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The selective modulation of endothelial cell mobility on RGD peptide containing surfaces by YIGSR peptides

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Abstract

The ability of the biomimetic peptides YIGSR, PHSRN and RGD to selectively affect adhesion and migration of human microvascular endothelial cells (MVEC) and vascular smooth muscle cells (HVSMC) was evaluated. Cell mobility was quantified by time-lapse video microscopy of single cells migrating on peptide modified surfaces. Polyethylene glycol (PEG) hydrogels modified with YIGSR or PHSRN allowed only limited adhesion and no spreading of MVEC and HVSMC. However, when these peptides were individually combined with the strong cell binding peptide RGD in PEG hydrogels, the YIGSR peptide was found to selectively enhance the migration of MVEC by 25% over that of MVEC on RGD alone ($p<0.05$). No corresponding effect was observed for HVSMC. This suggests that the desired response of specific cell types to tissue engineering scaffolds could be optimized through a combinatory approach to the use of biomimetic peptides.

Keywords: Microvascular endothelial cells; Peptide; Cell mobility; Polyethylene glycol; Tissue engineering

1. Introduction

Tissue engineering is principally concerned with the replacement of tissue that has lost function due to injury or disease. In general, it is possible to distinguish between two main approaches towards this goal. The one approach involves the in vitro culturing of cells on biodegradable polymeric scaffolds to form neo-organs that are then implanted into the body at the necessary anatomical site. In contrast to this more direct route, the second approach aims at in situ tissue regeneration through provision of an acellular, polymeric matrix that assists the ingrowth of remodeling cells. Ideally, the matrix should not only attract and enhance the growth of these cells but also selectively facilitate the ingrowth of preferred cells (e.g. endothelial cells in a vascular graft). Thus, in the quest to realize these goals for cellular interactions with ingrowth matrices, bioactive factors have been incorporated to optimize the adhesion, migration and proliferation of desired cells. These agents can either be added in a soluble form (e.g. growth factors [1]) or grafted to the matrix (e.g. adhesive peptides [2]).

Although surface grafting of bioactive molecules has been investigated for many years, new possibilities have arisen with the advent of fully synthetic ingrowth matrices. One of the most promising candidates for such synthetic ingrowth matrices is polyethylene glycol (PEG). Its inertness and notable lack of adhesiveness for cells makes it an ideal basic material for the incorporation of bioactive peptides [3]. The obstacle of incorporating such peptides into an inert hydrogel was overcome recently by a cross-linking method that generates PEG hydrogels from PEG-multiacrylates and PEG-dithiols through a Michael-type addition reaction [4]. This addition of thiols onto unsaturated

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esters also allows for the specific and quantitative coupling of cysteine-containing peptides, which can then act as degradation and/or adhesive sites [5].

The tripeptide RGD, present in all major extracellular matrix (ECM) proteins (fibronectin, collagen, laminin) has been the most intensively investigated cell-binding sequence. The RGD sequence has been shown to enhance adhesion and spreading of fibroblasts, endothelial cells and smooth muscle cells when the peptide was grafted onto different surfaces [6]. Migration of smooth muscle cells and fibroblasts has been found to be biphasically dependent on the concentration of RGD attached to the migratory surface with intermediate loadings of RGD displaying maximal mobility [7,8].

PHSRN, a cell binding sequence found only in fibronectin, has been shown to act synergistically with RGD for cell adhesion [9]. Applied individually, it stimulated invasion of serum-free extracellular matrices by keratinocytes and fibroblasts in vitro and enhanced the re-epithelialization and contraction of dermal wounds in healing-impaired diabetic mice [10].

YIGSR, a peptide derived from the laminin B1 chain, represents a class of adhesive peptides that, unlike RGD and PHSRN, does not interact with the integrin family of cell receptors but with the 67 kDa laminin binding protein (LBP) [11,12]. This peptide was found to promote adhesion and spreading of a large number of cell types including endothelial cells, fibroblasts and smooth muscle cells when grafted onto glass [13]. In addition, YIGSR was found to be pro-migratory for melanoma in a soluble form [14].

Thus, with cardiovascular tissue engineering in focus, RGD, PHSRN and YIGSR-derivatized PEG hydrogels were investigated regarding their ability to preferentially influence the adhesion and migration of MVEC and HVSMC.

