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RGD Island Spacing Controls Phenotype of Primary Human Fibroblasts Adhered to Ligand-Organized Hydrogels

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Introduction

The aim of tissue engineering is to provide man-made tissues or organs to patients, typically using a combination of a patient's own cells and polymeric scaffolds.[1,2] Controlling cell-polymer interactions is one of the most critical parameters in the design and tailoring of polymers for functional tissue regeneration.[3] Cellular adhesion ligands in the extracellular matrix (ECM) play a critical role in regulating cell phenotype, including adhesion, spreading, motility, proliferation, and differentiation.[4] The Arg-Gly-Asp (RGD) sequence is one widely exploited cellular adhesion ligand that is known to bind specifically to many integrin receptors of various cell types[5] and is frequently found in many ECM proteins.[6] The introduction of this specific ligand to tissue engineering scaffolds can enhance ligand-receptor interactions as well as improve tissue regeneration.[7,8]

Recently, the size of domains containing cellular adhesion ligands, at both the micrometer[9,10] and nanometer level,[11,12] has been shown to be critical in controlling the adhesion, spreading, and migration of certain cell types. We have previously reported that the distance between adhesion ligands in the nanometer size scale regulated the adhesion, spreading, proliferation, and differentiation of mouse cells such as MC3T3-E1 cells.[13] In the present study, we hypothesized that altering the distance between cellular adhesion ligands present in polymeric hydrogels at the nanometer level would regulate the phenotype of primary human cells, which are more clinically relevant for *in vivo* applications rather than a use of cell lines. Alginate hydrogels were chosen as a synthetic ECM because alginate forms biocompatible gels via ionic cross-linking with divalent cations such as Ca^{2+} and has been widely used in many biomedical applications.[14,15] An RGD-containing peptide was chemically coupled to the alginate backbone and used to generate various nanoscale ligand-organized hydrogels. The bulk RGD density in alginate gels was kept constant while the spacing between the ligands was changed. The adhesion and proliferation of primary human fibroblasts were investigated and compared to those of other cell types.

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Experimental

Sodium alginate (FMC Biopolymers) was modified with a peptide of the sequence $(glycine)_4$-arginine-glycine-aspartic acid-alanine-serine-serine-lysine (G_4RGDASSK) that was purchased from Anygen (Korea). Sodium alginate was dissolved in 2-(*N*-morpholino)ethanesulfonic acid buffer (pH 6.5, 0.3 M NaCl) at room temperature, and the peptide was added to the alginate solution in the presence of *N*-hydroxy sulfosuccinimide (Pierce) and 1-ethyl-3-(dimethylaminopropyl) carbodiimide (Aldrich).[16] The peptide-modified alginate was purified by extensive dialysis against deionized water for 4 days (molecular weight cut-off 3,500), activated charcoal-treatment, and sterilization through a 0.22-μm filter. The degree of substitution of the RGD peptides coupled to the alginate backbone was determined by amino acid analysis, which was performed at the amino acid analysis facility (Korea Basic Science Institute, Korea).

Hydrogel disks were prepared by ionic cross-linking of an alginate solution (2 wt%) with calcium sulfate slurry. The gel was cut into disks 10 mm in diameter and 1 mm thick and pre-swollen in Dulbecco's Modified Eagle Medium (DMEM; Gibco) overnight. Ligand-organized hydrogels were prepared using the inherent dimension of a single polymer chain. In brief, non-peptide-modified and RGD-modified alginates were mixed together at a fixed ratio to control the distance between RGD peptides in a gel. For example, a ten-fold dilution of RGD-modified alginate (nine parts of non-peptide-modified alginate with one part of RGD-modified alginate containing one or more RGD peptides per ligand island) increased the distance between RGD islands in the resultant gels. The distance between RGD peptides (RGD island spacing) was calculated using the specific volume of a single alginate chain in the gel under the assumption of even distribution of the peptides in the gel.[13]

Primary human fibroblasts were obtained from human foreskin using a previously described method[17] and cultured in DMEM containing 10% fetal calf serum (Gibco) and 100

units/mL penicillin-streptomycin (Gibco). Cells were seeded on the surface of gels at a density of 2×10^4 cells/cm^2 and cultured in DMEM. Photographs of fibroblasts adhered to the gels were taken using an optical microscope (Olympus, Japan). The projected area and aspect ratio of the cells were determined by means of ImageJ software (NIH). At days 1, 3, and 5 the disks were treated with 1 mL trypsin solution (0.05% in EDTA) for 10 min and placed in 5 mL 50 mM EDTA in PBS (pH 7.4, 37 °C) for 20 min to dissolve the gel disks and collect the cells. The number of cells was then counted using a haemocytometer (n=4).

