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LEUCINE-RICH AMELOGENIN PEPTIDE INDUCES OSTEOGENESIS BY ACTIVATION OF THE WNT PATHWAY

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Abstract

We previously showed that one of the amelogenin splicing isoforms, Leucine-rich Amelogenin Peptide (LRAP), induced osteogenic differentiation of mouse embryonic stem cells; however, the signaling pathway(s) activated by LRAP remained unknown. Here, we demonstrated that the canonical Wnt/ β -catenin signaling is activated upon LRAP treatment, as evidenced by elevated β -catenin level and increased Wnt reporter gene activity. Furthermore, a specific Wnt inhibitor sFRP-1 completely blocks the LRAP-mediated Wnt signaling. However, exogenous recombinant Wnt3a alone was less effective at osteogenic induction of mouse ES cells in comparison to LRAP. Using a quantitative real-time PCR array, we discovered that LRAP treatment up-regulated the expression of Wnt agonists and down-regulated the expression of Wnt antagonists. We conclude that LRAP activates the canonical Wnt signaling pathway to induce osteogenic differentiation of mouse ES cells through the concerted regulation of Wnt agonists and antagonists.

Keywords

leucine-rich amelogenin peptide; osteogenesis; Wnt signaling pathway; ES cell; osteogenic differentiation

INTRODUCTION

Wnts are an evolutionarily conserved family of secreted lipidated glycoproteins with well-established roles in cellular proliferation, differentiation, and polarity during embryogenesis [1,2]. With active Wnt signaling, stabilized β -catenin accumulates in the cytosol and translocates to the nucleus, where this transcriptional coactivator interacts with T cell factor/lymphoid enhancer binding factor (TCF/LEF) transcription factors to mediate many of the effects of Wnts on gene transcription [3]. Wnt signaling is tightly regulated by members of several families of antagonists.

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Amelogenins are a group of highly conserved enamel matrix proteins best known for their control over crystal growth during enamel development and biomineralization [4-6]. A number of alternatively spliced amelogenin isoforms are found in the enamel matrix, but the importance, function and abundance of each isoform is incompletely understood [4,7]. In the past decade, a mixture of enamel matrix proteins called Emdogain was identified and used with success in clinical dentistry to promote repair of hard and soft periodontal tissue [8,9]. Emdogain is composed principally of amelogenin, including the alternatively spliced amelogenin isoform called leucine-rich amelogenin peptide (LRAP) [10]. Investigators have shown that LRAP can induce cementogenesis and osteogenesis, cell differentiation fates that are associated with periodontal repair [11-13].

Previously, we showed that LRAP enhances osteogenic induction of mouse embryonic stem (ES) cells [14]. Our result supports the previously reported function of LRAP as a signaling molecule in other cell types [12,13,15,16]. Despite evidence in support of the osteo-inductive property of LRAP, studies to identify the mechanism of action by which LRAP induces bone formation have been limited. The involvement of canonical Wnt signaling pathway in the determination of naïve cells to commit to the osteogenic lineage [17] suggested to us that LRAP may exert its signaling property through activation of the Wnt signaling pathway. Here, we tested the hypothesis that LRAP exerts its signaling function through the activation of the canonical Wnt signaling pathway to induce bone formation. We showed that LRAP-treated ES cells exhibit elevated β -catenin protein level and increased Wnt reporter gene activity. Furthermore, a specific Wnt inhibitor sFRP-1 completely blocks the LRAP-mediated Wnt signaling. However, exogenous recombinant Wnt3a alone was less effective at inducing osteogenic differentiation of mouse ES cells in comparison to LRAP. We discovered that LRAP treatment results in the up-regulation of Wnt agonists and the down-regulation of Wnt antagonists.

MATERIALS AND METHODS

Cell culture and differentiation

Embryoid body (EB) formation of Mouse ES cells (RW4; Genome Systems) was induced either with the “hanging drop” method [14] or in rotary suspension culture of mouse ES cells (5×10^5 cells/ml) [18]. The two methods generated comparable results. Osteogenic differentiation of EBs was induced with control media or mineralization media with or without LRAP as previously described [14]. Recombinant human sFRP-1 (20ng/mL; R&D Systems), recombinant mouse Wnt3a (100ng/mL; R&D Systems) and purified canonical LRAP (MPLPPHPGSP GYINLSYEVLTPLKQWYQSMIRQPPLSPILPELPLEAWPATDKTKREEVD) or scrambled LRAP peptide (PPHMPLPGSPLSYEGYINVLTWEYQPLKSMRIRSPIKLQPPLELAWPPLEATDKEVD; GenWayBiotech Inc), was added to EB culture at day-5 when indicated.

