Institute of Experimental Morphology, Pathology and Anthropology with Museum
Bulgarian Anatomical Society

Acta morphologica et anthropologica, 22
Sofia ● 2015

Fibroblast Activation Protein α and Its Role in Cancer with a Focus on Breast Carcinoma: Review

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Fibroblast activation protein α (FAP-α) is a plasma membrane serine proteinase belonging to the S9b family of post-proline cleaving proteases. The enzyme is generally missing in normal tissues of adult humans and mammals but is up-regulated in the reactive stromal fibroblasts in tumors of epithelial origin and many types of sarcomas. For that reason, at present FAP-α is considered as an important marker molecule in oncology. However, the relationship between elevated enzyme activity/expression and prognosis is not always conclusive. Thus, in breast cancer, the connection between higher expression levels of FAP-α and prognosis varies from better to very poor in different studies. Obviously, despite the extensive studies by many research teams all over the world, the enzyme role in cancer is not elucidated yet. The aim of the present mini-review is to summarize the existing data about the role of FAP-α in cancer by focusing on its involvement in breast cancer.

Key words: fibroblast activation protein α, breast cancer, enzyme marker, Ehrlich ascites carcinoma, *in vivo* model.

General information about FAP-α

Fibroblast activation protein-α (FAP-α; EC 3.4.21.B28) is a serine type integral membrane protease belonging to the S9b family of post-proline cleaving enzymes. FAP-α is also known as Seprase (from ***S***urface ***E***xpressed ***PR***ote***ASE***). Its soluble form, which enters the blood plasma after shedding from cell surface [3], has been recently recognized to coincide with **A**nti**P**lasmin **C**leaving **E**nzyme (APCE) [22]. The active enzyme is a 170 kDa homodimer composed of two 97 kDa glycoprotein subunits [32].

FAP-α is a highly conservative protein, e.g. the mouse enzyme has 89% similarity to its human analogue [27]. Additionally, human and murine FAP-α genes show very similar genomic organization [28]. On the other hand, FAP-α is the most closely related to dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) which is the best studied member of S9b family. They share 50% sequence identity in the entire sequence and 70% identity in the catalytic domain [2] – a fact which impedes the design of specific substrates and/

or inhibitors for the two enzymes. Amino acids of the catalytic triads of FAP-α and DPPIV are arranged in one and the same manner and have very close positions: Ser624, Asp702, His734 for FAP-α and Ser630, Asp708, His740 for DPPIV [reviewed in 9]. The human FAP-α gene is located on chromosome 2q23 and is organized similarly to the DPPIV gene which maps to chromosome 2q24.3. For this reason, some authors believe that the two enzymes have a common origin, one arising by duplication of the other's gene [25]. Comparison of the crystal structures of FAP-α and DPPIV reveals that the major difference in the active sites is that FAP-α possesses Ala657 instead of Asp663 in DPPIV. This variation proves to be enough to lessen the acidity and increase the size of the active center pocket, thus making FAP-α capable of endopeptidase activity [26]. Substrates [21] and inhibitors [6] for FAP-α have been developed that utilize the above difference and are selective for the enzymes.

FAP-α is well known to possess a gelatinase activity; one of its most characteristic substrates is collagen type I [31]. The endopeptidase activity of soluble FAP-α cleaves α2-antiplasmin [22], which is involved in blood clotting. Amongst the most recently found enzyme's natural substrates are neuropeptide Y, B-type natriuretic peptide, substance P and peptide YY [14].

Tissue distribution of FAP-α

FAP-α is known to have a distinctive tissue distribution since it is usually absent from normal adult tissues as revealed by immunohistochemical analyses using specific monoclonal antibodies [7]. *In silico* electronic northern blot analysis also shows that normal tissues generally lack FAP-α mRNA expression [5]. In normal human and mammalian tissues, the enzyme activity is restricted to single reactive fibroblasts, glucagon producing A-cells in pancreatic islets and separated endometrial cells [reviewed in 38]. Comparatively high enzyme levels are found in mesenchymal cells during embryogenesis. However, FAP-α knockout mice have a normal phenotype in histological and hematological analysis [29]. Therefore, other compensatory pathways probably exists involving molecules with functional similarity to FAP-α, such as DPPIV or other S9b family members or matrix metalloproteases (MMPs).

