EMILIN-1 regulates the amount of oxytalan fiber formation in periodontal ligaments in vitro

Yuka Nakatomi,1 Eichi Tsuruga,2 Kazuki Nakashima,1 Yoshihiko Sawa,2 Hiroyuki Ishikawa1

Section of Orthodontics, Department of Oral Growth and Development, Division of Clinical Dentistry, Fukuoka Dental College, Fukuoka, Japan, 2Section of Functional Structure, Department of Morphological Biology, Division of Biomedical Sciences, Fukuoka Dental College, Fukuoka, Japan

Abstract

The elastic system fibers comprise oxytalan, elaunin, and elastic fibers, differing in their relative microfibril and elastin contents. Among them, human periodontal ligament (PDL) contains only oxytalan fibers (pure microfibrils). Elastin microfibril interface-located protein-1 (EMILIN-1) is localized at the interface between microfibrils and elastin. We hypothesized that EMILIN-1 may contribute to the formation of oxytalan fibers. We used a small interfering RNA (siRNA) for EMILIN-1 in PDL cell culture to examine the extracellular deposition of fibrillin-1 (the major component of microfibrils). EMILIN-1 was labeled on microfibrils positive for fibrillin-1 and was colocalized with fibrillin-1 upon immunoprecipitation assay. EMILIN-1 suppression reduced the level of fibrillin-1 deposition to 23% of the control, and this was responsible for the diminution of fibrillin-1 deposition revealed by immunofluorescence. These results suggest that EMILIN-1 may regulate the formation of oxytalan fibers and play a role in their homeostasis.

Keywords: EMILIN, fibrillin, microfibrils, oxytalan fiber, periodontal ligament

INTRODUCTION

Elastic system fibers are an integral part of the extracellular matrix, which gives flexibility and extensibility to tissues such as blood vessels, lungs, skin, and periodontal tissue [1]. These fibers consist of two distinct components: an amorphous core of cross-linked elastin and a peripheral mantle of microfibrils [2]. They can be classified into three types depending on their relative contents of these components. Elastic fibers contain a high proportion of elastin, whereas elaunin fibers contain very little elastin, and oxytalan fibers are bundles of pure microfibrils [3]. Elastic fiber formation is thought to begin with the formation of a scaffold of microfibrils on which the elastin is deposited. Interestingly, in the periodontal ligament (PDL), only oxytalan fibers, which are pure microfibrils, can be identified, whereas all three types of fibers are present in the gingiva [4]. In the PDL, oxytalan fibers are thought to have some functional roles, such as support of vascular orientation and regulation of vascular flow [5]. In our series of studies [6], we have demonstrated that human PDL fibroblasts express fibrillin-1 and fibrillin-2 (the major components of oxytalan fibers) without expression of tropoelastin (the precursor of mature elastin). By PDL fibroblast culture, we clearly showed that pure oxytalan fibers are formed in cell/matrix layers. However, the precise mechanism of oxytalan fiber development remains unclear.

Elastin microfibril interface-located proteins (EMILINs) are extracellular proteins characterized by a unique arrangement of structural domains, including a cysteine-rich domain known as the EM domain, at the N-terminus, followed by a coiled-coil domain and a GC1q-like domain [7]. So far, four family members have been identified: EMILIN-1, EMILIN-2, EMILIN-3 (multimerin-1), and EMILIN-4 (multimerin-2, endoglyx-1) [8–11]. Among them, the first protein of this family, EMILIN-1, initially named GP115, is present in a wide variety of connective tissues, particularly elastic tissues such as lung and blood vessels [10,12]. At the ultrastructural level, EMILIN-1 is located at the interface between the amorphous core of elastin and the surrounding microfibrils [13]. This is the reason why this protein was named EMILIN. EMILIN-1 can interact with tropoelastin [14]. Moreover, experiments using genetically targeted mice have shown that EMILIN-1 is necessary for the biogenesis of elastic fibers [14]. Therefore, EMILIN-1 is thought to be an adaptor for
binding tropoelastin to microfibrils for the formation of elastic fibers.

