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Cell Adhesion Ability of Artificial Extracellular Matrix Proteins Containing a Long Repetitive Arg-Gly-Asp Sequence

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We generated recombinant artificial extracellular matrix (ECM) proteins containing repetitive Arg-Gly-Asp (RGD) sequences: double (RGD2), 21 (RGD21) and 43 (RGD43) repeats of RGD. RGD43-coated glass slides promoted fibroblast NIH3T3 cell adhesion and spreading on the surface. Since actin stress fibers and focal contacts were also observed in cells adhering on RGD43-coated glass slides, it was suggested that the RGD peptides in RGD43 transmitted an adhesion signal via integrins and promoted cell adhesion. We coated recombinant ECM proteins, each containing repetitive RGD domains, on polystyrene plates and investigated the effects of RGD length on the cell adhesion ability using three different cell lines, namely, fibroblast NIH3T3, HeLa cancer and neuronal PC12 cell lines. The results indicated that RGD43 had a cell adhesion ability superior to those of natural extracellular matrix proteins, fibronectin and laminin, although the effects of RGD repeat length on the cell adhesion ability depended on the cell line. As an artificial three-dimensional scaffold for cell cultivation, we also prepared an RGD43 hydrogel by a cross-linking reaction using glutaraldehyde. On the RGD43 hydrogel scaffold, fibroblast cells also successfully adhered under serum-free conditions.

[**Key words:** cell adhesion, fibronectin, RGD motif, tissue engineering, protein polymer, overlap elongation PCR]

Integrin-mediated adhesion to extracellular matrix (ECM) proteins is required for the survival of many anchorage-dependent cells (1). Anchorage-dependent cells recognize ECM proteins by their integrins, cell surface receptors, the signal of which activates cytoskeletal remodeling and cell cycle progression (2). The disruption of the cell-ECM complex may be crucial for the regulation of the survival machinery, resulting in apoptosis (1, 3). Therefore, natural ECM proteins are required for various cell applications such as tissue engineering. However, natural ECM proteins are huge molecules (*e.g.*, fibronectin: 250 kDa); therefore, it is difficult to produce them in a full-length form using a microbial expression system. The differences in codon usage between mammals and bacteria are also a barrier to producing them using this system. To overcome these problems, artificial ECM-like materials with integrin ligand sequences have been developed as a substitute for natural ECM proteins because integrin recognizes a short peptide sequence itself (4, 5). As a protein-based material, an artificial ECM protein, in which a multiple Arg-Gly-Asp (RGD) integrin ligand was introduced between silk-fibroin motifs, Gly-Ala-Gly-Ala-Gly-Ser, has been reported (6). Synthetic polymer-based material with an RGD cell-adhesive-peptide sequence have also been reported (7, 8). These artificial materials can be used as plate modifiers due to their strong cell adhesive ability.

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Previously, we generated recombinant artificial ECM proteins containing RGD repeats of various lengths: 2, 21 and 43 repeat RGD sequences, designated as RGD2, RGD21 and RDG43, respectively (9). It was also shown that RGD43 formed a self-assembled fibrous structure and had an excellent cell adhesive ability for NIH3T3 cells. In this study, we quantitatively investigated the effects of RGD repeat length on the cell adhesive ability using three cell lines, namely, fibroblast NIH3T3, HeLa cancer and neuronal PC12 cell lines. We also prepared an RGD43 hydrogel by a chemical cross-linking method, and determined whether this hydrogel can be used as an artificial three-dimensional ECM-like scaffold.