Western immunoblot analysis

EBs at day-5 were collected after washing with PBS 2 times and the addition of M-PER mammalian extraction reagent (Pierce). Approximately 10 μ g of proteins from each experimental sample group was loaded to a 4-20% Tris-glycine SDS-PAGE gel. The size-resolved proteins were transferred to Immobilon-P membranes (Millipore) for 1 hour. The membrane was blocked with 5% non-fat milk in TBST (1xTBS, 0.1% Tween-20) for 1 hour at room temperature. Mouse anti β -catenin antibody (1:2000; BD Biosciences) was added to the TBST and the membrane was incubated at 4°C overnight. HRP-conjugated anti-mouse antibody (1:2000; Amersham Biosciences) was used as a secondary antibody and incubated with the membrane for 1 hour. The antigen-antibody signal was detected by ECL detection

system and normalized to the amount of β -actin from the same sample. Quantification of the signal was achieved by using ImageJ software (National Institutes of Health).

Detection of Wnt reporter activity

MC3T3-E1 cells grown in a 12-well culture dish were transiently transfected with the Wnt responsive TOPFLASH construct (1.6 μ g/well), which contains 16 copies of a TCF/LEF site. For control, parallel dish were transfected with the FOPFLASH construct, which contains 16 copies of a mutant TCF/LEF site. In each case, CMV-lacZ (0.16 μ g/well) was co-transfected to determine transfection efficiency. The conditioned medium from the EB culture groups was transferred to the MC3T3-E1 culture, replacing the transfection medium. Luciferase activity was detected using the Dual-Light reporter gene assay system (Applied Biosystems). Relative luciferase activity was calculated by normalization of the average luciferase activity to the β -galactosidase activity measured at 24 hour post-transfection.

RNA extraction, cDNA synthesis, quantitative real-time PCR analysis, Analysis of mineral deposition and Statistical analysis

These analyses were performed as described previously [14].

PCR array analysis

Approximately 5 μ g of total RNA was mixed with genomic DNA elimination buffer (SABiosciences), and subsequently mixed with RT cocktail (RT buffer, primer and control mix, RT enzyme; SABiosciences) for cDNA synthesis. The resulting cDNA reaction was mixed with SYBR green qPCR master mix and transferred into 96-well RT² Profiler PCR Array for mouse Wnt signaling pathway (SABiosciences), containing appropriate primers for genes associated with the Wnt signaling pathway and primers for house keeping genes. A two-step real-time PCR reaction was performed starting at 95°C (10 minutes) for 1 cycle and followed by 95°C (15 s) and 60°C (1 min) for 40 cycles. Normalized threshold cycle data from real-time instrument was calculated and interpreted using the PCR array data analysis web tool (SABiosciences). A subset of the genes whose expression was affected by LRAP was subject to RT-qPCR individually and the results from the PCR array were confirmed.

RESULTS

LRAP enhances β -catenin protein accumulation and Wnt activity during osteogenic differentiation of mouse ES cells

To determine whether canonical Wnt signaling is activated by LRAP, we initially assessed the effect of LRAP on cytosolic β -catenin levels in embryoid bodies (EBs). As shown by Western blot analysis, β -catenin level was elevated in EBs treated for 4 hours with LRAP as compared to untreated EBs (Fig. 1A, B). This effect was transient, and by 6 hours there was no difference between β -catenin levels in LRAP-treated and untreated EBs. To test the hypothesis that LRAP stimulates the secretion of Wnt agonists and/or inhibits the secretion of Wnt antagonists, we compared the ability of media conditioned by LRAP-treated versus untreated EBs for their ability to stimulate Wnt signaling in a heterologous culture system. EBs were treated with LRAP (10 ng/ml) for various periods of time and the conditioned medium from each time point was tested using MC3T3-E1 cells, which had been transiently transfected with the TOPFLASH Wnt reporter plasmid. MC3T3-E1 cells transfected with the non-responsive FOPFLASH plasmid were used as a control and the pCMV-lacZ reporter was co-transfected as an internal control for transfection efficiency. The relative luciferase values, normalized for the respective β -galactosidase activities, are presented in Fig. 1C. Treatment of the EBs with LRAP for as little as 1 hour increased the potential of the conditioned medium to activate Wnt signaling in the MC3T3-E1 reporter cells. Four-hour of LRAP treatment resulted in a stronger effect, with