On the contrary, the developmental expression of EMILIN-1 has not been investigated in elastin-free tissues. An ultrastructural study of EMILIN-1 immunolocalization revealed that the molecule was clearly labeled in oxytalan fibers of the corneal stroma [13]. Moreover, based on the fact that EMILIN-1 can bind with fibulin-5, which is expressed on oxytalan fibers in our PDL culture model [15,16], we hypothesized that EMILIN-1 might contribute to the formation of oxytalan fibers in PDL. In the present study, we used a culture model of pure microfibrils to investigate the role of EMILIN-1 in the development of oxytalan fibers and found that EMILIN-1 colocalizes with oxytalan fibers and regulates their biogenesis.

MATERIALS AND METHODS

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.

PDL fibroblasts were isolated from three different donors and cultured, as described previously [17]. Briefly, connective tissues were obtained surgically from the PDL of molar teeth extracted for orthodontic reasons. After washing in phosphate-buffered saline (PBS) supplemented with 100 μg/ml penicillin and 100 μg/ml streptomycin (Roche Diagnostics, Mannheim, Germany), PDL samples were cut into small pieces, plated in petri dishes, and incubated in Minimum Essential Medium (MEM) (Invitrogen, Grand Island, NY, USA) supplemented with 10% newborn calf serum (NCS; Invitrogen) at 37°C in humidified air containing 5% CO₂. When outgrowth of the cells reached confluence, they were harvested with 0.025% trypsin (Invitrogen) in PBS and transferred to plastic culture dishes at a 1:4 split ratio. For experiments, the cells were trypsinized and seeded at 1 × 10⁶ cells/ml per 35-mm culture dish (Corning Incorporated, Corning, NY, USA) in MEM supplemented with 10% NCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. PDL fibroblasts were used from the third to sixth passages in this study.

Western blot analysis

At 7 days of culture, cell/matrix samples were prepared as described previously [6]. The proteins (5 μg) were subjected to electrophoresis on 4–12% NuPAGE Bis-Tris gel (Invitrogen) for Western blot analysis, as described previously [18]. The primary antibodies used were those against human fibrillin-1 (Elastin Products Co., Owensville, MO, USA), human EMILIN-1 (Sigma, Saint Louis, MO, USA) and β-actin (Sigma) at 1:5000 dilution. Prestained molecular-weight markers (Invitrogen) were also run on each blot. Densitometric analysis of the signals was performed using the Image J program (National Institutes of Health, Bethesda, MD, USA) after finding the linear portion by sequential dilution of the proteins. Small variations in protein loading were corrected by normalization relative to the intensity of the corresponding band of β-actin. Each value presented is expressed as the mean ± standard deviation (SD) and all quantitative results represent at least three independent analyses. The unpaired Student’s t-test was used for analyzing the differences between experimental groups.

Immunoprecipitation

The cell/matrix layer on day 7 was washed 3 times in 1 ml of PBS and proteins were cross-linked with 2-mM 3, 3′-dithiobis(sulfosuccinimidylpropionate) (Pierce Biotechnology, Inc., Rockford, IL, USA) in PBS at 4°C for 30 min under gentle shaking. The cell/matrix layer was washed twice in 1 ml of PBS and taken up in 1 ml of lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, pH 7.5) with a proteinase inhibitor cocktail containing 5 mM ethylenediaminetetraacetic acid, 50 μM N-ethylmaleimide, and 50 μM phenylmethylsulfonyl fluoride. Centrifugation was performed to remove all debris. Aliquots of the cell/matrix extract were incubated with anti-human EMILIN-1 monoclonal antibody (Protein Tech Group Inc., Chicago, IL, USA) or control mouse IgG (Sigma), or without IgG, in the presence of 2% bovine serum albumin (BSA, fraction V; Sigma) for 2 hr at 4°C under agitation. Protein G-agarose beads (Pierce Biotechnology), prewashed with PBS containing 2% BSA, were then added to the sample–antibody mixture and incubated for 1 hr at 4°C. Then the beads were collected by centrifugation and washed 5 times extensively with lysis buffer, changing the tube for the last spin. The immunoprecipitated protein retained by the washed beads was then eluted with gel electrophoresis loading buffer (1% sodium dodecyl sulfate, 10 mM Tris, 5% glycerol, and 25% dithiothreitol, pH 7.0), and subjected to 4–12% polyacrylamide gel electrophoresis for Western blotting using an antibody against human fibrillin-1, as described above.

