Biomaterials 31 (2010) 5545–5551

Contents lists available at ScienceDirect

Biomaterials

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

The effect of spacer arm length of an adhesion ligand coupled to an alginate gel on the control of fibroblast phenotype

Jae Won Lee [a], Yoon Jeong Park [b], Seung Jin Lee [c], Sang Kyung Lee [a,d], Kuen Yong Lee [a,d,*]

[a] Department of Bioengineering, College of Engineering, Hanyang University, Seoul 133-791, Republic of Korea
[b] Department of Craniomaxillofacial Reconstructive Science, School of Dentistry, Seoul National University, Seoul 110-749, Republic of Korea
[c] College of Pharmacy, Ewha Womans University, Seoul 120-750, Republic of Korea
[d] Institute for Bioengineering and Biopharmaceutical Research, Hanyang University, Seoul 133-791, Republic of Korea

ARTICLE INFO

Article history:
Received 28 January 2010
Accepted 25 March 2010
Available online 20 April 2010

Keywords:
Hydrogel
Adhesion ligand
Spacer arm length
Cell–matrix interaction

ABSTRACT

Tissue engineering requires the use of polymeric scaffolds that mimic many roles of extracellular matrices (ECM) in the body. Controlling cell–scaffold interactions is one of the most critical parameters for regulating cell phenotype in tissue engineering, and a peptide with the sequence of RGD has been widely exploited for this purpose. We hypothesized that the spacer arm length of adhesion ligands coupled to synthetic ECMs could be vital for regulation of cell–scaffold interactions. We prepared alginate gels modified with RGD peptides containing varying spacer arm lengths and cultured primary human fibroblasts either on the gels (2-D) or within the gels (3-D). The spacer arm length of the RGD peptides significantly influenced the adhesion and proliferation of fibroblasts in both the 2-D and 3-D studies. We found that a minimum number of four glycine units in the spacer arm was essential for enhanced adhesion and growth of the cells *in vitro*. An optimal spacer arm length of the RGD peptides was also necessary for minimizing cellular stress responses as determined by analyzing expression of heat shock proteins and Bcl-2 in cultured cells. This approach to controlling cell phenotype using adhesion peptides with various spacer arm lengths could be useful for designing novel scaffolds in tissue engineering applications.

1. Introduction

The control of specific interactions between cell receptors and adhesion ligands within the extracellular matrix (ECM) is a critical aspect of tissue engineering approaches, including the regulation of many critical features of cellular adhesion, migration, proliferation, and differentiation [1,2]. While many interaction sites are available in ECM components and various ligands have been discovered via competitive adhesion assay and mutagenesis experiments [3,4], a peptide with the Arg-Gly-Asp (RGD) sequence has been extensively exploited to understand and improve ligand–receptor interactions in many tissue engineering applications [5,6]. This peptide sequence specifically binds to many transmembrane receptors (e.g., integrin), including the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ receptors [7,8] that recognize adhesion molecules on the surfaces of ECMs.

Cell adhesion ligands (e.g., RGD) can be introduced into various materials for use in tissue culture matrices or cell transplantation vehicles. Biomaterials of natural or synthetic origin that have an inherently poor ability to bind to anchorage dependent cells have been modified with adhesive ligands [9,10]. The bulk density of adhesion ligands in synthetic ECMs greatly influences the adhesion and proliferation of many cell types, such as fibroblasts [11], osteoblasts [12], and stem cells [13]. In addition, the domain size of cellular adhesion ligands in the nanometer size scale has been found to influence the adhesion and spreading of certain cell types [14,15]. However, few studies have reported how control of the spacer arm length of the adhesion ligand when it is coupled to synthetic ECMs can regulate cellular responses for tissue engineering applications.

The effect of spacer arm length and chemical structure on retention and binding affinity has previously been described in the context of chromatography systems [16]. In fact, the hydrocarbon spacer arm length has a direct impact on the binding and recovery of a variety of enzymes and carbohydrates in affinity chromatography separations [17,18]. Furthermore, using spacer arms with different degrees of hydrophobicity, length, and chemical composition greatly influenced interactions between agarose and an affinity ligand [19].

* Corresponding author. Department of Bioengineering, College of Engineering, Hanyang University, Seoul 133-791, Republic of Korea. Tel.: +82 2 2220 0482; fax: +82 2 2293 2642.
E-mail address: leeky@hanyang.ac.kr (K.Y. Lee).

