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Does Vitamin D play a role on Msx1 homeoprotein expression involving an endogenous antisense mRNA?☆

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Abstract

Msx1 homeobox gene, a member of Msx family, has been implicated in numerous organs. Its participation was established in different events, such as morphogenetic field determinism and epithelio–mesenchymal interactions. Most of Msx1 target organs are also known for their sensitivity to Vitamin D: such as bone, tooth germ, and hair follicle. Whereas, the expression of Msx2, another member of Msx family, has been shown to be controlled by Vitamin D, no information is available for Msx1. This study aims to analyze the potential relationships between Vitamin D and Msx1 through: (1) comparative analysis of Vitamin D receptor (VDR) and Msx1 protein expression, (2) investigation of Msx1 expression in VDR null mutant mice, and (3) study of Msx1 overexpression impact on osteocalcin VDR expression in immortalized MO6-G3 odontoblasts. Results show the existence of cross-talks between Vitamin D and Msx1 regulation pathways. In odontoblastic cells, Msx1 overexpression decrease VDR expression, whereas in rickets Msx1 sense transcript expression is decreased. These cross-talks may open a new window in the analysis of rickets mineralized tissues physiopathology. In Vitamin D null mutants, the study of the natural Msx1 antisense transcript which has been recently described should be informative.

Keywords: Msx1; Vitamin D; Tooth

1. Introduction

Msx1 homeobox gene is a member of the Msx family, orthologous to Msh of the *Drosophila* [1]. This homeogene has been implicated in the development of numerous organs [2–11]. Its participation was established in different events: morphogenetic field determinism [3–12], epithelio–mesenchymal interactions [13], regulation of cell cycle [14], and master gene expression inhibition (MyoD [15]; Cbfa1 [16]). Most of target organs for Msx1 are also known for their sensitivity to Vitamin D. Such is the case for bone, tooth germ, hair follicle, mammary gland, and muscle [17]. The expression of Msx2, an other Msx family member, has been shown to be controlled by the active metabolite of Vitamin D [18,19]), whereas no study aimed to analyze Msx1. This study aims to analyze the potential relationships between Vitamin D and Msx1 through: (1) comparative analysis of Vitamin D receptor (VDR) and Msx1 protein expression using an Msx1/nLacZ transgenic mouse line [8], (2) investigation of Msx1 expression in VDR null mutant mice [20], and (3) study of the impact of Msx1 overexpression on osteocalcin (OC) and Vitamin D receptor expression in immortalized MO6-G3 odontoblasts [16].

2. Materials and methods

2.1. Msx1/LacZ mice

Msx1/LacZ mouse model has been generated by insertion of the gene coding for the β-galactosidase inside the homeobox as previously described [8]. Heterozygous mice with a normal phenotype enabled to follow Msx1 homeoprotein expression by revelation of the β-galactosidase activity [21].

2.2. VDR KO mice

Vitamin D receptor knock-out mouse model (VDR (−/−)) has been generated by inserting the *neo*^{r} gene into exon 2 [20]. Mice homozygous for the mutation develop a severe type II genetic rickets showing bone defects,

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hypocalcemia, hypophosphatemia, and hyperparathyroidism [20,22]. Mandibular incisors of 56-day-old VDR (−/−) mice were extracted and dental mesenchyme was dissected out for further RT-PCR analysis.

2.3. In situ hybridization

Msx1 sense transcript expression in mandibular bone and hair follicle was analyzed in new-born C57B6 mice (Charles River, St Aubin les Elboeuf, France). After sacrifice, samples were fixed with 4% paraformaldehyde (PFA)–15% sucrose in PBS (Sigma, St Louis, USA), rinsed with 15% sucrose in PBS, and finally cut without decalcification with a cryostat at −25 °C (Bright, Instrument Company LTD, Huntington, England). Sections were deposited onto 50 mg/ml poly-L-lysine (Sigma) coated slides, then dehydrated in a graded ethanol series and finally stored at −20 °C. Msx1 sense digoxigenin-labeled probe was synthesized from a Bluescript-SK+ plasmid containing 350 bp of the second exon of the mouse Msx1 gene [8] after linearisation with *Bam*HI endonucleases (Boehringer Mannheim, Meylan, France) using T7 RNA polymerases (Boehringer Mannheim). In situ hybridization was performed as previously described [23] with minor modifications. Briefly, cryostat sections were hybridized with 30 μl of digoxigenin-labeled probes diluted 1/200 in hybridization buffer in a moist chamber overnight at 58 °C, and washed under high stringency conditions. To prevent non-specific binding of antibody, the anti-digoxigenin Fab alkaline phosphatase conjugate (Boehringer Mannheim) was diluted with 10% blocking reagent and 20% heat-inactivated sheep serum in maleic acid buffer. The sections were incubated in a moist chamber overnight at room temperature. The color development reactions were performed for 2–18 h. The sections were dehydrated and mounted under a coverslip.

