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How to cite

BRIOLAY, A. et al. Autocrine stimulation of osteoblast activity by Wnt5a in response to TNF- α in human mesenchymal stem cells. In: Biochemical and biophysical research communications, 2013, vol. 430, n° 3, p. 1072–1077. doi: 10.1016/j.bbrc.2012.12.036

This publication URL:https://archive-ouverte.unige.ch//unige:28234Publication DOI:10.1016/j.bbrc.2012.12.036

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Contents lists available at SciVerse ScienceDirect



Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Autocrine stimulation of osteoblast activity by Wnt5a in response to TNF- α in human mesenchymal stem cells

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ARTICLE INFO

Article history: Received 29 November 2012 Available online 22 December 2012

Keywords: Osteoblast Alkaline phosphatase Mineralization Inflammation TNF- α Wnt Ankylosing spondylitis

ABSTRACT

Although anti-tumor necrosis factor (TNF)- α treatments efficiently block inflammation in ankylosing spondylitis (AS), they are inefficient to prevent excessive bone formation. In AS, ossification seems more prone to develop in sites where inflammation has resolved following anti-TNF therapy, suggesting that TNF- α indirectly stimulates ossification. In this context, our objectives were to determine and compare the involvement of Wnt proteins, which are potent growth factors of bone formation, in the effects of TNF- α on osteoblast function. In human mesenchymal stem cells (MSCs), TNF- α significantly increased the levels of Wnt10b and Wnt5a. Associated with this effect, TNF-α stimulated tissue-non specific alkaline phosphatase (TNAP) and mineralization. This effect was mimicked by activation of the canonical βcatenin pathway with either anti-Dkk1 antibodies, lithium chloride (LiCl) or SB216763. TNF- α reduced, and activation of β -catenin had little effect on expression of osteocalcin, a late marker of osteoblast differentiation. Surprisingly, TNF-α failed to stabilize β-catenin and Dkk1 did not inhibit TNF-α effects. In fact, Dkk1 expression was also enhanced in response to TNF- α , perhaps explaining why canonical signaling by Wnt10b was not activated by TNF-a. However, we found that Wnt5a also stimulated TNAP in MSCs cultured in osteogenic conditions, and increased the levels of inflammatory markers such as COX-2. Interestingly, treatment with anti-Wnt5a antibodies reduced endogenous TNAP expression and activity. Collectively, these data suggest that increased levels of Dkk1 may blunt the autocrine effects of Wnt10b, but not that of Wnt5a, acting through non-canonical signaling. Thus, Wnt5a may be potentially involved in the effects of inflammation on bone formation.

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1. Introduction

The effects of inflammation on pathophysiological bone formation are contradictory. In rheumatoid arthritis (RA) for instance, TNF- α and interleukin (IL)-1 β reduce bone formation [1]. TNF- α and IL-1 β block expression of the osteoblast master transcription factor RUNX2 and trigger its degradation, provoking a decrease in collagen expression and consequently of bone formation [2,3]. On the opposite, excessive ossification is a hallmark of ankylosing spondylitis (AS), an inflammatory disease that affects the axial skeleton and the peripheral joints [4]. In AS, new bone formation initiates at entheses, the bony insertion of tendons and ligaments, and eventually leads to bone fusions, which provoke ankylosis. Although anti-TNF- α therapies have been shown to improve most symptoms in AS patients, they have proven unsuccessful to block the progression of bone spurs [5]. Interestingly, we have recently observed that in an organ culture model of mouse enthesis, TNF-

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 α inhibits tissue non-specific alkaline phosphatase (TNAP) activity and mineralization [6], indicating that TNF- α actually inhibits ossification in entheses as it does during developmental ossification. Recent MRI studies have shown that resolution of inflammation after anti-TNF treatment is associated with increased syndesmophyte formation, supporting the notion that TNF- α stimulates ossification early and indirectly, while later, it is inhibitory [7]. Strikingly, TNF- α seems to play the same kind of dual role during fracture healing. Indeed, a necessary early inflammation phase takes place during bone repair, whereas inflammation slows bone formation later on [8,9].

