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The effect of the DLTIDDSYWYRI motif of the human laminin α2 chain on implant osseointegration

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ABSTRACT

Considerable effort has been directed towards replacing lost teeth using tissue-engineering methods such as titanium implants. A number of studies have tried to modify bioinert titanium surfaces by coating them with functionally bioactive molecules for faster and stronger osseointegration than pure titanium surfaces. Recently, peptides have been recognized as valuable scientific tools in the field of tissue-engineering. The DLTIDDSYWYRI motif of the human laminin-2 α2 chain has been previously reported to promote the attachment of various cell types; however, the *in vivo* effects of the DLTIDDSYWYRI motif on new bone formation have not yet been studied. To examine whether a laminin-2-derived peptide can promote osseointegration by accelerating new bone formation *in vivo*, we applied titanium implants coated with the DLTIDDSYWYRI motif in a rabbit tibia model. The application of the DLTIDDSYWYRI motif-treated implant to tibia wounds enhanced collagen deposition and alkaline phosphatase expression. It significantly promoted implant osseointegration compared with treatment with scrambled peptide-treated implants by increasing the bone-to-implant contact ratio and bone area. These findings support the hypothesis that the DLTIDDSYWYRI motif acts as an effective osseointegration accelerator by enhancing new bone formation.

1. Introduction

Considerable effort has been directed towards regenerating defective tissues using tissue-engineering methods. Dental implants prepared from titanium (Ti) have been used to replace lost teeth through direct contact with surrounding bone, a process known as osseointegration. Modifications of Ti surfaces can cause faster and stronger osseointegration than on pure Ti surfaces [1–4]. Many researchers have tried to modify bioinert Ti surfaces of dental implants by coating them with bioactive molecules, such as calcium phosphate and bone morphogenetic proteins (BMPs), to enhance osseointegration and to shorten the healing period of the bone [5–7]. Although calcium phosphate-coated implants have shown clinical success with superior early bone healing response [5,7–10], there are still some problems, including coating fragmentation and osteolysis due to wear particles [9]. Similarly, implants coated with recombinant human BMP-2 are associated with complications such as early osteolysis and implant subsidence, although it usually results in excellent bone response at the interface between the bone and the implant [11].

The cell binding activity of laminin α2 chain has been reported to show preferential binding to integrins or syndecan-1 through differential interactions of the five carboxyl-terminal large globular (LG) domains of laminin [12–15]. This cell binding characteristics of laminin α2 chain may be utilized to help improve the bone response to the implant surface without the disadvantages of the osteolysis and implant subsidence of calcium phosphate and BMP-2

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coating [14,16,17]. To apply tissue-engineering methods for the treatment of patients, several problems, such as the functional activity and immune rejection of these engineered tissues, must first be resolved. Recently, peptides have been recognized as valuable scientific tools in the field of tissue-engineering because of their ability to improve the functional activity of the scaffold and to bypass or minimize immune rejection [18]. The surface modification of scaffolds with peptides has been tested extensively, and the application of these peptides to tissue regeneration is highly attractive [19].

The attachment of bone cells to implant surfaces is the first step in bone healing. Therefore, the cell attachment properties of laminin may improve biologic response at the bone-Ti-implant interface. Several synthetic peptides derived from the laminin α2 chain LG domains promote cell attachment [14,17]; however, investigations of laminin have mainly focused on epithelial attachment instead of bone-to-implant contact [20–22]. The potential roles of functionally bioactive peptides on osseointegration have not yet been elucidated.

We recently reported that the DLTIDDSYWYRI motif (amino acids 2221-2232; Ln2-P3) within the human laminin α2 LG1 domain mediates cell attachment through syndecan-1 by inducing phosphorylation and membrane localization of protein kinase Cδ (Fig. 1) [14]. Furthermore, Ln2-P3 has a broad spectrum of cell-type attachment activity [14]. These studies raised the hypothesis that a laminin-2-derived peptide, Ln2-P3, can promote new bone formation and osseointegration of Ti implants *in vivo*. In the present study, the effect of Ln2-P3 was examined in a rabbit implant model, as well as in human osteoblast-like cell culture system.

