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Fibroblast activation protein and chronic liver disease

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1. ABSTRACT

Fibroblast activation protein (FAP) is the member of Dipeptidyl Peptidase IV (DPIV) gene family that is most similar to DPIV. Four members of this family, DPIV, FAP, DP8 and DP9 possess a rare catalytic activity, hydrolysis of a prolyl bond two residues from the substrate N terminus. Crystal structures show that the soluble form of FAP comprises two domains, an alpha/beta-hydrolase domain and an 8-blade beta-propeller domain. The interface between these two domains forms the catalytic pocket, and an opening for substrate access to the internal active site. The FAP homodimer is structurally very similar to DPIV but FAP glycoprotein expression is largely confined to mesenchymal cells in diseased and damaged tissue, notably the tissue remodelling region in chronically injured liver. FAP peptide substrates include denatured collagen and alpha2-antiplasmin. The functional roles of FAP in tumors and fibrotic tissue are not fully understood. This review places FAP in the context of chronic liver injury pathogenesis.

2. INTRODUCTION

Membrane-bound proteases act both as effectors and as regulatory molecules in many physiological and pathological events, including protein turnover, ontogeny, inflammation, tissue remodelling, cell migration and invasion, by activation of proforms or degradation of biomolecules by cleavage. Membrane-bound proteases require tight regulation. Fibroblast activation protein (FAP) is a membrane-bound protease with a unique enzyme activity portfolio. FAP can hydrolyse a prolyl bond two or more residues from the N-terminus. Thus, FAP is both dipeptidyl peptidase and endopeptidase. Like its closest relative, the well-known proline-specific serine protease Dipeptidyl Peptidase IV (DPIV), FAP has a variety of biological roles.

Enzymes are increasingly proving to be useful targets of pharmaceutical research through the design of proteolytic inhibitors. FAP has attracted increased interest due to its unique expression in tumors, arthritis and

fibrosis. Unlike the ubiquitous expression of DPIV, FAP is only expressed at sites of tissue remodelling, but not in most normal human tissues. FAP is now a well-established marker of activated fibroblasts (1). In particular, only activated tumor stromal fibroblasts and activated hepatic stellate cells (HSC) and myofibroblasts strongly express FAP. Here we review the understanding of its biochemistry and biological roles with a focus on liver disease.

The DPIV gene family, consisting of DPIV, FAP, DP8, DP9, DP61 and DP10, is categorized as a subgroup of enzymes under the prolyl oligopeptidase (POP) family, which also includes prolyl endopeptidase (PEP). These enzymes are specialized in cleaving the prolyl bond, which is resistant to proteolytic cleavage due to its cyclic nature, and the presence of an imino rather than an amino group. The POP family enzymes are also unusual in that the catalytic pocket is buried inside the protein, a feature thought to restrict substrate size. As most peptide hormones and neuropeptides comprise one or more proline residues, this family of enzymes is useful for processing and degrading such peptides.

FAP was discovered in 1993 and DPIV was discovered in 1966 so DPIV is better understood. Therefore some DPIV background is provided here as an aid for understanding FAP.

3. DPIV

DPIV substrates include glucagon family members, neuropeptide Y (NPY) and chemokines. In addition to its enzymatic functions, DPIV has several protein ligands, adenosine deaminase, fibronectin (FN), plasminogen ε and the sodium-hydrogen exchanger isoform NHE3. Adenosine deaminase binding confers a role in T cell activation and proliferation.

Through cleavage, DPIV modulates its natural substrates. DPIV can hydrolyse nine chemokines. Chemokines have fundamental roles in the immune system via leucocyte trafficking regulation, maturation and homing of lymphocytes and the development of lymphoid tissues. By cleavage, DPIV reduces the inflammatory properties of chemokine targets and reduces the redundancy in their target specificity. The chemokines most rapidly cleaved by DPIV are CXCL12 (stromal derived factor -1 alpha and beta) and CCL22 (2-4). DPIV plays a role in the cleavage of the glucagon family of peptide hormones, including growth hormone releasing hormone, glucagon, glucagon-like peptide (GLP)-1 and GLP-2. GLP-1 is a gastrointestinal peptide hormone that stimulates pancreatic insulin secretion. Because GLP-1 is readily degraded by DPIV as it crosses into capillaries and in blood, DPIV inhibitors can increase the GLP-1 half-life and probably the half-lives of other hormones that influence metabolism, such as glucose-dependent insulinotropic polypeptide, vasoactive intestinal peptide, pituitary adenylate cyclase-activating peptide, gastrin-releasing peptide and GLP-2. Other important DPIV substrates include NPY, a neuropeptide involved in the control of appetite, energy homeostasis and blood pressure, and peptide YY, a peptide released in response to a meal that acts to inhibit several gastrointestinal functions such as gastric acid release (5).

DPIV is a validated therapeutic target in the metabolic disorder type 2 diabetes (6). The selective DPIV inhibitor Sitagliptin (Januvia / MK-0431) has been intensively investigated in clinical trials (7-9) and is licensed for sale in many countries. The potential for DPIV inhibitors to have wider therapeutic application in metabolic disorders has been reviewed previously (10, 11). DPIV inhibitors under development for diabetes therapy do not inhibit FAP.

4. FAP

FAP, Genbank accession number U09278, also known as seprase, is the closest relative of DPIV, having 52% amino acid identity. FAP and DPIV have similar gene sizes and are chromosomally adjacent to each other; they are 72.8 kb and 81.8 kb at 2q24.3 and 2q24.2 respectively, suggesting a gene duplication event. Both genes have 26 exons. Therefore, it is expected that FAP will prove to be similar to DPIV in having multiple protein binding partners in addition to and independent from the peptidase activity.

