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Cell specific ingrowth hydrogels

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ABSTRACT

Extracellular mimetic hydrogels formed from peptide crosslinkers and polyethylene glycol monomers permit cell-controlled invasion. The use of matrix metalloproteinase specific peptides might further allow for selective control of different cell-type invasion. In this study, the invasion of fibroblasts and vascular smooth muscle cells (VSMC) into hydrogels polymerised with either a peptide generally permissive for matrix metalloproteinase (MMP) degradation or peptides preferentially cleaved by MMP-14 or MMP-9 enzymes were compared. The two cell-types invaded the MMP permissive hydrogel equally. However, invasion of VSMC into MMP-14 selective peptide crosslinked hydrogels was diametrically opposite in nature to that of fibroblasts whereby VSMC showed a two-fold increase into these hydrogels relative to that observed in permissive hydrogels whilst fibroblasts had a relative two-fold decrease ($p < 0.01$). These findings are suggestive that invasion and growth of different cell-types in engineered synthetic extracellular matrix mimics may be controlled selectively by the choice of protease specific peptide crosslinker and this could have general utility in tissue regenerative and engineering approaches.

1. Introduction

An end goal of regenerative medicine is the replacement of temporary matrices or scaffolds by site-specific host tissue. It is critical for a regenerative matrix to be designed such that degradation occurs at the same rate as tissue ingrowth [1]. Further to this, an ideal ingrowth matrix should also allow for the preferential ingrowth of preferred cell-types. For example, it could easily be envisaged that a vascular graft designed to be replaced by appropriate host tissue might have a matrix within its walls that encourages vascular smooth muscle cell (VSMC) invasion.

There are a number of bioactive modalities that could be exploited towards the achievement of selective ingrowth. We ourselves initially explored selectively controlling cellular migration via the use of different combinations of adhesive peptide sequences on a PEG hydrogel surface [2]. Endothelial cell migration was enhanced on a surface derivatised with an integrin binding RGD sequence and YIGSR, a bioactive sequence that interacts with laminin binding protein while VSMC migration speed was unaffected by this peptide combination.

We have previously proposed another mechanism that might allow for selectively controlling cellular invasion, namely the targeting of the specific repertoire of proteases utilised by a desired cell type to degrade a pathway into a matrix [3]. In essence, through the engineering of a synthetic matrix with distinct protease specific degradation sites a milieu might be created that is more easily invaded by one cell-type over another. This concept stemmed directly from the original development of fully synthetic extracellular matrix (ECM) mimics that allow for the introduction of specific protease cleavage sites [4–6]. These mimics are based on the reaction of end-functionalized multiarm polyethylene glycol (PEG) macromers with bis-cysteine peptide sequences residues via Michael-type addition under physiological conditions, resulting in the formation of a peptide crosslinked network. Other bioactive molecules such as integrin binding peptides and growth factors can be appended via free cysteine moieties. In studies with these ECM mimics, it was shown that embedded fibroblasts were able to migrate within hydrogels crosslinked with matrix metalloproteinase (MMP) sensitive peptides but could not in hydrogels

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polymerised with plasmin sensitive peptides [7]. Therefore it was concluded that the fibroblasts lacked a key component of the plasminogen activation and that this substantially limited their movement in the plasmin sensitive gels. Thus, it is reasonable to extrapolate from these findings that such hydrogels may be designed to differentially alter cell-type ingrowth by varying the protease specificity of the crosslinking peptide sequences.

As certain MMPs appear to only be expressed by specific cell-types such as MMP-8 by neutrophils and MMP-12 by macrophages [8], they are potentially an ideal grouping of candidate proteases for cell specific hydrogels. There is other evidence of cell specificity for MMP expression with the general limitation of MMP-13 to bone and cartilage under physiological conditions, most likely due to the presence of a RUN-X2 site in the MMP-13 promoter and the preferential expression of the RUN-X2 protein in the bone and cartilage cells [9]. However, in most instances cellular patterns of MMP expression and their functional regulation by factors such as TIMPs will be extraordinarily complex. Indeed it has been suggested that MMPs form part of a "protease web" via their interconnections with other protease cascades and networks [10]. Therefore attempting to target cell specific proteases in order to determine their selective influence on a cell's ability to invade and migrate within a matrix might at present be overly challenging. It may make more sense to begin by empirically determining what influence peptide networks that have the ability to be cleaved preferentially by specific proteases might have on the invasion of different cell-types.