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doi:10.1016/j.biomaterials.2010.03.063

In this context, we hypothesized that varying the spacer arm length of an adhesion ligand could be a critical aspect of regulating the phenotype of human primary fibroblasts *in vitro* (Fig. 1). We prepared alginate gels modified with adhesion ligands containing various spacer arm lengths and then cultured primary human fibroblasts either on the gels (2-D) or within the gels (3-D). Alginate is a potential candidate for this type of research, due to its numerous applications as an injectable cell delivery vehicle in tissue engineering applications. Alginate has a number of advantageous features including excellent biocompatibility, low immunogenicity, and gentle gelling behavior, which may allow one to deliver cells and drugs to the body with minimal trauma [20]. However, alginate lacks the ability to bind to cells [21], and the introduction of adhesion ligands (e.g., RGD peptide) significantly enhances cellular adhesion and growth [9,10].

In this study, we investigated how adhesion peptides with various spacer arm lengths can influence cellular behavior (e.g., adhesion, proliferation) in both two-dimensional and three-dimensional environments using ligand-modified alginate gels. We also analyzed the expression of heat shock protein (HSP) and Bcl-2 protein, both of which are typical markers of cellular apoptosis, in order to elucidate the stress responses of cells cultured either on or within RGD-modified alginate gels with different spacer arm lengths.

Adaptation of synthetic ECMs for cell support in therapeutic applications is an important aspect of tissue engineering. Deviation from normal microenvironments provides cell stress, which elicits expression of stress related genes or induces cell membrane disruption and apoptosis. Therefore, cell stress is a good marker for analyzing the relevance of synthetic ECMs for their potential clinical use in tissue engineering applications. Among the cell stress markers, HSPs have been implicated in the regulation of apoptosis, as modulators of p53 protein function. The function of the HSPs is to protect cells from damage by allowing denatured proteins to adopt their native configuration as a molecular chaperone. Thus, increased HSP expression levels make cells more resistant to apoptosis [22–24]. HSP27 can be expressed in response to different types of stress, including thermal, mechanical, chemical, and pH-altering stimuli. Bcl-2 also plays an important role in cell survival, and is one of the cell death-regulating genes. The stress-induced apoptosis pathway is initiated by BH3 proteins, which inactivates Bcl-2 protein [25]; therefore, the stress apoptosis pathway reduces Bcl-2 gene expression [26]. Thus, we investigated the expression of the key players in apoptosis such as apoptotic (HSPs) and survival proteins (Bcl-2) in order to elucidate the stress responses of cells by using the RGD-modified gels with different spacer arm lengths.

2. Materials and methods

2.1. Peptide-modified alginate

Sodium alginate was purchased from FMC Biopolymers and dissolved in a 2-(N-morpholino) ethanesulfonic acid (MES, Sigma) buffer solution at room temperature (pH 6.5, 0.3 M NaCl). A peptide with the sequence of $(glycine)_n$-arginine-glycine-aspartic acid-serine-proline (G_nRGDSP) was purchased from Anygen (Korea) and added to the alginate solution in the presence of N-hydroxysulfosuccinimide (sulfo-NHS, Pierce) and 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC, Sigma). The peptide-modified alginate was purified by extensive dialysis against deionized water for four days (molecular weight cut-off = 3,500), activated charcoal treatment, and sterilization through a 0.22 μm filter. Non-modified alginate was also prepared in the same procedure as the peptide-modified alginate without peptide addition.

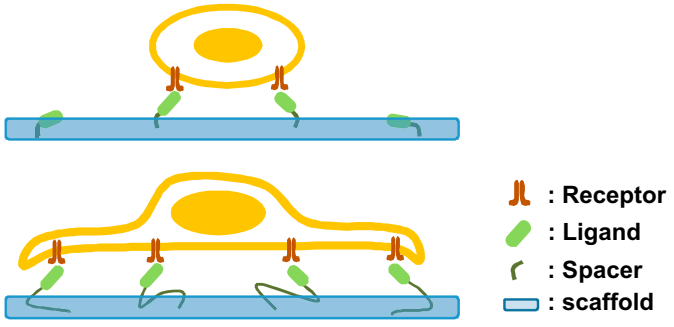

Fig. 1. Schematic description of cellular adhesion on ligand-modified synthetic ECMs with different spacer arm lengths.

2.2. Hydrogel preparation

Hydrogels were prepared by the ionic cross-linking of an alginate solution (2% wt) with calcium sulfate (Sigma). For the two-dimensional culture of cells, the gels were cut into disks (10 mm diameter, 1 mm thick) using a punch. For the three-dimensional experiments, an alginate solution (2% wt) was premixed with a cell suspension, and then cross-linked with calcium sulfate to form gels. The gels were then cut into disks (5 mm diameter, 0.5 mm thick). The gels were pre-swollen in Dulbecco's modified Eagle medium (DMEM, Gibco) before use [11].