MATERIALS AND METHODS

Preparation of expression plasmids The expression plasmids for RGD2, RGD21 and RGD43 were prepared as reported (9). We prepared a novel recombinant artificial ECM protein containing Ile-Gly-Asp (IGD) repetitive sequences as a negative control. It was reported that the IGD peptide sequence was first identified from fibronectin and exhibited cell migration, but not cell adhesion (10). To generate DNAs encoding a repetitive IGD peptide sequence, we used overlap elongation PCR (9). Briefly, we synthesized DNA oligonucleotides encoding double repeats of the IGD sequence: 5′ ATCGGTGATATCGGTGAT (sense strand) and 5′ ATCACCGATATCACCGAT (antisense strand). The thermal cyclic reaction was performed for 40 cycles with DNA oligonucleotides, dNTP and DNA polymerase (Vent DNA polymerase; New England Biolabs, Beverly, MA, USA). The resulting elon-

gated products were electrophoresed in an agarose gel, excised and purified from the gel. The purified DNA products (100–500 bp) were cloned into pBAD/ThioTOPO vectors (Invitrogen, Carlsbad, CA, USA) by the TA-cloning method. From the resulting repetitive DNA library, we isolated plasmids containing DNAs encoding 33- and 46-repeat IGD sequences. In this study, a 46-repeat IGD fused with thioredoxin (IGD46) was used as a negative-control ECM protein. The pBAD/Thio control vector (Invitrogen) was also used to prepare thioredoxin (TRX) as another negative-control protein.

Overexpression of recombinant proteins *Escherichia coli* TOP 10 (Invitrogen) harboring each of the expression plasmids was cultured overnight in 5 ml of LB medium containing 100 μg/ml ampicillin. An aliquot (2 ml) of the culture broth was then inoculated into 300 ml of fresh LB medium and cells were cultivated at 37°C with vigorous shaking. When OD_{600} reached approximately 0.6, recombinant protein expression was induced by the addition of L-arabinose to a final concentration of 0.1%. The culture was carried out for a further 20 h at 30°C with vigorous shaking, and the culture broth was then centrifuged at 2000×*g* for 20 min. The harvested cell pellet was resuspended in 30 ml of extraction buffer (50 mM potassium phosphate buffer, pH 7.0, containing 300 mM NaCl and 4 M urea) and disrupted by sonication. The cell debris was removed by centrifugation at 3000×*g* for 15 min. Metal affinity resin (3 ml of TALON metal affinity resin; BD Biosciences, San Jose, CA, USA), equilibrated with extraction buffer, was added to the supernatant fraction and mixed gently (100 rpm on a rotary shaker) for 60 min. The protein-resin complexes were washed 3 times with 10 ml of extraction buffer. The recombinant proteins were eluted with 5 ml of elution buffer (50 mM potassium phosphate buffer, pH 7.0, containing 300 mM NaCl, 4 M urea and 250 mM imidazole). Recombinant proteins were separated in 10% acrylamide gels, and stained with Coomassie Brilliant Blue. Perfect protein ladder markers (Promega, Madison, WI, USA) were used as standard protein molecular weight markers. The concentrations of the purified proteins were determined using a BCA assay kit (Bio-Rad, Hercules, CA, USA) with purified BSA as the standard.

Cell culture conditions Mouse NIH3T3 cells and HeLa cells, obtained from American Type Culture Collection (Rockville, MD, USA), were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and kanamycin (100 μg/ml) under a humidified 5% CO_2 atmosphere at 37°C. PC12 cells (kindly provided by Professor Y. Goto) were maintained in DMEM supplemented with 10% FBS and 5% horse serum (Gibco/BRL, Gaithersburg, MD, USA). Subconfluent cells were peeled from dishes using a trypsin-EDTA solution and used for subsequent cell adhesion studies.

Observation of NIH3T3 adhesion on RGD43-coated glass slides and staining of actin fibers and focal contacts in cells Diluted RGD43 solutions (100 μl, 50 μg/ml) were dried on glass-bottomed dishes (35 mm; MatTek, Ashland, MA, USA), and rinsed with phosphate-buffered saline (PBS, pH 7.0). As negative controls, thioredoxin- and IGD46-coated glass slides were also prepared by the same procedure. Next, 200 μl of NIH3T3 cell suspension in serum-free DMEM was added to the dishes and cell adhesion on protein-coated glass slides was examined after incubation for 3 h at 37°C. Nonadherent cells on RGD43-coated glass slides were removed by washing with PBS, and adherent cells were fixed using glutaraldehyde and cold acetone. Actin fibers were stained using rhodamine-labeled phalloidin (Molecular Probes, Eugene, OR, USA). Focal contacts were stained using a human anti-vinculin antibody (Sigma-Aldrich, St. Louis, MO, USA) (primal antibody) and an FITC-labeled anti-human IgG1 antibody (fluorescent secondary antibody). Stained cells were observed with an IX81 fluorescence microscope (Olympus, Tokyo) at ×100 magnification.