In this work, we sought to determine the involvement of Wnt family members in the indirect effects of TNF- α on human osteoblast activity. Wnt factors are suspected to relay the early indirect effects of TNF- α in AS [10] and during bone repair [11–13]. Wnt proteins are a family of 19 highly conserved secreted glycoproteins that play essential roles during development and tissue homeostasis. Some Wnt proteins such as Wnt3a and Wnt10b bind to Frizzled receptors, and recruit the LRP5/6 coreceptors to activate the canonical signaling pathway, leading to glycogen synthase kinase- β (GSK3 β) inhibition, β -catenin stabilization, translocation into the

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nucleus and regulation of TCF/LEF transcriptional activity. Binding of Wnt proteins to LRP5/6 is inhibited by secreted factors such as Dickkopf1 (Dkk1) [14]. Dkk1 binds to LRP5/6 causing the receptor to attract Kremen, and this interaction promotes clathrin-mediated internalization thereby inactivating LRP5/6.

The importance of the canonical Wnt signaling in bone is wellacknowledged. Genetic reports established that Wnt/β-catenin activity is essential for bone development [15]. Deficiency of Dkk1 [16] is associated with increased bone formation in mice and humans. In addition to its role in physiological bone remodeling, Dkk1 likely plays an important role in the inflammatory effects of TNF- α . Indeed, neutralization of Dkk1 activity in TNF- α transgenic mice switches the RA phenotype of these mice into a phenotype resembling to AS [10], and prevents TNF-mediated impaired osteoblast function [17]. Moreover, levels of functional Dkk1 predicts protection from syndesmophyte formation in patients with AS [18]. Taken together, these data suggest that TNF- α influence bone formation through modulation of canonical Wnt signaling. Wnt10b may be particularly important for bone formation. Wnt10b is expressed in the bone marrow by osteoblast progenitors [19], and transgenic overexpression of Wnt10b in mesenchymal derivatives leads to increased bone density and accelerated osteoblastogenesis in vitro, whereas Wnt10b^{-/-} mice have reduced trabecular bone [20]. Moreover, Wnt10b seems to stimulate osteoblast functions through a positive autocrine loop [21]. Furthermore, it was recently suggested that resolution of inflammation may stimulate osteoblasts in part through Wnt10b [22]. Interestingly, TNF- α stimulates the expression of Wnt10b in preadipocytes, resulting in the activation of the canonical Wnt pathway characterized by β-catenin activity and inhibition of adipogenesis [23].

Alternatively, non-canonical Wnt members may also be involved in the effects of TNF- α on ossification. In particular, Wnt5a seems to be the predominant Wnt expressed during osteoblastic differentiation of human MSCs [24]. Moreover, $Wnt5a^{+/-}$ mice present a reduced bone mass phenotype with decreased osteoblast number [25]. Finally and importantly, Wnt5a levels are increased in inflamed joints from patients with RA [26], and also in the early inflammation phase that takes place during fracture healing [27].

2. Materials and methods

2.1. Chemicals

Recombinant TNF- α was obtained from Immunotools (Friesoythe, Germany). Wnt5a, Dkk1 and anti-Dkk1 antibodies were purchased from R&D Systems (Lille, France). We used mouse Wnt5a,

Table 1	1
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Summary of primers used.

which shares 97% homology with human Wnt5a and is biologically active in human cells [28]. Anti-Wnt5a antibodies were from Santa-Cruz (Heidelberg, Germany).

2.2. Cell cultures

MSCs were obtained from two healthy donors [a 34-year old female and a 36-year-old male] (Lonza, Walkersville, USA) and also in MSCs obtained from one patient with Legg-Perthes-Calve disease [2]. MSCs were seeded at a density of 5000 cells per cm² and cultured in DMEM containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Eurobio, Les Ulis, France). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂ in air. Osteoblast differentiation was induced at confluence with 10^{-8} M 1,25(OH)₂VD3, 50 µM vitamin C and 10 mM β-GP (Sigma–Aldrich, St Quentin Fallavier, France) [2].

2.3. RNA extraction, reverse transcription and polymerase chain reaction (RT–PCR)

Total RNA was extracted using Trizol reagent according to the manufacturer's instructions. Contaminating DNA was removed in a 20 min digestion at 37 °C with DNase I. Six micrograms of each RNA sample were used for reverse transcription performed under standard conditions with Superscript II reverse transcriptase and random hexamer primers (Invitrogen, Cergy Pontoise, France). The reaction was carried out at 42 °C for 30 min and stopped with incubation at 99 °C for 5 min. Quantitative PCR was performed using a LightCycler system (Roche Diagnostics, Meylan, France), according to the manufacturer's recommendations. The primer sequences and PCR conditions are given in Table 1. All samples were quantified in duplicate and results are shown as the mean of three different experiments (three different MSCs). Relative quantification analyses were performed by RelQuant LightCycler software 4.1 (Roche Diagnostics, Meylan, France). Data are given as % variation as compared with maximal value.

