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Quantitation of fibroblast activation protein (FAP)-specific protease activity in mouse, baboon and human fluids and organs☆

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ABSTRACT

The protease fibroblast activation protein (FAP) is a specific marker of activated mesenchymal cells in tumour stroma and fibrotic liver. A specific, reliable FAP enzyme assay has been lacking. FAP's unique and restricted cleavage of the post proline bond was exploited to generate a new specific substrate to quantify FAP enzyme activity. This sensitive assay detected no FAP activity in any tissue or fluid of FAP gene knockout mice, thus confirming assay specificity. Circulating FAP activity was ~20- and 1.3-fold less in baboon than in mouse and human plasma, respectively. Serum and plasma contained comparable FAP activity. In mice, the highest levels of FAP activity were in uterus, pancreas, submaxillary gland and skin, whereas the lowest levels were in brain, prostate, leukocytes and testis. Baboon organs high in FAP activity included skin, epididymis, bladder, colon, adipose tissue, nerve and tongue. FAP activity was greatly elevated in tumours and associated lymph nodes and in fungal-infected skin of unhealthy baboons. FAP activity was 14- to 18-fold greater in cirrhotic than in non-diseased human liver, and circulating FAP activity was almost doubled in alcoholic cirrhosis. Parallel DPP4 measurements concorded with the literature, except for the novel finding of high DPP4 activity in bile. The new FAP enzyme assay is the first to be thoroughly characterised and shows that FAP activity is measurable in most organs and at high levels in some. This new assay is a robust tool for specific quantitation of FAP enzyme activity in both preclinical and clinical samples, particularly liver fibrosis.

Abbreviations: ALD, alcoholic liver disease; AMC, amino-4-methylcoumarin; DPP4, dipeptidyl peptidase 4; DMSO, dimethyl sulfoxide; EDTA, ethylene diamine tetra acetic acid; FAP, fibroblast activation protein-α; gko, gene knock out; HCV, hepatitis C virus; het, heterozygous; LDS, lithium dodecyl sulphate; LN, lymph node; mAb, monoclonal antibody; ND, non-diseased; PBC, primary biliary cirrhosis; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PEP, prolyl endopeptidase; PVDF, polyvinylidene fluoride; STLV, simian T-cell lymphotrophic virus; wt, wild type; yrs, years.

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1. Introduction

Proteases are increasingly recognised as important regulatory molecules [1]. Fibroblast activation protein (FAP) belongs to the S9 family of proteases, which also contains the similar enzymes dipeptidyl peptidase 4 (DPP4), DPP8, DPP9 and prolyl endopeptidase (PEP) [2]. All of these enzymes share the unique ability to cleave the post proline bond, which is usually resistant to degradation. Recently, this enzyme family has stimulated great pharmaceutical interest, as DPP4 inhibitors are a successful therapy for type 2 diabetes [3] and have the potential to treat other conditions [4,5].

FAP is a constitutively active serine protease that exists as a dimer both on the cell surface and in a soluble, circulating form in the blood. FAP can hydrolyse both dipeptidase and endopeptidase substrates, which include natural X-Pro-containing bioactive peptides [6] and denatured collagen [7,8] and α2-antiplasmin [9,10]. It is thought that FAP is generally absent from normal adult tissue but has increased expression during embryogenesis [11], tumourigenesis [12–14], tissue damage and wound healing, fibrosis [7,15] and inflammation [16,17]. As FAP is up-regulated in stromal fibroblasts of over 90% of malignant epithelial tumours but not in benign tumours [18], it has become a potential biomarker and therapeutic target for tumour stroma [19–21].

DPP4 is related transmembrane dimeric glycoprotein that cleaves the post proline bond but acts only as a dipeptidyl peptidase. It is a ubiquitous enzyme, found on most epithelial cells, especially in liver, kidney and gut, on capillary endothelial cells in most organs and on most lymphocytes in immune organs such as thymus, spleen and lymph node [22–25]. In contrast to FAP, many natural DPP4 substrates are known, including gastric hormones [26], neuropeptides [27,28], and chemokines [29,30]. Soluble DPP4 is present in plasma, serum, seminal and synovial fluids, as has been reviewed [22,31] and soluble DPP4 levels are associated with a variety of human conditions such as psoriasis [32], chronic fatigue [33], tuberculosis [34] and hepatitis C virus (HCV) [35,36]. Circulating DPP4 activity is elevated in some tumours [37,38] but reduced in others [39–41], so the level may be tumour-type dependent. It has also been reported to be both increased [42,43] and decreased [44,45] in type 2 diabetes patients. As with FAP, the regulatory process or sheddase activity by which the soluble form of the enzyme is released from the cell is unknown.

