Advances in Medical Sciences 60 (2015) 264–272

Contents lists available at ScienceDirect

Advances in Medical Sciences

journal homepage: www.elsevier.com/locate/advms

Original Research Article

Specific inhibition of fibroblast activation protein (FAP)-alpha prevents tumor progression in vitro

Volker Teichgräber [a,*], Carmen Monasterio [a], Krishna Chaitanya [b], Regina Boger [a], Katrin Gordon [a], Thomas Dieterle [c], Dirk Jäger [a], Stefan Bauer [a]

[a] National Center for Tumor Diseases, Department of Medical Oncology, University Hospital Heidelberg, Heidelberg, Germany
[b] Division of Oncology, University Hospital Zurich, Zurich, Switzerland
[c] Kantonsspital Liestal, Department of Medicine and Clinical Research, University Hospital Basel, Liestal, Switzerland

ARTICLE INFO

Article history:
Received 17 December 2014
Accepted 24 April 2015
Available online 11 May 2015

Keywords:
FAP
Tumor stroma
Activated fibroblasts
Cancer associated fibroblasts
Proliferation
Migration
Invasion

ABSTRACT

Purpose: Solid tumors modulate their environment to keep non-malignant stromal cells in a tumor-promoting state. The main cells in the stroma of epithelial derived tumors are cancer associated fibroblasts (CAF) that are critical to tumorigenesis and angiogenesis. CAFs also supply the tumor cells with growth factors and extracellular matrix (ECM) degrading enzymes. They are thus essential for tumor initiation as well as tumor progression and metastasis, suggesting that they represent an ideal cellular target of an integrative tumor therapy.

Fibroblast activation protein (FAP) is a well-defined marker, expressed at high levels on the cell surface of CAFs. FAP, a constitutively active serine peptidase with both dipeptidyl peptidase IV (DPP IV) and collagenase/gelatinase activity, promotes malignant and invasive behavior of epithelial cancers. High stromal expression levels of FAP correlate with poor prognosis. FAP is difficult to detect in non-diseased adult tissue, but it is generally expressed at sites of tissue remodeling.

Materials and methods: In our experiments, we aimed for a reduction of the pro-tumorigenic activities of CAFs by depleting FAP from fibroblasts growing in a composite environment with epithelial tumor cells.

Results: FAP depletion was achieved by two therapeutically relevant approaches: a novel internalizing anti-FAP IgG1 antibody and FAP gene knock-down by siRNA delivery. The antibody effectively removed FAP from the cell surface and was capable of reversing the FAP mediated migratory and invasive capacity. FAP RNA interference was equally effective when compared to the antibody.

Conclusions: Thus, targeting FAP on CAF suppresses pro-tumorigenic activities and may result in a clinically effective reduction of tumor progression and dissemination.

1. Introduction

Malignant tumors are composed of a variety of different cell types. Tumor cell-nests can maintain themselves by means of diffusion only up to size of a few cubic millimeters of tumor volume. Further growth requires the recruitment of adjacent tissue cells and precursor cells that are needed for the initiation and establishment of a functional supporting stromal structure, which ensures stability and supply. One critical component of the tumor stroma are activated fibroblasts, which can constitute up to 80 percent of the tumor mass [1]. Adjacent fibroblasts and immigrating mesenchymal precursor cells are attracted and recruited by the tumor microenvironment that transforms the fibroblasts into a distinct activated phenotype, i.e. cancer-associated fibroblasts (CAF) [2,3]. A hallmark of these activated fibroblasts is the expression of fibroblast activation protein alpha (FAP) [4,5]. This molecule is most prominently expressed on CAFs, but its surface expression has also been detected in bone and soft tissue sarcomas and in malignant melanomas [6,7]. It is a homodimeric type II transmembrane serine protease with post-prolyl cleavage specificity and harbors both dipeptidase and endopeptidase activity [8–11]. Due to its localization at the forefront of the pseudopodia of activated fibroblasts, FAP can digest and remodel the extracellular matrix (ECM) [12–16]. Thus, it