2. Materials and methods

All reagents were purchased from Sigma Chemical Co (St. Louis, MO), unless otherwise stated.

2.1. PEG acrylation

Polyethylene glycol (PEG, 20 kDa, 8-arm, Shearwater Corporation) was acrylated according to a method similar to that described by Elbert et al. [4]. Briefly, a 10 m% PEG solution in toluene was dried by azeotropic distillation (removing a third of the volume) and diluted back to original concentration through anhydrous addition of dichloromethane (DCM). After cooling in an ice bath and addition of 50% molar excess of triethylamine (TEA), acryloyl chloride (AcCl) (50% molar excess) was added dropwise over 5 min. The ice bath was removed and the reaction continued under an Argon atmosphere at room temperature (RT) for 24 h. Subsequent filtering, precipitation (3 times into cold hexane), extraction from an aqueous solution (10% PEG-8Ac, 0.5% NaCl, pH = 6) into DCM, final precipitation (hexane), and drying (in vacuo, 24 h, RT) resulted in the required product. The complete acrylation of the polymer was confirmed by NMR spectroscopy (Varian 400Mz), based on the absence of a $–CH_2–$OH peak in ^{13}C spectrum and on the 102% acrylation efficiency calculated from the ^{1}H peaks at 3.3–4.3 (97.76 H, $–CH_2–CH_2–O–CO–CH=CH_2$) and 5.8 ppm (dd, 0.45 H, $–CO–CH=CH_{cis}H_{trans}$).

2.2. Peptide synthesis and purification

The following peptides were synthesized. GCGGG**RGD**SPG (RGD), GCGGGV**PHSRN**SG (PHSRN) and GCGGG**YIGSR**G (YIGSR). Synthesis was performed by solid-state chemistry on resin using an automated peptide synthesizer (Perceptive Biosystems, Farmington, MA), with standard 9-fluorenylmethyloxycarbonyl chemistry. Peptides were purified by C18 chromatography (Perceptive Biosystems Biocad 700E) and analyzed by MALDI-TOF mass spectrometry.

2.3. Determination of swelling ratios

In order to determine the effect of acrylate capping on crosslink density, PEG-8Ac (62.5 mg in 250 μl 50 mM PBS) was reacted with ethanethiol (EtSH) (125 μl containing the equivalent of 0, 0.5, 2, 8 and 32 mol% of the available acrylate groups) for 1 h at 37°C. EtSH was substituted for the adhesive peptides in the swelling experiments due to the large quantity of peptides required. Crosslinking was subsequently performed by the reaction with PEG-dithiol (PEG-2SH, 3,4 kDa, in 125 μl 50 mM PBS 1 h, 37°C). The actual volumes of cylindrical gels ($n = 3$) were measured by volume displacement of hexane in a 10 ml burette, both directly after gellation (V_r) and after equilibration (V_s) in 50 mM PBS for 24 h (37°C). The swelling ratio (Q) was defined as V_s/V_r, and quoted as average ± standard error of the mean (SEM). A control experiment ($n = 3$) using the RGD peptide at 0.5 mol% capping showed the swelling ratio (2.89 ± 0.07) to be within the experimental deviation of, and not significantly different from, the gel produced after EtSH capping (0.5 mol%, 3.12 ± 0.13).

2.4. Cell culture

2.4.1. Microvascular endothelial cell isolation

Primary cultures of MVEC were prepared from neonatal foreskins as previously described [15].

Briefly the foreskin was obtained from operating theatre in a sterile container. All procedures were performed using sterile techniques in a Bio-Hazard

