Biomaterials 116 (2017) 118–129

Contents lists available at ScienceDirect

Biomaterials

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

Recapitulating the human tumor microenvironment: Colon tumor-derived extracellular matrix promotes angiogenesis and tumor cell growth

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ARTICLE INFO

Article history:
Received 5 October 2016
Received in revised form
22 November 2016
Accepted 23 November 2016
Available online 24 November 2016

Keywords:
3D cell culture
ECM
Decellularization
Tumor microenviroment
Tumor metabolism
Colon

ABSTRACT

Extracellular matrix (ECM) is an essential and dynamic component of all tissues and directly affects cellular behavior by providing both mechanical and biochemical signaling cues. Changes in ECM can alter tissue homeostasis, potentially leading to promotion of cellular transformation and the generation of tumors. Therefore, understanding ECM compositional changes during cancer progression is vital to the development of targeted treatments. Previous efforts to reproduce the native 3D cellular microenvironment have utilized protein gels and scaffolds that incompletely recapitulate the complexity of native tissues. Here, we address this problem by extracting and comparing ECM from normal human colon and colon tumor that had metastasized to liver. We found differences in protein composition and stiffness, and observed significant differences in vascular network formation and tumor growth in each of the reconstituted matrices, both *in vitro* and *in vivo*. We studied free/bound ratios of NADH in the tumor and endothelial cells using Fluorescence Lifetime Imaging Microscopy as a surrogate for the metabolic state of the cells. We observed that cells seeded in tumor ECM had higher relative levels of free NADH, consistent with a higher glycolytic rate, than those seeded in normal ECM. These results demonstrate that the ECM plays an important role in the growth of cancer cells and their associated vasculature.

1. Introduction

The extracellular matrix (ECM) is a complex network of proteins and glycosaminoglycans that provides biochemical and biomechanical signaling cues to the cells it surrounds. ECM disruptions can occur in the physical (porosity, fiber orientation), biochemical (composition and growth factor binding capacity), and biomechanical (stiffness) properties of the ECM, and some of these properties help to regulate cell differentiation, adhesion, survival, migration and proliferation rates [1].

There is a growing interest in the study of tumor cell-matrix interactions as these may contribute to cancer development by promoting cell dedifferentiation and cancer stem cell division. For example, the development of tumors has been linked to the desmoplastic reaction [2] which includes the production of large amounts of ECM by myofibroblasts, often in response to inflammation, leading to dramatic tissue remodeling. In response to tumor-derived signals, stromal cells express numerous ECM components, including collagens (I, III, IV, V, XII), proteoglycans and hyaluronan, which help create a more permissive environment for cancer propagation [3]. As a result of these changes in the ECM, the fiber conformation is modified as well as the tumor tissue stiffness [4]. Evidence suggests that these ECM changes can alter tumor growth and differentiation [5]. In particular, collagen can enhance cell proliferation and migration and, as a result, promote cancer cell proliferation [6].

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http://dx.doi.org/10.1016/j.biomaterials.2016.11.034
0142-9612/

Both *in vivo* and *in vitro* approaches have been used to study the process of cancer progression. *In vivo* tumor models are ideal as they more closely reproduce the native evolution of cancer and its microenvironment. However, it is difficult to control the environment in these models, and visualization of cell-cell and cell-ECM interactions, and quantification of results is problematic without specific imaging technology [7]. In contrast, *in vitro* models are easier to manipulate, offering the opportunity to probe key biological features of several of the stages of cancer progression. However, 2D monolayer cultures have predominated and these fail to model key aspects of the tumor microenvironment, including 3D geometry, porosity, density of ECM binding sites, ECM heterogeneity, and gradients of biochemical factors, among others.

Angiogenesis – the formation of new blood vessels from pre-existing vessels – is a critical feature of solid tumor growth that cannot be reproduced in a 2D environment. Without its own vasculature a tumor cannot grow beyond a few millimeters in size [8]. The angiogenic process starts when tumor cells become hypoxic and those cells and stromal cells (e.g. fibroblasts) secrete diffusible chemical signals, collectively called angiogenic factors. This process activates the expression of matrix metalloproteinases (MMPs) by endothelial cells, enabling their migration away from the parent vessel as a new sprout. ECM morphology and fiber orientation have strong effects on EC migration, sprout extension rate and vascular characteristics [9].

It is now well established that 3D *in vitro* experiments provide a better approximation of the *in vivo* tumor cell microenvironment than do 2D cultures. It has been shown that cancer cell morphology [10], cell migration [11], cell proliferation rates [12] and gene expression [13] are all different in 2D versus 3D cultures. In particular, 3D *in vitro* experiments of tumor spheroids showed upregulated expression of angiogenic factors compared to cells seeded in 2D [14], while other work demonstrated that cells in a 2D environment have lower IC_{50} to drugs for tumor cells than cells in 3D [15]. Finally, it is well known, from the results of Bissell and collaborators, that tumor cells de-differentiate when cultured in 2D, whereas in 3D cultures they adopt morphologies similar to the ones seen *in vivo* [5,16]. These are just some examples of the importance of studying tumor cells in the correct context.

