, that Xaa-Pro dipeptides which are products of substrate hydrolysis are competitive inhibitors of DP IV (Rahfeld 1989). Furthermore, the product analogous amino acid pyrrolidides (Pyrr), e.g. **1** (figure 1), and thiazolidides are also known as potent competitive inhibitors (Born *et al* 1994). Previously, we synthesized thioxo amino acid amides as amide bond isosteres of amino acid pyrrolidides and thiazolidides (Stöckel-Maschek *et al* 2000a). Moreover, analogous phosphonamidates **2** were synthesized as amide bond isosteres (figure 1).

Peptides containing a 3-amino-2-hydroxy acid like bestatin and amastain were described as inhibitors of different aminopeptidases (Umezawa *et al* 1976, Nishizawa *et al* 1977, Rich *et al* 1984). Peptides and amides containing a N-terminal 3-amino-2-hydroxy acid and a penultimate proline or proline-analogous structure were described as the first potent inhibitors of aminopeptidase P (APP) (Prechel *et al* 1995; Stöckel *et al* 1997). Recently, we showed, that these 3-amino-2-hydroxy acid containing APP inhibitors (**3**, figure 1) are also able to inhibit other aminopeptidases and dipeptidyl peptidase IV (Stöckel-Maschek *et al* in preparation). In a further study, the carbonyl group of the 3-amino-2-hydroxy acid was replaced by a tetrahedral phosphorus moiety. The concept of transition-state analogues has been very useful in designing potent inhibitors of proteolytic enzymes. Tetrahedral phosphorus derivatives are stable mimics of a tetrahedral intermediate that lies along the proteolytic reaction coordinate.

Thus, we have synthesized 2-amino-1-hydroxy phosphonic acid derived amides and peptides (compounds **4**, figure 1). The title compounds were tested as inhibitors of the aminopeptidases APP, aminopeptidase M (APM), leucine aminopeptidase (LAP) as well as DP IV.

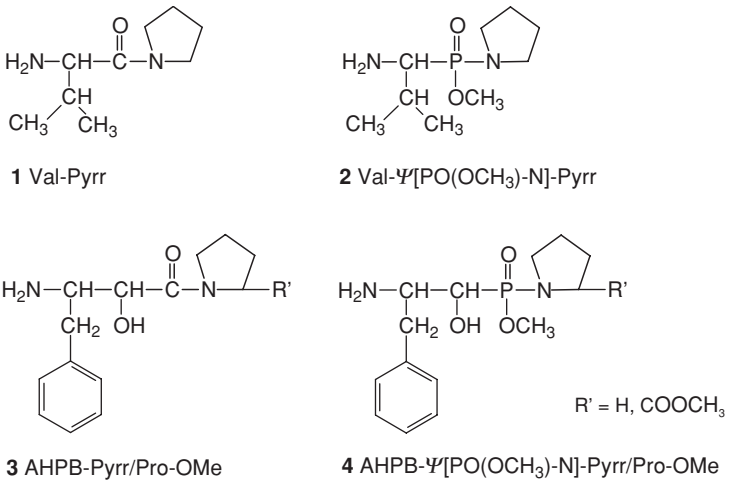


Figure 1. Structure of amino acid pyrrolidides and dipeptides as well as isosteric phosphonamidates - (AHPB, 3-amino-2-hydroxy-4-phenylbutanoic acid)

2. MATERIALS AND METHODS

The phosphonamidate **2** (figure 1) was synthesized starting from Z-protected phosphonovaline dimethylester, which was obtained by reaction of benzyl carbamate, isobutyraldehyde and dimethyl phosphite using the Oleksyszyn reaction (Oleksyszyn *et al* 1979). Partial dealkylation yielded the Z-protected monomethylester. Subsequently, methylester chloride was formed using $(COCl)_2$/DMF. The methylester chloride was converted into the desired phosphonamidate. It was obtained in a diastereomeric mixture and the diastereomers were separated by HPLC. Hydrogenolytic deprotection of the N-terminus provided the expected final compound Val-ψ[$PO(OCH_3)$-N]-Pyrr.

β-amino-α-hydroxy phosphonic acid derived amides and peptides **4** (figure 1) were synthesized starting from an appropriate Z-protected (*R*)- or (*S*)-α-amino alcohol. Oxidation followed by addition of dimethyl phosphite resulted in two diastereomers of Z-protected β-amino-α-hydroxy phosphonic acid dimethylesters (Patel *et al* 1990). After protection of the α-hydroxy group the dimethyl esters were converted into the corresponding amides/peptides *via* monomethylester and methylester chloride, as described above. The β-amino-α-hydroxy phosphonic acid derived amides and peptides were obtained in form of 4 diastereomers, because the compounds contain three asymmetric atoms. The configuration of the C2-atom is defined from the starting material. The diastereomers were separated by HPLC. The α-hydroxy group and the N-terminus were deprotected by well established methods.

DP IV from pig kidney was isolated according to Wolf *et al* (1978). LAP as well as APM both from pig kidney were obtained from Sigma-Aldrich. Recombinant APP from *E.coli* was a gift from Prof. T. Yoshimoto (Japan).

Except APP, the enzymatic hydrolysis of the substrates was monitored at a wavelength of 390 nm ($\varepsilon = 11\ 500\ M^{-1}cm^{-1}$) on a Beckman DU-650 UV/VIS spectrophotometer at 30 °C. Generally, the reaction was initiated by adding the enzyme and was followed over a time interval in which less than 10 % cleavage of substrate occurred.

The mechanism of inhibition of DP IV and the K_i values were determined from the enzyme-catalyzed hydrolysis of Gly-Pro-4NA (4NA: 4-nitroanilide) in the absence and the presence of inhibitor. The incubation mixture (1 ml) contained 40 mM Tris buffer pH 7.6, 0.125 mM NaCl, various concentrations of Gly-Pro-4NA and various concentrations of inhibitor around the expected inhibition constant. The final concentration of DP IV was 1.4 nM.

The activity of recombinant APP from *E.coli* was determined using the substrate Lys(Abz)-Pro-Pro-4NA (Abz, 2-aminobenzoyl, $K_m = 40.7\mu M$). The fluorescence of released Lys(Abz) was monitored at an excitation wavelength of 310 nm and an emission wavelength of 410 nm at 30 °C on a Perkin-Elmer LS-50B luminescence spectrometer (Stöckel-Maschek *et al* 2003). Lyophilized APP (0.1 mg) was dissolved in Tricine-citrate buffer (3 ml, 40 mM Tricine, 0.6 mM citrate, pH 7.4) containing $MnCl_2$ (3 mM). At first, APP was preincubated for 1 h at 30°C. A typical reaction mixture (1 ml) consisted of Tris buffer (40 mM, pH 7.4), $MnCl_2$ (0.75 mM), Lys(Abz)-Pro-Pro-4NA in various concentrations (4-15 μM) and different concentrations of inhibitor. The final enzyme concentration was 24 nM [MW: 49 650 Da (Yoshimoto *et al* 1989)].

The activity of LAP was determined from the enzyme-catalyzed hydrolysis of the substrate Leu-4NA ($K_m = 1.05$ mM) in the absence and presence of inhibitor. At first, LAP (100 μl) was incubated for 2 h at 40 °C in Tricin buffer (900 μl, pH 8.0) and $MnCl_2$ (100 μl, 15 mM). The reaction mixture (1 ml) contained Tris/HCl buffer (40 mM, pH 8.0), various concentrations of Leu-4NA (0.5–2 mM) as well as different concentrations of inhibitor around the expected inhibition constant. The final enzyme concentration was 24 μg/ml.

The substrate Ala-4NA ($K_m = 0.235$ mM) was employed for the determination of APM activity. The reaction mixture contained Tris/HCl buffer (40 mM, pH 7.2), various concentrations of Ala-4NA (0.12-0.5 mM) and different concentrations of inhibitor around the expected inhibition constant. The final enzyme concentration was 1.5 μg/ml.

The steady state kinetics were analyzed using the following equation for competitive and also linear mixed-type inhibition.

$$\frac{1}{v} = \frac{\left(1 + \dfrac{\alpha K_m}{[S]}\right)}{\alpha K_i V_{\max}}[I] + \frac{1}{V_{\max}}\left(1 + \frac{K_m}{[S]}\right) \tag{1}$$

K_i is the competitive inhibition constant and factor α multiplied with K_i represents the uncompetitive inhibition constant. For the distinction between competitive and linear mixed-type inhibition slopes and intercepts were replotted versus 1/[S] to complete the inhibition mechanism and the constants K_i as well as α (equations 2 and 3) (Segel 1993).

$$slope = \frac{K_m}{K_i V_{\max}}\frac{1}{[S]} + \frac{1}{\alpha K_i V_{\max}} \tag{2}$$

$$intercept = \frac{K_m}{V_{\max}}\frac{1}{[S]} + \frac{1}{V_{\max}} \tag{3}$$

In case of hyperbolic mixed-type inhibition the following equation was used to plot the data (Segel 1993).

$$\frac{1}{v} = \frac{\alpha K_m}{V_{\max}}\left(\frac{[I] + K_i}{\beta[I] + \alpha K_i}\right)\frac{1}{[S]} + \frac{1}{V_{\max}}\left(\frac{[I] + \alpha K_i}{\beta[I] + \alpha K_i}\right) \tag{4}$$

For the calculation of the inhibition constants K_i, α and β, the slopes and intercepts were replotted versus $[I]$ (equations 5 and 6) (Segel 1993).

$$slope = \frac{\alpha K_m}{V_{\max}} \left(\frac{[I] + K_i}{\beta[I] + \alpha K_i} \right) \tag{5}$$

$$intercept = \frac{1}{V_{\max}} \left(\frac{[I] + \alpha K_i}{\beta[I] + \alpha K_i} \right) \tag{6}$$

The progress curves of a slow-binding inhibition are described by equation (7)

$$P = v_s \cdot t + (v_i - v_s) \cdot (1 - e^{-k_{obs} \cdot t})/k_{obs} + d \tag{7}$$

Equation (7) allows the determination of the initial velocity v_i, the steady-state velocity v_s and the first-order rate constant k_{obs} for the approach of the steady-state velocity v_s of a single progress curve (Morrison & Walsh 1988, Szedlacsek & Duggleby 1995). A secondary plot of v_i and v_s versus [I] gives the dissociation constant K_i for the initial complex and the overall inhibition constant K_i*, respectively. The detailed type of the slow-binding inhibition as well as k_{off} and k_{on} were determined with equation (8) and (9), respectively.

$$k_{obs} = k_{off} + k_{on} \frac{[I]/K_i}{1 + [S]/K_m + [I]/K_i^*} \tag{8}$$

$$\frac{k_{on}}{k_{off}} = \left(\frac{K_i}{K_i^*} \right) - 1 \tag{9}$$

The kinetic data were analyzed with GraFit 3.0 (Erithacus Software Ltd., England) and SigmaPlot 5.0 (SPSS Inc., USA).

3. RESULTS AND DISCUSSION

Amino acid pyrrolidides (**1**) and thiazolidides are known as potent product analogous inhibitors of DP IV. Generally, these compounds inhibit DP IV competitively (Born *et al* 1994; Stöckel-Maschek *et al* 2000a). The two diastereomers of the isosteric Val-ψ[PO(OCH_3)-N]-Pyrr **2** are able to inhibit DP IV (figure 1, table 1). Interestingly, both diastereomers show different inhibition mechanisms for the inhibition of DP IV. Diastereomer 1 is a poor linear mixed-type inhibitor of DP IV with a K_i-value of 1800 μM, whereas diastereomer 2 is a more potent, competitive inhibitor with a K_i-value of 14.7 μM. Nevertheless, diastereomer 2 of Val-ψ[PO(OCH_3)-N]-Pyrr is an around 100 times less potent inhibitor of DP IV than the corresponding amino acid amide Val-Pyrr ($K_i = 0.255$ μM, Stöckel-Maschek *et al* 2000a). It seems, that the DP IV can not completely tolerate the chemical and stereochemical differences between amide and phosphonamide bond.

DP IV and APP have the same substrate specificity recognizing N-terminal Xaa-Pro dipeptides. APP is also inhibited by the both diastereomers of Val-ψ[PO(OCH_3)-N]-Pyrr **2** with inhibition constants in the micromolar range and the inhibition mechanisms are identical for both enzymes (table 1). In contrast to DP IV, APP is inhibited better by the phosphonamidates **2** than by the amino acid amides Ile-Thia and Phe-Thia or the isosteric thioxo amide Val-ψ[CS-N]-Thia, which have inhibition constants only in the millimolar range (Stöckel-Maschek *et al* 2000a).

Table 1. Inhibition by phosphonamidates Val-ψ[PO(OCH_3)-N]-Pyrr.

Enzyme	Diastereomer 1 K_i (μM)		Mechanism	Diastereomer 2 K_i (μM)		Mechanism
DP IV[1]	1800	$\alpha = 10$	linear mixed-type	14.7		competitive
APP	195	$\alpha = 0.5$	linear mixed-type	294		competitive
LAP	0.218	$\alpha = 5$ $\beta = 0.7$	hyperbolic mixed-type	7.8	$\alpha = 3$ $\beta = 0.3$	hyperbolic mixed-type
APM	341		competitive	$K_i = 546$ μM, $K_i^* = 291$ μM $k_{on} = 0.00015\ s^{-1}$, $k_{on}/K_i^* = 0.5\ s^{-1}M^{-1}$		slow-binding

[1] Stöckel-Maschek *et al* 2000b

Although, the investigated compounds do not correspond to the substrate specificity of LAP and APM, they were investigated as inhibitors of both enzymes, because it is known, that compounds containing a penultimate proline residue are able to inhibit both aminopeptidases (Cushmann *et al* 1977; Xu *et al* 1995; Prechel *et al* 1995). Contrary to our expectations, LAP was inhibited most efficiently from all investigated enzymes by Val-ψ[PO(OCH_3)-N]-Pyrr (**2**). The enzyme was inhibited by the hyperbolic mixed-type inhibition mechanism. This is a partial inhibition mechanism meaning, that the EIS-complex is catalytically active. The inhibition constants are in the low micromolar range (table 1). Otherwise, the well-known DP IV inhibitors Phe-Pyrr (1 mM) and Phe-Thia (1 mM) are not able to inhibit LAP.

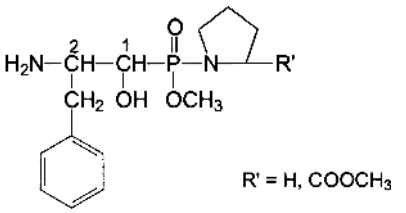
APM is inhibited according to the classical competitive inhibition mechanism by diastereomer 1 of Val-ψ[PO(OCH_3)-N]-Pyrr. On the other hand, the enzyme is inhibited in a time-dependent manner by diastereomer 2. The inhibition constants are in the high micromolar range. APM does not prefer one of the isomers. The amino acid pyrrolidides Ala-Pyrr (1 mM) and Ser-Pyrr (1 mM) are not able to inhibit the enzyme.

Generally, it seems, that inhibition of the metal-dependent aminopeptidases APP, LAP and APM by the phosphonamidate **2** is caused by chelation.

Recent studies showed, that 3-amino-2-hydroxy acid pyrrolidides **3** (figure 1) and thiazolidides as well as 3-amino-2-hydroxy acylproline derivatives are potent inhibitors of the aminopeptidases APP, APM, LAP and DP IV with inhibition constants in the micromolar up to the nanomolar range (Stöckel *et al* 1997; Stöckel-Maschek *et al* in preparation). These investigations were continued by the synthesis of analogous 2-amino-1-hydroxy-phosphonamidates **4** and the enzymatic characterization of the compounds as inhibitors of DP IV, APP, APM and LAP.

DP IV is inhibited by all diastereomers of AHPB-ψ[PO(OCH_3)-N]-Pyrr with inhibition constants in the micromolar range (table 2) according to the competitive inhibition mechanism. The inhibition constants differ around 20 times between the diastereomers. The inhibition constants of AHPB-ψ[PO(OCH_3)-N]-Pyrr are better or in the same range like the constant of (2*S*,3*R*)-AHPB-Pyrr ($K_i = 220$ μM, Stöckel-Maschek *et al* in preparation). The best inhibitors are the diastereomers 4 of (2*R*)- and (2*S*)-AHPB-ψ[PO(OCH_3)-N]-Pyrr with K_i-values of 8.8 μM and 13.7 μM, respectively. The enzyme does not show a preference for the (2*R*)- or (2*S*)-configuration, whereas in case of classical α-amino acids DP IV prefers the (*S*)-isomer. Kim *et al* (2005) have shown by X-ray crystal structure determination, that the β-amino acid-based DP IV inhibitor (2*R*)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-α]pyrazin-7,8*H*)-yl]-1-(2,4,5-trifluorophenyl) butan-2-amine (MK-0431) is bound exhibiting the amide

Table 2. Inhibition of DPIV and APP by 2-amino-1-hydroxy-phosphonamidates of the general structure AHPB–ψ[PO(OCH_3)-N]-R.



R	Configuration of C2-atom	Diastereomer	DP IV K_i (μM) competitive	APP K_i (μM) linear mixed-type	α
Pyrr	(S)	1	336	850	2.7
		2	23.9	1180	2.
		3	62.5	2240	3.2
		4	13.7	61.2	3.1
Pyrr	(R)	1	147	174	0.71
		2	76.7	1860	1.1
		3	22.5	181	0.97
		4	8.8	117	0.58
Pro-OCH_3	(R)	1	52.6	n.d.	
		2	11.5	n.d.	
		3	23.4	n.d.	
		4	57 %, ([I] = 1 mM, [S] = 0.1 mM)	n.d.	

moiety in the opposite orientation in comparison with substrates and inhibitors containing an α-amino acid. In case of the β-amino acid derived inhibitor MK-0431, the (*S*)-isomer is less potent.

The phosphonamidates **4** are 10 to 100 times less potent inhibitors of APP than 3-amino-2-hydroxy acid amides and dipeptides **3** (table 2). For example, (2*S*,3*R*)-AHPB-Pyrr inhibits APP with an inhibition constant of 19.8 μM and is a 3 times better inhibitor of APP than the best phosphonamidate diastereomer 4 of (2*S*)-AHPB-ψ[PO(OCH_3)-N]-Pyrr with an inhibition constant of 61.2 μM. Phosphonamidates **4** as well as 3-amino-2-hydroxy acid amides and dipeptides **3** are linear mixed-type inhibitors of APP.

APM and LAP are inhibited only in the millimolar range by single diastereomers (table 3). Most compounds are not able to inhibit the aminopeptidases efficiently. In contrast, (2*S*,3*R*)-AHPB-Pyrr inhibit LAP according to a competitive slow-binding mechanism with an overall inhibition constant K_i^* of 2.46 μM. APM was only slightly inhibited by (2*S*,3*R*)-AHPB-Pyrr.

4. CONCLUSIONS

The investigated enzymes DP IV, APP, LAP and APM are inhibited by both diastereomers of Val-ψ[PO(OCH_3)-N]-Pyrr. Unexpectedly, LAP is inhibited most efficiently with inhibition constants in the nanomolar and micromolar range. The other enzymes are inhibited with inhibition constants in the micromolar range.

Table 3. Inhibition of APM and LAP by 1-hydroxy-2-amino-phosphonamidates of the general structure AHPB-ψ[PO(OCH_3)-N]-R.

R	Configuration of C2-atom	Diastereomere	APM K_i (μM)	LAP K_i (μM)
Pyrr	(*S*)	1	n.I.	743 (competitive)
		2	560 (competitive)	n.I.
		3	n.d.	n.d.
		4	13% inhibition ([I] = 1 mM, [S] = 0.2 mM)	54% inhibition ([I] = 1mM, [S] = 1 mM)
Pyrr	(*R*)	1	n.I.	514 a = 4.5 (linear mixed-type)
		2	n.I.	1650 (competitive)
		3	4% inhibition ([I] = 1 mM, [S] = 0.2 mM)	30% inhibition ([I] = 1 mM, [S] = 1 mM)
		4	n.I.	32% inhibition ([I] = 1 mM, [S] = 1 mM)
Pro-OCH_3	(*R*)	1	54% inhibition ([I] = 1 mM, [S] = 0.12 mM)	18% inhibition ([I] = 1 mM, [S] = 1 mM)
		2	54% inhibition ([I] = 1 mM, [S] = 0.12 mM)	17% inhibition ([I] = 1 mM, [S] = 1 mM)
		3	54% inhibition ([I] = 1 mM, [S] = 0.12 mM)	26% inhibition ([I] = 1 mM, [S] = 1 mM)
		4	n.I.	51% inhibition ([I] = 1 mM, [S] = 1 mM)

DP IV is inhibited by most of the 2-amino-1-hydroxy-phosphonamidates with inhibition constants in the micromolar range. In contrast, these compounds are only very poor inhibitors of the aminopeptidases LAP and APM or they are not able to inhibit both enzymes. Originally, these compounds were designed as potential inhibitors of APP. Nevertheless, these compounds are less potent than the corresponding 3-amino-2-hydroxy acyl-proline derivatives and 3-amino-2-hydroxy acid amides. These results show, that it was not possible to get effective inhibitors of the metal-dependent aminopeptidases APP, LAP and APM using 2-amino-1-hydroxy-phosphonamidates as amide bond isosteres.

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CHAPTER 3

BIOCHEMICAL PROPERTIES OF RECOMBINANT PROLYL DIPEPTIDASES DPP-IV AND DPP8

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1. INTRODUCTION

Dipeptidyl peptidase IV (DPP-IV, also known as CD26; EC3.4.14.5) has emerged as an important and effective drug target for the treatment of type II diabetes. It is a serine protease involved in the in vivo inactivation of two insulin-sensing hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), by cleaving the first two amino acids from their amino termini (Mentlein, 1999; Mentlein *et al* 1993). DPP-IV knockout mice show improved metabolic controls, including higher levels of GLP-1 and insulin, improved insulin sensitivity, reduced pancreatic islet hypertrophy, and resistance to obesity and hyperinsulinemia (Conarello *et al* 2003; Marguet *et al* 2000). Inhibition of DPP-IV activity by chemical compounds in various animal models increases the concentration of active GLP-1 and GIP in circulation, stimulates insulin secretion, and improves glucose homeostasis (Ahren *et al* 2002; Deacon *et al* 2002; Mitani *et al* 2002; Pospisilik *et al* 2002; Reimer *et al* 2002). Recent success in human clinical trials using DPP-IV inhibitors to treat type II diabetes validates the use of DPP-IV as a drug target for this disease, and has kindled tremendous interest in this protease from both academia and pharmaceutical companies.

Its ability to cleave many hormones and chemokines in vitro and in vivo (Lambeir *et al* 2001; Mentlein, 1999; Proost *et al* 2000; Proost *et al* 1998; Zhu *et al* 2003) implicates DPP-IV in various cellular functions including immunological, neuronal, and metabolic regulation, and cancer. Apart from DPP-IV, several DPP-IV-homologous proteases such as DPP8, DPP9, FAP, and DPP-II, have been identified as having prolyl-cleaving dipeptidase activity in vitro (Rosenblum and Kozarich, 2003). This group of enzymes is unique in that they cleave the peptide bond after proline or alanine at the penultimate position from the amino terminus in vitro. Because many peptide hormones or chemokines contain proline to resist degradation and cleavage by cellular proteases, the prolyl-cleaving enzymes are important in regulating the activities of these hormones and chemokines in vivo. Even though these DPP-IV-homologous proteases have been implicated in various biological functions, including cancer invasion, metastasis, cell death, and apoptosis (Rosenblum and Kozarich, 2003), their functions in vivo are not known.

To study the biochemistry of DPP-IV and its homologous proteases and screen them as potential drug targets, it is essential to have sufficient amounts of pure and active proteins. Excitingly, in the past couple of years, DPP-IV and DPP8 have been successfully expressed and purified in considerable quantities from baculovirus-infected insect cells or the yeast *Pichia pastoris*, and their biochemical properties have been further studied. Moreover, DPP-IV has been crystallized in the presence of different inhibitors, extending our understanding of how this group of dipeptidases functions as enzymes. This also facilitates the design of novel, potent, and selective inhibitors for DPP-IV. Here, I summarize the progress made on understanding the biochemical properties of human DPP-IV and DPP8, expressed and purified from either baculovirus-infected insect cells or the yeast *Pichia pastoris*.

2. RECOMBINANT HUMAN DPP-IV

Human DPP-IV is an extracellular protease containing a short cytoplasmic tail (residues 1–6), a transmembrane domain (residues 7–29), and an ectodomain (residues 30–766). The expression of full-length DPP-IV in baculovirus-infected Sf9 insect cells resulted in uniform truncation of the 29 N-terminal amino acids (Dobers *et al* 2002; Weihofen *et al* 2004). Subsequently, the expression of DPP-IV was achieved by designing a vector with a secretion tag, fusing the extracellular domain of DPP-IV (residues 33 or 39 to 766) to the secretion tag, and expressing the protein in baculovirus-infected Hi5 insect cells (Aertgeerts *et al* 2004a; Aertgeerts *et al* 2004b; Chien *et al* 2004; Hiramatsu *et al* 2003; Rasmussen *et al* 2003). The secretion tags used were either the human CD5 or the baculovirus gp67 signal peptide. These secretion tags were cleaved when the proteins were secreted, resulting in DPP-IV protein containing the ectodomain only. Similarly, for its expression in the yeast *Pichia pastoris*, the ectodomain of DPP-IV (residues from 29, 31, or 37 to 766) was fused to the yeast secretion tag, the α-mating factor, and expressed in quantities sufficient for the crystallization study (Bär *et al* 2003; Oefner *et al* 2003; Thoma *et al* 2003).

One notable difference between the recombinant DPP-IVs and those purified from endogenous sources, such as human tissues, organs, or cell lines, is the extent and pattern of glycosylation. The calculated molecular weight for non-glycosylated DPP-IV (from residue 30 to 766) is about 85 kD, whereas the recombinant DPP-IVs are about 10 kD larger, with the additional mass contributed by glycosylation (Chien *et al* 2004; Rasmussen *et al* 2003; Thoma *et al* 2003). The extent of glycosylation in the recombinant protein is lower than in the DPP-IVs purified from endogenous sources, the molecular weights of which are usually around 110–120 kD (Chien *et al* 2004; De Meester *et al* 1992; de Meester *et al* 1996; Iwaki-Egawa *et al* 1998; Puschel *et al* 1982; Rasmussen *et al* 2003; Saison *et al* 1983; Thoma *et al* 2003). There are nine putative N-glycosylated Asn residues present in DPP-IV (Aertgeerts *et al* 2004a). Deglycosylation of the purified protease or mutations at these putative glycosylated Asn residues does not change the quaternary structure of the protein or its catalytic efficiency (Aertgeerts *et al* 2004a; Thoma *et al* 2003). Even though the recombinant DPP-IVs are glycosylated differentially at distinct Asn residues, the crystal structures are almost identical (Aertgeerts *et al* 2004a; Hiramatsu *et al* 2003; Rasmussen *et al* 2003; Thoma *et al* 2003). Interestingly, the crystal structure of DPP-IV purified from porcine kidney is tetrameric, differing from the dimeric structure of human recombinant DPP-IV (Aertgeerts *et al* 2004b; Chien *et al* 2004; Engel *et al* 2003; Hiramatsu *et al* 2003; Oefner *et al* 2003; Rasmussen *et al* 2003; Thoma *et al*

2003). It is not clear whether this difference is due to the species, the amino acid sequence, the extent and sites of glycosylation, or the conditions of crystallization. Nevertheless, the conformation and structure of the dimer are similar in the recombinant DPP-IVs and porcine DPP-IV.

Recombinant human DPP-IVs have biochemical properties similar to those of the endogenous proteins, in terms of quaternary structure, catalytic efficiency, and their interaction with adenosine deaminase (ADA) (Caporale *et al* 1985; Chien *et al* 2004; Daddona and Kelley, 1978; de Meester *et al* 1996; Iwaki-Egawa *et al* 1998; Kato *et al* 1978; Puschel *et al* 1982; Saison *et al* 1983). DPP-IV is homodimeric. One monomer of DPP-IV consists of two domains: a propeller domain and an α/β hydrolase domain. The catalytic triad Ser_{640}–Asp_{708}–His_{740} of DPP-IV is located in the hydrolase domain. This, together with Tyr_{547} and Glu_{205}–Glu_{206} from the propeller domain, defines the active site of DPP-IV with prolyl dipeptidyl peptidase activity (Abbott *et al* 1999; Bjelke *et al* 2004). The funnel-shaped propeller domain of DPP-IV consists of eight loose blades. Interestingly, the human DPP-IV protein exists only as the dimer after purification, completely devoid of monomer (Chien *et al* 2004). There are two dimer interfaces in DPP-IV: one is in the C-terminal region of DPP-IV involving residues 658–661, 713–736, and 746–757, and the other comprises the extended arm of blade 4 of the propeller domain involving residues 234–260 (Rasmussen *et al* 2003). The C-terminal region is highly conserved among these prolyl dipeptidases, including FAP, DPP8, DPP9, and DPP-II (Chien *et al* 2004). Therefore, it is possible that these proteases use the same regions for dimerization.

The kinetic properties of recombinant DPP-IVs, as determined by the measurement of the kinetic constants $k_{cat}$and K_m, are similar to those of endogenous DPP-IVs (Chien *et al* 2004; De Meester *et al* 1992,1996; Rasmussen *et al* 2003; Thoma *et al* 2003). The interaction between DPP-IV and ADA is critical for adenosine signaling and normal cellular function (Morimoto and Schlossman, 1998). DPP-IV is the anchoring molecule for ADA that regulates adenosine concentration and signaling on the cell surface (Morimoto and Schlossman, 1998). The affinities (K_d) between recombinant human DPP-IVs and human/bovine ADA, as measured by the surface plasma resonance technique, are around 0.4–3.1 nM (Aertgeerts *et al* 2004a; Bär *et al* 2003; Thoma *et al* 2003), which are close to the K_d values of 6–17 nM between rabbit DPP-IV and human/bovine ADA (Richard *et al* 2002; Schrader and West, 1990).

Further biochemical studies of DPP-IV have been facilitated by site-directed mutagenesis and the determination of crystal structures. Recently, we demonstrated that the C-terminal region of DPP-IV is crucial for DPP-IV dimer formation (Chien *et al* 2004). Single-site mutation at His_{750} in the C-terminal region converts dimeric DPP-IV into the monomeric form when the mutation is H750E, or into a mixture of dimeric and monomeric forms when the mutation is H750A (Chien *et al* 2004). There is no equilibrium between the monomeric and dimeric forms of the mutant proteins (Chien *et al* 2004). Interestingly, monomeric DPP-IV is not devoid of activity, as was previously inferred. Instead, it has 50–300-fold less activity, depending on the mutation (Chien *et al* 2004). These results indicate that the quaternary structure of DPP-IV is correlated with its enzymatic activity. Dimerization is thus essential to lock the active site into the configuration optimal for catalysis, and the C-terminal region of DPP-IV is important for its dimerization. Another kinetic study coupled with crystallization demonstrated that Tyr_{547} is critical for the enzymatic activity of DPP-IV, because it is involved in the formation of the tetrahedral oxyanion (Bjelke *et al* 2004). The enzymatic activity of the mutant protein Y547F is about 1/1500 that of the wild-type enzyme, even though the mutant protein remains dimeric with no gross conformational change at either the active site or in the whole molecule (Bjelke

et al 2004). This deficient catalytic efficiency is due to the loss of a water molecule positioned in the oxyanion hole and necessary for catalysis to occur (Bjelke *et al* 2004).

3. RECOMBINANT HUMAN DPP8 PROTEASE

Human DPP8 shares 51% homology or 27% identity with human DPP-IV, with prolyl-cleaving dipeptidase activity in vitro (Abbott *et al* 2000). DPP8 differs from extracellular DPP-IV in that it is a cytoplasmic protease (Abbott *et al* 2000). The biological substrates of DPP8, its structure, and its function in vivo are not known. Human DPP8 was expressed in baculovirus-infected Sf9 insect cells (Chen *et al* 2004). The substrate specificity of DPP8 for dipeptides was investigated and compared with that of DPP-IV (Chen *et al* 2004). Both DPP8 and DPP-IV prefer proline over alanine at the P1 site. Interestingly, the substrate preference for the P2 site is similar in both proteases, which prefer aromatic residues over charged ones, whereas acidic residues are the least preferred. Therefore, the sequence of preference at the P2 site for both proteases is aromatic > basic > acidic residues. Using chemical inhibitors as probes, we investigated whether the active site of DPP8 differs slightly from that of DPP-IV (Jiaang *et al* 2005). We found that five-ring structures, such as pyrrolidine and thiazolidide, are potent inhibitors of both DPP-IV and DPP8. The five-ring structure binds to the S1 site of the proteases, as shown by the co-crystallization of DPP-IV with five-ring inhibitors (Aertgeerts *et al* 2004b; Engel *et al* 2003; Hiramatsu *et al* 2004; Oefner *et al* 2003; Rasmussen *et al* 2003; Thoma *et al* 2003). Substitution of the five-ring structures with bulkier ones, such as piperidine, isoquinoline, or isoindoline, resulted in potent and selective DPP8 inhibitors with no inhibitory activity towards DPP-IV or DPP-II (Jiaang *et al* 2005). Therefore, the S1 site of DPP8 might be bigger than that of DPP-IV, and thus able to accommodate bulkier chemical structures (Jiaang *et al* 2005). Whether this is indeed the case awaits confirmation by determination of the crystal structure.

4. CONCLUSIONS

An expression system using baculovirus-infected insect cells or the yeast *Pichia pastoris* has been successfully used to produce large amounts of DPP-IV and DPP8 proteases for in vitro biochemical studies and the discovery of the chemical inhibitors. Recombinant DPP-IV protease has biochemical properties similar to those of endogenous DPP-IV proteases. Based on sequence homology, it is most likely that this group of prolyl-cleaving serine proteases shares a similar two-domain structure and a similar catalytic mechanism, that differ from those of other serine proteases, such as chymotrypsin or trypsin. Recently, a potent and selective DPP8 inhibitor with an IC_{50} value of 14 nM was reported (Jiaang *et al* 2005). The discovery of potent and selective chemical inhibitors targeting each individual protease will provide effective tools with which to study the biological function of these DPP-IV-homologous proteases in vivo.

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CHAPTER 4

PREDICTION OF DIPEPTIDYL PEPTIDASE (DP) 8 STRUCTURE BY HOMOLOGY MODELLING

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1. INTRODUCTION

Protein modelling provides a fast way of predicting the three-dimensional structure of a protein when only sequence data is available. The number of protein structures experimentally resolved lags far behind sequence data due to the substantial time required to prepare protein for crystallisation (Baker *et al* 2003). Typically this process includes protein expression, purification and finally crystallisation, where each step may take years to establish. Recently, DP8 was successfully purified, however, as yet its three-dimensional structure is not known (Chen *et al* 2004). Therefore our aim was to predict the structure of dipeptidyl peptidase 8 using homology modelling. A predicted model would be beneficial for analyzing catalytic mechanisms, and substrate and inhibitor specificity.

DP8 belongs to the S9b family of serine proteases which includes three proteins of known structure: DPIV; DP-like 1 (DPL1, DPX, DP6); and very recently, fibroblast activation protein (FAP). As the FAP structure (Aertgeerts *et al* 2005) was not available at the time this work was undertaken, the template structures used were DPIV and DPL1. DPIV and DPL1 are both homodimeric structures where each subunit contains two domains: an 8-bladed β-propeller domain and an α/β hydrolase domain (Rasmussen *et al* 2002, Strop *et al* 2004). The α/β hydrolase domain contains the conserved catalytic triad: Ser; Asp; His. In the β-propeller domain each blade is made up of four β-sheets. The conserved DW(V/L)YEEE motif of the S9b family lies in the β-propeller domain and is responsible for correct orientation of the substrate into the active site (Rasmussen *et al* 2002, Abbott *et al* 1999).

It has been statistically proven that if the level of sequence identity is above 30%, then up to 90% of the polypeptide conformation tends to be modelled well (Baker *et al*, 2003). Below 30% identity models are unreliable due to increasing alignment errors (Baker *et al*, 2003). Overall DP8 shares 27% sequence identity with DPIV (Table 1), however the sequence identity increases to 35% in the α/β hydrolase domain which is comfortably within the range for effective modelling. The second template DPL1 is a non-catalytic molecule which shares 24% overall sequence identity however, again this increases to 29% in the α/β hydrolase domain.

Table 1. Comparison of DP8 Sequence with Template Sequences Adapted from (Abbott and Correll, 2002).

DP8 Homolog	% Overall Identity	% Identity $-\alpha/\beta$ hydrolase domain	% Overall Similarity
DPIV	27	35	51
DPL1	24	29	52

As yet the biological function of DP8 is not known, however DP8 has dipeptidyl peptidase activity similar to DPIV, DP9 and FAP and binds some DPIV inhibitors (Abbott *et al* 2000, Park *et al 2005*). Recently, DP8 was shown to cleave the known prolyl endopeptidase (PEP) substrate Suc-Ala-Pro-pNA and acylaminoacyl peptidase (ACPH) substrate Ac-Ala-pNA (Park *et al* 2005). PEP cleaves at the carboxy groups of proline and alanine residues and has been implicated in Alzheimer's disease and amnesia while ACPH catalyses the removal of an N-acetylated amino acid from blocked peptides (Fulop *et al* 1998, Bartlam *et al* 2004). Therefore DP8 appears to behave like a dipeptidyl peptidase, prolyl endopeptidase and an acylaminoacyl peptidase. Comparison of the DP8 model with the DPIV, PEP and ACPH structures may uncover differences that provide DP8 with its multiple enzyme functionality.

2. METHODS

The DPIV and DPL1 protein structure files (PDB codes DPIV: =1N1M and DPL1:1XFD) were downloaded from the Protein Data Bank (www.rcsb.org/pdb/) (Rasmussen *et al* 2002, Strop *et al* 2004). Although five human DPIV structures were available, the structure with the highest resolution was selected (2.5 Å). All protein sequences of the DPIV-like family were obtained from National Centre for Biotechnology Information (NCBI) i.e. DPIV (Accession Number = M80536), DP8 (AF221634), DP9 (AAQ83119), DPL1 (M96859), DPL2 (A7307785) and FAP (Q12884). A multiple sequence alignment of the sequences was performed using the CLUSTALX program (Thompson *et al* 1997).

Sequences of DP8, DPIV and DPL1 were extracted from the multiple sequence alignment and used as input for the comparative protein modelling program MODELLER (Sali and Blundell, 1993). As the DP8 sequence was 80 amino acids longer at the N-terminus than the template sequences it was truncated according to the multiple sequence alignment with DPIV. The program uses the sequence alignment to thread the target sequence onto the backbone coordinates of the template structures (DPIV, DPL1) followed by restraint-based optimisation and simulated annealing for energy minimisation. The resulting model was output as a pdb file.

The CCP4 software suite was used for model assessment and structural alignments (Collaborative Computational Project, 1994). The PROCHECK module of CCP4 was used to detect unfavourable side-chain conformations and interactions. During analysis the DP8 model and the DP4 and DPL1 monomeric structures were superimposed using SUPERPOSE MOLECULES module of CCP4 to allow comparison in regions of variability. The Cα carbons of four structurally conserved regions were used for the alignment of the structures: (DP8:DP4:DPL1) Gly251-Glu260: Gly197-Glu205: Gly287-Glu296; Tyr315-Pro323: Tyr256-Pro264:

Tyr344-Pro352; Phe727-Leu749: Phe618-Leu640:Phe700-Leu722; and Leu810-Phe822: Leu701-Phe713: Leu785-Phe797.

Superpositioning of the active site of DP8 with the S9 members PEP (1H2W) and ACPH (1VE6) was performed using the Cα carbons of the three catalytic triad residues (DP8:DP4:DPL1:PEP:ACPH) Ser739: Ser630: Asp712: Ser554: Ser445; Asp817: Asp708: Asp792: Asp641: Asp524; His849: His740: His834: His680: His556 (Fulop *et al*, 1998; Bartlam *et al*, 2004). This process produces a root mean square deviation (RMSD) value, which is a measure of variance in the position of the Cα atoms of the corresponding residues in the selected regions and therefore an indication of the difference between the two models in the selected regions; the lower the RMSD the more similar the structures are in the selected regions. The models were assessed by visualisation in the molecular graphics program O (Jones *et al* 1999). Figures were produced using the PYMOL graphics program (DeLano 2002).

3. RESULTS

3.1. Comparison of DP8 Model with Template Structures

The DP8 model produced was compared separately to each of the template structures to validate and estimate the accuracy of the model. The model and template structures were compared by structural alignment using four structurally conserved regions. DP8 and DPIV aligned with a RMSD of 0.422 Å in these regions, while DPL1 aligned with a RMSD of 0.458 Å. Visualisation of the alignments confirmed that the DP8 model was structurally more similar to DPIV as there was less variation between the two structures (Figure 1).

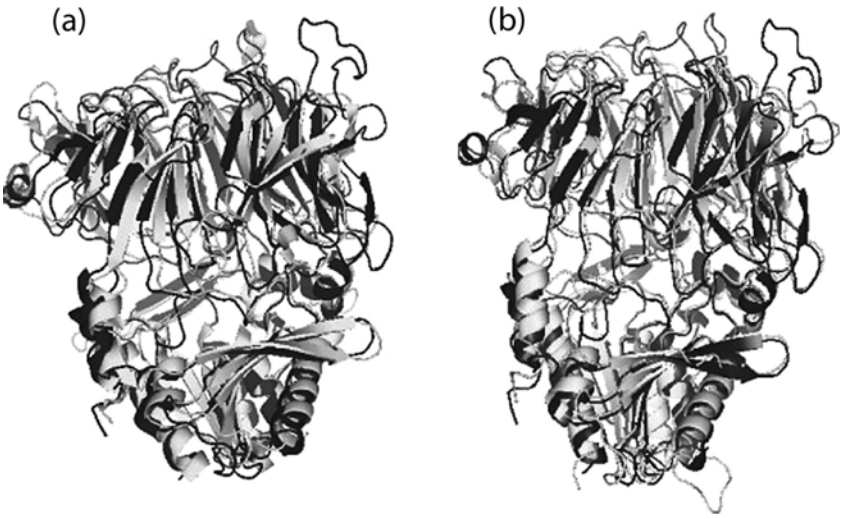

Figure 1. The DP8 model aligned with a) DPIV and b) DPL1 structures. The DP8 model was aligned with the template structures using four structurally conserved regions (DP8:DP4:DPL1) Gly251-Glu260: Gly197-Glu205: Gly287-Glu296; Tyr315-Pro323: Tyr256-Pro264: Tyr344-Pro352; Phe727-Leu749: Phe618-Leu640: Phe700-Leu722; and Leu810-Phe822: Leu701-Phe713: Leu785-Phe797. The DP8 structure is represented in black and the template structures are represented in grey.

The β-propeller domain of DP8 was poorly modelled and contained several longer loops and poorly aligned β-sheets compared to the template structures. Longer loops were observed between all the β-sheets of blades 4 and 6 and half of the sheets of blades 7 and 8 in the DP8 model. The α/β hydrolase domain modelled well and was tightly aligned to DPIV, however the alignment of the DP8 and DPL1 α/β hydrolase domains revealed several differing loops (Figure 1b).

3.2. Comparison of DP8 Active Site with DPIV and DPL1 Structures

To compare the active sites of the DP8 model and the other known S9b structures, DPIV and DPL1, we aligned the structures using the three catalytic triad residues. The DP8 active site Cα atoms aligned with the DPIV residues with a RMSD of 0.163 Å (Figure 2a), indicating that the catalytic triad residues in DP8 were placed in a similar position to the DPIV active site. However, there was a notable difference in the orientation of the side chain of the catalytic serine residue, where the side chain in DP8 was placed orthogonal to the corresponding DPIV side-chain. Of the three catalytic triad residues the most poorly aligned in the process was the Asp residue with a distance of 0.228 Å. The side chains of the Asp and His residues were aligned with very similar orientations in the two structures.

In the DPIV catalytic process, an essential Tyr547 residue adjacent to the catalytic Ser is responsible for stabilising the oxyanion intermediate (Bjelke *et al* 2004). From the alignment of the structures using only the three catalytic residues, the relative orientation of the Tyr653 in the DP8 model is very similar to the DPIV structure and the Cα atoms are only 0.52 Å apart, however the side-chain is orientated slightly closer to the serine. The conserved Glu residues responsible for aligning the substrates into the active site lie in an alpha helix inside the β-propeller tunnel (Rasmussen *et al* 2002). The Cα atoms of the corresponding Glu residues were aligned within 1 Å distance to the DPIV structure however the side chain of Glu260 in DP8 had an altered conformation to the corresponding Glu206 in DPIV.

DPL1 is enzymatically inactive due to substitutions of the catalytic Ser and possibly, the structurally adjacent Tyr, to Asp residues. Comparison of the DP8 catalytic triad and the corresponding DPL1 residues produced a RMSD value of 0.760 Å (Figure 2b). Compared to the low value obtained for the DPIV alignment, this value is relatively high. As only the Cα atoms were used to align the structures the Ser->Asp substitution in DPL1 would not have affected the RMSD value. The most closely aligned residue, with a distance of only 0.215 Å, was the DP8 Ser739 aligned with the DPL1 Asp712 residue, while the most poorly aligned residue was the His with a distance of 1.017 Å. The side-chain of the His residue had a similar orientation but was poorly aligned with the DP8 residue due to the distance between the Cα atoms. The DPL1 structure also has an Asp substitution at Tyr653, however the placement of the Cα atom was only 0.57 Å from the DP8 structure. Due to this substitution the DP8 side chain was larger and hence the carboxyl group of the Asp side-chain and the hydroxyl group of the Tyr side-chain were a considerable distance (4.68 Å) apart. The DP8 conserved Glu259 and Glu260 residues were also poorly aligned with both the Cα atoms approximately 1.7 Å distance from the corresponding DPL1 residues in the alignment.

3.3. Comparison of DP8 Active Site with PEP and ACPH Structures

In order to compare the DP8 model active site to other proteins with the same fold the DP8 catalytic triad was aligned with the S9a member PEP and the S9c member ACPH (Fulop *et al* 1998, Bartlam *et al* 2004).

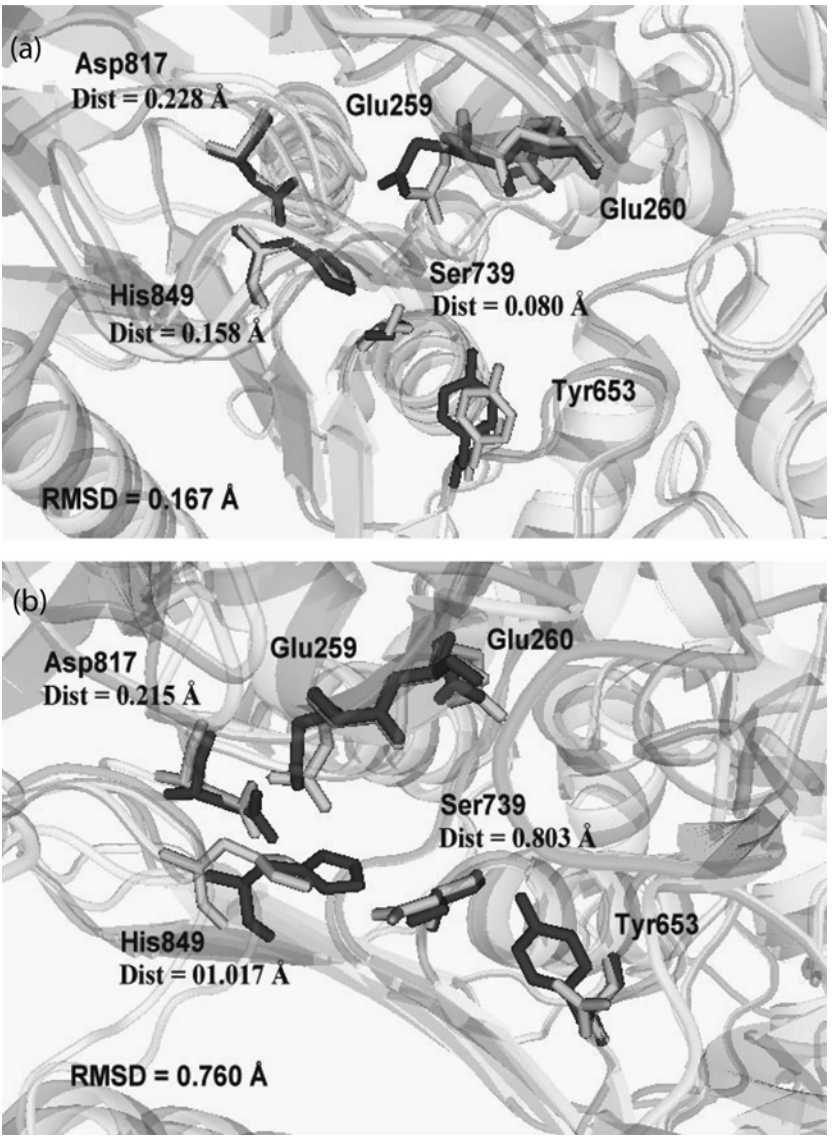

Figure 2. DP8 active site aligned with a) DPIV and b) DPL1. The structures were aligned using the catalytic triad (DP8:DP4:DPL1) Ser739:Ser630:Asp712; Asp817:Asp708:Asp792; His849:His740:His824. Dist = measured distance between the corresponding Cα atoms for the alignment. RMSD = calculated average variation of the average distances between the Cα atoms used in the alignment. The DP8 residues are represented in black; template structures are represented in grey. The residues are numbered according to the DP8 structure.

The DP8 active site Cα atoms aligned with the PEP residues with a RMSD of 0.180 Å (Figure 3a), indicating that the catalytic triad residues in DP8 were also closely aligned to the PEP active site. The side-chain of the catalytic serine in DP8 was also orthogonal to the PEP side-chain, as was observed with the DPIV alignment. Of the three catalytic triad residues the most poorly aligned in the process was the His residue with a distance of 0.244 Å. The side chains of the Asp and His residues aligned very closely in the two structures.

The PEP structure also contains a Tyr oxyanion stabilising residue adjacent to the catalytic serine (Fulop *et al* 1998). The DP8 Tyr653 aligned poorly with the PEP residue (0.91 Å) however the side chains had the same orientation. The orientation of the DP8 Tyr653 was therefore more similar to the PEP structure than the DPIV structure. The PEP structure does not contain the conserved Glu259, Glu260 residues in the β-propeller domain and no corresponding residues were observed that aligned well with these residues.

The alignment of the DP8 and ACPH active sites produced a RMSD of 0.403 Å (Figure 3b). This is comparatively higher than the values from the PEP and DPIV alignment however not as high as the DPL1 alignment. The smallest Cα distance of the aligned residues was Ser with a distance of 0.207 Å. Again the side-chain of the catalytic Ser in DP8 was orthogonal to the ACPH side-chain, as was observed with the DPIV and PEP alignment. Of the three catalytic triad residues the most poorly aligned in the process was the His residue with a distance of 0.558 Å. Despite the larger distances between the aligned Cα atoms the side-chains of both Asp and His residues had very similar orientations.

In the ACPH structure the oxyanion intermediate is stabilised by a Gly residue instead of a Tyr (Bartlam *et al* 2004). Therefore, when these residues were compared, the placement of the Cα atoms was very different, although the main-chain NH group of the Gly in ACPH, and the OH side-chain group of the Tyr in DP8 were still within 0.91 Å of each other. The conserved Glu259, Glu260 residues were also substituted in the ACPH structure and are instead a pair of non-adjacent Phe153, Phe155 residues. Despite large differences between the corresponding residues, the relative positions in the propeller opening are similar. The Phe153 Cα atom was closest to the Glu260 residue of DP8 with a distance of 4.74 Å while the smallest distance between the side-chains groups was 2.75 Å. The distance between the DP8 Glu259 and the ACPH Phe155 was much larger at 8.85 Å and the side-chains were also in very different positions.

4. DISCUSSION

A DP8 model was successfully made using the DPIV and DPL1 structures as templates. The β-propeller domain was modelled poorly, firstly due to the low sequence identity between the DP8 and template sequences in this region and secondly, because the DP8 sequence is approximately 100 amino acids longer than both the template structures in the β-propeller domain. Therefore, reliable analysis could not be performed on the β-propeller region of the model and our efforts mainly focused on the more reliably modelled α/β hydrolase domain. Due to the longer loops and the large N-terminal region that had to be truncated from the DP8 structure ($\sim$80 amino acids) it is possible that the actual N-terminal region of DP8 contains more secondary structural elements or longer β-sheets in the β-propeller region.

In all the S9 structures resolved, there are N-terminal elements before the β-propeller domain that interact with the α/β hydrolase domain. In the PEP, structure this region is large,

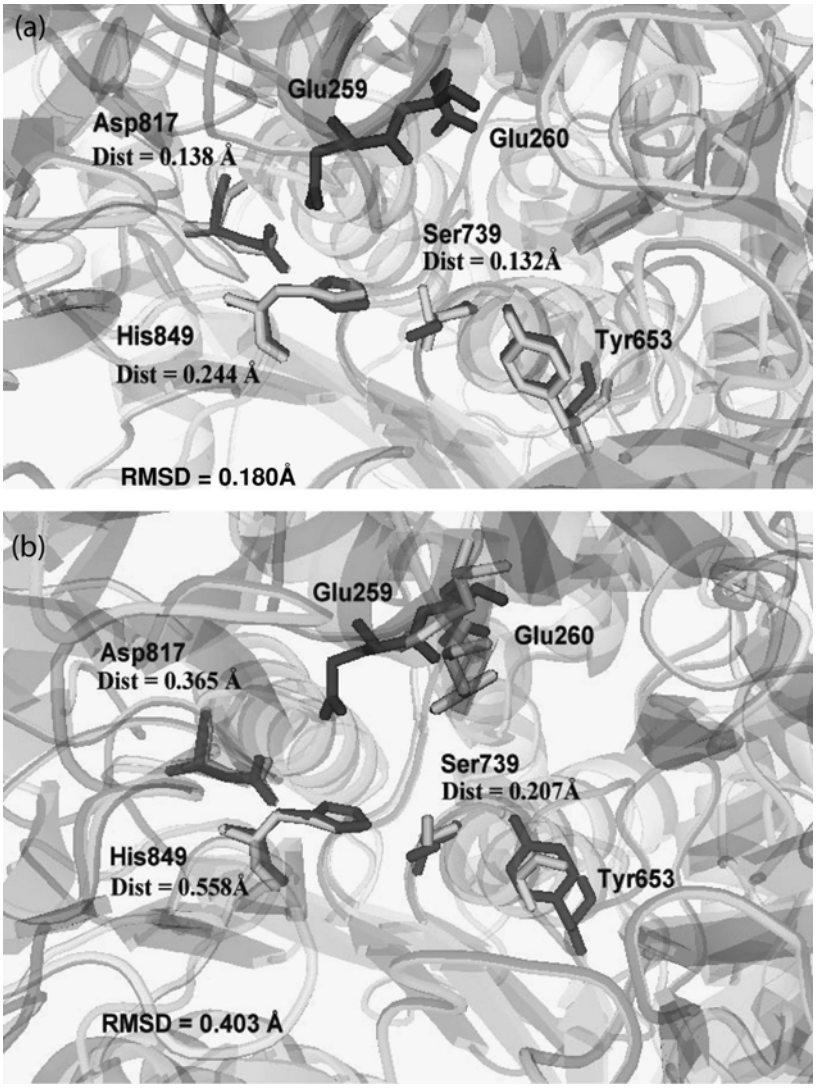

Figure 3. DP8 active site aligned with a) PEP and b) ACPH The structures were aligned using the catalytic triad (DP8:PEP:ACPH) Ser739:Ser554:Ser445; Asp817:Asp641:Asp524; and His849:His680:His556 His. Dist = measured distance between the corresponding Cα atoms for the alignment. RMSD = calculated average variation of the average distances between the Cα atoms used in the alignment. The DP8 residues are represented in black; PEP and ACPH structures are represented in grey. The residues are numbered according to the DP8 structure.

containing four secondary structural elements (Fulop *et al* 1998). However, in the DPIV and DPL1 structures, used as templates for the DP8 model, there is only one β-sheet before the β-propeller. Therefore, it is possible that, like PEP, this region contains more secondary structural elements that could not be predicted by homology modelling.

The α/β hydrolase domain modelled well and this was most likely due to the high level of sequence identity with the template structures in this region. The DPL1 structure did have some differing secondary structure when compared to the DP8 model, which indicated that the DP8 model was more closely based on the DPIV structure. The active site of the DP8 model was also shown to be more similar to the DPIV structure than the DPL1 structure. This is favourable because, like DP8, DPIV is an enzymatically active protein. The similarity of the DP8 model to DPIV was expected as DP8 has a closer evolutionary relationship with DPIV. Therefore, the DPL1 template was solely used to aid in the assignment of structurally conserved regions during production of the DP8 model.

The only obvious structural discrepancy in the DP8 active site was the side-chain conformation of the catalytic serine. The side-chain OH group was modelled orthogonal to the DPIV structure and this was also observed in the comparison of the PEP and ACPH structures. The modelling program placed the side-chain in the lowest energy conformation. In this conformation the hydroxyl group in DP8 is shifted 2.17 Å relative to the DPIV structure. However, if an alternative rotamer of this residue is selected the hydroxyl group is only shifted by approximately 1 Å. Hence, it is likely that this side-chain could readily adopt a catalytically active conformation.

