BBA - General Subjects xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

BBA - General Subjects

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

Transforming growth factor-β modulates pancreatic cancer associated fibroblasts cell shape, stiffness and invasion

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ARTICLE INFO

Keywords:
Atomic force microscopy
Desmoplasia
Cell mechanics
Collagen gels
Cell invasion
Stress fibers
Lamellipodia

ABSTRACT

Background: Tumor microenvironment consists of the extracellular matrix (ECM), stromal cells, such as fibroblasts (FBs) and cancer associated fibroblasts (CAFs), and a myriad of soluble factors. In many tumor types, including pancreatic tumors, the interplay between stromal cells and the other tumor microenvironment components leads to desmoplasia, a cancer-specific type of fibrosis that hinders treatment. Transforming growth factor beta (TGF-β) and CAFs are thought to play a crucial role in this tumor desmoplastic reaction, although the involved mechanisms are unknown.

Methods: Optical/fluorescence microscopy, atomic force microscopy, image processing techniques, invasion assay in 3D collagen I gels and real-time PCR were employed to investigate the effect of TGF-β on normal pancreatic FBs and CAFs with regard to crucial cellular morphodynamic characteristics and relevant gene expression involved in tumor progression and metastasis.

Results: CAFs present specific myofibroblast-like characteristics, such as α-smooth muscle actin expression and cell elongation, they also form more lamellipodia and are softer than FBs. TGF-β treatment increases cell stiffness (Young's modulus) of both FBs and CAFs and increases CAF's (but not FB's) elongation, cell spreading, lamellipodia formation and spheroid invasion. Gene expression analysis shows that these morphodynamic characteristics are mediated by Rac, RhoA and ROCK expression in CAFs treated with TGF-β.

Conclusions: TGF-β modulates CAFs', but not FBs', cell shape, stiffness and invasion.

General Significance: Our findings elucidate on the effects of TGF-β on CAFs' behavior and stiffness providing new insights into the mechanisms involved.

1. Introduction

The tumor microenvironment consists of the extracellular matrix (ECM), stromal cells and countless soluble factors in the extracellular milieu, whose importance in cancer progression and metastasis is indisputable [1,2]. Stromal cells, such as fibroblasts (FBs) are in close interaction with cancer cells [3] and are responsible for a desmoplastic reaction that results in extensive production and remodeling of tumor ECM. Pancreatic tumors are highly desmoplastic, being characterized by the intra-tumoral accumulation of excess amount of ECM, such as collagen type I and hyaluronan [4] that hinders effective treatment and it is responsible in large part for the low survival rates observed in these cancers.

FBs are mesenchymal-derived cells with a characteristic spindle-like morphology, and are usually present in the interstitial space of normal tissues, emedded whithin fibrillar ECM [5,6]. They were originaly identified as cells of the connective tissue that synthesize collagen and are now thought to be crucially involved in many vital cellular processes such as ECM deposition, secretion of ECM-degrading proteases (i.e. matrix metalloproteinases), regulation of epithelial cell differentiation, regulation of inflammation, and wound healing [5–7].

Interestingly, FBs can acquire an "*activated*" phenotype which can be induced by various stimuli, such as transforming growth factor-beta (TGF-β) and altered ECM composition [6,7]. Activated fibroblasts, also known as "*myofibroblasts*", express alpha-smooth-muscle actin (α-SMA), a cytoskeletal protein associated with smooth muscle cells, which is the most common marker of activated FBs [8], and are characterized by increased capacity to secrete ECM components, remodel the ECM and

Abbreviations: α-SMA, alpha-smooth-muscle actin; AFM, atomic force microscopy; DAPI, 4′,6-Diamidino-2-Phenylindole; DMSO, dimethylsulfoxide; EMT, epithelial to mesenchymal transition; ECM, extracellular matrix; FBs, fibroblasts; PBS, phosphate buffered saline; PCR, polymerase chain reaction; ROCK, Rho associated protein kinase; TGF-β, transforming growth factor beta; CAFs, cancer associated fibroblasts; PFA, paraformaldyhyde

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https://doi.org/10.1016/j.bbagen.2018.02.009
Received 3 October 2017; Received in revised form 19 January 2018; Accepted 14 February 2018

form contractile bundles composed of actin and myosin [5,8–10]. Although myofibroblasts were originally identified in wound healing, they are abundant in cancer [1]. Notably, in the case of cancer, myofibroblasts are also known as cancer associated fibroblasts (CAFs) [7], while terms such as carcinoma-associated fibroblasts, tumor-associated fibroblasts, peritumoral (myo-)fibroblasts, reactive stroma fibroblasts, may also be found in the literature [5,11]. It has been suggested that CAFs are recruited to the tumor microenvironment by cancer cells through various growth factors and cytokines in order to form a myofibroblastic microenviroment that supports malignancy, cell invasion and metastasis [1,12]. CAFs are also thought to exert mechanical forces on surrounding ECM [5,12]. It has also been demonstrated that CAFs enhance cancer cell proliferation and angiogenesis [1] and increase the invasiveness of originally non-invasive cancer cells [11]. Additionally, CAFs have the tendency to aggregate peritumorally and encircle carcinoma cells, being the first to invade through adjacent normal tissues [1].