Preparation of polystyrene plates coated with different amounts of recombinant proteins Each of the protein solutions (100 μl: 0.5, 1, 5, 25 and 50 μg/ml) in PBS was added into wells of untreated polystyrene plates (Iwaki, Chiba) and incubated for 12 h at 4°C. Protein solutions were removed and each well was washed twice with PBS. For a blocking reaction, 1% bovine serum albumin (Sigma-Aldrich) was added to each well, and incubated for 1 h at 37°C. The wells were washed twice with PBS, and used as protein-coated plates.

Evaluation of relative amounts of adsorbed recombinant proteins using enzyme-linked immunosorbent assay (ELISA) A horseradish peroxidase (HRP)-labeled anti-His antibody (Invitrogen) solution was added to wells of the polystyrene plates (Iwaki) coated with recombinant proteins, and reacted with His tags existing in the C-terminal of the adsorbed proteins for 6 h at 4°C. After discarding the antibody solution, each well was washed twice with PBS. *O*-Phenyrene diamine was added to each well as a colorimetric reagent (ELISA kit; Sumiron, Tokyo) following the manufacturer's protocol. Color development was measured using a UV-microplate reader (model 550; Bio-Rad) at 490 nm.

Cell adhesion on protein-coated polystyrene plates Cells peeled from the plate were washed twice with serum-free DMEM (Gibco/BRL) to remove cell adhesive factors derived from serum. Washed cells in serum-free DMEM (2.0×10^5 cells in 100 μl) were added to protein-coated polystyrene plates, and cultured for 3 h at 37°C. As positive controls, polystyrene plates coated with bovine plasma fibronectin (Gibco/BRL) and natural mouse laminin (Gibco/BRL) were also prepared by the same procedure. Non-adhesive and -spreading cells were removed by washing with serum-free DMEM, and DMEM with 10% FBS and a cell-counting reagent (Cell-Counting Kit 8; Promega) was added to each of the wells. After 1.5 h of incubation with the colorimetric reagent, the absorbance of each well was measured using a UV-microplate reader (model 550; Bio-Rad) at 490 nm. The number of cells adhering on a fibronectin-coated plate (50 μg/ml) was used to normalize adherent cell numbers and defined as 100% cell adhesion for each cell line.

Preparation of RGD43 hydrogel and NIH3T3 cell adhesion The purified RGD43 solution with 4 M urea was concentrated using an ultrafiltration membrane filter (30 kDa cut-off; Millipore, Billerica, MA, USA) to a final concentration of approximately 10 mg/ml. The concentrated protein solution was dialyzed in pure water for 24 h. The resultant protein solution had a high viscosity, but was not a solid product. The protein solution was poured into a 15-ml centrifugal tube, and glutaraldehyde (3.7% final concentration) was subsequently added to the top of the tube. A cross-linking reaction was performed under centrifugation (2000×*g*) for 10 min. After centrifugation, a solid hydrogel pellet was obtained at the bottom of the tube and washed twice with acetone. The hydrogel was sliced using a surgical knife, and soaked in pure water and PBS. NIH3T3 cells were cultured with the hydrogel under the serum-free condition described above, and stained using calsein-AM (Dojindo, Kumamoto) solution to observe living cells adhering on the hydrogel.

Scanning electron microscopy (SEM) of RGD43 hydrogel scaffold A sliced hydrogel particle was fixed onto the scanning electron microscope stub using carbon paste, and then dried using a vacuum dryer at room temperature. The fixed hydrogel was sputter-coated with platinum for 15 s and the sample plate was observed with an S-5000 scanning electron microscope (Hitachi, Tokyo) at 10 keV.