Alternatively, FAP-α expression is highly induced during different pathological processes. For example, the enzyme is up-regulated in inflammatory diseases like rheumatoid arthritis and osteoarthritis, where it is found in fibroblast-like synovial cells. Also, FAP-α is expressed by activated but not by resting hepatic stellate cells of cirrhotic liver. Additionally, the enzyme is highly expressed by fibroblasts at the remodeling interface in human idiopathic pulmonary fibrosis as well as in liver fibrosis [reviewed in 38].

The most characteristic feature of FAP-α, however, is that it is up-regulated in stromal fibroblasts of over 90% of human epithelial tumors and in lots of sarcomas, but not in benign tumors [reviewed in 9 and 18]. Although the enzyme name "FAP" implies that it is to be found only on reactive stromal fibroblasts, lots of studies show that this is not the case. In fact, FAP-α has been identified in many tumor cells like melanoma cells, human colorectal cancer cells, breast cancer cells, human gastric and intestinal carcinomas, etc. [18]. Moreover, in certain types of carcinomas, the enzyme has not been restricted to plasma membrane, but intracellular cytoplasmic pools have also been discovered.

The dual role of FAP-α in cancer

FAP-α as a tumor promoter

Epithelial cancers (carcinomas), just like other solid tumors, induce the formation of abundant stromal compartments which, in some cases, may comprise more than 50% of the mass of the tumor. Tumor stroma consists of newly formed blood vessels, connective tissue cells such as activated fibroblasts or cancer-associated fibroblasts (CAFs), infiltrating inflammatory cells and a network of matrix proteins. Resting fibroblasts of normal adult tissues usually do not synthesize proteins neither expresses FAP-α. On the contrary, CAFs are known to synthesize and secrete proteins which promote cancer cells' invasion and angiogenesis and regulate the immune response to the tumor growth. Additionally, a common characteristic of CAFs in almost all types of carcinomas and lots of sarcomas is that they express FAP-α. It has been shown that lack of FAP-α in the CAFs of lung tumors in FAP-α knockout mice causes a dramatically increased accumulation of type I collagen around tumor cells which impairs cell motility, tumor growth and angiogenesis [34]. These experiments are indicative for the important role of the enzyme in tumor progression.

Furthermore, FAP-α molecule of CAFs, endothelial cells of tumor blood vessels and cancer cells is situated in the invadopodia – membrane protrusions that contact and degrade extracellular matrix (ECM) – together with other proteolytic enzymes, mainly MMPs [15]. More recent works have shown that heterodimeric complexes FAP-α–DPPIV are formed in the invadopodia, which are able of hydrolyzing collagen type I and are responsible for the invasive phenotype of CAFs and cancer cells [19].

According to the recent studies [23], FAP-α expression by fibroblasts results in alterations of organization and composition of the ECM that favors invasion. Thus, the matrix produced by the FAP-α-positive cells promotes tumor cell motility and directs migration.

FAP-α has been shown to be highly expressed in endothelial cells of newly forming tumor blood vessels in different types of cancers [reviewed in 18]. Taken together with the enzyme ability to cleave off collagen type I, this fact is in favor of FAP-α supposed role in promoting angiogenesis. In fact, in an *in vivo* mouse model of human breast cancer expressing FAP-α, a much higher micro-vessel density has been reported in comparison to the same experiment but with human breast carcinoma cells with low levels of the enzyme [11]. Moreover, the lack of FAP-α-positive CAFs in FAP-α knockout mice carrying lung tumors results in a significant reduction of micro-vessel density in the tumor stroma [34]. All these findings confirm the involvement of FAP-α in the mechanisms underlying angiogenesis in solid tumors.

Various studies have shown that up-regulation of FAP-α in epithelial tumors is one of the main factors responsible for the inhibition of antitumor activity by the immune system. Thus, application of DNA vaccine to FAP-α which causes CD8+ T cells killing of FAP-α-positive CAFs has been shown to improve antitumor immune function and increase sensitivity of tumor cells to chemotherapeutics [24]. In another experiment with mice carrying lung or pancreatic carcinoma cells, the ablation of FAP-α expressing cells has led to a rapid necrosis of both cancer and stromal cells [20]. Hence, FAP-α seems to insert some kind of protective effect on tumor and stromal cells against the immune response.

