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3D extracellular matrix interactions modulate tumour cell growth, invasion and angiogenesis in engineered tumour microenvironments

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ABSTRACT

Interactions between tumour cells and extracellular matrix proteins of the tumour microenvironment play crucial roles in cancer progression. So far, however, there are only a few experimental platforms available that allow us to study these interactions systematically in a mechanically defined three-dimensional (3D) context. Here, we have studied the effect of integrin binding motifs found within common extracellular matrix (ECM) proteins on 3D breast (MCF-7) and prostate (PC-3, LNCaP) cancer cell cultures and co-cultures with endothelial and mesenchymal stromal cells. For this purpose, matrix metalloproteinase-degradable biohybrid poly(ethylene) glycol-heparin hydrogels were decorated with the peptide motifs RGD, GFOGER (collagen I), or IKVAV (laminin-111). Over 14 days, cancer spheroids of 100–200 μm formed. While the morphology of poorly invasive MCF-7 and LNCaP cells was not modulated by any of the peptide motifs, the aggressive PC-3 cells exhibited an invasive morphology when cultured in hydrogels comprising IKVAV and GFOGER motifs compared to RGD motifs or nonfunctionalised controls. PC-3 (but not MCF-7 and LNCaP) cell growth and endothelial cell infiltration were also significantly enhanced in IKVAV and GFOGER presenting gels. Taken together, we have established a 3D culture model that allows for dissecting the effect of biochemical cues on processes relevant to early cancer progression. These findings provide a basis for more mechanistic studies that may further advance our understanding of how ECM modulates cancer cell invasion and how to ultimately interfere with this process.

Statement of Significance

Threedimensional *in vitro* cancer models have generated great interest over the past decade. However, most models are not suitable to systematically study the effects of environmental cues on cancer development and progression. To overcome this limitation, we have developed an innovative hydrogel platform to study the interactions between breast and prostate cancer cells and extracellular matrix ligands relevant to the tumour microenvironment. Our results show that hydrogels with laminin- and collagen-derived adhesive peptides induce a malignant phenotype in a cell-line specific manner. Thus, we have identified a method to control the incorporation of biochemical cues within a three dimensional culture model and anticipate that it will help us in better understanding the effects of the tumour microenvironment on cancer progression.

1. Introduction

Interactions between tumour cells and their microenvironment are known to play a critical role in cancer progression [1–3]. More than 80% of human cancers originate from epithelial tissues; the most commonly affected tissues in women and men are breast and prostate, respectively [4]. The epithelium of normal breast or prostate acini is surrounded by the basal membrane, a highly organised and specialised extracellular matrix (ECM) layer rich in laminins, collagen type IV, entactin and proteoglycans such as perlecan [5,6]. Beyond the basal membrane a less structured underlying stromal compartment is located, which is a fibrous ECM

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predominantly composed of collagen I, and glycoproteins such as laminin and fibronectin, interwoven with a network made out of glycosaminoglycans (GAGs) [7,8]. The tumour stroma furthermore contains various cell types including fibroblasts, endothelial cells, adipocytes and immune cells. Interactions between tumour cells and these cell types as well as ECM molecules attribute to events relevant to cancer progression, such as uncontrolled proliferation, loss of apical-basal polarity, invasion of the basement membrane, and tumour angiogenesis [9–13]. During cancer progression the tumour ECM is manipulated by cancer cells themselves and/or by activated stromal cells. For instance, a study detected loss of laminin-332 and collagen IV and VII in prostate cancer specimens associated with poor prognosis [5,14,15]. In breast cancer specimens, low laminin [16] and high fibronectin [17] levels were detected. Also, increased expression of collagen type I and a more linearised fibril structure have been associated with breast [7,18] and prostate [19] cancer progression.

Similar to normal cells, invading cancer cells adhere to ECM proteins via various cell adhesion molecules such as integrins. Integrins are heterodimeric cell surface receptors recognising a specific set of ECM proteins. In response to signals provided by the cell microenvironment, they regulate intracellular signalling pathways that control essential cellular processes, such as migration, proliferation and survival [20,21]. Since these pathways are commonly altered in cancer, integrins are interesting targets for anti-cancer treatments [22,23]. Integrins are also very well-known mechanosensors: integrin downstream signalling can be potentiated by increased stiffness of the tumour microenvironment [24]. This is relevant in breast cancer, for instance, since aforementioned increase in collagen type I deposition and fibre straightening typically stiffen the tumour stroma [7,25]. Increased activation of integrin downstream signalling in response to stiffened tumour stroma has therefore been associated with increased cancer cell invasion *in vitro* and *in vivo* [24,26]. Taken together, there is strong evidence that altered cell-ECM interactions can drive tumour progression. Thus, understanding the basis of cancer cell-ECM interactions is vital to improving our understanding of tumour progression, and also offers opportunities to develop strategies to interfere with it.