In contrast to DPP4, little is known about the normal physiological function of either cellular or circulating FAP. Specifically identifying both the source and activity of soluble FAP has been difficult due to its dual enzyme activity, and to date all FAP synthetic substrates are also hydrolysed by DPP4 and/or PEP [46]. Up to now, analysis of FAP content in tissue samples has relied on mRNA measurements [47,48] or the use of antibodies, where few are reliable for mouse FAP [49]. FAP has been targeted in cancer models using chemical inhibitors [50], antibodies [51], toxins [52], pro-drugs [19], T-cells [53] and RNA interference [54], but basal, endogenous FAP expression has only recently been examined in normal tissue [55,56] and this has challenged the dogma that FAP is only expressed in diseased tissue. Thus, FAP's basal expression pattern and normal physiological activity profile require close examination to better understand the role of this protease.

Our hypothesis is that FAP is present and active in measureable amounts in some normal tissues and the aim of the present study was to utilise a new FAP-specific substrate, 3144-AMC, to quantify FAP expression in mouse, baboon and human fluids and tissues. We sought to show that this assay is specific, sensitive and efficient at quantifying both the soluble and cell-bound forms of FAP from a range of tissues in three different mammalian species. The poor availability of fresh human tissue was overcome by obtaining an extensive range of fresh tissues from a closely-related primate, the baboon. We also quantified FAP activity in diseased baboon and human tissue and plasma as a preliminary exploration of its use as a biomarker, while DPP4 activity was measured as a comparator. FAP is an intriguing protein and the heavy focus on targeting it in cancer therapy needs to be reconciled with its normal physiological function, which is not fully understood. Here we delineate locations and relative quantities of FAP enzyme activity in a wide range of settings to develop an understanding of the roles of this unique enzyme.

2. Results

FAP is present in a soluble form in human plasma [10]. A novel FAP substrate, 3144-AMC [57], was used in this study to quantify FAP activity in non-diseased plasma from three species. During optimisation of this soluble assay we found that the greatest FAP activity came from volumes of mouse, baboon or human plasma of 0.5–1 μl (Fig. 1A–C), so 1 μl was used for all subsequent assays. Clear inverse dose responses were produced by plasma volumes that were greater than optimal. All samples tested for enzyme activity in mouse, baboon and humans are listed in Supplemental Tables 1–3, respectively.

The second aim was to verify the specificity of substrate 3144-AMC for FAP, using FAP-deficient samples including plasma from FAP gene knockout (gko) mice [58] and plasma from humans carrying a FAP gene variant [59]. Similar quantities of FAP activity were detected in wt and DPP4 gko mouse plasma (Fig. 2A), which is consistent with our previous data showing no compensatory change in FAP expression in liver when DPP4 is absent [60]. In contrast, FAP gko mouse plasma had no detectable activity while heterozygous (het) mice had reduced FAP enzymatic levels (Fig. 2A). We separately identified FAP-deficient individuals expressing a Ser363Leu variant FAP protein that lacks functional FAP activity [59]. Plasma FAP activity levels from the heterozygous person (FAP+/−) were less than half that of healthy volunteers, whereas neither of the two FAP Ser363Leu homozygous individuals (FAP−/−) had detectable FAP activity (Fig. 2B). The four points on the graph for the mutation-affected individuals are from four separate blood draws over a 3-h period post glucose bolus ingestion. All FAP activity data for mouse, baboon and human fluids is given in Supplemental Table 4.

3144-AMC was then used to assess FAP activity in other primate fluids. Enzymatic levels in baboon plasma and serum were approximately 300 pmol AMC/min/ml but no detectable FAP activity was present in baboon urine or bile from three animals (Fig. 3A), despite various volumes of both fluids (0.1–50 μl) being tested (data not shown). Although there was ~2-fold variation between individuals, humans had greater average FAP activity than baboons. Interestingly, it was found that FAP activity levels were equal between plasma and serum in both baboons and humans (Fig. 3A), indicating that the presence of platelets, fibrinogen and other clotting factors do not affect this FAP assay. Subsequent FAP graphs contain average data for plasma and serum if both were analysed from one individual. The activity levels of DPP4 in these samples were then measured using the fluorogenic substrate H-Gly-Pro-AMC. Baboon plasma and serum had DPP4 activity of ~2785 pmol AMC/min/ml and human plasma and serum had similar levels to baboon (Fig. 3B). Baboon urine had barely detectable DPP4 activity, whereas bile had high levels. Further examination of bile from three species showed that mice had higher levels of bile DPP4 activity than a mixed cohort of baboons, with human bile from liver transplant recipients having the least DPP4 activity (one-way ANOVA p-value = 0.0024; Fig. 3C). Both soluble FAP and DPP4 quantitation was unaffected by 12 freeze/thaw cycles of plasma (Supplemental Fig. 1). All DPP4 activity data for mouse, baboon and human fluids is given in Supplemental Table 5.

A direct comparison shows that circulating FAP activity levels differ between species, with wt mice having approximately 19- and 15-fold greater FAP activity than baboons and humans, respectively (Fig. 4A). Baboon and human plasma, with average FAP activity levels of 315 and 404 pmol AMC/min/ml, respectively, are comparable. Total plasma protein quantitation in all three species was in line with