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Immobilization of Arg-Gly-Asp (RGD) Sequence in Sugar-Containing Copolymer for Culturing Fibroblast Cells

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Abstract The peptide Arg-Gly-Asp (RGD) was immobilized through their amino terminus to ends of a sugar bearing copolymer, producing a functional hybrid copolymer. Poly(N-p-vinylbenzyl-D-maltonamide-co-6-(p-vinylbenzamido)-hexanoic acid-g-GRGDS) [p(VMA-co-VBGRGDS)] promoted the attachment and growth of NIH fibroblast cells. The interaction between fibroblast cells and p(VMA-co-VBGRGDS) copolymer resulted in effective cell attachment, proliferation, and morphological changes by introduction of a GRGDS sequence. Moreover, when pretreated with soluble RGD monomer, attachment of fibroblast cells was suppressed approximately 50% from that of the p(VMA-co-VBGRGDS) surface.

Key words: RGD, copolymer, fibroblast cells, p(VMA-co-VBRGDS), sugar

Advances in our knowledge of biochemical interactions at the cell surface have led to structural elucidation of ligand molecules that bind to cell surface receptors and influence cell behavior [11]. In many cases, the development of novel materials for *in vitro* and *in vivo* biotechnology applications will include these ligands [1, 4]. The fabrication of these complex materials would be improved by the development of surface engineering techniques that permit the facile immobilization of high densities of ligands without degradation of either ligand or material. To date, the vast majority of ligand coupling to material is through covalent attachment of ligands to the bulk polymer.

To address the issue associated with cell adhesion, a wide variety of receptors on cell membranes can interact with their own ligands such as peptides [2, 12], proteins [7, 9], saccharides [10, 19], vitamins [8], and other biologically active substances [6, 12]. The cell recognizes various signals, such as ligands from its environment, instantaneously and accurately. To analyze the recognition mechanism of cells, it will be necessary to understand how the cells carry out multiple recognition tasks. It is possible to expect that materials carrying these ligands not only maintain inherent functions of the cells, but also increase their unique functions through the interactions. The analysis of these interactions between ligand and receptor and behavior of cells would be the key to understand cell function. Typically, a peptide containing the cell-binding domains found in the extracellular matrix (ECM) proteins is immobilized on the material to promote cell adhesion via ligand-receptor interactions. These interactions are mediated by cell surface receptors, such as integrin families and transmembrane proteoglycans, which bind to the peptide presenting the specific domain of the ECM protein. The most common cell-binding domain used as a candidate peptide to improve cell adhesion onto biomaterial surfaces is the Arg-Gly-Asp (RGD) sequence [15]. RGD recognition of the adhesive protein RGD sequence by cell integrins provides a signal for cell adhesion, spreading, and growth. RGD recognition within the cell-binding domain of fibronectin is a major mechanism for the attachment of a particular anchorage-dependent cell [16–18]. In order to develop RGD-based biomaterials, several studies have been focused on RGD-containing synthetic peptides as a substitute matrix for cultured surfaces coated with adhesive proteins [3, 5, 14].

We report here on the culturing of the fibroblast cells in a synthetic and biocompatible polymer. In order to design a specific culture for fibroblast cells, adhesion molecules of the RGD sequence in pendant-specific ligands was copolymerized with sugar-bearing polystyrene derivatives to provide the substrata that can be specifically recognized by the integrin families of fibroblast cells.