Laminar Flow Hood. The skin itself was removed, the remaining tissue cut to pieces of approximately 3 mm^3 and enzymatically dissociated (0.05% w/v collagenase, 0.05% w/v bovine serum albumin (BSA), 1 ml/g tissue) in a covered Petri-dish for 30 min at 37°C. The BSA solution was removed, the tissue sections were rinsed with PBS and subsequently covered with MVEC-medium (MCDB 131, 2 mM L-glutamine, 10 ng/ml epidermal growth factor (PeproTech Inc.), 5 ng/ml basic fibroblast growth factor (PeproTech Inc.), 1 μg/ml hydrocortisone, 10% foetal calf serum (FCS)). The tissue was then gently squeezed in order to extrude microvascular-associated cells and rinsed again with MVEC-medium. The cell containing medium was centrifuged, the pellet resuspended in MVEC-medium, re-centrifuged and finally seeded into a 25 cm^2 fibronectin-precoated (10 μg/ml) tissue culture flask (Corning Inc., Corning, NY). After 1 h, the MVEC-medium was replaced with MVEC-medium containing 5×10^{-4} M cAMP. Confluent stock cells were trypsinised (0.05% Trypsin/0.02% EDTA in PBS, pH 7.4), frozen at a concentration of 1×10^6 cells/ml and stored in liquid nitrogen (−196°C). For adhesion and migration experiments, cells from passage 3 to 5 were used.

2.4.2. Human vascular smooth muscle cell isolation

Primary cultures of HVSMC were prepared from the intimal-medial layer of an aorta from a heart transplant donor as previously described [16].

Briefly the aorta was aseptically dissected in theatre and placed in a sterile bottle containing MCDB 131. All procedures were performed using sterile techniques in a Bio-Hazard Laminar Flow Hood. The sample was cut down it's length and the luminal surface scraped using sterile cotton buds to remove the monolayer of endothelial cells. Subsequently the aorta was rinsed thoroughly with MCDB 131, further dissected into ~10 mm wide horizontal strips using scissors and placed in a petri dish containing MCDB 131. The adventitial layer was peeled away from the intimal-medial sections and discarded. Only the central portions of the intimal-medial strips were used for the explant. The trimmed aortic strips were cleanly cut to an explant size of ~1 mm. Explant fragments (100–150 fragments) were dispensed into 3 ml MCDB 131/10% FCS and transferred to a 75 cm^2 tissue culture flask. Minimal amounts of fresh MCDB131/10% FCS were added as needed, taking care not to dislodge explant tissue from the flask surface. Cell outgrowth from the explants was noted after 7 days and attached cells were trypsinised from the flask after 14 days. The cell suspension was spun down, resuspended in MCDB 10% FCS and transferred to a 75 cm^2 tissue culture flask. Confluent stock cells were trypsinised, frozen at a concentration of 1 × 106 cells ml and stored in liquid nitrogen (−196°C). For adhesion and migration experiments, cells from passage 3 to 5 were used.

MVEC and HVSMC cultures were identified with anti-human CD 31 (Dako A/S) and anti-HVSMC actin antibodies (Dako A/S) respectively, both by immunocytochemistry and flow cytometry analysis. Cell cultures were routinely found to be greater than 95% positive for their relevant cell marker by both methodologies.

2.5. Adhesion assay

Adhesion of MVEC and HVSMC was investigated on 20 kDa PEG-8Ac hydrogels (12.5% w/v, before swelling) after binding either a single peptide to 0, 0.5, 2, 8 and 32 mol% (RGD, PHSRN or YIGSR), or a combination of RGD (8 mol% of the available acrylate groups) with an equimolar loading of either PHSRN or YIGSR. Linear 3.4 kDa PEG-dithiol was subsequently used to crosslink the peptide-capped PEG-8AC.

Briefly, PEG-8Ac acrylate (50% of final volume), peptides (according to the dilution series mentioned above, 25% of final volume) and PEG-2SH (25% of final volume) were dissolved in 50 mM PBS, vortexed and sterilized by filtration (0.45 μm). PEG-8Ac and peptide aliquots were mixed and incubated for 1 h at 37°C. The reaction between PEG-acrylates and cysteine-containing peptides has previously been shown to go to completion [5]. After coupling the peptide to the PEG-8Ac, the PEG-2SH was added and the complete mixture immediately aliquoted into a 96-well dish (40 μl/well). After incubation for 1 h at 37°C, the crosslinked hydrogels were allowed to swell for 24 h in 50 mM PBS and then rinsed 3 times with PBS. The final peptide concentration, expressed in pmol/cm^2, was calculated to be equivalent to the number of peptide molecules in the top 10 nm of the hydrogel after swelling. This distance is considered to be the maximum effective depth for cells to bind to available adhesion sites [17]. The 0, 0.5, 2, 8 and 32 mol% peptide loadings thus correspond to 0, 0.08, 0.32, 1.24 and 3.9 pmol/cm^2.