This approach is facilitated by the recent emergence of a substantial number of peptide sequences that are highly specific and sensitive to individual MMPs [11–16]. The influence of protease specific peptides as crosslinkers for PEG hydrogels on cellular invasion has been systematically investigated by Patterson and Hubbell [17]. Here the impact of the crosslinking of PEG hydrogels with peptides optimised for cleavage by MMP-1, MMP-2, MMP-3, MMP-7, MMP-9 and MMP-14 (identified mainly by reiterative cleavage of oriented peptide libraries [16]) on 3D spreading and proliferation of fibroblasts was determined. The principal interest was the identification of hydrogels that were more rapidly invaded by fibroblasts and cells from aortic ring implants in order to distinguish peptide sequences that might facilitate hydrogel remodelling in tissue repair applications. It was also recognised that particular sequences could be incorporated to target individual cell-types such as endothelial cells. Most recently, the relative surface degradation of a PEG hydrogel polymerised with a cathepsin K specific peptide by osteoblasts and osteoclasts grown in 2D was determined [18]. In a finding suggestive of selective degradation, greater pitting was observed for osteoblasts.

However in order to truly determine selectivity, the invasion of two or more cell-types should be directly compared in two or more hydrogels crosslinked with different enzyme specific cleavage sequences. We have begun this process by choosing two sequences that are specifically cleaved by MMP-9 or MMP-14. The sequences, SGRSENIRTA (MMP-14 specific) and KGPRQIT (MMP-9 specific) were identified by a phage display strategy by Kridel et al. [12,13] to have very high k_{cat}/K_m values relative to those of other MMPs assayed. These two MMPs are of general interest with MMP-14 having been shown to be a critical MMP in cellular invasion of matrices [19] whilst MMP-9 appears to have relatively circumscribed expression in cells such as keratinocytes, monocytes and tissue macrophages [20]. We have directly compared the invasion of fibroblasts and VSMCs from 3-D cellular spheroids into PEG hydrogels polymerised with these sequences. Additionally, as a further comparative matrix, hydrogels were also formed with peptides containing the sequence GPQGIWGQ, a sequence that is cleaved by a wide range of MMPs [5] and has been utilised in a number of ECM mimic studies [5,6,17,21–23].

2. Methods

2.1. PEG gel formation

Vinyl sulfone derivatised 4-arm PEG of 20 kDa M_w (20PEG-4VS) was prepared as described previously [5]. Gels were formed by a Michael-type conjugate addition between equimolar ratios of vinyl sulfone groups of the derivatised PEG and thiols of cysteine residues on bis-cysteine MMP-sensitive peptides. The following bis-cysteine MMP-sensitive peptides were utilised: GCREGPQGIWGQERCG (Pan-MMP M_w 1733 Da); GCRESGRSENIRTAERCG (MMP-14 M_w 1982 Da) and GCREKGPRQITERCG (MMP-9 M_w 1690) (GenScript USA Inc., Piscataway, NJ, USA). Additionally the following mono-cysteine cell adhesion sequence was used: GCGYGRGDSPG (RGD M_w 1025 Da). Gels of 3.5% m/v were prepared by dissolving 87.5 nmoles of 20PEG-4VS in 7 μl phosphate-buffered saline (PBS, pH 7.5). This solution was then mixed with 7 nmoles RGD in 4.5 μl PBS and incubated at 37 °C for 30 min. For polymerisation 175 nmoles of a bis-cysteine peptide in 8 μl PBS were then added and the solution made up to a final volume of 20 μl with PBS.

