Biomaterials 52 (2015) 367–375

Contents lists available at ScienceDirect

Biomaterials

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

The phenotype of cancer cell invasion controlled by fibril diameter and pore size of 3D collagen networks

Jiranuwat Sapudom [a], Stefan Rubner [a], Steve Martin [a], Tony Kurth [b], Stefanie Riedel [b], Claudia T. Mierke [b], Tilo Pompe [a, *]

[a] Biophysical Chemistry Group, Institute of Biochemistry, Faculty of Biosciences, Pharmacy and Psychology, Universität Leipzig, Leipzig 04103, Germany
[b] Biological Physics Division, Institute for Experimental Physics I, Faculty of Physics and Earth Science, Universität Leipzig, 04103 Leipzig, Germany

ARTICLE INFO

Article history:
Received 19 December 2014
Accepted 2 February 2015
Available online 3 March 2015

Keywords:
Collagen
Cell morphology
ECM (extracellular matrix)
Microstructure
Mechanical properties
Invasion

ABSTRACT

The behavior of cancer cells is strongly influenced by the properties of extracellular microenvironments, including topology, mechanics and composition. As topological and mechanical properties of the extracellular matrix are hard to access and control for in-depth studies of underlying mechanisms *in vivo*, defined biomimetic *in vitro* models are needed. Herein we show, how pore size and fibril diameter of collagen I networks distinctively regulate cancer cell morphology and invasion. Three-dimensional collagen I matrices with a tight control of pore size, fibril diameter and stiffness were reconstituted by adjustment of concentration and pH value during matrix reconstitution. At first, a detailed analysis of topology and mechanics of matrices using confocal laser scanning microscopy, image analysis tools and force spectroscopy indicate pore size and not fibril diameter as the major determinant of matrix elasticity. Secondly, by using two different breast cancer cell lines (MDA-MB-231 and MCF-7), we demonstrate collagen fibril diameter – and not pore size – to primarily regulate cell morphology, cluster formation and invasion. Invasiveness increased and clustering decreased with increasing fibril diameter for both, the highly invasive MDA-MB-231 cells with mesenchymal migratory phenotype and the MCF-7 cells with amoeboid migratory phenotype. As this behavior was independent of overall pore size, matrix elasticity is shown to be not the major determinant of the cell characteristics. Our work emphasizes the complex relationship between structural-mechanical properties of the extracellular matrix and invasive behavior of cancer cells. It suggests a correlation of migratory and invasive phenotype of cancer cells in dependence on topological and mechanical features of the length scale of single fibrils and not on coarse-grained network properties.

1. Introduction

Leaving the primary tumor and invading the surrounding extracellular matrix (ECM) is a key step in cancer cell invasion. Similarly important are steps in metastasis, when single tumor cell migration into healthy tissues at distant sites forms a new tumor in another microenvironment. Unfortunately these processes of cancer cell dissemination, invasion and metastasis are still not well understood [1–5]. Reasons for that may lie in the fast and complex alteration of cancer cells gene expression and biochemical signaling, but also in the changes to the biochemical composition and biophysical characteristics of the surrounding microenvironment by the cells.

Cancer cells have a distinctive ability to adapt to and communicate with their microenvironment. Hence, tumor-associated alterations in the extracellular microenvironment such as the composition, pore size and elasticity have a feedback onto the behavior of tumor cells themselves [6–10]. In this context it is known that the mechanical properties of tumors and tumor microenvironments change during tumor progression [9,11–13]. Such mechanical changes of the surrounding ECM have been shown to trigger tumor cell behavior or even tumor-like behavior of non-tumorigenic cells [14]. Stiffening of tumor-associated ECM by deposition of collagen fibrils and increase of the fibril diameter are often observed in cancerous tissues suggesting a loss of control mechanisms in the regulation of collagen fibril formation [6].

* Corresponding author. Universität Leipzig, Institute of Biochemistry, Johannisallee 21-23, 04103 Leipzig, Germany. Tel./fax: +49 341 97 36931/9.
E-mail address: tilo.pompe@uni-leipzig.de (T. Pompe).

http://dx.doi.org/10.1016/j.biomaterials.2015.02.022
0142-9612/

However, a deep understanding of underlying mechanisms of the correlation between the mechanical properties and tumor formation as well as tumor progression is still hampered by the complexity of the involved signaling cascades, the heterogeneity of relevant cell populations and the shortcomings of model systems. While *in vivo* systems are difficult to investigate at high information depth, *in vitro* systems frequently lacking important biomimetic parameters of the *in vivo* situation like appropriate mechanics and dimensionality [15–18].