Table I. Comparison of Growth Rates of Various Cell Types Cultured on RGD-Alginate Hydrogels[a]

Cell Type	Growth Rate (day^{-1})	Reference
Mouse skeletal myoblast (C2C12)	1.14	(16)
Mouse calvarial preosteoblast (MC3T3-E1)	1.12±0.02	(13)
Primary rat calvarial osteoblast	0.51	(18)
Primary human dermal fibroblast	1.77±0.03	This study

[a][alginate] = 2 wt%, [RGD] = 12.5 mg/mg alginate, [cell] = 2×10^4 cells/mL.

Results and Discussion

Although alginate is widely used for biomedical applications, it inherently lacks the capacity for cellular interaction, and the introduction of cellular adhesion ligands can significantly alter cellular responses to alginate matrices. We introduced a peptide with the RGD sequence into alginate and tested the effect of the bulk RGD density on the adhesion of primary fibroblasts to the alginate gels and the proliferation of adhered cells. Fibroblasts were seeded on the surface of alginate gels containing varying RGD densities (number of peptides per unit volume). The adhesion and proliferation of fibroblasts cultured on alginate gels were dramatically enhanced by chemical conjugation of RGD peptides to the alginate backbone, compared with non-peptide-modified alginate gels (Figure 1). The number of cells adherent to the gels, as well as the growth rate, was strongly dependent on the bulk RGD density of the gels, consistent with previous studies using different cell types.[18,19] Specifically, the growth rate of fibroblasts was enhanced by an increase in the bulk RGD density in the gels up to 12.5 μg/mg alginate. Growth rates of various cell types cultured on alginate gels are compared in Table I. Interestingly, the proliferation rate of primary fibroblasts adhered to RGD-modified alginate gels was much greater than that of other cell types.

Modification of polymer scaffolds with cellular adhesion ligands has been an attractive approach towards tissue engineering. However, the distribution of the ligands in the scaffolds was random and there have been few reports on the spatial organization of the ligands in the synthetic ECMs. Gels with varied RGD island spacing were prepared to determine whether the spatial organization of adhesion ligands

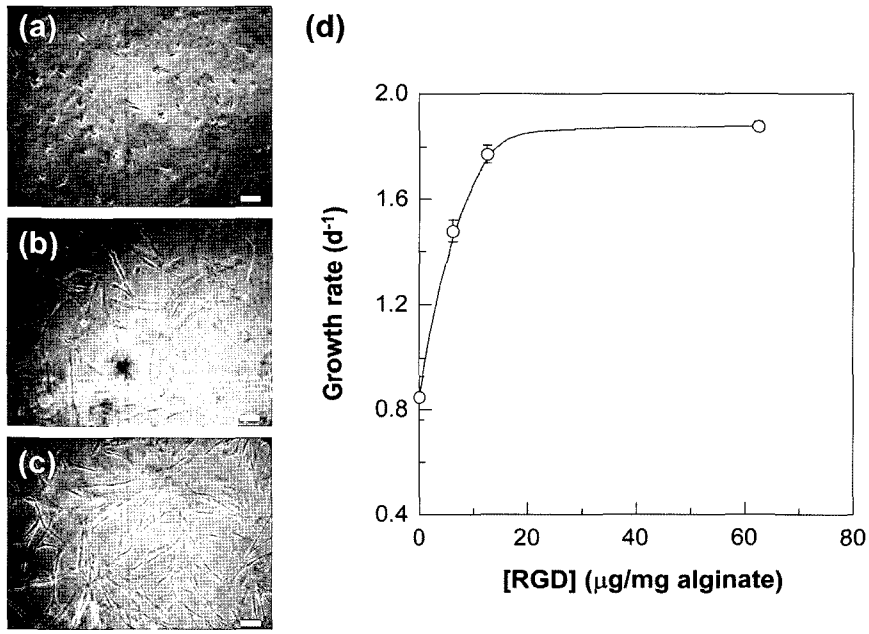

Figure 1. Effect of bulk RGD density on the adhesion and proliferation of primary human fibroblasts on alginate gels. Photomicrographs of fibroblasts adhered to the surface of alginate gels with different concentrations of peptides ([RGD]=(a) 0, (b) 12.5, (c) 62.5 μg/mg alginate; [cells]=2×10^4 cells/mL). Pictures were taken after 24 h culture at 100 × magnification (scale bar, 100 μm). (d) Growth rates of primary fibroblasts over 5 days after seeding on alginate gels with different bulk RGD densities (mean±standard deviation, n=4).