Cells were harvested enzymatically (0.05% Trypsin/0.02% EDTA in PBS, pH 7.4), resuspended in serum-free medium (MCDB 131, 0.1% BSA, 1% ITS, 5 ng/ml bFGF), seeded onto the hydrogels (20,000 cells in 150 μl medium), and allowed to adhere for 5 h. Cell adhesion was quantified photometrically by determination of AlamarBlue™ (Biosource Inc.) reduction [18]. The hydrogels were rinsed 3 times before AlamarBlue™ containing medium (1% v/v, medium as above) was added (150 μl/well). After 6 h, 90 μl/well of the supernatant was transferred to a new 96 well plate and read at 560 nm and 590 nm to determine the amount of reduced AlamarBlue™. Results were calculated according to a standard curve of cells on tissue culture-treated plastic.

2.6. Migration assay

Migration of MVEC and HVSMC was studied on the PEG-8Ac after coupling RGD alone (8 mol%) or in combination with equimolar amounts of either PHSRN or YIGSR. These concentrations represented the highest feasible loadings, as incorporation of higher concentrations did not allow for sufficient mechanical strength in the hydrogels formed. Hydrogels (50 μl) were produced in an identical manner to that employed in the adhesion study, with the exception that they were cast between 2 glass plates (0.4 mm spacing).

The resulting hydrogel discs were transferred into a 12-well tissue culture plate and immobilized at the bottom of the plate using Teflon rings. Cells were harvested enzymatically (0.05% trypsin/0.02% EDTA in PBS, pH 7.4), resuspended in serum-free medium (MCDB 131, 0.1% BSA, 1% ITS, 5 ng/ml bFGF), seeded onto the hydrogel discs (2200 cells/cm^2 in 1.5 ml medium), and allowed to adhere for 6 h. Subsequently, the medium was replaced and allowed to equilibrate for another 6 h before the plate was placed within a computer-controlled climatised chamber (PeCon GmbH, Erbach-Bach, Germany) maintained at 5% CO2, 37°C and 100% humidity) onto the microscope stage. Single cell migration was quantified using a time-lapse videomicroscopy setup on an inverted light microscope with computerized stage (Leica DM IRBE microscope, *x* and *y* stage; Leica microscopes, Wetzlar, Germany) similar to the method described by Kouvroukoglou et al. [19]. A videocamera (Sony XC-75E; Sony Corporation, Tokyo, Japan) on the microscope was interfaced with a frame grabber board (Meteor II; Matrox Graphics Inc, Dorval, Canada) of the controlling PC, allowing for software-controlled picture acquisition. Two macros were written for the Leica QWIN image software. The first allowed picture acquisition of up to 50 different locations at 15 min intervals over a period of 10 h by synchronizing the motion of the stage with the frame grabbing. The second allowed quantification of cell locomotion from the resulting stack of 41 pictures at each location by calculating the movement of the cell nucleus for every 15 min interval. Finally, migration speed was determined from the sum of quarterhourly distances of cell nuclei divided by the total time.

2.7. Statistical analysis

All experimental results were expressed as mean ± standard error of the mean. For the adhesion assay, three replicates for each condition were carried out; multiple comparisons were performed for the adhesion results of the peptide combinations and RGD alone at the concentration of 1.24 pmol/cm^2. Migration experiments were repeated 4 times ($n=4$) with minimum 12 samples per group. Since cells from a single passage were used to seed both RGD as well as RGD plus YIGSR or PHSRN derivatized hydrogels, a paired *t*-test using two-tailed *p*-values was used to determine the statistical significance of the effect of the substrate formulation on cell migration. A *p*-value of 0.05 was accepted as confirming significance.