While a lot of research on cell-ECM interactions has been conducted using tissue culture plastic coated with ECM proteins, it is increasingly acknowledged that artificial mechanical and geometrical cues are imposed on cells cultured in such 2D cultures, which are therefore far from the physiological conditions of native tissues [27–29]. Several studies have evaluated 2D and 3D cancer studies and highlighted the benefits of a 3D cancer model [30–33]. However, most of the currently used biomaterials for 3D culture models do not allow for the independent control of the microenvironment's mechanical and biochemical features. For instance, numerous studies have employed natural hydrogels, such as recombinant basement membrane extracts (e.g. Matrigel) [34] or collagen type I gels [24] as 3D culture system. For these gels, however, the range of stiffness values that can be obtained is limited. Also, collagen type I and Matrigel were recently compared regarding their effects on breast cancer cell invasion: while breast cancer organoids exhibited a less invasive phenotype within Matrigel, collagen type I induced the dissemination of single cells from the tumour mass [35]. The mechanical and biochemical properties of collagen type I and Matrigel, however, are difficult to control independently from each other, which complicates a systematic comparison of both hydrogels. Thus, in a further study, synthetic poly(ethylene) glycol (PEG)-based hydrogels were employed to more systematically test the effect of different ECM ligands [36]. Such PEG hydrogels can be equipped with specific integrin-binding sites and MMPs cleavage sites to allow for cell migration [37], such as shown in different studies on ovarian and prostate cancer cell lines [38,39]. Another more recent study has employed reconstituted basement membrane matrix-alginate gels to independently modulate ECM ligand density and gel stiffness and found that both factors cooperatively induced malignant phenotypes in MCF10A cells [40]. However, although these gels are highly suitable to test the effect of microenvironment stiffness on epithelial cells mimicking a very early stage of tumour growth and progression, the specific effect of ECM components in these processes cannot be unravelled.

In the present study, we set out to investigate the effect of biochemical cues of the microenvironment on early events of cancer progression in a more systematic way; for that purpose, we used matrix metalloproteinase-degradable biohybrid PEG-heparin hydrogels that we have recently developed (Fig. 1A) [41]. Briefly, the hydrogel network is formed via Michael-type addition between PEG-peptide conjugates containing free cysteine thiol groups at the peptide sequences and maleimide-functionalized heparin at neutral pH. The mechanical properties of the gels can be tuned by altering gamma, the molar ratio of PEG and heparin-maleimide components. The negatively charged heparin groups further serve as binding sites for growth factors provided within the cell culture medium [41]. We have recently shown that this hydrogel platform is suitable for the design of tumour angiogenesis microenvironments of breast and prostate cancer cells with vascular endothelial cells and mesenchymal stromal cells (MSCs) [42,43]. In the current study, we cultured breast and prostate cancer cell lines as monocultures or in co-culture with endothelial cells and MSCs within PEG-heparin gels, which were functionalised with specific cell adhesion motifs commonly found in the ECM surrounding the epithelium. We firstly show that within stiffness ranges relevant to breast and prostate cancer tissue, these integrin binding motifs had distinct effects on cancer cell morphology, invasion, metabolic activity and proliferation in a cell-type dependent manner. The results highlight the potential of our culture model for studies of cell-ECM interactions.

2. Materials and methods

2.1. Synthesis and purification of PEG-peptide conjugates

The hydrogel platform used consisted of four-arm poly(ethylene glycol) PEG-peptide conjugate (equipped with cell-adhesive peptide and MMP cleavable sites) and heparin, chemically modified with maleimide groups (Fig. 1A) [41]. The MMP-cleavable PEG-peptide conjugates PEG-$(MMP)_3$-$(GFOGER)_1$ (modified with a collagen(I)-mimetic cell-adhesive peptide [44,45]), PEG-$(MMP)_3$-$(SIKVAV)_1$ (modified with a laminin-derived adhesion peptide [46]), PEG-$(MMP\text{-}RGD)_4$ (modified with a fibronectin-derived adhesion site within the MMP sequence [47]) and PEG-$(MMP)_4$ were synthesized analogous to the given reference [41], which is further described in detail below. All organic solvents were purchased from IRIS Biotech GmbH (Marktredwitz, Germany). Ultra-high purity (UHP) water was prepared to 18.2 MΩ cm at 25 °C on a Milli-Q® Integral System (Merck Millipore, Darmstadt, Germany). All reagents were used without preliminary purification.

2.2. Synthesis and purification of cysteine-terminated PEG-$(MMP)_3$-$(GFOGER)_1$ & PEG-$(MMP)_3$-$(SIKVAV)_1$

All reaction steps were performed at room temperature (21 °C). 764.5 mg four-arm PEG-$(Maleimide)_4$ (PM, 0.072 mmol, molecular weight 10600 g/mol, JenKem Technology USA Inc., Allen, USA) was dissolved in 5 ml of 50/50 UHP water/acetonitrile (AcN) mixture.

PEG-$(MMP)_3$-$(GFOGER)_1$: While dissolving the polymer via gentle stirring, 93.4 mg (0.076 mmol, 5% excess) GFOGER peptide (H_2N-CWGPOGFOGER-$CONH_2$, O = L-hydroxyproline, Supplemental

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