2. Materials and methods

2.1. Cells and peptides

Osteoblast-like MG63 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS). All peptides were synthesized by the Fmoc (9-fluorenylmethoxycarbonyl)-based solid-phase method with a C-terminal amide by using a Pioneer peptide synthesizer (Applied Biosystems, Foster City, CA, USA), purified, and then characterized at Peptron (Daejeon, Korea). The purities of all peptides used in this study were greater than 95%, as determined by high-performance liquid chromatography.

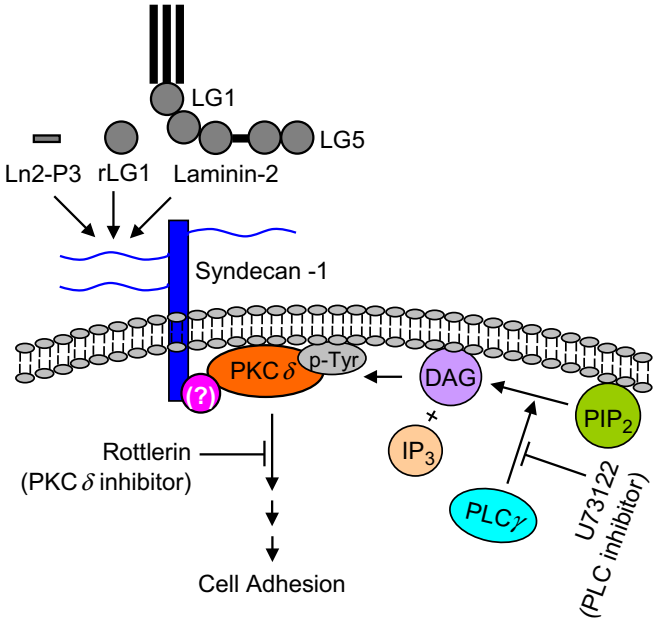

Fig. 1. Proposed pathway for the promotion of cell attachment of PC12 cells by either laminin-2 or the LG1 domain and the DLTIDDSYWYRI motif (Ln2-P3) within the human laminin α2 chain. The Ln2-P3 motif as well as the laminin-2 and LG1 domain promote cell attachment through syndecan-1 by inducing phosphorylation and membrane localization of PKCδ. PIP_2: phosphatidylinositol 4,5-bisphosphate, DAG: diacylglycerol, IP_3: inositol 1,4,5-trisphosphate.

2.2. Cell attachment and spreading assays

Twenty-four-well plates (Nunc, Roskilde, Denmark) were coated with human placental laminin (1.3 μg/cm^2; Sigma–Aldrich, St. Louis, MO, USA), scrambled peptide (21 μg/cm^2; SP), or Ln2-P3 (21 μg/cm^2) by drying for 18 h at room temperature and then washing with phosphate-buffered saline (PBS). These values were the lowest concentrations at which the maximum level of attachment activity of PC12 cells was determined from dose–response curves [14]. Cell attachment and spreading were assayed as previously described [23]. Briefly, MG63 cells were detached by trypsin digestion, 500 μl of a cell suspension containing 1×10^5 cells was placed in each well of a 24-well plate coated with peptide, and the cells were allowed to settle/adhere for 1 and 3 h at 37 °C in a 5% CO_2 atmosphere. Loosely adherent or unbound cells were removed by aspiration, and the wells were washed once with PBS. The remaining bound cells were fixed with 10% formalin in PBS for 15 min and stained with 0.5% crystal violet for 1 h. The wells were gently rinsed with double-distilled water (DDW) three times, photographed using an Olympus BX51 microscope at 100× magnification, and lysed with 2% SDS for 5 min. Absorbance was measured at 570 nm in a model 550 microplate reader (Bio-Rad, Hercules, CA, USA). Cell spreading was analyzed with photographs that were taken during the cell attachment assay. To ensure a representative count, each sample was divided into quarters and two fields per quarter were photographed with an Olympus BX51 microscope at 100× magnification. The area of each spreading cell was determined using a computer equipped with Image-Pro plus software (Media Cybernetics, Silver Spring, MD, USA). At least 200 cells were examined on each occasion. Averages and standard deviations were calculated from four independent experiments.