FAP-α as a Tumor Suppressor

Certain results of experimental research have shown that in some types of tumors FAP-α may play a role as a tumor suppressor. In that respect, particularly convincing

are the recent studies on mouse melanoma cells and on human non-small cells lung carcinoma (NSCLC) cells [36, 37]. Initially, it has been shown that the lack of DPPIV activity in the two types of cells is directly linked to the tumor phenotype. Restoration of DPPIV activity by virus vectors in both cases leads to a re-expression of FAP-α on the cell surface, followed by a profound suppression of the tumor phenotype and return to the dependence of the cell growth on exogenous growth factors. Moreover, the re-establishment of dependence on growth factors occurred even when a catalytically inactive mutant of DPPIV was expressed, which according to the authors may be due to the re-expression of endogenous FAP-α molecule. Other studies have provided direct evidence of FAP-α as a tumor suppressor at least in the case of malignant melanomas [33]. It has been observed that FAP-α re-expression resulted in decreased tumorogenicity of mouse melanoma cells and restored contact inhibition and growth factor dependence.

From the above-mentioned data it is obvious that a significant discrepancy exists between FAP-α function as tumor promoter or tumor suppressor. Thus, the role of FAP-α in cancer either depends on the tissue origin of the cancer cells or the mechanisms of the enzyme involvement in tumor diseases are much more complex and not well understood thus far.

FAP-α in breast cancer

FAP-α was first discovered in the mid-1980s as the protein binding the mouse monoclonal antibody F19 that labeled the reactive stromal fibroblasts of epithelial tumors, fibroblasts in fetal mesenchymal tissues and tumor cells of sarcomas. In one of the pioneer studies on FAP-α, using F19 antibody the enzyme was localized immunohistochemically in tissue sections of frozen malignant and benign breast tumors of 27 patients [7]. In this experiment, only reactive fibroblasts of malignant breast carcinomas were found to be FAP-α-positive, whereas stromal fibroblasts of the benign tumors as well as tumor epithelial cells were negative for the enzyme. After this work, many studies on FAP-α and its role in breast cancer have been carried out, however, with dissimilar results. For example, using the above antibody (F19) and three other clones recognizing monomeric or dimeric enzyme, retrospective immunohistochemical analyses were performed on tissue sections from invasive ductal carcinoma (IDC) of the breast of female patients who underwent mastectomy [1]. According to the authors, FAP-α was co-localized with type I procollagen, i.e. it was restricted to stromal fibroblasts adjacent to tumor-cell nests but not cancer cells. This result confirmed the early study on FAP-α in breast carcinoma [7]. On the other hand, FAP-α expression was reported in several human breast cancer cell lines [8]. Moreover, immunohistochemical analysis of the enzyme expression using FAP-specific antisera in 41 formalin-fixed and paraffin-embedded specimens of human breast tissue [16] revealed immunoreactivity also in malignant cells of invasive ductal carcinomas and in lymph node metastases. Neoplastic cells in ductal carcinomas in situ (DCIS) exhibited variable levels of staining, but epithelial cells of benign fibroadenomas and benign proliferative breast disease had no immunoreactivity. Epithelial cells of normal breast tissues were not stained, as well. These controversial results may be due to different antibodies used in the above studies. Obviously, F19 recognizes FAP-α on stromal cells, whereas the FAP-antisera may have a broader specificity [18].

Results from different studies on FAP-α expression and its connection with prognosis in breast carcinoma are also controversial. For example, retrospective clinicopathological analyses performed on 112 cases of IDC of the breast revealed that more

abundant FAP-α expression was associated with longer overall and disease-free survival [11]. The authors proposed the enzyme as an independent prognostic factor for mammary gland carcinoma. This result, however, contradicts all the later observations in breast cancer.

In a later study [11], to investigate the role of FAP-α in breast cancer, human mammary adenocarcinoma cells were engineered to over-express the enzyme. The cells were implanted into mammary fat pads of female immunodeficient mice and the xenografts were tested for tumorogenicity, growth rates and micro-vessel density. According to the results, control transfectants not expressing FAP-α grew slowly whereas cells over-expressing the enzyme formed fast growing highly vascular tumors. This observation is consistent with the hypothesis that FAP-α facilitates tumor growth and stimulates angiogenesis.