Comparison of the DP8 active site and PEP active site revealed remarkable similarities. Despite the fact that PEP has low homology with DP8 ($\sim$9% overall amino acid sequence identity), as it belongs to another subclass, and functions as an endopeptidase, the active site of the DP8 is very similar. The DP8 active site also aligned well with the ACPH triad with the only substantial difference being the previously mentioned Ser side-chain and the slightly different orientation of the His residue. In ACPH the NH group of a Gly residue is thought to perform the stabilizing function facilitated by the OH group of Tyr in the DPIV and PEP structures (Fulop *et al* 1998, Bartlam *et al* 2004). The backbones of these corresponding residues align well between the ACPH and the DP8 structures, however there is substantial distance between the functional groups in the Gly and Tyr residues. However, the DP8 Tyr is closely aligned to the PEP Tyr, hence this difference, if present in the actual DP8 structure, is not likely to prevent catalysis of ACPH substrates, which is further confirmed by DP8's ACPH activity (Park *et al* 2005).

Alignments of the DPIV catalytic triad with PEP and ACPH structures produce comparable RMSD values to the DP8 alignments, implying structural conservation between the enzymes. However, alignments of the conserved oxyanion stabilizing Tyr residues between DPIV and PEP reveal substantial variation in the placement of this residue suggesting less stringent structural conservation.

There was also some difference in the orientation of the two conserved Glu residues between the DPIV and DP8 structures. The small helix that holds the Glu residues narrows the active-site, leaving room for only two amino acids before the peptide substrate reaches the active-site Ser, conferring dipeptidyl peptidase specificity (Rasmussen *et al* 2002). It is possible that the differences in the orientation of the DP8 Glu residues may contribute to differing substrate specificity in DP8. However, as the conserved motif lies in the β-propeller domain, it is possible that the position of the Glu residues were affected by surrounding flaws. There were also substantial differences between the positioning of the DP8 Glu residues compared to the corresponding Phe residues in the ACPH structure. However, the relative overall position of these residues were quite similar and the side-chain of the Phe153 and Glu260 are within 3 Å of each other. The position of the Phe residues suggests that the substrate entry tunnel in ACPH is slightly larger than in DP8, as the Glu residues are closer to the catalytic triad. Further

analysis is required to determine whether this limits the size of the N-terminal acyl groups of the blocked peptide substrates DP8 can cleave.

5. CONCLUSIONS

The DP8 model produced was very similar to the DPIV structure in the α/β hydrolase domain. Analysis of the active site of the DP8 model revealed significant structural conservation in the catalytic triad between DPIV, PEP and ACPH. Further analysis is required to determine whether any differences in the substrate pockets or substrate access tunnel(s) may contribute to DP8's ability to act as a dipeptidyl peptidase, endopeptidase and acylaminoacyl peptidase. As the structure of fibroblast activation protein has recently been published, an alternative model may be made using this structure together with DPIV to make a model based on two enzymatically active proteins. Simulated docking of substrates and inhibitors into the model may uncover subtle differences between the structures. This may aid in determining the reason for DP8's multiple enzyme functionality and aid in the improvement of DPIV inhibitor specificity.

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TOPIC II

DPIV-RELATED ENZYMES

CHAPTER 5

STRUCTURE AND FUNCTION IN DIPEPTIDYL PEPTIDASE IV AND RELATED PROTEINS

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1. INTRODUCTION

Few proteinases are capable of cleaving the post-proline bond and very few can cleave a prolyl bond two positions from the N terminus. The latter small subset of serine proteinases, the post-proline dipeptidyl aminopeptidases, consists of the four enzymes of the dipeptidyl peptidase (DP) IV gene family, DPIV (E.C. 3.4.14.5), fibroblast activation protein (FAP), DP8 and DP9 (Abbott, Gorrell 2002) and DP-II (Leiting *et al* 2003) (E.C. 3.4.14.2). DPIV ligands include adenosine deaminase (ADA), kidney Na^+/H^+ ion exchanger 3 (Girardi *et al* 2001) and fibronectin (Cheng *et al* 2003). DPIV substrates include important chemokines, neuropeptides and incretins.

The prolyl oligopeptidase (POP) family contains the DPIV gene family, prolyl endopeptidase (PEP; EC 3.4.21.26) and acyl aminoacyl peptide hydrolase (ACPH; EC 3.4.19.1). The DPIV gene family is distinguished by a pair of glutamates that are distant from the catalytic serine in the primary structure (Abbott, Gorrell 2002), but within the catalytic pocket in the tertiary structure (Rasmussen *et al* 2003). These glutamates, at positions 205 and 206 in DPIV, are essential for DP activity (Abbott *et al* 1999a, Ajami *et al* 2003). The DPIV gene family has six members including FAP, DP8, DP9 and the two non-enzymes DPL1 and DPL2.

This overview comments on structural features and functions of the DPIV family.

2. DPIV

DPIV is a 110 kDa glycoprotein that is catalytically active only as a dimer. In addition to the integral membrane form, a soluble form of DPIV occurs in serum and semen. Lymphocytes and endothelial and epithelial cells, including acinar cells, express DPIV. CD26 cell surface

Table 1. Enzymes of the human DPIV family.

Characteristic	DPIV	FAP	DP8	DP9
Hydrolysis of H-Gly-Pro	√	poor	√	√
Hydrolysis of H-Ala-Pro	√	√	√	√
Hydrolysis of H-Arg-Pro	√	√	poor	poor
Gelatinase activity	×	√	×	×
Binding to adenosine deaminase	√	×	×	×
Ubiquitous mRNA expression in normal adult tissues	√	√	√	√
Protein expression by activated fibroblasts	√	√	?	?
Expression by foetal mesenchymal cells	√	√	?	?
Expression by lymphocytes	√	×	√	√

Key: √- yes; ×- no; ?- not known.

expression on T cells increases 5- to 10- fold following antigenic or mitogenic stimulation. Many epithelial tumours and cancer cell lines express DPIV but DPIV expression is downregulated or absent in tumour cells (Table 1). However, the stromal fibroblasts of solid tumours express DPIV and FAP (Abbott, Gorrell 2002).

3. FAP

FAP has a collagen type I specific gelatinase activity (Levy *et al* 1999, Park *et al* 1999). Like DPIV, catalysis depends upon dimerisation (Levy *et al* 1999, Pineiro-Sanchez *et al* 1997). DPIV and FAP can heterodimerise (Scanlan *et al* 1994). Controlling gelatinases is vital for organ structure. Unlike matrix metalloproteinases (MMPs), which have a proenzyme form, the gelatinase activity of FAP is constitutive so controlling its expression is crucial. FAP is expressed only by activated hepatic stellate cells (HSC), and by mesenchymal cells in other sites of tissue remodelling such as embryonic mesenchymal cells and stromal fibroblasts of epithelial tumors and healing wounds (Levy *et al* 1999, Rettig *et al* 1993). The FAP gene knockout (GKO) mouse has a normal phenotype for body weight, organ weights, histological examination of major organs and haematological analysis (Niedermeyer *et al* 2000). The HSC is important in the pathogenesis of cirrhosis because it is the major source of extracellular matrix (ECM) and various MMPs and their inhibitors. FAP immunostaining intensity strongly correlates with the histological severity of fibrosis in chronic liver disease (Levy *et al* 2002).

Plasminogen2ε associates with both ADA and DPIV (Gonzalez-Gronow *et al* 2004) and FAP associates with the urokinase plasminogen activator receptor annexin2 (Artym *et al* 2002). Thus, additional protein-protein interactions of DPIV and FAP have been identified and need further study.

4. DP8 AND DP9

DP8 and DP9 are ubiquitously expressed enzymes with DPIV peptidase activity (Abbott *et al* 2000, Ajami *et al* 2004). DP8 and DP9 have 26% amino acid identity with DPIV and FAP and 61% identity with each other (Abbott *et al* 2000, Gorrell and Yu 2005). However, some biochemical characteristics of DP8 are similar to PEP (Table 2). DP8 and DP9 are both

Table 2. Physical attributes of human DPIV and related proteins.

Attribute	DPIV	FAP	DP8	DP9	DPL1	DPL2	PEP	ACPH
Synonyms	CD26 ADAbp	Seprase			DPP6 DPPX	DPP10	POP PREP	ACP APEH
Gene Bank Accession	M80536	U09278	AF221634	AY374518 AF542510 AF452102	M96859 M96860	AY387785	AB020018 P48147	P13798
Reference		Scanlan *et al* 1994	Abbott *et al* 2000; Qi *et al* 2003	Ajami *et al* 2003; Qi *et al* 2003	Wada *et al* 1992	Allen *et al* 2003; Chen *et al* 2005; Qi *et al* 2003		Kobayashi *et al* 2004
Human gene Mouse gene	2q24.2 2	2q24.3 2	15q22.32 9	19q13.3 17	7q36.2 5	2q14.1 1	6q21 10	3p21.31 9
Transmembrane Domain	√	√	×	×	√	√	×	×
Monomer Mobility kDa	110	95	100	110	97	97	80	80
Number of Amino Acids	766	760	882	863,971	865,803	796,789	710	728
Glycosylation Sites	9	5	0	2	7	8	2	0

soluble proteins localised in the cytoplasm and are active as monomers. We recently observed PEP activity from DP8 (*see* Park *et al* chapter 10 of this book). Neither DP8 nor DP9 exhibit gelatinase activity and no natural substrates are known.

5. THE NON-ENZYMES: DPL1 AND DPL2

Two enzymatically inactive proteins closely related to DPIV lack the catalytic Ser and its neighbouring Trp. As restoring the enzyme activity of DPL1 or DPL2 would very likely require both the Ser and the Trp, as well as other residues such as Tyr547 (Bjelke *et al* 2004), their biological activities are probably exerted via binding interactions. DPL1 was previously called DPPX or DPP6. Despite the absence of DP activity, DPL1 exerts an important developmental function. The mouse rump white mutation, which lacks expression of the DPL1 gene, is embryonic lethal in homozygotes and causes a pigmentation defect in heterozygotes (Hough *et al* 1998). Intronic portions of the DPL2 gene link to asthma (Allen *et al* 2003). DPL2 mRNA expression occurs in brain, adrenal gland and pancreas (Chen *et al* 2005, Qi *et al* 2003). This is similar to the expression pattern of the long form of DPL1 (Wada *et al* 1992). Like DPL1, DPL2 associates with and modulates A-type potassium channels (Jerng *et al* 2004).

6. POTENTIAL FUNCTIONS OF DPIV-RELATED PEPTIDASES

It is likely that many DPIV inhibitors are not strictly selective for DPIV (*see* Park *et al* chapter 10 of this book). Therefore, some potential functions of DP8 and DP9 may be inferred from studies in which a function has been observed in cells or animals treated with a non-selective DPIV inhibitor. In several paradigms DPIV inhibitor treatment elicits similar responses in cells and animals irrespective of possession or lack of DPIV expression (Table 3). For example, DPIV inhibitors suppress collagen-induced and alkyldiamine-induced arthritis to similar extents in DPIV deficient German Fischer344 and wild-type rats (Tanaka *et al* 1997). These data imply that the peptidase activities of FAP, DP8 and/or DP9 have important functions in the pathogenesis of arthritis.

Table 3. *In vivo* effects of DPIV inhibitors.

Name of inhibitor	Outcome: wild type mouse	Outcome: DPIV GKO mouse	Reference
Ala-Pro-nitrobenzoyl-hydroxylamine	Suppression of experimental arthritis	Same as wild type	Tanaka *et al* 1997
Lyz-(Z-{NO_2})-thiazolidide			
Val-boro-Pro	Tumour Regression	Same as wild type	Adams *et al* 2004
Val-boro-Pro	Accelerates recovery from neutropenia	Same as wild type	Jones *et al* 2003

The DPIV/DP-II inhibitor Val-boro-Pro stimulates recovery from cyclophosphamide – induced neutropenia to similar extents in DPIV GKO and wild-type mice (Jones *et al* 2003), indicating that bone marrow expresses important DPIV-related enzymes. Effects of inhibiting FAP, DP8 and DP9 need to be understood.

7. CANCER AND ECM INTERACTIONS WITH DPIV

DPIV binds the ECM component fibronectin on rat hepatocytes and breast and lung cancer cells (Abdel-Ghany *et al* 1998, Cheng *et al* 2003), and this interaction is independent of its enzymatic activity. The ECM interactions of DPIV may help to explain its changes in expression levels in various human cancers because cancers differ in their ability to invade neighboring tissue. Following DPIV transfection, SKOV ovarian carcinoma cells exhibit increased cell-cell adhesion mediated by collagen or fibronectin (Kikkawa *et al* 2003). Moreover, ovarian carcinomas with greater DPIV expression were found to be less invasive, so perhaps the increased adhesion has an anti-invasive effect (Kajiyama *et al* 2002).

Either wild type or enzymatically-inactive DPIV overexpression in melanoma cell lines causes inhibition of tumor progression in nude mice, and inhibition of anchorage-independent growth, inhibition of cell growth and increased apoptosis *in vitro* (Wesley *et al* 1999). Invasive melanoma cells lack DPIV expression, so DPIV seems to suppress the malignant phenotype. An *in vitro* invasion study has shown that either wild type or enzymatically inactive DPIV overexpression can confer reduced invasiveness upon LOX melanoma cells in Matrigel (a basement membrane matrix) (Pethiyagoda *et al* 2001). Further understanding of the anti-invasion effect of DPIV might assist in the control of certain carcinomas.

The in vitro evidence suggests that DPIV - selective inhibitors would not influence tumor invasiveness and our in vitro data suggests that DPIV and related proteins can influence cell adhesion, movement and survival independently of DPIV enzyme activity (see Wang *et al* chapter 23, and Yu *et al* chapter 7 of this book). Indeed, the non-selective inhibitor Val-boro-Pro triggers tumor regression in DPIV GKO mice as effectively as in wild type mice (Adams *et al* 2004). The roles of DPIV, FAP, DP8 and DP9 in tumours and effects of selective enzyme inhibitors on tumours *in vivo* remain unclear. A comparison of FAP and catalytically inactive FAP expressing tumours suggests a small role for FAP enzyme activity in tumour progression (Ramirez-Montagut *et al* 2004), so perhaps FAP was the effective Val-boro-Pro target in the Adams *et al* study.

8. THE DPIV PROTEIN STRUCTURE

The many DPIV crystal structures recently reported reflect a sudden global interest in the pharmaceutical design of DPIV inhibitors (Table 4). The DPIV glycoprotein is a dimer (Figure 1). Each monomer subunit consists of two domains, an α/β-hydrolase domain (residues 39–51 and 501–766) and an eight-blade β-propeller domain (residues 59–497), that enclose a large cavity of $\sim$30–45 Angstroms in diameter. Access to this cavity is provided by a large side opening of 14 to 15 Angstroms (Rasmussen *et al* 2003). DPIV contains nine N-linked glycosylation sites that lie predominantly on the propeller domain near the dimerization interface (Engel *et al* 2003) and perhaps shield this trypsin—resistant extracellular protein from proteolysis.

Table 4. Crystal structures of DP-IV.

Inhibitor/ substrate	DP-IV Source	PDB Code[a]	Resolution Angstroms	Tag	Feature	Company	Reference
Val-Pyr	Baculovirus Bac-N-Blue	1N1M	2.5	CD5 signal	The first report	Novo Nordisk	Rasmussen *et al* 2003
None	Baculovirus Bac-to-Bac	1J2E	2.6	6xHis	PO comparison	Tanabe-Seiyaku	Hiramatsu *et al* 2003
Iodo-NVP DPP728	Yeast + endo-F	1PFQ	1.9	Secretion signal	Apo. Deglycosylate	Morphochem	Oefner *et al* 2003
None	Yeast	1NU6	2.1	6xHis	Tetrahedral intermediate	Roche	Thoma *et al* 2003
Diprotin A (Ile-Pro-Ile)		1NU8	2.5				
NPY (1-10)	Baculovirus Bac-to-Bac		2.1	6xHis	Tetrahedral intermediate	Syrrx	Aertgeerts *et al* 2003

[a] PDB: http://www.rcsb.org/pdb.

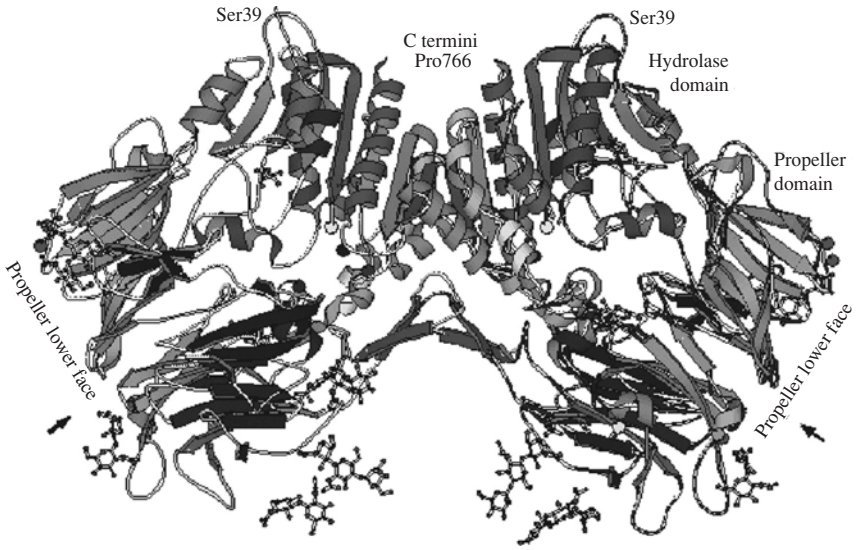

Figure 1. DPIV structure. The DPIV homodimer as a ribbon diagram with carbohydrates depicted in ball-and-stick representation and positions of important amino acids as spheres. Leu294 and Val341 (at bottom centre) are essential for ADA binding, Arg343 and Lys441 (at sides) are important in epitopes of antibodies that inhibit ADA binding (Abbott *et al* 1999b). Glu205 and Glu206 (at centre) are essential for enzyme activity (Abbott *et al* 1999a). Both domains contribute to the dimerisation interface, the β-propeller contributing two β-strands that protrude from blade 4. Substrate access to the catalytic site occurs via the side openings that face each other and are between the eight-blade β-propeller (bottom) and α/β hydrolase (top) domains. The transmembrane domain (residues 7 to 28) is above the molecule in this view of the extracellular portion, residues 39 to 766, of human DPIV. Figure prepared using the atomic co-ordinates having Protein Data Bank code 1N1M (Rasmussen *et al* 2003).

8.1. The Active Site and Catalytic Mechanism

The residues forming the catalytic triad are Ser630, Asp708 and His740. In addition, Tyr547 in the hydrolase domain is essential for catalytic activity and in the crystal structure appears to stabilise the tetrahedral oxyanion intermediate form of a substrate (Bjelke *et al* 2004). Two glutamic acids in the catalytic pocket, Glu205 and Glu206, align the substrate peptide by forming salt bridges to its N terminus, leaving room for only two amino acids before the peptide reaches the active serine residue, thus explaining its dipeptide cleaving activity. Furthermore, in the substrate second position only amino acids with smaller side chains such as proline, alanine and glycine can fit into the narrow hydrophobic pocket. Asp663 seems to constrict the catalytic pocket preventing hydrolysis of the N-terminally blocked PEP substrate Z-Gly-Pro (Aertgeerts *et al* 2005). In FAP this position is occupied by Ala, permitting the PEP activity of FAP. Thus, the crystal structures have helped to explain the substrate specificity of DPIV and the mutation data showing that Glu205 and Glu206 are essential for catalysis (Abbott *et al* 1999a, Ajami *et al* 2003).

Homodimerisation requires the hydrolase domain (Ajami *et al* 2003) and a protrusion from the fourth blade of the β-propeller (Figure 1). The dimerisation interface has been described in detail. A single amino acid point mutation near the C-terminus, His750Glu, is sufficient to prevent dimerisation (Chien *et al* 2004).

8.2. The POP Family Propellers

β-propellers have four to eight blades formed by a repeated subunit containing at least 30 and generally 50 amino acids in a β-sheet of four anti-parallel strands. Propellers commonly act as scaffolding for protein–protein interactions (Fülop, Jones 1999). The points of contact with ligand and antibodies are formed by loops contributed by adjacent propeller blades such that binding epitopes depend upon tertiary structure. DPIV has all of these characteristics and so this will probably be true of the other POP family members. POP family propellers lack molecular Velcro, which is a more rigid closure formed by overlapping a strand in the first and last blades. DPIV, DPL1 and FAP have an 8-blade propeller whereas PEP and ACPH have, like integrin α chain, a 7-blade propeller (Aertgeerts *et al* 2005, Bartlam *et al* 2004, Fülop *et al* 1998, Rasmussen *et al* 2003, Strop *et al* 2004). The eight-blade β-propeller domain of DPIV is more disordered than most propellers and more disordered than the PEP propeller. In the POP family only the DP8 and DP9 structures remain unknown. These proteins have larger N-terminal regions so may have either additional propeller protrusions or an additional element of tertiary structure.

Arg125 in DPIV propeller blade 2 contacts inhibitors and substrates (Aertgeerts *et al* 2004, Engel *et al* 2003, Hiramatsu *et al* 2004, Oefner *et al* 2003, Rasmussen *et al* 2003, Thoma *et al* 2003, Weihofen *et al* 2004). Arg125 is conserved in DPIV in all species from bacteria to human and occurs in FAP and DPL1 but is absent from DP8 and DP9. The sequence motif of the Glu205-containing α-helix is conserved in all six proteins of the DPIV gene family in all species (Abbott, Gorrell 2002).

8.3. The Adenosine Deaminase Binding Site on DPIV

Adenosine deaminase (ADA, EC 3.5.4.4) is a ubiquitous globular 43 kDa enzyme. ADA derived from rabbits, cattle and humans binds to human (K_A 4 to 20 nM) but not mouse DPIV.

We showed that DPIV propeller residues Leu294 and Val341 are essential for ADA binding (Abbott *et al* 1999b). The crystal structure of DPIV with ADA shows that the ADA binding site is located on the outer edges of the 4th and 5th blades near the lower face of the β-propeller domain of DPIV. The binding interface is predominantly hydrophobic and contains only one salt bridge. Most of the involved residues on DPIV are hydrophobic and most of the 13 involved residues on ADA, which are all polar, are charged (Weihofen *et al* 2004). Protein-protein binding generally primarily involves hydrophobic surfaces with some salt bridges that are usually peripheral in the binding interface. Thus, the amino acid composition of the ADA-DPIV binding site is unusual, perhaps due to the short evolutionary time that it has undergone selective pressure.

9. CONCLUSIONS

Potential therapeutic applications of DPIV inhibitors have fuelled interest in understanding the biological roles of DPIV and its relatives. Such efforts are confounded by the ubiquitous expression of DPIV, inhibitor selectivity questions and the variety of identified substrates. DPIV is not essential, but is such a useful enzyme that all animal species express it. The enzyme activity's ancient and primary function is probably nutritional, providing more complete proteolysis of food and recycled proteins. This function is unnecessary in well-fed humans. The development of selective inhibitors of proteolytic activity and identification of ligand binding activities in this gene family would lead to rapid advances in understanding the biology of the POP gene family.

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CHAPTER 6

EXPRESSION AND FUNCTION OF DIPEPTIDYL PEPTIDASE IV AND RELATED ENZYMES IN CANCER

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1. INTRODUCTION

Numerous biologically active peptides, systemic as well as local hormones, contain an evolutionary conserved proline residue as a proteolytic-processing regulatory element (Vanhoof *et al* 1995). Limited proteolysis of multiple neuropeptides, chemokines, incretins etc. by dipeptidyl peptidase-IV (DPP-IV) enzymatic activity (De Meester *et al* 2000) leads to both quantitative and in some cases due to the diversification of their receptor preference, also qualitative changes of their signaling potential (Busek *et al* 2004). Such hydrolytic activity was originally recognized as "canonical" DPP-IV/CD26 (EC 3.4.14.5). Work over the past years has demonstrated that DPP-IV has a multitude of physiological roles (Lambeir *et al* 2003). Although the greatest part of systemic DPP-IV activity probably resides in DPP-IV/CD26, further studies demonstrated that a significant amount of DPP-IV activity can be attributed to a growing panel of other proteins, including Fibroblast-activation protein α/Seprase (FAP), Quiescent cell proline dipeptidase (QPP/DPP-II/DPP-7), DPP8, DPP9 and Attractin. Moreover, several additional molecules devoid of the characteristic enzymatic activity, but possessing high degree of structural similarity to DPP-IV, for example DPP6 and 10, were included among DASH molecules too (Sedo and Malik, 2001). So far, DPP-IV, FAP and DPP-II/QPP have been shown to participate on the regulation of multiple important cellular programs, including cell growth, transformation, apoptosis, invasion and tumor metastasis. In contrast to proteases involved in cancer development and progression as the executors of tissue degradation, most DASH operate as regulatory molecules, modifying biologically active peptides. The DASH, their substrates and corresponding receptors of these substrates are jointly expressed in many tissues and their deregulation has been observed in the microenvironment of numerous malignancies (Table 1).

An antioncogenic effect of DPP-IV was demonstrated by experimentally induced expression in cell lines derived from melanoma (Wesley *et al* 1999), ovarian carcinoma (Kajiyama *et al* 2002, 2003), non-small cell lung carcinoma (NSCLC; Wesley *et al* 2004) and lately prostatic

Table 1. Examples of "DASH system" elements in human malignancies.

Tumor/ transformed cells	DASH enzymatic activity	DASH substrate(s)	DASH substrate receptors	Notes
Melanoma	↓DPP-IV[1] ↓FAP[2]	RANTES[3]	CXCR4[4]	Tumor suppressing effect of FAP[37]; FAP in invadopodia promotes invasivity[34]
Lung cancer	↓DPP-IV in NSCLC lines[5] ↑DPP-IV in tumor tissues[6]	NP Y, SP, VIP, GRP[7]	NK1[8] CXCR4[9] GRP- R[10]	Tumor suppressing effect of DPP-IV[5]
Ovarian cancer	DPP-IV[11]	CXCL 12[12]	CXCR4[13]	Tumor suppressing effect of DPP-IV[11]
Endometrial cancer	↓DPP-IV[14]	RANTES[15] CXCL 12[16]	CXCR4[17]	DPP-IV expression negatively correlates with tumor grade[14]
Colorectal cancer	↑FAP[18]	NP YY[19]	CXCR4[20] GRP-R[7]	CXCR4 promotes the outgrowth of lung micrometastases[20]
Prostate cancer	↑DPP-IV[21] ↓DPP-IV in metastases[22]	GRP, SP[23]	CXCR4[24] GRP-R[7]	Tumor suppressing effect of DPP-IV[35]
Breast cancer	FAP[25,36]	NP YY[19] CXCL 12[26,12] RANTES[27]	GRP-R[7] CXCR4[26,29] Y_1R[28]	In cancer cells FAP stimulates tumor progression[25]; In stromal cells associated with increased survival[36]
Thyroid cancer	↑DPP-IV in differentiated carcinomas[30]		CXCR4 in anaplastic carcinoma[31]	
Cervical cancer	↑FAP[32]			An effect via the gelatinase activity?
Gastric cancer	↑FAP[33]			An effect via the gelatinase activity?

Abbreviations: CXCL12-SDF 1α, stromal cell derived factor 1α; CXCR4-SDF 1α receptor; GRP-gastrin releasing peptide; GRP-R-GRP receptor; NK1-neurokinin 1 receptor; NP Y-neuropeptide Y; NP YY-neuropeptide YY; RANTES-regulated on activation normal T cell expressed and secreted, CCL5; SP-substance P; VIP-vasointestinal peptide; Y_1R-NPY/ NP YY receptor ↑ increased; ↓ decreased

References: [1,2]Wesley *et al* 1999; [3]Mrowietz *et al* 1999; [4]Murakami *et al* 2002; [5]Wesley *et al* 2004; [6]Sedo *et al* 1991; [7,8]Reubi 2003; [9]Kijima *et al* 2002, Burger *et al* 2003; [10]Jensen *et al* 2001; [11]Kajiyama *et al* 2003; [12,13]Hall and Korach 2003, Scotton *et al* 2002; [14,15]Khin *et al* 2003; [16,17]Mizokami *et al* 2004; [18]Iwasa *et al* 2003; [19]Tseng *et al* 2002; [20]Zeelenberg *et al* 2003; [21]Wilson *et al* 2000; [22]Bogenrieder *et al* 1997; [23]Nagakawa *et al* 2001; [24]Mochizuki *et al* 2004; [25]Kelly *et al* 1998, Huang *et al* 2004; [26]Lee *et al* 2004; [27]Luboshits *et al* 1999; [28]Reubi *et al* 2001; [29]Muller *et al* 2001; [30]Iwabuchi *et al* 1996; [31]Hwang *et al* 2003 ; [32]Jin *et al* 2003; [33]Mori *et al* 2004, Okada *et al* 2004; [34]Monsky *et al* 1994; [35]Wesley *et al* 2005 ; [36]Ariga *et al* 2001; [37]Ramirez *et al* 2004.

carcinoma (Wesley *et al* 2005). On the contrary, biochemical studies claim increased DPP-IV enzymatic activity in tumor extracts from prostate and lung cancer (Wilson *et al* 2000; Sedo *et al* 1991).

Also FAP possesses an antioncogenic effect in a melanoma model (Ramirez-Montagut *et al* 2004). Conversely, its overexpression in HEK293 xenografts enhances tumorigenicity and in cultured breast cancer cells alleviates the growth factor requirements (Cheng *et al* 2005; Goodman *et al* 2003).

On the basis of biochemical analyses, DPP-II/QPP was presumed to be present in transformed cells (Komatsu *et al* 1987), although its function there remains largely unknown.

Participation of DPP-8 and DPP-9 and Attractin in cancer has not yet been rigorously studied. Nevertheless, their broad tissue distribution and shared DPP-IV-like enzymatic activity (Qi *et al* 2003; Abbot *et al* 2000; Duke-Cohan *et al* 2004) suggest possible functional overlaps with other DASH molecules.

The aim of our study was to demonstrate some aspects of the functional interrelations within the hypothetical "DASH system" (Busek *et al* 2004), involving DASH, their substrates and corresponding receptors.

2. MATERIAL AND METHODS

The full-length cDNA of human DPP-IV/CD 26 (a generous gift from Prof. W.-T. Chen, SUNY, USA) was subcloned into the pGene vector of the mifepristone-inducible Gene Switch system (Invitrogen). The orientation of the insert was confirmed by DNA sequencing (Genomyx LR sequencer). DPP-IV negative T98G cells (ATCC, Middlesex UK) were transfected using lipofectamine reagent and stable clones were selected with zeocin and hygromycin. Resulting clones exhibited low, but measurable DPP-IV-like enzymatic activity and detectable DPP-IV mRNA expression, even without mifepristone stimulation. Such "leakage" transcription is an inherent property of the Gene Switch system (manufacturer's manual).

Inducible expression of DPP-IV mRNA was confirmed by real time RT-PCR (Figure 1A) using ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Cell surface DPP-IV-like enzymatic activity in suspensions of viable cells (Figure 1B) was assessed by continuous rate fluorimetric assay (Spectrofluorimeter Beckman L50B) using 7-(glycyl-prolylamido)-4-methylcoumarin (Bachem) as a substrate (Sedo *et al* 1989). The regulated expression of DPP-IV protein was further confirmed by immunohistochemistry (not shown).

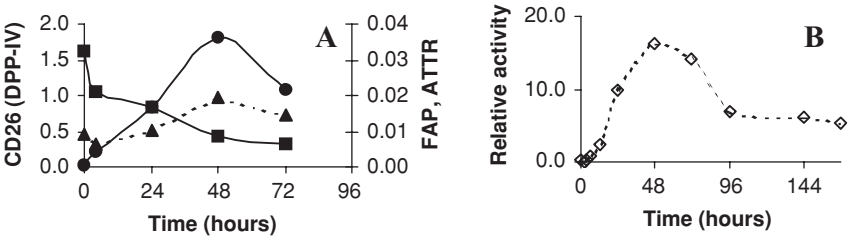

Figure 1. (A) Quantitative analysis of DASH transcripts expression normalized to β-actin mRNA (ΔCT). Circles: DPP-IV, Squares: FAP, Triangles: Attractin (ATTR). (B) Cell surface DPP-IV-like enzymatic activity. Time 0: induction of DPP-IV expression in transfected T98G cells by addition of mifepristone.

For growth curve determination, cells were seeded in 24 well plate in DMEM supplemented with 10% fetal calf serum, harvested daily in triplicate and counted on Coulter Counter Z2 (Beckman).

Expression of NK1 (preferential substance P receptor), Y1R and Y2R (members of neuropeptide Y receptor family) transcripts was assessed according to standard RT-PCR protocols (Dyad engine, MJ Research) optimized for the purpose in our laboratory. The specificity of the PCR products was confirmed by sequencing (ABI PRISM 310, Applied Biosystems) using the same PCR primers.

3. RESULTS AND DISCUSSION

3.1. Co-Expression of DASH Molecules

As shown in Figure 1A, robust up-regulation of DPP-IV mRNA in mifepristone stimulated transfectants was associated with about hundred-fold DPP-IV activity enhancement (Figure 1B). In compliance with the previous reports (Wesley *et al* 1999), DPP-IV transfected T98G cells exhibited higher FAP expression than the parental wild cells (not shown). The expression of FAP however declined during the course of mifepristone-induced DPP-IV activity upregulation, while there was only a slight change of attractin expression (Figure 1A). Downregulation of FAP, a plasma membrane protein with DPP-IV-like enzymatic activity, may hypothetically represent cell's reaction to the excessive DPP-IV-like activity.

3.2. DPP-IV and Cell Proliferation

Cell proliferation was substantially reduced in T98G cells upregulating DPP-IV compared to transfected, but unstimulated counterparts (Figure 2). Indeed, upregulation of DPP-IV has an antiproliferative as well as an antioncogenic effect in several other cell models possibly due to the degradation of local mediators (Wesley *et al* 1999, 2004, 2005).

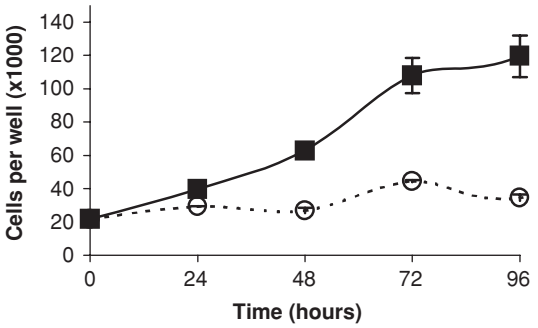

Figure 2. Growth curves of T98G cells with inducible DPP-IV expression. Squares: transfected cells; Circles: transfected cells upregulating DPP-IV after mifepristone induction at time 0. Mifepristone did not affect the growth of parental wild cells (not shown) Data expressed as a mean of triplicates.

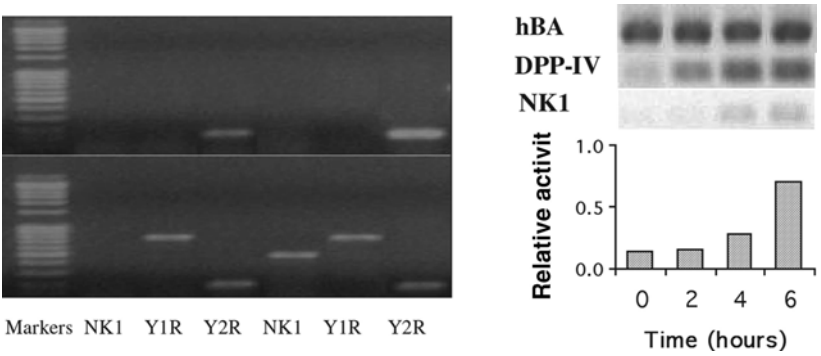

Figure 3. RT-PCR detection of biologically active DPP-IV substrate receptors (left). Panel identification: A, B: Parental "wild" T98G cells; C, D: DPP-IV transfected cells; A, C: mifepristone untreated cells; B, D: mifepristone treated cells. The identity of the resulting fragments was verified by sequencing. Relationship of DPP-IV and NK1 mRNA expression and DPP-IV enzymatic activity upregulation in transfected T98G cells (right). Time 0: induction of DPP-IV expression by addition of mifepristone. hBA:human β-actin.

3.3. Receptors of Biologically Active DASH Substrates

To better characterize further elements of the hypothetical "DASH system" and to demonstrate their possible coregulation, expression analysis of selected receptors of biologically active DPP-IV substrates was performed (Figure 3). DPP-IV transfected cells markedly expressed NK1 (preferential receptor of substance P) mRNA, following the Mifepristone-induced DPP-IV overexpression. Moreover, the NK1 upregulation was slightly delayed after the Mifepristone induced rise of DPP-IV mRNA, seeming to be more tightly linked to the boost of DPP-IV hydrolytic activity (Figure 3, right lower part). Immunocytochemical detection confirmed NK1 upregulation observed on transcriptional level (Busek *et al*, unpublished results). NK1 ligand substance P has a proven proliferation-promoting effect in glioma cells (Palma and Maggi 2000). NK1 upregulation could be interpreted as a compensatory facilitation of substance P signaling under conditions of its relative deficiency due to its augmented DPP-IV-mediated degradation.

The emergence of Y1R in transfected cells, regardless of the stimulation of DPP-IV expression, may be attributable to "low, but sufficient" DPP-IV leakage synthesis; it may however also be an artifact resulting from the transfection or selection procedures. All T98G derived cell populations—parental-wild, transfected and transfected-upregulating DPP-IV—express Y2R.

4. CONCLUSIONS

Upregulation of DPP-IV is apparently accompanied by decreased proliferation or survival of the glioma cells.

Co-expression of more enzymatically active DASH molecules may participate on the whole DPP-IV-like hydrolytic activity within one cell population. The observed changes of FAP expression associated with experimentally induced DPP-IV upregulation may suggest functional and/or regulatory relationships within the DASH group. Moreover, concomitant modulation

of NK1 and possibly also Y1R molecules, receptors of biologically active DPP-IV substrates, support existence of hypothetical integral and cooperating "DASH system" (Busek *et al* 2004), involving DASH, their substrates and corresponding receptors.

Cell or organ specific composition of the "DASH system" may explain different biological roles of particular DASH and diverse consequences of their pharmacological targeting, so far reported in literature.

Together, inhibition of DPP-IV-like enzymatic activity, an emerging therapeutic tool in numerous pathologies, should be considered from the perspective of "DASH system" complexity.

5. ACKNOWLEDGEMENTS

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CHAPTER 7

DP8 AND DP9 HAVE EXTRA-ENZYMATIC ROLES IN CELL ADHESION, MIGRATION AND APOPTOSIS

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1. INTRODUCTION

Cell adhesion and migration, proliferation and apoptosis are central to many pathological processes involving tissue remodeling, including liver fibrosis, inflammation, angiogenesis, cancer growth and metastasis. We have shown that DPIV and FAP exhibit altered expression in chronic liver injury (Levy *et al* 1999; Matsumoto *et al* 1992) and that FAP expression correlates with human liver fibrosis severity (Levy *et al* 2002).

DPIV-ECM-cell interactions are generally independent of enzymatic ability (Cheng *et al* 2003; Tanaka *et al* 1997). The observed variability of DPIV expression levels in human tumors seems to relate to tumor invasiveness, proliferation and/or apoptosis (Khin *et al* 2003; Wesley *et al* 2004). DPIV enzyme activity seems unrelated to DPIV roles in tumors (see review in chapter by Gorrell *et al*). FAP associates with $\alpha 3\beta 1$ integrin on activated cells (Mueller *et al* 1999).

DP8 and DP9 are recently cloned proteinases of the DPIV gene family. DP8 and DP9 are closely related peptidases of 61% amino acid identity and are ubiquitously expressed cytoplasmic molecules (Abbott *et al* 2000; Ajami *et al* 2004; Qi *et al* 2003). Human DP9 is prevalent in diseased tissues including tumors (Ajami *et al* 2004). The functions of DP8 and DP9 are unknown but DP9 contains the Arg-Gly-Asp (RGD) cell attachment sequence (Ajami *et al* 2004), which is the best characterized integrin binding motif.

In this first investigation of DP8 and DP9 functions in vitro, influences of DP8 and DP9 on cell-ECM interactions were examined and liver DP8 and DP9 expression levels were quantified. In order to obtain correlations between cell behaviors and peptidase expression levels, DP8 and DP9 overexpression in transfected cells was quantified by expressing green fluorescent protein (GFP) fusion proteins. This approach minimizes the behavioral prejudices that are exhibited by stably transfected clones due to their coincidental selection for adherence, survival and

proliferation. We found that, like DPIV and FAP, cells overexpressing DP8 and DP9 exhibit behavioral changes in the presence of ECM components. We demonstrated that these effects were independent of enzyme activity and of the RGD motif in DP9.

2. MATERIALS AND METHODS

The cDNAs of human DP8 and DP9 (GenBank accession numbers AF221634 and AY374518) were cloned in-frame upstream of C-terminal Green, Yellow and Cyan fluorescent proteins in the vectors pEGFP-N1, pEYFP-N1 and pECFP-N1 (BD Biosciences Clontech, Palo Alto, CA). This was achieved by PCR of the insert with Platinum Pfx Taq (Invitrogen, Carlsbad, CA) and primers containing incorporated *Sal*I and *Kpn*I restriction sites and stop codon removal.

Enzyme negative mutants of DP8 and DP9 were generated using point mutation primers for alanine replacement of the catalytic serine residues of DP8 at position 739 and DP9 at position 729 (Ajami *et al* 2003). The RGD → RAE (Arg-Ala-Glu) sequence substitution that ablates integrin binding was engineered into DP9 to produce ArgGlyAsp28ArgAlaGlu-GFP. The transfection methods have been described previously (Abbott *et al* 1999; Ajami *et al* 2003, 2004) and antibodies are listed in chapter 23 by Wang *et al*.

The cell adhesion assay followed a published method (Spizz, Blackshear 2001). Briefly, plates were coated with rat-tail collagen I (Sigma, St Louis, MO), human fibronectin (Sigma) or Matrigel (BD Biosciences, Bedford, MA) at 5 μg/well for 1 h at RT with gentle shaking. Plates were then rinsed, blocked with 1% BSA, rinsed and air dried. Forty hours after transfection, 293T cells were added in serum free media. Following incubation for 10 min at 37°C, non-adherent cells were gently removed. GFP expressing cells were enumerated by flow cytometry (Abbott *et al* 1999).

The wound healing assay was modified from a published method (Huttenlocher *et al* 1998). The transfected cell monolayer was scraped with a fine pipette tip to produce wounds of about 8 mm × 1 mm. After further incubation for 24–48 h, the cultures were imaged by bright field and fluorescence stereomicroscopy. KS400 image analysis software version 3.0 (Zeiss) with automatic threshold and lowpass filter was used to measure the total area covered by cells (bright field) and the area covered by fluorescence positive cells in wound and non-wound portions of each image. The formula used to calculate this estimate of the proportion of GFP positive cells was

$$\%\,\text{GFP positive area} = \frac{\textit{total area of GFP positivity}}{\textit{total area of cells by bright field}}$$

The cell migration assay was modified from previous methods (Itoh *et al* 2001). Following overnight serum starvation, cells transfected 40 h previously were placed in the upper chamber. The lower chamber contained conditioned medium with 1% fresh FCS. The percentages of fluorescent cells in the upper and lower chambers were enumerated by flow cytometry.

In the apoptosis assay, 293T cells were transfected with CFP fusion constructs and after 30 h replated, incubated overnight then incubated with 4 μM STS (staurosporine streptomyces) (Sigma) for 2 h, 4 h or 6 h. STS is a chemotherapeutic agent that induces cellular apoptosis.

The cells were then stained with PE conjugated Annexin V (1:50) and propidium iodide (PI) (100 ng/ml) to enumerate the apoptotic CFP-positive cells by flow cytometry. Proliferation was quantified by a 19 h tritiated thymidine uptake assay.

Each experiment was repeated three to six times. Results are expressed as means ± standard deviation. Data analysis used Student's t test, or the non-parametric test Mann-Whitney U for proliferation assays. P values < 0.05 were considered significant.

3. RESULTS

3.1. Expression of DP8 and DP9

3.1.1. Specific Recombinant Expression of DP8 and DP9

DP8 and DP9 transfected AD293 or 293T cells exhibited consistent high level transfection and significant specific dipeptidyl peptidase activity. DP8 and DP9 have a cytoplasmic localisation (Abbott *et al* 2000; Ajami *et al* 2004). Concordantly, in the DP8-GFP and DP9-GFP transfected 293T cells, the fluorescence was localised to the cytoplasm. Neither DP8 or DP9 transfection altered FAP or DPIV expression levels in 293 cells.

3.1.2. DP9 Overexpression Impaired In Vitro *Cell Adhesion*

DP9-GFP, but not DP8-GFP, expressing cells exhibited less cell adhesion on collagen I, fibronectin or Matrigel coated plastic compared to cells expressing GFP alone ($P < 0.05$) (Fig. 1A). Markedly more cells that expressed high levels of DP9-GFP were amongst the non-adherent than the adherent cell population (Fig. 1B, C). Apoptosis was not preferentially exhibited by these $DP9^{hi}$ expressing cells (see below). Moreover, adherent cell viability was

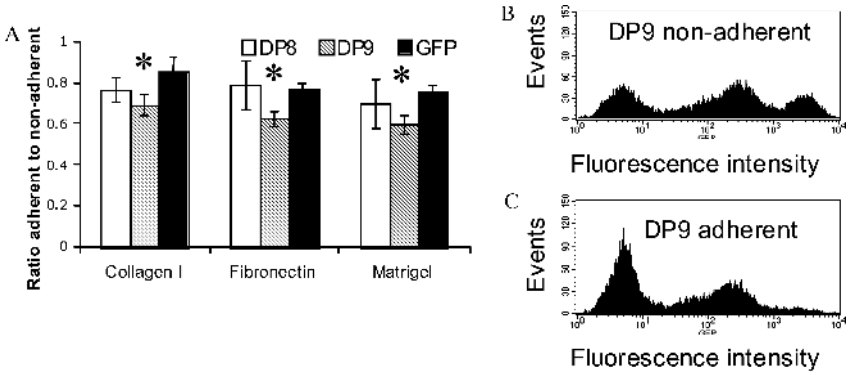

Figure 1. DP9-GFP overexpression decreased cell adhesion. In vitro cell adhesion expressed as a ratio of the percentage of fluorescent cells in the adherent population to the percentage of fluorescent non-adherent cells. Flow cytometry profiles of the non-adherent (B) and adherent (C) cell populations show that the non-adherent population contained far more DP9-GFP high-expressing cells.

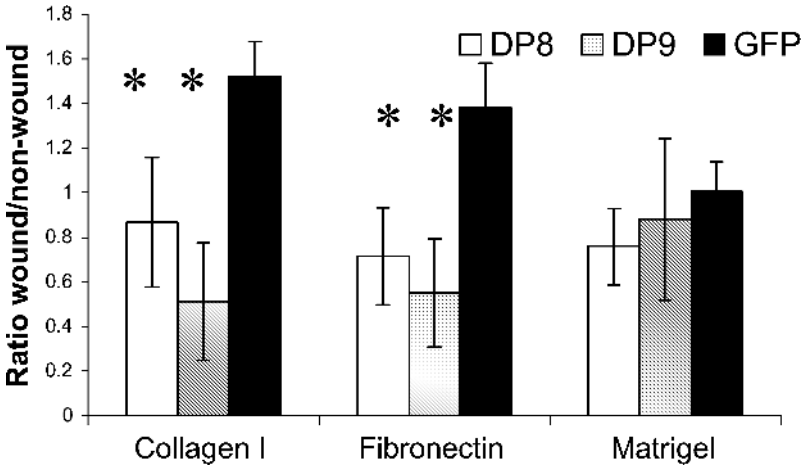

Figure 2. DP8-GFP and DP9-GFP reduced in vitro wound healing. Ratios of the percentage of fluorescent cells in the wound area to the percentage of fluorescent cells in non-wound regions of the monolayer.

only 1% to 2% greater than non-adherent cell viability. Thus, cell death did not measurably contribute to the reduced adhesion of DP9+ cells.

3.1.3. DP8 and DP9 Reduced Migration into Monolayer Wounds

In vitro wound healing assays indicate whether cells overexpressing a protein differ in their ability to repopulate a small area of coated plastic surface from which the cell monolayer has been scraped off. This is an assay of cell migration rather than proliferation (Nusrat *et al* 1992). DP8-GFP and DP9-GFP transfected cells exhibited reduced migration into wounds on collagen and fibronectin coated surfaces (Fig. 2), indicating an ability of DP8 and DP9 overexpression to impair monolayer wound healing on ECM.

3.1.4. DP8 and DP9 Impaired Cell Migration

Cells expressing DP8-GFP exhibited less migration across the transwell membrane towards collagen I (Fig. 3). DP9-GFP expressing cells exhibited less migration towards collagen I, fibronectin or Matrigel.

3.1.5. Peptidase Activity and the RGD Motif were not Required for DP9-Dependent Impairment of Cell Adhesion

To investigate the mechanism of DP9-dependent impairment of cell adhesion, an enzyme negative mutant of DP9-GFP, in which the catalytic Ser was mutated to Ala, was evaluated. In addition, whether the RGD integrin-binding motif of DP9 played a role was investigated. Neither the DP9 enzyme negative mutant nor the RGD RAE mutant differed from wildtype DP9 in impairing cell adhesion (Fig. 4A).

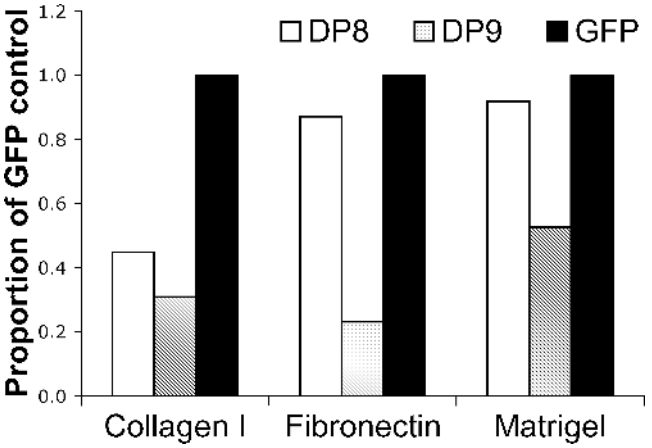

Figure 3. Cell migration was reduced by overexpression of DP9 or DP8. Each ratio of FP-derived fluorescence positive cells in the upper chamber to the lower chamber was normalised to the ratio obtained from GFP control transfected cells.

3.1.6. Peptidase Activity and the DP9 RGD Motif were not Required for DP8- or DP9-Dependent Impairment of Wound Healing

On a collagen I or fibronectin coated surface, the mutants behaved similarly to wildtype controls (Fig. 4B, C), demonstrating that the effects on wound healing were independent of enzyme activity and the DP9 RGD motif.

3.1.7. DP8 and DP9 Overexpression Increased Proliferation and STS-Induced Apoptosis

Cells transfected with DP8 or DP9 exhibited greater thymidine uptake than GFP alone. Increased percentages of apoptotic cells were observed amongst cells expressing any of the three DP9 constructs (Fig. 5). Scattergrams showed that neither annexin V nor PI levels were proportional to DP9-CFP levels, suggesting that all detectable levels of DP9 expression were associated with apoptosis.

In time-course experiments, both DP8-CFP and DP9-CFP transfected cells exhibited increased STS-induced apoptosis compared to cells transfected with CFP alone (Fig. 5). Furthermore, the same effect was seen using the DP8-GFP Ser739Ala and DP9-GFP Ser729Ala enzyme negative mutants, or the DP9 RGD RAE mutant, indicating that this effect was independent of enzyme activity or the RGD motif.

3.1.8. The Actin Cytoskeleton was Unaffected by DP8 or DP9 Overexpression

High magnification and resolution confocal microscopy showed that DP8 was visible throughout the cytoplasm (Fig. 6A) whereas DP9 was more localised (Fig. 6B). There was little or no colocalization of DP8 or DP9 with phalloidin - labelled actin cytoskeleton in AD293 cells

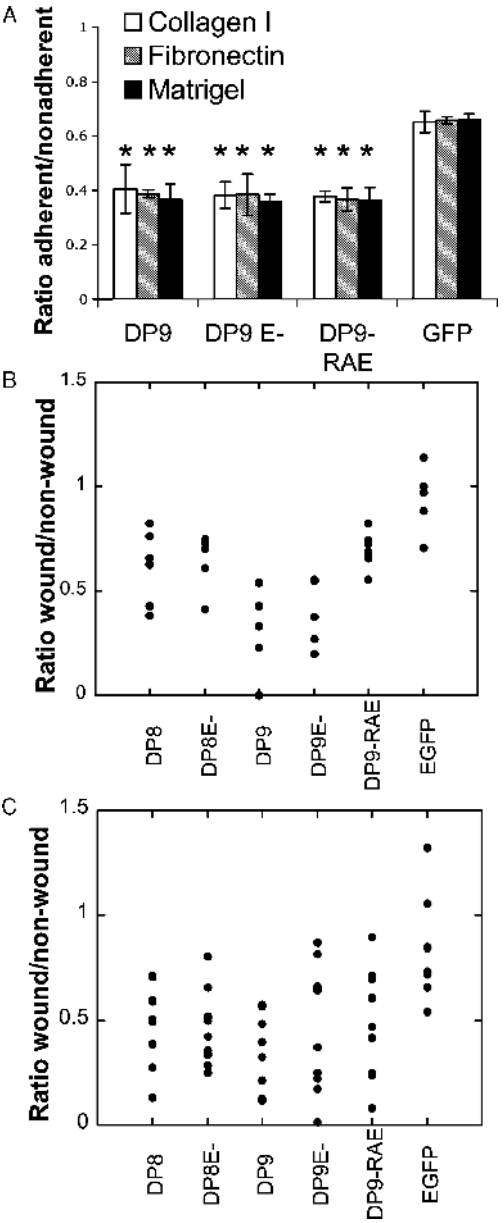

Figure 4. The DP8 and DP9 dependent impairment of adhesion and wound healing was independent of enzyme activity and the RGD motif. The RGD motif was mutated out of DP9 to make DP9 RGD → RAE. Enzyme-negative mutants of DP8 (DP8 E-) and DP9 (DP9 E-) were produced by alanine replacement of the catalytic serine. Cell adhesion was calculated as a ratio of the percentages of cells exhibiting GFP derived fluorescence in the adherent and non-adherent cell populations (A). Wound healing of transfected 293T cell monolayers on collagen I (B) and fibronectin (C) indicated no significant difference between DP9 mutants and wildtype.

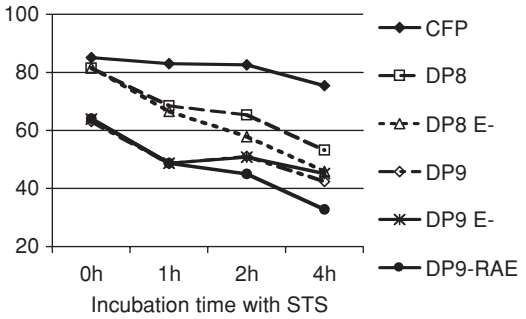

Figure 5. DP8 and DP9 enhanced STS-induced apoptosis independently of enzyme activity and the RGD motif. (A), Cells were exposed to staurosporine streptomyces (STS) at time zero and the non-apoptotic cells were enumerated by flow cytometry. The % viable is the percentage of cells CFP derived fluorescence positive, annexin V negative and PI negative. DP9 expressing cells exhibited more annexin V and PI positivity than did DP8-CFP or CFP expressing cells.

plated on collagen I, fibronectin or Matrigel coated slides. These data suggested no association between DP8 or DP9 and the cytoskeleton.

3.1.9. Molecular Phenotyping DP8 and DP9 Overexpressing 293T Cells

We investigated whether DP8 and DP9 overexpressing cells demonstrated changes in expression levels of an extensive panel of proteins associated with cell adhesion. Discoidin domain receptor (DDR) 1 is a non-integrin collagen receptor that stimulates adhesion and migration (Matsuyama *et al* 2004). The antibody to DDR1 is specific for an epitope in its cytoplasmic domain.Cytoplasmic levels of DDR1, E-cadherin and tissue inhibitor of matrix metalloproteinase (TIMP) 2 were reduced in DP9-CFP overexpressing cells compared to CFP or

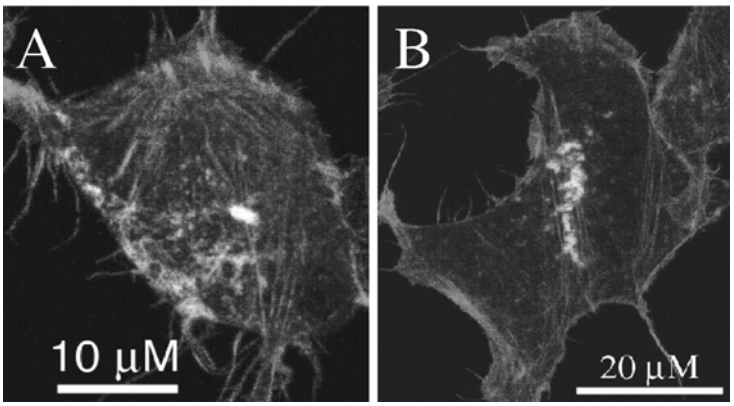

Figure 6. DP8, DP9 and the actin cytoskeleton. (A) DP8-GFP, (B) DP9-GFP transfected AD293 cells with confocal imaging. Phalloidin staining appears grey and GFP appears white.

Table 1. The molecular phenotype of DP8 and DP9 overexpressing 293T cells.