PVMA were synthesized as described previously [18]. *P*-vinyl benzoic acid (4 g, 27 mmol) was reacted with

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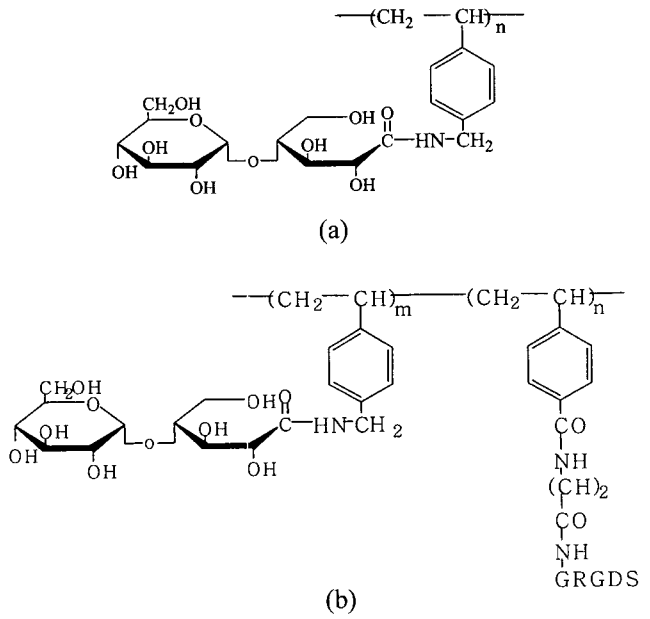

Fig. 1. Chemical structures used in this study.
(a): PVMA; (b): p(VMA-co-VBGRGDS).

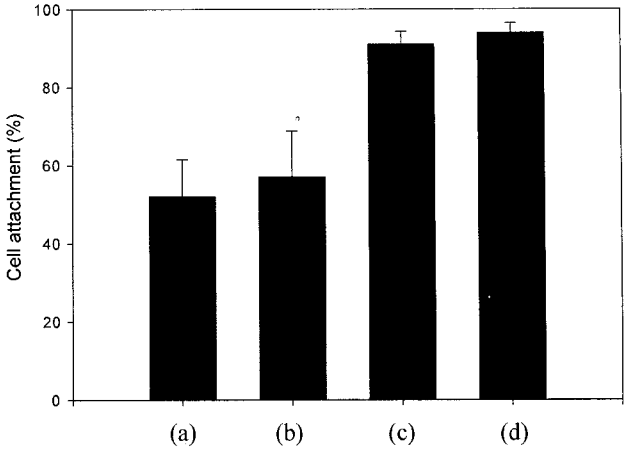

Fig. 2. Fibroblast cell attachment on various matrices-coated polystyrene dishes.
(a): control; (b): PVMA; (c): p(VMA-co-VBGRGDS); (d): collagen. The data represent the experimental data (mean±SD) (n=5).

N-hydroxy succinide (3.5 g, 30 mmol) and *N,N*-dicyclohexylcarboiimide (6 g, 30 mmol) in chloroform (10 ml) at 25°C for 24 h. The solution was filtered, and then the filtrate washed 3 times with water (30 ml), and solvent was evaporated to dryness. The product was purified by recrystallization from chloroform/ether (2/8 by vol) to yield 2.7 g. The resulting activated ester (o-succinimidyl p-vinyl benzoate, 1.5 g, 6 mmol) was treated with 6-amino-*N*-caproic acid (0.8 g, 6 mmol) in THF (10 ml) at room temperature for 4 days. 6-(p-vinylbenzylamido)-hexanoic acid was obtained by crystallization from chloroform in a yield of 1.2 g. Copolymerization of VMA (1 g, 2.1 mmol) with 6-(p-vinylbenzamido)-1-hexanoic acid (61 mg, 0.21 mmol) and 2.2'-azobis(isobutyronitrile) (AIBN) (20 mg) as an initiator dissolved in dimethylsulfoxide (DMSO; 10 ml) were poured into a glass ampoule. The sealed ampoule was placed in a thermostat at 60°C for 6 h, then the product was precipitated in methanol. The product was reprecipitated in methanol and freeze-dried. Yield of p(VMA-co-CapOH) was 800 mg. The copolymer composition was analyzed by ^{13}C-NMR spectroscopy (JEOL EX-300 MHz). p(VMA-co-CapOH) was activated with 1-ethyl-3-dimethylaminopropyl)carboiimide hydrochloride (WSC, Wako Pure Chemical, Tokyo, Japan) in 50 mM TEMED buffer at room temperature for 24 h. The activated copolymer was dialyzed against 10 l of distilled water and GRGDS was added to the solution. The reaction mixture was kept at 25°C for 3 days and then dialyzed against 10 l of distilled water. p(VMA-co-VBGRGDS) was isolated by freeze-drying of a water solution. The contents of GRGDS were measured by ^{13}C-NMR, and about 80% of the carboxyl groups were allowed to react with the amino terminal of GRGDS. The molecular weights of SU-incorporated copolymers were determined by means of a static light scattering study [Zimm's plot (Malvern Instruments, Series 4700, at 488 nm with a 40.140° scattering angle and at least 10 measurements)]. This method revealed that M_rs of PVMA and p(VMA-co-VBGRGDS) were 810 and 760 K, respectively. The structures and characteristics of prepared polymers are shown in Fig. 1 and Table 1, respectively. 3T3-L1 fibroblast cells were obtained from Korea Cell Bank (Seoul, Korea) and precultured in RPMI 1640 in 5% CO_2 and 95% air at 37°C. The medium was supplemented with 10% fetal calf serum, 50 mg/l streptomycin, and 75 mg/l penicillin sulphate. The cell morphology adhered on the copolymer was observed by phase-contrast microscope. The grown cell number was counted with a hemacytometer.