Due to that fact *in vitro* cell studies culture in 3D biomimetic matrices is a growing field as it offers a better representation of a natural cellular microenvironment in physiologically and pathologically relevant conditions. While synthetic systems frequently allow for well-defined preparation, they usually lack the complexity needed to address physiological questions. On the other hand naturally derived and reconstituted matrices are frequently hard to characterize and to reproduce in a well-defined manner [18]. In order to use matrices from naturally derived components good characterization of relevant parameters is a prerequisite for meaningful studies.

Type I collagen (Coll I) is a widely used ECM component due to its very high abundance in mammalian tissues. It is a gold standard for reconstituting 3D cell culture scaffolds regarding its fibrillar structure and ability to form networks of reasonable mechanical properties *in vitro* [19]. Coll I matrices facilitate cell attachment, proliferation and differentiation through receptor-mediated interactions predominantly via integrins [20–22]. Cell migration and invasion in 3D matrices are dynamical processes predominantly influenced by biophysical features of the surrounding matrix. Therefore, pore size and network elasticity are intensely discussed parameters to affect cell behavior [13,23–26]. However, less is known about how the Coll I fibril diameter specifically influences cell behavior, especially of cancer cells. In order to address such a question, 3D Coll I matrices with controlled fibril diameter are required. Recent reports emphasize the importance of such scaffolds as no direct correlation to matrix elasticity was found in contrast to previous reports [26,27]. *In vivo*, fibroblasts regulate the diameter of Coll I fibrils by controlling type III and V collagen concentrations as nucleators during the process of Coll I fibrillogenesis [28]. In contrast, the fibril diameter can be regulated *in vitro* by temperature and pH during Coll I self-assembly, as well as by type V collagen concentration [24,29–33]. Hence, there exist options for tuning topological features of Coll I matrices *in vitro* in order to generate well-defined 3D matrices. However, a comprehensive and independent modulation of those parameters and a correlated characterization of 3D Coll I matrices, i.e. pore size, fibril diameter and elasticity, is lacking. Hence, using well-defined 3D matrices with different topological and mechanical properties is envisioned to better clarify cancer cell behavior in a more physiological context.

2. Materials and methods

2.1. Reconstitution of 3D Coll I matrices

To immobilize 3D collagen matrices onto glass coverslips covalently, amine-functionalized glass coverslips of 13 mm diameter (VWR international, Leuven, Belgium) were coated with a thin layer of 0.14 wt.% poly(styrene-alt-maleic anhydride) (PSMA; MW30000 g/mol, Sigma–Aldrich, Steinheim, Germany) as published previously [34]. 3D collagen matrices were reconstituted using rat tail type I collagen (Coll I) (Corning, New York, USA). Final collagen concentrations of 2, 2.5 and 3 mg/ml were adjusted by mixing with 0.25 M phosphate buffer to achieve final pH values of 7, 7.5 and 8. Coll I solutions were prepared and kept on ice (4 °C) to prevent polymerization for 60 min pH value of Coll I solutions was determined prior to network formation using universal indicator solution pH (Sigma–Aldrich) (see also Fig. S1) and pH electrode (Mettler-Toledo, Gießen, Germany). Subsequently, 30 μl collagen solution was transferred onto PSMA-coated coverslips and polymerized at 37 °C, 5% CO_2 and 95% humidity for 90 min. Afterwards Coll I matrices were washed 3 times with phosphate buffered saline (PBS) (Biochrom, Berlin, Germany) and equilibrated at these neutral conditions. The reconstituted Coll I matrices, with a resulting thickness of about 250–300 μm, were kept in a hydrated state prior to topological and mechanical characterization as well as cell culture studies.

2.2. Kinetics of Coll I fibril formation

For analysis of Coll I fibril formation kinetics, 100 μl of prepared Coll I solution at different pH was transferred to a pre-chilled (4 °C) 96-well microplate (Greiner, Bahlingen, Germany) prior to polymerization. 96-well microplates were loaded into a pre-warmed (37 °C) plate reader (TECAN Infinite F200 Pro, TECAN, Grödig, Austria) and turbidity at 405 nm was measured at 1 min intervals for 90 min. Measurements were performed in three independent experiments.

