Biomaterials 34 (2013) 6539–6547

Contents lists available at SciVerse ScienceDirect

Biomaterials

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

Laminin-111-derived peptide-hyaluronate hydrogels as a synthetic basement membrane

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ARTICLE INFO

Article history:
Received 15 April 2013
Accepted 23 May 2013
Available online 10 June 2013

Keywords:
ECM (extracellular matrix)
Laminin
Peptide
Hyaluronic acid/hyaluronan
Cell culture

ABSTRACT

We have identified a number of cell-adhesive peptides from laminins, a major component of basement membranes. Cell-adhesive peptides derived from basement membrane proteins are potential candidates for incorporating cell-binding activities into scaffold materials for tissue engineering. Our goal is development of a chemically synthetic basement membrane using laminin-derived cell-adhesive peptides and polymeric materials. In this study, we used hyaluronic acid (HA) as a scaffold material and laminin-derived cell-adhesive peptides, A99 (AGTFALRGDNPQG, binds to integrin αvβ3), AG73 (RKRLQVQLSIRT, binds to syndecans), and an A99/AG73 mixture (molar ratio = 9:1) conjugated to two-dimensional (2D) HA matrices. As a result, it was found that the 2D A99/AG73-HA matrices have strong biological functions, such as cell attachment, cell spreading, and neurite outgrowth, similar to that of basement membrane extract (BME)-coated plates. Next, we developed three-dimensional (3D) peptide-HA matrices using the A99/AG73 mixture. The 3D A99/AG73-HA matrices promoted cell spreading and improved cell viability and collagen gene expression. Further, PC12 neurite extension was observed in the 3D A99/AG73-HA matrices. These biological activities of the 3D A99/AG73-HA matrices were similar to those of the 3D BME matrices. These results suggest that the peptide-HA matrices are useful as 2D and 3D matrices and can be applied for tissue engineering as a synthetic basement membrane.

1. Introduction

Three-dimensional (3D) cell culture matrices are essential in tissue engineering to maintain or improve cellular functions. Previous reports suggested that cell behavior in 3D matrices is different from that on two-dimensional (2D) matrices [1,2]. In native tissues, cells are three-dimensionally held within extracellular matrices (ECMs), which have critical roles in maintaining tissues, and guiding development, regeneration, and homeostasis [3]. Therefore, mimicking ECM is a common goal in biomaterial studies for tissue engineering, and synthetic or natural polymer hydrogels are widely used as 3D cell culture platforms [4–8].

Basement membrane is a thin layer of ECM and a soluble extract of basement membranes (BME) from the mouse Engelbreth-Holm-Swarm tumor, such as Matrigel (BD Biosciences, San Jose, CA) and Cultrex BME (Trevigen, Inc., Gaithersburg, MD), is commercially available. The major components of BME/Matrigel are type IV collagen, laminin-111, nidogen/entactin, and perlecan [9]. BME/Matrigel promotes the differentiation of cells, including hepatocytes, salivary cells, endothelial cells, and neurons, and has been used for various biological experiments, including 3D cell culture [9]. However, BME/Matrigel contains growth factors and small amounts of other proteins and cannot be used in humans. Fortunately, some of the biological functions of the basement membrane have been identified using purified protein components or their fragments [10,11]. These molecules are potential candidates for incorporating cell-binding activities to clinically compatible scaffold materials.

Laminins, major components of basement membrane, are large heterotrimeric glycoproteins consisting of α, β, and γ chains. Laminins play important roles in cell adhesion, migration, proliferation, neurite outgrowth, and angiogenesis, and laminin-111 is the major component of BME/Matrigel [11,12]. Using more than 600 synthetic peptides in a systematic screening, we previously identified various active sequences in laminin-111 [13–17]. About 60 peptides showed cell attachment activity and some of them were found to

Abbreviations: ECM, extracellular matrix; HA, hyaluronic acid; 2D, two-dimensional; 3D, three-dimensional; BME, basement membrane extract; FBS, fetal bovine serum; BSA, bovine serum albumin; HDF, human dermal fibroblasts; DMEM, Dulbecco's modified Eagle's medium; NHS, *N*-hydroxy succinimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; EDA, ethylenediamine; MBS, *N*-(*m*-maleimidebenzoyloxy)succinimide; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline.