2.3. Cell culture

Human primary fibroblasts were isolated from human foreskins and cultured in DMEM containing 10% fetal calf serum (Gibco) and 100 units/mL penicillin–streptomycin (Gibco) at 37 °C under a 5% CO_2 atmosphere. For two-dimensional studies, these cells were seeded on the surfaces of gel disks that were placed in a tissue culture plate at a density of 2.0×10^4 cells/mL, and incubated at 37 °C under a 5% CO_2 atmosphere. The cell density for three-dimensional experiments was 5.0×10^5 cells/mL, and gel disks containing the cells were cultured in a spinner flask (60 rpm). At predetermined time points, the gel disks were placed in 50 mM EDTA in PBS (pH 7.4, 37 °C) for 20 min to dissolve the gel disks. The cells were then collected by centrifugation and the number of cells was counted using a hemocytometer. Growth rates were calculated from cell counts comparing cell numbers at day 5 to cell numbers at 6 h post-seeding.

2.4. Image analysis

Photographs of fibroblasts that adhered to the gels were taken using an optical microscope (Olympus). The aspect ratio and projected area of the cells were determined using ImageJ software (NIH). The aspect ratio was defined as the ratio of the long axis relative to the short axis of an adherent cell, and the projected area of an adherent cell was determined by projecting its shape onto a gel substrate.

2.5. Immunostaining of vinculin

Fibroblasts cultured either on or within the gel were fixed with a 10% formalin solution, mixed with sucrose and cytoskeleton buffer for 20 min, and permeabilized with TBS buffer containing 0.5% Triton X-100 for 10 min. The cells were treated with AbDil for 10 min, followed by treatment with anti-human vinculin antibody (primal antibody, 1:200 dilution, Chemicon) for 5 min. The cells were then treated with rhodamine-conjugated donkey anti-mouse IgG as a secondary antibody (Jackson ImmunoLab). The gel disks were rinsed five times with TBS buffer containing 0.1% Triton X-100 and were mounted on a cover slip before being sealed with wax. Images of vinculin-stained cells were taken by confocal laser scanning microscopy (Olympus).

2.6. mRNA isolation and RT-PCR

RNA was isolated from cells after three days of culture using an RNAiso kit (TaKaRa) and dissolved in diethylpyrocarbonate (DEPC)-treated water (Sigma). The amount of RNA was determined by an UV–VIS spectrometer at 260 nm, and the concentration and quality were checked on an agarose gel. The RNA samples were reverse transcribed to cDNA using a Maxime RT PreMix kit (iNtRON Biotechnology). The primer pair sequences used were obtained from published sequences (NM001540) and purchased from Bioneer. PCR was carried out for 35 cycles (denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, elongation at 72 °C for 30 s). PCR products were analyzed by conventional agarose gel electrophoresis. The sequences of primers used are as follows: β-actin, 5′-ATCATGTTTGAGACCTTCAA, 3′-CATCTCTTGCTCGAAGTCGA (450 bp); HSP27, 5′-CAGCCAGCATGACCGAGCGC, 3′-CTGGTCGAAGAGGCGGTCAT (151 bp); HSP70, 5′-CTAGCCTGAGGAGCTGCTGCGACAG, 3′-GTTCCCTGCTCTCTGTCGGCTCGGCT (250 bp); Bcl-2, 5′-GGACAACATCGCCCTCTG, 3′-AGTCTTCAGAGACAGCCAGGA (148 bp).

2.7. Quantification of HSP secretion

To determine the amount of HSP27 secreted by cells over time, the media were collected at each time point, and the amount of HSP27 was determined by an enzyme-linked immunosorbent assay (ELISA, Biosource).

2.8. Statistical analysis

All data are presented as mean ± standard deviation. Statistical analyses were performed using Student's *t*-test. Values of $^{*}P < 0.05$ and $^{**}P < 0.01$ were considered statistically significant.

3. Results

3.1. Adhesion and spreading of cells on ligand-modified alginate gels (2-D)

We first investigated changes in cell phenotype of primary human fibroblasts cultured on the surface of alginate gels modified with RGD peptides containing different spacer arm lengths. The spreading of fibroblasts on the alginate gels was analyzed by calculating the aspect ratio and the projected area of the adherent cells (Table 1). The aspect ratio and projected area for the RGDSP and G_2RGDSP groups were significantly smaller than other experimental groups. However, the increase in the aspect ratio and the projected area of the cells was significant when a spacer arm length was above 4 glycine units. We found no statistically significant increase in cell spreading on gels modified with RGD peptides with a spacer arm of more than 12 glycine units. This result indicates that the introduction of a spacer arm to an adhesion peptide is vital in controlling the spreading of adherent cells, and at least four glycine units are required for proper adhesion of fibroblasts onto the RGD peptide-modified alginate gels.

3.2. Cell growth on ligand-modified alginate gels (2-D)

We next determined the growth rates of fibroblasts cultured on alginate gels modified with RGD peptides containing various spacer arm lengths. The growth of the cells was enhanced by increasing the spacer arm length of adhesion peptides (Fig. 2). The growth rate substantially increased when the peptide had a spacer arm of more than four glycine units. This finding was consistent with the results obtained from adhesion experiments. However, both the spreading and the growth of the cells leveled off when the gels were modified with peptides containing more than 12 glycine units.