2.2. Characterisation of PEG gels

In order to determine the swelling characteristics of the variously crosslinked gels, individual gel volumes ($n = 3$) were determined by weighing gels directly after gelation (Vg), after equilibrium swelling (Vs), and after drying (Vd) using a 5-decimal balance equipped with a density determination kit (Mettler Toledo). The molecular mass between crosslinks (Mc) was determined using the gelled and swollen-polymer fractions, and mesh size subsequently calculated as described by Andreopoulos et al. [24].

Storage and loss moduli (G' and G'') of swollen gels ($n = 4$) were obtained by small strain oscillatory shear rheometry on a TA ARES 1500 rheometer [4].

2.3. Determination of enzyme specificity

In order to be able to track enzymatic degradation of PEG gels, a far red fluorescent label was incorporated into the gels. PEG gels crosslinked by the respective MMP-sensitive sequences were formed as described above except the crosslinking peptide was labelled via a cysteine with Alexa Fluor® 660 maleimide (Invitrogen Molecular Probes, Eugene, Oregon, USA) prior to gel polymerisation. In brief, 175 nmoles of the relevant bis-cysteine peptide in 8 μl PBS was reacted with 0.35 nmoles Alexa Fluor® 660 maleimide in 1 μl PBS for 30 min at 37 °C (1 Alexa Fluor® 660 molecule per 1000 cysteine molecules). 5 μl gels were formed by aliquoting the gel solution after addition of the crosslinking peptide and prior to polymerisation. After 1 h polymerisation at 37 °C, each 5 μl gel was then washed in 2 changes of 400× volumes of Hepes buffered saline (HBS, pH 7.4) over 48 h at 37 °C to remove unincorporated label. For enzymatic digestion, the following enzymes were utilised: MMP-9 and the catalytic domain of MMP-14 (generous gift of Hideaki Nagase, Imperial College London). After washing, the gels were incubated with 100 nM MMP-9 or 200 nM MMP-14 enzymes in 20 μl Tris (50 mM), $CaCl_2$ (10 mM), NaCl 150 mM Brij 0.05% v/v, $ZnCl_2$ (50 μM) pH 7.5 at 30 °C with gentle agitation. MMP-9 enzyme was pre-activated with p-aminophenylmercuric acetate at 1 mM for 16 h at 37 °C. A 5 μl aliquot of supernatant was temporarily removed at the required time points from the gel digestions and fluorescence quantified in a Biorad fluorescent plate reader with excitation set at 663 nm and emission at 690 nm. The relative extent of gel degradation was also assayed by determining the swollen mass of the gels and by a modified fluorescamine assay [5]. In brief, the gels were non-enzymatically degraded by incubating in 1 M NaOH for 3 h at 70 °C, the resultant digest was reacted with fluorescamine (Sigma–Aldrich, Chemie GmbH, Steinheim, Germany) and fluorescence determined with excitation set at 400 nm and emission at 460 nm.

2.4. Human dermal fibroblast and vascular SMC isolation and culture

Both human dermal fibroblasts (HDF) and human vascular smooth muscle cells (VSMC) were isolated by explant culture release. The protocols have been described in detail for both HDF [25] and VSMC [2]. In brief, fibroblasts were isolated by culturing of 3 mm skin biopsies in minimal MCDB131 media/10% foetal calf serum (FCS) until sufficient fibroblasts had migrated out of the biopsy to allow for trypsinisation. Fibroblasts were positively characterised by their swirling growth pattern. VSMC were obtained from discarded portions of aortas from heart transplant donors. After removal of the adventitial layers, 1 mm tissue pieces were similarly cultured as described for fibroblasts. VSMC were identified by both their characteristic hill and valley growth pattern and positive staining for alpha-SMC actin. Cells were used between passages 3–5.

All experiments involving human materials were performed after approval from the University of Cape Town (Cape Town, South Africa) Human Ethics Committee, in accordance with the Declaration of Helsinki.

2.5. Spheroid based spout formation assay

Spheroids containing 750 cells were generated by the method described by Korff and Augustin [26,27]. In brief, trypsinised VSMC or HDF were resuspended in