4.1. Crystal structure of FAP

The crystal structure of proteins provides important information for their functions and specific inhibitor design. DPIV crystal structures have been reported by several groups and reviewed previously (10). The FAP structure has been solved (PDB ID 1Z68) and is very similar to that of DPIV (12). The FAP glycoprotein is a homodimer. Dimerisation of FAP is required for its catalytic function (13, 14). Each monomer subunit consists of two domains (Figure 1), an alpha/beta-hydrolase domain (residues 27-53 and 493-760) and an eight-blade beta-propeller domain (residues 54–492), that enclose a large cavity of ~30-45 Angstroms in diameter. A small pocket within this cavity at the interface of the beta–propeller and alpha/beta hydrolase domains contains the catalytic triad, composed of residues Ser624, Asp702 and His734. A large side opening of about 15 Angstroms provides access to this cavity (Figure 1C). However, only elongated peptides or unfolded or partly unfolded protein fragments can reach the small pocket within this cavity that contains the active site.

4.1.1. Beta-propeller domain

Beta-propellers have four to eight blades formed by a repeated subunit containing at least 30 and generally 50 amino acids in a beta-sheet of four anti-parallel strands. Propellers commonly act as scaffolding for protein–protein interactions. 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. Cocrystallisation of DPIV with a ten amino acid substrate fragment suggests that substrates enter the active site through the side opening (15). This feature very likely also occurs in FAP. A protrusion from the fourth blade of the beta-propeller strongly participates in dimerisation.

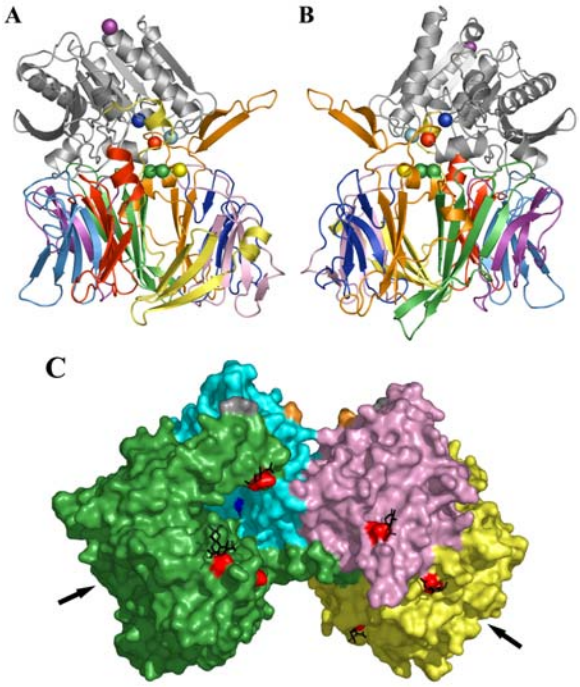

Figure 1. A, B. Ribbon diagram representing the overall structure of the FAP monomer. The beta–propeller and alpha/beta hydrolase domain form the catalytic pocket and a side opening at their interface. The side opening is at the right in A. The locations of active-site residues Ser624 (dark blue), Ala657 (red) and Asn704 (cyan) of the alpha/beta hydrolase domain, and Arg123 (gold), Glu203 and Glu204 (green) of the beta–propeller domain are indicated by spheres. The C-terminal Ser is shown as a purple sphere. The N-terminal transmembrane and cytoplasmic portions of FAP are not depicted; they would be above the molecule. The N-terminus to the end of propeller blade 1 is colored yellow. The propeller blades 2 to 8 are colored pink, dark blue, orange, green, purple, blue and red respectively. The hydrolase domain is grey. The molecule is turned 180 degrees around the vertical axis in image B. C. Space-filled representation of the FAP dimer highlighting potentially glycosylated Asn residues in red and sugars in black and oriented to show the side opening of this cavernous protein. Different colors were applied to the hydrolase (blue and purple) and propeller (green and gold) domains. The N- and C- termini are colored grey and orange respectively. The catalytic Ser624 is colored dark blue. An arrow points towards the opening in each beta-propeller lowerface. Image construction used PDB coordinates 1XFD (3.0 Angstrom resolution) and PyMOL.

DPIV contains nine N-linked glycosylated sites that lie predominantly on the propeller domain near the dimerisation interface (16). FAP contains six potential N-linked glycosylation sites (motif Asn-X-Ser/Thr) at Asn residues 49, 92, 99, 227, 314 and 679. All these sites are surface exposed and only one is on the hydrolase domain. Five of these potential glycosylation sites (not Asn99) are glycosylated in the baculovirus – expressed soluble human FAP (12). Interestingly, three of these sites border the side opening and all six sites are in the region of the inter-domain interface (Figure 1C). This location might restrict access by large molecules and might reflect a role for sugars in shielding FAP from proteolysis. This location means that the beta-propeller lower edge, which is the region usually involved in protein-protein binding by beta-propellers, lacks sugars.

4.1.2. Active site and catalytic mechanism

In addition to the residues forming the catalytic triad, Glu203 and Glu204 are essential for catalytic activity (17). These two glutamic acids in the catalytic pocket, Glu203 and Glu204 (Figures 1A, 1B and 2), 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. The equivalent conserved glutamic acids are essential for catalysis in DPIV and DP8 (18, 19). Comparison of the crystal structures of FAP and DPIV reveal one major difference in the vicinity of this Glu motif within the active site (Figure 2). Ala657 in FAP, instead of Asp663 as in DPIV, reduces the acidity in this pocket, and this change explains the lower affinity for N-terminal amines by FAP. Concordantly, the kinetic analysis of the mutant FAP Ala657Asp shows an approximately 60-fold increase in the catalytic efficiency for the cleavage of dipeptide substrates, and an approximately 350-fold reduction for cleavage of the endopeptidase substrate Z-Gly-Pro-7-amino-4-methylcoumarin (12, 20). Tyr656 is essential for catalysis and Asn704 and Arg123 greatly influence activity (21). All five conserved non-catalytic triad residues, at 123, 203-204, 656 and 704, appear to confer transition state stabilisation (21).