2.4. Western blotting

Cells were washed with PBS and rapidly frozen in liquid nitrogen. They were then scraped in 50 mM Tris (pH 7.4) containing 0.25 M sucrose, 2 mM Na₃VO₄ and a protease inhibitor cocktail and centrifuged at 100,000g for 45 min [29]. Proteins were subjected to 8% SDS–PAGE. Blots were probed by the anti- β -catenin antibody clone 5H10 (Chemicon, Molsheim, France).

5 1				
Gene	GenBank	Ta (°C)	Sequences	Lengths (bp)
TNAP	AB011406	60	F: 5'-CAAAGGCTTCTTCTTGCTGGT-3'	257
			R: 5'-AAGGGCTTCTTGTCCGTGTC-3'	
COX-2	M90100	55	F: 5'-TGATTGCCCGACTCCC-3'	162
			R: 5'-TTGAAAAACTGATGCGTGAAG-3'	
OC	NM1991734	57	F: 5'-ATGAGAGCCCTCACACTCCTC-3'	293
			R: 5'-GCCGTAGAAGCGCCGATAGGC-3'	
Wnt5a	NM003392.4	60	F: 5'-CAAGGGCTCCTACGAGAGTGC-3'	204
			R: 5'-GCCGCGCTGTCGTACTTCT-3'	
Wnt10b	NM003394	62	F:5'-CATCCAGGCACGAATGCGAAT-3'	218
			R:5'-AGGCTCCAGAATTGCGGTTGT-3'	
Dkk1	NM012242	55	F:5'-GTATCACACCAAAGGACAAG-3'	184
			R:5'-ACAGTAACAACGCTGGAA-3'	
RPLPO	M17885	60	F: 5'-CGACCTGGAAGTCCAACTAC-3'	289
			R: 5'-AGCAACATGTCCCTGATCTC-3'	

Shown are the primer sequences (F: forward; R: reverse), annealing temperatures (Ta), base pair (bp) lengths of the corresponding PCR products, and GenBank accession numbers (COX: cyclooxygenase; Dkk1: dickkopf-1; OC: osteocalcin; RPLPO: acidic ribosomal phosphoprotein PO; TNAP: tissue-non specific alkaline phosphatase).

2.5. Analytical methods

For the determination of TNAP activity by the method of Lowry [2], cells cultured in 6-well plates were harvested in 0.2% Nonidet P-40 and disrupted by sonication. Calcium deposition was determined using 2% Alizarin Red staining at pH 4.2 for 2 min, after quantification by extracting the stain with 100 mM cetylpyridinium chloride for 2 h [30].

2.6. Statistical analysis

All experiments were performed in triplicates and repeated at least twice. Results are expressed as mean \pm the standard error of the mean (SEM). For statistical analysis, a *t*-test was used. A difference between experimental groups was considered to be significant when P < 0.05.

3. Results

3.1. TNF- α modulates osteoblast differentiation from human MSCs

First of all, TNF- α from 1 ng/ml stimulated calcium deposition in culture after 14 days (Fig. 1A), confirming recently published results [2,31,32]. This stimulation in calcium deposition was due to increased TNAP activity (Fig. 1B), since inhibition of TNAP with levamisole blunted the effects of TNF- α (Fig. 1C) [30]. Moreover, TNF- α dropped the mRNA levels of osteocalcin after a 48-h treatment (Fig. 1D), but up-regulated levels of the inflammatory marker cycloxygenase-2 (COX-2) (Fig. 1E). Interestingly, these effects of TNF- α were associated with increased levels of Wnt10b (Fig. 1F) and Wnt5a (Fig. 1G).