siRNA design and transient transfection

siRNA for human EMILIN-1 (accession # NM_007046) was designed and synthesized by Sigma Aldrich Corp. (Tokyo, Japan). The synthesized siRNA corresponded to the 2199–2221 coding region of EMILIN-1. The siRNA sequence for fibulin-5 was sense 5′-GUGAACGGUUGGACACUGUGG-3′, antisense 3′-ACAGUGUCCACCCGGUGAC-5′. Negative control (scrambled order) was sense 5′-UCGGAUUGUGAGGCAGATT-3′, antisense 3′-CUUGCACUGCAUCACGGATT-5′. The sequence of the negative control was designed as a randomized...
sequence of the 2199–2221 coding region of EMILIN-1. BLAST searches of the database indicated that this siRNA is specific for EMILIN-1 and has no homology with other proteins. Transfection was performed at days 1 and 4 of culture continuously. The siRNA was transfected into PDL fibroblasts using X-treme GENE siRNA transfaction reagent (Roche, Mannheim, Germany). First, 237.5 μl of OptiMEM medium/dish (Invitrogen, Grand Island, NY, USA) and 12.5 μl of the transfection reagent were preincubated for 10 min at room temperature. During this time, 748 μl of OptiMEM medium was mixed with 2 μl of 100 μM siRNA. The two mixtures were then combined and incubated for 20 min at room temperature to allow formation of their complex. The entire mixture was added to the cells in one dish, resulting in a final concentration of 200 nM for the siRNAs. After 12 hr of incubation, the transfection medium was replaced with fresh complete medium (MEM with 10% FCS). Mock transfection of cultures with the transfection reagent alone was used as a control. PDL fibroblasts were transfected twice with the siRNA duplex (0, 100 nM), with a 72-hr interval between, and harvested at 7 days.

Northern blot analysis
Total RNA was prepared from the cultured PDL fibroblasts at 7 days using an RNasea Mini Kit (Qiagen, Hilden, Germany). One microgram of RNA was subjected to northern blot analysis, as described previously [17]. The probe for recognition of human fibrillin-1 was generated as described previously [6]. To generate the probe for human EMILIN-1, a cDNA template was obtained using the reverse-transcription polymerase chain reaction (RT-PCR) and RNA extracted from PDL fibroblasts. The primer sequences, which include SP6 or T7 RNA polymerase promoters at the 5′-end, used for PCR were (reading from 5′ to 3′): forward, CGTCTTCCACACCACGGCCC; reverse, CGCAGGCCTTGCAGCTCCTT. The PCR products were purified, and then SP6 RNA polymerase was mixed with digoxigenin (DIG)-labeled nucleotides (Roche Molecular Biochemicals, Mannheim, Germany) to generate the DIG-labeled 169-bp RNA probes (nucleotides 847–1015). The RNA probe for β-actin was from Roche Molecular Biochemicals. Densitometric analysis of the signals was also performed using the Image J program. Small variations in RNA loading were corrected by normalization relative to the intensity of the corresponding band of the β-actin probe.

Immunofluorescence
At 7 days of culture, PDL fibroblasts were fixed in ice-cold 4% paraformaldehyde for 15 min, followed by washing with PBS. Nonspecific immunoreactivity was blocked with 1% BSA in PBS for 1 hr at room temperature. The cell layers were then incubated for 2 hr at room temperature with the appropriate primary antibodies (polyclonal rabbit antibody against human fibrillin-1 diluted 1:1000; Elastin Products Co., Owensville, MO, USA, goat antibody against human EMILIN-1 diluted 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Controls included the use of preimmune normal goat or rabbit IgG for incubation with the primary antibody. After rinsing in PBS, the cells were incubated with Alexa Fluor® 568-labeled donkey anti-goat IgG antibody or Alexa Fluor®488-labeled donkey anti-rabbit IgG antibody (Molecular Probes, Eugene, OR, USA), diluted 1:1000 with blocking buffer, for 1 hr at room temperature. After the final washing, the cells were viewed using a confocal microscope (MRC-1024; Bio-Rad, Hemel Hempstead, UK), equipped with DAPI.