4.2. Activities of FAP

Besides its dipeptidyl peptidase activity (13, 22), FAP also has CN-I specific gelatinase activity (13, 14, 22) and recently reported endopeptidase activity (12, 20). Like DPIV, FAP catalysis depends upon dimerisation (14, 22). The k_{cat}/K_m values for cleavage of H-Ala-Pro-p-NA by FAP are about 100 times less than with DPIV (12). A single amino acid residue difference, Ala657, near the active site makes FAP capable of endopeptidase activity. DPIV has Asp663 at the corresponding site (12). Interestingly, this prolyl endopeptidase activity of FAP is restricted to Gly-Pro containing substrates (20). Thus, FAP has a unique endopeptidase activity on Gly-Pro derived substrates.

Should there be any natural substrates of FAP cleaved after the penultimate residue, ie DP cleavage, they are unknown. Important substrate-binding sites and key substrate-binding residues in both proteases are in very similar positions, suggesting that some DPIV substrates could also be FAP substrates. However, FAP has 100 fold less activity on H-Gly-Pro compared to DPIV, indicating that H-Gly-Pro is not a preferred substrate of FAP. FAP cleaves synthetic P_2-Pro_1-coumarins with broad P_2 specificity and *N*-acyl-P_2-Pro_1-coumarins with a P_2 Gly residue. Therefore, substrates likely to undergo detectable DP cleavage by FAP are probably a subset of DPIV substrates.

The narrow endopeptidase specificity of FAP has been exploited for inhibitor design. Recently, two FAP

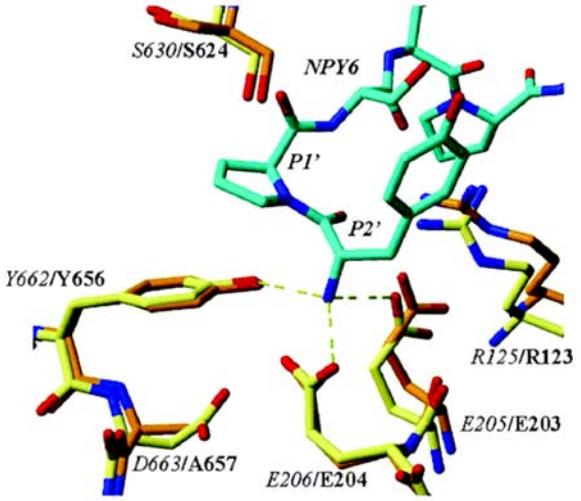

Figure 2. Superposition of FAP with DPIV showing a detailed view of the residues around the Glu motif, Glu203 and Glu204 of FAP (from (12)). The interactions in DPIV between active site residues and the N-terminal hexapeptide of the substrate NPY (NPY6; TyrProSerLysProAsp; *blue*), present in the crystal structure of DPIV (*yellow*), are shown as *dashed lines*. Residues in FAP are *orange*. Amino acid residues are labeled in *italic* and bold for DPIV and FAP, respectively.

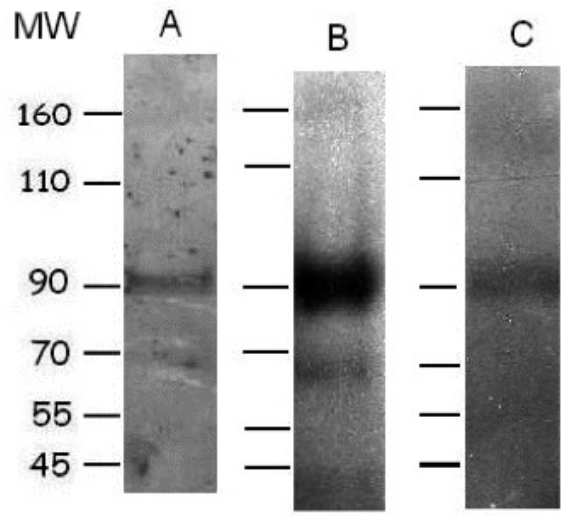

Figure 3. Purified human FAP. Baculovirus – expressed soluble human FAP (residues 39 – 760) polyhistidine-tagged at the C terminus was purified by metal affinity chromatography and visualised on silver-stained 3-8% SDS-PAGE (A). Immunoblot with MAb 1E5 (B) (Abnova, Taipei, Taiwan, Catalogue No. H00002191-M01) and with MAb F19 (ATCC, Manassas, VA, Hybridoma catalogue No. CRL-2733) (C) followed by horseradish peroxidase conjugated rabbit anti-mouse IgG (DAKO, Santa Barbara, CA, diluted 1:1000) showed that the 92 kDa band is FAP. In addition, MAb 1E5 recognized a 70 kDa band as a FAP fragment.

specific inhibitors, peptidyl-chloromethyl ketones and Ac-Gly-BoroPro have been reported (20). Both of these compounds inhibited FAP but not DPIV, suggesting that N-acyl-Gly-Pro-based inhibitors may be FAP-selective. Selective FAP inhibitors may aid in validating FAP as a therapeutic target, just as DPIV-selective inhibitors are a diabetes therapy, but non-selective inhibitors have other *in vivo* outcomes. Indeed, developing FAP, DP8 and DP9 selective inhibitors would be a very important step forward in understanding DPIV family biology.

All studies of FAP structure and enzymology use baculovirus – expressed soluble human FAP. We used soluble human FAP polyhistidine-tagged at the N- or C-terminus and purified by metal affinity chromatography (Figure 3). Enzyme activity on the substrate H-Ala-Pro-p-NA was comparable between N- and C- terminal polyhistidine-tagged FAP; the K_m was 0.86 mM, k_{cat} 2.8 sec^{-1} and specific activity 1.8 U/mg. With C- terminal tagged FAP we confirmed that Z-Gly-Pro-p-NA but neither Succinyl-Ala-Pro-p-NA nor Z-Ala-Pro-p-NA is hydrolysed by FAP. FAP hydrolysis on H-Ala-Pro-p-NA was about four times more than on Z-Gly-Pro-p-NA.