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http://dx.doi.org/10.1016/j.biomaterials.2013.05.044

recognize cellular receptors, such as integrins and syndecans. For example, peptide AG73 (RKRLQVQLSIRT, mouse laminin α1 chain 2719-2730), a syndecan-binding peptide, has various biological activities, such as promoting cell adhesion, migration, invasion, gelatinase production, neurite outgrowth, angiogenesis, and acinar-like development [13,18–22]. Peptide A99 (AGTFALRGDNPQG, mouse laminin α1 chain 1141-1153) binds to αvβ3 integrin and promotes cell adhesion, cell elongation with actin stress fibers, and neurite outgrowth [23–25]. Peptide EF1 (DYATLQLQEGRLHFMFDLG, mouse laminin α1 chain 2747-2765), an α2β1 integrin-binding peptide, also promotes cell adhesion, cell spreading, and organization of actin filaments [24,26]. Further, we conjugated the active peptides to polysaccharides, such as chitosan and alginate, and demonstrated that the peptide-polysaccharide matrices were biologically active and could potentially serve as bio-adhesives [23,27,28].

Hyaluronic acid (also called either hyaluronan, hyaluronate, or HA) is a biodegradable, non-cytotoxic, and non-immunogenic polysaccharide and a major component of the extracellular matrix, which has critical roles in cell proliferation, cell migration, and tissue repair. HA has been used for various medical applications, such as ophthalmic surgery, osteoarthritis of the joint, and also widely studied as a cell culture matrix for tissue engineering [29–33]. Several groups showed that Arg-Gly-Asp (αvβ3 integrin-binding motif) peptide-conjugated HA hydrogels are useful as a cell-adhesive matrix [34–37]. For example, Lei et al. reported that mouse mesenchymal stem cells (mMSC) proliferated in HA hydrogels, and the cells spread when the RGD peptide was present in the hydrogel [35].

In this paper, we conjugated laminin-derived cell-adhesive peptides to HA hydrogels and evaluated biological functions of the peptide-HA matrices as a cell culture scaffold. The A99 peptide and the AG73 peptide were covalently conjugated to the HA matrices as cell-adhesion molecules because the two peptides were identified to bind to integrin and syndecan, respectively, and promote receptor-specific biological activities. Additionally, A99/AG73 (molar ratio = 9:1)-HA matrices were also examined because the A99/AG73 mixture was reported to have stronger biological activities on a chitosan matrix [23,25]. First, we evaluated biological activities of 2D peptide-HA matrices. Then, to culture cells three-dimensionally, we developed 3D peptide-HA matrices and evaluated their biological functions.

2. Materials and methods

2.1. Synthetic peptides

All peptides were manually synthesized by the 9-fluorenylmethoxycarbonyl (Fmoc) strategy and prepared in the C-terminal amide form, as previously described [13]. For conjugation to HA, a Cys residue was added at the N-terminus and two Gly residues were used as a spacer between the Cys and the active peptide sequences. Purity and identity of the peptides were confirmed by analytical HPLC and electrospray ionization mass spectrometry at the Central Analysis Center, Tokyo University of Pharmacy and Life Sciences.

2.2. Cells

Human dermal fibroblasts (HDFs; Cell Applications Inc., San Diego, CA) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Rat pheochromocytoma cell, PC12, was purchased from ATCC (Manassas, VA) and cultured in DMEM containing 7.5% horse serum (Invitrogen), 7.5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.3. Preparation of MB-HA

Sodium hyaluronate was purchased from Wako Pure Chemical Industries, Ltd, (Osaka, Japan). Sodium hyaluronate (50 mg, 250 μmol of sugar unit) was dissolved in 0.1 M MES buffer containing 0.3 M NaCl (pH 3.6, 25 ml) and 1 M *N*-hydroxy succinimide (NHS; 500 μl, 500 μmol) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC; 500 μl, 500 μmol) was added. After a 15 min activation, 10% ethylenediamine (EDA; 50 μl, 75 μmol) was added and incubated for 24 h at pH 7–8. After reaction, the product was purified by dialysis (3500 MWCO) against 0.025 M NaCl aq. for 24 h and lyophilized. Then, the end of the reaction was confirmed with a ninhydrin test (positive). The HA product (10 mg) was dissolved in 30% acetonitrile aq. (9 ml) and *N*-(*m*-maleimidebenzoyloxy)succinimide (MBS; 8 mg, 25.5 μmol) in DMSO (1 ml) was added. The mixture was stirred at room temperature for 24 h. Then, the end of the reaction was confirmed with a ninhydrin test (negative). The maleimidobenzoyloxy (MB)-HA product was purified by dialysis (3500 MWCO) against 0.025 M NaCl aq. for 24 h and lyophilized. MB content in the MB-HA was determined by the absorbance at 280 nm and regulated by mixing the MB-HA with unmodified HA (final MB content: 1% HA sugar unit).