the conditioned medium inducing 2-fold higher luciferase activity in the MC3T3-E1 reporter cells when compared to the medium conditioned by the EBs in the absence of LRAP. Interestingly, the effect of LRAP was attenuated by six hours (Fig. 1C), reminiscent of the β -catenin protein profile (Fig. 1A, B). Taken together, these results suggest that LRAP increases the level of Wnt agonist(s) and/or decreases the level of Wnt antagonist(s) during osteogenic differentiation of EB cells.

Wnt antagonist Secreted Frizzled-related protein-1 (sFRP-1) inhibits LRAP-induced osteogenesis

To confirm the involvement of Wnt signaling in LRAP-mediated osteogenesis of embryoid bodies, we employed the Wnt antagonist, secreted frizzled (fzd)-related protein-1 (sFRP-1; 20 ng/ml), which inhibits the interaction between Wnt ligands and their frizzled receptors [19]. EBs were treated with LRAP (10 ng/ml), with or without recombinant sFRP-1 for 24 hours, and then subject to osteogenic conditions for 5 days, followed by real-time RT-PCR analysis of *Osx* and *BSP*, genes that mark development of the osteoblast phenotype. As shown in Fig. 1D, treatment with sFRP-1 abolished LRAP-stimulated up-regulation of *Osx* and *BSP* expression, suggesting that Wnt signaling is essential for the osteo-inductive effect of LRAP.

Canonical Wnt3a alone is not sufficient to mimic the osteogenic effect of LRAP

The observations that LRAP activates the canonical Wnt/ β -catenin signaling pathway and the Wnt antagonist sFRP-1 blocks LRAP-mediated osteogenesis (Fig. 1) indicate that canonical Wnt signaling is necessary for LRAP to exert its osteogenic effect. To determine whether the activation of canonical Wnt signaling is sufficient to mimic LRAP, cells were treated with either recombinant Wnt3a (100 ng/ml) or LRAP (10 ng/ml). A non-bioactive version of the LRAP peptide was produced by randomly scrambling the amino acid sequence without changing the amino acid composition and overall charge. This sequence-scrambled LRAP (scLRAP) was used as a control to eliminate non-specific signaling function(s) conveyed to the cells by the addition of a peptide such as the bioactive LRAP. Expression of marker genes (*Osx* and *BSP*) was analyzed with real-time PCR at two different stages of differentiation (Fig. 2A, B). The sequence-scrambled LRAP (scLRAP) had little effect on *Osx* and *BSP* mRNA levels. Wnt3a treatment resulted in increased expression of *Osx* and *BSP*, although the level was elevated to a lesser extent than that observed for LRAP treatment. Mineral deposition was analyzed with Alizarin Red staining (Fig. 2C) and quantification of calcium concentration (Fig. 2D). Consistent with marker gene analysis, LRAP strongly enhanced mineral deposition. scLRAP did not promote mineralization indicating that the osteogenic effect of LRAP is not random, but rather sequence specific. Exogenous Wnt3a, a canonical Wnt agonist, also promoted mineralization, although not as effectively as LRAP, demonstrating that LRAP stimulates the osteogenic program in mouse ES cells more effectively than the sole action of a canonical Wnt.