2.4. Msx1/LacZ transgene expression detection

Msx1/LacZ transgene expression in mandibular bone and hair follicle was analyzed in new-born heterozygous mice. After sacrifice, tail samples were collected for PCR genotyping [8], and mice were fixed by immersion in 4% PFA in PBS 15 min, rinsed in PBS, and stained overnight for β-galactosidase activity [21]. Samples were then fixed again for 4 h in the same PFA solution, rinsed in PBS with 15% sucrose, and cut without decalcification with a cryostat. Sections were finally stained according to a modified Van Gieson protocol [21].

2.5. MO6-G3 odontoblastic cell line transfection experiments

The mouse odontoblastic cell line MO6-G3 [24] was plated out in MEM medium supplemented with 15% fetal calf serum, at 0.8×10^6 cells per dish. After 72 h, cells were about 80% confluent, and transfected with 3 μg of the Msx1 expression vector and 12.5 μl lipofectamine per dish (Life Technologies, Cergy Pontoise, France) during 5 h, in 1 ml serum-free medium, according to the manufacturer procedure (Optimem, Life Technologies). One milliliter of medium containing 30% fetal calf serum was then added to stop the transfection reaction. After 72 h, the medium was removed, the cells were rinsed with PBS, and used for RNA analysis. Each experiment was made in triplicate. Control cells were transfected with the same plasmid containing no insert. To analyze the combined effect of Msx1 transfection and Vitamin D addition to the culture, 1,25-dihydroxy ($1,25(OH)_2$)Vitamin D_3 (10^{-7} M) was added every 24 h to the cell medium. Controls were realized with carrier ethanol addition with the same kinetic than $1,25(OH)_2$ Vitamin D_3 addition.

2.6. RNA analysis by RT-PCR

Total RNA from either microdissected lower incisor mesenchyme of VDR (−/−) and VDR (+/+) mice from the same litter or transfected and control MO6-G3 cells [25] and analyzed by RT-PCR. 1 μg of total RNA were reverse transcribed following the manufacturer protocol (Invitrogen, Carlsbad, USA) with random hexanucleotide primers. PCR was performed with the following primers: Msx1 sense RNA, 5′-CTCATGGCCGATCACAGGAA-3′ and 5′-TCAGGTGGTACATGCTGTAG-3′; VDR, 5′-CCAAGCTGTCTGAGGAGCA-3′ and 5′-TGGTTGGAGCGCAACATGAT-3′; OC, 5′-CTCACTCTGCTGGCCCTG-3′ and 5′-CCGTAGATGCGTTTGTAGGC-3′; GAPDH, 5′-TTCCAGTATGATTCCACTCA-3′ and 5′-CTGTAGCCATATTCATTGTC-3′. In all analysis, RNA without prior reverse transcription served as a control for genomic DNA contamination.

2.7. Immunocytochemistry

Msx1 protein expression was analyzed in MO6-G3 cells, transfected with Msx1 expression vector and control plasmid. Immunofluorescent labelling was performed using polyclonal rabbit antibodies directed against mouse Msx1 protein (BabCo, Richmond, CA, USA) and fluorescein-labeled secondary antibodies (Amersham Life Science, England). In immunolabelling controls, the primary antibodies were replaced by non-relevant rabbit antibodies.

3. Results

3.1. Msx1 sense RNA expression in VDR (−/−) mice dental mesenchyme

RT-PCR comparative analysis of Msx1 sense transcript expression between VDR (+/+) and VDR (−/−) mice dental mesenchyme was realized. These data show an

apparent decrease in Msx1 sense transcript expression level in rachitic hypocalcemic mice (Fig. 1).

3.2. Msx1 sense RNA and homeoprotein expression in known VDR expression tissues

The expression of Msx1 sense transcript and homeoprotein was analyzed in new-born mice respectively by in situ hybridization and β-galactosidase enzymology in Msx1/LacZ mice. Two Vitamin D target tissues were also analyzed, hair follicle, and mandibular bone. In hair follicle, Msx1 sense and protein are colocalized in cells of the basal region of the follicle (Fig. 2A and C). In the mandibular

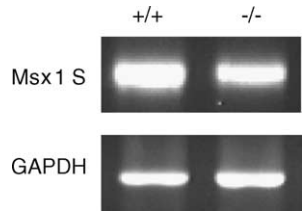

Fig. 1. RT-PCR comparative analysis of Msx1 sense transcript expression in the dental mesenchyme of wild-type and VDR (−/−) hypocalcemic mice. Msx1 sense transcripts decrease in the mesenchyme of VDR null mutant mice.