Transfected cDNA	E-cadherin	β-catenin	MMP2	TIMP2	CD44	CD29	CXCR4	CXCL12	DDR1
Cell Surface									
CFP	7.56*	0.97	1.8	0.47	13.6	4.18	6.61	4.13	0.52
DP8-CFP	9.72	0.6	1.94	0.92	14	5.84	7.51	2.83	0.66
DP9-CFP	7.63	0.58	1.54	1.21	11.3	5.12	7.78	4.33	0.69
Permeabilized									
CFP	30.63	184	3.14	63.7	ND**	ND	39.9	14.4	193
DP8-CFP	31	145	3.88	72.8	ND	ND	37	17.9	206
DP9-CFP	20.4	136	2	48.5	ND	ND	35	10	139

*Median fluorescence intensity. Values from each corresponding negative antibody control were subtracted. All data is derived only from cells in which expression of CFP was detected.

DP8-CFP overexpressing cells (Table 1). Both DP8 and DP9 overexpressing cells contained less β-catenin. Reduced TIMP2 and DDR1 levels could relate to disruption of cell-ECM interactions.

4. DISCUSSION

This is the first report on the biological significance of DP8 and DP9. A portfolio of elegant cell-ECM interaction assays revealed roles for DP9 in cell adhesion, in vitro wound healing, cell migration, proliferation and apoptosis, and for DP8 in wound healing, cell migration, proliferation and apoptosis enhancement (Table 2). DP9 overexpression impaired cell behaviour on a wider range of ECM components than for DP8. Despite their close sequence relatedness, DP8 and DP9 exert some differences in their cellular effects. Therefore these two proteins are likely to have different functions and ligands. The different patterns of cytoplasmic localisation may relate to their functional differences.

DP8 and DP9 have some overlapping properties with FAP and DPIV. DPIV-transfected LOX melanoma cells in the presence of Matrigel have reduced invasiveness compared to controls (Pethiyagoda *et al* 2001). DPIV-transfected non small cell lung carcinoma cells have shown inhibition of cell migration, increased apoptosis, inhibition of anchorage-independent growth, and suppression of tumor growth in nude mice (Wesley *et al* 2004). Our studies on DPIV and FAP in HEK 293T cells have further established these roles in cell-ECM interactions (*see* Wang *et al* chapter 23 of this book).

We showed here that the enzyme activities of DP8 and DP9 are not required for effects on adhesion, wound healing and apoptosis. Similarly, the enzyme activities of DPIV and FAP are

Table 2. Data summary.

Gene	Adhesion	Migration	Wound healing	Proliferation	Apoptosis	STS-induced apoptosis
DP8	N*	↓ collagen	↓ collagen ↓ fibronectin	↑	N	↑
DP9	↓ collagen ↓ fibronectin ↓ Matrigel	↓ collagen ↓ fibronectin ↓ Matrigel	↓ collagen ↓ fibronectin	↑	↑	↑

*N = no significant effect.

not required for their cell-ECM roles (Pethiyagoda *et al* 2001, Wesley *et al* 2004). Thus, the mechanisms of action likely involve protein-protein interactions, which most probably occur on the β-propeller domains of these proteins (Gorrell 2003). No ligand of DP8 or DP9 is known. The multifunctional aspect of these molecules both as enzymes and as interacting proteins highlights the need to understand the structure of these proteins (Gorrell, Yu 2004). It also suggests that specific enzyme inhibitors of the DPIV family might not influence cell-ECM interactions.

Many cytoplasmic events are involved in cell-ECM interactions causing changes to cell behaviour, so it is possible that cytoplasmic DP8 and DP9 influence such events. For example, integrin activation can be controlled by signaling pathways that involve protein-protein interactions (Hynes 2002). For example, nischarin is a cytoplasmic molecule that interacts with the cytoplasmic tail of integrins and thus influences cell migration (Alahari *et al* 2000). Cytoskeletal changes were not observed in cells overexpressing DP8 or DP9, so these proteins probably do not directly bind to the actin cytoskeleton. However, the observed decreases in DP9 overexpressing cells of the ECM interacting molecules DDR1, a kinase activated by collagen binding, and the MMP inhibitor TIMP2 suggest possible DP9 target pathways.

DP9 mRNA is ubiquitous and highly expressed in tumors (Ajami *et al* 2004). The reduced migration by DP9 overexpressing cells towards collagen I and fibronectin in transwells suggests that DP9 may reduce cell migration in tumors and injured liver. Thus, a function of increased DP9 expression may be to retain expressing cells in the tumor and in sites of expression in injured liver. Alternatively, by reducing ECM adhesion DP9 could be pro-invasive because the plasma membrane needs to release from the ECM during cell proliferation and tumor growth. It would be interesting to localize the DP9 expressing cells in tumors and cirrhotic liver.

5. CONCLUSIONS

The biological significance of the new DPIV family members DP8 and DP9 is unknown. In order to obtain correlations between cell behaviors and peptidase expression levels, DP8 and DP9 overexpression in transfected cells was quantified by expressing green fluorescent protein fusion proteins. We found that, like DPIV and FAP, cells overexpressing DP8 and DP9 exhibit behavioral changes in the presence of ECM components. We demonstrated that these effects were independent of enzyme activity, and of the RGD motif that occurs in DP9. This study is the first indication of some similarities as well as differences between DP8, DP9, DPIV and FAP in their cell biological roles.

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CHAPTER 8

IN VIVO EFFECTS OF A POTENT, SELECTIVE DPPII INHIBITOR

UAMC00039 is a possible tool for the elucidation of the physiological function of DPPII

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1. INTRODUCTION

The presence of proline residues gives unique structural features to peptide chains, influencing the susceptibility to protease cleavage (Vanhoof *et al* 1995). Previously dipeptidyl peptidase (DPP) IV was thought to be the only mammalian peptidase to cleave Xaa-Pro dipeptides from the N-terminus of peptides at neutral pH. Later, the existence of several DPPIV-like enzyme activity-bearing molecules has been noted. Among them are fibroblast activation protein α (FAPα), DPP8, DPP9 and DPPII. They are sometimes referred to as the 'DPPIV activity- and/or structure-homologues' (DASH) (Sedo *et al* 2001). More distant members include prolyl oligopeptidase (POP) and acylaminoacyl peptide hydrolase (ACPH). These proteases have important roles in regulation of signalling by peptide hormones, and their inhibitors have an interesting therapeutic potential in diabetes, oncology and other indications (Rosenblum *et al* 2003).

DPPIV (EC 3.4.14.5) is ubiquitously expressed in mammalian tissues and exists in both a membrane-bound and soluble form. At neutral pH it selectively removes N-terminal dipeptides from peptides with a penultimate Pro or Ala. DPPIV has received considerable attention since it truncates many bio-active peptides of medical importance. It is involved in glucose homeostasis through proteolytic inactivation of glucagon family peptides. DPPIV inhibitors are in phase III clinical trials for treatment of type 2 diabetes (Deacon *et al* 2004). The functions of DPPIV were recently reviewed (Lambeir *et al* 2003).

DPPII (EC 3.4.14.2) is a vesicular protease that also releases N-terminal dipeptides from oligopeptides with Pro or Ala in the penultimate position but preferably at acidic pH. We have recently reported kinetic data for a series of chromogenic and some fluorogenic dipeptide substrates of DPPII (Maes *et al* 2005). Natural substrates and the physiological role of DPPII still have to be elucidated.

DPPII and DPPIV are both distributed in a variety of mammalian tissues and they are closely related with respect to their catalytic mechanism and primary substrate specificity. In order to investigate the DPPII function, it is useful to develop potent and highly selective DPPII-inhibitors. Whereas inhibitors of DPPIV (reviewed in Augustyns *et al* 1999) are already known for some time, the development of inhibitors for the related enzymes like DPPII and DPP8 has only started recently (Senten *et al* 2002; Jiaang *et al* 2005). Structure-activity investigations starting from aminoacyl pyrrolidines and piperidines as lead compounds resulted in the development of γ-aminosubstituted analogues of 1-[(S)-2,4-diaminobutanoyl]piperidine as highly potent and selective inhibitors of DPPII (Senten *et al* 2004). The inhibitor *N*-(4-chlorobenzyl)-4-oxo-4-(1-piperidinyl)-1,3-(*S*)-butane-diamine dihydrochloride (UAMC00039) has an IC_{50} of 0.48 $\pm$ 0.04 nM and a high selectivity for DPPII compared to DPPIV ($IC_{50} = 165 \pm 9$ μM) and DPP activity not caused by DPPII or DPPIV (= DPP activity in cell homogenates after the removal of DPPII and DPPIV by affinity chromatography). Therefore, UAMC00039 seemed a promising tool to unravel the function of DPPII as well as to validate its potential as a therapeutic target.

In vivo efficacy and oral availability are important issues in the preclinical development of enzyme inhibitors. Therefore we tested this inhibitor *in vivo* using different routes of administration and different species. In a second step, the compound was evaluated in an *in vivo* pharmacology and toxicology profiling including central nervous system, cardiovascular and metabolism assays in rats and mice.

2. MATERIALS AND METHODS

2.1. Materials

N-(4-chlorobenzyl)-4-oxo-4-(1-piperidinyl)-1,3-(S)-butane-diamine dihydrochloride (M.W. 382.5 g/mol) (Fig. 1) was synthesised as described before (Senten *et al* 2004).

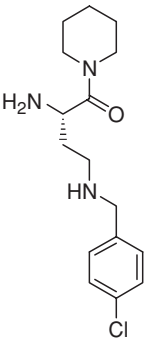

Figure 1. The DPPII inhibitor *N*-(4-chlorobenzyl)-4-oxo-4-(1-piperidinyl)-1,3-(*S*)-butane-diamine dihydrochloride (UAMC00039)

Lys-Ala-p-nitroanilide (-pNA) and Gly-Pro-pNA were obtained from Bachem. Bovine serum albumin (BSA) and cacodylic acid were from Sigma. All other chemicals were obtained from ICN Biomedicals, Inc.

Reference agents were obtained from Sigma, USA except for Clonidine HCl (RBI, USA), Dibucaine HCl (CIBA, USA), Dimaprit HCl (Tocris Cookson, UK), Propofol (AstraZeneca, UK), Carrageenan (Tokyo Chemical Industry Co, Japan), Cyproheptadine HCl (RBI, USA), Devazepide (Merck, USA), ethanol absolute (Merck, Germany), Evans blue (Fluka Chemie A.G., Switzerland), Morphine HCl (National Bureau of Controlled Drugs, R.O.C.), Ondansetron (Glaxo Wellcome Australia Ltd., Australia), Oxotremorine (Aldrich, USA), Pentagastrin (Peninsula, USA), Pentobarbital sodium salt (TCI, Japan), Pinacidil (Lilly, USA), Rauwolscine HCl (Tocris Cookson, UK) and WEB2086 (BoehringerIngelheim, Germany). Assay kits were purchased from Wako, Japan.

2.2. Oral Availability and Efficacy Tests

2.2.1. Animals

Male Swiss mice (∼0.023 kg), male Wistar rats (∼0.46 kg) and male CLO New Zealand White rabbits (∼4.00 kg) were obtained from Charles River Laboratories, Inc. The experiments were carried out following the guidelines of the local Ethical committee for animal experiments (University of Antwerp).

2.2.2. Administration of the Test Compound and Blood Sampling

The inhibitor was administered IV (intravenously) to the rabbits and by oral gavage to the rats using a 2 mM inhibitor solution in 50 mM sodium-phosphate buffer pH 7.4 (0.5 ml/kg). The mice received the compound by oral gavage in 2% Tween 80 at 10 ml/kg. Before oral administration of the compound, the mice were fasted for 24 hours with water ad libitum. Control animals received an equal volume of vehicle alone.

Blood samples were taken in the auricular artery of the rabbits and the tail vein of the rats at different time points after the administration of the compound. In the mice, blood was collected from the abdominal vena cava after anaesthesia. After centrifugation of the blood (1000× g, 15 minutes) the resulting serum was used to measure the enzyme activities.

2.2.3. Tissue Preparation

Three to five hours after a last dose of inhibitor was given, euthanasia of the rabbits was performed using 100 mg/kg pentobarbital and euthanasia of the rats was evoked by carbon dioxide asphyxiation. The mice were anaesthetised with diethyl ether and exsanguinated from the abdominal vena cava two hours after administration of the compound. The organs were collected, washed briefly in PBS and stored at −80°C until use. For measurement of enzyme activities, tissues were homogenised (Polytron (PT1200)-Aggregate, Kinematica AG, 25000 rpm) at 4°C in PBS containing 1% aprotinin and 1% octylglucoside. The homogenate was centrifuged at 1000 × g (20 min, 4°C) and the supernatant then collected and centrifuged twice at 18000 × g (10 min, 4°C). The resulting supernatant was used to measure the enzyme activities and the protein concentration.

2.2.4. DPPII and DPPIV Activity Assays

Enzyme activity was determined kinetically during 10 minutes at 37°C by measuring the initial velocities of p-nitroaniline release (405 nm) from the substrate using a Spectramax plus microtiter plate reader (Molecular devices).

DPPII enzyme activity was determined using Lys-Ala-pNA (1 mM) in 0.1 M cacodylic acid-NaOH buffer pH 5.5 containing 10 mM EDTA and 14 μg/ml aprotinin. DPPIV activity was determined using 0.5 mM Gly-Pro-pNA in 0.05 M Tris buffer pH 8.3.

One unit of enzyme activity was defined as the amount of enzyme that catalyses the release of 1 μmol p-nitroaniline from the substrate per minute under assay conditions.

2.2.5. Protein Concentration

Protein content was determined according to Bradford with BSA as the standard (Bradford 1976).

2.3. Pharmacology and Toxicology Profiling

2.3.1. Animals

Male or female ICR mice, Wistar and Long Evans rats and Duncan Hartley guinea pigs were provided by MDS Pharma Services-Taiwan Ltd. The experiments were performed by MDS Pharma Services in general accordance with the International Guiding Principles for Biomedical Research involving Animals (CIOMS Publication No. ISBN 92 90360194, 1985).

2.3.2. Treatment with the Test Compound

The compound was administered orally at 2 mg/kg (∼5 μmol/kg in a vehicle of 2% tween 80, 10 ml/kg) on a blind basis in all *in vivo* assays. For each assay, a reference compound and vehicle control was analysed concurrently. For the *in vivo* studies 3 to 5 animals per condition were tested.

For *in vitro* tissue assays, the inhibitor was dissolved in DMSO and diluted in the bath to a final concentration of 30 μM (0.1% DMSO).

The parameters measured experimentally are presented in Table 1 and Table 2 together with their corresponding literature reference.

3. RESULTS

3.1. Efficacy and Oral Availability Tests

3.1.1. Inhibition Kinetics of DPPII in Serum

Upon single IV bolus administration of the inhibitor (1 μmol/kg) to rabbits, the serum DPPII levels fell to less than 5% of the initial value (pretest) within 15 minutes (data not shown) and normalisation of DPPII activity was seen after 2 days. Reducing the dose to 0.2 μmol/kg resulted in a slightly less potent inhibition suggesting dose dependency. A second and third

Table 1. Methods for *in vivo* pharmacology and toxicology profiling. The compound was administered orally at ~5 μmol/kg on a blind basis in all assays.

Assay	Species	Reference compound	Reference
Maximum tolerated dose, autonomic signs, mortality	mice		Irwin 1968
Body temperature	mice	10 mg/kg chlorpromazine	Gylys 1963
Depression, behaviour	mice	10 mg/kg chlorpromazine	Irwin 1968
Motor coordination, Roto-Rod	mice	30 mg/kg chlorpromazine	Dunham 1957
Motor stimulation	mice	10 mg/kg DL-amphetamine	Irwin 1968
Respiration, rate and depth	mice	30 mg/kg diazepam, 10 mg/kg DL-amphetamine	Irwin 1968
Bleeding time	mice	100 mg/kg Aspirin	Dejana 1979
Cardiovascular, postural hypotension tilt response	rats	0.3 mg/kg prazosin	Lee 1982
Mydriasis	mice	10 mg/kg mecamylamine	Blackman, 1956
Cholesterol, normal serum, (total, HDL, triglyceride)	mice	10 mg/kg dexamethasone	Neuworth 1970
Hepatotoxicity	mice	300 mg/kg acetaminophen	Sippel 1990
Renal function (K^+ excretion, Na^+ excretion, urine volume increase or decrease)	rats	10 mg/kg hydroflumethiazide, 1 mg/kg pinacidil	Lipschitz 1943
Glucose, serum	fasted mice	3 mg/kg glibenclamide, 0.01 mg/kg clonidine	Root 1959
Gastric, acidity, basal increase	rats	30 mg/kg dimaprit	Hagiwara 1984
Gastric, irritation	rats	150 mg/kg aspirin	Cashin 1977
Gastrointestinal motility	mice	30 mg/kg metoclopramide, 100 mg/kg atropine	Omusu 1988
Analgesia, Phenylquinone writhing	mice	100 mg/kg aspirin	Siegmund 1957
Analgesia, Tail flick	mice	20 mg/kg morphine	D'Amour 1941
Cholinergic agonism, central/peripheral	mice	1 mg/kg oxotremorine	Lippman 1977
Cholinergic antagonism, central/peripheral	mice	10 mg/kg atropine	Lippman 1977
Convulsions, Maximal electroshock	mice	30 mg/kg diphenylhydantoin	Chen 1954
Convulsions, Metrazole	mice	1 mg/kg diazepam	Chen 1954
Sleep time, Hexobarbital potentiation	mice	10 mg/kg chlordiazepoxide	Turner 1965
Dopamine agonism/antagonism	non-climbing mice	1 mg/kg apomorphine, 1 mg/kg haloperidol	Protais 1976
Motor stimulation	mice	10 mg/kg DL-amphetamine	Irwin 1968
Serotonin 5-HTP potentiation	mice	30 mg/kg fluoxetine	Lippman 1977
Adenosine A_1 antagonism	rats	10 mg/kg theophylline	Gardner 1994
Adrenergic α_1 antagonism	mice	10 mg/kg prazosin	Freundt 1965
Adrenergic α_{2A} agonism	rats	0.1 mg/kg clonidine	Trolin 1975
Adrenergic α_{2A} antagonism	rats	10 mg/kg rauwolscine	Trolin 1975
Adrenergic β_1 antagonism	mice	30 mg/kg propranolol	Trolin 1975
Cardiac arrhythmia, Aconitine	mice	100 mg/kg quinidine	Nwangu 1977
Cardiovascular, blood pressure and heart rate (1, 2, 4 hrs)	spontaneously hypertensive rats	0.1 mg/kg clonidine	Yen 1978
Androgenic agonism	immature rats	1 mg/kg × 5 testosterone (for 5 consecutive days)	Hirotsu 1988

(*cont.*)

Table 1. (*Continued*)

Assay	Species	Reference compound	Reference
Androgenic antagonism	immature rats	3 mg/kg × 5 cyproterone (for 5 consecutive days)	Hirotsu 1988
Cholesterol, serum, (total, HDL, total/HDL ratio), diet-induced	mice	100 mg/kg × 3 bezafibrate (on days 5, 6 and 7)	Schurr 1976
Estrogenic agonism	immature mice	0.03 mg/kg × 3 β-estradiol (for 3 consecutive days)	Hirotsu 1988
Estrogenic antagonism	immature mice	1 mg/kg × 3 tamoxifen (for 3 consecutive days)	Hirotsu 1988
Glucose, serum, glucose-loaded	fasted mice	1 mg/kg glibenclamide	Ho 1979
Potassium channel [$K_{(ATP)}$] agonism/antagonism	fasted mice	30 mg/kg diazoxide, 3 mg/kg glibenclamide	Wilson 1988
Allergy, passive cutaneous anaphylaxis	rats	1 mg/kg cyproheptadine	Goose 1969
Histamine H_1 antagonism	rats	3 mg/kg cyproheptadine	Leibowitz 1984
Inflammation, Carrageenan	fasted rats	150 mg/kg aspirin	Winter 1962
Platelet activating factor antagonism	mice	10 mg/kg WEB-2086	Billah 1990
Cholecystokinin, CCK_A antagonism	fasted mice	0.03 mg/kg devazepide	Lotti 1989
Cholecystokinin, CCK_B agonism/antagonism (PO)	fasted rats	30 mg/kg histamine, 10 mg/kg cimetidine	Lotti 1986
Gastric ulcers, ethanol	fasted rats	300 mg/kg carbenoxolone	Robert 1979

injection of the compound after 5 and 40 days respectively resulted in the same profound DPPII inhibition and comparable recovery kinetics. In the same serum samples, the DPPIV activity was not inhibited. The effects of the inhibitor on the DPPII and DPPIV activity in serum are summarised in Fig. 2.

To check oral availability, the compound was given by oral gavage to mice (0.2, 1 and 5 μmol/kg) and rats (1 μmol/kg). Using our standard DPPII assay, the serum DPPII levels in

Table 2. Methods for *in vitro* pharmacology profiling. The inhibitor was tested at a concentration of 30 μM.

Assay	**Species**	**Reference compound**	**Reference**
cardiac inotropy in field stimulated left atria	guinea pigs	0.05 μM isoproterenol	Erjavek 1965
cardiac chronotropy in spontaneous beating right atria	guinea pigs	0.05 μM isoproterenol, 10 μM dibucaine	Black 1972
contractile agonism or antagonism of KCl-induced contractions of aorta, ileum, trachea (basal tone) and portal vein	rats guinea pigs rats	60 mM KCl and 0.03 μM nifedipine 0.1 μM nifedipine 1.6 μM epinephrine (no KCl) 0.1 μM nifedipine	Auget 1989; Paton 1968; Wasserman 1977; Hamilton 1986

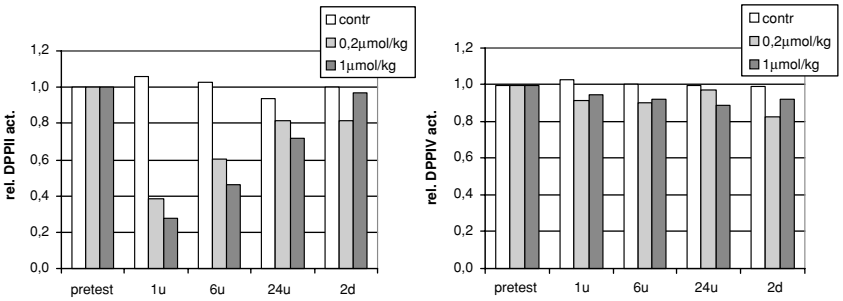

Figure 2. DPPII and DPPIV activity in the serum upon intravenous administration of compound UAMC00039 to rabbits. Blood samples were taken at the indicated timepoints after the injection of the compound or vehicle alone (control animal). The relative enzyme activity is given with the pretest value of each animal considered as 1. In the left panel the relative DPPII activity is shown and in the right panel the relative DPPIV activity

mice could not be detected. In the rats serum DPPII activity fell to less than 30% of the initial value within 1 hour after the administration.

The IV administration to rabbits and the PO (per os) administration to mice and rats up to a dose of 1 µmol/kg and 5 µmol/kg respectively, did not result in any observable acute toxicity.

3.1.2. Inhibition of DPPII and DPPIV in Different Organs

Two to five hours before euthanasia the animals received a last dose of compound. Specific activities of DPPII and DPPIV were determined in a number of organs including kidney, liver, spleen, lung, heart and brain. A dose dependent inhibition of DPPII but not of DPPIV was observed in the peripheral organs of both the rats and the mice (after oral administration) and the rabbits (after IV administration) (results not shown). The results of the experiments with the rats and mice are shown in Fig. 3.

3.2. Pharmacology and Toxicology Profiling

The assays performed in this study were selected to detect possible pharmacological activities in the animals treated with the DPPII inhibitor and in a number of important tissues (Table 1 and Table 2).

The compound tested orally at 2 mg/kg did not cause signs of acute toxicity and did not cause any significant changes in the following functions that were evaluated: general behaviour, body temperature, respiration, bleeding time, blood pressure, urine volume, liver function, fasting glucose and gastrointestinal parameters like acidity, motility and irritation. Also in the following mouse and rat assays no statistically significant effect could be observed: analgesia in phenylquinone (PQ) induced writhing and radio-heat induced tail flick in mice, central and peripheral cholinergic agonism and antagonism in mice, anti-convulsion activity versus maximal electroshock and metrazole induced seizures in mice, potentiation in hexobarbital induced sleep time in mice, dopamine agonism or antagonism in mice, motor stimulation in mice, serotonin potentiation in mice, adenosine A_1 antagonism in rats, adrenergic α_1 antagonism in mice

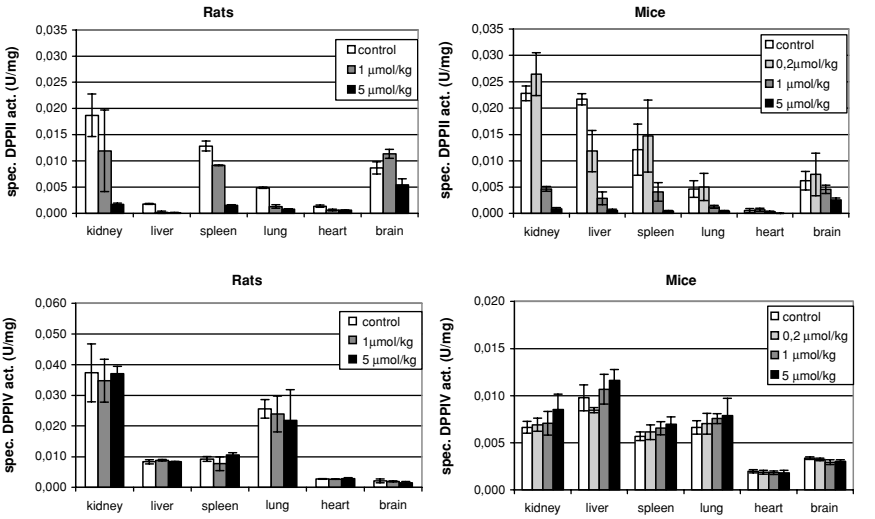

Figure 3. DPPII and DPPIV specific activity in the peripheral organs after oral administration of compound UAMC00039 to rats and mice. The data represent the mean ± standard deviation (rats n = 2, mice n = 3) for each condition. The control animal received vehicle alone. The upper panels show the specific DPPII activity; the lower ones the specific DPPIV activity in the rats (*left*) and the mice (*right*).

(no mydriasis was noted either), adrenergic α_{2A} agonism or antagonism in rats, adrenergic β_1 antagonism in mice, aconitine-induced cardiac arrhythmia in mice, androgenic agonism or antagonism in rats, estrogenic agonism or antagonism in mice, serum glucose in glucose loaded mice, potassium channel [$K_{(ATP)}$] agonism in mice, allergic passive cutaneous anaphylaxis (PCA) or histamine H_1 antagonism in rats, anti-inflammatory activity versus carrageenan-induced rat paw edema in rats, platelet activating factor (PAF) antagonism in mice, cholecystokinin CCK_A antagonism in mice, cholecystokinin CCK_Bagonism in rats, protection from ethanol-induced gastric ulcers in rats, serotonin 5-HT_3 antagonism in mice.

The compound tested at 30 µM in vitro did not exhibit significant activity (>50% change) in the following tissue assays: cardiac inotropy in field stimulated left atria, cardiac chronotropy in spontaneous beating right atria, contractile agonism or antagonism of KCl-induced contractions of aorta, ileum, trachea (relaxation of spontaneous tone) and portal vein.

4. DISCUSSION

Since the natural substrates and the physiological role of DPPII still have to be elucidated and since DPPII and DPPIV are very similar in distribution, catalytic mechanism and primary substrate specificity, there is a need for a potent and highly selective DPPII-inhibitor which can be used in *in vivo* experiments. This preliminary *in vivo* study of the potent DPPII inhibitor *N*-(4-chlorobenzyl)-4-oxo-4-(1-piperidinyl)-1,3-(*S*)-butane-diamine dihydrochloride (UAMC00039) demonstrates its *in vivo* efficacy and oral availability without evidence for acute toxicity. There was a dose-dependent inhibition of DPPII activity without affecting the DPPIV activity in the serum of rats and rabbits and in the peripheral organs tested in mice, rats and

rabbits. The high selectivity of the inhibitor will enable to differentiate between DPPII and DPPIV in biological systems and allows further investigation of the physiological function of DPPII and of the therapeutic potential of its inhibitors.

The control animals of these experiments reveal information on the tissue distribution of DPPII and DPPIV. Highest specific activities of DPPII were detected in the kidney and the spleen, whereas the heart and serum were rather poor sources. The DPPIV activity was highest in the kidney and the lung, which was also described by other authors (Mentlein 1999). In previous studies in literature, the kidney and the spleen were also shown to be rich DPPII sources (McDonald *et al* 1968a; Gossrau *et al* 1980). The ubiquitous distribution of DPPII is also reflected in the multiple sources that have been used for DPPII purification in literature (McDonald *et al* 1968; Hopsu-Havu *et al* 1970; McDonald *et al* 1980; Fukasawa *et al* 1983; Eisenhauer *et al* 1986; Mentlein *et al* 1989; Lynn 1991; Sentandreu *et al* 2001). As can be seen in Fig. 3, the inhibition of DPPII in the brain was less pronounced.

In a second step, the compound was evaluated (oral administration, 5 μmol/kg) in an *in vivo* pharmacology and toxicology profiling including central nervous system, cardiovascular and metabolism assays in rats, guinea pigs and mice. The inhibitor did not cause signs of acute toxicity and did not cause significant changes in the functions tested (Table 1). Even though not statistically significant, administration of the DPPII inhibitor at 5 μmol/kg did cause a decrease of 46% in sodium excretion and an increase of 149% in potassium excretion relative to the vehicle control. Repetition of this type of renal function tests using more animals and different species is necessary to definitely evaluate the influence of compound UAMC00039 on Na^+ and K^+ excretion, especially since DPPII expression among the different organs is highest in the kidney (McDonald 1968a; Gossrau 1980; Gossrau 1991; Araki 2001). Off-target effects of the compound can not be excluded in this preliminary investigation.

There is some inconsistency in literature concerning the localization of DPPII in the nephron. Gossrau et al. showed a high DPPII activity in the lysosomes of the proximal renal tubules (Gossrau 1980), while Araki et al reported intense staining of DPPII in the epithelial cells of distal and collecting tubules (Araki *et al* 2001). It is presumed that all kinds of peptide and propeptide hormones are endocytosed especially by proximal tubular cells and degraded by peptidases in their lysosomal system (Gossrau *et al* 1983). On the other hand, since DPPII is only believed to cleave very short peptides like tripeptides (Eisenhauer *et al* 1986; McDonald *et al* 1968b; Mentlein *et al* 1989; Brandt *et al* 2005 elsewhere in this volume), DPPII may have a role in amino acid homeostasis. Renal assimilation of short chain peptides plays an important role in systemic protein metabolism and amino acid homeostasis (Groneberg *et al* 2002). Furthermore, proximal tubular cells possess specific transport systems for di- and tripeptides. The peptidases and the peptide transport system in the renal brush border membrane play a significant role in the reabsorption of peptide-bound amino acid as well as in the regulation of plasma levels of small peptides (Ganapathy *et al* 1986).

In 1999 Chiravuri et al. observed that inhibition of QPP, shown to be identical to DPPII (Araki *et al* 2001; Leiting *et al* 2003; Maes *et al* 2005), led to cell death in resting lymphocytes (Underwood *et al* 1999; Chiravuri *et al* 1999). One can question the selectivity of the inhibitor ValboroPro used in these experiments. The molecular targets of ValboroPro in the hematopoietic system were identified as DPPIV and fibroblast activation protein α (FAPα) (McIntyre *et al* 2004). While Chiravuri et al excluded DPPIV as the target, since both $CD26^-$ and $CD26^+$ T cells were susceptible to the ValboroPro-induced cell death, FAPα involvement was not investigated. In 2004 Lankas et al. tested a series of inhibitors with similar pharmacokinetic profiles but with differing selectivity against DPPIV, DPP8/9 and DPPII in rat toxicity studies and in acute

tolerability studies in dogs (Lankas *et al* 2004). In rats, the DPPII inhibitor caused reductions in reticulocytes at high doses; in the dogs no acute effects were observed. The structure of the inhibitor used was not specified.

The selectivity of an inhibitor towards related enzymes may be important in predicting its side effects profiles. In a preliminary study, inhibition of DPP8/9 has been linked to profound toxicity, including anaemia, multiple histological pathologies and mortality (Lankas *et al* 2004). Compound UAMC00039 used in this study showed high selectivity for DPPII compared to DPPIV and DPP activity not caused by DPPII or DPPIV. Furthermore, the lack of significant pharmacological effects in this screening suggests that no severe side effects like e.g. cardiovascular effects are to be expected when this inhibitor will be investigated as a therapeutic tool in the future. However we should keep in mind the restrictions of this preliminary investigation. It is possible that effects are only seen when the compound is administered repeatedly during longer periods or when larger groups of animals are used. Moreover, certain therapeutic effects only become clear when specific disease models are used.

Even though the physiological role of DPPII couldn't be elucidated by this study, compound UAMC00039 proved to be a useful tool for further *in vivo* studies aimed at unravelling the role of DPPII in physiology and as a therapeutic target.

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CHAPTER 9

EXPRESSION OF DIPEPTIDYL PEPTIDASE IV-LIKE ENZYMES IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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1. INTRODUCTION

In the last years, inhibitors of DP IV-like activities became candidate substances for the treatment of several diseases. Thus, dipeptidyl peptidase IV (DP IV/CD26) and DP IV-like enzymes grew to interesting proteins not only for the scientific, but also for the pharmaceutical and medical communities.

Enzymes with DP IV-like activity are characterized by removing N-terminal dipeptides from peptides with proline or alanine in the penultimate position. DP IV-like enzymes are differentially distributed in the cell compartiments (Fig. 1). DPP2, DPP8 and DPP9 are intracellularely localised whereas DP IV, FAP and attractin are ectoenzymes existing in membrane-bound and secreted forms. From the latter, DP IV is expressed on the surface of T cells, B cells and NK cells and was discussed to play an important role during antigen-presentation and T cell activation. Inhibition of DP IV with Lys[Z(NO_2)]-thiazolidide (I49) and -pyrrolidide (I40) and a series of inhibitory Met-Trp-Pro- peptides suppresses proliferation of activated T cells in a way involving the immunosuppressive cytokine TGF-β1 (Kähne *et al* 1999).

Beside DP IV, attractin was described to be expressed on the surface of T cells and to be involved in interactions between T cells and monocytes (Duke-Cohen *et al* 1998). Although attractin is not structurally related to DP IV it exerts the similar substrate and inhibitor specificities as DP IV.

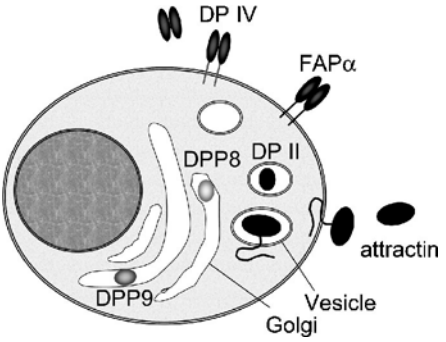

Figure 1. Schematic drawing of a cell demonstrating the localisation of enzymatically active DP IV-like proteins. For further details see text. DP II = dipeptidyl aminopeptidase II, DP IV = dipeptidyl aminopeptidase IV, DPP8 = dipeptidyl aminopeptidase 8, DPP9 = dipeptidyl aminopeptidase 9, FAPα = fibroblast-activating protein α.

Some years ago, monocytes were reported to express a DP IV-like enzyme activity on the cell surface though the monoclonal anti-CD26 antibodies TA1 and CB.1 neither bind monocytes nor inhibit the DP IV-like activities of monocytes (Bauvois *et al* 1992). To elucidate the nature of this DP IV-like activity we examined the expression of DP IV-like enzymes in human peripheral blood monocytes in the present study.

2. MATERIAL AND METHODS

2.1. Monocyte Preparation

Peripheral blood mononuclear cells (PBMC) were prepared from heparinised blood of healthy volunteers (Reinhold *et al* 1993). From these cells monocytes were enriched by counter-current centrifugal elutriation (Wahl *et al* 1984). Separated monocytes were resuspended in AIM-V serum-free culture medium. Staining and flow cytometric analysis of monocytes was carried out by using standard procedures. 90 $\pm$ 5% of these cells were positive for CD14.

2.2. Determination of Gly-Pro-pNA Hydrolysing Enzymes

Gly-Pro-pNA hydrolysing activities were determined according to the method described previously (Schön *et al* 1984). Briefly, separated peripheral blood monocytes were seeded in 48 well plates. After 2 h adhesion non-adherent cells were withdrawn and adherent cells were preincubated with 50 μM Lys[Z(NO_2)]-thiazolidide for 10 min at 37 °C. The enzymatic reaction was started by addition of Gly-Pro-pNA to a final concentration of 1.5 mM. After 2 h of incubation at 37 °C the samples were analysed at 392 nm. Cell viability was tested using CellTiter Blue assay (Promega, Mannheim, Germany) and by estimating LDH release. The effect of I49 on attractin activity was examined using purified human plasma attractin (Friedrich *et al* 2003).

2.3. Preparation of Total RNA from Monocytes and PBMC

Total RNA was isolated from cells using Nucleospin RNA II kit (Machery-Nagel, Düren, Germany). For RNA isolation, freshly prepared monocytes were pelleted and stored with lysis buffer RA1 at −80 °C. Frozen samples were thawed slowly and treated according to the Nucleospin RNA II purification protocol. For reverse transcription to cDNA, RNA was incubated with AMV reverse transcriptase.

2.4. RNA Amplification

For attractin mRNA detection the primers TGGCTCATTGAAGGAC-AGCC and CAGTTGTCTGTACAGTGAGG, were used. PCR was performed with HotStarTaq (Qiagen, Hilden, Germany).

3. RESULTS

Human peripheral blood monocytes hydrolysed 1.5 ± 0.5 pkat Gly-Pro-pNA/10^6 cells compared to 4.0 ± 0.5 pkat/10^6 cells for PBMC (data not shown). The Gly-Pro-pNA- hydrolysing enzyme activity doubles during culture for 72 h independently of monocyte activation with LPS (1 μg/ml). It was completely inhibited by 50 μM of the DP IV inhibitor I49 (Lys[Z(NO_2]-thiazolidide) (Fig. 2). Cell viability of monocytes was not impaired by I49 in concentrations up to 200 μM (data not shown).

To elucidate the nature of the DP IV-like activity of monocytes, we examined the surface expression and the mRNA levels of the enzymatically active DP IV-like proteins. Flow cytometric analysis of monocytes from different donors (n = 8) revealed an attractin cell surface expression of 81 ± 12%, whereas CD26 was barely detectable on monocytes (Fig. 3).

Attractin expression of monocytes was also observed on transcript level (Fig. 4).

Summarising, we identified attractin expression on monocytes and observed inhibition of Gly-Pro-pNA hydrolysis by the DP IV inhibitor I49. This implies that I49 could be able to inhibit attractin. To prove this, we measured kinetics with purified plasma attractin and found

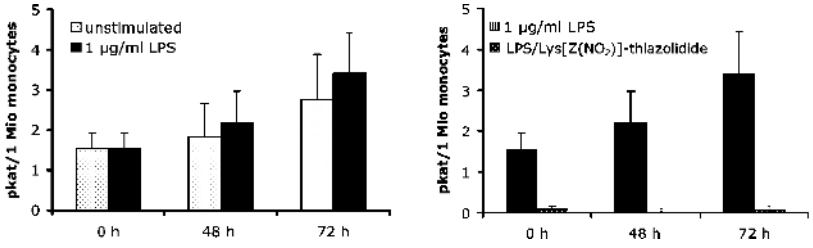

Figure 2. Gly-Pro-pNA hydrolysis of human peripheral blood monocytes: By elutriation isolated monocytes were further enriched by adhesion onto plastic surface. After 2 h non-adherent cells were removed and adherent cells were incubated with 1 μg/ml LPS and 50 μM Lys[Z(NO_2)]-thiazolidide for the time points indicated. Extracellular DP IV-like enzyme activities were measured by addition of Gly-Pro-pNA to the medium. Values are given in mean ± SD. Shown are results of 9 independent experiments.

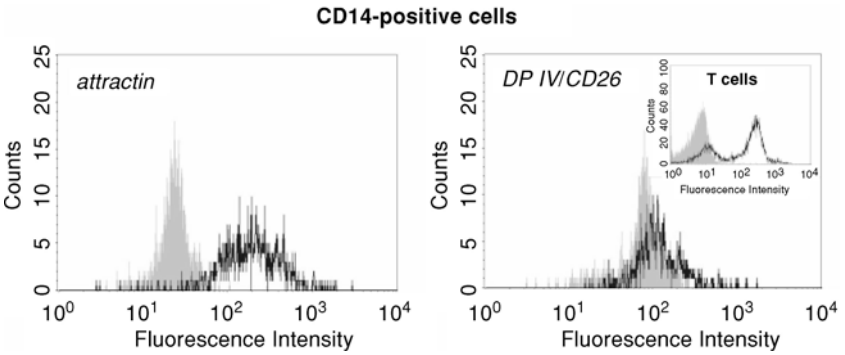

Figure 3. Attractin but not DP IV/CD26 is expressed on the cell surface of monocytes: Flow cytometric analysis of CD14-positive PBMC with anti-attractin and anti-CD26 monoclonal antibodies. The insert shows CD3-positive cells stained with anti-CD26 for positive control. Shown is one representative experiment of 8 experiments.

that I49, indeed, is a good inhibitor of attractin-catalysed Gly-Pro-pNA hydrolysis ($IC_{50} = 8.45 \cdot 10^{-9}$ M).

Using I49 in LPS-induced monocyte cultures, we observed increased TNF-α, IL-6 and TGF-β1 secretion, whereas the release of IL-1RA decreased down to control levels (data not shown). The modulation of monocyte cytokine production is a first indication for the influence of synthetic DP IV inhibitors, like I49, on monocyte function.

4. CONCLUSION

Peripheral blood monocytes hydrolyse the extracellularely administered DP IV substrate Gly-Pro-pNA. Thus, monocytes are a potential target of DP IV inhibitors administered for therapeutic intervention. The DP IV-like enzyme activity of monocytes is inhibited by the DP IV inhibitor Lys[Z(NO_2)]-thizolidide (I49). However, DP IV surface expression on monocytes could not be detected. Instead, monocytes express the DP IV-like enzyme attractin on the cell surface, which we demonstrated on protein and transcript levels. With purified human plasma attractin, we observed that the DP IV inhibitor I49 is not DP IV-specific but also inhibits

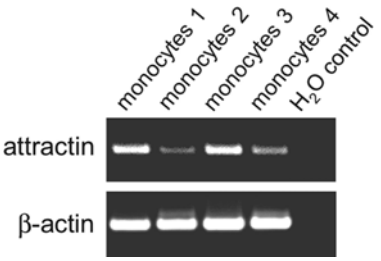

Figure 4. Determination of attractin transcript in human peripheral blood monocyte preparations of 4 different donors.

attractin-catalysed Gly-Pro-pNA hydrolysis. Incubation of LPS-stimulated monocytes with I49 modulates TNF-α, IL-6, IL-1ra and TGF-β1 cytokine release indicating an important role of attractin in the regulation of peripheral blood monocyte functions and the involvement of attractin in I49-mediated effects on monocyte functions.

Thus, monocytes have to be taken into account as a potential target for DP IV-inhibiting therapeutics. Further studies are planned to examine which beneficial or harmful monocyte reactions would arise.

ACKNOWLEDGMENTS

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CHAPTER 10

DIPEPTIDYL PEPTIDASE 8 HAS POST-PROLINE DIPEPTIDYL AMINOPEPTIDASE AND PROLYL ENDOPEPTIDASE ACTIVITIES

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1. INTRODUCTION

Very few proteinases can cleave the prolyl bond. Post-proline cleavage is often involved in changing the biological activity of a peptide substrate and has regulatory roles in many processes (Gorrell 2005). In the prolyl oligopeptidase (POP) gene family, dipeptidyl peptidase (DP) IV (EC 3.4.14.5), fibroblast activation protein (FAP), DP8, DP9 and prolyl endopeptidase (PEP; EC 3.4.21.26) have this catalytic property. The sixth enzyme of the POP family is N-acylaminoacyl peptide hydrolase (ACPH; EC 3.4.19.1), which removes an acylated N-terminal amino acid such that the peptide product has a free NH_2 terminus (Kobayashi, Smith 1987). Important DPIV substrates include chemokines, neuropeptides and incretins. Substrates of PEP include peptide hormones and neuropeptides such as substance P, oxytocin, vasopressin and angiotensin (Cunningham, O'Connor 1997).

We proposed that inhibitors thought to be DPIV specific might inhibit other enzymes of the DPIV gene family (Gorrell 2005). DPIV can hydrolyse a post-proline bond only when it is the penultimate residue at the N terminus of a substrate, so synthetic substrates are H-Xaa-Pro derived, optimally, H-Gly-Pro. In contrast, PEP can hydrolyse post-proline but is an endopeptidase unable to cleave if a free α-amine lies N-terminal to the proline. DP8 transfected mammalian cells and DP8 partially purified from such cells show that DP8 has DPIV activity (Abbott *et al* 2000, Qi *et al* 2003). Based on primary structure DP8, DP9 and FAP have been placed in the DPIV subfamily of the POP family (see chapter by Gorrell *et al*).

The crystal structures of DPIV, FAP, PEP and ACPH show two domains; one an α/β hydrolase domain and the other a β propeller domain that has seven blades in PEP and ACPH and eight blades in DPIV, DPL1 and FAP (Aertgeerts *et al* 2005, Bartlam *et al* 2004, Fülop *et al* 1998, Rasmussen *et al* 2003, Strop *et al* 2004). The structure of DP8 is unknown. DP8, PEP and ACPH are nonglycosylated, soluble, cytoplasmic proteins.

Here we report the purification, biochemical characterization, substrate specificity and inhibitor selectivity of recombinant DP8 and DPIV proteins expressed in the human embryonic kidney 293 epithelial cell line and by baculovirus-infected Sf9 cells. Interestingly, DP8 exhibited PEP activity as well as DPIV activity. The irreversible DPIV inhibitor ValboroPro inhibited both activities. These data indicate that DP8 is a multifunctional enzyme and that therapeutics based on DPIV inhibition should be counter-screened against DP8.

2. MATERIALS AND METHODS

Spodoptera frugiperda (Sf-9) cells, pMelbac transfer vector, BacNBlue linear DNA, Cell-FECTIN, anti-6xHis antibody and Sf-900 II medium were from Invitrogen (Gröningen, The Netherlands). Fluorogenic (AFC; 7-Amido-4-trifluoromethylcoumarin) and chromogenic (p-NA; p-nitroanilide) enzyme substrates and the PEP inhibitor benzyloxycarbonyl-ProProlinal acetal (Z-ProProlinal) (Compound 5) were from Bachem (Bubendorf, Switzerland). Ni-NTA resin was from Novagen (Madison, WI). Sepharose blue, Sephadex, DEAE and CM resins were from Pharmacia Biotech (Uppsala, Sweden).

For purification of DPIV, solid (NH4)2SO4 was added to insect cell culture supernatant, discarding the 40% cut and retaining the 80% saturation precipitate. This DPIV was applied to Sephadex G100 equilibrated with 10 mM sodium phosphate pH 7.6 containing 200 mM NaCl. Nickel resin was incubated with active fractions then washed with 10 mM sodium phosphate pH 7.6, 200 mM NaCl, 5 mM imidazole. The DPIV protein was eluted with 10 mM sodium phosphate pH 7.6, 200 mM NaCl, 150 mM imidazole.

The purification of DP8 was performed by CM cation exchange chromatography, Blue Sepharose chromatography and DEAE Sephacel anion exchange chromatography. CM Sepharose was equilibrated with 10 mM sodium phosphate pH 5.5. Bound protein was eluted by increasing salt concentration. Active fractions were applied to Blue Sepharose then DEAE Sephacel, both equilibrated with 10 mM sodium phosphate pH 7.6. Bound proteins were eluted from DEAE Sephacel by increasing the NaCl concentration stepwise to 200 mM.

DP8 activity was determined as described previously (Abbott *et al* 2000, Ajami *et al* 2004). DP-II assay used H-LysPro-pNA at pH 5.5 (Leiting *et al* 2003). Enzyme kinetics was measured at 37°C using chromogenic substrates in 0.1 M phosphate buffer pH 7.4 read at 405 nm. The steady state kinetic data were analyzed by non linear regression using Prism 4 (GraphPad Software, San Diego) or GraFit 5 (Erithacus Software, Surrey). The steady state parameters, kcat = Vmax/[E] and Km, were determined from initial velocity measurement, which were determined by non-linear fit to the Michaelis-Menten equation. The turnover number kcat was calculated using molecular masses of 101.4 kDa for DP8 and 88.3 kDa for DPIV.

The reversible competitive DPIV inhibitor diprotin A (IleProIle; compound 1) and (w-N-(O-acyl)hydroxy amid) aminodicarboxylic acid pyrrolidide (compound 4) were purchased (Merck, Whitehouse Station, NJ). The DPIV inhibitors amino boronic dipeptide Val-boro-Pro (Snow *et al* 1994) (compound 2) and 1-[[(3-hydroxy-1-1adamantylamino]acetyl]-2-cyano-(S)-pyrrolidine (Villhauer *et al* 2003) (compound 3) were synthesized following the published methods.

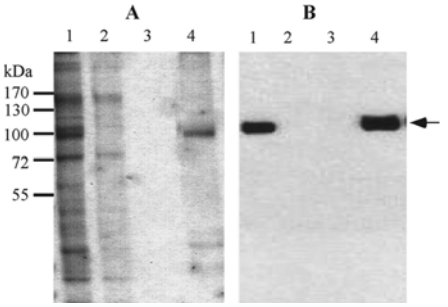

Figure 1. Purified recombinant human DPIV. A. SDS-PAGE. B. Immunoblot with anti- 6xHis antibody. DPIV monomer is arrowed. Lanes: 1) sample loaded onto Ni sepharose 2) initial flow-through 3) wash 4) protein eluted from nickel-sepharose with 150 mM imidazole.

3. RESULTS

3.1. Expression of DP8 and DPIV

Human DP8 and DPIV each with a C-terminal 6xHis tag were expressed in Sf9 cells using high titre recombinant virus stocks (5–10 x 10^8 pfu). The yield of purified DP8 was about 0.2 mg/litre and of DPIV 1.5 mg/litre cell culture supernatant. Cell culture supernatant containing DP8 retained activity for more than 5 months at 4°C. The purified 6xHis-DPIV was the expected molecular weight of 110 kDa (Fig. 1). The purification yield of DPIV was about 17% and specific activity 23,430 mU/mg.

Very little 6xHis-tagged DP8 bound to nickel sepharose or to anti-6xHis antibody in immunoblot so DP8 purification used ion exchange chromatography, giving 13% yield and 100-fold purification. The fractions of DP8 protein were shown by silver staining to include highly purified, 100 kDa DP8 (Fig. 2). There were no detectable contaminating proteins at the 80 kDa size of PEP. The DP8 specific activity was 621 mU/mg on H-AlaPro-pNA but 2,673 mU/mg on Z-GlyPro-pNA. Purified DP8 was stored in 20% glycerol at −20 °C.

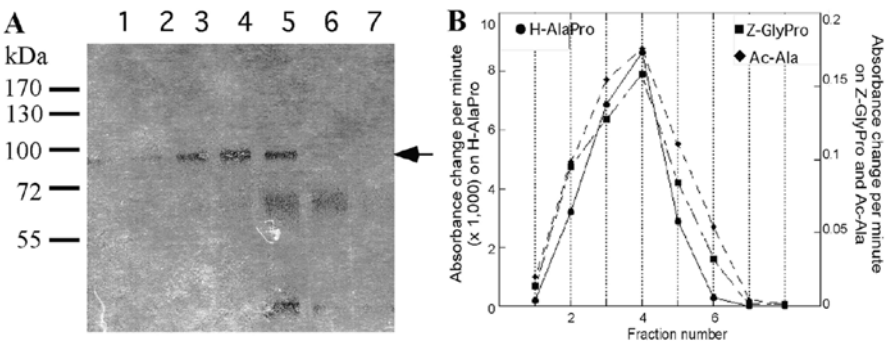

Figure 2. Anion exchange chromatography of recombinant human DP8. A. Silver stained 8% SDS-PAGE of fractions eluted from DEAE Sephacel. Arrow indicates intact DP8. B. Activities of eluted fractions.

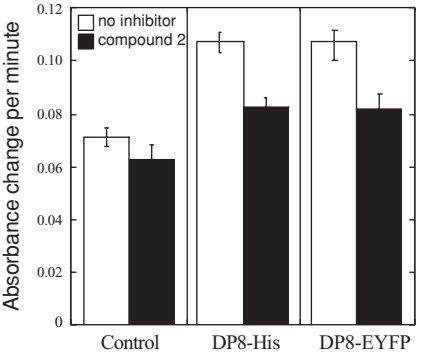

Figure 3. ACPH activity in recombinant DP8 containing cell culture supernatants. Cell culture supernatant from uninfected Sf9 cells or Sf9 cells expressing DP8-His or DP8-EYFP fusion protein was coincubated with ValboroPro at 100 μM before adding the ACPH substrate 0.8 mM Ac-Ala-pNA.

3.2. DP8 Peptidase Activities

Both DP8-6xHis and DP8-EYFP insect cell culture supernatants exhibited ACPH activity (Fig. 3). Purified DP8 exhibited tripeptidyl peptidase activity using H-AlaPhePro-pNA, ACPH activity using Ac-Ala-pNA and PEP activity using Z-GlyPro-pNA, Z-AlaPro-pNA or 3-Carboxy-propionyl-AlaPro-pNA (succinyl-AlaPro-pNA) (Table 1, Fig. 4). Confirming the DP8 endopeptidase activity, DP8 exhibited normal saturation kinetics with Z-GlyPro-pNA as a substrate and the pentapeptide Z-GlyProLeuGlyPro-OH demonstrated dose responsive inhibition (Fig. 4). Further suggesting that DP8 is foremost an endopeptidase, the catalytic constant (*k*cat) of DPIV was more than 10-fold greater than those of DP8 on dipeptidyl aminopeptidase substrates but the DPIV *k*cat on H-AlaPro-pNA was comparable to that of DP8 on Suc-AlaPro-pNA. Furthermore, DPIV had greater specificity constants (*k*cat/*K*m) than DP8 and the DP8 specificity constants were tenfold more on endopeptidase than dipeptidyl aminopeptidase substrates.

Table 1. DPIV and DP8 kinetics. Mean ± standard deviation. n = 3.

	Purified protein			Transfected cells	
Enzyme and Substrate (-pNA)	*K*m (μM)	*k*cat (sec^{-1})	*k*cat/*K*m (sec^{-1} mM^{-1})	*K*m (μM)	*V*max δA/min (x1000)
DPIV					
H-GlyPro	177 ± 28.5	30.9 ± 1.3	175 ± 18	347 ± 88	7.2 ± 0.49
H-AlaPro	130 ± 42.1	25.9 ± 2.0	199 ± 0.03	374 ± 134	9.6 ± 1.0
DP8					
H-GlyPro	924 ± 25.9	1.8 ± 0.2	1.98 ± 0.2	467 ± 64	12.4 ± 0.9
H-AlaPro	595 ± 18.2	1.2 ± 0.13	1.98 ± 0.3	991 ± 171	3.5 ± 0.14
H-AlaPhePro	122 ± 22.4	3.1 ± 0.13	26 ± 1.8		
Z-GlyPro	321 ± 54.3	4.8 ± 0.26	15 ± 0.7		
Z-AlaPro	312 ± 22.3	6.5 ± 0.17	21 ± 1.5	308 ± 249	1.67 ± 0.39
Suc-Alapro	312 ± 22.3	13.8 ± 0.5	33 ± 0.9		
Ac-Ala	517 ± 68.8	37.7 ± 2.3	73 ± 2.8		

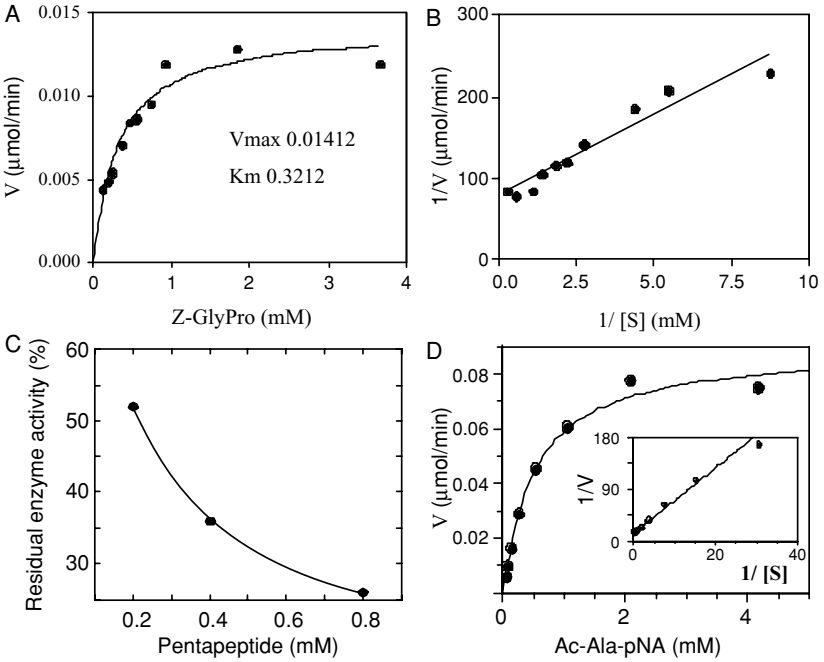

Figure 4. Kinetics of the DP8 PEP and ACPH activities. PEP substrate Z-GlyPro-pNA shown in Michaelis-Menten (A) and Lineweaver Burke (B) plots. C. Dose-response inhibition by the pentapeptide Z-GlyProLeuGlyPro-OH using 0.4 mM Z-GlyPro-pNA. D. Michaelis-Menten and Lineweaver Burke (inset) plots of ACPH activity. pH 7.6 Tris buffer.

