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Integrin αvβ3 regulates microfibril assembly in human periodontal ligament cells

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ABSTRACT

Fibrillin-1 is the major structural component of extracellular microfibrils. However, the mechanism by which extracellular fibrillin-1 assembles into microfibrils is not fully understood. Fibrillin-1 contains the Arg-Gly-Asp (RGD) motif, which may allow binding to RGD-recognizing integrins. We hypothesized that integrin αvβ3 on the cell surface of human periodontal ligament (PDL) fibroblasts may influence fibrillin-1 assembly into cell/matrix layers. We treated PDL fibroblasts with an integrin αvβ3-specific antagonist to examine fibrillin-1 assembly. Western blotting and immunofluorescence analysis showed that treatment with the integrin αvβ3 antagonist at 5 μM clearly abolished fibrillin-1 deposition. These results provide for the first time evidence that integrin αvβ3 regulates extracellular assembly of fibrillin-1, thereby modulating cell-mediated homeostasis of microfibrils.

1. Introduction

Fibrillin-1, a 350-kDa extracellular glycoprotein, has been widely recognized as the major constituent of fibrillin-rich microfibrils (Kielty, 2006). It contains 47 epidermal growth factor domains and 7 eight-cysteine-containing TB motifs (Pereira et al., 1993). Fibrillin-1 is known to mediate cell adhesion through binding to cell surface receptors of the integrin family (Pfaff et al., 1996; Sakamoto et al., 1996). This binding is at least partly mediated by the single Arg-Gly-Asp (RGD) cell adhesion motif within the fourth TB motif. Fibrillin–integrin interactions are likely to play an important role in the assembly of the microfibril network.

Human periodontal ligament (PDL) contains pure microfibrils that form bundles named oxytalan fibers (Sculean et al., 1999; Simmons and Avery, 1980). The oxytalan fibers in PDL form a vertically oriented interlacing network enclosing the molar root apex (Sims, 1973). We have previously demonstrated that human PDL fibroblasts express fibrillin-1 and form a microfibril network in the cell/matrix layers (Tsuruga et al., 2002b). The microfibrils in the cell/matrix layers of PDL fibroblasts are maintained through control of their degradation by matrix metalloproteinases as well as their synthesis (Tsuruga et al., 2007). Until recently, integrin αvβ3 was thought to be the major fibrillin-1 receptor in several cell lines (Kielty et al., 2002). Gene expression analysis has shown that human PDL fibroblasts express integrin αv and β3 subunits (Bolcato-Bellemin et al., 2000). Integrin αvβ3 has been shown to bind synthetic fibrillin-1 RGD peptides coated on the surface of culture plates, based on inhibition of fibrillin-mediated cell adhesion by the antibody LM609, which specifically blocks αvβ3 function (Bax et al., 2003). However, the relationship between integrin αvβ3 and extracellularly formed fibrillin-1-positive microfibrils has not been investigated.

In the present study, based on our hypothesis that PDL fibroblasts may control microfibril assembly in the cell/matrix layers by mediated by integrin αvβ3 on their cell surface, we applied a cyclic peptide antagonist of αvβ3, which selectively blocks αvβ3 function, to examine the dynamic relationship between the microfibrils and cells.

2. Materials and methods

2.1. Cells and culture

The protocol for these experiments was reviewed and approved by the Fukuoka Dental College Research Ethics Committee, and informed consent was obtained from the tissue donors (aged 17, 19 and 20 years).

PDL fibroblasts were isolated from three different donors and cultured in minimum essential medium (MEM; ICN Biomedicals Inc., Aurora, OH, USA) supplemented with 10% newborn calf serum (NCS; Life Technologies, Grand Island, NY, USA), using 35-mm culture dishes (Corning Co., Cambridge, MA, USA) as described

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previously (Tsuruga et al., 2002a). PDL fibroblasts were used from 3rd to 6th passages in this study.

2.2. Integrin αvβ3 antagonist treatment

The cells were treated with 5 or 10 μM integrin αvβ3 antagonist; cyclo[Arg-Gly-Asp-D-Phe-Val]RGDfV (Biomolecular International, Plymouth Meeting, PA) (Aumailley et al., 1991). The negative control peptide for the integrin αvβ3 antagonist was cyclo[Arg-Ala-Asp-D-Phe-Val]RADfV (Biomolecular International). These peptides were added to the media every 3 days, and the cell/matrix layer and media were harvested at 14 days.

2.3. Integrin αvβ3 immunoprecipitation from cell/matrix layers

The cell/matrix layers were taken up in 1 ml of RIPA buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS) (Sigma, St. Louis, MO, USA) with a mixture of protease inhibitors (Complete Mini, Roche, Mannheim, Germany) and lysed and centrifuged to remove cell debris at 4 °C. The supernatant was subjected to immunoprecipitation with anti-human αvβ3 monoclonal antibody LM609 (Chemicon International, Temeculla, CA, USA), as described previously (Tsuruga et al., 2002a). The precipitated immunocomplexes were resolved with NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA, USA), and boiled for 5 min in the presence of NuPAGE sample reducing buffer (Invitrogen).