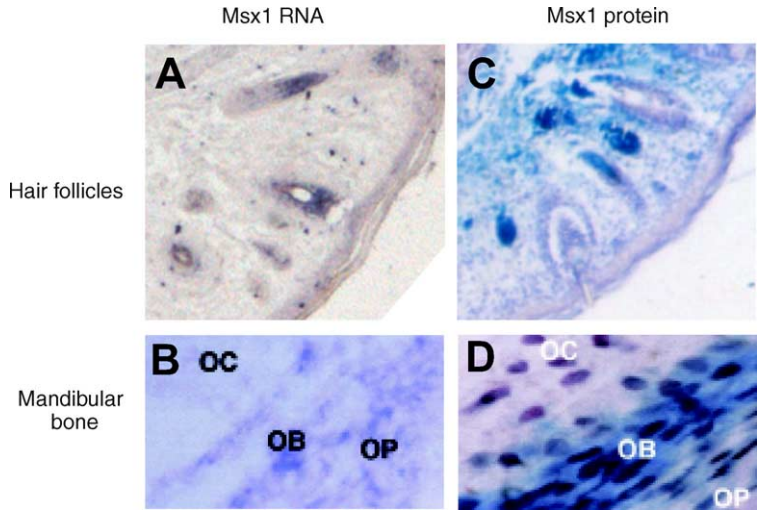

Fig. 2. Msx1 sense transcript (A and B) and homeoprotein (C and D) expression in hair follicles and mandibular bone. In hair follicle an Msx1 expression (sense transcript and protein) is detected at the base of the follicule in the region of cell proliferation (A and C). In bone, the Msx1 sense transcript is detected in preosteoblastic and osteoblastic cells (B). The homeoprotein shows a similar expression pattern with a graded extinction in osteoblasts (D). OB, osteoblasts; OC, osteocytes; and OP, osteoprogenitor cells.

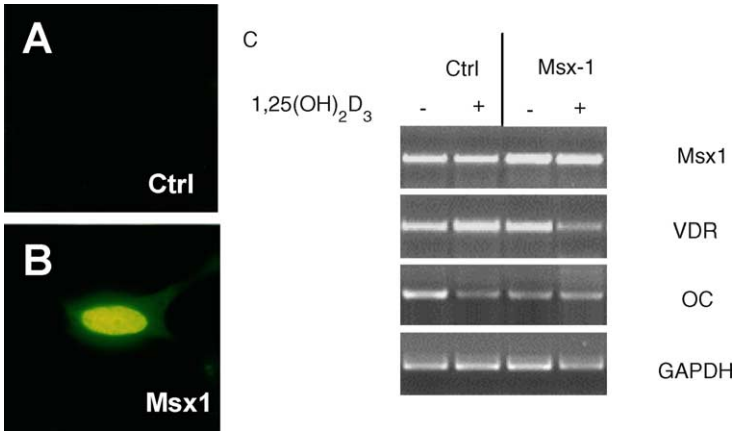

Fig. 3. Msx1 overexpression experiments in MO6-G3 cell line. Immunocytochemistry experiments (A and B) show an Msx1 homeoprotein expression in the nucleus after transfection. RT-PCR analysis (C) of Msx1 overexpression in MO6-G3 cells and effect of Vitamin D addition to the cell media. The transfection efficiency is confirmed by Msx1 sense transcript expression increase. Vitamin D addition efficiency to the media is confirmed by the VDR transcript increase and OC transcript decrease in untransfected cells. Single Msx1 overexpression induced a decrease of OC transcript expression and no effect on VDR transcript. The addition of Vitamin D to the Msx1 transfected cells induces a decrease of VDR transcript. The osteocalcin transcript expression is unchanged compared to simple transfection.

bone, Msx1 sense transcript and protein are detected in preosteoblastic and osteoblastic cells. In osteocytes, Msx1 expression is gradely weakened (Fig. 2B and D).

3.3. Transfection experiments

Transfections of 3 μg Msx1 vector in MO6-G3 cells induced a nuclear overexpression of Msx1 transcription factor (Fig. 3A and B) and a decrease in osteocalcin transcript expression, as shown detected by RT-PCR (Fig. 3C). In contrast to osteocalcin transcript, VDR transcript expression was unchanged by Msx1 overexpression alone. In contrast, the combined addition of 1,25$(OH)_2$ Vitamin D_3 and Msx1 overexpression induced a striking decrease in VDR transcript expression level (Fig. 3C). However, after the exclusive addition of 1,25$(OH)_2$ Vitamin D_3 in non-transfected MO6-G3 cells, Msx1 sense transcript expression was not affected, whereas, OC transcript expression was decreased and VDR one increased (Fig. 3C), as expected.