Later, using the above mouse model of human breast cancer, the authors investigated the role of proteolytic activity of FAP-α in promoting tumor growth, matrix degradation and invasion [12]. It was shown that breast cancer cells expressing a catalytically inactive mutant of FAP-α also produced rapidly growing tumors. Moreover, the FAP-specific inhibitor Val-boroPro (talabostat) given to the animals through oral gavage did not decrease substantially the growth rates of FAP-α expressing tumors. From these results it seems logical to conclude that proteolytic activity of FAP-α is not necessary for tumor promoting effects of the enzyme. In the same study [12], gelatin films with immobilized FITC-fibronectin and type I collagen gels were used for *in vitro* evaluation of the cells' capacity to degrade ECM and their invasive potential. Surprisingly, cells expressing inactive FAP-α molecules degraded gelatin films and invaded collagen gels with similar rates as did the cells expressing wild type FAP-α. The authors commented that the presence of FAP-α molecule, no matter active or inactive, in tumor cells invadopodia may induce the expression and secretion of MMPs like MMP-9 thus making the cells to acquire invasive phenotype. Actually, it was shown that FAP-α was co-localized with MMPs in tumor cells invadopodia [15, 19]. Nevertheless, the role of FAP-α activity in breast cancer remains controversial since other researchers have shown that FAP-α protease activity is abnormally high in extracts of patient tumors indicating that increased expression in real situations is always connected with increased FAP-α protease activity [17].

In another study [13], FAP-α was characterized in primary breast tumor samples and in cell lines, along with the potential interaction of FAP-α with other signaling pathways. These results showed that FAP-α once again was significantly increased in patients with poor outcome and survival. Another important observation was that the over-expression of FAP-α is closely related with the levels of focal adhesion kinase (FAK) – an enzyme known to coordinate adhesion and migration processes thus providing tumor cells migration. It was concluded that FAP-α promotes proliferation and migration of breast cancer cells, potentially by regulating the FAK pathway. These experiments tend to elucidate the intimate mechanisms of FAP-α over-expression on tumor growth.

Further on, attempts were made to specify the possible FAP-α diagnostic capacity for mammary gland carcinoma [10]. As it is well known, diagnosis of ductal carcinomas *in situ* (DCIS) in breast cancer is challenging for pathologists due to the large variety of artefacts, which could be misinterpreted as stromal invasion. Micro-invasion is usually detected morphologically by the presence of malignant cells outside the limits of the basement membrane and myoepithelium. In a recent study, FAP-α capacity as a diagnostic marker to distinguish DCIS from DCIS with micro-invasions (DCIS-MI) was evaluated. Evidence was provided that simultaneous immunostaining for FAP-α

(a cellular marker for invasiveness) and Calponin (a marker for myoepithelium layer integrity) could serve for pathologically diagnosing whether DCIS had micro-invasion. Thus, the presence of CAFs positive for FAP-α and interrupted myoepithelial layer (revealed by intermittent staining for Calponin), would indicate a high probability for DCIS-MI and vice versa.

Animal models for studying FAP-α in breast cancer

As it becomes clear from the above, most of the recent studies on the role of FAP-α in breast cancer have been performed using human breast cancer cells implanted in the fat pad of immunodeficient mice and a subsequent tracking of the development of the tumor formation. However, such xenografts in standard mouse models express stromal and vessels elements of murine origin. This limitation was recently overcome by the development of a human skin/mouse chimeric model established by transplanting human foreskin on to the lateral flank of severe combined immunodeficient mice (SCID). The subsequent inoculation of breast carcinoma cells within the dermis of the transplanted human skin resulted in the production of xenografts expressing stromal and vessel elements of human origin [35].

Another, yet unexploited possibility, are the mouse models of Ehrlich carcinoma (EC) – ascites or solid form. EC is a spontaneous murine mammary adenocarcinoma adapted to ascites form and carried in outbred mice by serial intraperitoneal (i.p.) passages. EC is used both as ascites and as solid form, that is, if tumor cells are injected i.p., the ascites form is obtained; if they are administered subcutaneously (s.c.), a solid form is obtained [30]. Thus, one of the main advantages of Ehrlich carcinoma is that same tumor cell line could be used in *in vivo* experiments both with ascites or solid form of the tumor. Moreover, EC is known to possess great resemblance to highly undifferentiated human mammary gland carcinomas and is widely used to evaluate innovative anti-cancer therapies [reviewed in 30]. EC (solid form) allows researchers to trace the volume of the tumor on a daily basis without sacrificing the laboratory animals. Also, it could be studied by the application of histology methods aiming to determine the crosstalk between tumor and surrounding tissues or the necrotic or apoptotic areas of the tumor. Additionally, changes in enzymatic activities of tumor cells or the extracellular matrix could be obtained.

Recently, we developed a fluorogenic substrate for the histochemical studies of FAP-α - 4-(β-Ala-Gly-Pro-hydrazido)-N-hexyl-1,8-naphthalimide (βAGP-HHNI) [4]. Using this substrate, we studied the localization of the enzyme in cryostat sections of normal mouse mammary gland and in EC-solid form, obtained by s.c. inoculation of 1×10^6 tumor cells in 0.2 ml PBS in mature female mice (**Fig. 1**).