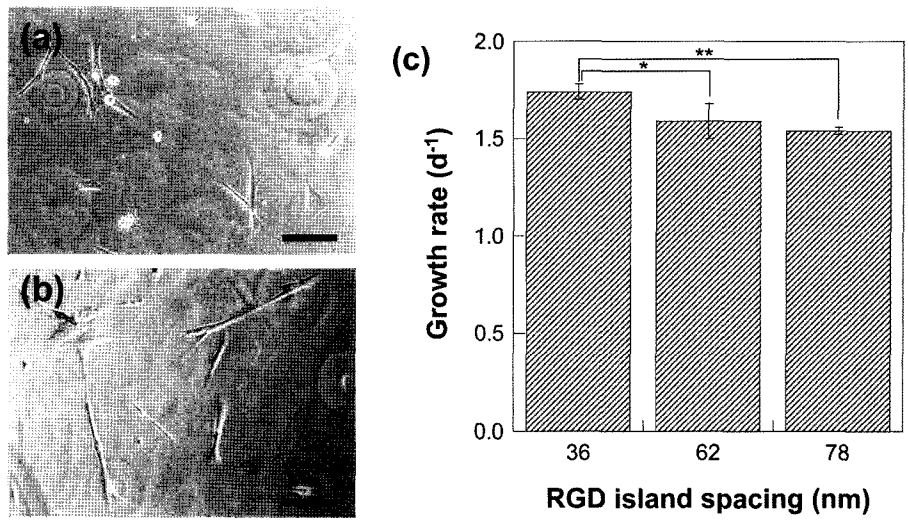

Figure 2. Effect of RGD island spacing on the adhesion and proliferation of primary fibroblasts on alginate gels. Photomicrographs of fibroblasts adhered to the surface of gels with RGD island spacings of (a) 36 and (b) 78 nm ([RGD]=12.5 μg/mg alginate; [cells]= 2×10^4 cells/mL). Pictures were taken after 24 h of culture at 100× magnification. (c) Growth rates of fibroblasts adhered to gels with differing RGD island spacings (mean±standard deviation, n=4, $*P<0.05$, $**P<0.01$).

in the nanometer size scale affects the phenotype of adhered primary fibroblasts. The bulk RGD density was maintained while the island spacing varied from 36 to 78 nm. Fibroblasts were seeded on the surface of gels with an average island spacing of 36, 62, and 78 nm, while maintaining a constant bulk RGD density of 12.5 μg/mg alginate. Cells adhered to gels with an island spacing of 78 nm showed a more extended shape than cells on gels with an island spacing of 36 nm, despite the same bulk RGD density in both gels (Figure 2). The projected area of adhered fibroblasts was not significantly influenced by the RGD island spacing. Surprisingly, the aspect ratio of the cells increased significantly as the island spacing increased (Table II), indicating that the distance between RGD islands in the synthetic ECM regulates the morphology and spreading of cells. The growth rate of the cells was suppressed as the RGD island spacing increased (Figure 2(c)), likely due to the lack of focal contact formation. These observations indicate that, in the concentration ranges used in this study, the RGD island spacing in the nanometer size scale is critical for the regulation of cell growth, rather than the bulk density of cellular adhesion ligands. It has previously been reported that the minimal ligand spacing required for integrin-mediated cell adhesion is in the range of several tens of nanometers, depending on the type of adhesion ligands and synthetic ECMs.[11-13,20-22]

Conclusions

We demonstrated that the nanoscale organization of RGD peptides in alginate gels, designed as a synthetic ECM, regulated the adhesion and proliferation of primary human fibroblasts. Cell spreading and growth were strongly correlated with the distance between islands of the peptides, irrespective of the same bulk RGD density in the gels. The approach of controlling the distribution and spacing of adhesion ligands at the nanometer level may be critical in the design and function of synthetic ECMs for tissue engineering applications.

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Table II. Characteristics of Primary Human Fibroblasts Adhered to Ligand-Organized Alginate Gels

[RGD] (μg/mg alginate)	Dilution Factor	Number of Peptides per Alginate Chain	Spacing (nm)	Aspect Ratio (a/b)	Projected Area (μm^2/cell)
12.5	1	2	36	5.3±0.9	6,530±990
12.5	5	10	62	8.9±1.4	6,600±1,170
12.5	10	20	78	10.3±1.9	6,350±860

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