Little hydrolysis of ArgPro and AspPro derived substrates by DP8 was observed. Neither DP8 nor DPIV displayed detectable DP-II activity.

Purified DPIV demonstrated enzyme activity over a broad pH range from 4.7 to 8.8, similar to previous reports. Purified DP8 was active above pH 6.5 and more active above pH 7.5 (Fig. 5A). Purified DP8 showed similar pH optima of about 8.3 on the PEP and DPIV substrates (Fig. 5B).

3.3. Inhibition Profile

The PEP and DP activities of DP8 were inhibited to similar extents by ValboroPro, shown by similar IC_{50} values (Table 3). Both ValboroPro and the PEP inhibitor Z-prolyl-prolinal inhibited the PEP activity of DP8. When ValboroPro or LAF237 were titrated on DP8 cell culture supernatant the IC_{50} data were similar to those obtained from purified DP8.

A reversible competitive DPIV inhibitor diprotin A inhibited DPIV but not DP8 (Table 2). DPIV inhibitor Val-boro-Pro inhibited enzyme activity of cell derived DPIV and DP8 with almost complete inhibition at 1 µM (Table 2) and purified DPIV was significantly inhibited (IC_{50} 6 nM) with complete inhibition at 10 µM. Purified DP8 was inhibited by ValboroPro (IC_{50} 10 µM) (Fig. 6a).

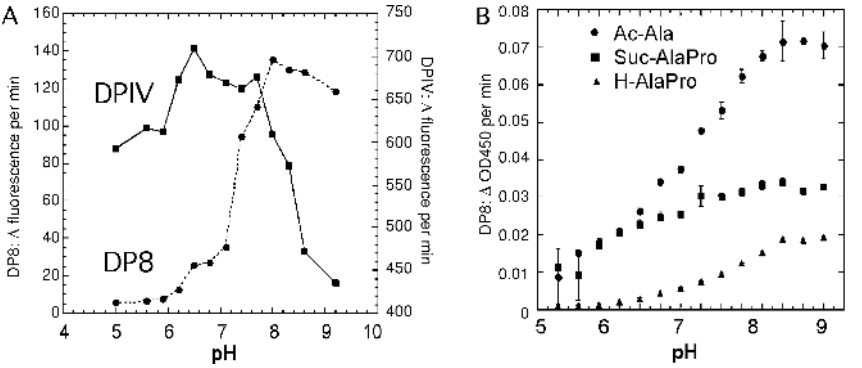

Figure 5. pH dependence of DP8 and DPIV enzyme activity. Purified proteins were used. A. 1 mM H-AlaPro-AFC with DP8 and DPIV. B. DP8 with 0.8 mM H-AlaPro-pNA, 0.8 mM Suc-AlaPro-pNA or 0.8 mM Ac-Ala-pNA to detect DPIV, PEP or ACPH activity respectively.

The selective DPIV inhibitor LAF237 inhibited cell derived DPIV (IC_{50} 600 nM), with complete inhibition at 5 μM. Cell derived DP8 was less sensitive to this inhibitor (IC_{50} 5 μM), with almost complete inhibition at 50 μM (Fig. 7). Purified DPIV showed similar results to the cell derived DPIV (IC_{50} 600 nM), with almost complete inhibition at 10 μM. In contrast, LAF237 did not inhibit purified DP8 (Fig. 6b).

Table 2. Inhibition profiles of DP8 and DPIV. Substrate 0.3 mM H-AlaPro-AFC, or 0.7 mM Suc-AlaPro-pNA if marked with an asterisk.

	Purified protein		Transfected cells	
	DP8	DPIV	DP8	DPIV
$IC_{50}(\mu M)$				
Compound 1	>100	93		
	*>100			
Compound 2	10	0.006	4	0.2
	*22			
Compound 3	>100	0.6	5	0.6
	*>100			
Compound 4	>100	153		
	*>100			
Compound 5	*41	>100		
% Residual Activity				
2 mM $ZnSO_4$	25	10	25	
	*26			
2 mM $MgCl_2$	66	56		
	*85			
2 mM $MgSO_4$	96	54		
	*87			
$CaCl_2$ (2 mM)	71	53		
	*83			
4 mM AEBSF	40	52		
0.4 mM Pentapeptide	*37			

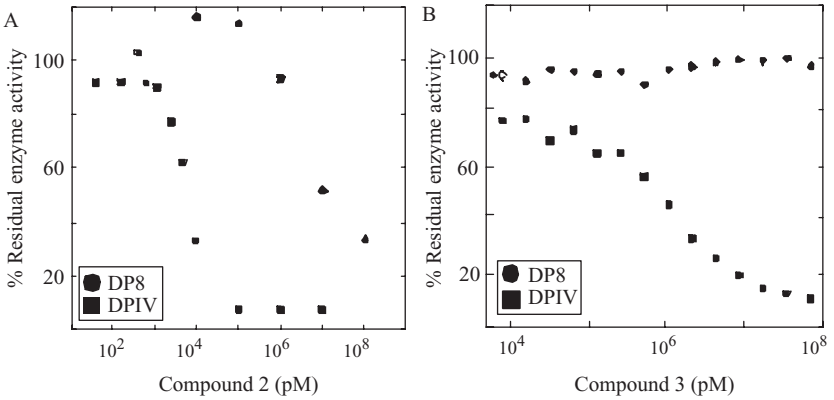

Figure 6. Inhibition of purified DP8 and DPIV. Val-boro-Pro (A) and LAF237 (B) were each incubated with purified proteins prior to assay with the substrate 1 mM H-Ala-Pro-AFC.

Subsequently, a similar purified DP8 preparation was subjected to gel filtration on sephadex G100. That experiment indicated that the observed ACPH activity was separable from the PEP/DPIV activity of DP8. ACPH is usually a tetramer of 80 kDa subunits. This suggests that the ACPH activity described above may be insect cell derived rather than human DP8 derived. Further investigations of this finding confirmed that the ACPH activity described here was not DP8 derived.

4. DISCUSSION

DP8 is a monomeric, soluble, cytoplasmic protein, which are also characteristics of PEP, but has more significant sequence homology with DPIV, FAP and DP9 than with PEP or ACPH (Abbott *et al* 2000, Ajami *et al* 2004). In this study, the full length human DP8 and a soluble form of DPIV (residues 29–766) were expressed by recombinant baculovirus infection of Sf9 insect cells and purified to homogeneity. Surprisingly, substrate and inhibitor kinetics showed DP8 to have more PEP activity than DPIV activity. The DP8 PEP activity was not detected above the high background PEP level in the insect cell culture supernatants. In contrast, subsequently we showed that the PEP and DPIV activities copurified at every chromatographic step (data not shown), so the PEP activity was not acquired during purification and was not separable from the DPIV activity.

Table 3. Inhibition of the three peptidase activities. The % of each peptidase activity remaining following pre- and co-incubation with a known inhibitor of DPIV or PEP.

Compound	Dipeptidyl peptidase H-AlaPro-pNA	Acylaminoacyl peptidase Ac-Ala-pNA	Prolyl endopeptidase Suc-AlaPro-pNA
100 μM ValboroPro	8	58	10
77 μM NVP-LAF237	62	86	69
150 μM Z-ProProlinal	20	85	11

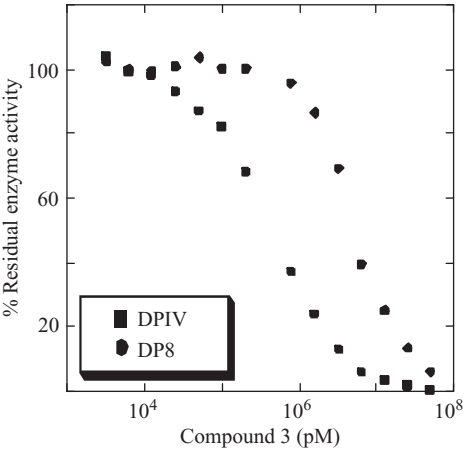

Figure 7. Inhibition of DP8 and DPIV in transfected 293 cells. LAF237 was incubated with permeabilised transfected cells, prior to assay with 1 mM H-Ala-Pro-AFC.

The DPIV and PEP activities of purified DP8 had the same pH optimum and were inhibited by ValboroPro. Moreover, the DP8 was purified to homogeneity at 100 kDa, which is larger than 80 kDa PEP. Furthermore, the DP8 kinetics on PEP substrates differed from published values on PEP. The Z-GlyPro-pNA Km was 321 μM on DP8 but is 24 μM on PEP (Polgár 1992). The DP8 pH optimum of 8.3 differs from that of PEP (Polgár *et al* 1993), which is 7.5. The inhibition of both peptidase activities by a small DP inhibitor, ValboroPro, indicates that the two catalytic activities reside in the same catalytic pocket of DP8.

It is very unlikely that an enzyme can gain catalytic activities by being deformed. Indeed, enzyme activity is routinely used in enzymology to show that a protein is correctly folded. The plasmid vector used to express DPIV and DP8 has the honey bee melittin (HBM) signal sequence at the N-terminus for secretion of recombinant protein into the medium. A 6xHis tag was placed after the C-terminus prior to a termination codon. The small 6xHis tag is unlikely to alter the biological activity of the expressed protein (Dobers *et al* 2002). Indeed, the specific activities of baculovirus (Table 1) and mammalian cell expressed DPIV (Oravecz *et al* 1997) are similar.

Curiously, the 6xHis tag on DP8 did not bind to metal-resin or antibody, perhaps because it was lost but probably because it was inaccessible. The C-terminus after the last α-helix is longer in DP8 than in other DPIV family members and this extension contains mostly hydrophobic residues. Therefore, the 6xHis tag or indeed any small tag on DP8 might be buried within the hydrolase domain.

DP8 contains 116 amino acids more than DPIV, so DP8 could have an additional element of tertiary structure at the N-terminus or within the β-propeller domain, such as additional protrusions in blade 4. The DPIV blade 4 protrusions bind adenosine deaminase, contact substrate and assist DPIV dimerization.

It is interesting that, in contrast to DPIV, DP8 inhibition kinetics differed between assays of whole cells and isolated protein. The underlying molecular basis of this phenomenon is unknown. Perhaps a cellular component renders DP8 more susceptible to inhibition. Whether inhibition of natural DP8 in vivo is similar to DP8-transfected mammalian cells or baculovirus-expressed culture supernatant and purified DP8 is unknown.

Potential applications of DP8 peptidase activities include celiac sprue therapy. PEP mediated proteolysis of the predominant immunogen involved in celiac disease, gliadin, depletes its immunogenicity (Piper *et al* 2004) and the PEP activity of DP8 could potentially be useful in a similar fashion. Alternatively, the discovery that substitution of Asp633 for Ala in DPIV confers PEP activity upon DPIV suggests that this modified DPIV has potential in a novel celiac disease therapy. DPIV has the advantages of being naturally expressed on enterocytes and possessing some resistance to the proteolytic gut lumen.

5. CONCLUSIONS

We report an expression and purification procedure to produce homogeneous, active human DP8 and DPIV. The most interesting discovery was that DP8 specifically has both prolyl dipeptidyl aminopeptidase and PEP activities. DPIV inhibitors varied in their selectivity against DP8 but all DP8 activities were inhibited by the irreversible DPIV inhibitor ValboroPro. These data indicate that DP8 is a multifunctional enzyme and that therapeutics based on DPIV inhibition should be counter-screened against DP8.

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CHAPTER 11

PROLYL ENDOPEPTIDASE CLEAVES THE APOPTOSIS RESCUE PEPTIDE HUMANIN AND EXHIBITS AN UNKNOWN POST-CYSTEINE CLEAVAGE SPECIFICITY

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1. INTRODUCTION

Prolyl endopeptidase (PEP; EC. 3.4.21.26) is the name giving enzyme of the peptidase-family S9A, prolyl oligopeptidases, within the clan SC Prolyl endopeptidase. The enzyme acts as an endopeptidase preferentially hydrolyzing the peptide bond on the carboxyl-side of proline, to a lesser extend of alanine residues (Polgar 1994). The ubiquitous distributed PEP belongs to the most abundant brain peptidases (Goossens *et al* 1996; Irazusta *et al* 2002), is highly conserved in mammals, essentially located in the cytosol, (Dresdner *et al* 1982), and involved in secretion processes (Schulz *et al* 2002). The physiological function of PEP was initially attributed to limited proteolysis of several peptide hormones including neuropeptides (Mentlein 1988; Welches *et al* 1993). However, administration of PEP-specific inhibitors on an ischemic rat model or cultured primary neurons led to protection against neuronal cell death or to an effectively deferment age-induced apoptosis, respectively (Shishido *et al* 1999; Katsube *et al* 1999). These results do not rule out the intracellular task of PEP modulating cellular rescue factors such as Humanin (HN). This peptide consists of 24 amino acids with a core of amino acids 3–19 responsible for a rescue function from neuronal cell death. Indeed, the HN core motif covers two potential cleavage sites of the highly specialized serine protease PEP (Figure 1). HN is able to preserve cortical neurons from prion-peptide or Aß (amyloid-ß) induced insults (Sponne *et al* 2004; Hashimoto *et al* 2001), improves impaired metabolic activity and prolongs survival of serum-deprived human lymphocytes (Kariya *et al* 2003).

HN binds to Bax *in vitro* and *in vivo* averting the translocation of Bax to mitochondria. Bax (Bcl2-assoziated X protein) is a member of the Bcl2 protein family. Bcl2 itself is an

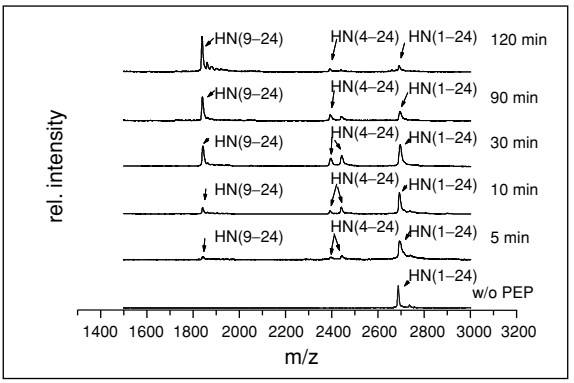


Figure 1. Hydrolysis of HN(1-24) by human recombinant prolyl endopeptidase. MALDI-TOF mass spectra recorded time dependent. In the presence of human recombinant prolyl endopeptidase proteolytic processing of HN(1-24) (MW: 2686.2 Da) to HN(4-24) (2386.6 Da) and HN 9-24 (1837.2 Da) occurs. The 48 Da greater twin peak of HN(4-24) (2386.6 Da) of 2434.7 Da represents the oxidation product of cysteine to sulfonic acid.

anti-apoptotic protein that prevents the release of cytochrome c and other intermembrane space proteins during apoptosis. In contrast, Bax triggers apoptotic mitochondrial protein release (Guo *et al* 2003). While the interaction between HN and Bax was shown, the intracellular homeostasis of the peptide remains unclear. An expression level based regulation or a enzymatic controlled turn-over are not described so far.

2. MATERIAL AND METHODS

2.1. Preparation and Assay Systems Used for Human Prolyl Endopeptidase and Dipeptidyl Peptidase IV

The expression and purification procedure of human PEP will be described elsewhere. Isolation of human recombinant dipeptidyl peptidase IV (DP4[1]) was performed as described by Bär (Bär *et al* 2003).

2.2. HN Degradation Assay

Degradation of 800 μg/ml HN dissolved in 1% DMSO/PEP-buffer was conducted at 37°C in PEP-buffer using 0.004 U/ml PEP. Samples (5 μl) were desalted using $ZipTip_{C18}$ technique (Millipore) and mixed with equal volumes of the matrix solution, sinapin acid, dissolved in 50% acetonitril/H_2O, and crystallized under vacuum. MALDI-TOF mass spectrometry was performed at a LD-TOF system G2025 provided from Hewlett Packard. The instrument was equipped with a 337 nm nitrogen laser, a potential acceleration source (5 kV) and a 1.0 m flight tube. Detector operation was in the positive-ion mode and signals were recorded and filtered using LeCroy 9350M digital storage oscilloscope linked to a personal computer. Calibration of the mass spectrometer was carried out using bovine serum albumin as standard.

[1] We propose here the following new straightforward abbreviations for dipeptidyl aminopeptidases: DPII = DP 2, DPP IV or DP IV = DP 4, DPP 8 = DP 8, DPP 9 = DP 9

2.3. Synthesis of HN

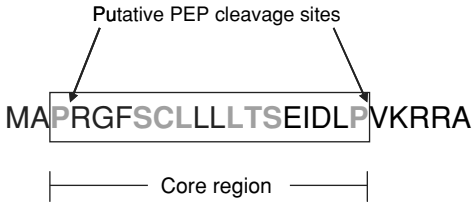
Synthetic HN as well as the inhibitor Fmoc-Ala-Cyanopyrrolidide (CNfmoc) and substrates were synthesized as follows: HN-peptides were produced with an automated synthesizer (Symphony; Rainin Instrument Co., USA) using a modified Fmoc-protocol. Cycles were modified by using five fold excess of Fmoc-amino acids (Novabiochem, Germany) and coupling reagent for 30 min in dimethylformamide (DMF; Roth, Germany). From Leu 18 double couplings were performed. The peptide couplings were performed by 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU; Novabiochem, Germany)/N-methylmorpholine (Merck, Germany) -activation using a 0.25 mmol/g preloaded Fmoc-Ala-NovaSyn TGA resin (Novabiochem, Germany) at 25 μmol scale. Fmoc-deprotection was accomplished with 20% piperidine (Merck, Germany) in DMF. To circumvent difficult couplings caused by the hydrophobic –Leu(9)-Leu(10)-Leu(11)-Leu(12)-sequence within the peptide, pseudo-proline building blocks were successfully incorporated for –Leu(12)-Thr(13)- and –Phe(6)-Ser(7)- (Fmoc-Leu-Thr($\Psi^{Me,Me}$ pro)-OH and Fmoc-Phe-Ser($\Psi^{Me,Me}$ pro)-OH, respectively, Novabiochem, Germany). Removal from the resin was carried out by a cleavage-cocktail consisting of 94% trifluoroacetic acid (TFA; Merck, Germany), 2.5% water, 2.5% ethanedithiol (Merck, Germany) and 1% triisopropylsilane (Fluka, Switzerland).

2.4. Preparation of U-343 Cell Extract

In order to prepare the cytosolic protein fraction of U-343 cells the medium was withdrawn from the cells and covered with 300 μl of PEP-puffer and 1 mM DTT (Dithiothreitol). Afterwards, three freezing (-80°C) and thawing steps were carried out followed by the removal of the cells from the incubation flask. Membrane and organelle fractions were separated and removed by a centrifugation step at 4°C and 3000x*g* for 15 minutes. 0.002 U/ml of the supernatant were used for HN degradation experiments. Inhibitors AEBSF, E64, pepstatin A and bestatin were from Sigma-Aldrich, Germany.

3. RESULTS

HN(1-24) hydrolysis by prolyl endopeptidase was determined by incubating HN-peptide and 0.004 U/ml PEP at 37°C applying an pH of 7.5. MALDI-TOF analysis revealed that HN(1-24) was cleaved in a time dependent manner and disappeared after 120 minutes incubation with PEP. We could detect a product peak with m/z = $[M + H]^+$ of 2387 Da corresponding to HN(4-24) representing the release of the N-terminal tripeptide MAP (Scheme 1).



Scheme 1. Amino acid sequence of Humanin. The core sequence and therein the essential amino acids (bold) to preserve apoptosis prevention as well as the predicted prolyl endopeptidase cleavage sites are shown in the box.

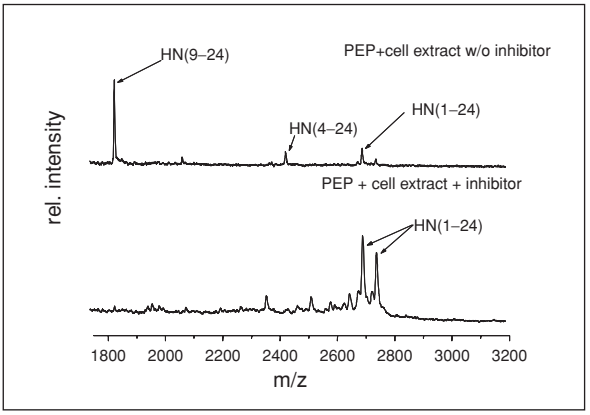


Figure 2. MALDI-TOF mass spectra recorded after 60 minutes incubation of HN(1-24) (MW: 2686.2 Da) and its twin peak (MW: 2734.1 Da, which represents the oxidation product of cysteine to sulfonic acid) together with U-343 cytosomal cell extract containing a PEP concentration of 0.002 U. HN was incubated in presence and absence of the PEP specific inhibitor CNfmoc.

A second product-peak at m/z = $[M + H]^+$ of 1837 Da appeared shortly after 5 minutes of incubation and represents HN(9-24) (Fig. 1). Surprisingly, only the unexpected hydrolysis of the peptide at the carboxy terminus of cysteine(8) could explain the detected mass peak. After 120 minutes incubation time the substrate and the HN(4-24) related peak had vanished and only the HN(9-24) representing peak remained. Thus, a total conversion of HN(1-24) to finally HN(9-24) was catalyzed by PEP (Fig. 1). In contrast to our expectations no product signal relevant for a post-proline cleavage of proline(19) was detectable.

To prove, whether this observation holds true for cell-based systems, a cytosolic fraction from a cell extract of human glial cell line U-343 was prepared. A PEP activity of 0.002 U/ml cell extract, which is comparable with the PEP activity used in the previously described *in vitro* studies was used for the following studies. The cell extract was incubated at 37°C in presence and absence of the specific PEP-inhibitor CNfmoc with 300 μM HN(1-24). It could be demonstrated by MALDI-TOF measurements, that cleavage products of HN(4-24) and HN(9-24) were apparent after 60 minutes (Fig. 2).

In contrast, the processing of HN(1-24) was totally prevented by the presence of the inhibitor. We could further show by applying different protease class specific inhibitors, that turnover of HN(1-24) was only inhibited by the serine protease inhibitor AEBSF (which unspecifically inhibits PEP), but not by cysteine protease inhibitors such as E64, by the aminopeptidase inhibitor bestatin, the aspartate protease inhibitor pepstatin A, or the metalloprotease inhibiting EDTA (results not shown).

In order to clarify, whether other members of the prolyl oligopeptidase family of enzymes may also be cysteine-specific, we investigated DP4. The exopeptidase belongs together with the endopeptidase PEP to the protease family S9. We analyzed whether DP4 as well as PEP processes dipeptide derivative substrates with cysteine in the P_1-position. The obtained kinetic data demonstrated, that the enzymatic specificity for cysteine in P_1-position was even superior to substrates containing alanine in P_1-position (Table 1).

We further asked whether other closely related enzymes, dipeptidyl peptidase 8 (DP8) and dipeptidyl peptidase 9 (DP9) also belonging to the S9 family, posses the so far unknown ability

Table 1. Kinetic parameters of recombinant human prolyl endopeptidase and dipeptidyl peptidase IV determined with enzyme specific substrates possessing either proline, alanine or cysteine in P_1-position

Enzyme	Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
PEP	Ala-Ala-Pro-AMC	19.6 ± 1.8	2.89 ± 0.07	$(147 \pm 4)^*10^3$
	Ala-Ala-Cys-AMC	800 ± 78	0.89 ± 0.04	$(1 \pm 0.03)^*10^3$
	Ala-Ala-Ala-AMC	500 ± 37	2.35 ± 0.07	$(4.7 \pm 0.1)^*10^3$
DP4	Ala-Pro-AMC	19 ± 1	32.3 ± 0.9	$(1680 \pm 130)^*10^3$
	Ala-Cys-AMC	244 ± 9	7.5 ± 0.3	$(30 \pm 0.9)^*10^3$
	Ala-Ala-AMC	550 ± 34	5.7 ± 0.2	$(10 \pm 0.3)^*10^3$

to release peptides after cysteine. As expected we found that both enzymes revealed the same post-cysteine-cleaving features as shown for DP4 and PEP.

In addition, dipeptidyl peptidase II (DP2) which displays a similar specificity as the proline-specific proteases investigated, but belongs to the distinct structural family S28 was also found to hydrolyze peptide bonds on the carboxy-terminus of cysteine (results not shown).

4. CONCLUSION

The data presented, demonstrate for the first time PEP turnover of HN by a limited post-cysteine as well as the expected post-proline proteolysis demonstrated in cell extract resulting in the inactivation of this potentially apoptosis-related factor. These findings lead to the hypothesis of a PEP-mediated control of HN homeostasis maintaining neuronal cell survival. This implicates a novel use of PEP inhibitors potentially preventing intracellular HN digestion. Consequently, PEP-inhibition might be a new target for apoptosis prevention.

This study further uncovered a so far unknown enzymatic specificity for a post-cysteine cleavage of the mammalian exopeptidases DP2, DP4, DP8 and DP9 and the endopeptidase PEP.

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CHAPTER 12

DISTRIBUTION OF DIPEPTIDYL PEPTIDASE IV-LIKE ACTIVITY ENZYMES IN CANINE AND PORCINE TISSUE SECTIONS BY RT-PCR

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1. INTRODUCTION

Dipeptidyl peptidase IV (DP 4[1], CD26, EC 3.4.14.5) is a serine protease that hydrolyses dipeptides from the *N*-terminus of oligopeptides and small peptides with either proline, alanine or to a lesser extent cysteine and serine, at the penultimate position. Substrates include gastric hormones GLP and GIP, neuropeptides endomorphin-1, peptide YY, neuropeptide Y and substance P as well as chemokines RANTES and SDF-1α. Therefore, the enzyme is involved in various physiological and immunological processes such as peptide metabolism, cell adhesion, chemotaxis and T-cell activation. Consequently, DP4 activity is associated with several pathological conditions like diabetes mellitus, arthritis, transplantation rejection, HIV infection, psoriasis, cirrhosis and various forms of cancers (Lambeir *et al* 2003), and for that reason inhibitors have been designed as possible drug candidates (Augustyns *et al* 2003).

Nevertheless, a number of enzymes have been discovered also reported to display DP4 activity (Abbott and Gorrell 2002; Sedo and Malik 2001). These enzymes include DP4, fibroblast activation protein alpha (DP5)(Levy *et al* 1999), dipeptidyl peptidase 8 (DP8) (Abbott *et al* 2000) and dipeptidyl peptidase 9 (DP9) (Olsen and Wagtmann 2002; Qi *et al* 2003), all of them belonging to family S9b; dipeptidyl peptidase II (DP2) (Araki *et al* 2001) alias quiescent cell proline dipeptidase (QPP) (Underwood *et al* 1999) of S28; as well as attractin, of which the mechanism of catalysis has not been elucidated yet (Duke-Cohan *et al* 2004).

Families S9b and S28 belong to the serine protease clan SC, having the catalytic residues in the order of Ser, Asp and His (Abbott and Gorrell 2002). Recently, the crystal structure of

[1] We propose here the following new straightforward abbreviations for dipeptidyl aminopeptidases: DPP I = DP1, DPII = DP2, DPP IV or DP IV = DP4. FAP = DP5, DPL-1 = DP6, DP7 = DP2, DPP 8 = DP8, DPP 9 = DP9, DPL-2 = DP10.

dipeptidyl peptidase 4 has been elucidated, revealing a topology comprised of a *N*-terminal propeller domain and an α/β-hydrolase domain, made up from the *N*-terminal and *C*-terminal portion of DP4 (Engel *et al* 2003; Oefner *et al* 2003; Thoma *et al* 2003). Sequence analysis and site directed mutagenesis suggest similar topology for all members of family S9b (Ajami *et al* 2003). Attractin shows no structural homology to DP4, but contains a CUB domain, a Kelch domain, EGF-like domain, C-type lectin domain and laminin-EGF-like domain (Duke-Cohan *et al* 1998; Duke-Cohan *et al* 2004; Tang *et al* 2000).

DP4, DP5 and some attractin isoforms are cell surface glycoproteins (Levy *et al* 1999; Tang *et al* 2000), while DP8 and DP9 are found in the cytosol (Abbott *et al* 2000; Abbott and Gorrell 2002; Olsen and Wagtmann 2002; Qi *et al* 2003) and DP2 in the secretory vesicles (Chiravuri *et al* 2000a). DP9 contains no transmembrane sequence, two *N*-Glycosylation sites and an Arg-Gly-Asp cell attachment motif (Abbott and Gorrell 2002). Therefore it is believed to be either cytoplasmic or secretory. DP2, like DP4 and DP5 undergoes *N*-glycosylation and is functionally active as a dimer (Chiravuri *et al* 2000b; Chiravuri and Huber 2000). Except for DP5, all dipeptidyl peptidase 4-like enzymes are reported to be expressed ubiquitously (Abbott and Gorrell 2002). DP5 expression is more associated with pathogenic tissues of remodeling like activated stellate cells and myofibroblasts in cirrhotic liver, activated stromal fibroblasts of epithelial tumours and healing wounds as well as embryonic mesenchymal cells (Abbott and Gorrell 2002). In addition to its DP4 activity, DP5 also hydrolyzes gelatin and denatured collagen I (Ghersi *et al* 2003; Kelly 1999). While DP4-like activity organ and tissue distribution has been investigated intensively over the past 30 years, little is known about localization and distribution of the recently discovered new DP4-like enzymes, contributing to this activity. Hence, we initiated a study to investigate tissue distribution and expression of DP4-like enzymes using RT-PCR in different mammalian species.

2. MATERIALS AND METHODS:

2.1. Tissue Isolation

Porcine tissue was obtained from the abattoir at the Physiology Institute of the Veterinary faculty of the University of Leipzig. A $\pm$ 14 month old pig was bolted and exsanguated. Tissue was removed immediately, cut into its sections, shock-frozen in liquid nitrogen and stored at -80° C until required. Canine tissue was obtained from LPT in Hamburg. Beagles were anaethesised, tissue was removed, cut into its sections, shock-frozen, sent via courier on dry ice within 3 hours and stored at -80° C until required. The following sections were analyzed: Kidney cortex, outer stripe and inner stripe of outer medulla of the kidney; duodenum, jejunum and ileum of the small intestine; alveolar sacs and bronchial epithelium of the lung and head, body and tail of the pancreas.

2.2. Enzymatic Assay

DP4 activity was measured spectrophotometrically using the Micro-plate reader SUNRISE TECAN (Crailsheim, Germany) at $\lambda = 405$ nm and 37° C, using 0.4 mM Gly-Pro-pNA·HCl as substrate, buffered in 40 mM HEPES with an ionic strength of 0.125 at pH 7.4. One enzyme unit was defined as the amount of enzyme necessary to hydrolyze one μmol substrate per minute.

2.3. Protein Determination

BCA protein determination assay (Sigma, Deisenhofen) was implemented, using the microtiter plate procedure descibed by (Redinbaugh and Turley 1986).

2.4. Primer Design

Primers were designed with the aid of Vector NTI and ordered from Metabion (Munich). All primers produced PCR fragments that covered at least 3 exon-intron junctions. The templates used for primer design had the following accession numbers: **AY198323** (DP4), **AF221634** (DP8), **AF452102** (DP9), **NM_004460** (DP5), **AF154502** (DP2), **AF106861** (attractin), **AF054837** (Porcine β-actin), **Z70044** (Canine β-actin).

2.5. RNA Isolation and Reverse Transcription

RNA was isolated using TRIzol reagent from Gibco Life Technology. Transcription kit from Gibco Life technology was employed, using Superscript II transcriptase. Except for the random hexamer primer from Promega, all reagents were part of a 3' RACE kit. The protocol was performed according to the description of the 3' RACE kit, using 10 μg of total RNA and 300 ng/μl random hexamer primer.

2.6. PCR

PCR was performed with an Eppendorf PCR mastercycler, using 1 Unit of enhanced Herculase polymerase (Stratagene) and 0.4 nM of each primer. All other reagents and PCR conditions were according to the instruction manual, except for an annealing step of 30 seconds with a $57 \pm 5.5^{\circ}$C temperature gradient.

2.7. Nested PCR

For nested PCR, 1 μl PCR was added to a final 100 μl solution composed of 0.4 nM of each primer, 200 μM NTP mix (Promega), 20 mM Taq polymerase buffer and 2 Units of Taq polymerase (Promega). PCR was performed with an Eppendorf PCR mastercycler, having a denaturation cycle for 2 minutes at 94°C, 35 cycles comprised of a denaturation step for 45 seconds at 94°C, an annealing step for 45 seconds with a $55 \pm 5.0^{\circ}$C temperature gradient and an elongation step for 50 seconds at 68°C, and an extension cycle of 7 min at 72°C. The positive PCR bands were either gel extracted or PCR purified and sent to SeqLab (Göttingen) to verify the sequence.

2.8. Semi-Quantitative PCR

Three sets aliquots per DP4-like enzyme were prepared according to the PCR method described above. However, after 30 cycles, the PCR was put on hold at 80°C and the first set of aliquots was removed immediately, followed by 5 PCR cycles to remove the second set. After additional 5 PCR cycles, the last set of aliquots was removed and analyzed.

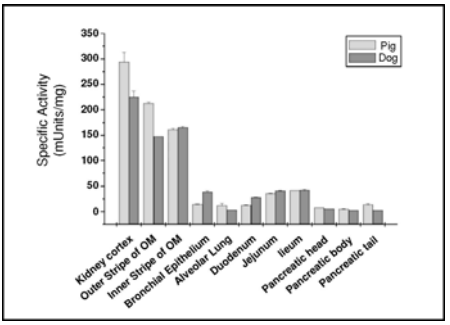


Figure 1. Specific activities of porcine and canine tissue extracts, analysed with 0.4 mM Gly-Pro-pNA. OM, outer Medulla. Error bars represent SEM with n = 3.

3. RESULTS

3.1. Enzyme Activity

The specific activities of DP4 of the various porcine and canine tissue sections can be depicted in Figure 1. It illustrates that most of the specific activity is found in the kidney, decreasing from kidney cortex to outer stripe to inner stripe of the outer medulla. In the small intestine the activity increases from duodenum to jejunum to ileum in both species. Lower specific activities of DP4 between 1.7 mUnits and 9.7 mUnits were found in lung and pancreas tissues from pig and dog respectively, except for canine lung epithelium.

3.2. Primer Design

An overview of the annealing sites of the primers towards their respective templates is given in Figure 2.

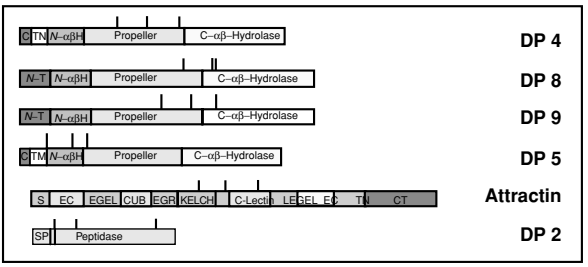
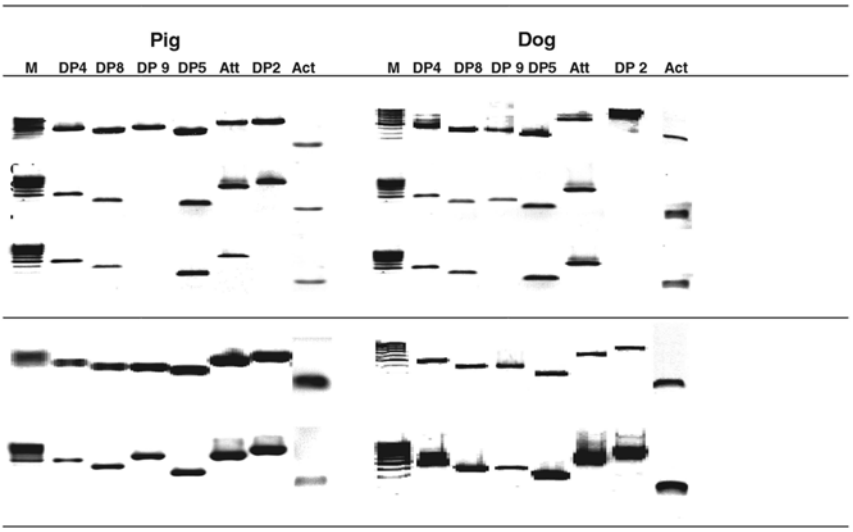


Figure 2. Scheme of primer annealing sites on their respective templates for the various DP4-like enzymes. CT/C = cytoplasmic tail; TM = transmembrane region; *N*-$\alpha\beta$H = *N*-terminal portion of α/β-hydrolase domain; Propeller = propeller domain; C–α/β-hydrolase = C-terminal portion of α/β-hydrolase domain; *N*-T = N-terminus; S = signal peptide; EC = extracellular region; EGF-L = EGF-like domain; CUB = Cub domain; KELCH = KELCH domain; C-Lectin = C-type Lectin domain; LEGFL = Laminin-EGF-like domain; P = propeptide.

Table 1. Results of nested RT-PCR of DP4-like enzymes obtained from porcine and canine tissue sections from lung and kidney.



3.3. Nested RT-PCR

In the kidney, a decrease of expression of DP4-like enzymes is observed from cortex to outer and inner stripe of outer medulla (Table 1). While all DP 4-like enzymes were expressed in the cortex, the outer stripe of the outer medulla lacked DP9 in the porcine and DP2 in the canine tissue section. In the inner stripe of the outer medulla, the number of DP4-like enzymes was reduced to DP4, DP8, DP5 and attractin. Similar to the kidney, the number of DP4-like enzymes decreased in the small intestine from duodenum, jejunum to ileum (data not shown). DP4, DP8, DP9 and attractin were present in all three tissues from both pig and dog, whereas DP5 was only present in duodenum from both species and porcine jejunum (data not shown). A great abundance of DP4-like activity enzymes were expressed in the lung as illustrated in Table 1. All DP4-like enzymes were present in bronchial epithelium and alveolar tissue from dog and pig. Likewise, all DP4-like enzymes except for DP8 were found in the head, body and tail of the pancreas from both animal species (data not shown).

3.4. Semi-Quantitative RT-PCR

The results of the semi-quantitative analysis are shown in Figure 3 and Table 2. It clearly indicates, that there is different relative abundance of expression of DP4-like enzymes. In the kidney cortex, DP4, DP9 and DP2 already appeared at cycle 30 in both species, attractin only in dog. However, after cycle 40 all DP4-like enzymes were present, resembling the pattern of nested RT-PCR. Semi-quantitative PCR analysis of DP4-like enzymes in the small intestine, revealed that DP9 was mostly expressed, detected already at 30^{th} cycle in all tissue sections, whereas signals of DP8 showed up at 30^{th} cycle in porcine duodenum and jejunum and at 35^{th} in the remaining tissue sections. DP4 in turn, gave signals at 35^{th} cycle in the ileum and canine duodenum, whereas jejunum and porcine duodenum had bands at 40^{th} cycle. DP2 was

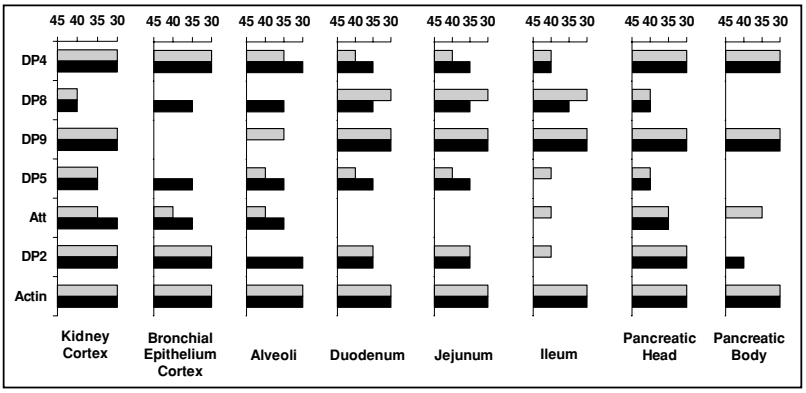
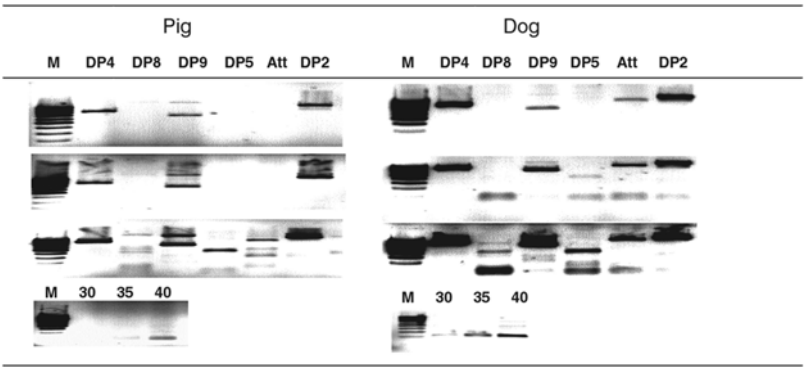


Figure 3. Results of semi-quantiative RT-PCR after 30, 35 and 40 PCR cycles, using transcribed cDNA from various porcine and canine tissue sections and primers specific for DP4, DP8, DP9, DP5, attractin and DP2, respectiviely. □, pig, ■, dog.

Table 2. Semi-quantitative RT-PCR analysis of DP4-like enzymes from porcine and canine kidney cortex after 30, 35 and 40 PCR cycles.



only found in the duodenum and at small levels in porcine jejunum, while only small levels of attractin could be detected in porcine jejunum and ileum. Semi-quantitative PCR in the lung demonstrated that DP4 and DP2 were the most abundant enzymes, except for DP2 in canine alveolar tissue, which expressed DP9 instead. DP4 and DP9 were the most abundantly expressed DP4–like enzymes in the pancreas, followed attractin in porcine pancreas and DP2 in porcine pancreatic head and canine pancreatic body. Generally, Figure 3 implies, that DP4 and DP9 are most abundantly expressed, followed by DP5, attractin and DP2, whereas DP8 appeared to have the lowest mRNA levels.

4. CONCLUSION

Nested RT-PCR revealed the co-localisation of DP4-like enzymes in the tissue sections investigated. Although similar results were obtained with Northern blot analyses (Abbott *et al* 1994; Abbott *et al* 2000; Hildebrandt *et al* 1991; Hong *et al* 1989; Olsen and Wagtmann

2002), the ubiquitous expression of DP5 in healthy tissues could be demonstrated for the first time.

According to semi-quantitative RT-PCR analysis however, the RNA levels of DP5 appeared to be low (Fig. 3). Likewise, the ubiquitous distribution of DP4-like enzymes in lung, pancreas and duodenum (Table 1) could be reduced to certain sets of enzymes showing signals at different cycles as outlined in Fig. 3. Generally, semi-quantitative RT-PCR analysis revealed different relative abundance of expression of DP4-like enzymes, with DP4, DP9 and attractin having the highest expression, followed by DP5, DP2 and DP8 (Fig. 3).

In contrast, nested RT-PCR in the kidney and small intestine showed that DP4-like enzymes were highly associated with specialized microstructures of a particular tissue sections (Table 1), since nested RT-PCR is highly sensitive and would detect a single molecule. Especially in the kidney, it was shown that the number of DP4-like enzymes decreased from the cortex to the outer stripe to the inner stripe of the outer medulla, corresponding to the decrease of microstructures.

While the highest specific activity of DP4 (Fig. 2) in the kidney cortex agrees with the presence of all DP4-like enzymes determined semi-quantitatively (Table 2 and Fig. 3), the lower number, but high specific activity in the medulla tissue would suggest a contribution to the overall activity by only few DP4-like enzymes. Similarly, the decrease in number of DP4-like enzymes (Table 1) and concurrent increase of specific activity from duodenum, to jejunum to ileum (Fig. 1) implies either a dominant DP4-like enzyme or post-transcriptional regulation of expression.

Post-transcriptional regulation of DP4-like enzymes were reported for DP5 (Abbott and Gorrell 2002), attractin (Tang *et al* 2000) and DP4 in the lung (Hildebrandt *et al* 1991) due to their difference of their expression at mRNA and protein level. Association of DP4 activity and protein expression with highly differentiated tissues has already been confirmed by histochemical activity and immunohistochemical analysis of DP4 (Dikov *et al* 1999; Hartel-Schenk *et al* 1990), implying important physiological functions of the DP4-like enzymes. Surprisingly, very few species-related differences of the expression of DP4-like enzymes could be detected between pig and dog.

The study might help, identifying specific sites for the action of selective inhibitors by discriminating DP4-like enzymes and therefore may contribute to the understanding of a distinct pharmaco-dynamic response to the application of drug compounds *in vivo*.

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TOPIC III

METABOLIC DISORDERS

CHAPTER 13

RELATIVE CONTRIBUTION OF INCRETINS TO THE GLUCOSE LOWERING EFFECT OF DP IV INHIBITORS IN TYPE 2 DIABETES MELLITUS (T2DM)

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1. INTRODUCTION

The incretin effect, augmentation of insulin release via enterally absorbed carbohydrates, is mediated by the intestinal hormones GIP (glucose-dependent insulinotropic polypeptide) and GLP-1 (glucagon-like peptide-1). The majority of insulin secretion following a mixed meal is thus stimulated by GIP and GLP-1, resulting in appropriate and rapid disposal of circulating sugars. The use of incretins directly as anti-diabetic agents was an optimistic prospect, however, early data showed little therapeutic promise because of lack of efficacy and reduced beta cell responsiveness to incretins in T2DM. The discovery that dipeptidyl peptidase IV was responsible for the rapid degradation and inactivation of both incretin hormones partly explained the earlier failures in their direct application, but provided additional possibilities for harnessing the incretin effect as a therapy for diabetes. Thus, enzyme-resistant synthetic analogues of GIP and GLP-1 behave as "super-agonists" in vivo, and are able to reduce glycaemic excursions in healthy and diabetic models, and in humans for selected compounds. Similarly, proof-of-concept studies in rodents, pigs and dogs have shown that DP IV inhibition is capable of preserving intact biologically active GIP and GLP-1, thus improving glucose tolerance and insulin sensitivity. Data from clinical trials indicate DP IV inhibition to be one of the most promising new drug classes to treat human diabetes and complement existing oral therapies. There is little doubt that incretin-induced insulin release is reduced in T2DM, yet it is presumed that the incretins are involved in the mechanism of action of DP IV inhibition. Results from transgenic mice in which the incretin receptors were ablated, singly or in combination, indicate the glucose lowering effect of DP IV inhibitors is mediated entirely by these two

hormones. The question then becomes which of the two hormones is primarily mediating the effect or are both necessary? Insight can be gained from existing published data, however, in vivo experiments with specific antagonists of the GIP and GLP-1 receptors in combination with DP IV inhibitors will likely resolve this matter in animal models and the human disease state.

2. THE INCRETIN EFFECT

Two concepts emerged from clinical studies in the late 1960s, that examined glycaemic and insulin responses elicited from different routes of glucose administration. The term 'enteroinsular axis' (Unger and Eisentraut 1969) was introduced to describe the intrinsic connections between the intestine and the cells of the endocrine pancreas: (i) direct stimulation by absorbed nutrients, (ii) a neural link between gut and pancreas, and (iii) bloodborne hormones released from gastrointestinal endocrine cells acting on surface receptors of islet cells. Hormones mediating the latter bridge of the enteroinsular axis were subsequently designated as "incretins", provided two criteria were met: intestinal hormone release was stimulated by luminal glucose, and physiological concentrations of these factors potently stimulated insulin release only in the presence of elevated blood glucose (Creutzfeldt and Ebert 1985). The "incretin effect" thus describes the difference between the insulin response to enterally and parenterally administered sugar resulting from release of intestinal incretins (Nauck *et al* 1986).

2.1. GIP and GLP-1

Glucose-dependent insulinotropic polypeptide/gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are now believed to be the dominant, if not the only, hormones that mediate the incretin effect. GIP is released from K-cells and GLP-1 is released from L-cells, enteroendocrine cells of the small intestine. Mature GIP is a 42 a.a. polypeptide, whereas GLP-1 is processed from proglucagon to either a 31 a.a. or 30 a.a. amidated form. The receptors for both hormones have been identified and sequenced, and belong to the family B/class II category of serpentine 7-TM G-protein coupled receptors (Mayo *et al* 2003). Both couple to stimulation of adenylyl cyclase and an increase in [Ca2+]i, as well as pleiotropic signaling cascades. Incretin receptor activation not only acutely regulates β-cell insulin release proportional to the nutrient load, but is also thought to contribute to the plasticity of β-cell function by modulating gene expression and protein translation (Hinke *et al* 2004).

2.2. The Incretin Effect in Type 2 Diabetes Mellitus

From initial observations of the incretin effect in humans, it was evident that it was curtailed in non-insulin dependent diabetes (Perley and Kipnis 1967). The underlying cause of the reduction has since been limited to the combination of two phenomena: inadequate release of GLP-1 from L-cells and blunted end-organ responsiveness to GIP (Holst and Ørskov 2001). Thus, infusion of exogenous GLP-1 produced potent insulin responses from diabetic patients, in contrast to exogenous GIP (Nauck *et al* 1993). β-cell responsiveness to most insulin secretagogues is diminished in T2DM (Kahn and Porte 1988), however, possibly even more so for GIP. Clinical studies suggest that the defect in GIP effectiveness mainly alters the late phase

of insulin release in diabetic patients (Vilsbøll *et al* 2002). The result has been the extensive study of GLP-1 based therapeutics, with little interest in GIP over the last decade. However, recent clinical studies comparing the mode of incretin administration, by continuous infusion versus bolus injection, gave different results for GIP and GLP-1. Bolus doses of GIP gave more pronounced responses than continuous infusion, which produced poor results in both healthy and T2DM patients (Meier *et al* 2004). In contrast, GLP-1 needed to be given as a continuous infusion in order to observe beneficial glycaemic effects, and bolus injections were ineffective (Todd *et al* 1997; Larsen *et al* 2001). Hence, it is unclear whether it is appropriate to compare the efficacy of these two hormones, as differences observed seem to be highly dependent upon the administration protocol.

2.3. Degradation of Incretins

In addition to the circulation dynamics of peptide hormones governed by secretory rate and elimination from the serum via renal filtration and organ extraction, GIP and GLP-1 are degraded by soluble and membrane bound proteases. Parallel studies on the hormones identified the common enzyme, dipeptidyl peptidase (DP) IV as the primary enzyme in serum able to cleave their N-terminal dipeptides, rendering them biologically inactive (Mentlein *et al* 1993; Kieffer *et al* 1995; Pauly *et al* 1996). Secondary contribution of neutral endopeptidase (NEP) in the degradation of GLP-1 has also been observed, however, GIP appears to be a poor substrate for this enzyme Hupe-Sodmann *et al* 1995). Following the discovery of the importance of DP IV in GLP-1 degradation, it was concluded that the requirement for continuous infusion in human experiments was to overcome the rapid inactivation of the hormone by this enzyme (Holst and Ørskov 2001). As both incretins show similar DP IV degradation kinetics in vitro and in vivo (Kieffer *et al* 1995), but GIP responses to continuous infusion in healthy (or diabetic) subjects are less pronounced than bolus injections (Meier *et al* 2004), the initial conclusion regarding GLP-1 may to have been premature.

2.4. Ablation of Incretin Signaling

Following the discovery of GIP, a number of studies were conducted to establish if other incretins existed. Immunoneutralization studies indicated that indeed, other incretins were present in intestinal epithelium (Ebert *et al* 1983) and this was later confirmed by the identification of GLP-1 and its demonstrated bioactivity (Lund *et al* 1981; Weir *et al* 1989). Immunoneutralization experiments were the first attempts to block GIP signaling, resulting in acute glucose intolerance (Ebert and Creutzfeldt 1982. Later, Helodermaditae lizard venom peptide fragment, exendin-4[9-39], was shown to be a relatively specific antagonist of the GLP-1 receptor, with only very weak effects on the GIP receptor (Raufman *et al* 1992; Thorens *et al* 1993; Göke *et al* 1993). Whereas use of truncated fragments of GLP-1 as receptor antagonists has largely met with failure (Montrose-Rafizadeh *et al* 1997), GIP fragments with high binding affinity but low or non-existent agonist activity were the first specific GIP receptor antagonists available (Gelling *et al* 1997; Hinke *et al* 2001; Tseng *et al* 1996). In the meantime, a GIP receptor antibody directed against an extracellular domain of the receptor could be used to effectively block GIP signal transduction (Lewis *et al* 2000), indicating GIP primarily exerts its insulinotropic effect upon the first phase of insulin release, consistent with studies using exogenous hormone (Hinke *et al* 2002). In vivo experiments in rodents have been performed using single incretin

antagonists or a combination, confirming the major contribution to the incretin effect is derived from GIP, although GLP-1 also plays an important role. Additionally, GIP antagonist administration paradoxically reduced the glycaemic excursion in response to intragastric glucose (Tseng *et al* 1999), consistent with the hormone's function to regulate intestinal sugar absorption (Cheeseman and Tsang 1996). Hence, it is unclear whether ablation of GIP signaling may be used to reduce uptake of ingested carbohydrates, or as an anti-obesity agent (Miyawaki *et al* 2002), or how the two effects may be related.

GLP-1 receptor null mice were generated by the Drucker laboratory (Scrocchi *et al* 1996), and have proven invaluable in the identification of the many physiological functions of GLP-1, in addition to its role in postprandial insulin secretion. One complication of the phenotype of these mice, however, is the compensation for the deficit by upregulation of the GIP component of the enteroinsular axis (Pederson *et al* 1998). Later, GIP receptor null mice were similarly generated (Miyawaki *et al* 1999), also showing compensatory effects mediated by GLP-1 (Pamir *et al* 2003). Hence, chronic ablation of incretin signaling may yield different results compared to acute use of receptor antagonists. Interbreeding these two knockout mice strains have generated double incretin receptor knockout (DIRKO) mice, which are significantly more glucose intolerant than single knockout animals (with some evidence of sexual dimorphism), but still do not present overt diabetes (Hansotia *et al* 2004; Preitner *et al* 2004). Thus it is possible that either an unidentified incretin is again compensating for the deficiency in GIP and GLP-1, or alternative modes of compensation are coming into play via other glucoregulatory mechanisms (hormonal and/or neural).

3. INCRETIN BASED THERAPEUTICS

Because of the unique therapeutic potential of incretins, the fact that they are both degraded by the same mechanism has been exploited for the treatment of human diabetes. Two main strategies have been used: synthetic or chemical modifications of the peptides rendering them resistant to DP IV degradation (Hinke *et al* 2003; Green *et al* 2004), and use of selective DP IV inhibitors to preserve the intact bioactive incretins (McIntosh *et al* 2005), both having the same desired endpoint: the restoration of a normal/enhanced incretin effect in T2DM patients (Figure 1). Additional therapeutic applications for these strategies may also emerge. One such speculative use for incretins (native or modified), either alone or in combination with DP IV inhibitors, is for preservation of the integrity of human islets during their isolation and culture for transplantation in type I diabetes mellitus patients. Incretins are well known for rendering islets glucose competent, however, more recent studies also indicate important roles in regulating gene transcription resulting in β-cell expansion/neogenesis and/or protection from apoptosis (Hinke *et al* 2004).

Clearance half-life and delivery are limiting factors for direct incretin based therapies. The peptides require injection unless effective alternative formulations can be produced (oral, nasal or transdermal), although modifications facilitating binding to serum proteins can reduce the frequency of injection (Kieffer 2004). Safety and tolerance of GIP and GLP-1 are advantageous, as the therapy consists of utilising an existing physiological mechanism. However, although reduction in gastric emptying is thought to contribute to reduction in glycaemic excursions for GLP-1, it is also thought to result in the undesirable nausea and vomiting at higher doses (Ritzel *et al* 1995); GIP lacks effects on gastric emptying (Meier *et al* 2004), and thus does not share this drawback.

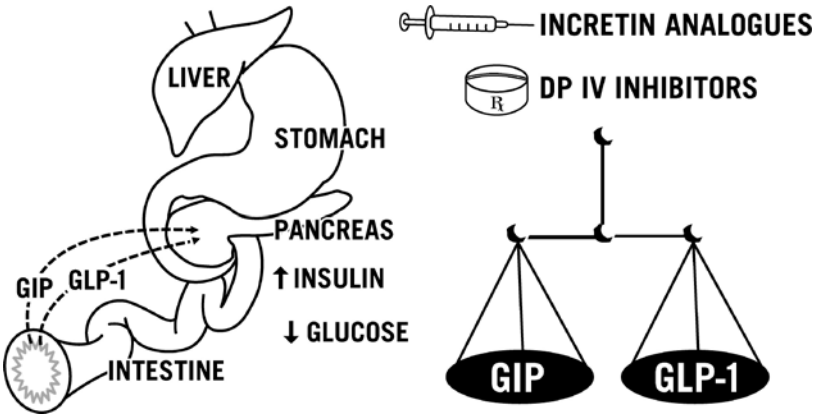

Figure 1. Therapeutic strategies exploiting the enteroinsular axis. Incretin hormones are normally released from the small intestine on ingestion of a mixed meal; they act on the endocrine pancreas to augment nutrient-induced insulin secretion. Oral administration of dipeptidyl peptidase IV inhibitor compounds preserves the N-terminally intact bioactive forms of GIP and GLP-1, allowing a greater proportion to reach target tissues. Injection of GIP and GLP-1 analogues with modifications to render them resistant to DP IV proteolysis can be used as enhanced potency incretin mimetics. It is currently unknown to what degree GIP vs. GLP-1 contributes to the anti-hyperglycaemic effects of DP IV inhibition, nor is the actual therapeutic potential of injectable incretin analogs in the treatment of human diabetes known.

