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Study on Fibroblast Activation Protein-a Activity in an *In Vivo* Mouse Model of Ehrlich Ascites Carcinoma

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Fibroblast activation protein-a (FAP-a) is a membrane-associated serine type post-proline cleaving protease with a very restricted normal tissues distribution but highly active in tumor tissues. In human breast cancer, FAP-a is expressed both in the reactive stromal fibroblasts and tumor cells. However, the enzyme role in pathogenesis of the breast cancer is unknown and its association with the prognosis is controversial varying from better to very poor. Animal models could help in elucidating the enzyme involvement in mammary gland carcinoma. In this study we present our results for FAP-a imaging in normal mouse mammary gland and in a murine *in vivo* model of Ehrlich ascites carcinoma (EAC) using a fluorogenic enzyme histochemical method recently developed by us. We show that FAP-a is not expressed in normal mouse mammary gland but is highly active in EAC cells. This result proves that mouse *in vivo* EAC model can be useful in studies of FAP-a diagnostic/prognostic value for human breast cancer.

Key words: fibroblast activation protein-a, Ehrlich ascites carcinoma, *in vivo* model, mouse, enzyme cytochemistry.

Introduction

Fibroblast activation protein-a (FAP-a; EC 3.4.21.B28) is a 170 kDa integral membrane protease belonging to the S9b subfamily of post-proline cleaving enzymes [14]. This class of peptidases is known to modify bioactive peptides thus changing their cellular functions and to have important roles in cancer [16]. Some of the natural FAP-a substrates include collagen type I, α2-antiplasmin, neuropeptide Y, B-type natriuretic peptide, substance P and peptide YY [7]. Both immunohistochemical [15] and mRNA expression [3] studies have shown that in humans and mammals FAP-a has a highly restricted normal tissue distribution. It has been found only in fetal mesenchymal tissues as well as in healing wounds, single reactive fibroblasts and a distinct set of glucagon producing pancreatic islet cells (A cells) in adults. On the other hand, FAP-a is present in stromal fibroblasts of over 90% of epithelial tumors and some sarcomas [for review see 17]. Based on the above findings, the enzyme is largely considered to be a valuable marker and a potential therapeutic target for different types of carcinomas. Although malignant epithelial, neural and haematopoietic cells have generally been found to be

FAP-a-negative, some studies have revealed that the enzyme is expressed also in neoplastic cells of mesenchymal origin [15] and epithelial tumor cells of breast, gastric, and colorectal cancers [reviewed in 9]. It is commonly believed that the over-expression of FAP-a contributes to the tumor invasion capacity and facilitates sprouting of tumor blood capillaries [9, 13, 17].

In one of the earliest studies on FAP-a expression in human breast cancer using the F19 monoclonal antibody, the enzyme molecule has been identified only in the reactive tumor stroma [5]. Later on, by applying FAP-specific antisera, it has been shown that the enzyme is expressed in breast cancer cells, as well [8]. Presently, despite the widely recognized role of FAP-a in human breast cancer, its value as a prognostic marker is greatly controversial and varies from better [1] to very poor outcomes [6, 8]. Therefore, more studies on the marker importance of the enzyme in mammary gland carcinoma are needed, including use of appropriate animal models.

Mouse Ehrlich ascites carcinoma (EAC) is one of the commonly used *in vivo* animal models of mammary gland cancer, due to its resemblance with the most rapidly growing undifferentiated and chemotherapy sensitive human breast tumors [12]. It represents a spontaneous murine mammary adenocarcinoma adapted to ascites form and carried in outbred mice by serial intra-peritoneal (i.p.) passages. This model has been widely used for the evaluation of alternative approaches in cancer therapy [10] as well as the therapeutic significance of natural extracts and synthetic compounds [reviewed in 12]. However, FAP-a expression has not been studied in EAC, thus far.

The aim of the present paper is to compare FAP-a distribution in normal mouse mammary gland and in tumor cells of EAC in an *in vivo* mouse model using the fluorescent enzyme cytochemical method recently developed by us.

Materials and Methods

The FAP-α substrate 4-(β-Ala-Gly-Pro-hydrazido)-N-hexyl-1,8-naphthalimide (βAGP-HHNI) was synthesized according to the previously described reaction scheme [2]. The precise chemical syntheses as well as the spectral analyses of the compounds will soon be published elsewhere.

Animals and Tissue Treatment

All the experiments were performed in compliance with the Institutional Guidelines for Animal Experiments of IEMPAM – Bulgarian Academy of Sciences.