4.2.1. FAP and Plasmin

Tissue repair involves coagulation, which results in fibrin deposition. The fibrin of a clot is usually lysed, primarily by plasmin, which is converted from its inactive form, plasminogen, by plasminogen activators. Fibrinolysis is inhibited by plasminogen activator inhibitor-1, plasminogen activator inhibitor-2 and alpha2 antiplasmin, which are induced by tissue trauma. FAP converts alpha2 antiplasmin to a more active form, Asn-alpha$_2$antiplasmin (23) and associates with the uPA receptor annexin 2 (24). Thus, a selective FAP inhibitor might enhance wound healing and reduce adhesions via increased fibrinolysis. It will be interesting to investigate some peptide hormone or neuropeptide PEP substrates, such as alpha-melanocyte-stimulating hormone, substance P, angiotensin and bradykinin, as candidate substrates of FAP.

4.3. FAP expression

Controlling gelatinases is vital for organ structure. Unlike matrix metalloproteinases (MMPs), which have a proenzyme form, the gelatinase activity of FAP is constitutive. FAP and DPIV exhibit different patterns of expression and tissue distribution. FAP mRNA expression and immunohistochemistry in normal human tissues show definite signals in some organs, including breast tissue, cervix, endometrium, pancreas, placenta, and skin (25). However, intense FAP expression is generally only on activated fibroblasts and embryonic mesoderm at the sites of tissue remodelling, including resorbing tadpole tail (26), stromal fibroblasts of epithelial tumors (27, 28), healing wounds (27, 29), mesenchymal cells in the embryo and newborn foreskin fibroblasts (29). Recently FAP has been colocalized with MMP1, MMP13, CD44 and alpha–smooth muscle actin in rheumatoid arthritis synovium (30). *In vitro,* FAP expression includes some sarcoma and glioma cell lines, phorbol ester-stimulated melanocytes and cultured fibroblasts (22, 29, 31). FAP has also been

localised to the advancing portion (invadopodia) of cultured melanoma cells in conjunction with MMP2 (14, 32). FAP is in a subset of glucagon - producing A cells in pancreatic islets (27).

A soluble form of FAP has been isolated from normal bovine and human serum but, interestingly, despite the abundance of serum DPIV and the ability of DPIV and FAP to heterodimerise (28), serum FAP is homodimeric (23, 33). Many diseases have been associated with altered plasma levels of DPIV, including autoimmune disease, HIV infection, hepatitis C infection, cancer and liver cirrhosis (10). Similar studies of serum FAP would be interesting.

FAP is not detected in normal adult liver (22). However, FAP-positive cells are present in early stages of liver injury and FAP immunostaining intensity strongly correlates with the histological severity of fibrosis in chronic liver disease (34). FAP is notably near lipid accumulation, called steatosis, in liver. Its expression is only on myofibroblasts and activated HSC at sites of tissue remodelling, which is the portal-parenchymal interface of cirrhotic liver (22). Similarly, FAP is expressed by fibroblasts at the tissue remodelling interface in human pulmonary fibrosis (35). The FAP gene knockout (GKO) mouse has a normal phenotype for body weight, organ weights, histological examination of major organs and haematological analysis (36).

4.4. Roles of FAP in cancer

Fibroblasts, considered a key cellular component of tumors, have a prominent role in the progression, growth and spread of cancers. Normal stroma in most organs contains few fibroblasts in association with a physiological ECM. However reactive stroma is associated with many more fibroblasts, increased capillary density, and CN-I and fibrin deposition. In addition, activated fibroblasts/myofibroblasts, associated with cancer at all stages of tumor progression, are known as carcinoma-associated fibroblasts. Their production of growth factors, chemokines and ECM facilitates the angiogenic recruitment of endothelial cells and pericytes. They might facilitate angiogenesis and cancer progression by providing oncogenic signals to the transformed epithelia in a peregrine fashion and secreting and organising altered ECM within the tumor stroma (1). Therefore, fibroblasts are a key determinant in the malignant progression of cancer and represent an important target for cancer therapies.

FAP expression is associated with wound healing and malignant tumor growth and is strongly expressed by stromal fibroblasts in most epithelial cancers and in primary stromal cells from human non-small cell lung cancer (37), suggesting its potential role in cancer development. Cancer growth and metastasis involves cell adhesion and migration processes, as well as rapid degradation of ECM components. Peptide processing immediately outside the cell at the pericellular interface provides key control points for cell proliferation, adhesion and migration. Release of ECM-bound or shedding of membrane-bound factors can extend their effect from local to systemic (1). The CN-I specific gelatinase activity of FAP suggests FAP could play a significant role in degrading ECM proteins and therefore contribute to cancer development. Fibroblasts interact with their surrounding microenvironment through integrins.

4.4.1. FAP in cancer and ECM interactions

Reports of FAP as a marker for various cancers have provided the possibility of exploiting FAP as a target for cancer therapeutics (25, 38-41). FAP is upregulated in most human tumors. Interestingly, a large colon cancer study found that stromal cell FAP expression inversely correlated with tumor stage (41). A melanoma study has found FAP-positive fibroblasts in reactive stroma of all melanocytic nevi, with 30% having additional FAP on a melanocytic cell subset, but no FAP was detected in melanoma cells from primary or metastatic melanomas (31). FAP may be a useful marker for expression profiling of stromal fibroblasts at different stages of melanoma progression, particularly if FAP influences tumor cell growth and proliferation. Antibodies to human FAP have been identified that have potential for such clinical exploitation (42).