3.1. GIP Analogues

The initial studies showing weak effects of continuous GIP infusion into T2DM patients (Nauck *et al* 1993; Elahi *et al* 1994) all but halted investigation into the potential therapeutic action of GIP in diabetes. A few researchers countered this notion and continued with basic research to address the potential shortcomings of GIP as a therapeutic agent by focusing on the underlying basis for: (i) blunted responsiveness to GIP in T2DM, (ii) its unfavourable pharmacokinetic profile (biological and circulating), (iii) route of administration-dependent differences in effects, and (iv) the activity of small molecular weight bioactive fragments. Similar shortcomings also concern GLP-1 based therapeutics although, because of the greater research interest, many of these issues have been solved. However, there have been many advances in addressing these potential problems for GIP, including critical studies duplicating phenotypic blunted responses in animal models of diabetes and that GIP analogues with improved plasma stability were still effective in these animals (Hinke *et al* 2002; Lynn *et al* 2001). Continued work is needed on the points mentioned above to further enhance the therapeutic potential of GIP, however, given the progress which has been made already, it seems that proof-of-concept studies using these enhanced agents in diabetic patients are required. Pending the outcomes of these studies, the true therapeutic potential of GIP based compounds will be known.

Initially, synthetic analogues of GIP1-42 or GIP1-30 with modifications to amino acids 1, 2, 3, or 4, were synthesized, and the peptides demonstrating the greatest resistance to DP IV, while retaining potency at the GIP receptor, were identified (Kühn-Wache *et al* 2000; Hinke *et al* 2003). Lead compounds were selected for further in vivo testing (Hinke *et al* 2002; Hinke *et al* 2004). In parallel, studies were able to show that the VDF Zucker rat, a rodent T2DM model, exhibited a reduction in responsiveness to GIP infusion at rates giving physiological concentrations

(Lynn *et al* 2001), similar to the case found in the human disease. This phenomenon was found to result from a direct downregulation of the GIP receptor gene via hyperglycaemia and hyperlipidaemia in these animals (Lynn *et al* 2001, 2003). Nevertheless, bolus pharmacological doses of [D-Ala2]GIP were effective in these same animals (Hinke *et al* 2002), providing proof-of-concept and adding credence to the possibility of GIP's utility in diabetes therapy. As predicted from the data regarding DP IV degradation of native GIP, [D-Ala2]GIP behaved as a super-agonist in vivo due to improved serum stability; notably, this synthetic analogue primarily augmented the first phase of insulin release. Fragment analysis of GIP also identified a bioactive domain within the N-terminus of the molecule, opening up the possibility of designing more potent small molecular weight agonists of the GIP receptor (Hinke *et al* 2001, 2004a).

An independent parallel series of studies on chemically modified GIP were undertaken by the research group of Flatt and O'Harte. These studies examined in vitro glycation of the N-terminus of GIP ([Tyr1-glucitol]GIP), and the biological effects of this molecule. N-terminal glucitol modification produced differential effects on in vitro potency; GIP was more potent than glycated GIP in the stimulation of glucose uptake and oxidation, and glycogenesis in striated muscle strips (O'Harte *et al* 1998), but when comparing insulinotropic activity using clonal insulin producing cells, the situation was reversed (O'Harte *et al* 1998a). In a follow-up study of this compound, it was recognized that glycation conferred the ability to resist N-terminal dipeptidyl peptidase IV degradation (O'Harte *et al* 1999). Although studies of the GIP receptor expression and regulation have not been performed in the ob/ob mouse model, the glycated analogue was shown to have greater in vivo potency in both normal rodents (O'Harte *et al* 1999) and obese (ob/ob) mice (O'Harte *et al* 2000) during bioassay experiments. Thus, regardless of differential tissue sensitivity to glycated GIP in vitro, the enhancement in potency resulting from reducing degradation in vivo resulted in better therapeutic potential as an antidiabetic agent. Subsequently, many studies examining in vitro stability and in vivo bioactivity of additional N-terminally modified synthetic GIP analogues have been reported (Gault *et al* 2003).

The data from basic research in vitro and in animal models of diabetes strongly supports a similar potential of GIP to GLP-1 in human therapy, mediated by overlapping and distinct physiological means. As GIP mainly stimulates secretion during the initial phase of insulin release (Lewis *et al* 2000; Hinke *et al* 2002, 2004), whereas human diabetes is characteristically deficient in early insulin release (Del Prato 2003) and the defect in the response to GIP affects only the late phase (Vilsbøll *et al* 2002, 2003), strategies based on GIP may restore defective early phase release in these patients. However, clinical data using DP IV resistant compounds are needed to establish if GIP has therapeutic potential. Because patients suffering from T2DM become refractory to monotherapy treatment, having an extra therapeutic option is worth pursuing.

3.2. GLP-1 Analogues

As mentioned previously, initial favourable human data on insulin secretory responses to exogenously administered incretins reported preservation of $\sim 71 \pm 17\%$ of the β-cell response to GLP-1 in T2DM. In contrast, GIP was found to retain only $\sim 47 \pm 9\%$ in the same study (Nauck *et al* 1993; Meier *et al* 2003), thus favouring GLP-1 based approaches. A naturally occurring peptide found in Gila monster venom, exendin-4, exhibited full agonist activity at the GLP-1 receptor, and already resisted proteolytic degradation, also giving an advantage to GLP-1 mimetic therapy (Nielsen and Baron 2003). The quantity of research in this field goes beyond the scope of this review, and has been summarized comprehensively elsewhere. The pace of

research examining GLP-1 analogues has proceeded at a remarkable pace, such that exendin-4 is well into clinical trials, and several additional novel strategies to extend the serum half-life of GLP-1 have been well studied. These include addition of an acyl chain to the native molecule to facilitate binding to serum proteins, as well as chemical linkage of a DP IV resistant form to albumin. Such modifications appear to produce long term beneficial effects via extending their duration of action (Joy *et al* 2005). The clinical trials which have been reported thus far are quite promising, but the role that injectable GLP-1 based peptides will have in treatment of human disease relative to existing and developing oral therapies remains to be seen.

4. DIPEPTIDYLPEPTIDASE INHIBITORS

Parallel studies of GIP and GLP-1 degradation in serum supported the conclusion that DP IV was the primary protease responsible for cleavage of N-terminal dipeptides from each (Mentlein *et al* 1993; Kieffer *et al* 1995; Pauly *et al* 1996), rendering them biologically inactive (Hinke *et al* 2002; Knudsen and Pridal 1996). This observation drew the interest of physiologists studying incretins and biochemists examining DP IV catalysis, and their combined efforts demonstrated the potential utility of this strategy to treat human diabetes. Thus, oral or intravenous administration of isoleucine-thiazolidide was able to preserve endogenous incretins release following a glucose challenge, in normal weight rats and obese VDF Zucker rats (Pederson *et al* 1998; Pauly *et al* 1999). Additional studies showing similar results using exogenously infused incretins have also been published (Deacon *et al* 1998, 1997), and the effectiveness of these agents to lower glycaemic excursions has been extended to several species, including humans (McIntosh *et al* 2005).

Unlike incretin therapies which rely on injection of peptides, DP IV inhibitors have a clear advantage since they are orally bioavailable. Most DP IV inhibitors are structurally modified dipeptides, and as such, can be absorbed rapidly from the gut via amino acid transporters (Foltz *et al* 2004). Decades of research had already been spent examining the structure-function relationships of different classes of DP IV inhibitors, and thus much is already known regarding their mechanism of action (Demuth 1990). One important point is the duration of inhibition which is necessary to achieve glycaemic control using these compounds, a topic which is still undecided. The foundation of knowledge regarding kinetics of DP IV inhibitors allows selection of long acting slow binding inhibitors, or a choice of equally potent shorter acting compounds. Both types appear to result in similar levels of glycaemic control and thus this debate may be a moot point, however, there are two rationales for the use of the different compounds. On the one hand, short acting DP IV inhibitors taken with a meal are less likely to result in undesirable side effects—the agent is only present during the time which they are needed to reduce glycaemia, and at the same time which the incretins are released from enteroendocrine cells. Slow binding inhibitors are suitable for once a day administration, however, DP IV is completely blocked also during fasting, when inhibitors may block proteolysis of other bioactive peptides (McIntosh *et al* 2005). DP IV also functions as an immune marker, and thus trials must carefully monitor immune function; thus far, many inhibitor compounds tested appear safe and well tolerated.

4.1. Mechanism of Glucose Lowering Action

Because of the broad number of substrates of DP IV, it would be difficult to definitively state the exact mechanism of action by which DP IV inhibitors are able to reduce blood glucose.

However, clear evidence has been provided at least for the known bioactive substrates involved in glucose homeostasis, and the effect of DP IV inhibition has followed what would be expected. In this regard, the development of techniques capable of recognizing the intact N-terminus of GLP-1 or GIP have proven highly useful (Deacon *et al* 1995, 2000; Wolf *et al* 2004). Both endogenously released and exogenously infused GIP and GLP-1 are preserved in their intact biologically active form in animal models and in humans during DP IV inhibitor treatment (Deacon *et al* 1998, 2001; Ahren *et al* 2004; Marguet *et al* 2000). Inhibitor administration to mice also enhances the insulin stimulating effects of exogenous PACAP and GRP (Ahren and Hughes 2005). Glucagon can also be degraded by DP IV, although with slower kinetics than incretin hormones, and thus inhibition of this enzyme may also preserve bioactive glucagon, but the physiological consequence or relevance is not presently known (Pospisilik *et al* 2001; Hinke *et al* 2000).

Long term DP IV inhibitor therapy also produced several beneficial effects, indicating that acute enhancement of incretin action, and/or that of other DP IV substrates, may also produce chronic changes in glucose homeostasis. In obese diabetic rats, 12-week twice daily oral administration of P32/98 significantly improved glucose tolerance following drug washout, and this is thought to be the consequence of improved peripheral insulin sensitivity in these animals (Pospisilik *et al* 2002, 2002a). In the STZ rodent β-cell injury model, inhibition of DP IV reduced the severity of β-cell destruction, protecting the insulin secreting cells from apoptosis. In the same model, an increased incidence of small islets were observed in animals treated with DP IV inhibitors, suggesting substrates of DP IV are involved in stimulation of islet precursor cells or expansion of a reserve pool of insulin secreting cells (Pospisilik *et al* 2003).

Regardless of their exact mechanism of action, whether it is via the incretins, or other biologically active peptides, or even if the DP IV inhibitor compounds may similarly inhibit related enzymes or those with similar specificity (although it should be noted that DP IV inhibitors are ineffective in DP IV null mice (Marguet *et al* 2000), the outcome is the same. DP IV inhibitors appear to be a safe and effective means of controlling glycaemia. As clinical trials proceed, and more basic research is performed on the mediators of these drugs, it is sure that the mechanism of action of DP IV inhibition in glucose homeostasis will be clarified (McIntosh *et al* 2005).

4.2. Combination Therapy

There have yet to be any contraindications reported for combined use of DP IV inhibitors with existing oral therapies for T2DM. Most studies, in fact, report an additive effect of DP IV inhibition with biguanides, glitazones, or sulphonylureas (Ahren *et al* 2004; Hoffmann *et al* 2001). The combination of DP IV inhibitors with other novel strategies for treatment of diabetes have not yet been examined. Despite low availability of human data for DP IV inhibitors in combination with other drug classes, several studies examining the effect of exogenously infused GLP-1 show that this incretin exerts an additive effect compared to oral monotherapy (Zander 2001, 2004), and thus, given the presumed mechanism of action of DP IV inhibitors, it follows that similar results will be found.

Combination therapy also raises interesting questions, as the traditional pharmaceutical agents may also exert effects on the enteroinsular axis, thus influencing the effectiveness of DP IV inhibition. Although biguanides and glitazones are generally thought to affect peripheral tissue responsiveness to insulin, via transcriptional pathways mediated by AMPK and PPAR-γ, direct effects of these compounds on enteroendocrine and pancreatic endocrine cells have been

reported. Meneilly et al reported that glyburide treatment improved the β-cell response to GIP in T2DM, where GIP's potency is blunted (Meneilly *et al* 1993). On the other hand, it has also been reported that the glucose lowering effect of the same compound is enhanced by GIP via an effect on hepatic insulin extraction (Kindmark *et al* 2001). Hence, use of DP IV inhibitors in such patients would be expected to result in further amplification of the contribution of GIP. Another situation is associated with biguanide stimulation of GLP-1 release from L-cells (Hinke *et al* 2002a,b; Yasuda *et al* 2002); it can be inferred that DP IV inhibition with co-application of metformin would augment the contribution of GLP-1. At the same time, DP IV inhibition alone appears to initiate a negative feedback loop in dogs limiting the secretion of GIP and GLP-1 (Deacon *et al* 2002). As such, it is difficult to predict the mechanism behind the additive combined effects of DP IV inhibition and other oral agents used to treat diabetes, however, this area will certainly be clarified upon the full disclosure of ongoing clinical trials.

4.3. Relative Contribution of GIP and GLP-1 to the Glucose Lowering Effects of DP IV Inhibitors

Until recently, the relative contribution of the incretins in mediating the glycaemic actions of DP IV inhibitors was not questioned. GLP-1 is one of the most potent insulin secretagogues identified to date, and because DP IV inhibition was shown to preserve biologically active intact GLP-1, it was assumed that it mediated the glucose lowering effects. Only recently, has the role of GLP-1 been debated, prompted by the observation that DP IV inhibition does not mimic all of the known physiological actions of GLP-1 (Nauck and El-Ouaghlidi 2005). The relatively lower potency of GIP in T2DM has all but excluded it as a mediator of DP IV inhibitor effectiveness in public opinion. This is but one defect contributing to the reduced incretin effect in T2DM, the other being insufficient GLP-1 secretion. There is little scientific evidence that enhancement of the reduced GLP-1 levels is capable of underlying the observed effects on glycaemic control, or exclusively mediates this effect (or, for that matter, that GLP-1 is the more effective endogenous incretin in T2DM). Combination therapy in human T2DM is a complicating factor, which could clearly shift the balance between the contribution of GIP and GLP-1, and therefore pre-clinical experiments should be performed to aid in the interpretation of eventual human data.

Recent use of transgenic and normal animals has provided insight into the role of incretin hormones in DP IV inhibitor therapy. In several animal studies, DP IV inhibition resulted in preservation of both intact GLP-1 and GIP, thus improving glucose tolerance (Pederon *et al* 1998; Pauly *et al* 1999; Deacon *et al* 1998, 2001; Marguet *et al* 2000; Ahren *et al* 2000). In GLP-1 receptor null mice, valine pyrrolidide treatment reduced the glycaemic excursion in response to oral glucose, presumably mediated by enhancing GIP action (Marguet *et al* 2000). Similarly, in a murine model lacking functional GIP receptors, the same experiment gave the same result, this time presumably due to improved GLP-1 action (Hansotia *et al* 2004). These animals have already compensated to a degree for the loss of the single incretins (Pederson *et al* 1998; Pamir *et al* 2003), and thus the observed result was expected. However, in mice lacking both incretin receptors, DP IV inhibitors were ineffective at altering glucose tolerance (Hansotia *et al* 2004), suggesting for the first time that the entire glucose lowering effects of these agents is mediated by the two incretin hormones, GIP and GLP-1.

Specific tools exist which may conclusively demonstrate the relative contribution of the two hormones, but have not been employed. As mentioned in section 2.4, in addition to genetic mouse models of incretin receptor ablation, specific receptor antagonists of both the GIP and GLP-1 receptor exist. Rat and mouse studies using immunoneutralizing antibodies against

the extracellular domains of the GIP receptor and GIP fragments have been used to measure the contribution of GIP to the enteroinsular axis (Tseng *et al* 1996, 1999; Lewis *et al* 2000; Baggio *et al* 2000). Similarly, exendin[9-39] has been employed to demonstrate that GLP-1 is a physiological incretin, and its relative contribution to the enteroinsular axis, including in man (Gault *et al* 2003; Wang *et al* 1995; Kreymann *et al* 1987). Unfortunately, GIP antagonism experiments have not been performed in healthy or diabetic humans to date, nor have studies using a combination of incretin antagonists been reported. Such an experiment should be able to reveal the quantitative contribution of GIP and GLP-1 in health and disease, and potentially identify if they are the only contributors to the incretin effect (although similar studies in rodents have been complicated due to the effect of these antagonists on glucose absorption from the gut, and gut motility, emphasizing the need for careful experimental design). These experiments need to be performed to quell speculation as to the relative contribution of the incretins to the effectiveness of DP IV inhibitors in humans.

5. CONCLUSIONS

Incretin based therapies for type 2 diabetes mellitus are quite promising. Presently, basic research places both GIP and GLP-1 based approaches on an equal standing. Recently, there has been renewed interest into the physiology of GIP in humans, and thus it is possible that DP IV resistant GIP analogues will be administered to human diabetics, perhaps reconciling differences between clinical and pre-clinical studies. Development of injection-delivered GLP-1 derivatives continues to meet expectations as a therapeutic option for the future. Particular attention to severity of diabetes and age must be considered when examining the effectiveness of either GIP or GLP-1 based analogues in human patients. Despite some uncertainties as to the precise mediators of DP IV inhibitors, data from mouse models indicate that the beneficial effects are conveyed by the known incretin hormones. Predictions of the relative contributions of GIP and GLP-1 to the glucose lowering activity of DP IV inhibition have been made, however, experimental data is required for conclusive resolution of this point. Only specifically designed studies can answer this question using selective antagonists of either the GIP or GLP-1 receptor, alone or in combination, in conjunction with DP IV inhibitors in healthy and diabetic humans.

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CHAPTER 14

DIPEPTIDYL PEPTIDASE IV: A MOLECULAR SWITCH OF VASCULAR ACTIONS OF NEUROPEPTIDE Y

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1. INTRODUCTION

Neuropeptide Y (NPY) is a 36 amino acid amidated peptide and a good substrate for dipeptidyl peptidase IV (DPPIV) since it possesses proline in the penultimate position, preceded by tyrosine in the first position. NPY belongs to a family of peptides which includes gastrointestinal peptides, peptide YY (PYY) and pancreatic polypeptide (PP). They share high a degree of homology, a tight 3-dimensional structure, so-called PP-fold, and ability to activate similar G-i/p-coupled receptors, designated Y1-Y6. NPY is the most abundant in the central nervous system. In the periphery, it is a ubiquitous sympathetic co-transmitter and adreno-medullary neurohormone, stored and co-released with catecholamines during nerve activation in conditions such as stress (Zukowska 1995; Zukowska-Grojec and Wahlestedt 1993). Circulating NPY levels are also elevated in many diseases where sympathetic hyperactivity is present, for example, in some forms of hypertension, congestive heart failure and renal insufficiency (Zukowska 1995; Zukowska-Grojec and Wahlestedt 1993). Increased plasma NPY levels are also seen in some proliferative diseases such as lymphoblastic leukemia, due to peptide's expression in immune cells (von Hörsten *et al* 1996), and in pheochromocytomas (Grouzmann *et al* 1989) and neuroblastomas (Grouzmann *et al* 1989), due to its production by these tumors (see Kitlinska *et al*, chapter 24 of this book).

Other extra-neuronal sources of NPY are endothelial cells (Zukowska-Grojec *et al* 1998) and also megakaryocytes and platelets (Myers *et al* 1988) and in rats and in some murine strains, where the peptide is an autocrine and paracrine modulator of cells' behavior, such as migration, proliferation and/or platelet aggregation (Myers *et al* 1988). Regardless of its neuronal or extraneuronal origin, any NPY1-36 circulating in the bloodstream is exposed to actions of soluble and/or membrane-bound DPPIV and expressed on the endothelium or immune cells.

As a result of its cleavage, NPY3-36 is formed and this changes the peptide's affinities for its receptors and its biological activities.

2. NPY, DPPIV AND VASOCONSTRICTION

Only mature NPY1-36 is capable of binding with high affinity to the Y1 receptor, since it requires intact tyrosines on both the N-and C-terminal (Grundemar and Hakanson 1993). The Y1 receptor is the major vascular receptor of the NPY family of peptides. It is also the only one which mediates vasoconstriction of small resistance arteries in such vascular beds as cerebral, coronary and splanchnic (Wahlestedt *et al* 1990, Zukowska-Grojec *et al* 1996). Activation of these receptors causes a slow-onset, long-lasting vasoconstriction and leads to protracted hypertensive responses (Wahlestedt *et al* 1990; Zukowska-Grojec *et al* 1996). Males, both rats (Zukowska-Grojec *et al* 1991) and humans (Lewandowski *et al* 1996) are more responsive than females to NPY- and stress-induced vasoconstriction, due to a strong positive regulation of the NPY gene expression by androgens (Zukowska-Grojec 1997). Whether or not greater NPY-Y1-mediated constrictive responses in males are due to differences in DPPIV-like activity between genders has never been tested. However, we have found that recombinant human DPPIV can mimic the effects of the Y1 antagonist while the peptidase inhibitor acts similarly to NPY—in modulating blood pressure recovery from hypotensive shock (Qureshi *et al* 1998). This indicates that in regard to the NPY-mediated vasoconstriction, DPPIV acts as an endogenous Y1-receptor antagonist.

3. NPY, DPPY AND ANGIOGENESIS

Once NPY1-36 is cleaved by DPPIV, it can no longer bind to the Y1 receptor but retains affinity for its Y2 and Y5 receptors. These receptors, which, like DPPIV, are expressed on endothelial cells where they mediate NPY's angiogenic activities (Zukowska-Grojec *et al* 1998). During the activation of the angiogenesis in conditions such as cell wounding (Ghersi *et al* 2000) or skeletal muscle ischemia (Lee *et al* 2003), the Y2/Y5 receptors and DPPIV are up-regulated (Lee *et al* 2003), and the enzyme becomes translocated to pseudopodia of migrating endothelial cells (Ghersi *et al* 2000). The blockade of DPPIV markedly impairs the endothelial cell migration, not only in response to NPY but also the spontaneous one but not migration induced by NPY3-36, the product of DPPIV (Ghersi *et al* 2000). Similarly, the Y2R knockout reduces NPY-mediated retinal angiogenesis (Koulu *et al* 2004) and ischemic muscle revascularization (Lee *et al* 2003), indicating that the DPPIV-Y2 system is critical. The loss of the expression of this system with aging markedly impairs NPY's angiogenesis, and in general, the body's ability to form new vessels (Kitlinska *et al* 2002).

4. NPY, DPPIV AND VASCULAR SMOOTH MUSCLE GROWTH

In addition to being vasoconstrictive via the Y1 receptor and angiogenic via the Y2/Y5Rs, NPY is also a potent mitogen for vascular smooth muscle cells (Zukowska-Grojec *et al* 1993; Pons *et al* 2003). We have studied the receptors and the mechanisms underlying vascular growth-promoting activities as well as the role of DPPIV, but the emerging picture is a complex

one. Like in the endothelial cells, NPY's effect in vascular smooth muscle cells (VSMC) is multi-receptor mediated, and it involves Y1 and Y5Rs, which are up-regulated during cell proliferation, and by growth factors, including NPY itself (Pons *et al* 2003). Both the Y1 and Y5 receptor antagonist inhibit NPY-induced VSMC proliferation but both are required to eliminate the response completely (Pons *et al* 2003). Theoretically, DPPIV, should act similarly to the Y1 receptor antagonist and *inhibit* the responses. However, the recombinant human DPPIV *increased* and inhibitor (Li *et al* 1995) decreased both the spontaneous and NPY-mediated VSMC proliferation *in vitro* (Kitlinska *et al* 2003). This, in part, could be due to alterations in the expression of the endogenous DPPIV, which changed in the opposite direction e.g. was increased by the DPPIV inhibitor (Oravecz *et al* 1997; Kitlinska *et al* 2003). Also, since DPPIV interacts with numerous peptides, including those with growth factor activities, e.g. RANTES (Oravecz *et al* 1997), some of these interactions may impact the VSMC proliferation. Finally, DPPIV may be acting not only as an enzyme but also as a co-receptor presenting NPY's molecule to its receptors, which may be less dependent on DPPIV-like enzymatic activity and more dependent on the expression of the endogenous, membrane-bound enzyme, which increased with the administration of the inhibitor. To determine the role of DPPIV in vascular smooth muscle growth and NPY's mitogenic activities, we turned to *in vivo* studies using an established model of VSMC proliferation—the formation of neointima in the artery injured by balloon angioplasty.

5. NPY, DPPIV AND RESTENOSIS/ATHEROSCLEROSIS

Within 14 days angioplasty itself activated the NPY system—the Y1 and Y5 receptors, DPPIV and the peptide - in the injured rat carotid artery and elevated plasma NPY levels (Li *et al* 2003). The exogenous administration of a slow release NPY pellet (1–10 μg/14 days) at the side of the injured artery only slightly augmented these changes, but markedly increased neointima formation. At the highest dose, NPY occluded the vessel with a neointimal highly vascularized lesion, which contained thrombus, matrix, macrophages and lipids (Li *et al* 2003). The NPY-induced lesion resembled an advanced atherosclerotic plaque in spite of being formed in rats without any lipid abnormalities (Li *et al* 2003). Remarkably, a similar post-angioplasty occlusion developed in injured vessels in rats that were subjected to chronic stress of daily exposure to cold water (standing in 1-cm ice cold water, 2 hrs/day/14 days, starting with the period before angioplasty). Both the NPY- and stress-induced atherosclerotic-like lesions were completely prevented by an infusion of a specific Y1 receptor antagonist (H409/22) (Li *et al* 2003). The Y1 antagonist also inhibited 50% of the neointima formation stimulated by angioplasty alone, indicating that the endogenous NPY-Y1 receptor system plays an important role on vascular remodeling after vascular interventions (Li *et al* 2003).

6. EFFECT OF DPPIV INHIBITOR, P32/98, ON ANGIOPLASTY-INDUCED NEOINTIMA FORMATION

To determine if DPPIV acts as an endogenous Y1 receptor inhibitor that attenuates neointima formation after angioplasty, we repeated the same studies using the DPPIV inhibitor, P32/98 (Probiodrug, Germany). P32/98 or its vehicle was administered at 16–20 mg/kg/day,

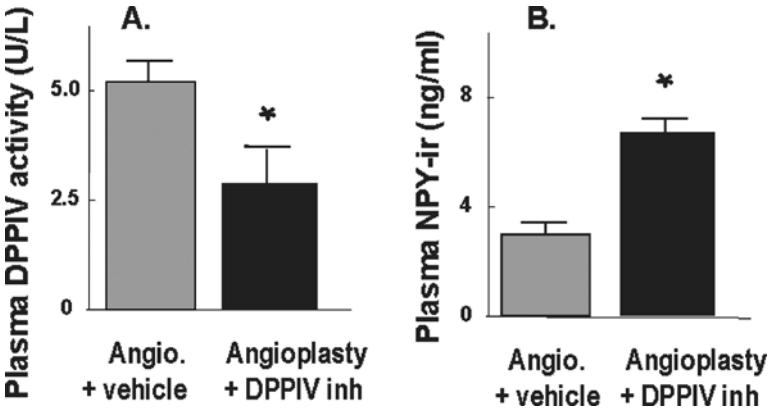

Figure 1. Effects of a DPPIV inhibitor, P32/98 (16–20 mg/kg/day for 14 days, i.v.) on plasma enzyme activity (A) and NPY-ir levels (B) in rats subjected to balloon angioplasty of carotid artery ($n = 6$ each). $^{*}P < 0.01$ by as compared to vehicle-treated rats by Student t-test.

i.v. via osmotic minipump for 14 days to rats subjected to angioplasty, with or without the NPY pellet. NPY was administered at a dose, which was previously found to stimulate neointima formation (1 μg), but not a complete vessel occlusion (Li *et al* 2003).

P32/98 (20 mg/kg/day, 0.55 + 0.05 mg/ml plasma concentrations) decreased plasma DPPIV activity in rats subjected to angioplasty, with or without NPY (which had no effect of its own), by up to 45% (Figure 1A). The reduction in enzyme activity was associated with 2-fold increased plasma NPY-immunoreactivity levels (by ELISA, Peninsula Labs, Belmont CA) ($P < 0.1$, Figure 1B). These changes were associated with augmented angioplasty-induced neointima formation in rats that were treated with P32/98, with or without the NPY pellet (Figure 2). The DPPIV inhibitor also increased the NPY-stimulated neointima as compared to the group of rats studied previously (Li *et al* 2003). Importantly, in rats that were subjected to angioplasty and NPY-treated, P32/98 often caused vessel occlusion, in spite of the fact that the NPY dose used was low and a non-occlusive (Li *et al* 2003). The nature of the lesion induced by the DPPIV inhibitor resembled an atherosclerotic-like plaque, which is characteristic of the effect of the high dose of NPY (Li *et al* 2003)—one that contains thrombus, matrix and immune cells.

7. CONCLUSION

DPPIV plays different roles in various NPY-mediated processes. In angiogenesis—DPPIV is essential: it acts as a converting enzyme for NPY1-36, forming an angiogenic form of the peptide, NPY3-36, and is required for NPY-evoked endothelial cell migration and differentiation. In regulation of the vascular tone—DPPIV acts as an endogenous inhibitor of Y1 receptor-mediated vasoconstriction and is a critical modulator in stress-induced hypertensive responses. In vascular remodeling and atherosclerosis, the role of DPPIV is complex and probably involves both its enzymatic and co-factor properties for NPY receptors. However, prolonged inhibition of DPPIV, using P32/98 (20 mg/kg/day for 14 days) leads to the augmentation of NPY's vascular growth promoting and pro-atherosclerotic activities in rats following carotid

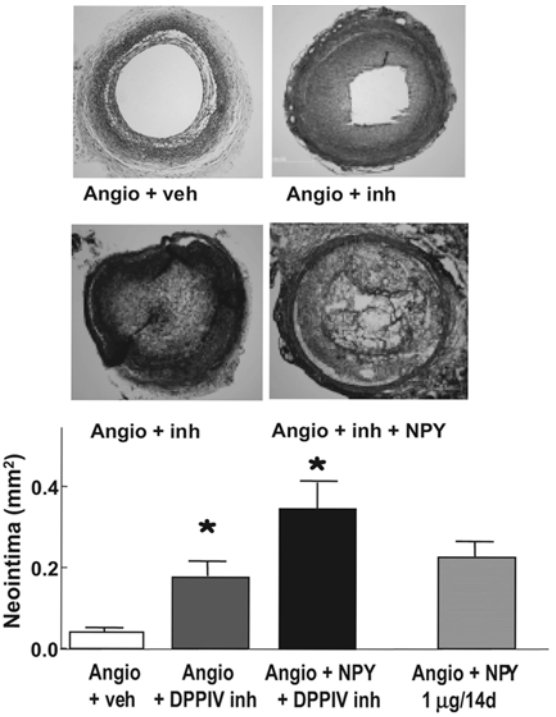

Figure 2. P32/98-induced augmentation of angioplasty-induced neointima formation and induction of an occlusive atherosclerotic-like lesion in vessels additionally treated with the NPY (1 mg/14 days) pellet, placed on the side of the injured artery (representative cross sections of rat carotid arteries, hemotoxylin-eosin staining).

artery balloon angioplasty. Therefore, our study raises concern of deleterious vascular effects of DPPIV inhibitors—the actions, which would be of particular importance to patients at high risk for atherosclerosis such as diabetics.

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TOPIC IV

IMMUNE DISORDERS

CHAPTER 15

DIPEPTIDYLPEPTIDASE IV (DPIV) AND ALANYL-AMINOPEPTIDASES (AAPs) AS A NEW TARGET COMPLEX FOR TREATMENT OF AUTOIMMUNE AND INFLAMMATORY DISEASES—PROOF OF CONCEPT IN A MOUSE MODEL OF COLITIS

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1. INTRODUCTION

Dipeptidylpeptidase IV (DPIV) and membrane alanyl-aminopeptidase (mAAP) have been shown previously to co-operate in the regulation of T lymphocyte activation. *In vitro* results demonstrated that inhibition of DPIV as well as of AAPs by competitive inhibitors affects the proliferation response and cytokine production by T helper cells (Kähne *et al* 1999; Lendeckel *et al* 1999). The simultaneous inhibition of both peptidases strongly enhances the immunosuppressive effects observed by inhibition of either AAPs or DPIV alone (Lendeckel *et al* 2003; Biton *et al*, chapter 19 of this book). Most importantly, the inhibition of both peptidases *in vitro* promotes potent endogenous immunosuppressive mechanisms, in particular the production of immunosuppressive cytokines such as transforming growth factor-β1 (TGF-β1) and IL-10. Recent data suggest that $CD4^+CD25^+$ regulatory T cells are the main source of these immunosuppressive cytokines (Bukowska *et al* 2003). Lack of function or insufficient numbers of regulatory T cells facilitate the development of autoimmune diseases.

Here we demonstrate that these effects of peptidase inhibitors are partially due to a direct action on natural regulatory T cells. *In vitro*, the pre-treatment of these cells with inhibitors

of alanyl-aminopeptidase substantially enhanced their expression of immunosuppressive cytokines and their suppressive activity in co-culture experiments with peripheral blood mononuclear cells (PBMNC). Extending these findings we also demonstrate that the combined administration of inhibitors of AAP and DPIV ameliorates acute colitis in mice via targeting regulatory T cells or effector T cells, respectively, at the same time.

2. EFFECTS OF DPIV AND AAP INHIBITION ON $CD4^+CD25^+$ REGULATORY T CELLS *IN VITRO*

2.1. Characteristics of Enriched $CD4^+CD25^+$ Regulatory T Cells

We analysed human $CD4^+CD25^+$ T cells from the peripheral blood of healthy donors using flow cytometry and quantitative RT-PCR; the characteristics of the cells are given in Table 1 and Figure 2. According to their surface expression of CD4, CD25, CD45RB, and TGF-β1, the $CD4^+CD25^+$ T cells were enriched to more than 94% purity. The lack of IL-2 and IFN-γ mRNA expression in freshly isolated cells (not shown) indicated the absence of any significant contamination by activated effector T cells.

2.2. Expression of mAAP and DPIV in $CD4^+CD25^+$ T Cells

$CD4^+CD25^+$ T cells express both mAAP and DPIV as detected by RT-PCR. About two-thirds of the cells were CD26-positive, whereas they appeared CD13-negative in flow-cytometric analysis (Table 1). This raised the question of whether APN is localised intracellularly or whether access of anti-CD13 monoclonal antibody to surface-bound APN is prevented by its inclusion in, for example, 'lipid-raft' domains. Here, by immunoprecipitation analysis using the anti-CD13 monoclonal antibody, clone LeuM7, we clearly demonstrate the presence of APN immunoreactivity in $CD4^+CD25^+$ T cells (Fig. 1). It is of note that clone LeuM7 is distinguished from other clones, including WM15 and My7, by its lack of cross-reactivity with cytosolic alanyl aminopeptidase (cAAP) (Lendeckel *et al* 2004). Furthermore, immunoprecipitated APN showed alanine-*p*-nitroanilide-cleaving enzymatic activity that was almost completely inhibited (12% residual activity) by the addition of phebestin (10^{-6} mol/l), but hardly affected by PAQ-22, a selective inhibitor of cAAP (95% residual activity).

Table 1. Surface markers of enriched $CD4^+CD25^+$ T cells

Surface antigen	Relative amount of positive cells (%, mean $\pm$ Se, $n = 4$)
CD25	97.6 ± 0.6
CD4	93.7 ± 1.1
TGF-β_1	96.9 ± 16.1
CD45RB	98 ± 0.6
CD13	<5
CD26	67 ± 14.4

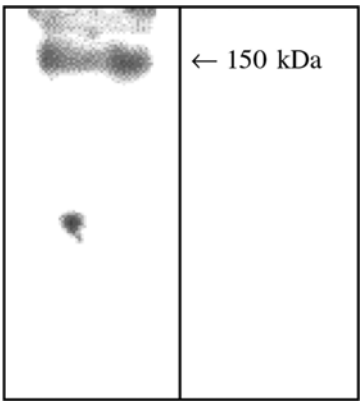

Figure 1. Detection of the molecular target of phebestin, mAAP (CD13), in human $CD4^+CD25^+$ T cells by immunoprecipitation from total $CD4^+CD25^+$ T cell lysate. The anti-CD13 mab, clone LeuM7, precipitated a 150-kDa isoform, corresponding to the mature glycosylated APN.

2.3. Inhibition of AAP and DPIV Differently Affects $CD4^+CD25^+$ T Cell-Specific mRNA Expression

Expression of TGF-β1 has been implicated as an essential phenomenon in the suppression of responder T cells by $CD4^+CD25^+$ T cells. Interestingly, activation of $CD4^+CD25^+$ T cells by phytohemagglutinin (PMA)/phorbol 12-myristate 13-acetate (PMA) led to a slight reduction of TGF-β1 mRNA and surface expression that was accompanied by a decrease in the release of both latent and active TGF-β1 into the culture medium (Table 2). Phebestin, when added simultaneously with the activation, not only attenuated the activation-dependent decrease of TGF-β1 expression at all expression levels investigated, but also provoked expression levels that exceeded those of freshly isolated $CD4^+CD25^+$ T cells. In particular, the TGF-β1 mRNA content of untreated $CD4^+CD25^+$ T cells, surpassing that of $CD4^+CD25^-$ T cells by more

Table 2. Effect of phebestin on TGF-β1 expression of human $CD4^+CD25^+$ T cells after 24 h. Data given as absolute amounts in pg/ml as well as relative amount based on activated $CD4^+CD25^+$ cells.

Substance applied	TGF-β1 surface expression (mfi)	TGF-β1 secreted into medium (pg/ml) [%]	
		Latent	Active
None	99.6 ± 17.4	499 ± 32.9	296 ± 31
		[115.5 ± 6.6]	[125.4 ± 13.1]
PHA/PMA	93.5 ± 28.4	432 ± 39.6	236 ± 11.1
		[100 ± 9.2]	[100 ± 4.7]
PHA/PMA + phebestin	115 ± 31	567 ± 16.6	369 ±49.3
		[131.3 ± 3.8]	[156.4 ± 20.9]
	$n = 7$	$n = 8$	$n = 10$

than 20-fold, were further elevated by phebestin or RB3014, respectively. In contrast, the administration of the DPIV inhibitor Lys-Z[NO_2]-pyrrolidide did not alter TGF-β1 mRNA levels in $CD4^+CD25^+$ T cells (Fig. 2). Immunoblot analysis confirmed at the protein level the induction of TGF-β1 in response to the administration of phebestin (Fig. 3).

Amounts of IL-10 mRNA appeared not changed upon activation of $CD4^+CD25^+$ T cells or administration of Lys-Z[NO_2]-pyrrolidide. However, the inhibitor of AAPs, RB3014, led to a marked increase of IL-10 mRNA expression after 24 hours (Fig. 2).

Next, we assessed the expression of FOXP3, a transcription factor that is believed to be exclusively expressed in $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells, and the expression of which correlates with their suppressive phenotype in humans and mice (Khattri *et al* 2003; Hori *et al* 2003, Walker *et al* 2003, Sakaguchi *et al* 2003, O'Garra *et al* 2004). In this study, as expected, we observed that FOXP3 mRNA was abundantly expressed in $CD4^+CD25^+$ T cells, but not expressed in $CD4^+CD25^-$ T cells (not shown), and only weakly expressed in ΔMNC (not shown). Upon activation by PHA/PMA, there was a significant decrease in FOXP3 mRNA expression, a finding that is in full accordance with data reported by others (Khattri *et al* 2003, Hori *et al* 2003, Sakaguchi *et al* 2003). Remarkably, the observed decrease in levels of FOXP3 mRNA in response to activation was partly reversed by the administration of either phebestin or Lys-Z[NO_2]pyrrolidide (Fig. 2). This finding suggests that inhibitors of AAPs exert a stabilizing effect on the suppressive phenotype of $CD4^+CD25^+$ T cells via induction of both immunosuppressive cytokine production and FOXP3 expression. Inhibition of DPIV, on the contrary, although capable of partly preserving the FOXP3 expression, does not increase the expression of either TGF-β1 or IL-10 in $CD4^+CD25^+$ T cells.

2.4. The 'Suppressive Phenotype' of $CD4^+CD25^+$ T Cells is Lost Upon Activation *In Vitro* but Preserved by Inhibition of AAP

The observed decrease in TGF-β1 and FOXP3 mRNA expression in $CD4^+CD25^+$ T cells, together with increased amounts of interferon (IFN)γ and IL-2 mRNA in response to PHA/PMA (not shown), suggested an activation-dependent loss of suppressive activity. Indeed, the co-culture of $CD4^+CD25^+$ T cells that had previously been exposed to PHA/PMA for 24 h with depleted ΔMNC failed to inhibit the proliferation of the MNC at both cellular ratios studied (1:1 and 4:1), but, instead, the proliferation rate was further increased (Fig. 4). Under the same conditions, freshly isolated $CD4^+CD25^+$ T cells significantly inhibited cell proliferation (Fig. 5).

A similar activation-dependent loss of suppressive activity was found in response to providing CD28 co-stimulation or high amounts of IL-2 to a maximal anti-CD3 stimulus (Baecher-Allan *et al* 2003). Furthermore, in other studies, murine $CD4^+CD25^+$ T cells suppressed the proliferation of co-cultured effector T cells upon stimulation with soluble anti-CD3, whereas highly cross-linked plate-bound anti-CD3 failed to induce suppression (Thornton *et al* 1998, Itoh *et al* 1999). In contrast, $CD4^+CD25^{high}$ T cells, a recently described subset within the circulating human $CD4^+CD25^+$ T-cell population, were capable of inhibiting the proliferation and cytokine production induced by TCR cross-linking of $CD4^+CD25^-$ effector T cells in a contact-dependent manner (Baecher-Allan *et al* 2003).

Addition of freshly isolated $CD4^+CD25^+$ T cells to the ΔMNC resulted in inhibition of PHA-induced proliferation that was directly correlated to the number of regulatory T cells included in the co-culture (Fig. 5).

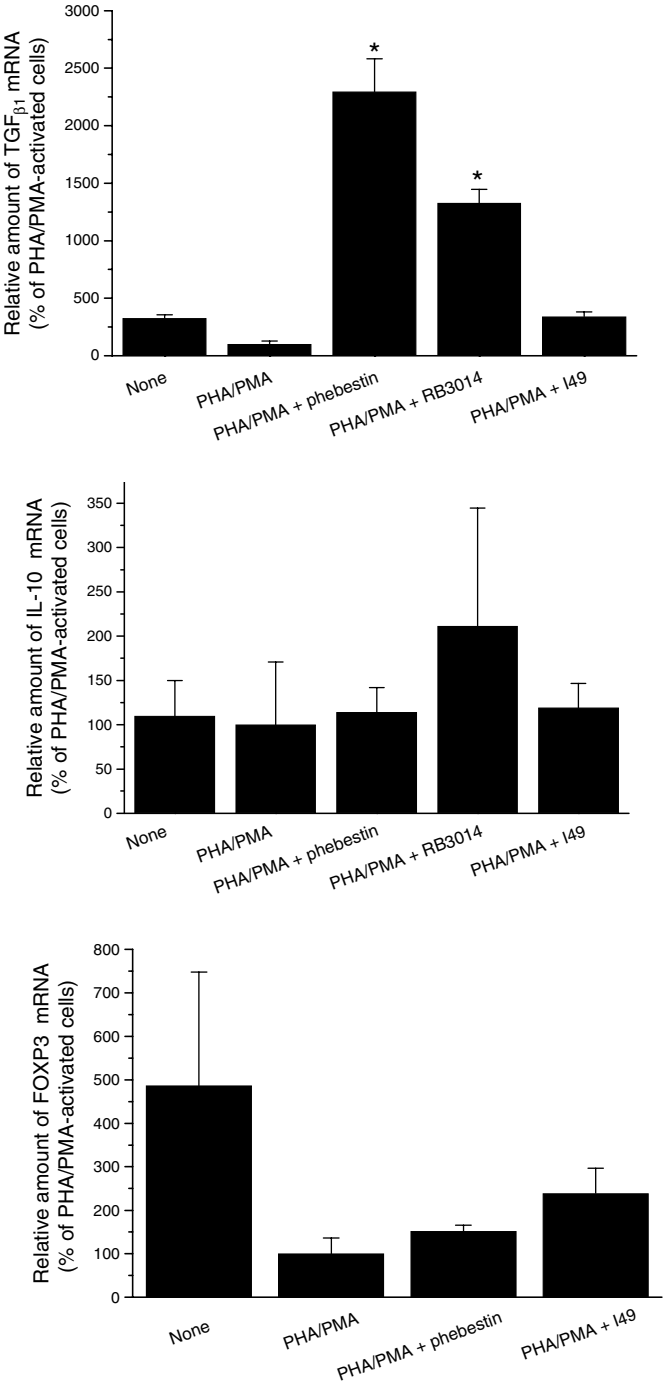

Figure 2. Effect of APN and DPIV inhibitors on TGF-β1(top), IL-10 (middle), and FOXP3 (buttom) mRNA expression of human $CD4^+CD25^+$ T cells (after 24 h).

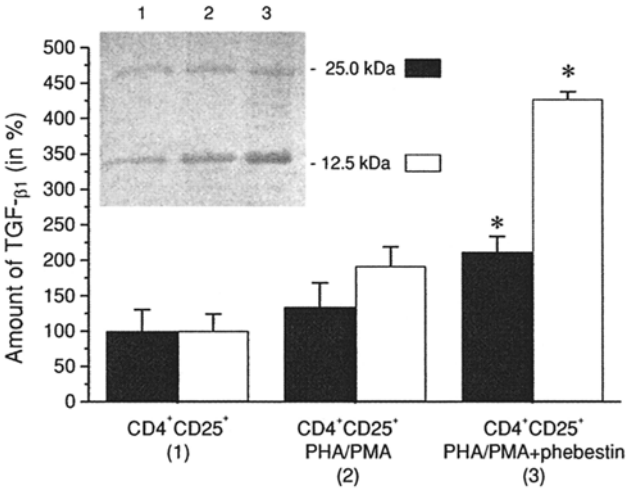

Figure 3. Detection of TGF-β1 in total lysates of $CD4^+CD25^+$ T-cells by immunoblot analysis. Phebestin (1 μM) significantly increased the amounts of TGF-β1 after 24 h ($P < 0.05$ *vs. control and vs. PHA/PMA, n = 3).

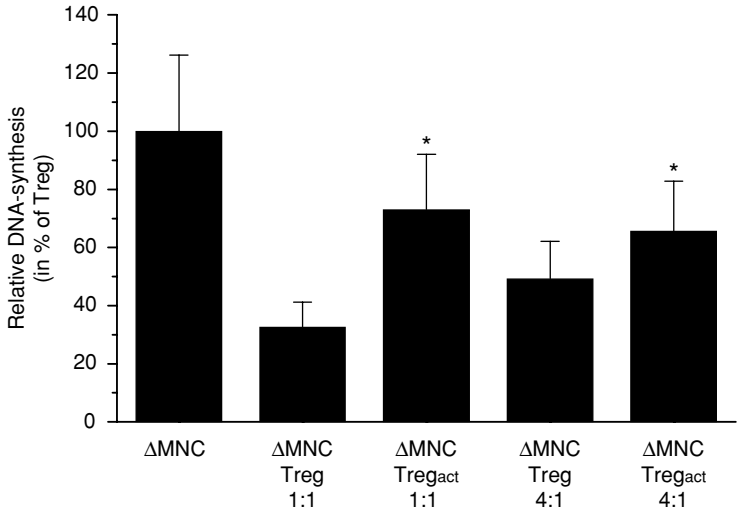

Figure 4. The reconstitution of PHA stimulated ΔMNC with $CD4^+CD25^+$ T cells (Treg) at the ratios 1:1 and 4:1 decreased the DNA synthesis. However, $CD4^+CD25^+$ T cells activated by PHA for 24 h prior to the co-culture ($Treg_{act}$) led to a complete loss of their suppressive activity, which resulted in a much more pronounced proliferative response (mean of two independent experiments with six replicates each; $P < 0.05$, *vs. ΔMNC/Treg).

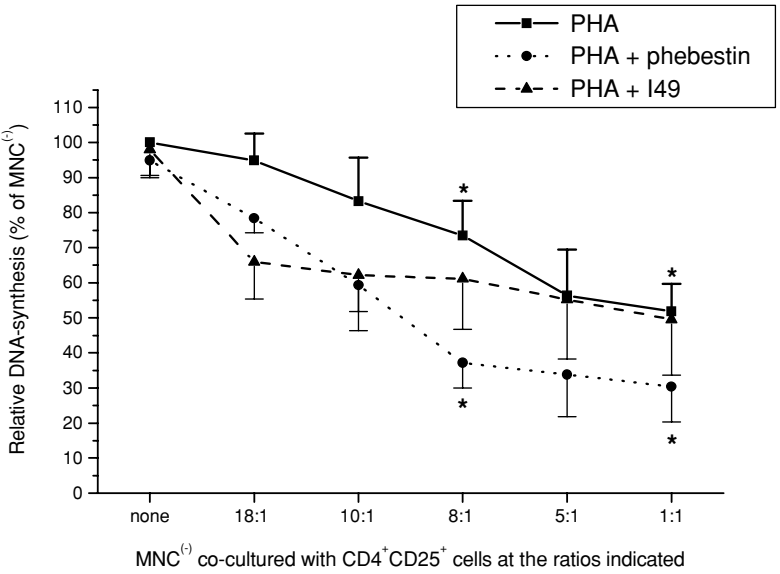

Figure 5. Phebestin, but not Lys-Z[NO_2]-pyrrolidide (I49), preserves the suppressive activity of $CD4^+CD25^+$ T cells in co-culture with human mononuclear cells. The suppressive activity was further increased by the addition of 1 μM phebestin at various cellular ratios ($n = 4$, $^*P < 0.05$).

$CD4^+CD25^+$ T cells that had been exposed to phebestin (10^{-6} M, 30 min) prior to their co-culture with ΔMNC exhibited a significantly increased suppressive activity when compared with untreated cells. In contrast, pre-treatment of the cells with Lys-Z[NO_2]-pyrrolidide (I49) did not lead to a comparable growth inhibition (Fig. 5).

3. INHIBITION OF DPIV AND AAPs AMELIORATES ACUTE COLITIS IN MICE

To prove the therapeutic effects of the ectopeptidase inhibitors *in vivo*, we used a mouse model of the human ulcerative colitis—a sodium dextran sulphate induced TH1-driven colon inflammation. Disease activity was assessed by weight loss and faeces parameters. Colitis in Balb/c mice was induced by free administration of 3% DSS with the drinking water through the experimental period of 7 days. After onset of clear clinical symptoms and under contineuos administration of DSS, the treatment with specific inhibitors started at day 4.

While the treatment with DPIV or AAP inhibitors (up to 50 mg/kg) alone had marked but no significant therapeutic effects, the simultaneous administration of both peptidase inhibitors (0.5 mg/kg) was found to cause a significant reduction of disease activity by more than 50% in comparison to placebo-treated control mice (Fig. 6).

The anti-inflammatory effect of the peptidase inhibitors was also reflected by a decrease in the number of circulating polymorphonuclear neutrophils as well as in the systemic concentration of the proinflammatory cytokines IFN-γ and TNF-α (not shown).

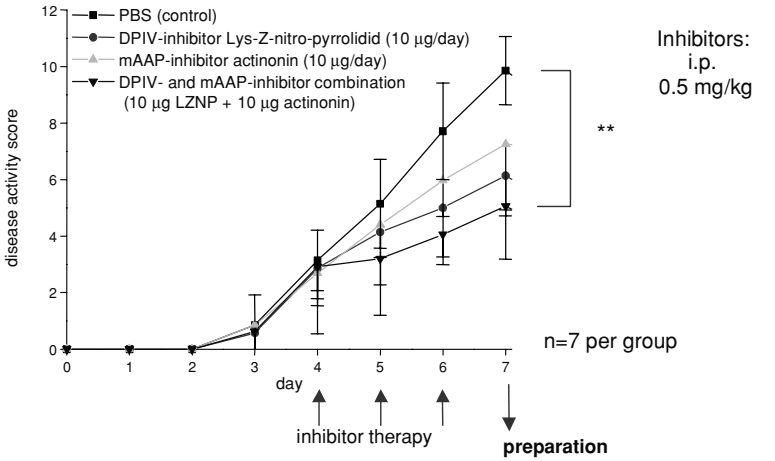

Figure 6. Effect of AAP-inhibor actinonin, DPIV-inhibitor Lys-Z[NO_2]-pyrrolidide or the combination of both peptidase inhibitors on the desease activity in a mouse model of ulcerative colitis (DSS-induced). Inhibitor therapy started at day 4 after the clear onset of colitis symptoms. Disease activity was assessed by daily analysis of weight, faeces consistency and the detection of colon bleedings.

Extending the *in vitro* findings regarding the effects of AAP or DPIV inhibitors on the expression of genes crucial for Treg function, we analyzed the FoxP3 and TGF-β1 expression in colon tissue of placebo-treated and inhibitor-treated mice. As demonstrated in figure 7, the amounts of FoxP3 and TGF-β1 mRNA was found to be significantly increased in response to inhibitor treatment in comparison to the control.

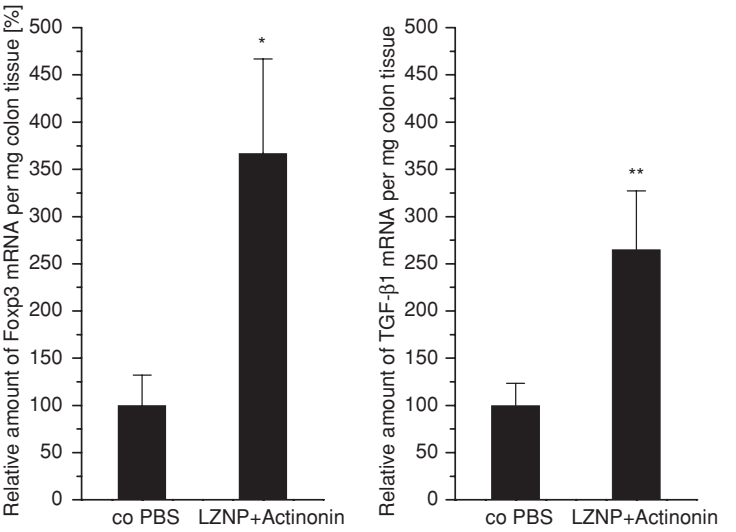

Figure 7. Local expression of TGF-β1 and foxp3 mRNA in the colon of colitis mice treated with either LZNP + actinonin or placebo (co PBS).

4. CONCLUSIONS

In summary these results strongly support the idea that AAPs and DPIV represent a promising target complex for the pharmacological therapy of T cell-mediated diseases by preserving and enhancing endogenous immunosuppressive mechanisms. Whereas inhibitors of AAPs appear to preferentially act on $CD4^+CD25^+$ regulatory T cells by preserving their immunosuppressive activity via enhanced expression of immunosuppressive cytokines and FOXP3, inhibition of DPIV leads to increased production/release of TGF-β1 and inhibition of cellular proliferation of predominantly activated effector T cells. Thus, specific inhibition of DPIV and AAPs via small molecular compounds provides a new approach for the pharmacological treatment of autoimmune and inflammatory diseases that simultaneously interferes with two major axis of T cell function.

ACKNOWLEDGMENTS

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METHODS

Cells. Human MNC were isolated from heparinized venous blood of healthy donors using density gradient centrifugation over Lymphocyte Separation Medium (PAA Laboratories, Pasching, Austria) (Ansorge *et al* 2003). $CD4^+CD25^+$ T cells were positively selected from human MNC using CD25 MicroBeads (Miltenyi Biotech, Bergisch-Gladbach, Germany). Cells were suspended in AIM-V Medium (Gibco-BRL) with 33 μg/ml refobacin. For stimulation over a period of 24 h, PHA (1 μg/ml, Roche-Diagnostics, Heidelberg, Germany) and PMA (20 nmol/l, Sigma, Steinheim, Germany) were added. To examine possible effects of inhibitors, cells were cultured in the absence or in the presence of phebestin (10^{-6} M) (Sigma), RB 3014 (10^{-6} M) (B. P. Roques, Paris) or Lys-Z[NO_2]-pyrrolidide (10^{-6} M)(K Neubert, J Faust). **RNA preparation**. Total RNA from $1*10^6$ regulatory T cells was prepared using the RNeasy Mini kit (Qiagen, Hilden, Germany). Contaminating DNA was removed by DNase I digestion (RNase-free, Boehringer Mannheim). RNA amounts were determined spectrophotometrically using the Genequant (Pharmacia LKB, Freiburg, Germany). **Quantitative PCR**. cDNA was generated from 1 μg total RNA, and 1/20th of the cDNA mixture was used for quantitative RT-PCR in the iCycler (Bio-Rad, Munich, Germany). A typical 25-μl reaction mixture contained 12.5 μl HotStarTaq Master Mix (Qiagen), 0.25 μl of a 1:1000 dilution of SYBR Green I (Molecular Probes, Eugene, Oregon), 025 μl of a 1:1000 dilution of Fluorescein Calibration Dye (Bio-Rad) and 0.5 μmol of the specific primers (BioTeZ). The following primers were used: human FOXP3-US, 5′-TGTCAGTCAACTTCACCAAG-3′; human FOXP3-DS, 5′-AGCTGGTGCATGAAATGTGG-3′; human TGF-β1-US, 5′-CAGAAATACAGCAACAATTCCTGG-3′; human TGF-β1-DS, 5′-TTGCAGTGTGTTATCCGTGCTGTC-3′; human IL-10-US, 5′-GCCTA ACATGCTTCGAG-ATC-3′; human IL-10-DS, 5′-TGATGTCTGGGTCTT GGTTC-3′. An initial denaturation-activation-step (15 min 95 °C) was followed by 40 cycles

(30 s 95 °C, 30 s 58 °C, 45 s 72 °C). The amounts of mRNA were normalised to α-tubulin mRNA (human α-tub-US, 5′-CATTTCACCATCTGGGCTGGCTC-3′; α-tub-DS, 5′-CACCCGTCTTCA GGGCTTCTTGGTTT-3′) and analyzed using a one-way analysis of variance.