Female BALB/c mice were sacrificed by cervical dislocation 10 days after weaning of the pups. Thoracic and abdominal mammary glands were extracted and immediately frozen in liquid nitrogen. Cryostat sections (10 μm) were cut on cryotome Reichert-Jung (Germany) at –26 °C and mounted on gelatinized glass slides. The sections were air-dried, covered with 0.5% collodion (Sigma-Aldrich) in acetone: diethyl ether: absolute ethanol (4:3:3) for a minute at room temperature and used for the histochemical visualization of FAP-α activity.

Ehrlich tumor cell line was maintained in female albino mice, 5 to 7 weeks old with live body weight 25 g to 30 g, throughout serial weekly intraperitoneal injections of 1×10^6 viable tumor cells suspended in 0.2 ml phosphate buffered saline (PBS) per mouse. Mice were sacrificed by cervical dislocation 8-10 days after inoculation and Ehrlich ascites carcinoma (EAC) cells were collected by a syringe from the abdominal cavity together with the ascitic fluid. Smears of the aspired EAC cells were made on gelatinized glass slides, air-dried and fixed in paraformaldehyde vapors for 4 minutes at

room temperature. Some of the smears were stained with haematoxylin-eosin according to the classical methods of histology. Other smears were covered with 0.5% collodion as above and used for the visualization of FAP-α activity.

Incubation Solutions and Controls

Tissue sections and EAC smears were incubated in solutions containing 0.5 mM FAP-α substrate βAGP-HHNI and 0.5 mg/ml piperonal in 0.1 M phosphate buffer, pH 7.4, supplied with 100 mM NaCl at 37 °C for 16 h. After the incubation, they were post-fixed in 4% neutral formalin for 15 min at room temperature, slightly counter-stained with haematoxylin and embedded in glycerol/jelly. Control samples were treated in the same manner but in the lack of substrate in the incubation solutions. All the preparations were observed under the fluorescent microscope Leica DM5000B (USA).

Results and Discussion

Fibroblast activation protein-a is widely recognized as one of the most valuable tumor markers and is considered an important target for developing innovative and more effective anti-cancer therapies. Although the enzyme is mainly known as a common characteristic of tumor stromal fibroblasts, a huge number of recent studies have revealed that it is expressed in many types of epithelial tumor cells [for review see 9]. FAP-a capacity to hydrolyze collagen type I and a2-antiplasmin is believed to contribute to tumor invasiveness and metastatic ability not only by opening free spaces in extracellular matrix, but also by releasing different growth factors in the tumor vicinity thus recruiting them in outspread of the tumor cells and blood vessels [9, 17].

In human breast cancer, FAP-a expression has been documented both in the reactive stromal fibroblasts [1, 5] and tumor cells [6, 8]. However, the precise role of the enzyme in this type of cancer is largely unknown and the enzyme association with the prognosis is controversial varying from better [1] to very poor [6, 8]. Animal models of human breast cancer could help in elucidating the enzyme involvement in the development of mammary gland carcinoma. The most commonly used mouse model of breast cancer is Ehrlich ascites carcinoma (EAC) [12]. EAC firstly appeared as a spontaneous breast cancer in a female mouse and was used for sub-cutaneous transplantations to produce solid tumors. Later, it has been adapted to a liquid form and injected i.p. to produce ascites form of the mouse breast cancer. EAC is known to possess great resemblance to highly undifferentiated human mammary gland carcinomas and is widely used to evaluate innovative anti-cancer therapies [for review see 12]. However, FAP-a distribution in EAC has not been studied yet. Additionally, it is well known that FAP-a is a highly conservative protein and mouse enzyme has 89% similarity to its human analogue [11].

Recently, we developed a novel fluorogenic substrate for FAP-a-4-(β-Ala-Gly-Pro-hydrazido)-N-hexyl-1.8-naphthalimide (βAGP-HHNI) and used it successfully for the *in situ* imaging of the enzyme activity in normal and tumor mouse fibroblasts [2]. In this paper we present our results from FAP-a distribution studies in a mouse *in vivo* model of EAC. In our experiments we used female mice, which were inoculated i.p. by EAC cells. The animals were examined every day and their abdominal circumferences were compared to those of non-treated controls. The experiments showed that 8-10 days after inoculation the mice developed ascites cancer in the peritoneal cavity. After the animal sacrifice, tumor cells were aspired and FAP-a activity was visualized in cell smears using our novel substrate βAGP-HHNI according to the cytochemical proce-

dure described before [2]. Histochemical preparations of freshly frozen healthy mature animals' mammary glands were used as controls by applying the same fluorogenic substrate. No FAP-a activity was detected in the healthy mouse mammary gland (**Fig. 1**).