Genes associated with Wnt signaling pathway are regulated by LRAP

To analyze the effect of LRAP on components and regulators of the Wnt signaling pathway, we profiled the expression of 84 genes related to Wnt signaling using the Mouse Wnt Signaling Pathway RT² Profiler^{TM} PCR Array (SABiosciences). First strand cDNAs were synthesized from RNA recovered from day-5 embryoid bodies cultured in mineralization medium (Min), mineralization medium with 10 ng/ml of LRAP (Min+L), or control medium (control) for 1- or 4-hours. The relative expression levels for selected Wnt-responsive target genes were plotted in clustergrams (Fig. 3). After 1-hour treatment with LRAP we observed altered gene expression evidenced by enhanced expression of *Wnt3*, *Wnt3a*, *Wnt6*, *Wnt7b* and *Wnt8b*. In contrast, genes encoding sFRP-1, sFRP-4, *Wnt8a*, *Fzd2* and the solute carrier family 9 (sodium/hydrogen exchanger) member 3 regulator-1 (*Slc9a3r1*) were down-regulated upon treatment

with LRAP (Fig. 3). After 4 hours of LRAP treatment, expression levels of Wnt3a, Wnt7b and Wnt8b remained elevated. The mRNA for sFRP-2 and sFRP-4 were also elevated. Fzd2 and Slc9a3r1 remained down-regulated as they were after 1-hour treatment. sFRP-1 and Dkk1 were also down-regulated by LRAP at the 4-hr time point.

DISCUSSION

The function of leucine-rich amelogenin peptide (LRAP) as a signaling molecule to induce osteogenic differentiation has been demonstrated in different cellular contexts [8,12-15]. Despite the identification of LRAP receptor and attempts to address mechanistic function(s) for LRAP for its osteo-inductive property [20], the signaling pathway responsible for the osteogenic effect of LRAP remains largely unclear. In this study, we demonstrated that LRAP activates the canonical Wnt signaling pathway during osteogenic differentiation of mouse ES cells, as evidenced by elevated β -catenin level (Fig. 1A, B) in LRAP-treated embryoid bodies and the increased Wnt-activating potential of media collected from these cells (Fig. 1C). LRAP-induced up-regulation of bone marker gene (Osx and BSP) is abolished by a secreted Wnt antagonist, sFRP-1 (Fig. 1D). Furthermore, we demonstrated that LRAP is more potent than exogenous Wnt3a at osteogenic induction of mouse ES cells, as evidenced by the expression level of Osx and BSP as well as mineral deposition (Fig. 2). We then profiled by RT-qPCR the expression of 84 genes associated with Wnt signaling during the osteogenic differentiation of LRAP-treated embryoid bodies and found that Wnt agonists are up-regulated whereas Wnt antagonists are down-regulated (Fig. 3). Taken together, these data indicate that LRAP activates the canonical Wnt/ β -catenin signaling pathway during ES cell differentiation to the osteogenic lineage. In response to LRAP treatment, the impact of Wnt signaling activation on osteogenic induction is maximized by the concerted regulation of Wnt agonists and antagonists.

The function of the canonical Wnt signaling pathway during osteoblast differentiation has been well studied [21]. The observation that conditioned media collected from LRAP-treated embryoid bodies activated the Wnt reporter suggests that LRAP treatment gives rise to higher concentration of canonical Wnt proteins in the media, which in turn results in the elevated β -catenin level in response to LRAP treatment. This is consistent with the data from these experiments that genes encoding Wnt proteins in Wnt1 family, including Wnt3, Wnt3a, Wnt7b and Wnt8b, are up-regulated after LRAP treatment. Wnt signaling is tightly regulated by antagonists. The LRAP-induced down-regulation of Wnt antagonists (sFRP-1 and Dkk1), coupled with the up-regulation of Wnt agonists, most likely results in an enhanced effect on osteogenic differentiation of mouse ES cells, as compared to that of Wnt3a alone. Interestingly, expression of sFRP-4 is up-regulated 4-hour post-LRAP treatment. The increased expression of sFRP-4 may be due to a feedback mechanism in response to the increased Wnt signaling activated by LRAP. It has been shown that sFRP-4 acts as a Wnt antagonist to inhibit osteoblast proliferation and decrease bone formation in mice [22], but recent findings also suggest that sFRPs have other functions during the development and disease progression [23] as well as the regulation of other signaling pathways [24]. The expression levels of Fzd2 and Slc9a3r1 are also reduced 4 hour after LRAP treatment. Like Wnt antagonists, Frizzled homolog 2 (Fzd2) has an antagonistic function in the canonical Wnt pathway. The inhibition of Fzd2 expression has been reported to increase TOPFLASH Wnt reporter activity in lung epithelial cells [25]. Slc9a3r1 has been shown to function at the late stage of bone formation [26], as opposed to the immediate early stage of osteogenic differentiation of ES cells that have been sampled in this study.