3. Results

3.1. Hydrogel swelling

The effect of EtSH loading on the swelling of PEG-8AC gels can be seen in Fig. 1. The non-capped

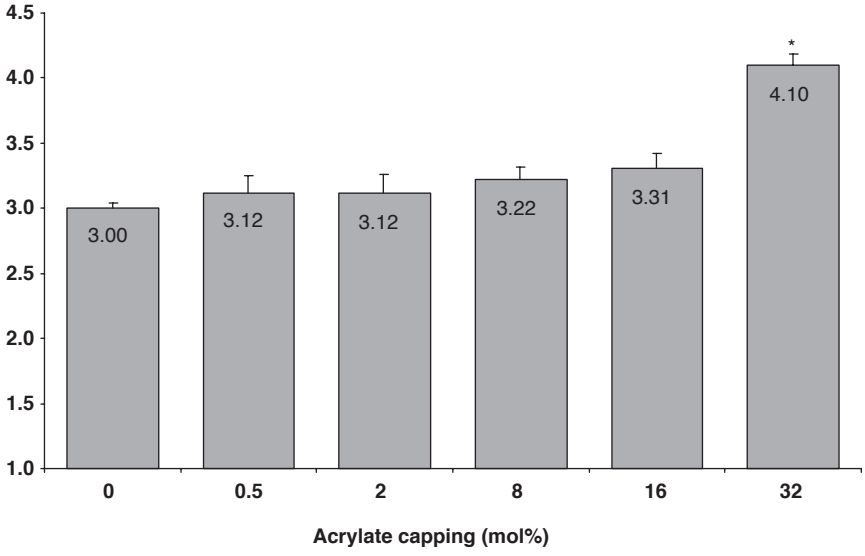

Fig. 1. Swelling ratios of PEG-8Ac depending on mol% capping of the acrylated groups. Significant difference compared with no acrylate capping at $^*p<0.05$.

hydrogels increased to 3 times their original volume upon equilibration in 50 mM PBS. Of the derivatised hydrogels, only the PEG with 32 mol% capping of acrylate end-groups showed a significant increase in swelling ratio (36.6%, $p<0.05$).

3.2. Adhesion and spreading of MVEC and HVSMC on hydrogels with immobilized peptides

On RGD-derived PEG, both cell types achieved maximal adherence at 0.32 pmol/cm^2 peptide content and did not increase with greater loadings (Fig. 2a,b). The adhesion to the other two peptides (PHSRN, YIGSR) increased with increasing peptide concentration for HVSMC and MVEC though the overall binding was significantly reduced compared to hydrogels derivatised with RGD ($p<0.05$). When hydrogels were derivatised with equimolar amounts of RGD and PHSRN or RGD and YIGSR (1.24 pmol/cm^2 of each peptide), HVSMC showed a significant increase in adhesion on the RGD/YIGSR surface relative to RGD/PHSRN and RGD alone ($p<0.05$). The adhesion of MVEC was not significantly altered by either combination of peptides relative to RGD alone.

Though adhesion was observed on YIGSR and PHSRN modified hydrogels, there was no spreading of the two cell types even at the maximal possible loading of these peptides (Fig. 3a,b). However, both MVEC and HVSMC were well spread on RGD modified hydrogels at 1.24 pmol/cm^2, a third