In vitro overexpression studies have provided a more detailed understanding of the effects of FAP on cancer cell behaviour. A stable FAP-transfected HEK293 cell line has exhibited a significantly greater incidence of tumor development and growth in SCID mice (43). FAP overexpression in melanoma cells can suppress a malignant phenotype in cancer cells, specifically cell cycle arrest at G0/G1 phase, increase susceptibility to stress-induced apoptosis, and restore contact inhibition (44). Similar to DPIV studies, overexpressing FAP abrogated tumorigenicity in nude mice and, surprisingly, enzymatically inactive FAP further abrogated tumorigenicity, demonstrating that enzyme activity is not essential for these effects. This study also linked FAP-induced apoptosis to caspase activation, and suggested that tissue remodeling processes that involve apoptosis may depend upon FAP expression (44).

Determining the contribution of FAP to metastasis, cancer progression and invasion is difficult. Studies in recent years indicate that FAP expression in cancer is complex and dependent on the cancer and tissue type. FAP can behave like a tumor suppressor in the development of melanoma (31, 44). However, its expression level correlates with breast cancer progression (45, 46). Therefore, the effect of FAP on tumor growth by influencing cell adhesion is unclear. It could help restrain cells at the initial tumor site, thus reducing their ability to spread and metastasize.

Like FAP, DPIV appears to act as a tumor promoter or suppressor in various studies. Recently, the roles of growth factors and chemokines in cancer have become better understood. For instance, the chemokine DPIV substrate stroma-derived factor (SDF-1/CXCL12) has multiple roles in tumor pathogenesis. CXCL12 promotes tumor growth and malignancy, enhances tumor angiogenesis, participates in tumor metastasis, and

contributes to immunosuppressive networks within the tumor microenvironment (47). This chemokine is potentially a natural substrate of FAP. Additionally, the cell surface expression of FAP and DPIV probably confers effects on cell behaviour via FN and integrin binding.

The gelatinase activity of FAP, specifically, collagenolytic activity towards CN-I suggests FAP could play a significant role in degrading many of the ECM proteins. In cirrhotic liver FAP co-localises with FN and CN, and with CN fibres alongside activated HSC (17, 48). In invadopodia, integrin $alpha_3beta_1$, a FN receptor, interacts with FAP when induced with CN (49). This may involve the DPIV-FAP heterodimer complex, which is known to exist in invadopodia (50). FAP is associated with integrin $alpha_3beta_1$. This indicates that FAP and ECM interactions may be closely related to cancer cell behaviour by influencing their adhesion, migration, proliferation and apoptosis possibly through an integrin pathway. We have found significant effects of overexpressed FAP on cell adhesion, migration, proliferation and apoptosis in epithelial and fibroblastic cell lines, which further support a role of FAP in cancer growth (17).

Thus, FAP may function coordinately to modulate the growth, differentiation, adhesion, and metastasis of tumor cells.

4.4.2. Role of FAP enzyme activity

Whether enzyme activity is required for the various roles of FAP is a complex question. In many cases, it is not required for either its tumor suppressor or promoter properties. In our hands, enzyme activity was not required for the *in vitro* effects of FAP on cell adhesion, migration, proliferation and apoptosis, suggesting that FAP most likely exerts these effects by interaction with other surface molecules (17). The interaction of $alpha_3beta_1$ integrin with FAP may contribute to this role as it helps to localise FAP at the ECM, thereby permitting cell invasion and migration.

Cells transfected with an enzyme negative mutant of FAP can abrogate tumorigenicity as effectively as the wild type FAP control (44). However, some studies support a contention that inhibition of the protease activity of FAP and DPIV through inhibitor therapy suppresses the growth and metastasis of tumor cells in some cancer types. Studies using the inhibitor Val-Boro-Pro, which inhibits DPIV, FAP, DP8, DP9 and DPII, in epithelial carcinoma cells (51), fibrosarcoma cells, lymphoma cells, melanoma cells and mastocytoma cells (52) have shown that inhibition of protease activity can promote anti-tumor effects of Rituximab and Trastuzumab therapies. Thus, particular emphasis should be placed on understanding the roles of each dipeptidyl peptidase in individual cancer types.

4.4.3. FAP as a therapeutic target for the treatment of cancer

Since FAP was identified 24 years ago as a putative tumor marker the use of FAP as a cancer diagnostic and/or therapeutic target has been investigated. FAP is induced in tumors, is not detected on most normal adult tissues, and is physically attached to tumor cells. These properties make it an appealing molecule to provide targeting specificity for delivering reagents designed to kill tumor cells. Inhibition of FAP protease activity promotes anti-tumor activity of the immune system (52), so inhibiting FAP protease activity is another appealing target for slowing tumor growth. Disruption of the signalling of FAP complexes with other surface molecules is another potential therapeutic target. Perhaps disrupting protein-protein interactions of FAP may be the more significant activity in the progression/suppression of cancer.

Further research into the biological roles of FAP in the progression of individual cancer types will aid in translating biological knowledge of FAP in cancer into the clinical setting.

5. LIVER CIRRHOSIS

Liver cirrhosis is a significant worldwide health problem for which no simple therapy exists. It often leads to hepatocellular carcinoma or liver failure. Hepatic failure due to cirrhosis is caused by a progressive fibrosis that ultimately results in nodular regeneration with loss of function. Even though liver cirrhosis can be initiated by chronic metabolic, autoimmune, postnecrotic, or toxic injury, all causes produce very similar end stage disease and involve similar end stage pathogenic pathways. Early fibrotic events lead to conversion of the basement membrane-like matrix into one rich in fibril-forming matrix such as CN-I, CN-III and FN (53).

5.1. Liver structure and the ECM

The structural unit of the liver is the hepatic lobule in which interconnecting plates of hepatocytes are arranged around a central vein. Portal triads are enclosed in a connective sheath and are comprised of a branch of the hepatic artery, a branch of the portal vein, a bile duct and a lymphatic vessel. The liver parenchyma is composed of several cell types: (a) hepatocytes, which is an epithelial component, (b) sinusoidal endothelial cells, (c) HSC, which are perisinusoidal mesenchymal cells, and (d) Kupffer cells, which are tissue macrophages. The space between the endothelium and the hepatocytes is known as the space of Disse, where HSC are located. The cellular elements of liver parenchyma are organised within and around the sinusoids, and the perisinusoidal space of Disse separates the hepatocytes from the sinusoidal endothelium.