2.4. Western blot analysis

At 14 days of culture, samples of PDL fibroblast cell/matrix and media were prepared as described previously (Tsuruga et al., 2007). Proteins in immunoprecipitated samples or cell/matrix lysates (5 μg) or medium (10 μg) were subjected to Western blot analysis, as described previously (Tsuruga et al., 2002b). The antibodies used were anti-human integrin αv polyclonal antibody (AB1930; Chemicon International, Mannheim, Germany), anti-human α5 polyclonal antibody, anti-human vitronectin monoclonal antibody and anti-human fibronectin polyclonal antibody (Chemicon International, Mannheim, Germany), anti-human integrin β1 polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-human fibrillin-1 polyclonal antibody (Elastin Products Co., Owensville, MO, USA), and anti-β-actin polyclonal antibody (Sigma, Saint Louis, MO).

Densitometric analysis of the signals was performed using the Image J program (National Institutes of Health, Bethesda, MD, USA). Small variations in protein loading were corrected by normalization relative to the intensity of β-actin. Student's *t*-tests were used to determine the statistical significance of the difference. A level of $p < 0.05$ was considered significant.

2.5. Northern blot analysis

Total RNA was prepared from the cultured PDL fibroblasts at 14 days using an RNeasy Mini Kit (Qiagen, Hilden, Germany). One microgram of RNA was subjected to northern blot analysis, which was performed as described previously (Tsuruga et al., 2002a). The probe for recognition of human fibrillin-1 was generated as described previously (Tsuruga et al., 2002a). The RNA probe for β-actin and RNA markers were from Roche Molecular Biochemicals (Mannheim, Germany). Densitometric analysis of the signals was performed using the Image J program (National Institutes of Health, Bethesda, MD, USA). Small variations in RNA loading were corrected by normalization relative to the intensity of β-actin. All results represent at least three independent analyses.

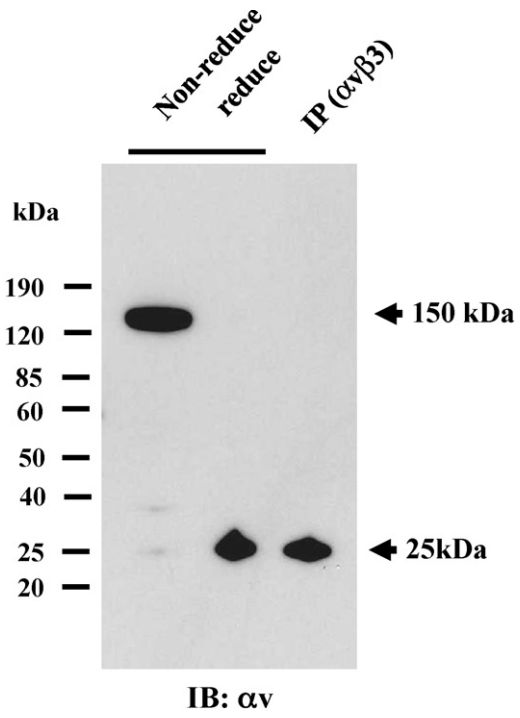

Fig. 1. PDL fibroblasts express integrin αvβ3. Cell lysates of PDL fibroblasts cultured for 14 days were subjected to Western blotting under non-reducing and reducing conditions. Integrin αv is composed of a disulfide-bonded heavy (125-kDa) and a light (25-kDa) chains. This antibody for αv recognizes COOH-terminal of the light chain. Therefore, it recognizes a 150-kDa band when intact, whereas it recognizes a 25-kDa band when denatured. The cell lysates under non-reducing and reducing conditions contain a 150-kDa (left lane) and 25-kDa (middle lane) band, respectively. Moreover, the immunoprecipitated materials obtained using an antibody against integrin αvβ3 contains a 25 kDa band of the integrin αv subunit under reducing conditions (right lane), showing that PDL fibroblasts express integrin αvβ3.

2.6. Immunofluorescence

At 14 days of culture, PDL fibroblasts were fixed in ice-cold 4% paraformaldehyde for 15 min. Nonspecific immunoreactivity was blocked with 1% bovine serum albumin in PBS for 1 h at room temperature. Then the cell layers were incubated for 1 h at room temperature with the primary antibodies against human fibrillin-1 (1:100, Elastin Product Company). After rinsing in PBS, the cells were incubated with Alexa Fluor® 568-labeled goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR, USA), diluted 1:100 with blocking buffer, for 1 h at room temperature.

Following a rinse in PBS, SYTOX® Green (Molecular Probes) was added at 100 nM for nuclear staining. Then immunoreactivity was observed using a confocal microscope (MRC-1024; Bio-Rad, Hemel Hempstead, UK).

3. Results

3.1. PDL fibroblasts express integrin αvβ

The αv protein (150 kDa) is composed of a disulfide-bonded heavy chain (125 kDa) and a light chain (25 kDa). The antibody for αv we used recognizes the C-terminal of the light chain. We detected the disulfide-bonded αv protein (150 kDa) under non-reducing conditions, and the light chain (25 kDa) under reducing conditions (Fig. 1). To confirm that PDL fibroblasts express integrin αvβ3, we performed immunoprecipitation using an antibody against αvβ3 from PDL fibroblast cell/matrix layers. As expected, the immunoprecipitated materials contained αv light chain,