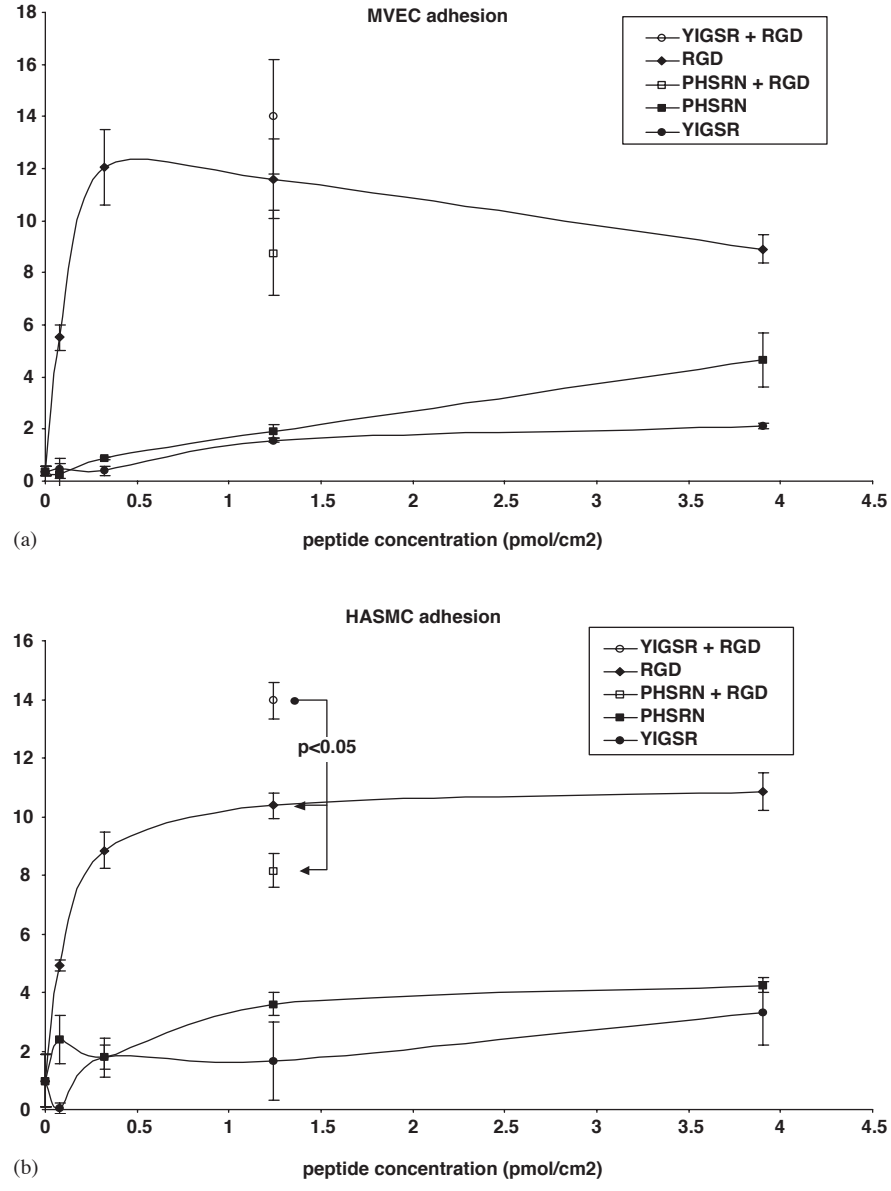

Fig 2. Adhesion of MVEC (a) and HVSMC (b) to PEG-8Ac derivatised with RGD, PHSRN, YIGSR alone or combinations of RGD plus PHSRN or YIGSR.

of the maximal possible loading (Fig. 3c,d). The equimolar combinations of RGD and YIGSR or RGD and PHSRN did not effect the spreading of MVEC and HVSMC relative to that on RGD alone (data not shown).

3.3. Single cell migration of MVEC and HVSMC on hydrogels with immobilized peptides

To determine the effect of the YIGSR and PHSRN peptides on the migration of MVEC and HVSMC whilst