Animals. We performed all animal studies in compliance with international and local animal welfare legislations. Female Balb/c mice were purchased from Harlan-Winkelmann (Borchen, Germany) and housed under standard conditions (25 °C and 12 h light–12 h dark cycle) for >1 week before starting the experiments. Mice were fed with standard pellets ad libitum. Acute colitis was induced by adding 3% (w/v) dextran sulphate sodium (DSS, MW 36,000–50,000; ICN Biomedicals, Aurora, Ohio) to the drinking water for 7 days. This concentration of DSS has previously been shown to induce a severe colitis, but with a low risk of death, within 7 days. The animals had free access to the DSS solutions, which were changed every other day. Severity and progress of colitis was monitored by daily examination for the general state of health (activity, grooming behaviour, mean food/water consumption). Weight changes were recorded daily. Feces were visually inspected for signs of diarrhoea and rectal bleeding (Hemoccult sensa; Beckmann-Coulter, Krefeld, Germany). The disease activity index (DAI) was determined by summarising the scores for weight loss (0 points <5% weight loss, 1 point = 5–10% weight loss, 2 points = 10–15% weight loss, 3 points = 15–20% weight loss, 4 points = more than 20% weight loss), stool consistency (0 points = well-formed pellets, 2 points = pasty/semi-formed stool, 4 points = liquid stool) and bleedings (0 points = no rectal bleedings, 2 points = hemoccult positiv, 4 points = visible gross bleeding). Tissue sampling and histological analysis. Animals were killed by an overdose of carbon dioxide. Blood was collected post-mortem by cardiac puncture and platelet-free plasma was obtained using a two-step centrifugation as described previously (Lendeckel et al 2004). The entire colon plus cecum was dissected and the length from the colocecal junction to the anus was measured as marker of inflammation.

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CHAPTER 16

DIPEPTIDYL PEPTIDASES AND INFLAMMATORY BOWEL DISEASE

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1. INTRODUCTION

Inflammatory bowel disease (IBD) is a collective term for a group of idiopathic inflammatory disorders affecting the gastrointestinal tract, typified by Crohn's disease and ulcerative colitis, which often affect children, adolescents or young adults (Rutgeerts *et al* 2003). Although both environmental and genetic factors have been implicated in IBD the actual cause is unknown. Current conventional treatments for IBD include 5′aminosalicylic acid, corticosteroids, antibiotics and immunosuppressants. These therapies are not always effective (eg 20% of patients are refractory to corticosteroid therapy) and can have serious side effects (Rutgeerts *et al* 2003). There is a clear need for the development of novel agents for treating patients with IBD and in recent years many biological therapies have emerged including antibody therapies, probiotics and native/recombinant peptide therapy. In the last few years, glucagon like peptide-2 (GLP-2) has emerged as one of the most intriguing and potent modulators of intestinal growth and function. However, *in vivo,* GLP-$2_{1\text{-}33}$ is rapidly degraded by a dipeptidyl peptidase (DP) to an inactive form (GLP-$2_{3\text{-}33}$) lacking growth-promoting properties.

2. GLP-2 AND DPIV

2.1. GLP-2_{1-33} and Its Action in Experimental Models of Intestinal Injury

GLP-2_{1-33}, a member of the glucagon-like peptide family, is a 33 amino acid peptide which is secreted into the bowel lumen by L-type enteroendocrine cells of the small intestine and colon (Drucker *et al* 2000). Intestinal injury or resection are associated with increased levels of the proglucagon mRNA transcripts in the intestinal remnant, as a result of increased proglucagon RNA content in the remaining enteroendocrine cells (Drucker 2001). The gastrointestinal tract, from the stomach to the colon, is the principal target for GLP-2_{1-33} action. Current data demonstrate that GLP-2_{1-33} regulates motility, nutrient absorption, epithelial permeability, cell proliferation, and apoptosis in the gastrointestinal tract (Drucker 2001). Exogenous GLP-2_{1-33} administered to normal mice and rats increases growth of the mucosal epithelium in small and large intestine (Drucker *et al* 1997; Tsai *et al* 1997). The increase in small bowel mass is attributable in part to activation of crypt cell proliferation and inhibition of enterocyte apoptosis (Tsai *et al* 1997). The cytoprotective and reparative effects of exogenous GLP-2_{1-33} are evident in rodent models of experimental injury. GLP-2_{1-33} reduces mortality and decreases mucosal injury, cytokine expression, and bacterial septicemia in the setting of small and large bowel inflammation (Boushey *et al* 1999). GLP-2_{1-33} also enhances nutrient absorption and gut adaptation in rodents (Scott *et al* 1998; Sigalet and Martin 2000). The actions of GLP-2_{1-33} are transduced by the GLP-2 receptor, a G protein-coupled receptor expressed in gut endocrine cells of the stomach, small bowel and colon (Munroe *et al* 1999). Activation of GLP-2 signalling in heterologous cells promotes resistance to apoptotic injury *in vitro* (Yusta *et al* 2000).

2.2. GLP-2 in Humans

Elevated plasma GLP-2_{1-33} levels have been described in patients with short bowel syndrome (Jeppesen *et al* 2000) resulting from either ulcerative colitis or Crohn's disease (Xiao *et al* 2000). The cytoprotective, reparative and energy-retentive properties of GLP-2_{1-33} in rodent models suggest that GLP-2_{1-33} may potentially be useful for the treatment of human disorders characterised by injury and/or dysfunction of the intestinal mucosal epithelium (Drucker 2001). Thus, GLP-2_{1-33} is currently being developed as a therapeutic for clinical use in humans. Evidence of a potential role for GLP-2 in the treatment of IBD has been described in a study by Alavi *et al* using a rodent model of spontaneous IBD induced by the microinjection of human HLA-B27 and β-2-microglobulin genes (Alavi *et al* 2000). This study described a marked reduction in gross and histologically-assessed bowel lesions following treatment with GLP-2. In addition, exogenous GLP-2_{1-33} treatment has been shown to improve intestinal absorption and nutritional status in short-bowel patients with impaired postprandial GLP-2 secretion, in whom the terminal ileum and the colon have been resected (Jeppesen *et al* 2001). However, one of the major problems associated with therapeutic use of GLP-2_{1-33} is its rapid degradation and its inactivation by the protease dipeptidyl peptidase IV (DPIV) (Drucker *et al* 1997).

2.3. DPIV and GLP-2

GLP-2_{1-33} is secreted as a 33 amino acid peptide, but it rapidly degrades at the N terminus to inactive GLP-2_{3-33}. Both functional and binding studies have demonstrated that GLP-2_{3-33}

acts as a partial agonist with potential competitive antagonistic properties on the GLP-2 receptor (Thulesen *et al* 2002). This degradation is thought to occur through the action of DPIV (Hartmann *et al* 2000a). DPIV inhibitors prevent GLP-$2_{1\text{-}33}$ degradation both *in vitro* and *in vivo* (Brubaker *et al* 1997; Drucker *et al* 1997; Hartmann *et al* 2000a). DPIV was further implicated in controlling the biological activity of GLP-$2_{1\text{-}33}$, as studies in rats demonstrated considerably greater intestinotrophic activity when a GLP-$2_{1\text{-}33}$ analogue that was resistant to DPIV mediated inactivation was exogenously administered (Drucker *et al* 1997; Scott *et al* 1998). Similarly, the co-administration of a DPIV inhibitor has been reported to enhance the trophic activity of exogenous native GLP-$2_{1\text{-}33}$ in rats (Hartmann *et al* 2000b). Recent work in our laboratory has focused on further investigating the role that DPIV plays in IBD using either DPIV knockout mice or DPIV inhibitors and the dextran sulphate colitis (DSS) model of experimental murine colitis.

3. DEXTRAN SODIUM SULPHATE (DSS) COLITIS AS A MODEL OF IBD

The ulcerative colitis variant of IBD in humans is characterised histopathologically by a chronic, relapsing focal ulcerative process, primarily affecting the distal colon (Hanauer and Baert 1994). The DSS experimental animal model is a model of intestinal damage and inflammation with symptomatic and histopathological similarities to ulcerative colitis in humans so it has been chosen for use in our work. When DSS, a synthetic polysaccharide, is orally fed to mice and rats, it is known to inhibit crypt cell proliferation and promote apoptosis, leading to epithelial damage, ulceration, crypt loss and inflammation, predominantly localised to the distal colon (Kullmann *et al* 2001). DSS treatment has also been shown to cause rectal bleeding, diarrhoea, a decrease in colon length and a reduction in total body weight, all of which are common symptoms of human ulcerative colitis (Egger *et al* 2000). While the DSS method can also be manipulated to achieve chronic experimental ulcerative colitis, in our experiments we are using an acute and resolving colitis model. In a pilot study we found that 2% DSS provided ad libitum in the drinking water for 6 days, induces colitis in both $DPIV^{+/+}$ and $DPIV^{-/-}$ male mice on a C57BL6 background (Geier *et al* 2005). In our model normal drinking water is substituted for DSS on day 6, and mice continue to drink regular water for differing periods of time depending on protocol.

4. EXPERIMENTAL COLITIS IN DPIV KNOCKOUT MICE

Given the evidence that DPIV mediates GLP-2 bioactivity we hypothesised that DPIV deficient mice would display an increased resistance to and an enhanced recovery from, DSS-induced colitis compared to wildtype mice. $DPIV^{+/+}$ and $DPIV^{-/-}$ mice consumed 2% DSS for 6 days, followed by a 15 day recovery period (Geier *et al* 2005). Mice were assessed daily using a disease activity index (DAI) which monitors the progression and resolution of DSS-induced colitis (Vowinkel *et al*, 2004). Using this index mice are scored daily on the basis of weight loss, stool consistency, rectal bleeding and overall condition of the animal as described previously (Murthy *et al* 1993; Howarth *et al* 2000). The only significant difference between DAI in $DPIV^{+/+}$ and $DPIV^{-/-}$ mice was observed at day 14 (Figure 1).

In addition, mice were killed at days 0, 3, 6, 9, 14 and 21 ($n = 6$–8) and the small intestine and colon removed for histological assessment of villus height, crypt depth, and crypt area

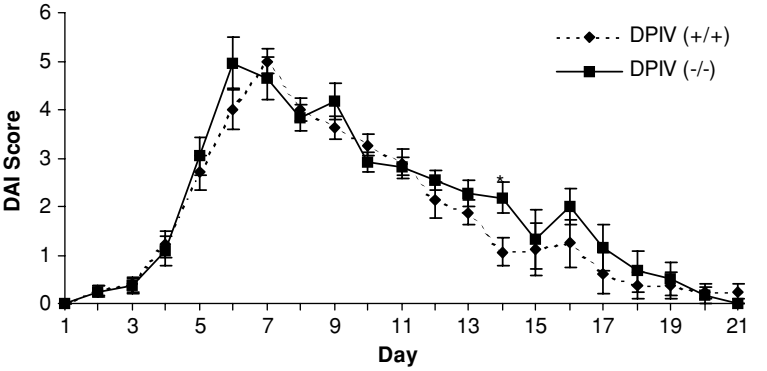

Figure 1. Comparison of disease activity between DPIV$^{+/+}$ and DPIV$^{-/-}$ mice during the induction and resolution of DSS-induced colitis.

(Figure 2). The epithelial cell proliferative labelling index was determined by proliferating cell nuclear antigen (PCNA) immunostaining. No significant differences in small intestine, colon and total body weight, distal colon, crypt depth or PCNA labelling index were observed between wildtype and DPIV$^{-/-}$ mice. Therefore we concluded that loss of DPIV activity does not increase resistance to experimental colitis. However, we could still find measurable levels of DP levels in plasma from DPIV$^{-/-}$ mice, suggesting that another DPIV family member may also be involved in the cleavage of GLP-2. In order to further investigate the role of DPIV and other DPs in the IBD setting we next examined the efficacy of DP inhibition in the DSS-colitis model.

5. DP INHIBITION AS A POTENTIAL THERAPEUTIC FOR IBD

We have begun preliminary studies to investigate the potential for different DPIV inhibitors to modify the course of disease activity in DSS-colitis. DPIV is structurally homologous to other proteases of the DPIV gene family (FAP, DP8, and DP9) and if these proteases are present in gut tissue the inhibitors may also bind to them. Mice were orally gavaged twice daily with 0.9% saline, 10 mg/kg isoleucyl-cyano-pyrrolidine (Ile-cyano-pyrr) or 10 mg/kg isoleucyl-thiazolidine (Ile-thia) (n = 6). Mice consumed DSS for 6 days to induce colitis, followed by a 3 and 8-day recovery period. Groups of mice were killed at day 9 and day 14. Disease severity was assessed again using the DAI index, together with histological assessment of crypt area and depth in the distal colon. Epithelial cell proliferative labelling index (LI) was determined by proliferating cell nuclear antigen immunostaining.

DP activity was significantly inhibited ($p < 0.05$) following inhibitor treatment in both 9 and 14-day trials (data not shown). Mice treated with Ile-thia or Ile-cyano-pyrr had significantly lower DAI scores compared to saline treatment (Figure 3). At day 9, crypt depth was significantly lower ($p < 0.05$) by 17% in Ile-thia treatment compared to saline treatment. At day 14, crypt area was significantly higher ($p < 0.05$) in mice treated with Ile-cyano-pyrr or Ile-thia compared to saline control. At day 14, LI in the Ile-cyano-pyrr treatment group was significantly higher

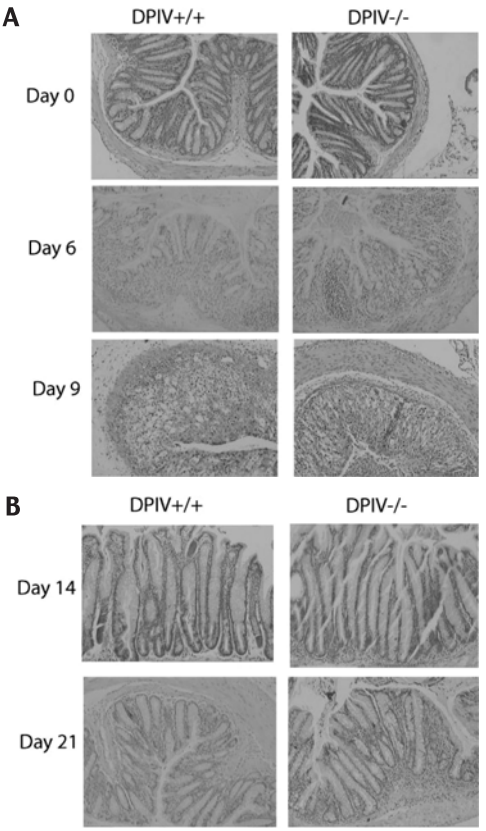

Figure 2. No differences in crypt damage were observed between DPIV$^{+/+}$ and DPIV$^{-/-}$ mice during the induction and resolution of DSS-induced colitis measured. Photomicrographs (20x) are of 4 μm sections of distal colon stained with haematoxylin and eosin. No differences in crypt damage were observed between DPIV$^{+/+}$ and DPIV$^{-/-}$ mice during the induction and resolution of DSS-induced colitis measured. Photomicrographs (20x) are of 4 μm sections of distal colon stained with haematoxylin and eosin.

($p < 0.05$) by 60% compared to saline treatment. Thus this preliminary study shows, for the first time, inhibition of DPIV-like activity appears to have a significant effect in attenuating the effects of experimentally induced colitis in mice.

The inhibitors used in the current study possess differing inhibitory properties. While both inhibit DPIV in the nM-range, Ile-cyano-pyrr is a slow-binding inhibitor, binding covalently to the active site (the DPIV enzyme will reactivate over a time period of about 10 hrs), thus Ile-cyano-pyrr will inhibit DPIV activity for a longer period of time than Ile-thia, which is a competitive inhibitor whose effects are shorter lasting due to the metabolic clearance of the non-enzyme-bound compound. Moreover, if the effect of the inhibitors unfolds preferentially after absorption, both absorption rate and metabolic clearance concerning inhibitor efficacy come into the picture (Folz *et al* 2004). However, if different DP-like activities other than that of DPIV or even a mixture of them are the target of the inhibition attenuating colitis disease symptoms,

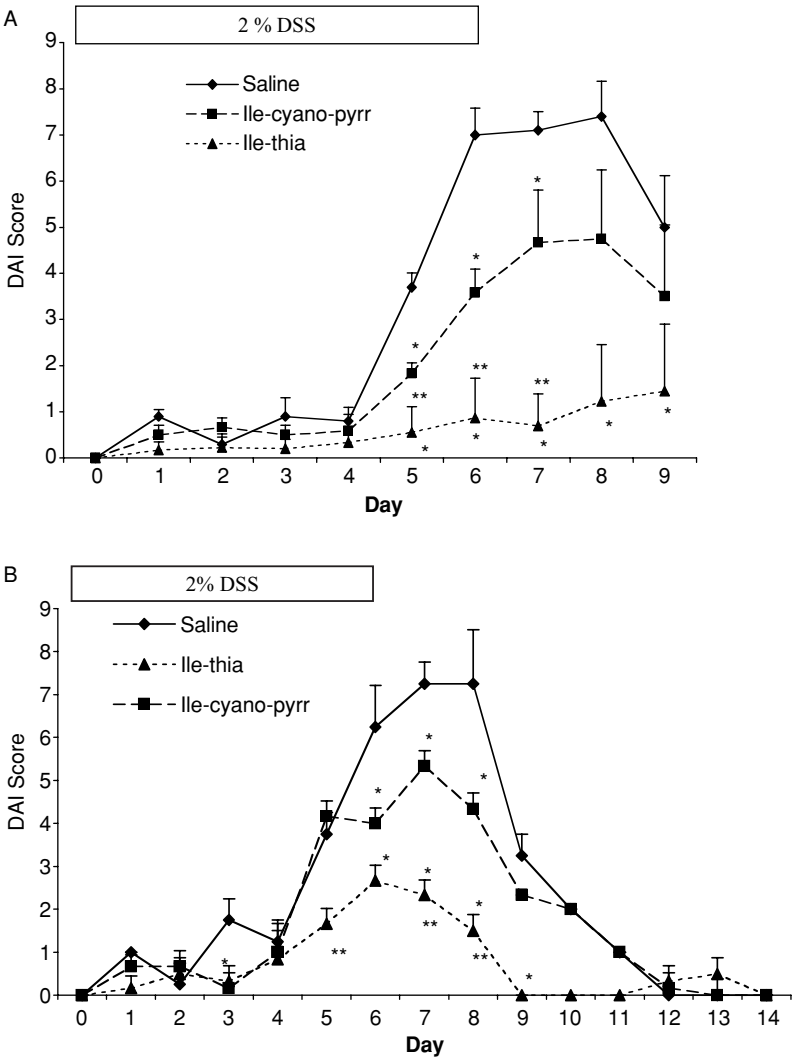

Figure 3. Reduced disease activity in inhibitor treated mice compared to saline treated mice. Over a 9-day period mice were given saline, Ile-cyano-pyrr or Ile-thia (n = 12 for all groups) and disease activity was assessed (A). Disease activity was then monitored over a 14-day period in saline treatment, Ile-cyano-pyrr group and Ile-thia group (n = 6 for all groups) (B). Data are expressed as mean ± SEM. $^{*}p < 0.05$ for DAI scores in Ile-cyano-pyrr and Ile-thia treatment groups compared to saline treatment. $^{**}p < 0.05$ for DAI scores with Ile-cyano-pyrr treatment compared to the Ile-thia administration.

the different results might be due to different inhibitory specificities of the compounds tested versus these different enzymes. In addition, while DPIV cleavage acts to negatively regulate GLP-2 action, the role of DP enzyme activity in regulating peptides involved in immune and other cellular functions is still unclear. It is therefore possible that it is advantageous to have DP activity present at some levels during a 24 hr period in our disease model to help balance

DPIV-mediated immune and other cellular effects and that DPIV inhibition is only required around feeding when GLP-2 is secreted.

6. CONCLUSIONS

In our study, both DPIV inhibitors, Ile-cyano-pyrr and Ile-thia, decreased colitis disease activity and helped maintain intestinal integrity. However, Ile-thia possessed a greater protective effect. It is possible that greater intestinal growth may have been observed had GLP-$2_{1\text{-}33}$ levels been elevated either directly by exogenous GLP-$2_{1\text{-}33}$ administration or indirectly, via metformin treatment. Hartmann *et al* demonstrated that endogenous GLP-$2_{1\text{-}33}$ levels appear to be sufficient to promote intestinal growth following intestinal damage as GLP-2 immuno-neutralization in a rat model of intestinal damage resulted in a significant decrease in intestinal growth (Hartmann *et al* 2002). The co-administration of metformin and DPIV inhibitor may be a more feasible therapeutic approach as Yamazaki *et al* recently showed that plasma GLP-2 levels were significantly increased by 1.4 to 1.6-fold in fasted F344 rats 1 hour after a dose of the oral biguanide, metformin (300 mg/kg) (Yamazaki *et al* 2004). Although DPIV inhibitors were protective in the colitis model, the mechanism of their action is unclear. We observed no difference in the development and resolution of DSS-colitis between wildtype and DPIV knockout mice suggesting that DPIV does not play a role in regulating GLP-2 levels. Therefore, further investigations are required in order to elicit the role that other DPs such as FAP, DP8 and DP9 may play in this process. It would also be very interesting to investigate the efficacy of inhibitor therapy in models of small intestine damage where higher levels of DPIV are observed. In summary, this study is the first to demonstrate that inhibition of DPIV-like activity confers a protective effect against the symptoms of experimentally-induced colitis. We propose that inhibition of DPIV or other DPIV family members possessing DPIV-like activity may provide a new treatment strategy for IBD.

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CHAPTER 17

POSSIBLE ROLE OF DP IV INHIBITORS IN ACNE THERAPY

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1. INTRODUCTION

Acne is a very common disease with a prevalence of 80–85% among adolescents (Stern 2000). It exhibits a peak incidence at 15–18 years and can persist throughout adulthood in 12% of women older than 25 years and 3% of persons aged 35–44 years (White 1998, Goulden *et al* 1999). To prevent physical or psychological scarring, as many as 15–30% of patients with acne need intense medical treatment, thus representing the largest patient group seen by dermatologists worldwide. The costs for physicians' consultations and prescriptions for acne in the USA are estimated to exceed $1 billion per year, whereas additional economic implications include higher unemployment rates among acne subjects and increased psychiatric comorbidity (Gupta 1998).

Acne occurs in the pilosebaceous units localized on the face, chest and back, and is characterized by clinically noninflammatory (comedones) or inflammatory lesions (papules pustules or nodular lesions). Several main factors contribute to the pathogenesis of this complex disorder (Gollnick 2003), among them androgen-controlled sebaceous hyperplasia with hyperseborrhea and follicular hyperkeratosis resulting in both increased keratinocyte proliferation and disturbed desquamation, microbial hypercolonization with *Propionibacterium acnes* as

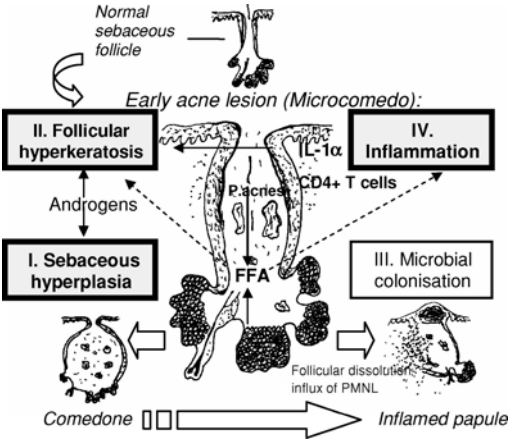

Figure 1. DP IV inhibitors can target three of the four major pathogenetic factors of acne lesion development (FFA = free fatty acids).

well as inflammation and immunological host reaction (Figure 1). Pro-inflammatory cytokines and CD4+ T cells are implicated in the development of early acne lesions (Jeremy *et al* 2003).

Due to the complex pathogenetic events in acne, efficient treatment is often achieved only by combining two or more therapeutic agents that target different pathogenetic factors in a concerted action (Gollnick *et al* 2003). Therefore, the development of safe new compounds with the ability to target more than one major pathogenetic factor in parallel is clinically required.

Inhibitors of dipeptidyl peptidase IV affect proliferation and function of immune cells and show therapeutic potential in inflammatory disorders. Moreover, it was shown that the presence of the inhibitors influenced keratinocyte proliferation and differentiation *in vitro* (Reinhold *et al* 1998, Vetter *et al* 1999) and *in vivo* (Thielitz *et al* 2002).

The ability of DP IV inhibitors to suppress hyperproliferation and disturbed differentiation of skin cells in combination with their immunosuppressive effects gives reason to further investigate their possible role in future acne therapy.

2. HOW CAN DP IV INHIBITORS TARGET ACNE PATHOGENESIS?

The microcomedone as the first detectable acne lesion develops as a result of androgen-stimulated hyperseborrhea and follicular hyperkeratosis in the pilosebaceous infundibulum showing features of both hyperproliferation and disturbed differentiation (Figure 1). The disease is aggravated by inflammatory events and immunological host reaction which are partially caused by microbial colonization, but can initially occur without bacterial influence. The perifollicular amounts of the proinflammatory cytokine IL-1α and CD4+ memory T cells are significantly increased in early acne lesions and unaffected follicles of acne patients compared to non-acne subjects. Free fatty acids (FFA) in the follicle produced by sebocytes and released from sebum triglycerides triggered by bacterial lipase have pro-comedogenic and proinflammatory properties.

The microcomedone as clinically non-visible lesions develops either to non-inflammatory open (blackhead) or closed comedones (whitehead) and then consecutively or directly to inflammatory lesions such as papules, pustules or nodes.

An ideal therapeutic agent in acne would be capable to reduce sebocyte proliferation and consecutive hyperseborrhea, suppress keratinocyte proliferation and restore disturbed desquamation and have direct antiinflammatory activity against immune cells involved in acne pathogenesis. An additional antibiotic activity agains *P. acnes* would be desirable but also bears the danger of inducing bacterial resistance.

2.1. Antiproliferative Activity Against Keratinocytes and Sebocytes

Keratinocytes express ectopeptidases such as dipeptidyl peptidase IV (DP IV, CD26) and membrane alanyl aminopeptidase (mAAP; CD13) constitutively on their surface. *In vivo*, a significant upregulation of CD26 and CD13 immunoreactivity has been reported in the epidermis of patients with hyperproliferative and neoplastic skin diseases such as psoriasis, lichen planus or cutaneous T-cell lymphoma (Novelli *et al* 1996, Hunyadi *et al* 1993) We have shown previously that DP IV is involved in the regulation of keratinocyte growth *in vitro* and application of DP IV inhibitors partially restores disturbed keratinization in the mouse tail model of psoriasis *in vivo*. Thus DP IV inhibitors are promising agents to normalize also follicular hyperkeratosis and hyperproliferation which is present in acne (Figure 1).

Moreover, we have investigated human SZ 95 sebocytes (an immortalized human sebaceous cell line) and found a high expression of DP IV on their surface as detected by flow cytometry, RT-PCR and enzymatic assays (CD26-Expression 92 $\pm$ 4 %, enzymatic activity 37,3 $\pm$ 9,7 pkat/10^6 cells). The synthetic DPIV inhibitors Lys[Z(NO_2)]-thiazolidide and –pyrrolidide supress DNA-synthesis of these cells in a rapid and dose-dependent manner (IC50: 55 μM and 92 μM; IC25: 2,5 μM and 5 μM after 24 h of incubation), measured by ^{3}H-Thymidine incorporation. The differentiation of sebocytes, characterized by increasing cell size and lipid content, was detected by nile red fluorescence intensity. Differentiation was dose-dependently increased after 48 h of incubation, which possibly indicates an accelerated maturation process in the presence of inhibitors before sebocytes undergo holocrine secretion in the last maturation step.

Furthermore Lys[Z(NO_2)]-thiazolidide significantly induced IL-1RA protein determined in SZ 95 supernatants by ELISA after 24 and 48 h of incubation with a maximum 2-fold increase after 48 h. Quantitative mRNA analysis confirmed these results with a maximum increase after 4 h. IL-1α was also detectable in low amounts but was not significantly changed in the presence of the inhibitor.

The pro-inflammatory cytokine IL-1α is another central hallmark in acne pathogenesis. Its levels are already increased in uninvolved follicles and early acne lesions, and are abundant in extracted mature comedones (Ingham *et al* 1992). Although the cascade inducing the increased expression *in vivo* of IL-1α is not yet elucidated, its functional relevance was studied in an *in vitro* organ model, where it induced a follicular hyperkeratosis in the isolated sebaceous infundibula. This process could be blocked or reversed by the application of IL-1RA, indicating that the ratio of IL-1α/IL-1RA is involved in regulating growth and differentiation of keratinocytes.

In HACAT keratinocytes (a spontaneously immortalized cell line), IL1-RA levels were also increased on the protein and mRNA levels in the presence of the DP IV inhibitor, in a ratio comparable to sebocytes.

The results are summarized in Table 1.

Table 1. Synopsis of DP IV inhibitor effects on cells involved in acne pathogenesis

		Keratinocytes (KC)		*P. acnes*.-stimulated
	SZ 95 sebocytes	HACAT	Primary KC	T cells
Proliferation	↓↓	↓↓	↓	↓↓↓
Cytokines (protein)	IL-1RA ↑ (48 h)	IL-1RA ↑ (48 h)	IL-1RA ↑ (48 h)	TGF-β1↑, IL-2↓ (24 h)
Cytokines (mRNA)	IL-1RA ↑ (4 h)	IL-1RA ↑ (4 h)	n.d.	n.d.
Differentiation	↑	↑ (*in vivo*-mouse tail model)		n.d.

2.2. Antiinflammatory Activity Against *P. acnes*-Stimulated T Cells

Recent evidence suggests that inflammatory processes occur in uninvolved skin of acne patients prior to hyperproliferative or abnormal differentiation events (Jeremy *et al* 2003), among them significantly increased numbers of CD4+ T cells and macrophages around pilosebaceous follicles. Within the increased perifollicular CD4+ T cell population, the majority consisted of memory/effector (CD45RO) cells, with a similar proportion exhibiting a skin-homing phenotype; this finding suggests a specific antigenic response. *P. acnes* antigen has been identified as one stimulus that induces a prototypic T helper 1 immune response in animals (Matsui *et al* 1997) and this observation was confirmed in human T cell lines isolated from lesional acne skin (Mouser *et al* 2003). These cells showed a significant increase in proliferation and interferon (IFN)-γ production in response to *P. acnes* stimulation, which could not be achieved with psoriatic T cell lines or in presence of other skin commensals. We therefore isolated peripheral blood mononuclear cells (PBMC) from acne patients and subjects with a history of acne, and stimulated them with *P. acnes* antigen. We found a significantly increased but varying proliferative response of PBMC of acne patients/subjects with acne history compared with control subjects without a history of acne. PBMC of acne patients/subjects with acne history and control subjects showed equal rates of DNA synthesis after stimulation with the mitogen PHA.

Moreover, we studied the effects of the DP IV inhibitors on *P. acnes* antigen-stimulated DNA synthesis of acne patients/subjects with acne history. In accordance to previous data obtained from mitogen-stimulated PBMC and T cells (Reinhold *et al* 1997, 2002), we found very strong antiproliferative activities for each of the four inhibitors used in a concentration range of 1–20 μM on *P. acnes* antigen-stimulated PBMC. Furthermore, we demonstrated a decrease in IL-2 production and a significant increase in the immunosuppressive cytokine TGF-ß1 after 24 h in the presence of DP IV inhibitors used in concentrations of 10 μM.

Thus our data provide evidence for the action of a possible novel therapeutic agent which may prevent initiation of the acne lesion at a very early stage, as increased perifollicular amounts of CD4+ T cells in acne are already found in uninvolved follicles prior to any detectable signs of keratinocyte hyperproliferation.

3. CONCLUSIONS

The presented data provide evidence that DP IV is expressed on human sebocytes and most likely involved in regulation of sebocyte proliferation and cytokine production. The aminopeptidase inhibitors Lys[Z(NO_2)]-thiazolidide and Lys[Z(NO_2)]-pyrrolidide suppress proliferation of three cells types involved in acne initiation; moreover, they induce the anti-inflammatory

cytokine IL-1RA in sebocytes and keratinocytes being capable to follicular hyperkeratosis, and they up-regulate the immunosuppressive cytokine transforming growth factor-β_1 in *P. acnes*-stimulated T cells.

In summary, DP IV inhibitors affect proliferation, differentiation and inflammation processes in acne-involved cells in a therapeutic manner (Table 1). Thus they might in the future act as promising new agents for acne therapy.

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CHAPTER 18

DIPEPTIDYL PEPTIDASE-IV ACTIVITY AND/OR STRUCTURE HOMOLOGS (DASH): CONTRIBUTING FACTORS IN THE PATHOGENESIS OF RHEUMATIC DISEASES?

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1. INTRODUCTION

Dipeptidyl peptidase-IV (DPP-IV/CD26, EC 3.4.14.5) is a multifunctional molecule, executing its physiological functions via the receptor (non-hydrolytic) mechanisms as well as by limited proteolysis of biologically active peptides. By the latter mechanism, DPP-IV was shown to play a role in many physiological and pathological events, mostly due to cleavage of multiple neuropeptides and cytokines (for review, see Sedo and Malik 2001, Aytac and Dang 2004). Such proteolytic processing can either terminate biological effect of the peptide or change its receptor preference, leading to its signaling potential modification. Subsequently, a number of molecules constituting a group of "Dipeptidyl peptidase-IV Activity and/or Structure Homologues" (DASH) was defined (Sedo and Malik 2001). This group comprises for example fibroblast activation protein α/seprase (FAP), DPP8, DPP9, attractin, N-acetylated-α-linked-acidic dipeptidases I, II and L, quiescent cell proline dipeptidase (QPP, formerly DPP-II or DPP-VII), thymus-specific serine protease and DPP-IV-β. Because of the similar substrate specifity it is tempting to speculate on their possible functional crosstalk.

Changes of DPP-IV enzymatic activity as well as alterations of its biologically active substrates in various diseases, including rheumatic ones, were observed by several authors (Aytac and Dang 2004; Busek *et al* 2004, Gotoh *et al* 1989; DeMester *et al* 2000). Consequently, a possible role for "canonical" DPP-IV in the development of rheumatoid arthritis and even its potential to become a new therapeutic target in these diseases has been suggested recently (Williams *et al* 2003).

The aim of our study was to analyze expression patterns and resulting DPP-IV-like enzymatic activity of DASH molecules in inflammatory rheumatic diseases, represented by rheumatoid arthritis (RA) and psoriatic arthritis (PsA). Considering possible different roles of particular DASH in the regulation and perpetuation of inflammatory processes on systemic and local levels, we studied the DPP-IV-like enzymatic activity in blood plasma, synovial fluid and DASH expression in mononuclear cells separated from both above mentioned compartments.

2. METHODS

Patients with RA and PsA were diagnosed using standard criteria (Gladman *et al* 1990; Arnett *et al* 1988). The control group comprised patients with osteoarthritis (OA).

Blood mononuclear cells (BMNC) and synovial fluid mononuclear cells (FMNC) were isolated by discontinuous Lymphoprep density centrifugation. Isolated cells were counted on Coulter cell counter Z2, and their viability was determined by trypan blue exclusion.

Enzyme activities of all samples were measured by continuous fluorimetric assay using 7-(glycyl-prolylamido)-4-methylcoumarin or 7-(lysyl-alanyl)-4-methylcoumarin (Bachem, Bubendorf, Switzerland), final concentration 50 μmol/l as substrates. Aminomethylcoumarin release was monitored on a fluorimeter LS50B (Perkin-Elmer, Ueberlingen, Germany) at excitation and emission wavelengths 380 and 460 nm, respectively. Cell surface activity (CS) was recorded in whole cell suspension, total activity (T) after the addition of Triton X-100 in the same sample (Sedo *et al* 1998).

Sephacryl S-300 (Pharmacia Uppsala, Sweden) column (1.5 × 95 cm) was used for gel chromatography to separate DPP-IV-like enzymatic activity bearing molecules from blood plasma and synovial fluid.

To determine the presence of particular DASH transcripts, reverse transcription-polymerase chain reaction was used. Total RNA was isolated from BMNC and FMNC samples using MINI kit (Qiagen). Reverse transcription was performed according to the manufacturer's instruction (Roche). The following primers were used: Attractin: forward: 5′- ACA CAG ATT GTT ACA GCT GCA C –3′; reverse: 5′- CCT TTC ACA GAC ACT ACC ATT G –3′. DPP IV: forward: 5′- GTA GCT AGC TTT GAT GGC AGA –3′; reverse: 5′- GGT TAC GTA CCC TCC ATA TGA C –3′. QPP: forward: 5′- GTT CTA GCT GTG GCA GGC CTC – 3′; reverse: 5′- TAT CGG TCA CAT TGT TGC TGG C – 3′. FAP: forward: 5′- GTA TTT ATG CTG GTC GCC TG – 3′; reverse: 5′- ACG AAA TGG CAT CAT AGC TG – 3′. DPP8: forward: 5′- GTC ACG AAG AGG AAA TTG AG – 3′; reverse: 5′- CAC CAT ATA TGA ACA GCA CAG – 3′. The specificity of the PCR products was confirmed by cyclic sequencing (ABI Prism 310, Applied Biosystems, Foster City, CA) using the same PCR primers.

Mann-Whitney test was used to compare non-paired sets of data. Correlation between quantitative variables was assessed by Spearman's correlation coefficient.

3. RESULTS

3.1. Blood Plasma and Synovial Fluid

As shown in Figure 1A, the total blood plasma DPP-IV-like specific activity was significantly lower in patients with RA and PsA compared to the controls. In the case of RA,

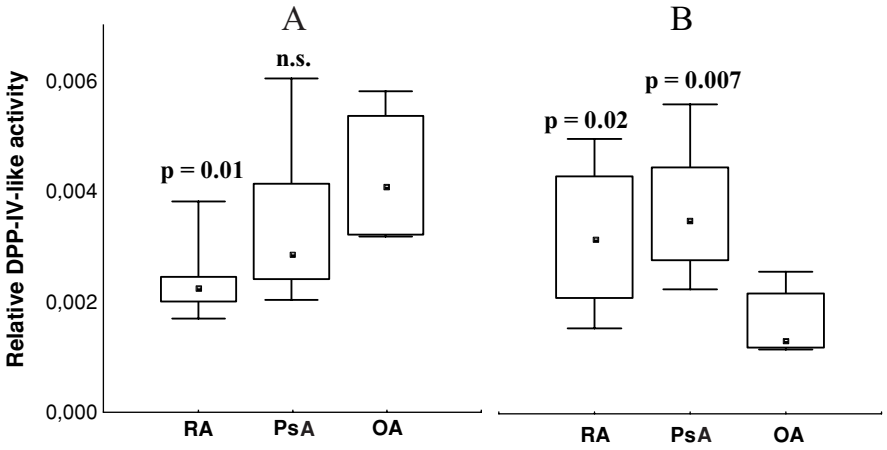

Figure 1. DPP-IV-like enzymatic activity in: **(A)** blood plasma; RA (n = 9), PsA (n = 7) and OA (n = 6) and **(B)** Synovial fluid; RA (n = 11), PsA (n = 7) and OA (n = 6); Squares: Medians; Boxes: middle 25–75% of measured values; Bars: Extreme measured values. Indicated probability of difference (p) compared to OA in particular biological material; n.s. Non-significant.

DPP-IV-like activity negatively correlated with the plasma C-reactive protein and with the standard Disease Activity Score 28 (DAS28; Prevoo *et al* 1995). Such trend is consistent with previously published observations (Cuchacovich *et al* 2001). Surprisingly, in contrast with the previous literature evidence (Gotoh *et al* 1989), DPP-IV-like specific activity in the synovial fluid from patients with RA and PsA was significantly higher than that of OA in our experimental group (Figure 1B).

Gel chromatography of blood plasma as well as synovial fluid revealed different patterns with markedly varying proportion of two molecular weight (MW) forms of DPP-IV-like activity with the MW of about 400 and 250 kDa. Considering MW of all so far identified DASH molecules, the higher one may represent previously described tetrameric form of DPP-IV (Engel *et al* 2003), however, copurification of some DPP-IV-like active DASH in complex with other molecule should be considered as well. The lower MW form of DPP-IV-like enzymatic activity corresponds to DPP-IV/CD26. Relative proportion of both MW isoforms in individual patients markedly varied but did not revealed any relation to particular diagnosis so far.

3.2. Synovial Fluid and Blood Mononuclear Cells

Studies of FMNC revealed significantly lower total (T) and especially cell surface (CS) DPP-IV-like enzymatic activity in RA patients compared to OA (Figure 2). Lower CS/T ratio of the enzymatic activity in patients with RA than that of OA suggests prevailing reduction of the plasma membrane DASH molecules over the intracellularly localized ones in RA. The presence of DPP-IV and attractin, both known to be typically localized in plasma membrane, has been preliminarily revealed by flow immunocytochemistry in both FMNC and BMNC. Absence of FAP transcript in all studied cell types (Table 1) and no effect of bestatin, an attractin enzymatic activity specific inhibitor (Duke-Cohan *et al* 1995) on the detected DASH hydrolytic

Table 1. DASH transcripts in BMNC and FMNC of RA, PsA and OA patients.

Code	Dg	Cells	DPP IV	Att	FAP	QPP	DPP 8
KH	RA	BMNC	+	−	−	+	+
		FMNC	+	−	−	+	+
KI	RA	BMNC	+	+	−	+	+
		FMNC	+	+	−	+	+
HM	RA	BMNC	+	+	−	+	+
		FMNC	+	−	−	+	−
KI2	RA	BMNC	+	+	−	+	+
		FMNC	+	+	−	+	+
PH	RA	BMNC	−	−	−	+	+
		FMNC	−	−	−	+	+
FJ	RA	BMNC	−	+	−	+	+
		FMNC	−	+	−	+	+
MR	RA	BMNC	+	+	−	+	+
		FMNC	ND	ND	ND	ND	ND
KH	RA	BMNC	+	+	−	+	+
		FMNC	+	+	−	+	+
BM	RA	BMNC	+	+	−	+	+
		FMNC	+	+	−	+	+
RJ	PsA	BMNC	+	+	ND	ND	ND
		FMNC	+	−	ND	ND	ND
SP	PsA	BMNC	+	+	−	+	+
		FMNC	+	+	−	+	+
KP	PsA	BMNC	+	−	−	−	−
		FMNC	+	+	−	+	+
SI	PsA	BMNC	−	+	−	+	+
		FMNC	−	+	−	+	+
DV	PsA	BMNC	+	+	−	−	+
		FMNC	−	+	−	+	+
KS	PsA	BMNC	ND	ND	ND	ND	ND
		FMNC	+	+	ND	+	ND
SJ	PsA	BMNC	+	+	−	+	+
		FMNC	+	−	−	+	−
EI	OA	BMNC	+	+	−	−	+
		FMNC	+	+	−	+	−
PL	OA	BMNC	+	−	−	+	−
		FMNC	ND	ND	ND	ND	ND
BV	OA	BMNC	+	+	−	+	+
		FMNC	ND	ND	ND	ND	ND

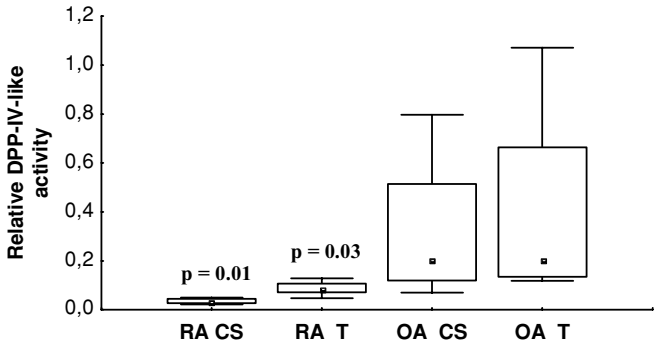

Figure 2. DPP-IV-like enzymatic activity in FMNC. CS: cell surface; Squares: Medians; Boxes: middle 25–75% of measured values; Bars: Extreme measured values. T: total cellular. RA (n = 6) and OA (n = 6); Indicated probability of difference (p) compared to OA in particular biological material.

activity suggest that at least the major part of DPP-IV-like activity in FMNC from OA patients may be attributed to the canonical DPP-IV. In RA, substantial involvement of intracellularly localized DASH molecules (DPP8, 9) should be considered, although the major part of plasma membrane DASH activity is probably carried by DPP-IV. Seemingly contradictory presence of mRNA transcripts and no inhibitory effect of bestatin on the DPP-IV-like activity of BMNC and FMNC may be explained either by the low final effective concentration of an attractin protein itself or by its low specific activity, compared to DPP-IV.

Since there was almost a complete inhibition of DPP-IV-like enzymatic activity at acidic pH 4.5–5 (not shown), participation of another typically intracellularly localized DASH, QPP/DPP-II, seems not probable, although QPP transcripts were detected (Table 1).

We observed no apparent differences in the DPP-IV-like enzymatic activity in BMNC among all experimental groups.

RT-PCR analysis demonstrated differential, patient specific, but yet not disease specific, expression patterns of several DASH transcripts in BMNC and FMNC. DPP-8 and QPP mRNA transcripts were detectable in virtually all, DPP-IV and attractin in the overwhelming majority, while FAP transcripts, as mentioned above, were not detected in any of the studied samples (Table 1).

4. CONCLUSIONS

Deregulation of DPP-IV-like activity was observed in synovial fluid and blood plasma of patients with RA and PsA. The cellular source of soluble DPP-IV-like enzymatic activity remains unclear.

Our results suggest significant contribution of intracellular DASH molecules to the total DPP-IV-like enzymatic activity, at least in FMNC from rheumatoid arthritis patients and in BMNC from both rheumatoid arthritis and osteoarthritis.

DASH subcellular distribution was different in FMNC in patients with rheumatoid arthritis compared to degenerative osteoarthritis.

Altered expression pattern and resulting distorted properties of whole DASH enzymatic activity in both systemic—blood—as well as local—joint—environments may impair processing of mediators involved in the inflammatory processes.

Together, DASH molecules, due to their enzymatic activity, may participate on the pathogenesis of RA and PsA and deserve attention as possible future therapeutic targets.

NOTES

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TOPIC V

NEURONAL DISEASES

CHAPTER 19

DIPEPTIDYL PEPTIDASE IV (DP IV, CD26) AND AMINOPEPTIDASE N (APN, CD13) AS REGULATORS OF T CELL FUNCTION AND TARGETS OF IMMUNOTHERAPY IN CNS INFLAMMATION

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1. INTRODUCTION

The ectoenzyme dipeptidyl peptidase IV (DP IV, E.C. 3.4.14.5) was discovered by Hopsu-Havu and Glenner (Hopsu-Havu and Glenner 1966) and is identical with the leukocyte surface antigen CD26 (Fleischer 1994). The critical role of DP IV/CD26 in inflammation is associated with its enzymatic activity as well as its cellular functions. Initial work focused on the enzymatic activity of this molecule. DP IV/CD26 is an exopeptidase catalyzing the release of N-terminal dipeptides from oligo- and polypeptides preferentially with proline, hydroxyproline and, with less efficiency, alanine in the penultimate position. The post proline cleaving substrate specificity makes DP IV/CD26 relatively unique among other proteases. Among the rare group of proline-specific proteases, DP IV was originally believed to be the only membrane-bound enzyme specific for proline as the penultimate residue at the amino-terminus of the polypeptide chain. However, recent studies have identified a number of additional molecules bearing DP IV-like enzymatic activity, which are designated DPP-IV activity- and/or structure-homologues (DASH) (Sedo and Malik 2001).

In 1977, Lojda demonstrated for the first time DP IV activity on human peripheral blood lymphocytes (Lojda 1977). Within the hematopoietic system, DP IV/CD26 is expressed on

the surface of resting and activated T, B, and NK cells. DP IV/CD26 expression is upregulated following stimulation of T cells with mitogen, antigen, anti-CD3 antibodies or IL-2, B cell stimulation with staphylococcal proteins and IL-2 stimulation of NK cells. Thus, activated antigen-specific $CD4^+$ T cell clones express high levels of DP IV/CD26 (Kähne *et al* 1999), while some reports indicate preferential expression on T helper type 1 (Rogge *et al* 2000) and T regulatory cells (Cavani *et al* 2000). The wide range of DP IV/CD26 activity in inflammation is related to soluble mediators and cellular functions. DP IV exopeptidase activity has been shown to play a role in the activation or inactivation of biological peptides (De Meester *et al* 1999, 2003; Lambeir *et al* 2001, 2003; Wrenger *et al* 1996, 1997, 2000, 2000a, 2000b; Lorey *et al* 2003). DP IV/CD26 has attracted great interest due to its ability to process and either inactivate or alter the specificity of many chemokines, including CXCL10 (IFN-inducible protein 10 kDa; IP-10) and CXCL12 (stromal cell-derived factor-1; SDF-1) (Lambeir *et al* 2001). Processing of CXCL10 and CXCL11 (IFN-inducible T cell α-chemoattractant; I-TAC) by DP IV/CD26 results in reduced CXCR3-binding and more than 10-fold reduced chemotactic potency, while cleavage products act anti-chemotactically (Proost *et al* 2001). Similar mechanisms were observed for CXCL12 (De Meester *et al* 1999, 2003). On the other hand, DP IV/CD26 differentially regulates the chemotaxis of T cells and monocytes toward the chemokine CCL5 (regulated on activation, normal T expressed and secreted; RANTES), enhancing directed T cell migration by a factor of two (Iwata *et al* 1999). Several observations support the notion of a role *in vivo* for the enzymatic activity of DP IV/CD26 on leukocyte membranes. For example, anergic T cells have been reported to exert antigen-independent inhibition of cell-cell interactions via chemokine metabolism. Instrumental to this effect may be the increased cell surface expression and enzymatic activity of CD26, which may act by metabolizing chemoattractants bound to the endothelial/epithelial cell surface (James *et al* 2003). Apart from processing polypeptides, DP IV/CD26 can interact either as a receptor or ligand with various proteins playing a role in immune responses. It thus became increasingly clear that in addition to its enzymatic activity, cellular DP IV/CD26 is associated with a variety of leukocyte functions. The enzyme has been described as a collagen receptor, adenosine deaminase (ADA) binding protein and a ligand for CD45 (Ishii *et al* 2001; Kameoka *et al* 1993, 1995; Torimoto *et al* 1991; Hanski *et al* 1985). These insights and observations summarized in the next section led several groups to focus on the key role of DP IV/CD26 in regulation of activation, differentiation and growth of T cells (Torimoto *et al* 1991; Dang *et al* 1990; Ansorge *et al* 1997).

2. THE ROLE OF DP IV/CD26 IN T CELL FUNCTIONS

The development of specific synthetic DP IV inhibitors facilitated the study of the cellular functions of this enzyme on the surface of immune cells. In 1985 Schön and coworkers (Schön *et al* 1985) were the first to show that N-Ala-Pro-O-(nitrobenzoyl-)hydroxylamine, which irreversibly inhibits DP IV, is capable of suppressing the proliferation of human peripheral blood mononuclear cells (PBMC) stimulated with mitogens. This was the first evidence that DP IV plays a critical role in the regulation of DNA synthesis of immune cells and that the enzymatic activity of DP IV is involved in this process.

In the meantime, many biochemically distinct synthetic inhibitors of DP IV have been studied under different stimulation conditions using a variety of target cells. Among the most

potent reversible inhibitors were Lys[Z(NO_2)]-thiazolidide, piperidide, and -pyrrolidide which cause fifty percent inhibition of DP IV in the range of 2–3 μM concentration.

Subsequently, expression of DP IV/CD26 as well as inhibitory effects of DP IV-specific inhibitors on leukocyte proliferation were seen in different human and mouse T cell populations (Reinhold *et al* 1997, 1997a, 1998; Vivier *et al* 1991; Steinbrecher *et al* 2000, 2001).

Besides their influence on DNA synthesis, DP IV inhibitors also exhibit strong suppressive effects on the production of cytokines. It was shown that Lys[Z(NO_2)]-thiazolidide, -piperidide, and -pyrrolidide inhibit the production of IL2, IL-10, IL-12, and IFN-γ of PWM-stimulated PBMC and purified T cells (Reinhold *et al* 1997) as well as the production of IL-2, IL-6 and IL-10 of PHA-stimulated mouse splenocytes and ConA-stimulated mouse thymocytes (Reinhold *et al* 1997). These inhibitors also reduce in a dose-dependent manner IFN-γ, IL-4, and TNF-α production of myelin basic protein (MBP)-stimulated T cell clones from MS patients (Reinhold *et al* 1998). As shown by competitive RT-PCR, also the levels of IL-2 and IFN-γ mRNA in mitogen-stimulated T cells are decreased after exposure of cells to Lys[Z(NO_2)]-thiazolidide (Arndt *et al* 2000).

In contrast to their suppression of most pro-inflammatory cytokines, DP IV inhibitors elicit enhanced production and secretion of latent transforming growth factor-β1 (TGF-β1). In an attempt to determine the relationship between production of immunosuppressive TGF-β1 and inhibition of DNA synthesis and cytokine production mediated by DP IV inhibitors, the concentrations of latent TGF-ß1 in supernatants of PWM-stimulated PBMC and T cells were assayed in the presence or absence of DP IV inhibitors. In these experiments, DP IV inhibitors induced a 3- to 4-fold increase in latent TGF-β1 secretion (Reinhold *et al* 1997). These results correlated well with the observed elevation of TGF-β1 mRNA levels in T cells treated with Lys[Z(NO_2)]-thiazolidide (Kähne *et al* 1999). In agreement with previous reports on the immunosuppressive role of TGF-β1 (Reinhold *et al* 1995; Reinhold *et al* 1994), a neutralizing chicken anti-TGF-β1 antibody abolished DP IV inhibitor-induced suppression of DNA synthesis of PWM-stimulated PBMC and T cells. These observations suggest that TGF-β1 may be responsible for the molecular action of DP IV/CD26 inhibitors on T cells, i.e. suppression of DNA synthesis and cytokine production. In addition to their effects on T cell proliferation and cytokine production, TGF-β1 as well as DP IV inhibitors are involved in a negative feedback mechanism by negatively regulating mRNA expression of DP IV/CD26 (Arndt *et al* 2000).

3. TARGETING DP IV IN CNS INFLAMMATION

Due to their blocking effects on T cell activation and function, DP IV inhibitors were tested for immunosuppressive therapy *in vivo*. The role of DP IV/CD26 and the effect of synthetic DP IV inhibitors were examined in several animal models of human diseases (Steinbrecher *et al* 2000, 2001; Tanaka *et al* 1997, 1998; Korom *et al* 1997, 1997a, 1999). Based on results of these studies, DP IV/CD26 has attracted major interest as a potential target in developing anti-inflammatory therapy for MS. As with other T cells, CD26 is expressed on myelin-reactive T cell lines from patients with MS (Reinhold *et al* 1998). In addition, patients with MS have an increased median percentage of $CD26^+$ cells among $CD4^+$ T cells in the peripheral blood (Jensen *et al* 2004; Sellebjerg *et al* 2000, 2000a; Constantinescu *et al* 1995; Hafler *et al* 1985). CD26 expression decreases somewhat in peripheral blood and cerebrospinal fluid after oral

high-dose methylprednisolone treatment. Others found correlations between changes in the CD26-expressing T cells and lesion activity on magnetic resonance imaging in MS patients with relapsing-remitting and chronic progressive disease, consistent with the function of CD26 in T cell activation (Khoury *et al* 2000). Jensen et al. recently studied $CD4^+$ T cell activation in patients with clinically isolated syndromes suggesting an initial attack of MS (Jensen *et al* 2004). They demonstrated that the percentage of blood $CD26^+$ $CD4^+$ T cells was increased in these patients, and correlated with magnetic resonance imaging disease activity and clinical disease severity. In contrast, the percentage of $CD25^+$ $CD4^+$ T cells, a phenotype consistent with naturally occurring regulatory T cells, in cerebrospinal fluid correlated negatively with the cerebrospinal fluid concentration of myelin basic protein and the presence of IgG oligoclonal bands. Finally, gene expression profiling in MS patients and healthy controls identified higher average expression of DP IV/CD26 in peripheral blood mononuclear cells of MS patients (Bomprezzi *et al* 2003).