3.3. Focal contact formation (2-D)

We used confocal laser scanning microscopy to detect immunostained vinculin from fibroblasts that had adhered to the alginate gel (Fig. 3). In this work, we clearly observed the focal adhesion of fibroblasts on G_{12}RGDSP-modified alginate gels, compared with RGDSP-modified alginate gels. This finding indicates that an increase of the spacer arm length of adhesion peptides can promote cellular binding to polymer scaffolds, a finding that is likely due to the sufficient formation of focal adhesion, and which consequently improves the growth of the cells.

Table 1
Characteristics of primary human fibroblasts cultured on alginate hydrogels modified with adhesion ligands containing different spacer arm lengths ($n = 5$).

Peptide	Aspect ratio (a/b)	Projected area (μm^2/cell)
RGDSP	1.16 ± 0.15	672.2 ± 72.3
G_2RGDSP	1.75 ± 0.33	748.1 ± 40.9
G_4RGDSP	3.13 ± 0.52	4439.3 ± 506.0
G_8RGDSP	4.25 ± 0.55	4809.3 ± 341.3
G_{12}RGDSP	5.21 ± 0.41	6280.1 ± 302.4
G_{16}RGDSP	5.34 ± 0.43	6376.2 ± 456.2
G_{20}RGDSP	5.68 ± 0.49	6879.1 ± 501.5

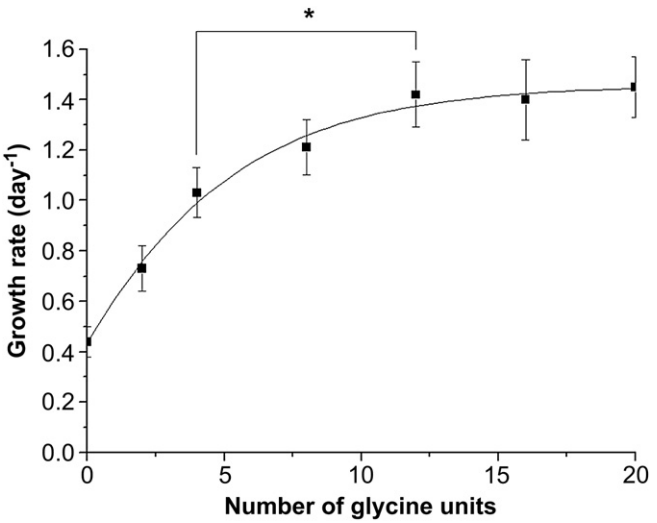

Fig. 2. Effect of the spacer arm length of adhesion peptides on the growth rate of fibroblasts cultured on alginate gels (2-D). Growth rates were calculated from changes in cell numbers over time ($^{*}P < 0.05$, $n = 4$).

3.4. Stress response of cells to gels (2-D)

When cells are exposed to several types of environmental stress, cellular apoptosis may occur. We next tested whether the spacer arm length of adhesion peptides coupled to alginate gels could influence apoptosis of cells that were adherent to the gels (Fig. 4). Expression of HSPs has been implicated in the regulation of apoptosis [22], as HSP can mediate many cytoprotective events. The HSP expression in fibroblasts cultured on non-peptide-modified alginate gels was observed more clearly than those cultured on G_4RGDSP- and G_{12}RGDSP-modified alginate gels. Bcl-2 is one of the anti-apoptotic genes and also functions to protect a stress apoptosis pathway. The Bcl-2 expression of fibroblasts cultured on G_{12}RGDSP-modified alginate gels was prominent compared with other experimental groups. This finding clearly demonstrates that an increase in the spacer arm length of RGD peptides could provide cells with less stressful environments and reduce cellular apoptosis.

We next quantitatively monitored the HSP27 secretion of fibroblasts cultured on RGD peptide-modified alginate gels with various spacer arm lengths over time (Fig. 5). The initial expression of HSP27 in cells cultured on non-peptide-modified and RGDSP-modified gels was significantly high, indicating that cells experienced much stress from these synthetic ECMs. Surprisingly, this stress was significantly reduced by the introduction of adhesion ligands to alginate gels with a proper spacer arm length.

3.5. Proliferation and focal contact formation inside ligand-modified alginate gels (3-D)

We cultured fibroblasts within alginate hydrogels (3-D) modified with RGDSP, G_4RGDSP, and G_{12}RGDSP. Non-peptide-modified alginate gels were also used as a control. The number of cells within non-peptide-modified alginate gels increased slightly until the third day of culture, and then slowly decreased. However, the number of cells within G_{12}RGDSP-modified alginate gels increased significantly and was maintained for three weeks (Fig. 6). This result indicates that an increase of the spacer arm length significantly influences the viability of cells cultured within three-dimensional synthetic ECMs and is consistent with the results obtained from the two-dimensional culture of cells on the gels.