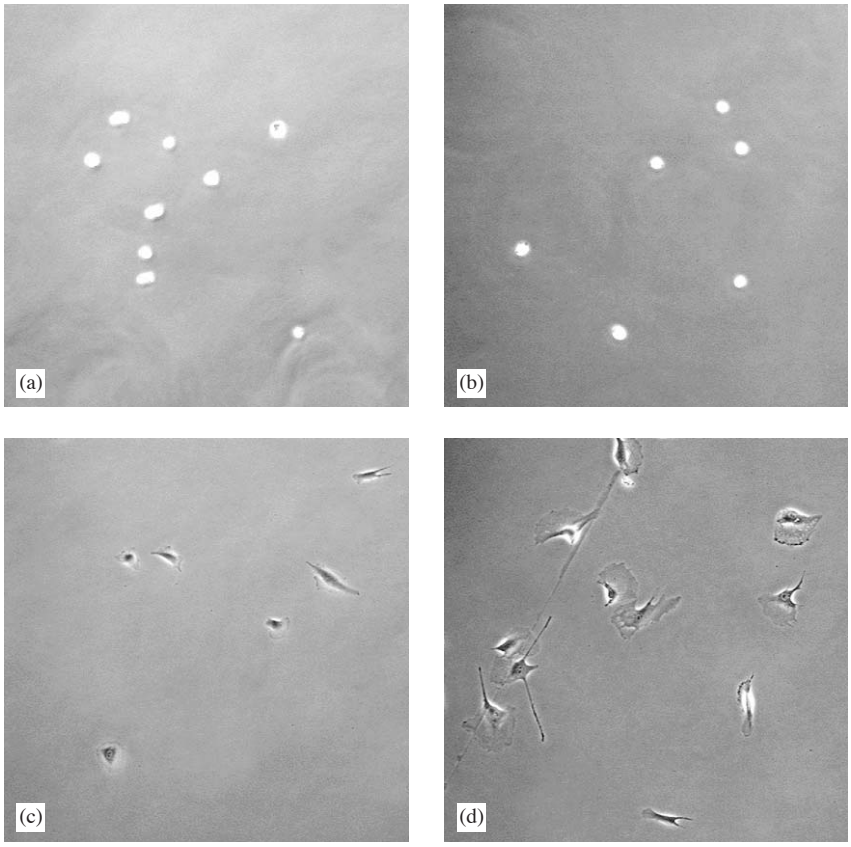

Fig. 3. Phase contrast microscopy pictures of cell spreading on single peptide derivatised PEG-8Ac: MVEC on (a) YIGSR (3.9 pmol/cm^2) and (b) PHSRN (3.9 pmol/cm^2; HVSMC similar, pictures not shown), (c) MVEC and (d) HVSMC on RGD (1.24 pmol/cm^2).

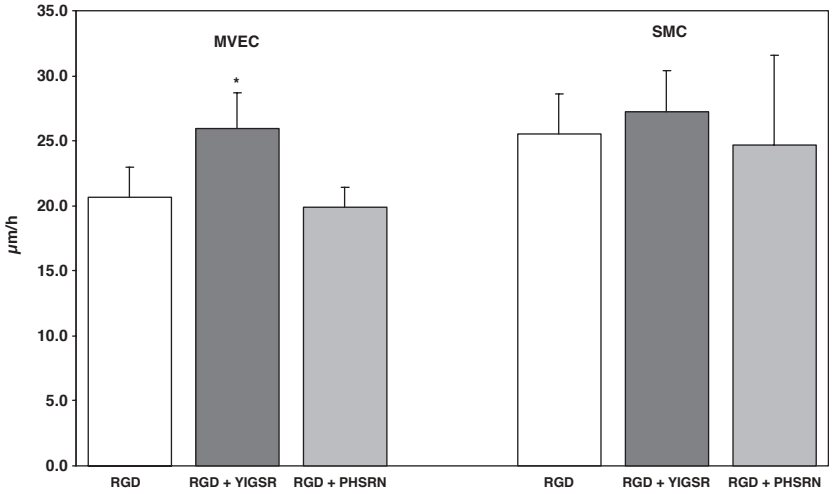

Fig. 4. Single cell migration speed on peptide derivatised PEG-8Ac: comparison of cell motility (µm/h) of MVEC and HVSMC on RGD alone (1.24 pmol/cm^2) against equimolar combinations with YIGSR or PHSRN. Significant difference compared with RGD alone at $^*p<0.005$.

these cells were interacting with the RGD peptides, time lapse video-microscopy of individual cells moving on PEG hydrogels derivatised with the relevant combination of peptides was employed. Due to the lack of spreading on either YIGSR or PHSRN modified PEG hydrogels, it was not possible to quantify migration on these peptides alone.

A migration speed for MVEC of $20.3 \pm 3.7\,\mu m/h$ was observed on RGD ($1.24\,pmol/cm^2$) modified PEG-8Ac hydrogels (Fig. 4). The addition of an equimolar amount of YIGSR resulted in an increase in speed ($25.3\,\mu m/h \pm 4.2$; $p < 0.005$), whereas the similar addition of PHSRN did not influence MVEC mobility ($20.2\,\mu m/h \pm 2.7$).

The additional derivatisation of RGD containing PEG hydrogels with YIGSR or PHSRN did not influence the mobility of HVSMC ($25.6\,\mu m/h \pm 3.0$ vs $27.0\,\mu m/h \pm 2.6$ and $24.1\,\mu m/h \pm 1.9$).