For these reasons, we addressed the role of DP IV/CD26 in murine experimental autoimmune encephalomyelitis (EAE), a well characterized $CD4^+$ T-cell mediated autoimmune disease leading to CNS inflammation and demyelination in susceptible strains of mice (Steinbrecher *et al* 2000, 2001). We demonstrated that the clinical signs of EAE can be suppressed by DP IV inhibition *in vivo* both in a preventive and therapeutic fashion. CNS inflammation associated with acute EAE was reduced. DP IV inhibition *in vivo* did not eliminate autoreactive T cells as encephalitogenic T cells were isolated from both healthy mice treated with Lys[$Z(NO_2)$]-thiazolidide and from diseased control mice. In addition, DP IV inhibition *in vivo* did not suppress antigenic priming. We could show, however, that the protective effect of DP IV inhibition is caused by a modulation of T cell effector function. DP IV activity was detected on the cell surface of all autoreactive T cell clones examined. The DP IV inhibitors Lys[$Z(NO_2)$]-thiazolidide and -pyrrolidide had strong antiproliferative effects *in vitro* on the T cell clones examined, on both an encephalitogenic Th1 clone and a nonencephalitogenic Th2 clone. Lys[$Z(NO_2)$]-thiazolidide and -pyrrolidide also suppressed the proliferation of lymphe node cells and, importantly, their secretion of TNF-α and, to a lesser extent, IFN-γ . It is widely accepted that EAE can be mediated by Th1 $CD4^+$ T cells typically secreting IFN-γ, TNF-α , and lymphotoxin. We therefore suggest that the inhibition of T cell proliferation and effector functions including proinflammatory cytokine secretion may in part be responsible for the *in vivo* effect. More importantly, we found an up-regulation of latent TGF-β 1 production *in vivo* both in spinal cord tissues and in plasma from Lys[$Z(NO_2)$]-pyrrolidide-treated mice as compared to mice treated with PBS or Lys[$Z(NO_2)$]-OH. We demonstrated furthermore that Lys[$Z(NO_2)$]-pyrrolidide increases the secretion of latent TGF- β1 by antigen-stimulated lymphe node cell populations. We extended these findings, demonstrating for the first time that anti-TGF-β1 can also block the effect of DP IV inhibition on antigen-specific T cell proliferation. Our *in vitro* data suggested that the cell types induced to secrete latent TGF-β1 by Lys[$Z(NO_2)$]-pyrrolidide treatment include T cells. One can assume that TGF-β1 secretion is increased both by the encephalitogenic T cells initiating the infiltrate and by T cells that are attracted and activated during the later stages of lesion formation in a bystander fashion. Not surprisingly, macrophages and microglia, in addition to a majority of T cells, appear to produce TGF-β1 in acute EAE lesions. Whether those or other cell types are susceptible to regulation of DP IV/CD26 remains to be investigated. Taken together, our data suggest that the therapeutic effect of DP IV inhibitors may be mediated by upregulation of the immunosuppressive cytokine TGF-β1 *in situ* and the inhibition of T cell effector functions.

4. TARGETING CNS INFLAMMATION WITH INHIBITORS SPECIFIC FOR DP IV/CD26 AND AMINOPEPTIDASE N (APN/CD13)

Our previous work suggests that DP IV/CD26 inhibition represents a therapeutic approach protecting from autoimmune disease by a mechanism that includes an active TGF-β1-mediated antiinflammatory effect at the site of pathology. In addition, recent *in-vitro*-studies have shown that inhibitors of DP IV/CD26 and aminopeptidase N (APN/CD13) act in concert suppressing proliferation and effector functions of proinflammatory T cells (Lendeckel *et al* 2003). Based on these observations, we evaluated in the present study the combined use of DP IV/CD26 and APN/CD13 inhibitors as a potential therapy of EAE.

4.1. Materials and Methods

4.1.1. Proliferation Assay

T cells were enriched by nylon wool adherence from mononuclear cells isolated from peripheral blood of healthy donors by Ficoll-Paque gradient centrifugation. T cells were stimulated in serum-free AIM-V medium (GIBCO) with PHA (1 μg/ml) in the presence of different concentrations of the synthetic DP IV inhibitor Lys[Z(NO_2)]-thiazolidide and actinonin (3-((1-((2-[Hydroxymethyl]-1-pyrrolidinyl) carbonyl)-2-methylpropyl) carbamoyl) octanohydroxamic acid, C19H35N3O5), an inhibitor of APN/CD13. Cells were cultured for 96 hours and DNA synthesis determined by standard ^{3}H-thymidine uptake assay.

4.1.2. Animals

Female SJL/JolaHsd mice, age 5–6 weeks, were purchased from Harlan Israel. Mice were housed in the animal facility of the Hebrew University-Hadassah Medical School (HUJI). All procedures were conducted according to protocols approved by the HUJI Institutional Animal Care and Use Committee.

4.1.3. Peptide

$PLP_{139-151}$ (proteolipid protein peptide, aa 139–151, sequence HSLGKWLGHPDKF) was synthesized by the synthetic peptide facility at HUJI. Amino acid composition of the peptide was verified by HPLC, and purity was >85%.

4.1.4. Induction of EAE by Active Immunization

Mice were immunized subcutaneously (s.c., distributed over four spots across the flanks) with 200 μg $PLP_{139-151}$ in 0.2 ml emulsion consisting of equal volumes of PBS and CFA (Difco, Detroit, MI) and containing 4 mg/ml of mycobacterium tuberculosis H37Ra (Difco). To induce a severe disease, in some experiments 200 ng pertussis toxin (PTX) (List Biological Laboratories, Campbell, CA) was administered intraperitoneally (i.p.) at days 0 and 2.

4.1.5. Clinical Evaluation of EAE

Mice were examined daily for signs of disease and graded on a scale of increasing severity from 0 to 5 as follows: 0, no signs; 1, limp tail and/or slight slowing of righting reaction; 2,

hind limb weakness (paresis) and/or marked slowing of righting reaction; 3, complete paralysis of at least one hind limb; 4, hind limb paralysis and forelimb weakness; 5, moribund or dead animals. Mice with intermediate clinical signs were scored in 0.5 increments. Mean clinical scores at separate days were calculated by adding scores of individual mice and dividing the sum by the number of mice in each group.

4.1.6. Reversible Inhibitors of Ectopeptidases and EAE Treatment

Actinonin and the DP IV inhibitor Lys[Z(NO_2)]-pyrrolidide were used for treatment *in vivo* as indicated in the legends. Mice injected with equal volume of vehicle served as controls.

4.2. Results

4.2.1. Combined Action of DP IV and APN Inhibitors on T Cell Proliferation

As previously reported, T cell growth is controlled by DP IV and APN since inhibitors to both molecules inhibit T cell proliferation. Here, we tested a combined effect of targeting both ectopeptidases on T cells. Interestingly, the DP IV inhibitor Lys[Z(NO_2)]-thiazolidide and the APN inhibitor actinonin act in combination to fully abrogate mitogen-induced T cell proliferation (Fig. 1). Based on these in vitro findings, we decided to measure inhibitor effects in vivo in an established Th1 autoimmune model, EAE.

4.2.2. Treatment of EAE with DP IV/CD26 and APN/CD13 Inhibitors

Results of treatment of EAE with actinonin and Lys[Z(NO_2)]-pyrrolidide are summarized in Figure 2. Treatment of animals (5 mice/group) was initiated at or around the peak of the

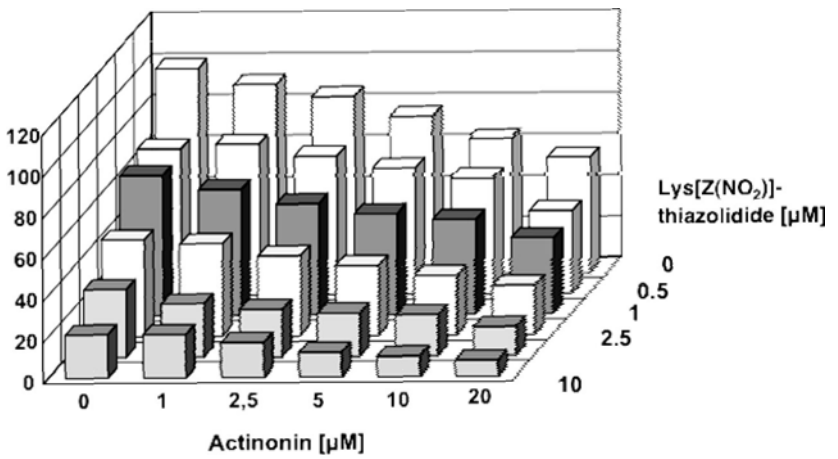

Figure 1. DNA synthesis of PHA-stimulated human T cells is inhibited by DP IV and APN inhibitors alone and in combination. The combined use of both inhibitors leads to total abrogation of mitogen-induced T cell proliferation.

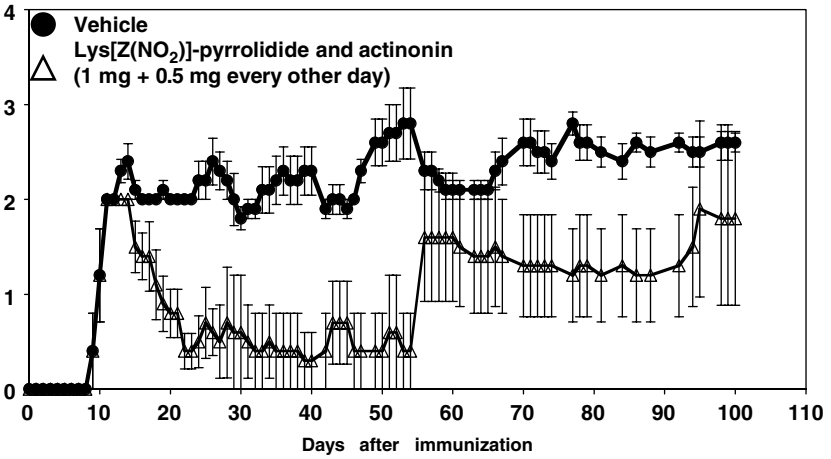

Figure 2. Treatment of EAE with a combination of Lys[Z(NO_2)]-pyrrolidide and actinonin. Treatment was started at day 11 after immunization near the peak of acute clinical disease in chronic EAE induced by immunization of SJL/J mice with $PLP_{139-151}$. Treatment was continued until day 46. Mice were treated with initially 1 mg Lys[Z(NO_2)]-pyrrolidide and actinonin followed by a dose of 0.5 mg every other day in PBS (open triangles). Vehicle (PBS; closed circles) was administered to mice in the control group. N = 5 mice per group.

first episode of acute clinical disease. Combined treatment with Lys[Z(NO_2)]-pyrrolidide and actinonin reduced the clinical severity of chronic EAE induced by immunization of SJL/J mice with $PLP_{139-151}$.

5. CONCLUSIONS

In conclusion, these findings from *in vitro* and *in vivo* studies demonstrate that DP IV activity associated with CD26 plays an important role in the activation of autoreactive T cells. Moreover, inhibition of both DP IV/CD26 and APN/CD13 activity *in vitro* and *in vivo* provides a new approach to modulate T cell functions and tissue-specific autoimmunity in the CNS. These results may have important implications for the treatment of human diseases with a putative autoimmune pathogenesis. At present, major research efforts are directed at the investigation of DP IV/CD26 and APN/CD13 as potentially powerful and safe pharmacological targets. Our preliminary data raise the possibility that simultaneous inhibition of DP IV/CD26 and APN/CD13 may be advantageous over targeting a single ectopeptidase, and support the development of inhibitors with dual specificities for both ectopeptidases.

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CHAPTER 20

CD26/DP IV IN T CELL ACTIVATION AND AUTOIMMUNITY

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1. INTRODUCTION

The ectoenzyme dipeptidyl peptidase IV (DP IV, CD26, EC 3.4.14.5) was first described by Hopsu-Havu and Glenner (1966). Among the rare group of proline-specific proteases, DP IV was originally believed to be the only membrane-bound enzyme, specific for proline as the penultimate residue at the amino-terminus of the polypeptide chain. However, recent studies have identified a number of additional molecules bearing DP IV-like enzymatic activity (Sedo and Malik, 2001).

In 1977, Lojda demonstrated for the first time DP IV activity on human peripheral blood lymphocytes. Later, it was discovered that DP IV is identical with the leukocyte surface antigen CD26. Subsequently, at the 4th Workshop on Leukocytes Differentiation Antigens a number of monoclonal antibodies recognizing DP IV were subsumed under the term CD26 (Fleischer 1994).

Within the hematopoietic system, DP IV is expressed on the surface of resting and activated T cells, activated B cells, and activated NK cells. In general, CD26 expression is upregulated following stimulation of T cells with mitogens, antigens, anti-CD3 antibodies or IL-2, stimulation of B cells with staphylococcal proteins and stimulation of NK cells by IL-2. Moreover, activated antigen-specific $CD4^+$ T cell clones express high levels of DP IV/CD26 (Kähne *et al* 1999).

Several groups have shown a key role of DP IV in the regulation of differentiation and growth of T lymphocytes (Torimoto *et al* 1991; Ansorge *et al* 1991; Dang *et al* 1990; Reinhold *et al* 1997).

The development of synthetic DP IV inhibitors facilitated the study of the cellular functions of this enzyme on the surface of immune cells. In 1985 Schön and coworkers showed for the first time that N-Ala-Pro-O-(nitrobenzoyl-)hydroxylamine, which irreversibly inhibits DP IV, is capable of suppressing the proliferation of human peripheral blood mononuclear cells (PBMC) stimulated with mitogens.

Later, the effects of DP IV inhibitors on leukocyte proliferation and cytokine production were confirmed in different T lymphocyte models. The reversible inhibitors Lys[Z(NO_2)]-thiazolidide, -piperidide, and -pyrrolidide were shown to suppress dose-dependently DNA synthesis of pokeweed mitogen (PWM)- and phytohemagglutinin (PHA)-stimulated human PBMC and purified T cells (Reinhold *et al* 1997). The same holds true for myelin basic protein (MBP)-stimulated T cell clones derived from patients with multiple sclerosis (MS) (Reinhold *et al* 1998). These cells also express high levels of DP IV/CD26 on their surface.

Besides their influence on DNA synthesis, DP IV inhibitors also exhibit strong suppressive effects on the production of various cytokines. It was shown that Lys[Z(NO_2)]-thiazolidide, -piperidide, and -pyrrolidide inhibit the production of IL2, IL-10, IL-12, and IFN-γ of PWM-stimulated PBMC and purified T cells (Reinhold *et al* 1997). These inhibitors also reduce IFN-γ, IL-4, and TNF-α production of MBP-stimulated T cell clones from MS patients in a dose-dependent manner (Reinhold *et al* 1998).

Interestingly, DP IV inhibitors elicit the enhanced production and secretion of the immunosuppressive cytokine transforming growth factor-β1 (TGF-β1).

Multiple sclerosis is the most frequent, demyelinating disease of the central nervous system in Northern Europeans and North Americans. DP IV/CD26 is discussed as a potential target in developing anti-inflammatory therapy for MS. CD26 is highly expressed on myelin-reactive T cell clones from patients with MS (Reinhold *et al* 1998). In addition, patients with MS have an increased median percentage of $CD26^+$ cells among $CD4^+$ T cells in the peripheral blood. CD26 expression decreases somewhat in peripheral blood and cerebrospinal fluid after oral high-dose methylprednisolone treatment (Constantinescu *et al* 1995; Hafler *et al* 19985; Sellebjerg *et al* 2000a; Sellebjerg *et al* 2000b). Others found correlations between changes in the CD26-expressing T cells and lesion activity on magnetic resonance imaging in MS patients with relapsing-remitting and chronic progressive disease, consistent with the presumed function of CD26 in T cell activation (Khoury *et al* 2000). Jensen *et al*. (2004) studied $CD4^+$ T cell activation in patients with clinically isolated syndromes suggesting an initial attack of MS. They demonstrated that the percentage of blood $CD26^+$ $CD4^+$ T cells was increased in these patients, and correlated with magnetic resonance imaging disease activity and clinical disease severity. In contrast, the percentage of $CD26^+$ $CD4^+$ T cells in cerebrospinal fluid correlated negatively with the cerebrospinal fluid concentration of myelin basic protein and the presence of IgG oligoclonal bands. Finally, gene expression profiling in MS patients and healthy controls identified higher average expression of DP IV/CD26 in peripheral blood mononuclear cells of MS patients (Bomprezzi *et al* 2003).

Recently, we addressed the role of CD26/DP IV in mouse experimental autoimmune encephalomyelitis (EAE) and demonstrated that the clinical and histopathological signs of the disease can be suppressed by CD26/DP IV inhibition *in vivo* (Steinbrecher *et al* 2000; Steinbrecher *et al* 2001). Suppression of Th1 memory T cell effector functions was identified as part of the mechanism by which CD26/DP IV inhibition attenuates EAE. These findings from *in vitro* and *in vivo* studies show that targeting CD26/DP IV enzymatic activity with synthetic inhibitors modulates autoimmune disease, but do not address the intrinsic role of CD26/DP IV during immune responses *in vivo*.

To obtain a more definitive answer on the role of CD26/DP IV in Th1 immune responses and autoimmunity, in this study, we examined the effect of CD26 gene disruption on the development of EAE using CD26 gene knockout mice on the C57BL/6 background (Marguet *et al* 2000).

We demonstrate that the lack of the CD26/DP IV molecule leads to systemic deregulation of Th1 immune responses *in vitro*.

2. MATERIALS AND METHODS

2.1. Animals

In all experiments, 8–12 weeks old homozygous CD26−/− mice on C57BL/6 genetic background and wild-type C57BL/6 mice were used. The animal experiments were approved by the local authorities (IM/G/1/00) and performed according to the institutional guidelines.

2.2. Peptide

MOG p35-55 peptide corresponding to mouse sequence was synthesized on a peptide synthesizer by standard 9-fluorenylmethoxycarbonyl chemistry, and purified by high-performance liquid chromatography (HPLC).

2.3. Immunization

Active EAE was induced in 8–12 weeks old mice by immunization with MOG p35-55 peptide in complete Freund's adjuvant (CFA, Sigma-Aldrich, Taufkirchen, Germany). Briefly, 200 μg of MOG p35-55 peptide and 800 μg of killed *Mycobacterium tuberculosis* (Difco Laboratories, Detroit, USA) were emulsified in CFA and injected s.c. by means of four injections over the flanks. In addition, 200 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) dissolved in 200 μl PBS were injected i.p. on day of immunization and again the day after.

2.4. Proliferation Assays

Splenocytes were obtained on day 9–12 after immunization by trituration of spleen using cell strainers (Falcon, Heidelberg, Germany). Cells were washed twice in serum-free AIM-V culture medium (Invitrogen, Eggenstein, Germany) and resuspended in AIM-V culture media supplemented with 10^{-5} M 2-mercaptoethanol (2-ME; Merck, Darmstadt, Germany). For proliferation assays, splenocytes (3×10^5 cells/well) were plated in triplicate in 96-well microtiter culture plates (Falcon, Heidelberg, Germany). MOG p35-55 was added to cell cultures to final concentrations of 0.5, 5 or 50 μg/ml, while medium was added to control cultures. Culture plates were incubated for 72 h at 37°C. Proliferation was assessed by adding [^{3}H]thymidine (0.2 μCi/well) for the last 16 h. Cells were harvested and radioisotope incorporation as index of lymphocyte proliferation was measured in a betaplate liquid scintillation counter (Wallac, Turku, Finland).

2.5. Cytokine Measurements

For determination of cytokine secretion, splenocytes were cultured in AIM-V medium (Invitrogen, Eggenstein, Germany) supplemented with 10^{-5} M 2-ME. Cells were stimulated

with MOG p35-55 (0.5, 5 or 50 μg/ml) and cell culture supernatants were harvested after 48 h and stored at −70°C until cytokine determination. The IL-2, and TGF-ß1 concentrations of the cell culture supernatants were determined with commercially available sandwich enzyme-linked immunosorbent assays (ELISA, R&D Systems, Wiesbaden, Germany) according to the manufacture's instructions.

3. RESULTS

3.1. *In Vitro* Investigations of Proliferation and Cytokine Production on Splenocytes of MOG-Immunized CD26−/− and Wild-Type Mice

To address the immunological mechanisms in CD26−/− mice, we analyzed the proliferative response to MOG p35-55 (DNA synthesis) in MOG-primed lymphocytes from wild-type and CD26−/− mice.

Splenocytes from CD26−/− mice and wild-type mice were isolated on day 11 to 12 following immunization with MOG p35-55 in CFA. Cells were cultured with MOG p35-55 for 72 h, and proliferation was measured using a [^{3}H]-thymidine incorporation assay. Compared to wild-type mice, MOG p35-55-immunized CD26−/− mice showed an increased proliferative response of lymphocytes from spleen when activated with MOG p35-55 *in vitro* (Figure 1).

Moreover, we determined the concentrations of IL-2 and of latent TGF-ß1 in cell culture supernatants of MOG p35-55-primed splenocytes from CD26+/+ wild-type and CD26−/− mice. Splenocytes were isolated after immunization with MOG p35-55 and cultured for 48 h in presence of increasing concentrations of MOG p35-55. The levels of IL-2 and TGF-β1 in these cultures were measured by specific ELISA.

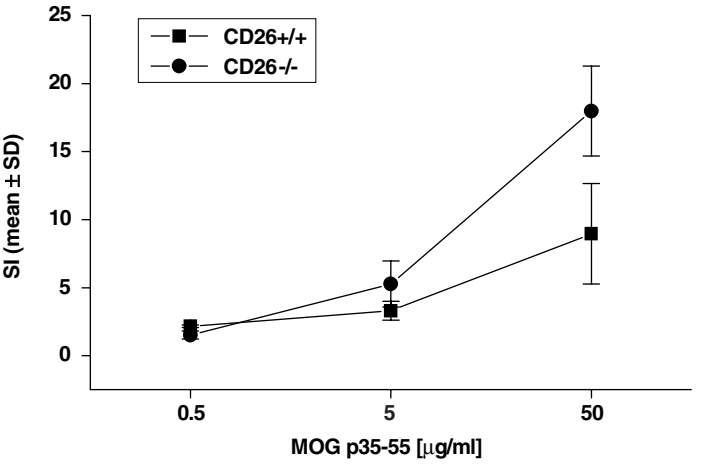

Figure 1. Proliferative response to MOG p35-55 in MOG-primed splenocytes from wild-type (CD26+/+) and CD26−/− mice. The cell proliferation is shown as stimulation index (mean ± SD) of three independent experiments. The [^{3}H]-thymidine incorporation in cultures of splenocytes stimulated with 50 μg/ml MOG p35-55 were 7,740 ± 1,270 cpm (CD26+/+) and 13,536 ± 3,380 cpm (CD26−/−), respectively.

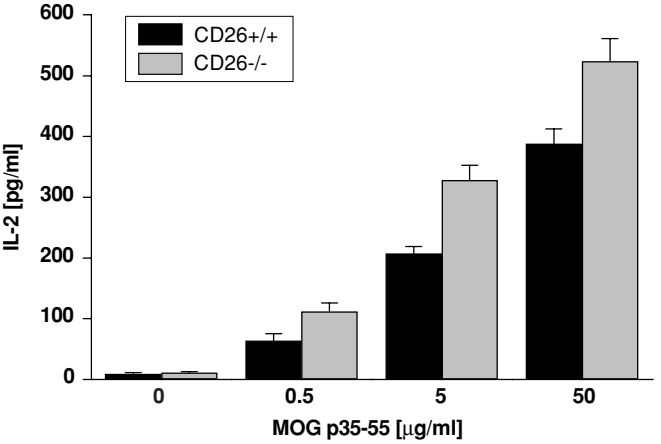

Figure 2. IL-2 production to MOG p35-55 in MOG-primed splenocytes from wild-type (CD26+/+) and CD26−/− mice. IL-2 production is shown as mean ± SEM of 4 independent experiments.

Enhanced MOG p35-55-induced proliferation was associated with increased production of IL-2 in culture supernatants of MOG-primed splenocytes from CD26−/− mice in comparison to wild-type mice (Figure 2).

Regarding immunomodulatory cytokines, the production of latent TGF-ß1 was diminished in culture supernatants of MOG p35-55-stimulated splenocytes obtained from MOG-primed CD26−/− animals in comparison to cells of CD26+/+ wild-type mice (Figure 3).

The data presented here support the hypothesis that ablation of the CD26/DP IV gene *in vivo* results in a general Th1 immune deregulation which is the basis for enhanced autoimmunity observed in the EAE model. Based on our previous data and the results presented in this study, we postulate that increased EAE severity seen in CD26−/− mice is due at least in part to a deficit of TGF-β1-mediated immune response shut-off mechanisms in these mice.

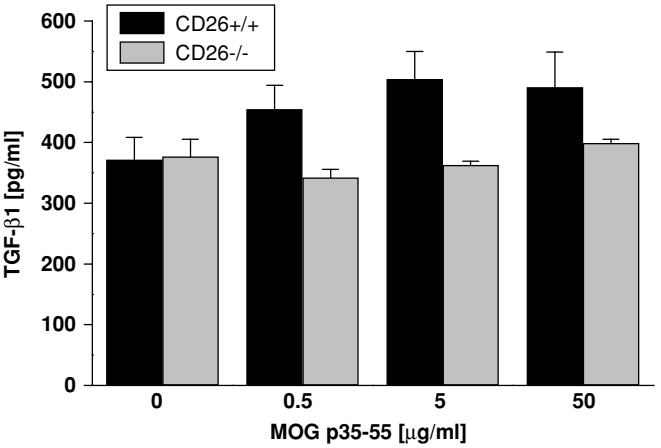

Figure 3. TGF-β1 production to MOG p35-55 in MOG-primed splenocytes from wild-type (CD26+/+) and CD26−/− mice. TGF-β1 production is shown as mean ± SEM of 4 independent experiments.

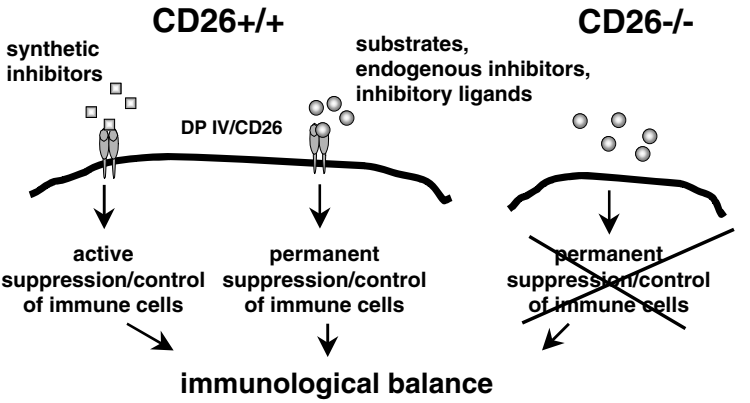

Figure 4. Model of the hypothetical function of DP IV/CD26 on immune cells.

It appears that in CD26+/+ wild-type mice proteins and peptides exist that bind to CD26/DP IV and function as endogenous DP IV inhibitors (Wrenger *et al* 1997, 2000). These inhibitory effectors could induce a permanent TGF-β1-mediated suppression/control of immune cells, which leads to regulation of the immunological balance. CD26−/− mice are lacking these regulatory mechanisms.

In addition to this permanent physiological regulation of T cell responses by natural ligands of CD26/DP IV *in vivo*, targeting CD26/DP IV by external synthetic inhibitors or inhibitory peptides leads to an active strong TGF-β1-mediated suppression/control of CD26-expressing cells (Figure 4).

4. CONCLUSIONS

In summary, the present study demonstrates a critical role for CD26/DP IV in balancing Th1 immune responses. We observed a deregulation of the autoimmune process in EAE *in vitro*, the prototypic model of an immune-mediated CNS disorder. Our results support the notion of a critical regulatory role for the CD26/DP IV molecule within the immune system.

ACKNOWLEDGMENTS

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TOPIC VI

CANCER

CHAPTER 21

DPIV/CD26 AND FAP IN CANCER: A TALE OF CONTRADICTIONS

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1. INTRODUCTION

Current research into the role of the DPIV gene family in cancer has focused on two enzyme members, DPIV and FAP. DPIV and FAP are members of the prolyl oligopeptidase (POP)/S9 enzyme family of serine proteases having two domains; an α/β hydrolase domain that operates as an enzyme, and an 8 bladed propeller domain that is able to bind extracellular matrix components such as integrins and fibronectin (Mueller *et al* 1999; Ajami *et al* 2003). The focus of this review will be to link the biological properties of DPIV and FAP to current evidence in the literature that demonstrate the involvement of DPIV and FAP in a variety of cancer-derived cell lines and tissues, including ovarian, breast, lung, colon, melanoma, and more recently, prostate cancer.

2. DPIV AND FAP: TUMOUR SUPPRESSORS OR TUMOUR PROMOTERS?

Conflicting lines of evidence presented in the literature have shown DPIV and FAP to both promote and suppress metastasis and tumour growth, i.e. they can act both as tumour suppressors and tumour promoters. Tumour promoters promote the malignant phenotype of a cancer cell by (i) reducing the dependency of tumour cells on external growth factors; (ii) reducing the sensitivity of tumour cells to external growth inhibitory signals; (iii) preventing apoptosis; (iv) sustaining angiogenesis or (v) aiding in tissue invasion and metastasis, including anchorage-independent growth (Yan *et al* 2004). Tumour suppressors prevent cancer progression by suppressing one or more of these activities.

Consistent with their tumour suppressor/promoter properties, DPIV and FAP expression is significantly reduced or lost in some tumours, while clearly up-regulated in other cancer types. In order to elucidate the potential roles of DPIV and FAP in tumour development, much effort

has been put into examining their expression and activity across a wide range of malignancies. The next two sections describe experimental data describing the up and down regulation of DPIV and FAP, suggesting either the tumour promoter or tumour suppressor roles of DPIV and FAP in selected cancer types. These findings are linked to the different biological properties of DPIV and FAP, to endeavour to provide explanation as to why these proteases are denoted as tumour promoters in some cancer types, and as tumour suppressors in others. What is interesting, although somewhat confusing about the roles of DPIV and FAP in cancer, is how the same biological property can contribute to tumour progression and suppression depending on the type of cancer.

3. DPIV IN CANCER CELL LINES

3.1. DPIV in Melanoma/Skin Cancer

DPIV is expressed *in vitro* by normal melanocytes, but not in melanoma, their malignant counterpart (Wesley *et al* 1999). Early studies first showed that DPIV expression is lost at an early stage during the transformation of normal melanocytes into melanoma cells (Houghton *et al* 1988), thus paving the way for the more recent investigations into the role of DPIV in the transformation of melanocytes into melanoma cells.

Wesley *et al* (1999) were the first to investigate a potential biological role for DPIV in suppressing the malignant phenotype of melanocytic cells. Human melanoma cell lines derived from metastatic lesions of patients were transfected with a control vector, wild type DPIV cDNA or enzyme negative mutant DPIV cDNA (enzyme negative mutants have no serine protease activity due to a Ser→Ala substitution in the catalytic domain). Re-expression of wild type DPIV at levels comparable to those found in normal melanocytes, led to reduced differentiation of cells, apoptotic cell death and cell cycle arrest, and produced major phenotypic changes in the melanoma cell that were characteristic of a normal melanocyte. Wesley *et al* (1999) proposed that the catalytic domain, and therefore the dipeptidyl peptidase activity of DPIV was essential for suppressing the malignant phenotype of melanocytic cells. However, when nude mice were transfected with wild type and enzyme negative mutant DPIV-transfected melanoma cells there was no difference in tumour progression. Furthermore, cell cycle arrest was demonstrated in 32% of cells expressing mutant DPIV, which was intermediate between wild type DPIV expressing melanoma cells, and control cells (Wesley *et al* 1999). Taken together, these results suggest that some other function other than the DP activity of DPIV is involved in suppressing the malignant phenotype of melanocytic cells.

Pethiyagoda *et al* (2001) later confirmed this using a Matrigel invasion assay to demonstrate that enzyme negative mutant DPIV-transfected melanoma cells demonstrated an anti-invasive effect similar to that seen with wild-type DPIV transfected cells. Invasion and migration through a Matrigel matrix is routinely used to measure the invasiveness and possible metastatic potential of cultured cells (Pethiyagoda *et al* 2001). Using the same Matrigel invasion assays, and a cytoplasmic domain deletion mutant (created through PCR amplification), this group also demonstrated that the 6 aa DPIV cytoplasmic domain was also not required for its anti-invasive effect (Pethiyagoda *et al* 2001).

One possible explanation for the anti-invasive effect of DPIV re-expression is its ability to form heterodimers with FAP. Recent studies have suggested that FAP is also a tumour suppressor in the context of melanoma (see section 4.1), down-regulating proliferating

melanocytes (Ramirez-Montagut *et al* 2004), and that FAP expression is also lost in the transformation of normal melanocytes into transformed melanocytic cells. Wesley *et al* (1999) showed that re-expression of both wild type and mutant DPIV by the melanoma cell lines up-regulated the cell surface expression of FAP. Pethyiagoda *et al* (2001) hypothesised that reintroducing DPIV into FAP-expressing melanoma would promote the formation of DPIV/FAP heterodimers. Therefore, DPIV may act as a tumour suppressor in the context of melanoma by rescuing FAP expression.

Another possibility to explain how DPIV is acting as a tumour suppressor is through its ability to bind to adenosine deaminase (ADA). ADA binding is unique to DPIV in higher mammals, as ADA does not bind to mouse or rat DPIV, nor FAP (Abbott *et al* 1999). ADA is present mainly in the cytosol, but is also present on the cell surface of B and T cells when bound to DPIV (also known as the ADA binding protein) (Iwata and Morimoto, 1999). ADA catalyzes the transformation of adenosine and deoxyadenosine to inosine and deoxyinosine respectively. Adenosine signals through four different receptors: A_1, A_{2A}, A_{2B} and A_3, which are all present in cultured human melanoma cells (Merighi *et al* 2002), and has been proposed to have multiple potential effects on the fate of a tumour, including suppression of the immune response, promotion of angiogenesis, stimulation of cell motility and both stimulation and inhibition of cell growth (Tan *et al* 2004). Consequently, it may be that the ADA-binding property of DPIV may have a role in suppressing the growth of melanoma cells. DPIV binds to ADA, localizing it on the cell surface. This localization would decrease cell surface levels of adenosine (as ADA would catalyse its deamination to deoxyadenosine), which would in turn result in decreased binding of adenosine to its receptors. This may lead to activation of the immune response, a decrease in angiogenesis, inhibition of cell motility and inhibition of cell growth due to decreased signalling through its receptors, hence suppressing the malignant phenotype of melanoma cells.

3.2. DPIV in Non-Small Cell Lung Cancer

Cell surface expression of DPIV and its enzymatic activity is greatly reduced in human non-small cell lung cancer cell lines compared with cells derived from the normal lung (Asada *et al* 1993; Wesley *et al* 2004). Recent work by Wesley *et al* (2004) has sought to investigate the functional role of DPIV in regulating the growth of non-small cell lung cancer cells. This study supported their previous findings reported above that DPIV acts as a tumour suppressor in the transformation of normal melanocytes into melanoma cells (Wesley *et al* 1999) as they found that re-expression of DPIV also suppresses the malignant phenotype of non-small cell lung cancer cells (Wesley *et al* 2004). Again, functional comparison of enzyme negative mutant DPIV transfected lung cancer cells and wild type transfected DPIV lung cancer cells showed that the catalytic domain of DPIV is not required for its tumour suppressor properties in human non-small cell lung carcinoma cells, as mutant DPIV transfected cells inhibited *in vitro* cell migration, differentiation and anchorage-independent growth in soft agar, and promoted apoptosis (Wesley *et al* 2004).

Transfection of DPIV into non-small lung cancer cells again rescued expression of FAP (Wesley *et al* 2004) therefore the ability of DPIV to upregulate FAP may also contribute to the DPIV suppressor role observed in lung cancer cells. However, it is still unclear what property of FAP allows it to act as a tumour suppressor (see Section 4.1). In addition, the ability of DPIV on the surface of lung cancer cells to bind ECM components may also play a role in its tumour suppressing property, as it does in ovarian cancer (see section 3.5). As yet no one has

investigated the ability of DPIV to bind ECM or ADA in the context of non-small cell lung cancer.

3.3. DPIV in Colon Cancer

Little is known about the biological roles of DPIV in colorectal cancer. Analyses of the serum levels of DPIV in patients with colorectal cancer have shown strong variations (Cordero *et al* 2000; de la Haba-Rodriguez *et al* 2002). In one study, patients with advanced colorectal cancer were shown to have higher serum levels than those in the early stages of cancer development (de la Haba-Rodriguez *et al* 2002), suggesting a shedding of membrane DPIV into the circulation. However, previous work showed no correlation between loss of membrane DPIV and increases in soluble DPIV (Cordero *et al* 2000). Tan *et al* (2004) proposed that changes in DPIV cell surface expression and DPIV serum activity may be a consequence of local influences such as adenosine levels. For example, treating HT-29 cells with adenosine markedly reduces the cell surface expression of DPIV, and at the same time decreased binding to the ECM, and enhanced tumour spread are observed (Tan *et al* 2004). However, the mechanism by which adenosine down-regulates cell surface expression of DPIV is unclear. Tan *et al* propose the tumour suppressor role for DPIV may be dependent on its ability to bind to components of the ECM. A major component of the ECM is fibronectin, which is able to bind DPIV through a number of DPIV binding domains (Cheng *et al* 2003). DPIV-mediated binding of fibronectin may suppress tumour metastasis by helping to restrain cells at the initial tumour site, thus preventing their spread. However, if cells are able to detach from the tumour site, their ability to adhere to other sites will contribute to the course of metastatic spread. This adhesion is also mediated by ECM binding so if a DPIV expressing cell escapes from the tumour site, DPIV-mediated ECM binding would then contribute in a positive manner to tumour spread thus in this instance DPIV would start acting as a tumour promoter.

Another major property of DPIV is its peptidase activity. Tan *et al* (2004) have correlated a decrease in DPIV enzyme activity with an increase in chemokine activity, as the chemokines would not be subject to proteolytic attack by DPIV. This may result in increased binding of active pro-inflammatory chemokines to their receptors, resulting in either increased proliferation or migration of tumour cells. In particular, the receptor for the chemokine SDF-1α (stromal cell-derived factor 1α) is highly expressed in colorectal cancer cells and its activation has been linked to progress of the metastatic process in breast cancer (Muller *et al* 2001; Tan *et al* 2004). If the peptidase activity of DPIV were required for its role in the suppression of colorectal cancer, then this would be in contrast to previous studies that have shown that the catalytic domain of DPIV is not required for its tumour suppressor role (Wesley *et al* 1999; Pethiyagoda *et al* 2001; Wesley *et al* 2004). Further functional studies are needed to elucidate the biological role of DPIV in colorectal cancer.

3.4. DPIV in Prostate Cancer

DPIV protein levels are decreased in metastatic prostate tissues compared to normal prostate tissue (Bogenrieder *et al* 1997). As a consequence of this, a tumour suppressor role for DPIV in prostate cancer has been proposed whereby a decrease in DPIV expression is a necessary condition for tumour spread. Recent studies by Wesley *et al* (2005) have sought to reveal a possible mechanism by which DPIV suppresses the malignant phenotype of prostate cancer cells. Using a variety of assays, they have shown that a loss of DPIV is correlated with increased

in vitro cell proliferation and invasion, and that re-expression of DPIV led to induction of apoptosis and cell cycle arrest, and inhibition of the *in vitro* cell proliferation and invasion. Further, using RNA silencing techniques they showed that a loss of DPIV in these prostate cancer cell lines is directly associated with an increase in nuclear levels of basic fibroblast growth factor (bFGF) production, and that re-expression of DPIV decreases bFGF levels (Wesley *et al* 2005). bFGF is a multifunctional molecule that migrates to the nucleus through its N-terminal nuclear localization signal. Nuclear bFGF signals through the MAPK-ERK1/2 pathway, and also increases levels of urokinase-type plasminogen activator (uPA), which converts plasminogen to plasmin, leading to plasmin-mediated breakdown of ECM components, and cellular proliferation, migration, cell survival, wound healing, angiogenesis and tumour progression through the MAPK/ERK 1/2 pathways (Wesley *et al* 2005). The exact mechanism by which DPIV suppresses bFGF has not yet been determined, and is yet to be confirmed by functional experiments that mutate various DPIV domains.

Gonzalez-Gronow and colleagues (2001) have demonstrated a role for plasminogen 2ε in the tumor-promoting property of DPIV in prostate cancer. Plasminogen 2ε was shown to selectively bind to DPIV and to initiate a Ca^{++} influx. Several target molecules appear to depend on the interaction between Plasminogen 2ε and DPIV. For instance, matrix metalloproteinase 9 (MMP-9) expression was enhanced following their interaction, resulting in an increase in tumor cell invasion (Gonzalez-Gronow *et al* 2001). Antibodies directed against DPIV blocked the effects observed upon binding of DPIV to plasminogen 2ε. Interestingly, the degradation of plasminogen 2ε generates a negative feedback mechanism. Angiostatin 2ε, a fragment of plasminogen 2ε, competes selectively with its precursor molecule for binding to DPIV, inhibits Ca^{++} influx, impedes MMP-9 expression and abrogates the enhanced matrigel invasion of tumor cells observed upon interaction between DPIV and plasminogen 2ε (Gonzalez-Gronow *et al* 2005).

3.5. DPIV in Ovarian Cancer

Mesothelial cells are specialized epithelial cells that extend over the surface of the abdominal cavity. Once carcinoma cells attach to these mesothelial cells, they may invade the mesothelial cell layer and extend into the peritoneum. Peritoneal dissemination is the main metastatic process of ovarian cancer (Kajiyama *et al* 2002a; Kajiyama *et al* 2002b), initiated by adhesion of tumour cells to mesothelial cells. Using fluorescence-activated cell sorting (FACS), Kajiyama *et al* (2002a) demonstrated that DPIV showed a high and selective expression on the cell surface of human mesothelial cells, and that this expression correlated with an increased adhesion rate of SKOV ovarian carcinoma cells to the mesothelial cell layer. From these results alone, it would be reasonable to speculate that DPIV acted as a tumour promoter by facilitating adhesion of ovarian carcinoma cells to the mesothelial cell layer. However, further studies by the same group demonstrated that ovarian carcinoma cell lines with higher DPIV expression were less invasive and led to a decrease in tumour size, and inoculating nude mice with these same cell lines showed that the mean survival of these mice was correlated with an increase of DPIV expression suggesting a conflicting role for DPIV in tumour suppression in this cell type (Kajiyama *et al* 2003).

Later studies found that this increased adhesion potency of tumour cells to the mesothelial cell layer was through DPIV-mediated binding to components of the ECM such as fibronectin and collagen (Kikkawa *et al* 2003). Therefore, DPIV may act as a tumour suppressor by forming tight cell-cell adhesion junctions, rendering the detachment of cancer cells from the original

tumour difficult and thus reducing their metastatic potential (Kikkawa *et al* 2003). However, DPIV can in turn mediate attachment to the mesothelial cell layer through its same ECM binding property, and thus could then act as a tumour promoter as suggested previously in colon cancer (see section 3.3).

Another way that DPIV may contribute to tumour suppression is through its ability to regulate the expression of E-cadherin. Immunoblot analysis of ovarian carcinoma cell lines expressing differing amounts of DPIV have shown that the expression of DPIV is positively correlated with expression of E-cadherin and α- and β-catenin (Kajiyama *et al* 2003). A loss of E-cadherin expression correlates with invasiveness and metastatic potential (Kajiyama *et al* 2003). E-cadherin contributes to the maintenance of cell-cell contacts (adherin junctions), preventing the detachment of tumour cells from their primary tumour site and therefore preventing their metastasis. E-cadherin also down-regulates matrix metalloproteinases (MMP) which have known roles in tumour invasion, angiogenesis and metastasis. Thus, in ovarian cancer, DPIV may act as a tumour suppressor by regulating levels of E-cadherin in the cell, although the mechanism is unclear.

4. FAP IN CANCER CELL LINES

Research into the role of the FAP in cancer has mainly focused on melanoma and breast cancer cell lines.

4.1. FAP in Melanoma

FAP, like DPIV, is a tumour suppressor in the development of melanoma (Huber *et al* 2003; Ramirez-Montagut *et al* 2004). Immunohistochemical analysis of surgical tissue sections revealed that proliferating normal melanocytes express FAP, while primary and metastatic human melanomas do not (Rettig *et al* 1993; Huber *et al* 2003) suggesting that FAP is down-regulated when normal melanocytes undergo malignant transformation. Recently, Ramirez-Montagut *et al* (2004) explored this possibility, attempting to elucidate the biological role of FAP in the suppression of malignant melanoma development. Using mouse melanoma derived cell lines, it was demonstrated *in vitro* that B78H1 melanoma cells transfected with catalytically active FAP led to cell cycle arrest and increased apoptosis following stress, suggesting a role for FAP in checking uncontrolled tissue growth. Injecting FAP transfected cells into mice showed either no tumour growth, or decreased tumour growth with delayed tumour progression compared with control-transfected mice. Thus, expression of FAP may provide a 'brake' for otherwise uncontrolled proliferation and tumourigenicity (Ramirez-Montagut *et al* 2004). These findings are in contrast to those of FAP in breast cancer cells (see Section 4.2), which suggest expression of FAP in breast cancer cells promotes tumour growth (Goodman *et al* 2003; Huang *et al* 2004).

Interestingly, this study demonstrated that the serine protease activity of FAP was not required for its tumour suppressor properties, as cells transfected with FAP cDNA mutated at the catalytic domain also abrogated tumorigenicity. Therefore, neither the dipeptidyl peptidase nor the gelatinase activity of FAP is required for suppressing the malignant phenotype of melanocytic cells. This discovery is linked to previous findings that neither the proteolytic activity nor the C- terminal cytoplasmic domain of DPIV is required for its tumour suppressor properties in the transformation of normal melanocytic cells to malignant tumour cells (de la Haba-Rodriguez *et al* 2002). Also interesting is the finding that FAP expression does not rescue

DPIV expression in mouse melanoma cells; therefore, none of the tumour suppressor properties can be linked to an increase in DPIV expression. It is therefore difficult to propose a biological property of FAP that is responsible for the tumour suppressor role in melanoma.

4.2. FAP in Breast Cancer

FAP promotes breast cancer progression by alleviating the serum growth requirement of breast cancer cells, promoting rapid tumour growth and promoting angiogenesis by increasing microvessel density (Goodman *et al* 2003; Huang *et al* 2004). When FAP expression is suppressed breast cancer cells became sensitive to serum starvation, causing them to grow more slowly (Goodman *et al* 2003). In contrast to DPIV (Kajiyama *et al* 2003), expression of FAP was apparently not related to E-cadherin expression. In addition, Huang *et al* (2004) injected FAP-expressing breast cancer cells into the mammary fat pads of immunodeficient mice and demonstrated the tumour growth promoting properties of FAP. They found that the size of the tumours produced (weight), the number of tumours produced per injection sites, the percentage of FAP-positive tumour cells, the number of metastases and the microvessel density increased in mice that had been injected with FAP-expressing cells compared to those injected with control vector only expressing cells.

The reduced dependence of tumour cells on serum growth factors, and the increased microvessel density obtained could be a result of the proteolytic activity of FAP. The catabolism of neuropeptide Y (NPY) and peptide YY by DPIV or FAP can be assumed to, by a shift in the receptor specificity, contribute to angiogenesis (Ghersi *et al* 2001), providing an explanation to the observation that FAP-expressing tumours were larger in size than non-FAP expressing tumours.

Tumour cells expressing FAP can also degrade collagen in the ECM due to the gelatinase activity of FAP. Immunoprecipitation, immunofluorescence and cell surface cross-linking experiments have shown that FAP and $\alpha 3\beta 1$ integrin associate with each other at invadopodia in a collagen dependent manner (Mueller *et al* 1999). The attachment of FAP to $\alpha 3\beta 1$ integrin may contribute to its growth-promoting role as it helps to localize FAP at the ECM, therefore permitting the invasion and migration of tumour cells.

5. CONCLUSIONS AND KEY UNANSWERED QUESTIONS

As discussed in this review, the role of DPIV and FAP in cancer is complex, with their role changing depending on cancer and tissue type. When it comes to designing DPIV or FAP-directed therapies for suppressing tumour development and metastasis, it must be stressed that each tumour type should be considered individually.

This review has highlighted the fact that in many cases, the catalytic domains of both DPIV and FAP are not required for either its tumour suppressor or promoter properties. Therefore, it would be logical to assume that in some cancer types, inhibiting DPIV/FAP activity would not be successful in the suppression of cancer development. Many authors have demonstrated that other domains such as the ADA binding domain of DPIV or the ECM binding domains (DPIV and FAP) (through fibronectin, collagen and integrin binding) are more significant to the progression/suppression of cancer, and therefore peptide therapies which block certain binding domains may be a more suited type of therapy to target DPIV and FAP in cancer. However, to complicate matters more, these domains have differing roles in the suppression/progression of cancer depending on tissue type. For example, the ECM binding domain of DPIV has been

proposed as a suppressor of tumour metastasis in colon cancer and ovarian cancer by helping to form tight cell-cell adhesion junctions so that tumour cells cannot detach. Yet, this same function could also be acting as a tumour promoter by mediating attachment to the mesothelial cell layer therefore, promoting metastatic events. The multifunctional nature of these binding domains may make it difficult to predict the consequences of their inhibition through peptide therapies. Further, the almost ubiquitous expression of DPIV would further complicate any therapeutic strategies designed at blocking any of the binding domains of the peptide. Consequences of FAP inhibition may be easier to predict, as it is not expressed in normal adult tissue (Huber *et al* 2003). Yet, there is reason to believe that DPIV acts primarily as a tumour promoter in prostate cancer. The mechanisms that are employed in this process, especially the interaction between DPIV and plasminogen 2ε, pave the way for conceivable therapeutic approaches by the development of angiostatin 2ε like competitive inhibitors that would limit the tumour growth.

There are recent studies however, which support inhibition of the protease activity of DPIV and FAP through inhibitor therapy in suppressing the growth and metastasis of tumour cells in some cancer types. Preliminary studies using a rat model of adenocarcinoma, showed that slow-binding and irreversible inhibitors to DPIV inhibited tumour growth and metastasis by 50–60% (Hoffman *et al* 2003). In addition, a more recent study using the inhibitor Val-Boro-Pro (against both DPIV and FAP) in epithelial carcinoma cells (Cheng *et al* 2005) or various other tumours (Adams *et al* 2004) have shown that inhibition of FAP/DPIV protease activity can promote anti-tumour effects. It remains to be elucidated whether this effect can be attributed to the tumour or rather to the surrounding tissue, given the fact that the presence of an active immune response forms an essential part of the observed effect of the inhibitor (Adams *et al* 2004).

As an aside, it is also worth considering the involvement of other DPIV family members in tumour progression/suppression. DP8 and DP9 share high homology with DPIV and FAP (Abbott and Gorrell, 2002). It is therefore feasible that these enzymes may have similar roles to DPIV and FAP in the invasion of different cancer cells. Additionally, although DPL1 and DPL2 are not proteases they share many structural features with the enzymes, thus have the potential to interact with ECM components such as collagen, fibronectin and integrins. Therefore, they may also play a role in the suppression/promotion of tumour cell invasion through these domains in the tissues in which they are expressed.

Due to the differing roles of DPIV and FAP highlighted in this review, particular emphasis should be placed on understanding their role in individual cancer types. Future research into the biological roles of DPIV and FAP in the progression of individual cancers, as well as extensive knockout animal work will help us to understand the implications for inhibitor/peptide therapy design, and will aid in translating biological knowledge of the dipeptidyl peptidase family in cancer into the clinical setting.

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CHAPTER 22

TYPE-II TRANSMEMBRANE PROLYL DIPEPTIDASES AND MATRIX METALLOPROTEINASES IN MEMBRANE VESICLES OF ACTIVE ENDOTHELIAL CELLS

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1. INTRODUCTION

Activation of micro-vascular endothelial cells involves increased expression of multiple cell surface proteases that occur during tumor angiogenesis and metastasis. These proteases include families of matrix metalloproteinases (MMPs) (Hotary *et al* 2002; *et al* Hiraoka 1998; Brooks *et al* 1998) and serine proteases (Pepper *et al* 1993). In addition, $\alpha_v\beta_3$ integrin (Brooks *et al* 1994) and $\alpha_3\beta_1$ integrin (Mueller *et al* 1999; Ghersi *et al* 2002; Pineiro-Sanchez *et al* 1997) are involved in docking of cell surface proteases to promote the activation of the cell for migration and invasion in collagenous matrices.

Recently, homologues of serine-type of prolyl dipeptidases, dipeptidyl peptidase IV (DPP4/CD26) and seprase/fibroblast activation protein alpha (FAP-α) that contain extracellular, non-classical serine protease domains and can degrade macromolecules such as gelatin, are thought to augment the essential cellular properties exhibited by MMPs and integrins (Netzel-Arnett *et al* 2002). DPP4 and seprase are absent on the cell surface in differentiated endothelial and stromal cells but they are expressed to the cell surface of invasive cell types in cancerous tissues and healing wounds (Chen and Kelly 2003). DPP4 expression is altered in cells of several pathological conditions including cirrhotic human liver cells (Levy *et al* 1999) and in follicular thyroid carcinoma cells (Hirai *et al* 1999). DPP4 is re-distributed to sites interfacing the basement membrane, supporting its role in degradation of collagenous matrices. In addition, chemokines were shown to be natural substrates of DPP4 and their activities could be regulated by DPP4 (Lambeir *et al* 2001). Furthermore, neuropeptide Y (NPY) when processed by DPP4 becomes pro-angiogenic (Zukowska-Grojec *et al* 1998) and its product $NPY_{3\text{-}36}$ is involved in

activation of endothelial cell migration (Ghersi *et al* 2001). Migration of fibroblasts occurring during a healing wound was shown to involve formation of the DPP4—seprase complex at invadopodia of the cell (Ghersi *et al* 2002).

Previous studies have shown that invasive cells release membrane vesicles in a manner similar to virus budding (Dainiak 1991). These vesicles, ranging from 100 to 1000 nm in diameter (Dainiak 1991a; Dolo *et al* 1995), are suggested to play a role in cell migration and tumor invasion (Poste and Nicolson 1980) and major proteases associated with these vesicles have been identified (Dolo *et al* 1994, 1998; Zucker *et al* 1987; D'Angelo *et al* 2001). When human umbilical vein endothelial cells (HUVEC) were treated with FGF-2, VEGF, and Thrombospodin-1, there was an increased secretion of vesicles and associated proteases (Taraboletti *et al* 2002). Here, we summarize profiles of major serine- and metallo-proteases isolated from membrane vesicles shed by HUVEC and their role in endothelial cell migration.

2. PROTEOLYTIC ENZYMES IN ENDOTHELIAL CELLS

2.1. Expression of Proteolytic Enzymes in Compartments of Endothelial Cells

To evaluate gelatinolytic activities present in the cell body, plasma membrane and extracellular vesicles, we have analyzed non-ionic detergent extracts derived from endothelial cells in confluent and sparse cultures by gelatin zymography. Endothelial cells in confluent culture are less migratory than these in sparse culture, as the former is regulated by contact-inhibition of cell migration. When isolated proteins, with 20 μg of proteins per lane, were loaded for gelatin zymography, the cell body fractions of confluent HUVEC exhibited very low level of gelatinolytic activities, whereas these of sparse endothelial cells showed detectable MMP-2 and MMP-9. In the plasma membrane fractions, MMP-2 and MMP-9 were detectable in both confluent and sparse cells; moreover, in sparse or migratory cells, the plasma membrane showed additional EDTA-resistant and benzamidine-sensitive serine proteases. In membrane vesicles, gelatinases similar to these present in the plasma membranes were identified; these in sparse cultures showed 10-fold higher activity than these in confluent culture. These protease were identified by immunoblotting that included MMP-2, MMP-9; MT1-MMP, uPA and its receptor uPAR, seprase and DPP4.

2.2. RNA Expression in Endothelial Cells

Endothelial cells during vascular morphogenesis and angiogenesis were examined by reverse trascriptase mRNA analyses and up-regulated several serine- (Aimes *et al* 2003) and metallo- (Nawrocki-Raby *et al* 2003) proteases. Notably, serine type of proteases, including seprase and DPP4, and MMPs, including MMP-2, MMP-9 and MT1-MMP, were regulated in endothelial cells under different culture conditions.

2.3. Localization in Endothelial Cells Cultured in 3D Collagen Gels

Localization of proteases in endothelial cells should be best examined in cell migration in 3D collagen gels, as cells in 3D collagen culture mimic their *in vivo* stromal microenvironment.

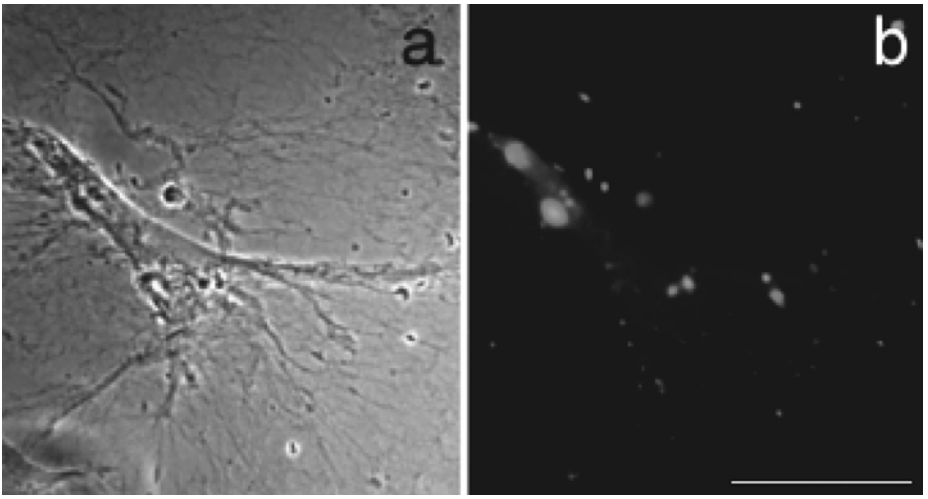

Figure 1. Distribution of MT1-MMP in endothelial cells cultured in a 3D collagen gel. (**a**) Phase contrast image. (**b**) Immunofluorescent distribution of MT1-MMP in the cell and membrane vesicles in collagen gel. Bar = 10 μm.

We have examined protease localization in endothelial cells by culturing cells at low cell density in 3D collagen gels, fixation with paraformaldehyde, and stained doubly with two separate fluorochromes that were conjugated to different mAbs against seprase, DPP4, β_1-integrin, MMP-2, MMP-3, MMP-9, MMP-13 or MT1-MMP. We found that most antigens listed above, except MMP-3 and MMP13, localized on the plasma membrane and in membrane vesicles (Figure 1). Moreover, direct immunofluorescence performed with mAbs against seprase and DPP4 show their co-localization on the plasma membrane invadopodia and membrane vesicles.

