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Identification of a bioactive core sequence from human laminin and its applicability to tissue engineering

In-Sung Yeo [a, 1], Seung-Ki Min [b, 1], Hyun Ki Kang [c], Taek-Ka Kwon [d], Sung Youn Jung [c], Byung-Moo Min [c, *]

[a] Department of Prosthodontics, Seoul National University School of Dentistry, 101 Daehak-Ro, Jongno-Gu, Seoul 110-744, Republic of Korea
[b] Department of Oral and Maxillofacial Surgery, Seoul National University School of Dentistry, 101 Daehak-Ro, Jongno-Gu, Seoul 110-744, Republic of Korea
[c] Department of Oral Biochemistry and Program in Cancer and Developmental Biology and Dental Research Institute, Seoul National University School of Dentistry, 101 Daehak-Ro, Jongno-Gu, Seoul 110-744, Republic of Korea
[d] Department of Dentistry, St. Vincent Hospital, Catholic University of Korea, Ji-Dong, Paldal-Ku, Suwon 442-723, Republic of Korea

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Finding bioactive short peptides derived from proteins is a critical step to the advancement of tissue engineering and regenerative medicine, because the former maintains the functions of the latter without immunogenicity in biological systems. Here, we discovered a bioactive core nonapeptide sequence, PPFEGCIWN (residues 2678–2686; Ln2-LG3-P2-DN3), from the human laminin $\alpha2$ chain, and investigated the role of this peptide in binding to transmembrane proteins to promote intracellular events leading to cell functions. This minimum bioactive sequence had neither secondary nor tertiary structures in a computational structure prediction. Nonetheless, Ln2-LG3-P2-DN3 bound to various cell types as actively as laminin in cell adhesion assays. The *in vivo* healing tests using rats revealed that Ln2-LG3-P2-DN3 promoted bone formation without any recognizable antigenic activity. Ln2-LG3-P2-DN3-treated titanium (Ti) discs and Ti implant surfaces caused the enhancement of bone cell functions *in vitro* and induced faster osseointegration *in vivo*, respectively. These findings established a minimum bioactive sequence within human laminin, and its potential application value for regenerative medicine, especially for bone tissue engineering.

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1. Introduction

Complications, including graft rejection, donor site morbidity, and inadequate tissue formation, depending on the health status of each patient, limit current grafting methods to repair damaged tissues [1,2]. Tissue engineering is an exciting new approach that can overcome the limitations of tissue transplantation, providing man-made tissues to patients in need [3,4]. Several challenges have to be solved for a tissue-engineered material to be clinically applied; achieving appropriate functional activity without triggering an immune reaction is one of the most important goals. An example of an undesirable immune reaction is the recombinant human bone morphogenetic protein (rhBMP)-2, which is associated with early osteolysis, a kind of immunologic reaction, despite its enhanced bone formation effect [5,6]. The result is the sinking of an adjacent implanted material into the marrow [6]. Peptides are attractive as novel therapeutic reagents, since they are flexible in adopting and mimicking the local structural features of proteins. Their smaller molecular weight, synthetic versatility, and economical production mean that peptides share many drug targeting, potency, stability, and bioavailability challenges with other protein-based biological therapeutics such as antibodies and growth factors [7]. Finding short bioactive peptides is anticipated to overcome the obstacles that entire proteins have in regenerative medicine, because shorter amino acid sequences lower antigenicity [8–10]. A peptide found to have desirable bioactivity without immunogenicity is therefore fascinating in the field of tissue regeneration and regenerative medicine.

Laminins are major proteins in the basal lamina, a structural base for most cells and organs, and they contribute critically to cell adhesion, differentiation, and migration as well as tissue survival

* Corresponding author.
E-mail addresses: pros53@snu.ac.kr (I.-S. Yeo), snowmanonmars@gmail.com (S.-K. Min), kang1978@snu.ac.kr (H.K. Kang), tega95@naver.com (T.-K. Kwon), jsy1618@snu.ac.kr (S.Y. Jung), bmmin@snu.ac.kr (B.-M. Min).
[1] These authors contributed equally to this work.

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[11,12]. They are biologically active with a number of diverse cell types. A very interesting feature is that the cell functions of laminins are diverse, depending on cell types and cell-specific intracellular events [11,13]. This feature suggests that laminins and some derivative molecules should have the potential for broad applicability to the regeneration of various tissues. Structurally, laminins are trimeric proteins of an α, a β, and a γ chain that intersect to form a cross-like structure binding to some cell transmembrane molecules; this binding triggers intracellular events [14]. Laminin α chains contain a C-terminal large globular (LG) domain consisting of five globular modules (LG1-LG5) that are implicated as active regions for biological functions [11,15]. Specifically, the LG domains of the laminin α2 chain bind to the transmembrane molecules, integrins or syndecan-1, for the intracellular events and cell functions [15–18].

A bioactive short peptide would solve the immunologic reaction problem that every protein has by lowering antigenicity. Laminin has various biological effects according to cell type; intracellular events are promoted when the laminin binds to a membrane receptor. Therefore, if the core binding sequence of laminin is found and this short peptide is still bioactive for the cell functions, it will become a very useful tool for tissue regeneration and engineered tissue therapy with multiple desirable effects for each cell and tissue type with minimal immune reaction.

We previously found that the RNIPPFEGCIWN (residues 2675–2686; Ln2-LG3-P2) sequence within the human laminin α2 LG3 domain is a major ligand for α3β1 integrin, which mediates cell functions by inducing the membrane recruitment and phosphorylation of protein kinase Cδ (PKCδ) [17]. This study identified the minimum bioactive sequence that actively stimulated the intracellular events, and confirmed its applicability in the tissue engineering field by demonstrating that this peptide accelerated bone regeneration and healing without recognized side effects.