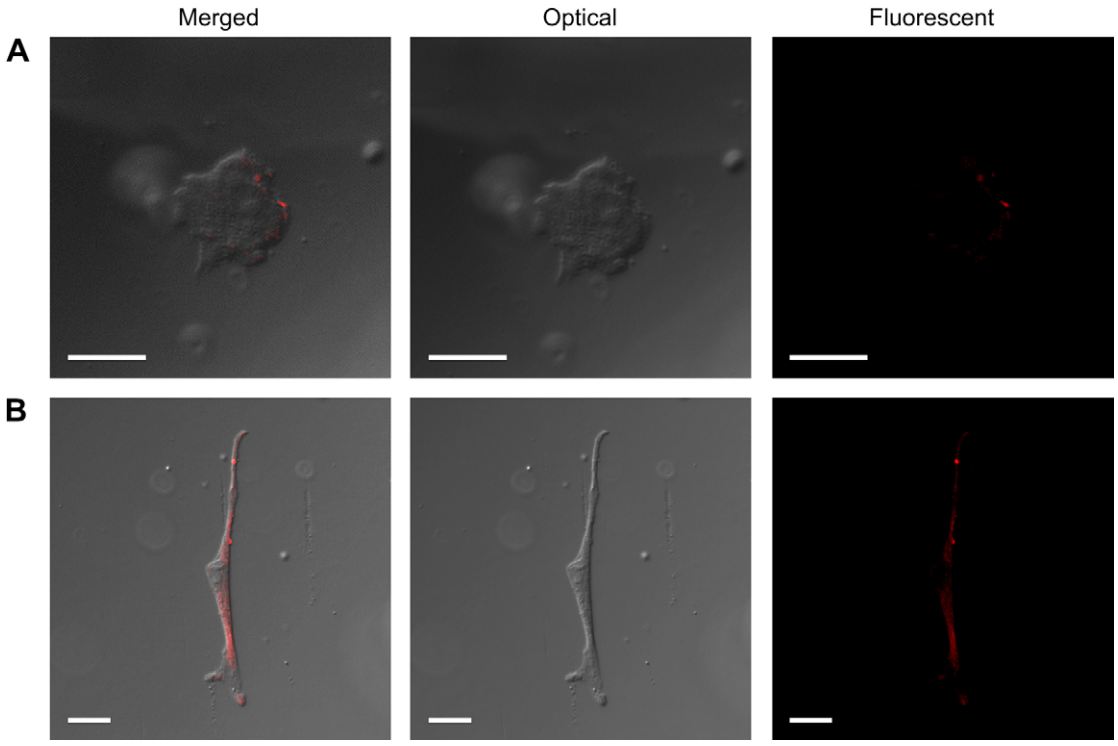

Fig. 3. Images of primary human fibroblasts cultured on (A) RGDSP- and (B) G_{12}RGDSP-modified alginate gels (scale bar, 20 μm). Cells were treated with anti-vinculin antibody, followed by treatment with rhodamine-conjugated donkey anti-mouse IgG as a secondary antibody.

Our visualization of vinculin from fibroblasts adhering to the gels also supported this finding. Using confocal laser scanning microscopy, we clearly observed immunostained vinculin from fibroblasts cultured within RGD peptide-modified alginate gels with a spacer arm of G_4 and G_{12}, compared to fibroblasts cultured within non-peptide-modified alginate gels (Fig. 7). Formation of a proper focal contact might be one critical parameter for cell proliferation in three-dimensional experiments, as shown in the two-dimensional studies.

3.6. HSP expression of cells inside ligand-modified alginate gels (3-D)

Since we found that HSP secretion, which is related to the apoptosis of cells, can be regulated by the introduction of a spacer arm to an adhesion peptide in 2-D experiments, HSP expression of fibroblasts cultured in 3-D conditions was also monitored over time. Therefore, we considered HSP27 to be a stress marker and quantitatively analyzed its secretion from fibroblasts cultured within alginate gels (Fig. 8). The initial HSP27 expression of cells cultured within non-peptide-modified alginate gels was significantly high, and the expression was greatly reduced by the introduction of G_4RGDSP and G_{12}RGDSP to the gels, indicating that environmental stress to cells in three-dimensional studies can be substantially diminished by an adhesion ligand with a spacer arm of the proper length.