4. Discussion

The major extracellular matrix proteins (laminin, fibronectin and collagen) that directly interact with cells and are necessary for crucial functions such as adhesion, migration and proliferation have been found to contain several distinct and different bioactive regions [20]. It is, therefore, reasonable to assume that combinations of these bioactive peptides will be needed to confer increased biological activity to biomaterials [21]. We have investigated the effect of the 67 kDa LBP ligand, YIGSR [12] and the PHSRN motif from fibronectin domain FIII-9 [22] on the migration of the major vascular cells (MVEC and HVSMC) on surfaces modified with the potent cell binding RGD peptide [11].

We utilized a recently developed PEG hydrogel that allows for quantitative, covalent attachment of thiol-containing peptides for our migration experiments [5]. The Michaels addition reaction permits the attachment of a peptide via a cysteine residue placed at the amino terminal, thereby potentially maximizing exposure of the functional region.

The non-adhesivity of PEG hydrogels [17] was demonstrated here by the ability of RGD to increase cell adhesion by at least 10-fold for both cell types over that found on non-derivatised hydrogels. Though the two other peptides (YIGSR, PHSRN) investigated allowed cellular adhesion that was proportional to the amount of peptide loaded, adhesion was at a much lower level than that exhibited on RGD. In addition, no spreading of either cell type was observed on these peptides in contrast to the complete spreading observed on the RGD containing hydrogels. The lack of spreading was expected for PHSRN alone [23], but not for YIGSR. Though adhesion but no spreading was observed when YIGSR was adsorbed on either tissue culture treated plastic or glycophase glass [12,13], complete spreading occurred for a wide range of cell types when YIGSR was grafted via its amino terminal glycine onto glycophase glass [13]. The difference in behaviour between the latter model and our experimental setup may be ascribed to possible differences in precise orientation of the peptides due to similar, but not identical attachment loci of the peptides.

Interestingly, we observed a significant increase in adhesion for HVSMC to peptide modified PEGs when YIGSR was combined with RGD in comparison to either RGD or the combination of PHSRN and RGD. No synergism of adhesion was observed for PHSRN with RGD but the requirement for precise spacing between the two peptides, necessary for this effect, has been well characterized [24,25].

When the migration rates of the two cell types on RGD plus YIGSR or PHSRN were compared to that on RGD alone, only MVEC showed a significant difference in motility, where an increase was observed on the YIGSR/RGD combination. It is unlikely that this is caused by a change in the structure of the PEG hydrogel as no similar effect was observed for PHSRN/RGD hydrogels on the MVEC. In addition, no significant difference was found for the swelling of the hydrogels at the two levels of derivatisation used in the migration assay. Though it is not possible from these results to determine the cellular mechanism/s by which this increase in migration is achieved, it is worth noting that the YIGSR peptide has been found to co-localize LBP with α-actinin and vinculin, two crucial components of focal adhesion sites [13]. It is well established that RGD modified surfaces allow the formation of focal adhesion sites with concomitant cell spreading [26]. As stated above, proper cell spreading on our PEG hydrogels was dependent on the presence of RGD. When RGD and YIGSR were combined in our model, it is possible that the rate of assembly and disassembly of the focal adhesion sites, that has been shown to regulate cell mobility [27], might have been influenced by the YIGSR driven association of LBP with the RGD containing focal adhesion sites. YIGSR has also been shown to cause the phosphorylation of a range of proteins of molecular mass 115–130 kDa. The phosphorylation of cytoplasmic focal adhesion kinase (FAK) is believed to be central to the regulation of cell motility [28] and it is interesting that FAK was found to co-migrate with the above group of phosphorylated proteins [29].

5. Conclusion

We analysed the migration rates of MVEC and HVSMC on PEG gel surfaces derivatised with either the cell binding peptide RGD, RGD plus the laminin

derived peptide YIGSR or RGD plus the fibronectin derived peptide PHSRN. The combination of YIGSR plus RGD was found to significantly increase MVEC migration rates relative to RGD alone, whilst the combination of PHSRN plus RGD had no effect on MVEC migration. Neither of the peptide combinations influenced the motility of HVSMC relative to that on RGD alone. Therefore, this suggests that the tissue engineering of a scaffold with a higher specificity for a particular cell type might require the use of combinations of bioactive moieties. Very recently, a similar conclusion was drawn from a study on the adhesion of corneal epithelial cells to surfaces containing combinations of bioactive peptides [30].

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