3. ROLES OF PROTEOLYTIC ENZYMES IN DEGRADATION OF EXTRACELLULAR MATRIX AND REGULATION OF CELL MIGRATION

3.1. Extracellular Matrix Degradation by Membrane Vesicles of Endothelial Cells

Proteases present in membrane vesicles of the endothelial cells in confluent and sparse (migratory) cultures have been evaluated for their capability in degrading extracellular matrix components, including type-I and type-IV collagens, laminin and fibronectin. These macromolecules were incubated with isolated vesicles; cleaved products were then analyzed by SDS-PAGE. In vesicles from migratory endothelial cells, type-IV collagens, laminin and fibronectin were extensively degraded during the 18 hour incubation. The degradation of type-I collagen by vesicles from migratory endothelial cells occurred during the first 2 hours of incubation, whereas that from confluent endothelial cells became detectable after 24 hours of incubation.

3.2. Involvement of Serine- and Metallo-Proteinases in Extracellular Matrix Degradation and Cell Migration

To determine the role of the proteolytic enzymes in endothelial cell migration, we overlaid a thin layer of type-I collagen on a cell monolayer wound model (Ghersi *et al* 2002) for morphological examination. Cell migration and extracellular matrix degradation were evaluated by counting the area of cell migration/collagen removal using phase contrast and fluorescence microscopy and image analysis (NIH Image 1.62b4/fat program). We observed that endothelial cells at the wound edge migrated into the gel and close the 1-mm wound within 48 hours. Addition of the inhibitory mAbs against DPP4 and inhibitors of serine-(AEBSF) or metallo-(CT1847) proteases blocked collagen removal and cell migration, whereas other antibody controls and cysteine protease inhibitors did not. Migratory endothelial cells showed time-dependent collagen degradation and cell migration, and the antibody inhibitory effect could be reverted by antibody removal from the culture. Moreover, membrane vesicles added to the model promote both collagen degradation and cell migration, and serine- and/or metallo- protease inhibitors blocked the effect of vesicles.

4. AUTOCATALYTIC ACTIVATION

4.1. Low Molecular Weight Proteolytic Fragments of Proteases in Membrane Vesicles of Endothelial Cells

Proteolytic activities present in membrane vesicles could be products of specific mRNA or from cleaved products of existing proteases. In order to examine these possibilities, we have prepared membrane vesicles in presence of a cocktail of protease inhibitors to block possible proteolytic processing of existing enzymes. In addition, a combination of gelatin zymography and Western immunoblotting analyses were used to identify major proteases involved. Table 1 shows summary of results, in which gelatinases migrated with low molecular weights in gelatin zymograms indicate that they are products of autocatalytic processing of proteases of metallo- and serine-classes.

5. CONCLUSIONS

Endothelia cells in sparse culture are migratory and increase the production of gelatinases of serine- and metallo-classes in membrane vesicles. Collectively, proteases associated with membrane vesicles degrade extracellular matrix components including type-I and type-IV collagens, laminin and fibronectin. Inhibitor studies suggest the existence of small gelatinases that were derived from these serine- and metallo-proteases. Thus, further studies are warranted to demonstrate the cooperative action of metallo- and serine proteases on cell surfaces and in extracellular vesicles during endothelial cell migration in 3D collagenous matrices, and potential proteolytic activation mechanism for these cell surface proteases.

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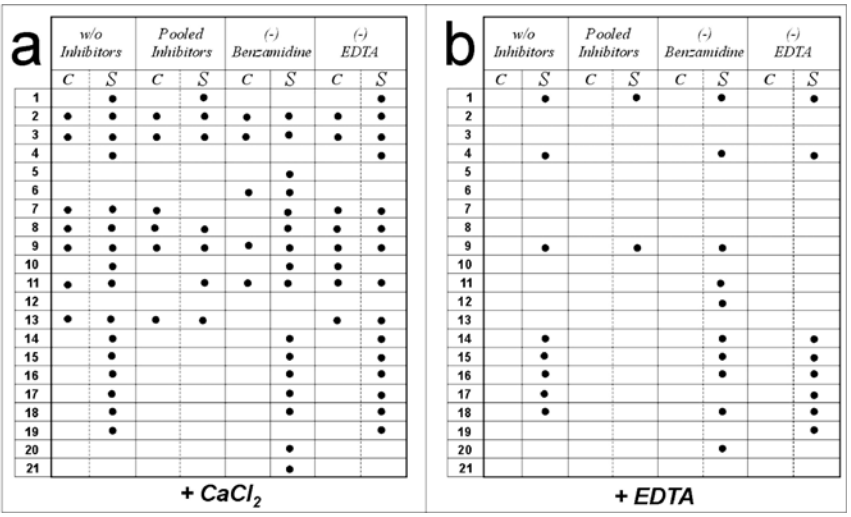
Table 1. Major serine- and metallo-proteases observed in membrane vesicles derived from endothelial cells grown in confluent (C) and sparse (S) cultures.



Gelatin zymography was used to examine general protease profiles (bands 1–21) of vesicles in the medium conditioned by HUVEC cultures in confluence (C) or in sparse (S) cultures. Before separation of proteins by gelatin zymography, isolated membrane vesicles were prepared without protease inhibitors [w/o Inhibitors], with a cocktail of protease inhibitors, including benzamidine 20 μM, EDTA 10 μM, pepstatin 1 μM and leupeptin 10 μM, final concentration) [Pooled Inhibitors], with pooled inhibitors except serine-protease inhibitors [(-) benzamidine], or with pooled inhibitors except metallo-protease inhibitors [(-) EDTA]. The presence of serine- and metallo-proteases in bands 1–21 on zymograms were identified by incubation of gelatin zymograms in the presence of buffer containing $CaCl_2$ or EDTA. In addition, Western immunoblotting was used to identify the proteases.

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CHAPTER 23

EXTRA-ENZYMATIC ROLES OF DPIV AND FAP IN CELL ADHESION AND MIGRATION ON COLLAGEN AND FIBRONECTIN

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1. INTRODUCTION

Fibroblast activation protein (FAP) is a plasma membrane-bound, constitutively active enzyme that has DPIV activity, prolyl endopeptidase activity and type I collagen (CN-I) specific collagenase activity (Aertgeerts *et al* 2005, Levy *et al* 1999, Park *et al* 1999).

Hepatic stellate cells are a key mediator of progressive liver fibrosis. In the normal liver stellate cells are quiescent, but following liver injury stellate cells become activated and generate fibril-forming matrix that primarily incorporates type I and type III collagens and fibronectin (FN). In cirrhotic liver, FAP is strongly expressed by activated stellate cells in the portal-parenchymal interface (PPI) and by myofibroblasts in the septum (Levy *et al* 1999). FAP expression intensity correlates with the severity of liver fibrosis (Levy *et al* 2002). These data suggest a profibrotic role for FAP in chronic liver injury.

Several cell types express DPIV including epithelial cells, endothelial cells, and activated T cells in liver. Abherent basolateral DPIV expression occurs on hepatocytes in liver cirrhosis (Matsumoto *et al* 1992). DPIV interacts with extracellular matrix (ECM) via FN binding (Cheng *et al* 2003) and interferes with hepatocyte spreading on FN and collagen (Hanski *et al* 1985). The ECM interactions of DPIV may help to explain its changes in expression levels in various human cancers because cancers differ in their ability to invade neighboring tissue. Most studies suggest that DPIV expression by tumor cells is disadvantageous to the tumor and that the enzymatic activity is not involved in tumor cell-ECM interactions (reviewed by Gorrell *et al* Chapter 5 of this book).

Cell adhesion and migration, proliferation and apoptosis are central to many pathological processes involving tissue remodeling, including liver fibrosis, inflammation, angiogenesis, cancer growth and metastasis. This study demonstrated that FAP and DPIV influence apoptosis

and proliferation and cell adhesion, *in vitro* wound healing and cell migration on ECM substrata. No role for FAP or DPIV enzymatic activity was indicated in experiments that used catalytically inactive forms of FAP and DPIV.

2. MATERIALS AND METHODS

Matrigel matrix (BD Biosciences, Bedford, MA), collagen type I (CN-I), FN, staurosporine streptomyces (STS) and 4',6-diamidine-2'-phe-nylindole dihydrochloride (DAPI) (Sigma, St Louis, MO) were used. Immunofluorescence (antibodies in Table 1), enzyme histochemistry and flow cytometry have been described previously (Ajami *et al* 2003, Ajami *et al* 2004).

Human FAP (GenBank code U09278) was cloned in-frame downstream of N-terminal Green Fluorescent Protein (GFP) or Cyan Fluorescent Protein (CFP) in the vectors pEGFP-C2 and pECFP-C1 (BD Biosciences) while human DPIV (M80536) was cloned in upstream of the fluorescent proteins in the vectors pEGFP-N1 and pECFP-N1. The two FAP enzyme negative mutants were engineered using point mutation primers for alanine (GCC) replacement of the catalytic serine (TCC) at position 624 and alanine replacement of the glutamic acids at 203 (GAG to GCG) and 204 (GAA to GCA). Plasmid DNA extraction, site-directed mutagenesis,

Table 1. Antibodies

Antibody	Isotype	Supplier	Catalogue No.	Working Dilution
Primary Antibody				
FAP	Mouse IgG1	W. Rettig (Levy *et al* 1999)	ATCC, CRL-2733	1:5
DPIV	Mouse IgG1	T. Kähne (Ajami *et al* 2003)	Clone B10/EF6	1.7 μg/ml
Alexa Fluor 594 Phalloidin	NA	Molecular Probes	A12381	1:40
MMP-2	Mouse IgG1	Santa Cruz	SC-13595	20 μg/ml
TIMP-2	Rabbit IgG	Santa Cruz	SC-5539	10 μg/ml
E-cadherin	Mouse IgG1	Santa Cruz	SC-21791	10 μg/ml
β-catenin	Mouse IgG1	Transduction Laboratories	C19220	0.6 μg/ml
Integrin β1 (CD29)	Mouse IgG2a	BD Biosciences	556048	2.5 μg/ml
Phycoerythrin (PE) CD44	Mouse IgG2b	BD Biosciences	55479	1:10
PE Annexin V	NA	BD Biosciences	556421	1:50
CXCR4	Rabbit IgG	Chemicon	AB1846	10 μg/ml
CXCL12	Mouse IgG1	R&D systems	MAB310	50 μg/ml
DDR-1	Rabbit IgG	Santa Cruz	SC-532	6.7 μg/ml
Secondary Antibody				
Alexa Fluor 488 anti-rabbit	Goat IgG	Molecular Probes	A11034	1:400
Alexa Fluor 594 anti-mouse	Goat IgG	Molecular Probes	A11032	1:400
PE anti-mouse	Goat IgG	Molecular Probes	P852	1:400
PE anti-rabbit	Goat IgG	Molecular Probes	P2771	1:400

NA not applicable

restriction enzymes and human embryonic kidney (HEK) 293T cell line transfection have been described previously (Ajami *et al* 2003, Ajami *et al* 2004).

The cell adhesion assay, in vitro monolayer wound healing assay, cell migration in transwell assay and apoptosis and proliferation assays are described by Yu *et al* (chapter 7 of this book).

Statistical Analysis. Results are expressed as means ± SD. Differences among groups were analysed using the Student's t test or Wilcoxon rank sum test. P values <0.05 were considered significant.

3. RESULTS

Expression of the constructs FAP-GFP, DPIV-GFP, GFP-FAP, GFP-FAP Ser624Ala and GFP-FAP Glu203/204Ala. Influences of FAP and DPIV on cell adhesion and migration were examined using the readily transfected HEK 293 epithelial cell line. Transient transfection was used to avoid the behavioral prejudices towards adherence, survival and proliferation that can be imposed by selecting a stably transfected cell line (Wesley *et al* 1999). GFP or CFP derived fluorescence was used to identify and analyze the behaviour of the peptidase expressing cells and all assays enumerated GFP/CFP vector transfected cells as the principal negative control. DPIV trafficking is unaffected by fusion to GFP (Slimane *et al* 2001).

The fusion protein of GFP onto the C-terminus of DPIV exhibited both green fluorescence and peptidase activity. In contrast, fusions of GFP onto the C-terminus of FAP conferred fluorescence but not peptidase activity upon transfected cells. However, with GFP at the N-terminus of FAP both green fluorescence and peptidase activity were seen. GFP was not placed on the cytoplasmic N-terminus of DPIV because DPIV tends to be shed from the cell surface.

Glu205 and Glu206 of DPIV and the homologous residue in dipeptidyl peptidase 8, Glu259, are essential for peptidase activity (Abbott *et al* 1999, Ajami *et al* 2003). The homologous residues of FAP, Glu203 and Glu204, and the catalytic Ser624, were substituted with Ala and found to be essential for peptidase activity (data not shown). All of the DPIV and FAP constructs were cell surface expressed (Fig. 1).

Endogenous expression of FAP or DPIV in 293T cells. In order to interpret effects of FAP or DPIV overexpression, measuring endogenous expression of FAP and DPIV by 293T cells is important. FAP was not detected either on the surface (Fig. 1C) or intracellularly (Fig. 1L). DPIV was not detected on the cell surface (Fig. 1B) but exhibited some endogenous intracellular expression (Fig. 1K). In contrast to SKMEL28 melanoma cells, which express both DPIV and FAP following transfection with DPIV (Wesley *et al* 1999), in 293 cells neither FAP nor DPIV transfection induced expression of the other enzyme (Fig. 1E, I, N, R).

FAP and DPIV in cell adhesion. GFP-FAP expressing cells exhibited less cell adhesion on FN, CN-I or Matrigel coated plastic compared to cells expressing GFP alone ($P < 0.05$) (Fig. 2). However, probably because DPIV binds to fibronectin (Cheng *et al* 2003), DPIV-GFP expression increased cell adhesion on FN ($P < 0.01$, Fig. 2A). The two enzyme-inactive GFP-FAP constructs indicated that the enzyme activity of FAP is not necessary for the FAP-associated reduced cell adhesion ($P < 0.05$, Fig. 2B).

FAP and DPIV reduced wound healing. Cells expressing either GFP-FAP or DPIV-GFP, compared to GFP, were less prevalent ($P < 0.05$) in the wound area than the non-wound area on CN-I, FN or Matrigel (Fig. 3). This under representation of DPIV+ and FAP+ cells in the healing wound indicates that overexpression of either peptidase impairs migration on ECM.

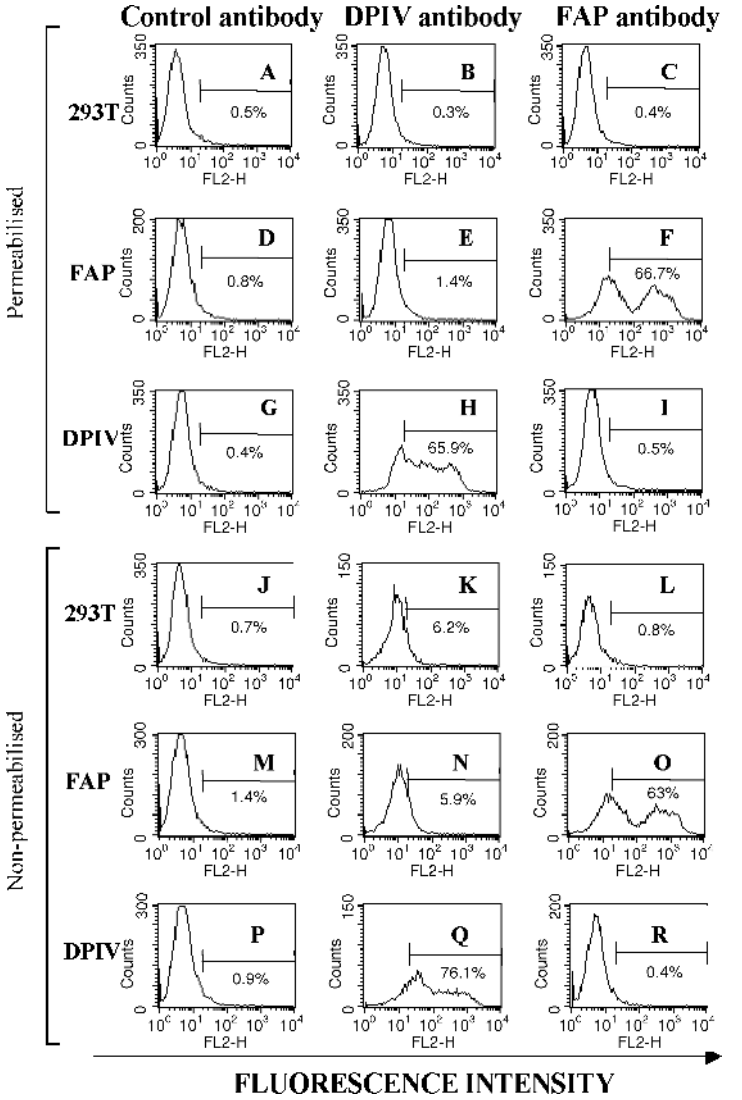

Figure 1. Expression of FAP and DPIV by 293T cells. More than 63% of 293T cells transfected with GFP-FAP (D-F, M-O) or DPIV-GFP (G-I, P-R) expressed detectable recombinant fusion protein on the cell surface (F, H). Untransfected 293T cells were FAP negative (C, L) but exhibited a low level of endogenous DPIV expression intracellularly (K) but not on the surface (B). FAP or DPIV transfection did not induce expression of the other enzyme (E, I, N, R). Transfected cells were fixed then half were permeabilized. Cells were stained with MAbs to DPIV (B, E, H, K, N, Q) or FAP (C, F, I, L, O, R) followed by goat anti-mouse PE.

FAP and DPIV reduced cell migration. A cell invasion assay further revealed impairment by FAP and DPIV of cell migration on ECM. Cells expressing either GFP-FAP or DPIV-GFP exhibited less migration across the transwell membranes towards CN-I, FN or Matrigel than did GFP expressing cells ($P < 0.05$, Fig. 4).

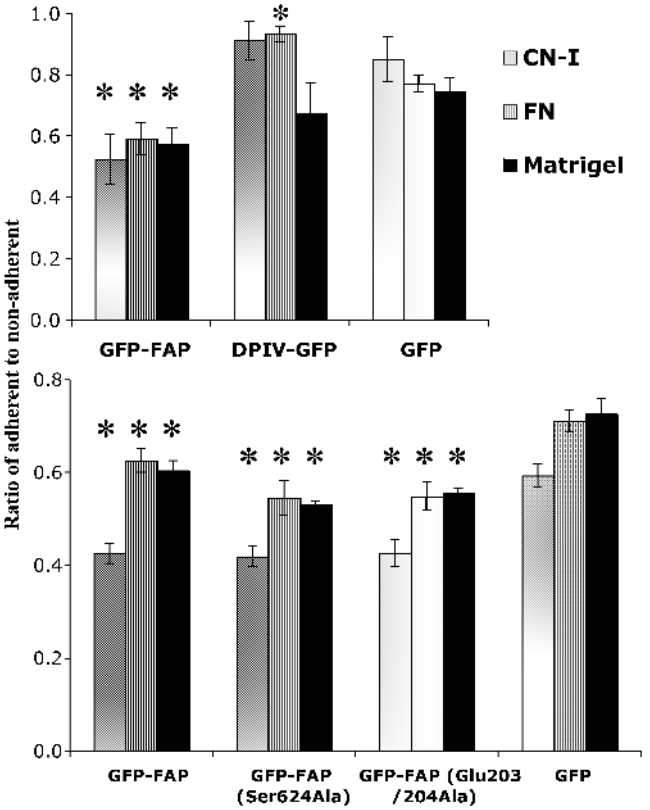

Figure 2. Cell adhesion of FAP and DPIV overexpressing 293T cells on ECM components. *Indicates $P < 0.05$ compared to GFP transfected cells.

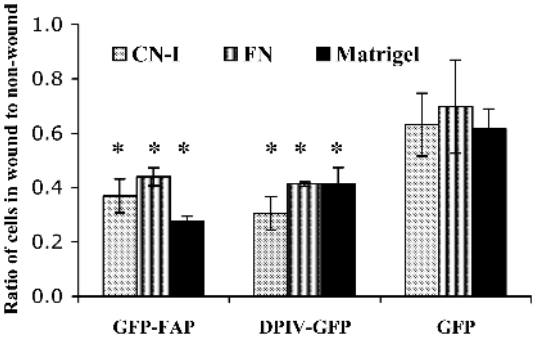

Figure 3. FAP or DPIV overexpression inhibited *in vitro* wound healing. The ratios of relative fluorescence positivity in the wound to the non-wound area. *$P < 0.05$ compared to GFP transfected cells.

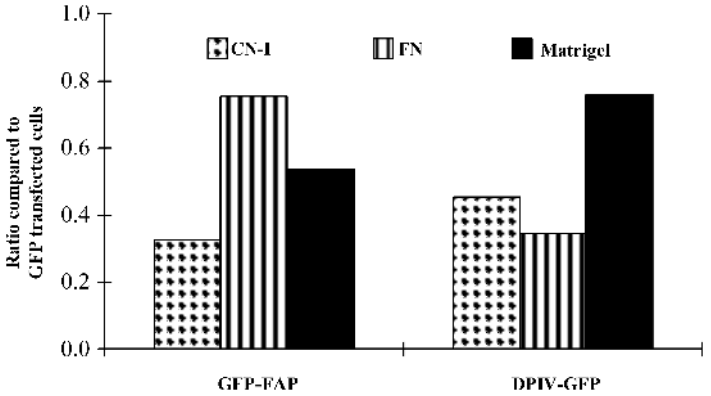

Figure 4. FAP or DPIV overexpression reduced cell migration. Transfected cells were placed above CN-I, FN or Matrigel coated wells. The percentages of green fluorescence-positive cells in the upper and lower chambers were enumerated to calculate the ratios of migrated to non-migrated recombinant protein—expressing cells. The figures depict each ratio obtained from GFP-FAP and DPIV-GFP fusion proteins as a proportion of the corresponding ratio obtained from GFP vector transfected cells.

FAP, DPIV and the cytoskeleton. The cytoskeleton is essential for cell motility in wound healing and tissue remodeling, Alexa Fluor 594-conjugated phalloidin, which binds to F-actin filaments, was used to visualize the actin cytoskeleton. Phalloidin exhibited very little co-localization with FAP in the cytoplasm. FAP was located in Golgi, endoplasmic reticulum, some small cytoplasmic vesicles and on the cell surface (Fig. 5). Similar results were obtained from DPIV transfected cells (data not shown). These data indicate that FAP and DPIV are exported to the cell surface via the trans-Golgi in the usual manner without associating with the cytoskeleton.

Cellular proliferation and apoptosis. DPIV expression can influence cell proliferation and apoptosis (Aldinucci *et al* 2004, Mizokami *et al* 2004, Sato *et al* 2003, Wesley *et al* 2004) but no such data on FAP is available. The thymidine uptake assay detected robust 293T cell proliferation, in the range 5000–15000 cpm. Cells transfected with DPIV or FAP exhibited greater thymidine uptake than GFP alone. GFP expression was an important control because it caused decreased proliferation compared to untransfected cells. The proliferation ratio of transfected to untransfected cells was 0.46 ± 0.09 for GFP, 0.55 ± 0.07 for FAP and 0.62 ± 0.11 for DPIV transfected cells ($P < 0.001$).

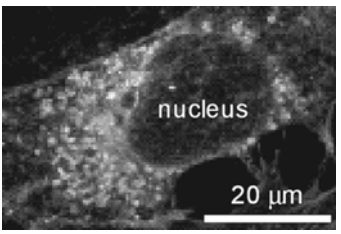

Figure 5. Confocal image of a 293AD cell transfected with GFP-FAP.

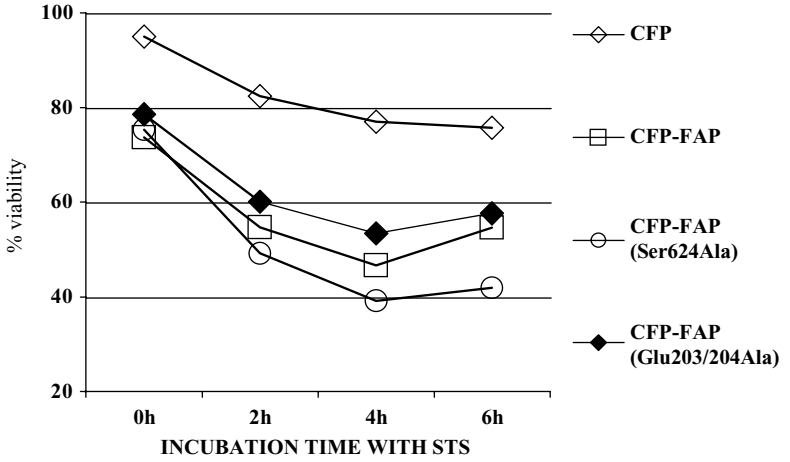

Figure 6. Overexpression of FAP increased STS induced cell apoptosis. The cyan fluorescence-positive cell population was selected for analysis. The annexin V-PE and propidium iodide (PI) double negative cell population was enumerated as nonapoptotic.

Nonapoptotic CFP positive 293T cells were enumerated by flow cytometry. In the absence of STS, cells overexpressing FAP (Fig. 6) or DPIV (data not shown) exhibited a small increase ($<$10%) in apoptosis. However, in the presence of STS, cell viability decreased about 20% to 30% within 2 hours. The FAP enzyme negative mutants were as effective as wildtype FAP at increasing STS induced apoptosis.

The molecular phenotype of FAP and DPIV overexpressing 293T cells. Expression levels of some proteins that have roles in cell adhesion may provide insights into the molecular mechanisms of the phenomena reported above. In recent studies of carcinoma cell lines DPIV overexpression was associated with increased MMP2 and CD44 (Kajiyama *et al* 2003, Wesley *et al* 2004). Similarly, we observed that overexpression of either FAP or DPIV was associated with increased expression of MMP2 and CD44. Discoidin domain receptor (DDR)-1 is a non-integrin collagen receptor that stimulates adhesion and migration (Matsuyama *et al* 2004) and is upregulated in cirrhotic liver (Shackel *et al* 2002). Decreased expression of DDR-1 was associated with DPIV overexpression. However, the flow cytometry profiles of the most prominent differences indicate that these changes were small (Fig. 7), which suggests that the mechanism is multigenic.

4. DISCUSSION

FAP and DPIV were found to influence cell-ECM interactions in elegant *in vitro* models. FAP reduced cell adhesion and migration on ECM proteins, DPIV reduced migration and both proteins enhanced STS-stimulated apoptosis. Thus, DPIV and FAP expression may influence cell behaviour in tissue remodelling microenvironments. We showed that the glutamates at positions 203 and 204 in FAP are essential for peptidase activity. FAP peptidase activity was not essential for FAP—mediated cell adhesion impairment or apoptosis enhancement.

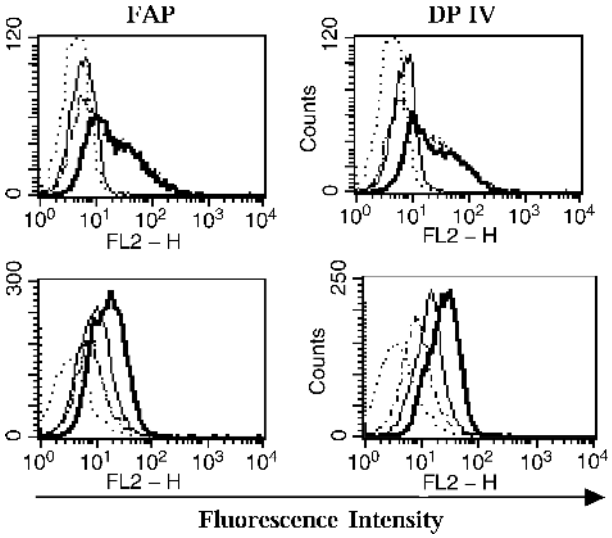

Figure 7. Flow cytometry of CFP fluorescence positive cells cell surface immunostained for CD44 (top) or β1-integrin (lowerpanel), two days after CFP-FAP (left), DPIV-CFP (right) or CFP transfection. Showing small increases in CD44 and β1-integrin expression. CFP transfected cells stained with antibody (_·_·_) or control antibody (. . . . .); FAP or DPIV transfected cells stained with antibody (_____) or control antibody (_____).

Although FAP is a cell surface molecule, most FAP molecules lie in cytoplasm, where they might interact with cytoskeleton associated proteins, such as vinculin, actinin, talin and paxillin. Indeed, nischarin inhibits cell motility by binding to an integrin cytoplasmic domain (Alahari *et al* 2000). However, FAP did not co-localize with the actin cytoskeleton or alter cytoskeletal morphology so it appears unlikely that FAP directly interacts with the cytoskeleton.

FAP is an integral membrane glycoprotein that associates with α3β1 integrin in the presence of CN-I (Mueller *et al* 1999). Therefore, interactions between the extracellular domain of FAP and extracellular molecules potentially have a role in inhibiting adhesion and migration on ECM. Cell adhesion and migration are associated with up-regulation of MMPs that proteolytically degrade ECM (Luo *et al* 1999). CD44 is a hyaluronic acid receptor and thus has roles in cell migration and adhesion. Integrins are the major class of cell surface molecules linking the cytoskeleton to the ECM. We found that overexpressing FAP upregulated MMP2 and CD44 and downregulated β1-integrin. These data indicate possible pathways for impairing adhesion and migration by FAP in 293T cells. FAP significantly enhanced STS-stimulated apoptosis using an enzyme activity independent process. FAP also increases apoptosis in the mouse B16 melanoma cell line (Ramirez-Montagut *et al* 2004). The low level of apoptosis induced by FAP expression in the absence of STS indicates that the observed deficiencies in adhesion and migration exhibited by FAP—expressing 293 cells were not due to apoptosis.

The DPIV data concords with prior in vitro studies showing FN—DPIV binding (Cheng *et al* 2003) and DPIV mediated inhibition of invasion by melanoma cells (Pethiyagoda *et al* 2001) and enhanced apoptosis (Sato *et al* 2003, Wesley *et al* 2004). For DPIV to inhibit invasion, neither its protease activity nor its cytoplasmic domain are required (Pethiyagoda *et al* 2001).

5. CONCLUSIONS

FAP and DPIV overexpression in the HEK293 cell line reduced cell adhesion, migration and invasion on ECM components, increased proliferation and promoted apoptosis, independently of enzyme activity. These observations may aid an understanding of the roles of FAP and DPIV in tissue remodeling.

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CHAPTER 24

ROLE OF NEUROPEPTIDE Y AND DIPEPTIDYL PEPTIDASE IV IN REGULATION OF EWING'S SARCOMA GROWTH

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1. INTRODUCTION

Ewing's sarcoma family of tumors (ESFT) is a group of aggressive malignancies which develop in children and adolescents and often lead to the death of patients despite intensive therapy. The ESFT includes several phenotypic variants, such as Ewing's sarcomas arising in bones, peripheral primitive neuroectodermal tumors (pPNET) of soft tissues and Askin tumors of the thoracopulmonary region (de Alava and Gerald 2000; Kovar 2003; Rodriguez-Galindo *et al 2003*). The characteristic feature of ESFT tumors is a translocation resulting in the fusion of the EWS gene located on chromosome 22 with an ETS transcription factor (de Alava and Gerald 2000; Beltinger and Debatin 2001; Whang-Peng *et al* 1986; Burchill 2003). The EWS-ETS protein acts as an aberrant transcription factor and alters mRNA splicing (de Alava and Gerald 2000; Chansky *et al* 2001). The abnormal functions of this chimerical molecule are believed to be the major mechanism leading to the malignant transformation of ESFT cells (Lessnick *et al* 1995).

Due to their cholinergic properties, ESFT are thought to originate from parasympathetic neurons (Biedler *et al* 1973). The tumors exhibit variable degrees of neuronal differentiation and express neuron-specific markers, such as neuropeptide Y (NPY) (Kitlinska *et al* 2005). NPY is a 36-amino acid sympathetic neurotransmitter, present also in parasympathetic nerves (Colmers and Wahlestedt 1993). The peptide acts via multiple $G_{i/o}$ protein-coupled receptors designated Y1–Y5 (Larhammar *et al* 1992; Rose *et al* 1995; Gerald *et al* 1996). Its actions are also modified by dipeptidyl peptidase IV (DPPIV), which cleaves the full length NPY_{1-36} to NPY_{3-36} (Mentlein 1999). This shorter form of NPY is no longer able to activate Y1 receptors. Thus, DPPIV changes the affinity of NPY to its receptors and shifts its actions from those mediated by Y1 receptors, to those dependent on other receptor types.

The best known functions of NPY include inhibition of neurotransmitter release, vasoconstriction and regulation of appetite (Colmers and Wahlestedt 1993; Zukowska-Grojec and Wahlestedt 1993; Grundemar *et al* 1997). However, there is a growing line of evidence that the peptide is also a potent growth-regulatory factor for a variety of cells, including vascular smooth muscle cells (Zukowska-Grojec *et al* 1993, 1998; Pons *et al* 2003), neuronal precursors (Hansel *et al* 2001) and neural crest-derived tumor cells such as neuroblastoma (Kitlinska *et al* 2005). Furthermore, the peptide is also a potent angiogenic factor which stimulates proliferation and migration of endothelial cells (Zukowska-Grojec *et al* 1998a; Lee *et al* 2003). NPY-mediated angiogenesis is mainly Y2 receptor-dependent, since the angiogenic activities of the peptide are severely impaired in Y2 knockout mice (Lee *et al* 2003a; Koulu *et al* 2004; Ekstrand *et al* 2003).

2. ROLE OF NPY IN REGULATION OF ESFT GROWTH

ESFT cells express NPY and high levels of its Y1 and Y5 receptors (Kitlinska *et al* 2005; van Valen *et al* 1992). This receptor pattern distinguishes these tumors from other neuroendocrine tumors of childhood, such as Y2 receptor-expressing neuroblastomas of the sympathetic origin. Moreover, expression of NPY and its receptors in ESFT cells is accompanied by the presence of high levels of DPPIV mRNA (Kitlinska *et al* 2005).

Due to its high Y1 receptor expression, ESFT cell lines, such as SK-N-MC, have been extensively used to study its signaling and trafficking (Shorter and Pence 1997; Fabry *et al* 2000; Langer *et al* 2001). However, sparse and conflicting data have been reported regarding the function of NPY in these cells (van Valen *et al* 1992; Shorter and Pence 1997; Reubi *et al* 2001). Recently, we have found that the peptide stimulates apoptosis in SK-N-MC cells, which is associated with increased caspase 3/7 activity. The growth-inhibitory effect of NPY is mediated by both Y1 and Y5 receptors. In addition, Y1 and Y5 receptor antagonists not only block the NPY-mediated apoptosis, but also increase the basal proliferation levels above the control, which indicates the role of endogenous NPY in regulation of ESFT growth (Kitlinska *et al* 2005). The growth-inhibitory effect of NPY observed in vitro has been also confirmed in vivo. In nude mice, exogenous NPY administered as a slow release pellet inhibited growth of SK-N-MC xenografts, which was associated with increased apoptosis (Kitlinska *et al* 2005). However, despite dramatic growth impairment, the NPY-treated tumors were better vascularized than the control group. Thus, due to its angiogenic properties, NPY stimulates vascularization of the tumors, which in turn can augment tumor growth. Therefore, in ESFT tumors, NPY exerts two opposite effects—the Y1/Y5 receptor-mediated apoptosis of the tumor cells and Y2 receptor-dependent angiogenesis. The overall effect of the peptide on the growth of ESFT tumors depends on the balance between these two processes.

3. ROLE OF DPPIV IN REGULATION OF ESFT GROWTH

Although both in vitro and in vivo results obtained on SK-N-MC were very promising, another study indicated that not all ESFT cell lines were equally responsive to the NPY treatment (Fig. 1B). Since there were no significant differences in the expression of NPY and its receptors between the ESFT cell lines, we investigated the possible role of DPPIV in modifying the peptide's apoptotic actions. The protease was expressed in all investigated ESFT cell lines.

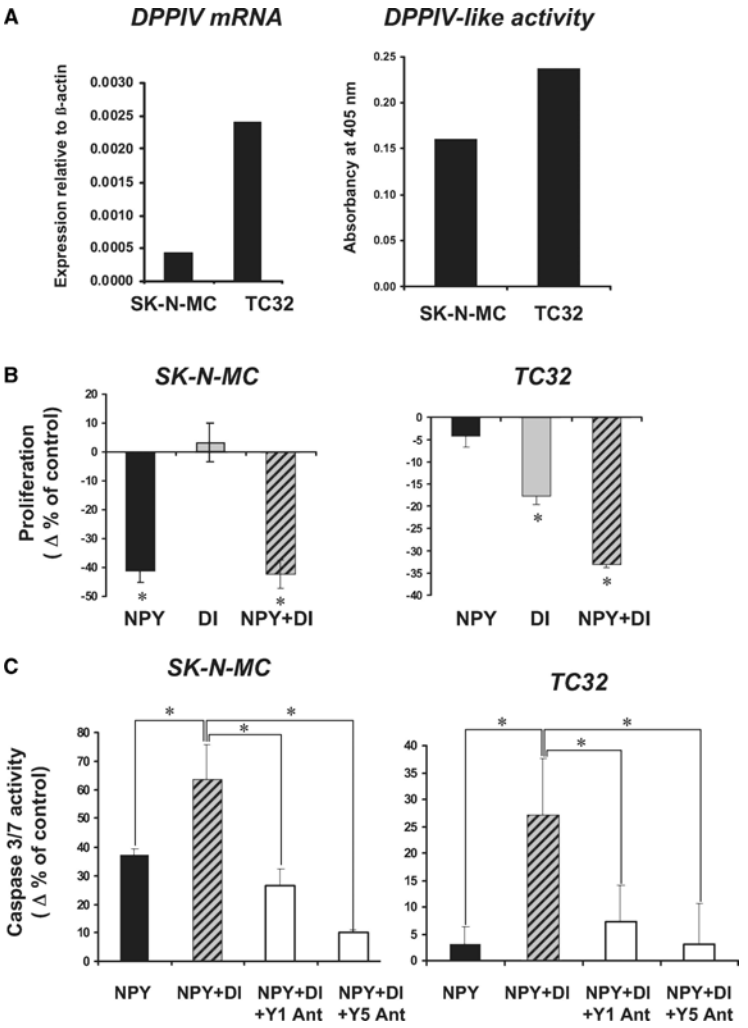

Figure 1. DPPIV inhibitor augments growth-inhibitory effects of NPY. A. Real time RT-PCR. TC32 cells express 5-fold higher levels of DPPIV mRNA, as compared to SK-N-MC, which is accompanied by increased proteolytic activity B. MTS assay. In SK-N-MC cells, NPY alone inhibits cell proliferation and this effect is not augmented by DPPIV inhibitor, P32/98. In TC32 cells, NPY alone has no effect on cell proliferation, however its inhibitory effect are restored in the presence of the DPPIV inhibitor. C. Caspase 3/7 activity. In SK-N-MC cells, NPY increases caspase 3/7 activity, whereas no effect is observed in TC32 cells. DPPIV inhibitor increases NPY apoptotic effect in both cells and this effect is blocked by Y1 and Y5 antagonists. DI—DPPIV Inhibitor, P32/98. Agonists at concentration 10^{-7}M, antagonists—10^{-6}M, DPPIV inhibitor—10^{-5}M.

However, DPPIV mRNA levels and DPPIV-like activity was higher in non-responsive TC32 cells, as compared to the responsive SK-N-MC (Fig. 1A). Since, according to our previous study, both Y1 and Y5 receptors seemed to be necessary for NPY's apoptotic actions in ESFT cells (Kitlinska *et al* 2005), elevated DPPIV activity could potentially prevent this effect by converting the peptide to the non-Y1 agonist. Indeed, in TC32 cells, which did not respond to the

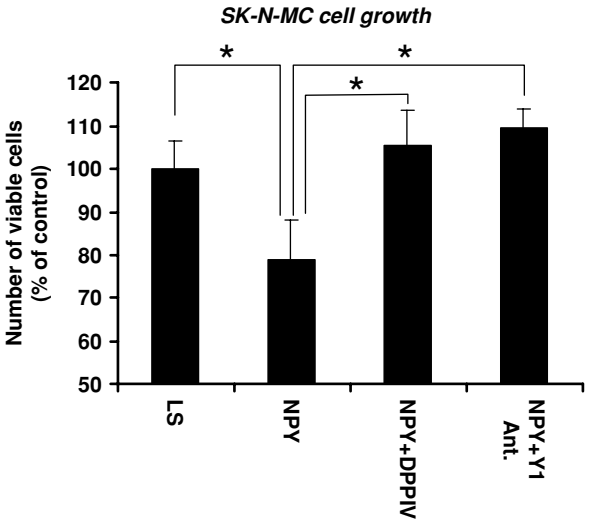

Figure 2. Recombinant DPPIV prevents NPY-induced apoptosis. MTS assay. In SK-N-MC cells, NPY inhibits growth, which is blocked by Y1 antagonist. This effect is mimicked by DPPIV, which cleaves NPY to the NPY3-36. This short form of the peptide is not able to bind to the Y1 receptor and stimulate SK-N-MC cell apoptosis. NPY at concentration10^{-7}M, antagonists—10^{-6}M, DPPIV—100 ng/ml.

NPY treatment, the DPPIV inhibitor P32/98 decreased growth which was further augmented by the addition of exogenous NPY (Fig.1B). Thus, inhibition of DPPIV activity restored apoptotic activities of both the endogenous and exogenous peptide. In SK-N-MC cells, NPY alone inhibited growth, whereas P32/98 had no effect, which could be associated with lower DPPIV-like activity. The above results were confirmed by the caspase 3/7 activity assay (Fig. 1C). NPY alone induced apoptosis in SK-N-MC cells, whereas no effect was observed in TC32 cells. P32/98 inhibitor augmented the apoptotic activity of the peptide in both cell lines and this effect was blocked by Y1 and Y5R antagonists. Moreover, growth inhibitory actions of NPY in SK-N-MC cells were completely blocked by the addition of the recombinant DPPIV, which mimicked the effect of the Y1 receptor antagonist (Fig. 2). Thus, dipeptidyl peptidase activity is an important regulator of NPY actions in ESFT cells. The DPPIV-mediated cleavage of the peptide prevents its binding to the Y1 receptor and suppresses its apoptotic actions. In this way, DPPIV acts as a survival factor for ESFT cells, protecting them from the inhibitory effect of endogenous NPY.

Another aspect of the potential role of DPPIV in the regulation of ESFT growth is associated with the angiogenic activities of NPY. Formation of new blood vessels is, along with proliferation of tumor cells, one of the crucial processes determining tumor growth and spread. As mentioned before, NPY stimulates angiogenesis via its Y2/Y5 receptors (Lee *et al* 2003a; Koulu *et al* 2004; Ekstrand *et al* 2003). DPPIV, the protease that converts NPY to the Y2-preffering agonist, is an integral element of the NPY angiogenic system. The enzyme is abundant in endothelial cell and up-regulated with NPY and its Y2 receptors in ischemia (Lee *et al* 2003; Ghersi *et al* 2001). Moreover, DPPIV activity is crucial for NPY-induced endothelial cell migration (Ghersi *et al* 2001). As described above, NPY synthesized by ESFT cells can exert two opposite effects on tumor growth— Y1/Y5-mediated apoptosis of ESFT cells and Y2/Y5-dependent angiogenesis. Thus DPPIV, by cleaving NPY, can not only prevent its apoptotic activities, but can also enhance

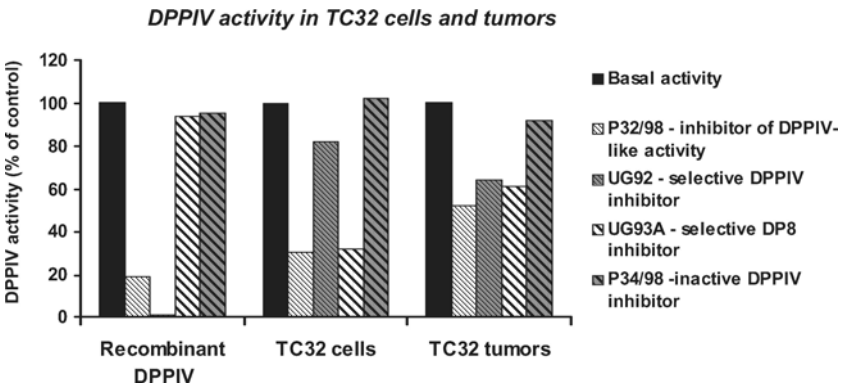

Figure 3. Dipeptidyl peptidase activity in TC32 cells is dependent on DPPIV and DP8. DPPIV activity assay. The enzymatic activity of TC32 cell extracts is blocked only in 20% by the specific DPPIV inhibitor, UG92. The effect of DP8 selective inhibitor, UG93, is more pronounced, which indicates a role of this peptidase in regulation of ESFT cell growth. The percent of the proteolytic activity blocked by the selective DPPIV inhibitor increases in TC32 tumor tissues, which could be associated with the presence of endothelial cells. The proteolytic activity was detected by cleavage of ?? and colorimetric measurement at 405 nm.

the peptide's angiogenic effect at the same time. In this way, the protease can shift NPY's activities from inhibition to stimulation of tumor growth.

4. DPPIV-LIKE PROTEASES IN ESFT

Although all investigated ESFT cells expressed DPPIV mRNA, the results obtained when inhibiting the protease with P32/98 were puzzling. Since this inhibitor is not specific for DPPIV, it can potentially inhibit other DPPIV-like peptidases. Thus, to determine if DPPIV is the only protease active in ESFT cells, the following selective inhibitors were used: DPPIV-specific UG92 and DP8-specific UG93. Surprisingly, the DPPIV selective inhibitor reduced DPPIV-like activity in TC32 cells by only 20%, whereas DP8-specific UG93 was much more effective (Fig. 3). Thus, DPPIV is not the only peptidase and also not the most powerful one in ES cells. Interestingly, in tissue samples obtained from TC32 xenografts, the percent of DPPIV-dependent proteolytic activity was higher than in cells grown in vitro (Fig. 3). This may be due to higher levels of DPPIV present in vascular endothelial cells (Ghersi *et al* 2001). Thus, further studies are necessary to determine the role of other dipeptidyl peptidases in the regulation of NPY's actions in ESFT tumors. However, even if DPPIV is not the most important dipeptidyl peptidase in ESFT, its activity may be still important in these tumors, due to the presence of DPPIV in vascular endothelial cells.

5. CONCLUSIONS

DPPIV-like proteases are important factors in the regulation of NPY actions in ESFT cells. By cleavage of NPY, dipeptidyl peptidases prevent NPY-induced apoptosis of ESFT cells mediated by Y1/Y5 receptors and shift the peptide's activity toward Y2 receptor-dependent angiogenesis (Fig. 4). Thus, targeting DPPIV-like enzymes, possibly in combination with NPY

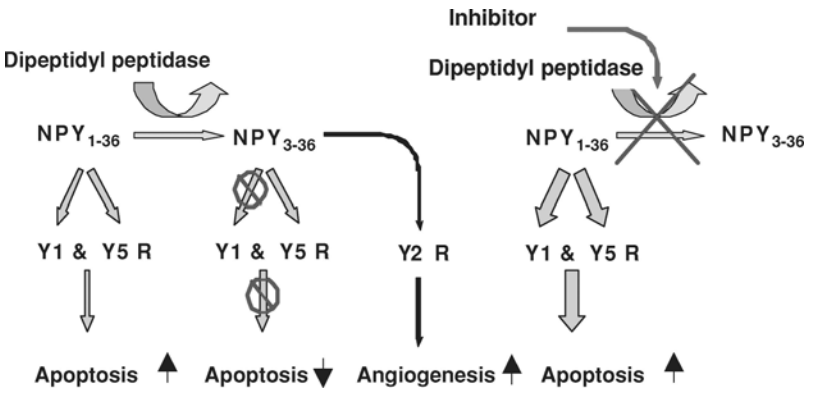

Figure 4. Proposed mechanism of DPPIV actions in ESFT cells. Activation of both Y1 and Y5Rs is necessary for NPY growth-inhibitory effects. Dipeptidyl peptidases convert NPY to NPY_{3-36}, which is inactive at Y1Rs and in this way suppress NPY-induced inhibition of proliferation/apoptosis and shift activity of the peptide to Y2-mediated angiogenesis. Blocking DPPIV-like activity directs NPY actions toward Y1/Y5-mediated growth inhibitions.

or Y1/Y5 specific agonist, may be an effective strategy in treatment of ESFT tumors. However, further studies are required to determine which proteases should be targeted, considering their expression and functions in both tumor and endothelial cells.

ACKNOWLEDGEMENTS

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CHAPTER 25

THE ROLE OF CD26/DPP IV IN PRESERVATION OF EARLY PULMONARY GRAFT FUNCTION

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1. INTRODUCTION

CD26 plays a pivotal role in thymic differentation/maturation (Simeoni *et al* 2002), costimulation (Morimoto 1998), migration (Ikushima *et al* 2003) and the T cell memory response (De Meester *et al* 1999). In addition, it possesses enzymatic activity (dipeptidyl peptidase IV, DPP IV), which is linked to its costimulatory efficacy (Tanaka *et al* 1993, 1994), and has been correlated with immunological competence in vivo (Vanham *et al* 1993). Circulating DPP IV enzymatic activity is increased in various immune disorders (Fujita *et al* 1978; Constantinescu *et al* 1995). We have previously shown that the course of acute cardiac allograft rejection in rats is associated with a significant increase in DPP IV serum activity. Specifically inhibiting DPP IV circulating activity in transplant recipients impaired cellular and humoral immune responses, significantly prolonging graft survival in models of acute cardiac rejection (Korom *et al* 1997). Following successful kidney transplantation (Tx) in patients on maintenance immunosuppression, systemic DPP IV catalytic activity was markedly reduced for twelve months, and CD26 surface expression on circulating lymphocytes displayed a significant down-regulation for eighteen months post Tx (Korom *et al* 2002). To further analyze the influence of DPP IV targeted inhibition on the course of early allograft performance, we chose lung Tx in the rat as an alternative model of perfused organ engraftment. In contrast to the heart, the lung constitutes a more challenging immunological set up, due to its vast area of interaction with the milieu exterieur.

In this study we demonstrate that specifically targeting circulating DPP IV enzymatic activity in LEW recipients of LBNF1 pulmonary grafts maintains pulmonary macromorphological architecture and preserves ventilatory parameters for up to five days following Tx.

2. MATERIALS AND METHODS

2.1. Animals and Grafting Technique

Inbred male rats (Harlan, Horst, Netherlands) weighing 250 to 300 g were used. Left pulmonary grafts were harvested (Lewis [LEW] x Brown Norway [BN] F_1 hybrids ($LBNF_1$) and transplanted into LEW (RT1′) recipients using a cuff technique (Mizuta *et al* 1989) for vascular and bronchial anastomosis.

2.2. Experimental Setup and DPP IV Inhibitor Treatment

Experimental LEW recipients (group I) of LBNF1 orthotopic left lung transplants received peri-/postoperative care as described, and were treated in addition with a Pro-Pro-diphenyl phosphonate derivative (AB 197) which acts as an irreversible inhibitor of DPP IV. The initial dose at day of engraftment (d0) was 100 mg/kg/bodyweight sc., followed by 60 mg/kg/bw/d sc. until the fourth postoperative day (d4). Control animals (group II) consisted of four LEW recipients which were treated accordingly to group I, yet, instead of AB 197, they received 0.5 ml saline/d sc. from d0–d4.

2.3. Measurement of DPP IV Serum Levels

DPP IV activity was determined fluorometrically, as previously described [13], with modifications. Briefly, the enzyme-containing serum was incubated at 37°C with 1.4mmol/L Gly-Pro-4-methoxy-2-naphthylamide in 50 mmol/l Tris-HCl (pH 8.3). The reaction was stopped by the addition of 0.1mol/l citrate buffer (pH 4.0), after which the fluorescence of the formed 4-methoxy-2-naphthylamine was measured. Hydrolysis of L-Ala-4-methoxy-2-naphthylamide (2 mmol/l) in 60 mmol/l phosphate buffer (ph 7.4) was chosen for determination of membrane alanyl aminopeptidase (EC 3.4.11.2) activity. One unit of enzymatic activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of assay-product per minute under conditions used. The DPP IV activity determined before any invasive procedure was defined as 100%.

2.4. Assessment of Transplant Function

At day 5 post Tx, the recipient animals were anaesthesized, intubated and ventilated as previously described. A median sternotomy was performed, and peak airway pressure (PawP) measured and recorded in mmHg. All structures of the right hilum were clamped, to assess the oxygenation capacity of the transplanted left lung. Three minutes after occlusion, when a steady state was reached, 300 μl of blood was aspirated from the ascending aorta for blood gas assessment. Next, the IVC was incised, the left atrial appendage cut to vent the perfusing solution, and a 14-gauge cannula placed into the PA through an anterior incision in the right ventricular outflow tract. Following flushing with 20 ml of 0.9% saline solution with a pressure of 20 cm H_20, the heart-lung-block was explanted and samples were placed in a 10% formalin solution for further histological analysis.

3. RESULTS

3.1. Profound DPP IV Serum Enzymatic Activity Inhibition

A stable and reproducible inhibition of DPP IV serum activity was achieved by initial administration of 100 mg of AB 197/kg. We noted a rapid serum activity suppression of $\leq$6% following injection, which was maintained below 10% remaining serum activity by maintenance therapy of 60 mg/kg/animal/d (data not shown).

3.2. DPP IV Serum Activity Inhibition Preserves Early Pulmonary Graft Function

DPP IV enzymatic activity inhibition with AB 197 preserved the gas exchange capacity of the engrafted lung in the early phase following transplantation. Isolated ventilation of the transplanted lung in hosts undergoing DPP IV serum activity inhibition displayed a markedly higher pO_2, in comparison to control animals (mean $pO_2 \pm$ sd mmHg; controls: 28.7 ± 6.5 vs. inhibited: 153.5 ± 26.4; $n = 4$/group). Accordingly, pCO_2 in treated recipients was profoundly reduced for 5 days after Tx as compared to rejecting controls (mean $pCO_2 \pm$ sd mmHg; controls: 60.3 ± 12.4 vs. inhibited: 41.0 ± 8.8; $n = 4$/group).

3.3. DPP IV Enzymatic Activity Inhibition Macroscopically Preserves Allograft Structure

All treated pulmonary allograft recipients, at time of harvest (d5 post-Tx), showed a macroscopically unalterated graft without the typical signs of acute rejection (Fig. 1). Grafts were well ventilated, with good perfusion and without signs of infiltration or atelectasis. The macroscopic appearance correlated well with the functional performance, as seen during isolated ventilation/perfusion of the transplanted lung.

4. CONCLUSION

For the first time we have shown that early pulmonary allograft function can be preserved by selectively targeting CD26/DPP IV serum enzymatic activity. In line with our previous data [10, 11], where acute and accelerated allograft rejection was successfully abrogated, these preliminary findings stress the importance of targeting the enzymatic moiety of this intriguing lymphocyte surface molecule.

The observed effect of preserving ventilatory function (in a more immunogeneic perfused organ transplantation model) may be attributed to modulation of the immune cascade, triggered by allo-Ag exposure. In addition, inhibiting DPP IV activity may ameliorate otherwise ischemia/reperfusion-induced injuries within the pulmonary parenchyma. Further studies are needed to decipher the mode of action of this heterogenous molecule.

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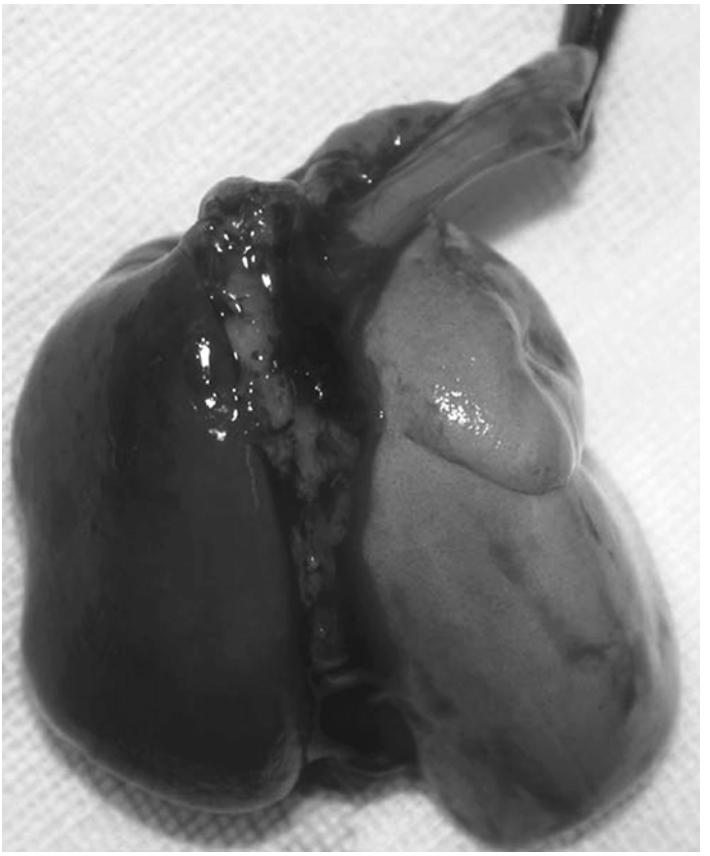

Figure 1. Rat bilateral pulmonary block (dorsal view), at day 5 post-Tx. Transplanted lung (Tx) in untreated control (A) vs. DPP IV inhibited recipient (B). Note: Preservation of macroscopic structure of the pulmonary graft in treated recipients.

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