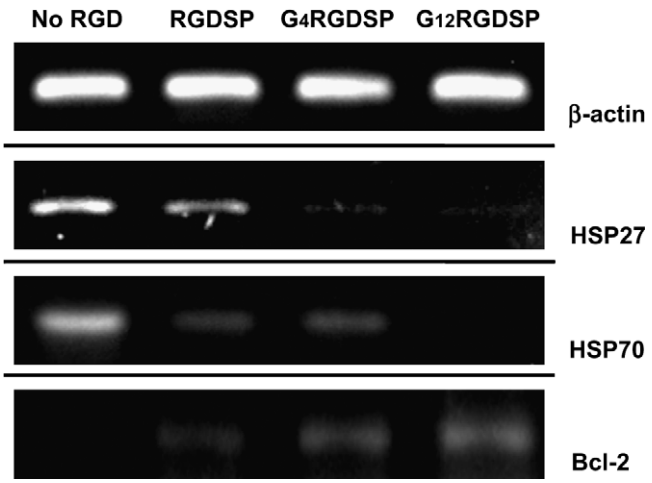

Fig. 4. Expression of heat shock proteins (HSPs) and Bcl-2 in primary human fibroblasts cultured on ligand-modified alginate gels with different spacer arm lengths.

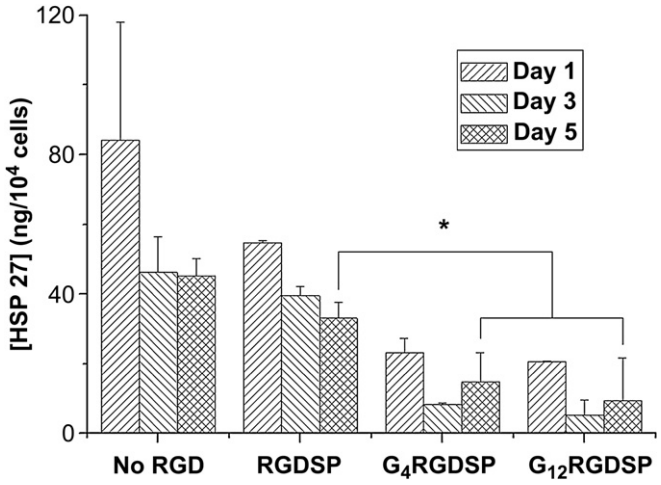

Fig. 5. Quantitative analysis of heat shock protein 27 secretion from fibroblasts cultured on ligand-modified alginate gels with different spacer arm lengths (*$P < 0.05$, $n = 4$).

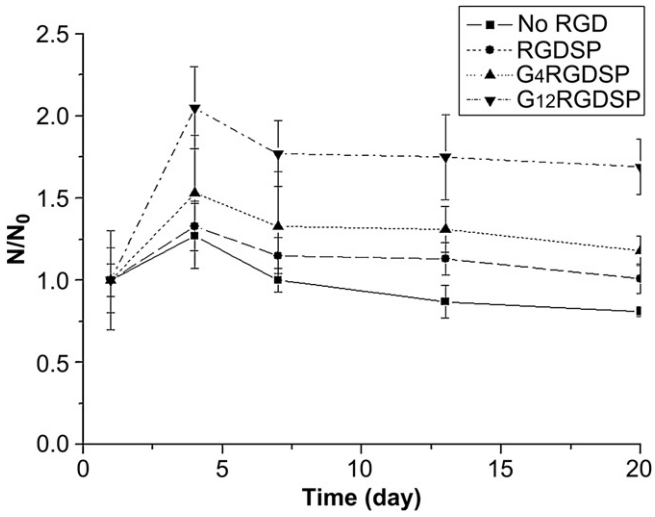

Fig. 6. Effect of the spacer arm length of adhesion peptides on proliferation of fibroblasts cultured within alginate gels (3-D). N_0 and N represent cell numbers at time 0 and t, respectively.

4. Discussion

In this study, we aimed to demonstrate how cell phenotype can be controlled by the introduction of various spacer arms to adhesion ligands conjugated to synthetic ECMs. In order to regulate cell phenotype in tissue engineering approaches, clinically relevant 3-D scaffolding systems should be designed properly and prepared precisely to mimic the extracellular matrices in the body that are capable of displaying adhesion ligands. Modification of polymer scaffolds with cell adhesion ligands has been an attractive approach in tissue engineering. 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 gels. Previous work has shown that the adhesion and proliferation of certain cell types cultured on alginate gels containing various bulk RGD densities are dramatically enhanced compared with non-peptide-modified alginate gels [11]. In addition, studies have also shown that the ligand distribution in the scaffolds (i.e., spatial organization in the matrix) is also very important in regulating cell functions in tissue engineering approaches [15,27]. We previously demonstrated that