3.2. Canonical Wnt signaling stimulates TNAP in human MSCs

We next questioned whether canonical Wnt signaling mimics TNF- α effects in MSCs. Since in addition to Wnt10b, TNF- α increased the expression of other activators of the canonical pathway such as Wnt3a (data not shown), we embarked on strategy to activate canonical Wnt with anti-Dkk1 blocking antibodies, which are expected to specifically activate the canonical pathway [29]. Anti-Dkk1 antibodies slightly stimulated the expression of TNAP in MSCs, although this effect did not reach significance (Fig. 2A). A similar slight effect was however observed in MG63 cells (data not shown), suggesting that canonical Wnt signaling is a slight positive regulator of TNAP. This hypothesis was strengthened by the use of LiCl and SB216763, two synthetic activators of canonical signaling. Indeed, low doses of LiCl (0.2 mM) and SB216763 (1 µM) stimulated TNAP activity, whereas higher doses had no positive effect, or were even inhibitory (Fig. 2B). These dose-dependent effects of LiCl and SB216763 also occurred in human SaOS-2 osteoblast-like cells (Fig. 2C), and convincingly, were quite similar to the dose-dependent effects of the GSK3ß inhibitor 6-bromoindirubin-3'-oxime [33]. Collectively, these data suggest that the canonical Wnt pathway activates TNAP expression and activity in MSCs. On the other hand, anti-Dkk1 antibodies did not modulate osteocalcin nor COX-2 levels in human MSCs (Fig. 2D and E). Activation of the canonical pathway with LiCl and SB216763 also failed to modulate these transcript levels (data not shown).

3.3. Canonical Wht signaling is not involved in the stimulation of TNAP by TNF- α in human MSCs

The fact that anti-Dkk1 treatment stimulated TNAP suggests that human MSCs constitutively secreted Dkk1 and Wnt members.



Fig. 1. Effects of TNF- α on osteoblasts differentiating from human MSCs. MSCs were treated or not at confluence with TNF- α and levamisole. (A) Calcium deposition after 14 days of treatment; (B) TNAP activity after 7 days of treatment; (C) calcium deposition after 14 days of treatment (A, B and C repeated at least 4 times with similar results; one representative experiment is shown). Levels of (D) osteocalcin (OC), (E) cyclooxygenase-2 (COX-2), (F) Wnt10b and (G) Wnt5a as determined by quantitative PCR after 48 h of treatment in MSCs from three different donors. **P* < 0.05 as compared with untreated cells; **P* < 0.05 as compared with TNF- α treated cells.



Fig. 2. Effects of canonical Wnt signaling on osteoblasts differentiating from human MSCs. Human MSCs, MG63 and SaOS-2 cells were treated at confluence with 1 μ g/ml of anti-Dkk1, LiCl or SB216763. (A) TNAP transcripts in human MSCs after 48 h of treatment; (B) and (C) TNAP activity after 7 days of treatment; levels of (D) OC and (E) COX-2 in human MSCs after 48 h of treatment. (B) and (C) repeated 3 times with similar results; one representative experiment is shown; A, D and E are results in MSCs from three different donors. **P* < 0.05 as compared with untreated cells.

In fact, endogenous Dkk1 likely prevented constitutive activation of canonical Wnt signaling since exogenous Dkk1 failed to reduce TNAP activity (Fig. 3A). Moreover, we hypothesize that endogenous Dkk1 levels also inhibited the increase in β -catenin protein levels in response to TNF- α (Fig. 3B) and IL-1 β (data not shown and [34]). Interestingly, in addition to Wnt10b levels, Dkk1 levels were increased by TNF- α in MSCs (Fig. 3C). Time-course experiments revealed that Dkk1 levels increased as early as 8 h after TNF- α treatment (data not shown).

3.4. Autocrine Wnt5a stimulates TNAP in response to TNF- α in MSCs

Since its expression was up-regulated by TNF- α (Fig. 1G), we questioned whether Wnt5a, which activates non-canonical pathways, may be involved in TNF- α effects. Wnt5a at 10 ng/ml induced a 2.5-fold increase in TNAP levels after 48 h (Fig. 4A). However, 100 ng/ml of Wnt5a were necessary to significantly increase TNAP activity after 7 days (Fig. 4D). Interestingly, anti-Wnt5a blocking antibodies slightly but significantly reduced TNAP activity (Fig. 4E). In contrast, Wnt5a (Fig. 4B) and anti-Wnt5a (data not shown) did not show noticeable effects on osteocalcin levels. Finally, Wnt5a increased the levels of COX-2 in human MSCs (Fig. 4C), but anti-Wnt5a antibodies did not reduce COX-2 levels, either in presence or absence of TNF- α (data not shown).