RESULTS
EMILIN-1 localization on microfibrils
We first examined whether EMILIN-1 was localized on microfibrils (Figure 1A). Positive staining for fibrillin-1 was observed on microfibrils, which appeared as networks of fiber patterns. EMILIN-1 was labeled on a proportion of fibrillin-1-immunolabeled microfibrils of human PDL fibroblasts cultured for 7 days. Control immune serum produced no labeling (not shown). These results indicated that EMILIN-1 was colocalized with fibrillin-1 on microfibrils.

We then evaluated protein–protein interaction between EMILIN-1 and fibrillin-1 by carrying out immunooprecipitation of EMILIN-1 in the presence of a reversible crosslinking agent and examining whether fibrillin-1 was coprecipitated by immunoblotting. As shown in Figure 1B, anti-EMILIN-1 clearly immunooprecipitated its own antigen. Fibrillin-1 was coprecipitated only with EMILIN-1, but not with the control mouse IgG or protein G-agarose beads alone, indicating that the immunoprecipitation was specific.

Decrease of microfibril assembly by knockdown of EMILIN-1 expression
To investigate the function of EMILIN-1, we used siRNA to suppress EMILIN-1 expression. We transfected PDL fibroblasts with 100 nM EMILIN-1 siRNA. Densitometric analysis based on the northern blot showed that when 100 nM siRNA was used for transfection, the siRNA effectively reduced the level of EMILIN-1 gene expression to about 20% level of the control (vehicle only) in the PDL fibroblasts at 7 days (Figure 2A). Additionally, transfection with EMILIN-1 siRNA did not affect fibrillin-1 gene expression, as expected. Moreover, scrambled siRNA had no effect on EMILIN-1 expression, and no difference from the control was evident (data not shown), proving that this siRNA was specific for EMILIN-1.

In matrix proteins at 7 days, densitometric analysis based on the Western blot shown identified a 77 ± 8%
significant decrease of fibrillin-1 ($p = 0.021$) (Figure 2B). EMILIN-1 deposition was decreased to about 40% of the control, coinciding with the decrease of EMILIN-1 gene expression.

The immunolabeling data also supported the data from Western blotting that the EMILIN-1 suppression reduced the level of fibrillin-1 to 23% of the control. In cells transfected with 100 nM siRNA, fibrillin-1 staining was diminished in comparison with the control cells (Figure 3).

**DISCUSSION**

In the present study using the RNA interference technique, we have demonstrated for the first time that EMILIN-1 has molecular interaction with fibrillin-1 and affects the amount of extracellular deposition of fibrillin-1. This is the first report to have focused on a function of EMILINs in relation to oxytalan fibers.

Developmental expression of EMILIN-1 has been investigated in chicken [8] and mice [19]. It is reported that EMILIN-1 is expressed not only in elastic-rich tissues such as lung, skin, heart, and blood vessels, but also in nonelastic tissues, from the postnatal to adult stage [20]. First, EMILIN-1 was detected in elastic fibers at the ultrastructural level, being located at the interface between microfibrils and elastin [13]. Therefore, this protein was named EMILIN and consequently became a focus of attention with regard to its relationship with elastic fibers. It has been shown

---

**Figure 1** Immunolocalization of EMILIN-1 to microfibrils. (A) Double immunofluorescence of fibrillin-1/EMILIN-1 in PDL fibroblast cultures. Human PDL fibroblasts were cultured for 7 days, then simultaneously labeled with fibrillin-1 (green, left panel), EMILIN-1 (red, middle panel), and superimposition of both labels (right panel). Nuclei are blue. Bar: 100 μm. (B) Immunoblotting against anti-fibrillin-1 (upper panel) and anti-EMILIN-1 (lower panel) antibodies following immunoprecipitation of PDL culture cell/matrix lysates using anti-EMILIN-1 antibody in the presence of a reversible cross-linking agent. Cell lysates were immunoprecipitated with anti-EMILIN-1 antibody conjugated with protein G-agarose beads. The immunoprecipitates were resolved by SDS-PAGE, and proteins coprecipitated with EMILIN-1 were analyzed by immunoblotting with anti-fibrillin-1 antibody. Lane 1, protein G-agarose beads alone; lane 2, control mouse IgG; lane 3, anti-EMILIN-1 antibody.