The ECM is a complex network that provides structural support to connective tissue, regulates the biological functions of some molecules, such as integrins, serves as storage for a variety of growth factors, including transforming growth factor (TGF)-beta, hepatocyte growth factor (HGF), vascular endothelial growth factor, interleukin (IL)-3, tumor necrosis factor (TNF) -alpha and platelet-derived growth factor (PDGF), and provides cells with signals that direct polarization, adhesion, migration, proliferation, survival and differentiation. The composition of the ECM can be broadly subdivided into the following groups: collagens, divided into fibrillar (type I, III, V and XI) and non-fibrillar CN (such as type IV, VI, VII, VIII and

X); structural glycoproteins, such as FN and laminin and proteoglycans, such as heparin and decorin (54).

In normal liver, the space of Disse contains small amounts of ECM that can be subdivided into the pericellular matrix in direct contact with the cells, the basal lamina-like material that forms a support for the endothelium and the stellate cell processes, and the interstitial collagen bundles that reinforce the architecture of the space of Disse.

The three cell types in the space of Disse, hepatocytes, endothelial cells and HSC, all express ECM components. Quiescent HSC appear to express mainly CN-III, IV and laminin, whereas endothelial cells express mainly CN-IV, and hepatocytes express mainly FN. These three quiescent cell types express only small amounts of CN-I. The communication between cells and ECM is mediated by integrins that include $alpha_1beta_1$, $alpha_2beta_1$ and $alpha_3beta_1$ integrin, through the connection of ECM to the actin cytoskeleton of the cells. The synthesis and accumulation of the ECM is tightly regulated by matrix metalloproteinases (MMP). MMP activity is in turn monitored by tissue inhibitors of matrix metalloproteinases (TIMP). This coordination is critical to ensure appropriate organ homeostasis (55).

5.2. ECM in liver injury

After a single insult, this ECM ecosystem can completely reconstitute itself in a process resembling physiological wound healing, proceeding via formatting of early granulation tissue that contains, for example, FN, vitronectin, tenascin-C, and CN-III, CN-IV, CN-V, and CN-VI, to deposition of a mature ECM that contains larger amounts of, for example, CN-I, CN-XIV, and decorin. Fibrillar collagens (type I, III and IV), especially CN-I, are the most abundant proteins associated with fibrosis. In chronic liver diseases, this ECM deposition eventually disrupts regular wound healing and leads to progressive scarring. *In vivo,* myofibroblasts are stress-activated cells responsible for rapid wound closure, generating a contractile force and filling the defect with ECM (56). As the liver becomes fibrotic, significant qualitative changes of the ECM occur, predominantly in the periportal and perisinusoidal space, while the total content of CN and non-CN proteins increases up to tenfold. This abnormal, predominantly interstitial perisinusoidal matrix compromises hepatocyte function and leads to activation of more HSC and myofibroblasts.

5.2.1. ECM Regulation

In most normal tissues there is some turnover and remodelling of ECM, with an overall balance between the rates of matrix synthesis and degradation. Regulation of ECM is accomplished, in part, by MMPs, a diverse family of zinc- and calcium-dependent endopeptidases involved in degrading a variety of ECM proteins. For instance, the interstitial collagenases (MMP1, 8 and 13) degrade CN-I, II and III, while the gelatinases (MMP2 and 9) digest denatured CN, along with CN-IV, V and VII (57, 58). The MMP expression levels are transcriptionally regulated by cytokine and growth factor signalling, including TGF-beta, IL-1, IL-4, fibroblast growth factor (FGF), epidermal growth factor, connective tissue growth factor (CTGF) (59) and insulin-like growth factor (60). MMP regulation relies upon TIMPs and the matrix itself via integrin-linked pathways.

5.3. HSC

HSC, also known as Ito cells, vitamin A-storing cells, fat-storing cells, interstitial cells or lipocytes, were first described in 1876. HSC represent 10 to 15% of liver cells. HSC can be derived from the neural crest since HSC express glial fibrillary acidic protein and nestin, and that neural crest stem cells can differentiate into myofibroblasts expressing alpha–smooth muscle actin (61). The localisation and long cytoplasmic processes of HSC promote interaction with neighbouring cells. HSC have direct contact with endothelial cells and hepatocytes.

HSC is distinguished from other liver cell types by its unique marker and tissue distribution pattern. Three neurotrophin receptors (p75, Trk-B and Trk-C) and neural-cell adhesion molecule have been found expressed on both quiescent and activated HSC of human and rat tissue (61, 62). Five membrane proteins: PDGF receptor subunits alpha and beta, insulin-like growth factor -II/ mannose-6-phosphate receptor, the cellular prion protein and FAP are present on activated HSC. Activated HSC express several integrin $beta_1$-associated alpha subunits, particularly $alpha_1beta_1$ and $alpha_2beta_1$. Some quiescent HSC in rat and human liver contain synaptophysin, HGF and tenascin. Many HSC express laminin and desmin. The neurotrophins nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4/5 are expressed in subsets of both quiescent and activated rat and human HSC (62).

The distribution of HSC in the liver is heterogenous. In rodents, the extent of arborisation of cytoplasmic processes, vitamin A storage, desmin content, and glial fibrillary acidic protein content are dependent on the location within the lobule (63). In the portal tracts, HSC contain few, small lipid droplets while in periportal areas HSC contain more and larger lipid droplets.