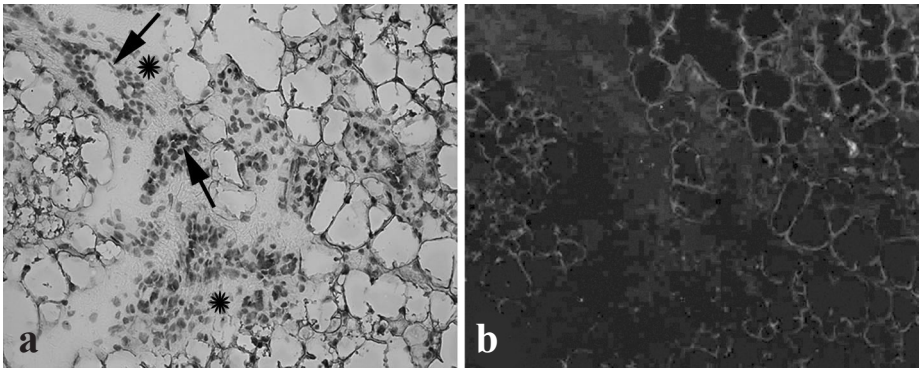

Fig. 1. Lack of FAP-α activity in the mammary gland of a mature female mouse as revealed using the FAP substrate βAGP-HHNI. Ductal epithelial cells (arrows) and dense connective tissue elements asterisks are all FAP-α-negative. a – light microscopy; b – fluorescent microscopy (× 200)

Both alveolar epithelial cells and dense connective tissue elements proved to be FAP-a-negative. This result is in compliance with previous findings that normal adult tissues of humans and mammals do not express the enzyme [3, 15].

The haematoxylin-eosin staining of EAC cells revealed the well-known previously observed morphological features of this type of tumor cells [4]. EAC cells possessed comparatively large nuclei and moderate amounts of basophilic vacuolated cytoplasm (**Fig. 2**). Different stages of mitosis were also to be seen in the smears.

In our enzyme histochemical study, all the EAC cells proved to be highly FAP-a-positive (**Fig. 3**). This result is to show that mouse breast carcinoma cells are FAP-a-positive just like human mammary gland carcinoma cells.

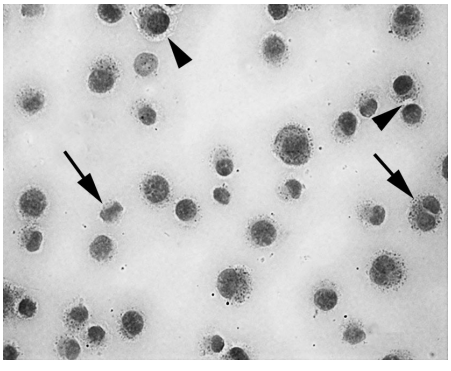

Fig. 2. Haematoxylin – eosin stained smear of Ehrlich ascites tumor cells. Cells in different stages of mitosis (arrows); cytoplasmic vacuoles (arrowheads) (× 400)

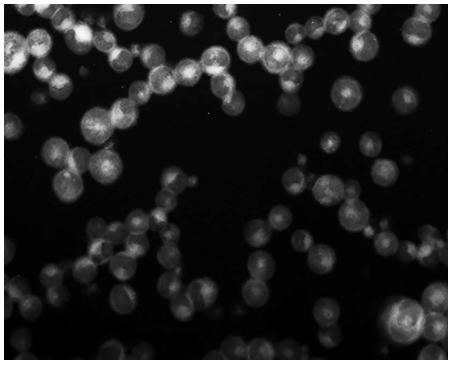

Fig. 3. FAP-a activity in a smear of Ehrlich ascites tumor cells visualized by means of the newly developed fluorogenic substrate βAGP-HHNI. All the cells are highly positive for the enzyme (× 400)

In conclusion, our results show that tumor cells of mouse breast carcinoma express FAP-a, which represents one more similarity between mouse EAC and human breast tumors. On the other hand, this result proves that mouse *in vivo* EAC model could be useful in the studies of FAP-a diagnostic/prognostic value for human mammary gland carcinoma.

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