Recent work looking at tumor cells in a 3D setting *in vitro* have focused on tumor cells or tumor spheroids, grown in Matrigel, collagen or fibrin [17,18]. Matrigel is highly enriched for laminin 111, but lacks the substantial amounts of collagen normally seen in tumor matrix [19]. Collagen and fibrin, while effective *in vitro* matrices, do not capture the complexity of native matrix. Recent advances in 3D matrices for cell culture include artificial hydrogel systems (e.g. cross-linked peptide-based gels) [20], tissue extracted ECM gels [21], and cell derived matrices [22]. Importantly, hydrogels containing ECM extracted from tissues using decellularization techniques contain almost all of the protein of native tissues and in the correct protein ratios. Furthermore, these gels allow for other tunable features such as rheological properties and fiber density, [23]. Additionally, through polymerization, these gels form three-dimension scaffolds similar to collagen and fibrin gels [21].

Here, we have focused on colorectal cancer (CRC) as a model system as it is the world's fourth most deadly cancer and every year in America 150,000 people are diagnosed with CRC, and 50,000 die of the disease. We have previously published on the importance of the 3D microenvironment when studying CRC drug responses [24] and we extend those studies here. Specifically, we tested the hypothesis that normal and tumor ECM affect both blood vessel and tumor growth and that this can be recapitulated using reconstituted ECM. We focused on reproducing the native ECM microenvironment by decellularizing, both normal and tumor tissues for incorporation into 3D hydrogels. We found that normal (n)ECM and tumor (t)ECM have different protein compositions, and that significant differences in vascular characteristics and tumor growth are seen in the different matrices. Additionally, using Fluorescence Lifetime Imaging Microscopy (FLIM), we noted a dramatic shift to a more glycolytic metabolism in tumor cells growing in tECM versus nECM.

2. Materials and methods

2.1. Materials, cells and animals

Endothelial growth medium-2 (EGM-2) was obtained from Lonza (Basel, Switzerland), DMEM was from Corning (Corning, New York), Fetal Bovine Serum (FBS) was from Gemini Bio Products (Sacramento, California). Sodium deoxycholate (SDC), Triton-X100 and pepsin were from Sigma-Aldrich (St. Louis, Missouri), sodium dodecyl sulfate (SDS) was from Bio-Rad (Hercules, California), DNAse was from Worthington (Lakewood, New Jersey), Antibiotic-Antimycotic, 1X and 10X Dulbecco's phosphate buffered saline (DPBS) and Trizol were obtained from Life Technologies (Carlsbad, California). Fibrinogen 90% clottable bovine was from MP Biomedicals (Santa Ana, California), thrombin (1.3 Units/ml) was from Sigma-Aldrich (St. Louis, Missouri) and polydimethylsiloxane (PDMS) was from Dow Corning (Midland, Michigan). Gene primers were synthesized by Integrated DNA Technologies (Coralville, Iowa). Carboxylated 2 μm beads were purchased from Bangs Laboratories (Fishers, Indiana).

Endothelial colony forming cells (ECFC) were isolated from human umbilical cords obtained from the University of California (UC), Irvine Medical Center, under an approved Institutional Review Board according to [25]. The normal human lung fibroblasts (NHLF) were purchased from Lonza (Basel, Switzerland) and colon tumor cell lines (SW620, SW480, HCT116) were obtained from UC Irvine's Chao Family Comprehensive Cancer Center. ECFC were cultured in EGM-2 and used between passages 4–8. NHLF (used between passages 4–8), SW620, SW480 and HCT116 were cultured in DMEM containing 10% FBS. All cells were cultured at 37 °C, in a 5% CO_2 incubator. The ECFC and cancer cells were transduced with lentivirus expressing mCherry (LeGO-C2 (plasmid # 27339)) or green fluorescent protein (GFP), (LeGO-V2 (plasmid # 27340), from Boris Fehse (Addgene, (Cambridge, Massachusetts)). Human, anonymous, discarded normal colon tissue and tumor tissue were obtained from the Medical Center, UC Irvine, following an Institutional Review Board-approved protocol.

NOD/SCID mice were purchased from Jackson Laboratories (Bar Harbor, Maine). All *in vivo* studies were completed under the approval of the IACUC committee at UC, Irvine. All the animal work was carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Human colon submucosa decellularization

Human discarded colon tissue was obtained from the UC Irvine Medical Center and frozen at −80 °C for at least 48 h to induce cell lysis. The tissue was then thawed in sterile ddH_2O for 1 h, and the colon submucosa was isolated by mechanically removing the fat, mucosa and the colon muscle layers. The submucosa was cut into small pieces (approximately 3 × 3 mm length) and washed with ddH_2O for 1 h. The tissue was then washed with 2X phosphate buffered saline (PBS) for 1 h and then washed with 2% SDC for six days (our previous studies with porcine and human colon submucosa using 1% SDS resulted in low collagen content compared with the 2% SDC-treated (Fig. S1), and recent decellularization studies showed that SDC is preferred for thin tissues because it is