2.4. Preparation of 2D peptide-HA matrices and 2D BME matrices

The 2D HA matrices were prepared by cross-linking of MB-HA chains via EDA and drying onto the plates. Briefly, the MB-HA (1 mg, 5 μmol of sugar unit) was dissolved in 0.1 M MES buffer containing 0.3 M NaCl (100 μl). Then 1 M NHS (3 μl, 3 μmol) and 1 M EDC (10 μl, 10 μmol) were added. After a 15 min activation, 1% EDA (6.7 μl, 1 μmol) was added. The solution was diluted by Milli-Q water (200 μg/ml) and a 50 μl of the solution was added to the 96-well plates (Thermo Fisher Scientific, Roskilde, Denmark) and dried at room temperature for 2 days (300 ng/mm^2, MB: 15 pmol/mm^2). After washing with PBS (100 μl × 2 times), peptide solutions in 0.1% trifluoroacetic acid (TFA) aq. were prepared. For conjugation to the MB-HA, 0.2 mM peptide solutions (50 μl) and an equal amount of 1% $NaHCO_3$ aq. were added into the wells and incubated for 2 h. The A99/AG73 solution (molar ratio = 9:1, total 0.2 mM) was prepared by mixing of the A99 and AG73 solutions and coupled to the MB-HA. Obtained 2D peptide-HA matrices were washed with PBS and used for the biological assays. Growth Factor-Reduced Cultrex BME (Trevigen, Inc., Gaithersburg, MD), diluted 1:100 in PBS, was added to the 96-well plates and incubated for 1 h at 37 °C. Then the plates were washed with PBS and used for the biological assays.

2.5. 2D HDF attachment assay

Human dermal fibroblasts (HDFs) were detached with 0.02% trypsin-ethylenediaminetetraacetic acid (EDTA) solution and resuspended in serum-free media. Then, HDFs (2 × 10^4 cells/100 μl) were added to each well and incubated at 37 °C in 5% CO_2. After washing off the unattached cells, the attached cells were stained with 0.2% crystal violet in 20% methanol aq. (50 μl) for 15 min. After washing with Milli-Q water (200 μl × 2 times), the attached cells in three randomly selected fields were counted using a BioZero microscope (Keyence, Osaka, Japan). All assays were carried out in triplicate with each experiment repeated at least three times.

2.6. Inhibition assay

For inhibition of cell attachment with EDTA or heparin, HDFs (2 × 10^4 cells/100 μl) were added to the wells and incubated for 1 h at 37 °C in the presence of either 5 mM EDTA or 10 μg/ml heparin. The attached cells were stained with 0.2% crystal violet in 20% methanol aq. (50 μl) and counted as described above. All assays were carried out in triplicate with each experiment repeated at least three times.

2.7. 2D neurite outgrowth assay

After priming with nerve growth factor (100 ng/ml; Invitrogen) for 24 h prior to use, PC12 cells were resuspended in DMEM/Ham's F-12 containing 30 nM of $NaSeO_3$ (Wako), 100 μg/ml transferrin, 20 nM progesterone (Sigma–Aldrich), 5 μg/ml insulin (Invitrogen), and 100 ng/ml NGF. The cells were added to the wells (3 × 10^3 cells/100 μl/well) and incubated at 37 °C for 24 h in 5% CO_2, then fixed with 4% formaldehyde in PBS for 10 min, and stained with 0.2% crystal violet in 20% methanol aq. After the wells were washed with Milli-Q water (150 μl × 2 times), the cells were analyzed under a BioZero microscope. The cell that had neurites more than double the length of the cell body was defined as an extended cell. Each condition was tested in triplicate and the assays were repeated at least three times.

2.8. Preparation of 3D peptide-HA matrices

The 3D peptide-HA matrices were prepared as follows. MB-HA (10 mg, 50 μmol of sugar unit) was dissolved in 0.1 M MES buffer containing 0.3 M NaCl (425 μl). 1 M NHS (12.5 μl, 12.5 μmol) and 4 M EDC (12.5 μl, 50 μmol) were added. After a 15 min activation, 1% EDA (50 μl, 7.5 μmol) was added and incubated for 24 h at pH 7–8 to allow the gel to set. The hydrogels were mechanically crushed using a homogenizer and washed with acetone. After drying, MB-HA powder was obtained. For conjugation to the MB-HA, the A99/AG73 solution (molar ratio = 9:1, total 0.2 mM, 1 ml) and an equal amount of 1% $NaHCO_3$ aq. were added and incubated for 2 h. Then, the peptide-HA hydrogels were washed and dehydrated by acetone and peptide-HA powder was obtained. The peptide-HA powder was carefully divided into five tubes (MB-HA: 2 mg/tube) and re-swelled with cell suspension media (100 μl) and used as a 3D peptide-HA matrix.