4. Discussion

In our study, some relationships of Msx1 and Vitamin D regulation pathways was shown: (1) Msx1 and VDR are co-expressed in the same tissues here, hair follicle, bone, and tooth germ; (2) Msx1 expression is disturbed in dental mesenchyme of VDR (−/−) mice compared to controls; and (3) reversely, VDR transcript expression is decreased in odontoblastic cells when, Msx1 is overexpressed in the presence of Vitamin D active metabolite. Such a Vitamin D control of Msx homeogenes (Msx2 [18,19]), suggests a potential implication of both Msx1 and Msx2 genes in defects associated with rickets [19,26,27], and rise the question of hierarchy of Vitamin D and Msx cross-talks. Msx1 overexpression in MO6-G3 odontoblasts showed here an impact on OC expression as previously reported for Msx2 in odontoblasts [19] and osteoblasts [28]. Such in vitro data are supported by the reverse expression patterns for Msx1 [29], Msx2 [30] and osteocalcin. Msx1 and Msx2 homeogenes are known to control the terminal differentiation of odontoblasts which culminate with OC expression. The present data also show that Vitamin D may control Msx1 gene expression as shown for Msx2 [18]. The studies on Msx1 sense transcript (here) and Msx2 [18] suggest that 1,25$(OH)_2$ Vitamin D_3 could act on odontoblast differentiation through the control of Msx homeogene expression. Interestingly, Vitamin D is known to control odontoblasts differentiation [26]. Thus, Vitamin D may contribute to odontoblast terminal differentiation in part through Msx homeogene regulation. On the other hand, the data show that Msx1 overexpression induced decreased steady state level of VDR transcript when 1,25$(OH)_2$ Vitamin D_3 was added to culture medium of odontoblasts. This finding suggests that Vitamin D action on dental cells may be mediated through the reversed regulation of VDR with or without the associated expression of Msx1. Such findings are supported by the complementary expression patterns of Msx1 [16] and VDR [31] in dental cells.

Recent reports, have underlined the importance of an endogenous Msx1 antisense transcript in the control of the homeoprotein expression in the same tissues, teeth, and bone [16,32]. Increased ratio of antisense/sense transcripts inhibits Msx1 homeoprotein expression in vitro. However, the molecular mechanism underlying this control is unclear. Our data reporting decreased Msx1 sense transcript level in VDR (−/−) dental mesenchyme show that in this tissue which express the antisense transcript [16,32], Msx1 transcripts ratio could be modified. Therefore, the role for sense and antisense mRNA would be relevant to analyze in the physiopathology of rickets. Observations in perinatal mice have outlined a complementary expression of Msx1 sense/antisense transcript and protein in tooth ([16]; unpublished data) contrasting with the present homogeneity observed in hair follicles and mandibular bones of new-born mice (Fig. 2). At the studied stage of tooth development (late-bell stage and initial-mineralization stage), a low Msx1 sense/antisense transcript ratio has been reported in both dental epithelium and mesenchyme ([16]; unpublished data) that could explain the absence of Msx1 homeoprotein expression [21]. In contrast, in earliest stages of tooth development (initiation, bud and cap stages), sense/antisense Msx1 transcripts ratio appear to allow homeoprotein expression in the dental mesenchyme ([16]; Robert, personnal communication) in which the VDR is expressed [31,33]. At these developmental stages, a similar decrease of Msx1 sense transcript in dental mesenchyme of rachitic animal, may have an impact on the Msx1 transcripts ratio and locally disturbed Msx1 homeoprotein expression. Such a hypothesis, could be of dramatic impact on tooth morphogenesis and partially explains some morphogenesis defects reported in rachitic mouse teeth [19,26,27].

5. Conclusion

Msx homeobox gene and Vitamin D are jointly involved in the development of numerous organs. In tooth germ and probably other organs, Msx, and Vitamin D regulation pathways appeared to cross-talk. Such observations open a new window in the understanding of defects associated to rickets. However, the exact molecular modality of Vitamin D and Msx should be delineated more precisely. Regarding Msx1 homeoprotein expression control, the recently described, endogenous antisense transcript appears to be considered as a key element probably throught interference with sense transcript. A comparative study of sense and antisense expression in normal and VDR (−/−) mice should provided some new data for the understanding of molecular events related to Vitamin D and Msx1 homeobox gene cross-talks.

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