Table 1. Characteristics of prepared polymers.

Polymers	Feed ratio of VMA to CapOH (wt/wt)	Composition of GRGDS to VMA (CapOH) (wt%)[a]	Molecular weight	$[\eta]^b$	Yields (%)
PVMA	100:0	0	810,000	0.15	91
P(VMA-co-CapOH)	90:10	8.9	760,000	0.25	88

[a]Composition of GRGDS in the copolymer estimated from ^{13}C-NMR measurement.
[b][η] was measured in H_2O at 25°C.

Table 2. Effects of RGD sequence on inhibition of interaction between RGD-conjugated polymer and fibroblast cells.

Peptides \ Substrata	PS dishes (contriol)	PVMA	p(VMA-co-VBGRGDS)	Collagen
GRGDS	48±7.5	55±8.5	57±3.5	65±3.6
GREDS	51±7.5	53±8.5	84±3.5	88±3.6

The overall goal of this study was to develop materials that could rapidly be assembled for screening potential cell-attractive applications. In this viewpoint, the effect of the synthetic RGD peptide sequence in cell attachment is shown in Fig. 2. To investigate the specific interaction between the synthetic extracellular matrix and fibroblast cells, the cell adhesion assay was carried out on the polystyrene dish coated with a synthetic extracellular matrix and conventional polystyrene dishes as a control. Fibroblast cells (cell density: 2×10^4 cells/100 μl) suspended in RPMI1640 were seeded into the polymer-coated dishes (96 wells). The media were removed after 30 min. The cells were washed once with PBS buffer containing 0.5% BSA and 5 mM glucose. The number of attached cells were then determined by MTT assay method. This result shows that fibroblast cells adhered highly to collagen-coated dishes having the RGD peptide sequence. Moreover, the synthetic RGD peptide conjugated copolymer showed no difference in cell attachment, compared to the natural extracellular matrix of collagen. Thus, it would appear that recognition of the adhesion peptide by the cell-surface integrin families recognize strongly the RGD conjugated copolymer.

In order to evaluate the interaction mediated by integrin families, soluble RGD sequence was used as a competitive inhibitor between RGD-conjugated matrix and fibroblast cells. Fibroblast cells (cell density: 2×10^4 cells/100 μl) were pretreated with soluble RGD in RPMI1640, and then seeded into the polymer-coated dishes (96 wells). The media were removed after 30 min, and the cells were washed once with PBS buffer containing 0.5% BSA and 5 mM glucose. Then, the number of attached cells was determined by the MTT assay method. The effects of the RGD sequence on attachment for various matrix-coated surfaces are shown in Table 2. Pretreatment of fibroblast cells with pentapeptide GRGDS before seeding to p(VMA-co-VBGRGDS) and collagen surfaces significantly suppressed the attachment of cells onto matrices, but not onto control and GRGDS-free surfaces. Furthermore, the attachment of fibroblast cells onto RGD-conjugated matrix and extracellular matrix of collagen was strongly blocked by pentapeptide GRGDS, an analog of the RGD sequence recognized by