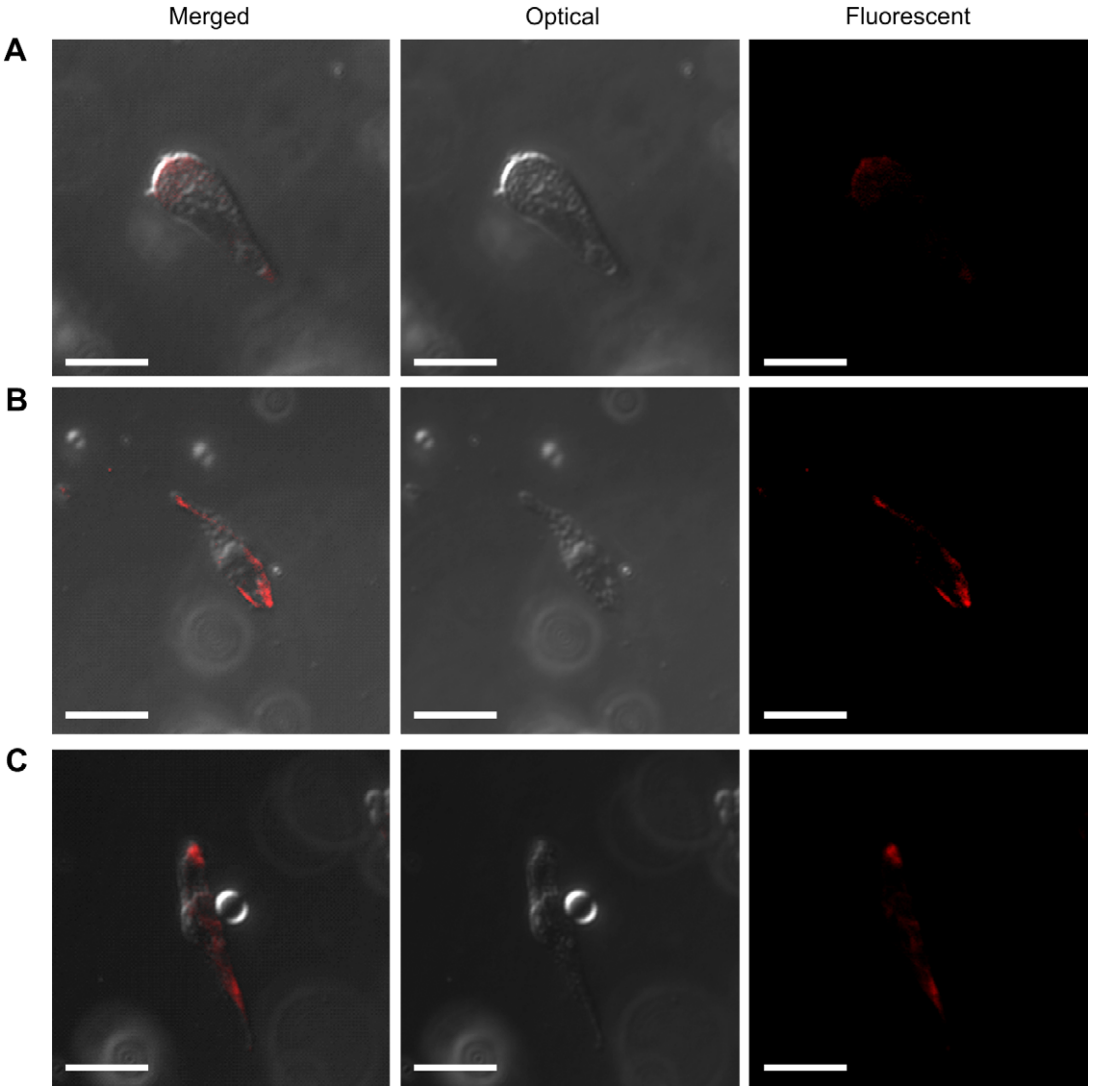

Fig. 7. Images of primary human fibroblasts cultured within alginate gels modified with (A) RGDSP, (B) G_4RGDSP, and (C) G_{12}RGDSP (scale bar, 20 μm). Cells were treated with anti-vinculin antibody, followed by treatment with rhodamine-conjugated donkey anti-mouse IgG as a secondary antibody.

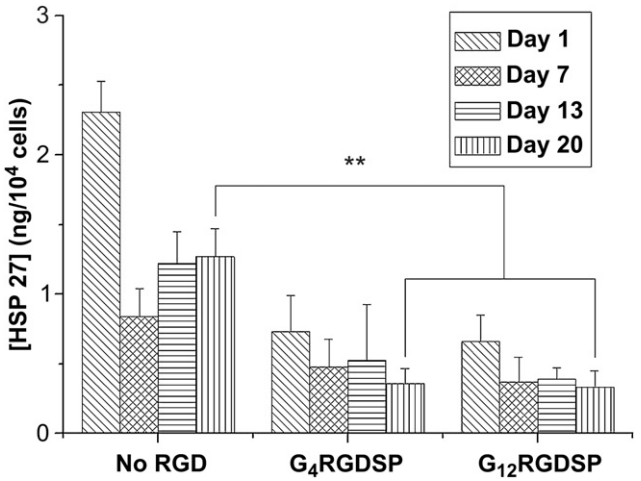

Fig. 8. Quantitative analysis of heat shock protein 27 secretion from fibroblasts cultured within alginate gels modified with RGD peptides with different spacer arm lengths ($^{**}P < 0.01$, $n = 4$).

the spatial organization of adhesion ligands in alginate gels at the nanoscale can control the phenotype of adherent fibroblasts [11].