* Corresponding author at: National Center for Tumor Diseases, Department of Medical Oncology, University Hospital Heidelberg, Im Neuenheimer Feld 460, 69120 Heidelberg, Germany. Tel.: +49 6221 567229; fax: +49 6221 567225.
E-mail address: volker.teichgraeber@nct-heidelberg.de (V. Teichgräber).

http://dx.doi.org/10.1016/j.advms.2015.04.006
1896-1126/

may play an important role in tumor progression by promoting tumor cell division and motility as well as lymphatic and hematogenous vessel infiltration and dissemination [17–21]. Physiologically, FAP is produced transiently by activated stromal fibroblasts during embryogenesis, wound healing, and occasionally on pancreatic α-cells [22,23]. FAP is not characteristically found on normal tissues or benign tumors [1,10]. Recent studies strongly suggest additional modulatory effects of FAP in the absence of its enzymatic activity by yet unknown mechanisms [24]. FAP-expressing stromal cells have been shown to suppress antitumor immunity, adding another layer of functionality to FAP that could contribute to tumor progression [25]. Because of its manyfold influence on tumor physiology, restricted expression pattern within diseased areas and correlation with poor clinical outcome in patients with cancer, FAP has emerged as a promising target for cancer therapy [4,5,24,26,27]. Expression of FAP has been connected to poor prognosis in several epithelial tumors [27–29]. In particularly aggressive tumor types, such as pancreatic ductal adenocarcinoma (PDAC) and non-small cell lung cancer (NSCLC), FAP has been described as an independent negative prognostic marker. Here, FAP expression seems to correlate closely with a more aggressive disease biology and tumors containing marked FAP-positive CAFs and stromal tissue identify patients at high risk for early recurrence and poor overall outcome [30–33]. The investigation of targeted FAP inhibition as a novel therapeutic approach for those poorly treatable cancers carries high clinical relevance. Therefore, we further investigated the biological functions of FAP in CAF in those two tumor diseases.

Employing murine tumor models of PDAC and NSCLC, we established an improved in vitro setup that closely resembles the conditions of tumor initiation and establishment found in vivo. Primary fibroblasts from the pancreas or the lung are cultivated in a composite environment (co-culture, mixed culture) with pancreatic or bronchial carcinoma cells, respectively. Since the remodeling capacities of FAP are thought to be of central importance for promoting sustainable tumor growth and invasion, the cell–cell interactions of tumor cells and fibroblasts could be instrumental to overall function and efficacy of FAP-mediated processes. The impact of a composite environment on FAP function and associated core features of aggressiveness like proliferation, migration, and invasion are focus of this investigation.

2. Material and methods

2.1. Isolation of fibroblasts

Primary fibroblasts were isolated from whole lung and pancreas of 6–8-week old C57BL/6 mice as described in previous studies [34]. In brief, to reduce blood cell contamination the left heart was fenestrated and lungs perfused in situ via injection of the right ventricle with 10 ml ice-cold DPBS before removal. The pancreas was removed in total after separating it bluntly from its surroundings. Extracted tissues were immediately transferred in DPBS supplemented with trypsin inhibitors into a petri dish situated in an ice bath. Here, the parenchyma was further dissected and shred into small cubicles using a scalpel. Tissue particles of ~1 mm^3 were gently transferred into collagen-coated 6-well-plates, pinned down onto the surface with the tip of a scalpel and then immobilized by weighing them down with a cover slip before growth medium was added. After 3–4 days, fibroblasts started radial outgrowth from the fixed particles. Tissue particles were subsequently removed, wells thoroughly rinsed and fibroblasts further expanded. The fibroblast cultures were maintained in DMEM low glucose supplemented with 20% FBS, 1% penicillin–streptomycin (100 U/l), gentamicin (50 μg/ml), 1% L-glutamine (200 mM) and 1% HEPES (1 M). For our experiments, bronchial and pancreatic fibroblasts of culture passages 5–7 were used.