According to the results, alveolar epithelial cells and dense connective tissue in healthy mice were FAP-α-negative (**Figs. 1a, b**). Haematoxyline-eosin staining of cryosections from EC showed tumor cells in different stages of cell division as well as necrotic foci within the mass of cell (**Fig. 1c**). All the tumor cells, however, were FAP-α-positive (**Fig. 1d**). The result indicates that normal mouse mammary glands do not express the enzyme just like in humans, whereas mouse breast carcinoma cells do express FAP-α. This is additional evidence that the mouse model of EC can be successfully used to study FAP-α role in breast cancer.

In conclusion, research results obtained thus far show beyond any doubt that FAP-α is an important marker for many types of tumors including breast cancer. The enzyme is involved in tumor growth and dissipation throughout different mechanisms which are

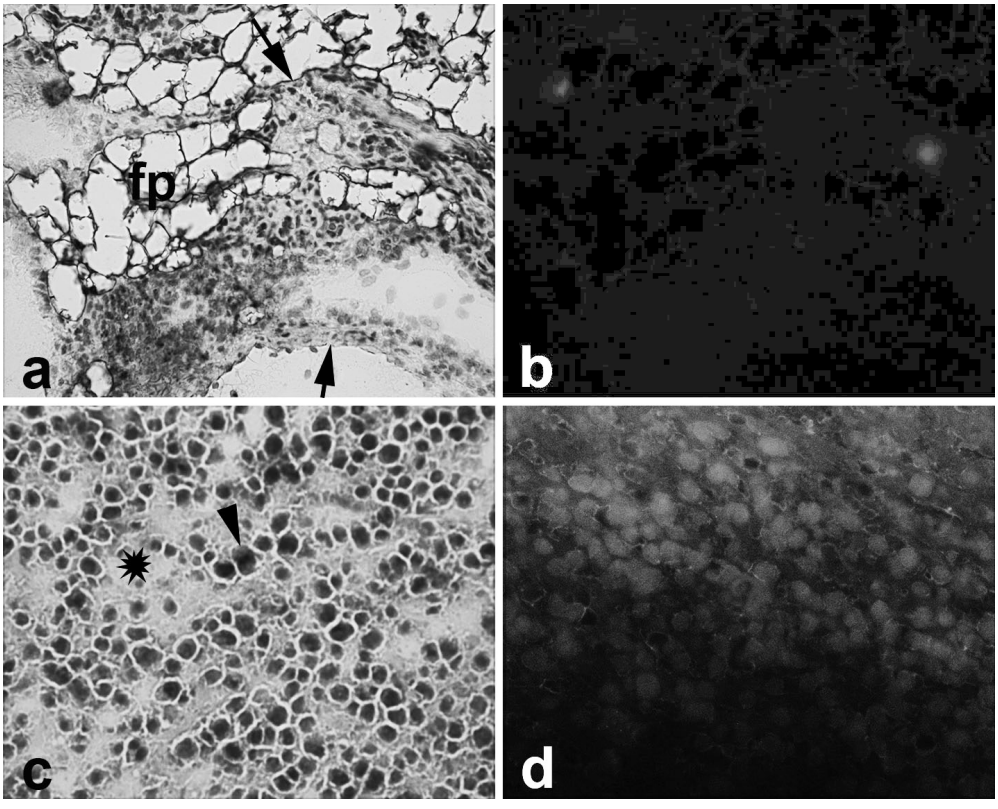

Fig. 1. FAP-α activity in normal mouse mammary gland (a, b), and in a mouse model of EC – solid form (c, d); a, b – No FAP-α activity in healthy mouse mammary gland (fp – fat pad; arrows – glandular epithelium and surrounding dense connective tissue); c – haematoxylin-eosin staining of solid EC with cells in mitosis (arrowhead) and necrotic foci (asterisk); a, c – light microscopy; b, d – fluorescent microscopy. a, b –200×; c, d – 400×

not fully elucidated yet. The use of animal models might be useful in biomedical studies on FAP-α involvement in cancer pathology as well as to evaluate the enzyme capacity as diagnostic and/or prognostic marker in cancer.

Acknowledgements: This work is financially supported by the National Science Fund of the Bulgarian Ministry of Education and Science, Grant No T02/25 2014.

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