2.3. Preparation and characterization of the Ti discs

Ti discs (20 mm and 50 mm in diameters, 0.5 mm thick) were prepared from commercially pure grade 4 Ti. Four types of disc surfaces were then prepared. First, pure Ti surfaces without any surface modification serving as controls. The second type of disc was a Ti surface that was sandblasted with large alumina (Al_2O_3) particles and etched with hydrochloric acid solution (SLA surface; Dentium, Seoul, Korea), rinsed, ultrasonically washed, and dried. The third type of surface was an anodized Ti disc (Dentium) that was anodically oxidized in an aqueous electrolyte solution under a pulsed direct current field at 660 Hz. The electrolyte was prepared by dissolving 0.15 M calcium acetate monohydrate [$Ca(CH_3COO)_2 \cdot H_2O$] and 0.02 M calcium glycerophosphate ($CaC_3H_7O_6P$) in DDW. Anodic oxidation was carried out at 270 V for 3 min in a water-cooled bath. The thickness of the oxide layer was approximately 2 μm. The fourth type of surface was calcium phosphorus (Ca–P)-coated. First, the Ti surface was sandblasted and acid-etched, like the third type of surface. Then, Ca–P thin film (~300 nm thickness) was deposited on the surface with an electron beam. The Ca–P evaporants for electron beam deposition were prepared by sintering a mixed hydroxyapatite (Alfa Aesar, Johnson Matthey, London, UK) and calcium oxide (Sigma–Aldrich) powder at 1000 °C for 2 h. Finally, the surface was heat-treated at 350 °C for 1 h. Electron probe micro-analysis (EPMA; JXA-8900R, Jeol, Tokyo, Japan) and confocal laser scanning microscopy (CLSM; LSM 5-Pascal, Carl Zeiss AG, Oberkochen, Germany) were performed to determine the chemical composition and roughness of each surface.

2.4. Scanning electron microscopy

SEM was used to examine the cell attachment of MG63 cells cultured onto the Ti surfaces. Ti discs (20 mm in diameter, 0.5 mm thick) were placed in 12-well plates (Nunc), with one disc per well. The culture plates containing the Ti discs were coated with synthetic peptides (21 μg/cm^2) by drying for 18 h at room temperature, blocked with/without 1% heat-inactivated bovine serum albumin (BSA) for 1 h at 37 °C, washed with PBS, and then seeded with 1 ml of a cell suspension containing 1×10^5 cells. The cultures were incubated for 1 h at 37 °C in 5% CO_2. The loosely adherent or unbound cells from the experimental wells were removed by aspiration, the wells were washed once with PBS, and the remaining bound cells were fixed in 4% paraformaldehyde in PBS for 15 min. The fixative was then aspirated. After washing in the buffer, the Ti discs were dehydrated in a graded series of ethanol solutions. After critical point drying (HCP-2, Hitachi, Tokyo, Japan), the samples were sputtered with Au/Pd using an SEM coating system (Quorum Q150T-S, Quorum Technologies, West Sussex, UK), and the probes were examined by field emission-SEM (FE-SEM; Hitachi S-4700, Hitachi) at 15 kV. To ensure a representative count, each Ti disc was divided into quarters and one field per each quarter was photographed using FE-SEM and counted. Averages and standard deviations were calculated from three independent experiments.