5.3.1. HSC activation

Liver injury is associated with the activation of HSC. HSC undergo a process of activation towards a phenotype characterised by increased proliferation, motility, contractility, and synthesis of ECM components. In the final and common pathway of the wound healing response of the liver, resident HSC within the space of Disse migrate to areas of inflammation in increased numbers and transdifferentiate to myofibroblasts with the expression of CN-I and CN-III. Activated HSC and myofibroblasts express a wide range of ECM molecules, and their synthetic capacity largely determines the surrounding fibrotic matrix both quantitatively and qualitatively. Activated HSC and myofibroblasts also secrete most of the MMP and TIMP. The final result of this process is that any liver injury that results in HSC activation, particularly if chronic, leads to an increase in overall numbers of myofibroblast-like activated HSC that are actively producing matrix, while simultaneously

preventing degradation of the matrix through expression of TIMP1 and TIMP2. Myofibroblasts have multiple origins including HSC, fibroblasts, epithelial cells and bone marrow cells (64-66). Myofibroblasts can derive from bile duct cells or hepatocytes by epithelial-mesenchymal transition (EMT) (67, 68). EMT, HSC transdifferentiation and FAP expression are all driven by TGF-beta1.

5.3.2. Regulation of HSC activation

HSC activation and proliferation is regulated by several soluble factors, including growth factors, cytokines, chemokines, and products of oxidative stress (69). These soluble factors, that are potentially released in adjacent areas of injury by inflammatory cells, activated HSC and hepatocytes, induce resident HSC to migrate into the areas with the major structural constituent of fibrotic ECM, CN-I, and also induce HSC to invade the fibrillar matrix, which is capable of further perpetuating HSC activation (70). These different factors can be grouped according to their class of receptors (69): (a) factors promoting HSC proliferation, migration and survival: PDGF, CTGF, FGF; (b) factors promoting fibrillar ECM accumulation: TGF-beta1; (c) factors with a prevalent contractile effect on HSC: ET-1, thrombin, angiotensin-II and vasopressin; (d) proinflammatory cytokines and chemokines; (e) cytokines with a prominent anti-inflammatory/ anti-fibrogenic activity: IL-10 and INF-γ.

TGF-beta1 is a multifunctional cytokine having many roles, including tissue response to injury in parenchymal organs such as the kidney and liver (71, 72). The role of TGF-beta1 in the liver is of particular interest, because this cytokine is believed to be the principal contributor to fibrosis (71, 73). TGF-beta1 overexpression leads to increased matrix deposition. Nearly all liver cell types produce TGF-beta1 but HSC is the major source (73).

PDGF is the most potent mitogenic cytokine for HSC. Expression of both PDGF and its receptor (PDGF-R) are upregulated during HSC activation. PDGF stimulates autocrine growth, chemotaxis and loss of retinoids. Similarly, CTGF has been shown to induce cell migration, proliferation and adhesion of HSC and increase expression of CN-I (74). Peroxisome proliferator-activated receptor gamma (PPAR-gamma) is markedly reduced in culture-activated human and rat HSC. PPAR-gamma ligands suppress DNA synthesis, CN production, alpha–smooth muscle actin expression and PDGF-induced HSC migration (75).

In liver fibrosis the ECM becomes unbalanced leading to a continuous wound healing process and ultimately scar formation. CN-I and FN can serve as chemoattractant stimuli for HSC (70). Activities of HSC can be regulated by integrins, including integrins $alpha_1beta_1$, $alpha_2beta_1$, $alpha_vbeta_1$ and $alpha_6beta_4$ (69).

5.3.3. HSC and liver inflammation

HSC have an active role in hepatic inflammation (76). Activated HSC migrate in response to cytokines and secrete a number of proinflammatory cytokines and chemokines (69, 76). Toll-like receptor 4, CD14 and myeloid differentiation protein have been shown to mediate inflammation with up-regulated expression during HSC activation (77). Importantly, activated HSC express CD40, a receptor whose cognate ligand is present on immune effector cells, thereby providing a direct mechanistic link between inflammatory and fibrogenic cells (78).

5.3.4. HSC apoptosis

Apoptosis governs the controlled loss of cells in physiological and pathological situations. The complex relationship between HSC apoptosis and fibrosis is critical to understanding the pathogenesis of cirrhosis. HSC not only proliferate, they also transdifferentiate into myofibroblasts. Myofibroblasts become responsive to apoptotic stimuli, including the death receptor Fas and tumor necrosis factor-related-apoptosis-inducing-ligand receptor-2 (79). As liver injury resolves, the number of activated HSC decreases mainly through apoptosis (65). HSC apoptosis results in decreased TIMP1 and TIMP2 and facilitates degradation of fibrotic matrix by MMP (55). Some growth factors such as nerve growth factor induce apoptosis in HSC.

On the other hand, several survival factors such as TIMP play an important role in preventing HSC apoptosis. Persistent *in vivo* expression of TIMP1 has been associated with persistence of activated HSC. Its anti-apoptotic effect on HSC is independent of its MMP inhibiting ability (80). TNF-alpha and TGF-beta, both of which are released in liver injury, promote HSC survival, as does $alpha_vbeta_3$ integrin (81).

6. FAP IN LIVER FIBROSIS

Liver fibrosis is a wound-healing response with significant functional changes, including large alterations in ECM production (70). HSC play a central role in the pathogenesis of fibrosis. During fibrosis, HSC acquire an activated phenotype, which includes increased proliferation, contractility, fibrogenesis, matrix degradation, chemotaxis, and cytokine release. FAP is only expressed on activated HSC in the tissue remodelling area, tightly colocalised with fibrillar matrix, CN-I and FN (17, 48). In addition, FAP overexpression enhances HSC adhesion, migration, proliferation and apoptosis on fibrillar ECM substrata *in vitro* (17). These data suggest that FAP has a critical role in liver fibrosis, most likely by influencing the functions of activated HSC. FAP is co-localised with CN fibres and FN in human cirrhotic liver (17). FAP and DPIV influence apoptosis, proliferation, cell adhesion, *in vitro* wound healing and cell migration on ECM substrata. Most notably, FAP overexpression by the LX-2 human HSC cell line increased cell adhesion and migration on CN and FN (17). Thus, FAP might act by interacting with ECM and/or regulating HSC migration, proliferation and apoptosis.