2. Materials and methods

2.1. Peptides, reagents, and preparation of titanium discs and implants

All peptides were synthesized by the 9-fluorenylmethoxycarbonyl-based solid-phase method with a C-terminal amide on a Pioneer peptide synthesizer (Applied Biosystems, Foster City, CA, USA). Peptides were purified and characterized by Peptron (Daejeon, Korea). The purities of all of the peptides used in this study were greater than 95%, as determined by high-performance liquid chromatography. The structures of the peptides were analyzed by the PSIPRED method for secondary structure prediction and by an *ab initio* technique for computational structure prediction [19,20]. Human placental laminin and bone morphogenetic protein (BMP)-2 were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Pepro-Tech (Rocky Hill, NJ, USA), respectively. Titanium (Ti) discs (20 mm and 50 mm in diameter, 0.5 mm thick) and Ti implants (3.5 mm in diameter, 8 mm long) were prepared from commercially pure grade 4 Ti (Warrantec, Seoul, Korea).

2.2. Cell cultures and directed differentiation from skin-derived precursors to osteogenic cells

The PC12 cell line from a transplantable rat pheochromocytoma was purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in RPMI 1640 medium (BioWhittaker Cambrex, Walkersville, MD, USA) containing 10% fetal bovine serum (FBS). The mouse embryo fibroblast cell line NIH/3T3, normal African green monkey kidney fibroblast cell line CV-1, and murine osteoblastic MC3T3-E1 cell line were purchased from the ATCC and cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Carlsbad, CA, USA) containing 10% FBS. For osteogenic differentiation, MC3T3-E1 cells were cultured in α-minimal essential medium (α-MEM; Gibco BRL) supplemented with 0.1 μM dexamethasone, 280 μM ascorbic acid, 10 mM β-glycerol phosphate, and 10% FBS for two weeks. The medium was changed every 2 d, and cultures were maintained for two weeks without passaging. Primary normal human epidermal keratinocytes (NHEKs), normal human oral keratinocytes (NHOKs), normal human dermal fibroblasts (NHDFs), and normal human oral fibroblasts (NHOFs) were prepared and maintained as described previously [21,22].

Human osteogenic cells were prepared and maintained as reported previously [23]. Briefly, for the directed differentiation of skin-derived sphere-forming cells into osteogenic cells, multipotent skin-derived precursors (SKPs) were isolated from the human foreskins of patients (1–3 years old) undergoing surgery [24,25]. Next, SKPs were differentiated into mesenchymal cells, as previously described [24]. Briefly, the tertiary spheres were dissociated into single cells with accutase and cultured in α-MEM supplemented with 10% FBS. Attached cells were subcultured upon reaching 80% confluence, and third passage cells were used as SKP-derived mesenchymal cells. For osteogenic differentiation, SKP-derived mesenchymal cells were cultured in α-MEM supplemented with 10 μM dexamethasone, 200 μM ascorbic acid, 10 mM β-glycerol phosphate, and 10% FBS for two weeks. The medium was changed every 2 d, and cultures were maintained for two weeks without passaging [24]. All procedures for obtaining human tissue specimens were performed in accordance with the guidelines of the Institutional Review Board on Human Subjects Research and the Ethics Committee at Seoul National University Dental Hospital, Seoul, Korea (approval number: CRI12004G).

2.3. Cell adhesion, spreading, and migration assays

Cell adhesion assays were performed as described previously [26]. Briefly, 24-well culture plates (Nunc, Roskilde, Denmark) were coated with bovine serum albumin (BSA; 1%), human placental laminin (1.1 $\mu g/cm^2$), recombinant LG3 (rLG3; 5.7 $\mu g/cm^2$), and synthetic peptides (11.4 $\mu g/cm^2$) by drying for 24 h at room temperature. The concentrations of the synthetic peptides were determined from a dose–response curve, and the lowest concentration necessary to achieve maximum adhesion to human osteogenic cells was used [17]. Substrate-coated plates were blocked with 1% heat-inactivated BSA in PBS for 1 h at 37 °C and were then washed with PBS. Cells were detached with trypsin/ethylenediaminetetraacetic acid (EDTA) and resuspended in serum-free culture medium. Cells (1×10^5 cells/500 μl) were added to a plate coated with peptide and incubated for 1 h for cell adhesion assays and 3 h for spreading assays at 37 °C in a 5% CO_2 atmosphere. After incubation, unattached cells were removed by rinsing the plates twice with PBS. Attached cells were fixed with 10% formalin in PBS for 15 min and then stained with 0.5% crystal violet for 1 h. The plates were gently washed three times with double-distilled water (DDW), after which the contents of each well were solubilized in 2% sodium dodecyl sulfate (SDS) for 5 min. Absorbance was measured at 570 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). Similar cell adhesion assays were also performed with the Ln2-LG3-P2-DN3 motif (11.4 $\mu g/cm^2$), NHEKs (passage 2), NHOKs (passage 2), NHDFs (passage 4), NHOFs (passage 4), human Schwann cells, MC3T3-E1, NIH/3T3, and CV-1 cells. For the cell spreading assays, cells (3×10^4 cells/250 μl) were added to each substrate-coated plate and incubated for 3 h at 37 °C. Attached cells were fixed with 10% formalin and then stained with 0.5% crystal violet for 1 h. Plates were gently washed three times with PBS. Cell spreading

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