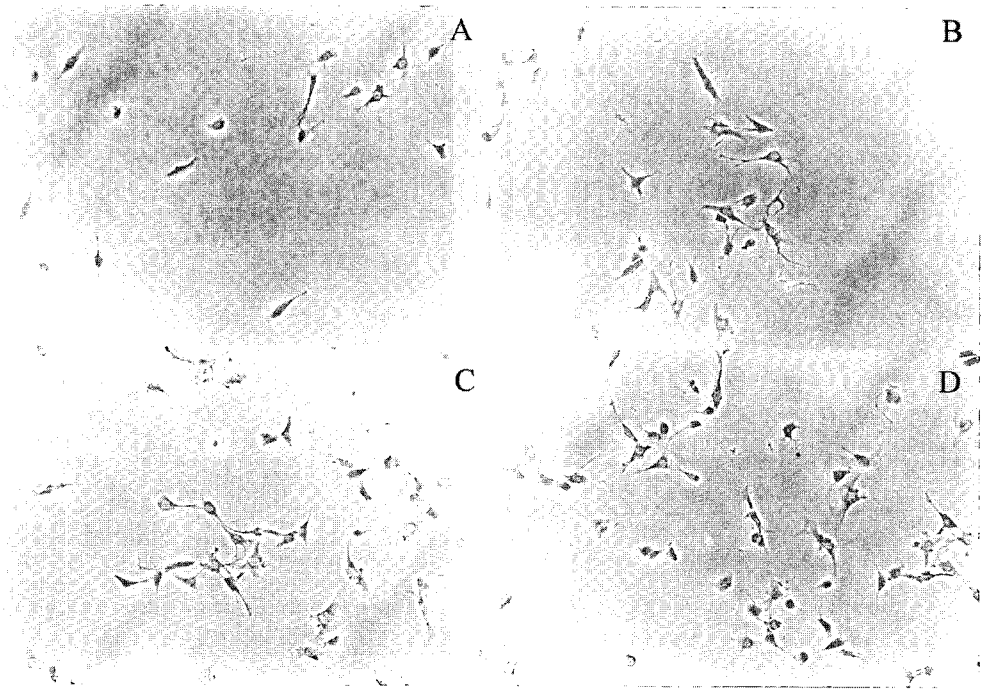

Fig. 3. Phase contrast photographs of fibroblast cells attached to the various polymer-coated surfaces.
a): control; (b): PVMA; (c): p(VMA-co-VBGRGDS); (d): collagen.

various integrins, but not by GREDS, the biologically inactive sequence. This means that the pentapeptide GRGDS interacted preferentially with integrin families expressed on the cell surface: The specific binding sites of RGD-conjugated substrata or collagen matrix for fibroblast cells were not available because of their occupation by pentapeptide GRGDS. This indicates that the RGD sequences of p(VMA-co-VBGRGDS) copolymer and collagen specifically interact with integrin families on fibroblast cell membrane.

Morphological change of fibroblast cells attached to the matrix-coated dishes for 1 day was also examined. No marked differences in morphology of attached fibroblast cells were found among polymers coated on PS dishes. Interestingly, significant proliferation of cells was observed for the p(VMA-co-VBGRGDS) and collagen matrix, while a little proliferation was found for the homopolymer (PVMA) or conventional polystyrene dishes (Fig. 3). As for the cell proliferation, it may be expected that cell-matrix interactions occurred through ligand-receptor specific interaction. Integrin families play an integral role in regulating differentiation and migration of neuronal and glial cells. It means that GRGDS-conjugated copolymer interacts with integrin families on fibroblast cell membrane in an RGD-dependent manner.

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