Although the effect of ligand spacer arm length on binding affinity has been reported for many chromatographic systems [16–19], few have been reported in tissue engineering applications. The surface concentration of biotin, a model ligand, was influenced by the presence of a poly(ethylene glycol) (PEG) spacer arm in poly (propylene fumarate-*co*-ethylene glycol) gels, which could be important for the adhesion, migration, and function of anchorage dependent cells [28]. However, more thorough and systematic studies on this issue may be required to more efficiently regenerate tissues or organs. In this study, we demonstrated that the adhesion and growth of fibroblasts could be dramatically influenced by the spacer arm length, while the total concentration of the peptides in the gels was kept constant. In addition, an increase of the spacer arm length significantly enhanced the spreading and growth of fibroblasts in both 2-D and 3-D systems. At least four glycine units are required for RGD peptides to properly bind to the cellular integrin receptors. It is likely that as the spacer arm length increases, the cells have enhanced accessibility to the ligand (Fig. 1). This finding can be supported by the results obtained from the adhesion and focal contact formation of the cells (Table 1, Fig. 3). However, no more than 12 glycine units were effective in enhancing adhesion and growth of human fibroblasts in 2-D systems.

In our study, the HSP27, HSP70, and Bcl-2 genes were used to investigate the biological responses of fibroblasts cultured on the gels modified with adhesion peptides containing various spacer arm lengths. The stress apoptosis pathway increases the expression of HSPs and reduces Bcl-2 gene expression. An increase of the spacer arm length of RGD peptides could diminish steric hindrance of conjugated ligands and provide sufficient accessibility of cells to the ligands coupled to the synthetic ECMs, resulting in reduced environmental stress to the cells. Our analyses of the cell stress markers such as HSP27, HSP70, and Bcl-2 support our finding that the spacer arm length of the adhesion ligand is critical for cells to adapt to the synthetic extracellular matrix (Fig. 4). Our finding that HSP27 and HSP70 have reduced expression in G_4RGDSP- and G_{12}RGDSP-modified alginate gels clearly demonstrates that a spacer arm length greater than 4 did not induce cell stress. In addition, expression of Bcl-2 in G_4RGDSP- and G_{12}RGDSP-modified alginate gels indicates that the spacer arm length is a very important factor for cell survival in cell culture systems. Other assays such as Annexin V staining or activation of caspase 3 protein can be used as definitive markers for cell apoptosis. However, these assays only detected responses once cells are committed to apoptosis that culminates in cell death. On the other hand, the expression of HSP27 and HSP70 can be considered as early markers for cell stress and function to protect cells from damage during periods of stress [29]. Therefore, we believe our analysis of stress related genes when testing various spacer arm lengths in cell culture systems is a better approach for testing novel scaffolds to be used in tissue engineering. Further analyses regarding the biological responses of cells including stem cells, are required before these scaffolds are used in therapeutic tissue engineering applications.

5. Conclusions

Our study demonstrates that the adhesion and proliferation of human primary fibroblasts can be regulated by controlling the spacer arm length of RGD peptides coupled to alginate gels. At least four glycine units as a spacer arm are required for sufficient focal adhesion and substantial growth of fibroblasts. The longer the spacer arm length of RGD peptide, the more the cells proliferated in both 2-D and 3-D studies. However, we found that a spacer arm of more than 12 glycine units did not significantly increase cell growth *in vitro*. Expression of typical fibroblast stress markers indicated that alginate gels modified with RGD peptides with a spacer arm provided the most suitable synthetic ECMs for human primary fibroblasts cultured in both 2-D and 3-D environments. This approach of controlling cell phenotype by regulating the spacer arm length of adhesion ligands may be critical in the design of scaffolds using novel synthetic ECMs and may be useful in many tissue engineering applications.

Acknowledgements

This work was supported by National Research Foundation of Korea grant funded by the Korean Government (2009-0065528, 2009-0072339, F01-2007-000-10016-0, 2009-0079989).

Appendix

Figures with essential color discrimination. Figs. 1, 3, 7 in this article have parts that are difficult to interpret in black and white. The full color images can be found in the online version, at doi:10.1016/j.biomaterials.2010.03.063.

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