2.3. Topological characterization of 3D Coll I matrices

3D Coll I matrices were stained with 50 μM 5-(and-6)-Carboxytetramethylrhodamine succinimidyl ester (5(6)-TAMRA-SE) (Invitrogen, Carlsbad, USA) at room temperature for 1 h and rinsed 3 times with PBS. Collagen matrices were imaged with confocal laser scanning microscope LSM700 (Zeiss, Jena, Germany) using 40 × /NA 1.3 oil immersion objective. Acquired images were 16-bit color depth, 1024 × 1024 pixels in resolution and a vertical stack size of 200 images (equivalent to 100 μm). The voxel size of the acquired images was 0.13 × 0.13 × 0.5 μm (x × y × z). Representative sample images of the microarchitecture were generated from summed z-stacks of 50 μm in-depth using Imaris (Bitplane, Zurich, Switzerland). Pore size and fibril diameter were analyzed as described by Franke et al. [23] using a home-built image processing procedure with erosion algorithm and autocorrelation method, respectively. This topology analysis was performed at least in triplicates with 6 positions per sample. Fibril length of 40 fibrils in total from 3 independent experiments was manually analyzed using the ImageJ fibril tracking tool (NIH, USA).

2.4. Colloidal probe force spectroscopy of 3D Coll I matrices

Elasticity of 3D Coll I matrices was determined using colloidal probe force spectroscopy (NanoWizard 3 AFM, JPK Instruments, Berlin, Germany). The colloidal probes were prepared by attaching a 50 μm glass microbead (Polyscience Europe GmbH, Eppelheim, Germany) to a tipless MLCT rectangular cantilever (μMash, Wetzlar, Germany) using an epoxy glue (Mettler-Toledo, Gießen, Germany). The spring constant of MLCT cantilever (approx. 60 nN/m) was evaluated by the thermal noise method [35]. All experiments were performed in PBS buffer at room temperature. At least 50 force-distance curves were measured at 3 positions of each Coll I matrix with 3 independent experiments. The Young's modulus of the 3D collagen matrices was determined from the force-distance curves by fitting the Hertz model with an indentation of 3–7 μm.

2.5. Cell culture

3D Coll I matrices were placed into 24-well plate (Greiner, Bahlingen, Germany). 1×10^4 cells of MDA-MB-231 (human breast carcinoma cell line) and MCF-7 (human breast adenocarcinoma cell line) were seeded on top of 3D Coll I matrices and cultured in DMEM (Biochrom, Germany) supplemented with 10% fetal calf serum (Biochrom, Germany) and 1% ZellShield (Biochrom, Germany) at 37 °C, 5% CO_2 and 95% humidity.

Cell proliferation was determined after 4 days by means of commercial WST-1 assay (Roche, Germany). Cells were rinsed 3x with Hanks' balanced salt solution (HBSS; Sigma–Aldrich, Germany) with Ca^{2+} and Mg^{2+} and were subsequently incubated for 2 h with 500 μl WST-1 solution (1:10 dilution with cell culture medium) at cell culture condition. Supernatants were collected and 100 μl of each supernatant were transferred to 96-wells plates. The absorbance was measured at a wavelength of 450 nm with a multi-well plate reader.

2.6. Analysis of cell invasion and morphology

Cell invasion and morphology were analyzed after 4 days of culture. For analysis, cells were fixed in 4% paraformaldehyde (Roth, Karlsruhe, Germany) for 10 min at room temperature and rinsed 3 times with PBS. Afterwards, cells were permeabilized with 0.1% Triton X100 (Roth, Germany) for 10 min at room temperature and rinsed 3 times with PBS. For analysis of cell invasion and morphology, cells were stained with DAPI (Invitrogen, Germany) and Alexa Fluor 488 Phalloidin (Invitrogen, Germany) for 30 min at room temperature and rinsed 3 times with PBS. Cells were imaged using an AxioObserver.Z1 with scanning stage (Zeiss, Jena, Germany) using a 10 × objective. Images were 1388 × 1040 pixels in resolution with 5 μm z-distance. At least 200 cells per experimental condition (3 positions from 3 independent experiments) were analyzed using DAPI signal from individual cell nuclei. Cells located >10 μm below the Coll I layer surface were counted as invasive cells. Cell morphology was imaged using Alexa Flour 488 Phalloidin signal. Aspect ratio (major axis/minor axis) was determined using ImageJ. Cells with an aspect ratio <1.5 were characterized as round cells. Cluster formation was manually counted, whereby cells without contact to other cells were counted as single cells (single object). For morphological and cluster formation analysis, at least 360 cells of 3 positions from 3 independent experiments were analyzed per experimental condition. For each cell