2.2. Cell lines and antibodies

The C57BL/6 syngeneic murine tumor cell lines Lewis Lung carcinoma (LL2) and pancreatic adenocarcinoma (Panc02) were a gift from the cell repository of German Cancer Research Center, Heidelberg. LL2 cells were maintained in DMEM High Glucose supplemented with 10% FBS and penicillin–streptomycin (100 U/l). Panc02 cells were cultivated in RPMI-1640 without L-glutamine supplemented with 10% FBS and penicillin–streptomycin (100 U/l). HEK293huFAP, HEK293muFAP were generated by cloning human FAP, or murine FAP cDNA into the pEAK8 vector (Edge Biosystems) and stable transfection of HEK293 c-18 cells (A TCC CRL-10852) followed by monoclonalization by limited dilution. The cell lines were maintained in DMEM supplemented with 10% FBS, penicillin–streptomycin (100 U/l), and puromycin (3 μg/ml). HT1080 and HT1080FAP, huFAP expressing HT1080 (ATCC CCL-121), were maintained in RPMI 10% FBS and in RPMI 10% FBS supplemented with 200 μg/ml G418, respectively. Humanized murine antibody mF19 against FAP and human anti-A33 antibody [35] were obtained from Ludwig Institute for Cancer Research (Melbourne, Australia). A33 is structurally identical to ESC11 but is directed against the unrelated human A33 antigen, a surface differentiation antigen that is uniformly expressed in 95% of colorectal cancers. For control experiments, anti-fibroblast activation protein alpha antibody was purchased from abcam (ab53066, abcam pic, Cambridge, UK). We described the engineering of the fully human huFAP-muFAP specific IgG1 antibody ESC11 recently [36].

2.3. Confocal microscopy

Cells were seeded on sterile 20 mm fibronectin-coated (Sigma F1141-1MG, at 10 μg/ml) coverslips (Carolina Biological) in 12-well plates and grown to 20–30% confluency. Cells were fixed with 2% paraformaldehyde on ice and permeabilized using Cytofix/Cytoperm solution (51-2090KZ; BD Biosciences). Primary antibody (DyLight549 conjugated ESC11) was diluted in 1× Perm/Wash (51-2091KZ; BD Biosciences) for a final concentration of 10 μg/ml. Cells were fixed with 4′,6-diamidino-2-phenylindole anti-fade (Invitrogen). All images were acquired on Leica SP5 UV/Vis confocal microscope (Leica Microsystems) at the Center for Microscopy and Image Analysis (University of Zurich, Zurich, Switzerland) using LAS AF software. Images were kept in 1024 × 1024 formats and acquired with a zoom factor of 4 at 700 Hz frequency with HCxAPO Lambda blue 63.0× oil UV objective lens at a numerical aperture of 1.40. Images were stored in Tagged Image File Format (TIFF) and further processed using ImageJ software. For internalization experiments, cells were incubated with 30 μg/ml ESC11 for the indicated time. FAP was detected with directly conjugated ESC11-DyLight 549. Alternatively, internalization was directly induced and monitored by DyLight 549-conjugated human IgG1 (30 μg/ml).

2.4. FAP surface depletion

Sub-confluent fibroblast and HT1080FAP cultures were trypsinized, washed with PBS and resuspended in RPMI, 10% FBS at 5×10^5 cells and 0.5 ml per well seeded in 12 well cell culture plates and the indicated concentration of ESC11 IgG was added. After over night incubation, cells were trypsinized, washed, and stained with 10 μg/ml F19 followed by FITC-conjugated polyclonal goat anti-mouse and analyzed by flow cytometry. % FAP expression was calculated (100/(m.f.i. 0 μg/ml ESC11 IgG − m.f.i. background)) × (m.f.i. with ESC11 IgG − m.f.i. background).