It is generally known that members of Wnt1 family are activators of the canonical Wnt pathway [27], however several studies have shown that these Wnt1 family members also are implicated in the noncanonical Wnt pathway. For example, the canonical Wnt3a has been reported to activate Rho-associated kinase, which are the downstream effectors in Wnt planar cell polarity

(PCP) pathway [28]. Wnt7b can induce osteoblast differentiation via the PKC delta-mediated pathway [29]. Therefore, it is possible that the up-regulation of Wnt gene expression observed in this study reflects an increase in the activity of Wnt proteins implicated in the activation of both canonical and noncanonical Wnt pathway.

The discovery that LRAP activates Wnt/ β -catenin signaling pathway to stimulate osteogenesis makes LRAP amenable to novel therapies and interventions to treat Wnt-related bone diseases. Furthermore, delineating the signaling pathway(s) activated by LRAP may shed light on the mechanism by which Emdogain stimulates periodontal tissue regeneration in clinical dentistry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. LRAP activates canonical Wnt signaling pathway

(A) Western immunoblot analysis shows increased steady-state protein level of β -catenin in mouse embryoid body (EB) cells cultured in the mineralization medium containing LRAP (L) for 4 hours when compared to the protein level of β -catenin in mouse EB cells cultured in mineralization medium (M) alone. The level of β -catenin in mouse EB cells cultured with LRAP for 6 hours was unchanged when compared to that of mouse EB cells cultured in mineralization medium. β -actin was used as an internal control. (B) Quantification of band intensity from the Western immunoblot result in (A) shows 2.5-fold increase in β -catenin protein level in mouse EB cells cultured in mineralization medium containing LRAP (10 ng/mL) for 4 hours when compared to the β -catenin protein level in EB cells cultured in

mineralization medium alone. (C) EB cells were treated with LRAP (10 ng/ml) for various time periods. The conditioned medium from each time point was then collected and transferred to MC3T3 cells that had been transiently transfected with TOPFLASH or FOPFLASH reporter. Luciferase activity was measured 24 hours later. “Control FOPFLASH”, FOPFLASH-transfected MC3T3 cells with conditioned medium from untreated EB cells; “LRAP FOPFLASH”, FOPFLASH-transfected MC3T3 cells with conditioned medium from LRAP-treated EB cells; “Control TOPFLASH”, TOPFLASH-transfected MC3T3 cells with conditioned medium from untreated EB cells; “LRAP TOPFLASH”, TOPFLASH-transfected MC3T3 cells with conditioned medium from LRAP-treated EB cells. (** $P < 0.05$; * $P < 0.01$) (D) Wnt antagonist sFRP-1 abolishes LRAP-mediated marker gene upregulation. EB cells were treated with recombinant sFRP-1 (20 ng/ml), along with LRAP (10 ng/ml), for 24 hours and subsequently induced to osteogenic differentiation. Expression levels of Osx and BSP were analyzed by real-time PCR.

Figure 2. Recombinant Wnt3a alone is not sufficient to mimic the osteogenic effect of LRAP
 EBs were induced to osteogenic differentiation and maintained in mineralization medium, or in mineralization medium containing LRAP (10 ng/mL), or in medium containing Wnt3a (100ng/mL) or in medium containing scrambled LRAP (scLRAP) (10 ng/mL) for 24 hours. After 24 hours, the medium for all culture groups was replaced with mineralization medium throughout the remainder of the experiment. (A) Quantitative real-time PCR analysis of *Osx* and *BSP* at day-10. (B) Quantitative real-time PCR analysis of *Osx* and *BSP* at day-20. (C) Mineral deposition at day-20 analyzed by Alizarin red S staining. (D) Quantification of calcium concentration at day-20.

Figure 3. Genes associated with Wnt signaling pathway are regulated by LRAP

Clustergrams illustrating the magnitude of gene expression in LRAP-treated EBs at 1 hour (Min+L1h), comparing to EBs cultured in mineralization medium (Min 1h) and EBs cultured in the control medium (Control 1h); and LRAP-treated EBs for 4 hours (Min+L4h), comparing to EBs cultured in mineralization medium (Min 4h) and EBs cultured in the control medium (Control 4h).