Interestingly, FAP expression is stimulated by TGFbeta and retinoic acid (29), which also stimulate HSC and myofibroblasts. Moreover, TGFbeta1 is the major stimulus for EMT, which is a major contributor of myofibroblasts in chronic liver injury (64, 67, 68). It would

be useful to establish whether FAP is a marker of all myofibroblasts of whichever origin.

Besides the possible effects of FAP on HSC, FAP and DPIV could contribute to liver fibrosis by modulating chemokines. Recently, serine and metalloproteinases, the two largest classes of proteases, have been recognised to orchestrate the delivery of signals crucial to cell division and to bring about structural ECM remodelling in liver regeneration (82). Similarly, many growth factors and cytokines are thought crucial for liver fibrosis by interacting with ECM in the hepatic environment including vascular endothelial growth factor, HGF, FGF and TGFbeta. Many of them require proteolytic processing for activity. Many chemokines are DPIV substrates and so potentially are substrates of FAP. More importantly, unlike DPIV, FAP is capable of cleaving large substrates such as type I CN and alpha2-antiplasmin, so other large proteins including growth factors and cytokines could be cleaved by FAP. Furthermore, FAP is involved in fibrinolysis. Thus, FAP deficiency might enhance wound healing and reduce adhesion via increased fibrinolysis.

It is known that fibroblasts play a significant role in attracting and retaining inflammatory leucocytes within sites of inflammation through their production of cytokines, chemokines, and other biologically active factors (37). FAP may have some effects on these cells by modulating their soluble factor secretion and activities to influence leucocyte movement.

6.1. FAP as a therapeutic target for the treatment of liver fibrosis

The understanding of the cellular and molecular mechanisms underlying liver fibrogenesis has dramatically advanced in the last decade. The discovery of activated HSC as the major fibrogenic cell type in the injured liver has facilitated the design of promising new anti-fibrotic therapies. These therapies are aimed at inhibiting the accumulation of activated HSC at the sites of liver injury and preventing the deposition of ECM. The ideal anti-fibrotic therapy would be one that is liver specific, well tolerated, and effective in attenuating excessive CN deposition without affecting normal ECM synthesis. Furthermore, HSC also play an active role in hepatic inflammation. The development of liver fibrosis in most human liver diseases is preceded by chronic inflammation of the hepatic parenchyma, so treatments inhibiting liver inflammation may also attenuate the progression of liver fibrosis. Therefore, the use of substances with combined anti-fibrotic and anti-inflammatory effects should be considered in anti-fibrotic therapy.

As a therapeutic target FAP has several favourable features that cause it to be considered. Firstly, in liver FAP is only expressed on activated HSC, but not in normal adult liver. Therefore, it specifically targets activated HSC, but not quiescent HSC. Secondly, it is dominantly expressed in areas of tissue remodelling, which are regions with active fibrogenesis.

7. CONCLUSION

Liver fibrosis is a multi-genic process. FAP is a multifunctional protein and has pro-inflammatory and pro-fibrotic roles in liver injury, as well as potential roles in energy and lipid metabolism. There may be several mechanisms by which FAP has an impact on liver fibrosis. Most likely, FAP executes its biological functions in a cell-context dependent manner through a combination of its protease activity and its ability to form complexes with other cell-surface molecules on HSC and therefore influence fibrotic processes. Studies on tissue remodelling models of FAP GKO mice and on the FAP signalling pathway may help to further elucidate its roles in ECM interactions, liver fibrosis and cancer.

The natural substrates of FAP are unknown. Identification of the natural substrates of FAP and the functions of FAP-hydrolysed peptides remains an important area to be investigated. Purified FAP protein could be used to discover natural substrates, ligands and selective inhibitors of FAP. The narrow endopeptidase activity of FAP is useful for designing a selective inhibitor for FAP. Specific areas for *in vivo* testing of FAP-selective inhibitors will include not only liver and lung fibrosis but also other biological processes in which FAP may act, such as cancer, haematopoiesis, arthritis and diabetes.

FAP and DPIV may function co-ordinately to regulate pathological processes and both enzymes are appealing targets for therapeutics designed to inhibit liver fibrosis and cancer. The roles of FAP protease activity and FAP complex formation with DPIV and other cell surface molecules in activating cell signalling need to be elucidated since these represent potential targets for therapeutic intervention.

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Enzymes: fibroblast activation protein GenBank U09278; dipeptidyl peptidase IV GenBank P27487, EC 3.4.14.5; prolyl endopeptidase GenBank P48147, EC 3.4.21.2.

Abbreviations: CN: collagen; CTGF: connective tissue growth factor; DP: dipeptidyl peptidase; ECM: extracellular matrix; FAP: fibroblast activation protein; EMT: epithelial-mesenchymal transition; FGF: fibroblast growth factor; FN: fibronectin; GKO: gene knockout; GLP: glucagon-like peptide; HGF: hepatocyte growth factor; HSC: hepatic stellate cell; IL: interleukin; MAb: monoclonal antibody; MMP: matrix metalloproteinase; NPY: neuropeptide Y; PDGF: platelet-derived growth factor; p-NA: p-nitroanilide; PEP: prolyl endopeptidase; POP: prolyl oligopeptidase; PPAR: peroxisome proliferator-activated receptor; TGF: transforming growth

factor; TIMP: tissue inhibitor of MMP; TNF: tumor necrosis factor.

Key Words: Fibroblast Activation Protein, CD26, Serine Proteinase, Chemokine, Cancer, Beta Propeller, Hydrolase, Aminopeptidase, Fibroblast Activation Protein; Dipeptidyl Peptidase, Prolyl Oligopeptidase, Review

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