4. Discussion

In the present study, TNF- α stimulated the expression of Wnt10b, which is known to activate the canonical Wnt signaling pathway. Since TNF- α seemed to increase the levels of other

canonical Wnt proteins, we investigated the activation of the canonical Wnt signaling pathway with anti-Dkk1 antibodies. Stimulatory effects of anti-Dkk1 antibodies have recently been reported in mouse primary osteoblasts [17], and are consistent with the beneficial effects of anti-Dkk1 antibodies on bone formation in vivo [10,17,35,36]. In our experiments, blocking Dkk1 slightly increased TNAP expression in osteoblasts differentiating from human MSCs. This effect was confirmed with both LiCl and SB216763, two β -catenin activators. The fact that TNF- α stimulated the expression of Wnt10b and that canonical Wnt signaling activated TNAP and mineralization in human osteoblasts seems to suggest that Dkk1 may represent a possible treatment to inhibit syndesmophyte formation. Importantly, levels of functional Dkk1 predict protection from syndesmophyte formation in patients with AS [18]. Moreover, adenoviral overexpression of Dkk1 was shown to strongly reduce bone healing in mouse, in association with a complete prevention of Runx2 expression and TNAP activity [37]. However, in our hands, Dkk1 failed to reproducibly inhibit TNAP or mineralization. Since TNF- α was unable to stabilize β -catenin despite stimulating Wnt10b expression, we suspect that the effects of TNF- α on the expression of canonical activators were totally masked by the stimulation of canonical inhibitors such as Dkk1.

Besides Wnt10b, we also particularly focused on Wnt5a, an important Wnt factor in bone biology [24]. Interestingly, Wnt5a is expressed in response to a panel of inflammatory stimuli and exerts inflammatory functions [38]. Wnt5a is for instance expressed in joints of patients with RA [39]. Like in RA, inflammation in AS seems driven by TNF- α ; it is therefore likely that Wnt5a is expressed in inflamed joints in AS. In the present study, Wnt5a levels increased in response to TNF- α in human differentiating osteo-blasts. We also report that Wnt5a stimulated TNAP activity in hu-



Fig. 3. Effects of TNF- α on β -catenin and Dkk1 levels in osteoblasts differentiating from human MSCs. Human MSCs and MG63 cells were treated or not at confluence with 250 ng/ml of Dkk1, 1 ng/ml TNF- α , LiCl or SB216763. (A) effects of Dkk1 on TNAP activity quantified after 7 days of treatment (mean of 3 experiments with MSCs from three different donors). (B) β -Catenin protein levels after 24 h of treatment in MG63 cells (repeated three times with identical results; one representative experiment is shown); (C) Dkk1 transcripts after 48 h of treatment, as determined by quantitative PCR in MSCs from three different donors. *P < 0.05 as compared with untreated cells.



Fig. 4. Effects of Wnt5a on osteoblasts differentiating from human MSCs. Human MSCs were treated or not at confluence with Wnt5a or anti-Wnt5a antibodies. Transcript levels of (A) TNAP, (B) OC and (C) COX-2 after 24 h of treatment, as determined by quantitative PCR in *MSCs from three different donors*; (D) TNAP activity quantified after 7 days of treatment; (E) effects of anti-Wnt5a antibodies on TNAP activity in human MSCs. *P < 0.05 as compared with untreated cells. (D) and (E) are the mean of 3 experiments with MSCs from three different donors.

man osteoblasts [28,40,41]. We hypothesize that Wnt5a stimulates TNAP and mineralization through inhibition of PPAR activity in osteoblasts [25,30]. More importantly, we also report that Wnt5a blockade decreased basal TNAP activity indicating that Wnt5a is a constitutive autocrine activator of mineralization [34,41]. Wnt5a may therefore represent a possible target to block ossification in AS. In addition to preventing excessive bone formation, inhibition of Wnt5a may also reduce the neighboring bone resorption that characterize patients with AS, since osteoblast-derived Wnt5a stimulates osteoclastogenesis [42]. Finally, Wnt5a blockade may

also impact the secretion of inflammatory mediators such as IL-6, IL-1 β and IL-8 [38]. In our hands, Wnt5a stimulated the expression of COX-2, an important enzyme in inflammation and bone repair.

In conclusion, we report that albeit TNF- α increased the levels of Wnt10b in human osteoblasts, it did not activate canonical signaling and Dkk1 failed to inhibit mineralization. In contrast, TNF- α stimulated the expression of Wnt5a and blockade of Wnt5a reduced mineralization by osteoblasts. Wnt5a may therefore represent a possible target to prevent syndesmophyte formation in AS.

Acknowledgments

This study was supported by the "Société Française de Rhumatologie" and the "Association France Spondylarthrites".

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