Although, FBs, CAFs and TGF-β are generally thought as key players in desmoplasia development in cancer, the exact mechanism of their action as well as their interplay is still not well-defined. Also the effect of TGF-β on the morphodynamic characteristics of CAFs as well as their invasive phenotype is not yet fully studied. Furthermore, as desmoplasia has been proposed to inhibit drug delivery and enhance tumor progression and metastasis [13], defining the mechanistic interactions between CAFs and TGF-β can provide a new basis for the development of an improved treatment [14–17]. In the present work, optical and fluorescence microscopy, atomic force microscopy (AFM), image processing techniques, three dimensional (3D) in vitro invasion assays and real-time polymerase chain reaction (PCR) were employed to investigate the effect of TGF-β on normal pancreatic FBs and CAFs with regard to several cellular morphodynamic characteristics and relevant gene expression.

2. Materials and methods

2.1. Cell culture

Native human FBs derived from pancreas (Cat.# SC00A5) and pancreatic CAFs (Cat.# CAF08) cell lines were purchased from Neuromics (Edina, MN) and cultured in MSC-GRO (VitroPlus III, low serum, complete) medium. The experiments were performed in cells without any treatment (parental cells), cells treated with 5 ng/ml TGF-β for 2 days (TGF-β treated cells) [18–20] and cells treated with the same amount of solvent (4 mM HCL 1 mg/ml with 1 mg/ml Bovine Serum Albumin) as the one in which TGF-β was dissolved (control cells).

2.2. Assessment of lamellipodia formation

For quantifying the number of cells forming lamellipodia, cells were stained with phalloidin (see *Supplement*), and observed under an Olympus BX53 fluorescent microscope. Multiple pictures were taken and the number of cells forming lamellipodia compared to the total number of phalloidin-stained cells was counted manually. At least 250–300 cells were used for quantification per condition and the mean ratio of lamellipodia-forming cells compared to total in each condition was assessed from 3 independent experiments.

2.3. α-SMA quantification

To quantify α-SMA levels in cells, a previously described method was used [21,22]. Briefly, after obtaining images from α-SMA stained cells (see *Supplement*) using the fluorescent microscope, an outline was drawn by hand around each cell using ImageJ (NIH, Bethesda, MD) software and circularity, area, and mean fluorescence were measured. Also three background areas per cell were measured in order to calculate the mean fluorescence of the background. The total corrected cellular fluorescence = integrated density − (area of selected cell × mean fluorescence of background readings), was calculated.

2.4. Cell elongation

Cell elongation was assessed using optical microscopy images from live cells. Pictures of individual cells (not forming clusters) were taken using a Nikon Eclipse TS100 inverted microscope equipped with a digital camera (Olympus XC50 Color CCD camera, 5 megapixel) and a Nikon Ph1 DL 10 × 0.25 phase microscope objective lens. ImageJ software was used to automatically measure factor *E* from cells [23]. Factor *E* equals to the long axis divided by the short axis minus one. The *elongation factor E* describes the extent in which the equimomental ellipse is lengthened or stretched out [24]. Thus, *E* is zero for a circle, and one for an ellipse with an axis ratio 1:2. The cells that presented *E* values 0.0–0.5 were considered as spherical, 0.5–1.0 as ellipsoid, and *E* values higher than 1.0 as elongated [25].

2.5. Stress fibers

For the characterization of the actin stress fibers, the *FilamentSensor* tool was used [26]. After taking images with an Olympus BX53 fluorescent microscope from cells stained with phalloidin, the actin filament structure of TGF-β-treated and untreated FBs and CAFs was reconstructed using the filament sensor tool. In the reconstructed images each color corresponds to a different fiber orientation.

2.6. Spreading

Cells were plated on 24-well culture plates and incubated at 37 °C for 30 and 40 min for FBs and CAFs, respectively. Cells were then fixed in 4% paraformaldehyde (PFA, Sigma P6148) and the cell morphology was observed under optical microscope. Unspread cells were defined as round cells, while spread cells were defined as cells with extended processes [27,28]. The percentage of spread cells was quantified by analyzing at least 300 cells from 5 randomly selected fields. Three independent experiments were performed and results represent mean values from all three of them.

2.7. Atomic force microscopy (AFM)

For AFM characterization of cells and collagen I gel (see *Supplement* for gel formation methodology), a Molecular Imaging-Agilent PicoPlus AFM system was used. Imaging of fixed cells was performed in contact mode in air and under phosphate buffered saline (PBS) with silicon and V-shaped silicon nitride probes (SICON-Applied Nanostructures and PNP-TR-Nanoword). Force spectroscopy on live cells was performed with V-shaped soft silicon nitride probes (MLCT, Bruker) and the collected force curves were analyzed by AtomicJ [29] so as to calculate the sample's Young's modulus using the Hertz model. Collagen I gels were characterized with V-shaped PNP-TR probes in contact mode. The AFM image processing from all the samples was performed by using the PicoView software (Agilent) and the freeware scanning probe microscopy software WSxM 5.0 dev.2.1 [30]. A detail representation of the AFM methods is presented in the *Supplement*.

2.8. Invasion assay

The “hanging drop” technique was used for the formation of FB and CAF cell spheroids (see *Supplement*), as described previously [31–34]. Immediately after transferring the spheroids into wells of a 96-well plate containing collagen I gel, images were taken (time zero) with an optical microscope. Spheroids were then incubated at 37 °C for 6 h and new images were taken. Cell invasion through surrounding collagen was assessed using ImageJ software and spheroids' size (average of the major and minor axis length) after 6 h was compared to the initial size