**Figure 2** EMILIN-1 siRNA suppresses deposition of fibrillin-1. (A) Northern blots of RNA samples (1 μg) extracted from PDL fibroblasts cultured for 7 days after transient transfection with EMILIN-1 siRNA. PDL fibroblasts were transiently mock-transfected (lane 1), or transiently transfected with 100 nM siRNA for EMILIN-1 (lane 2). The blots were probed with fibrillin-1 (upper lanes) and EMILIN-1 (middle lanes). Each of the probes recognized a 9.7- and 3.9-kbp band, respectively. β-Actin was used as an internal control in each lane (lower lane). The intensity of each band was normalized relative to that of β-actin, and quantified using the Image J program. (B) Western blots of cell/matrix lysates (10 μg) of PDL fibroblasts cultured for 7 days after transient transfection with EMILIN-1 siRNA. The blots were probed with anti-fibrillin-1 (upper lanes) and EMILIN-1 (middle lanes) antibodies. Each of the probes recognized a 350- and 120-kDa band, respectively. The intensity of each band was normalized relative to that of β-actin, and quantified using the Image J program.
histologically that in EMILIN-1 null mice the alignments of elastic fibers in the aorta and tropoelastin (the precursor of cross-linked elastin) extracted from the aorta are degraded biochemically, suggesting that EMILIN-1 may play a role in correct deposition of microfibrils.

It remains unclear whether EMILIN-1 affects the formation of oxytalan fibers, which are pure microfibrils. We demonstrated that EMILIN-1 was colocalized on a proportion of oxytalan fibers in vitro. In a previous immunoelectron microscopy study, EMILIN-1 was shown to be labeled on oxytalan fibers in Descemet’s membrane of the chick corneal stroma [13]. Although the mechanism of EMILIN-1 molecule assembly has been clarified [21], the function of EMILIN-1 on oxytalan fibers has not been investigated previously. Besides the fact that EMILIN-1 is located at the interface between microfibrils and elastin, it is speculated that EMILIN-1 plays a role in the process of oxytalan fiber formation. In present study, we demonstrated that EMILIN-1 is a crucial molecule that regulates the quantity of oxytalan fiber formation.

It has been reported that EMILIN-1 can bind with tropoelastin and fibulin-5 [14]. We showed previously that fibulin-5 localizes with oxytalan fibers to control their coalescence [16]. In the present study, we showed by immunoprecipitation that EMILIN-1 colocalizes with fibrillin-1. Fibrillin-1 is the principal structural component of microfibrils [2]. This provides strong confirmation that EMILIN-1 is localized on oxytalan fibers. In vitro binding assays have revealed molecular interactions involving fibrillin and microfibril-associated molecules, such as MAGP, fibulins and proteoglycans [2]. Therefore, other molecules such as fibulin-5 may be present between EMILIN-1 and fibrillin-1, and the molecular mass including EMILIN-1 may regulate the quantity of oxytalan fibers.

We previously demonstrated that integrin $\alpha_v\beta_3$ on PDL fibroblasts controls the formation of oxytalan fibers [22]. During pericellular events, fibroblasts in connective tissues may retain a proportion of oxytalan fibers through cell surface receptors for integrins. PDL fibroblasts express $\alpha_v\beta_3$ and $\alpha_5\beta_1$ [23]. Considering that EMILIN-1 does not contain an RGD motif [24], it may not make direct contact with the cell membrane. However, some integrins may contact the RGD motif in fibrillin-1 and fibulin-5, which form complexes with EMILIN-1, and these complexes including EMILIN-1, as a whole, may exert the regulation of oxytalan fiber formation.

Figure 3 Decrease of oxytalan fibers by suppression of EMILIN-1. Immunofluorescence staining was performed with anti-fibrillin-1 antibody (upper panels), anti-EMILIN-1 antibody (middle panels), and superimposition of both labels (lower panels). Nuclei are blue. Bar: 100 $\mu$m.
In summary, we have demonstrated that EMILIN-1 is colocalized with microfibrils and may regulate the formation of oxytalan fibers. Our findings may provide a new insight into the homeostasis of oxytalan fibers.

ACKNOWLEDGMENTS
This work was supported by the Advanced Science Research Center and by Grants-in-Aid for Scientific Research (No. 22592325) from the Ministry of Education, Science, Sports, and Culture of Japan.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES