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CYP24A1-deficient mice as a tool to uncover a biological activity for vitamin D metabolites hydroxylated at position 24☆

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ABSTRACT

The CYP24A1 enzyme (25-hydroxyvitamin D-24-hydroxylase) not only is involved in the catabolic breakdown of 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] but also generates the 24,25-dihydroxyvitamin D [24,25$(OH)_2$D] metabolite. The biological activity of 24,25$(OH)_2$D remains controversial. While *in vitro* studies suggest that primary cultures of rat rib chondrocytes respond to 24,25$(OH)_2$D in a maturation-specific manner and that the metabolite is necessary for the cells to progress from a proliferating, immature status to a differentiated, 1,25$(OH)_2$D-responsive stage, *in vivo* evidence to support this putative role remains lacking. Studies in chicken showed increases in serum levels of 24,25$(OH)_2$D and of the renal mRNA levels of *Cyp24a1* following fracture, suggesting a role for 24,25$(OH)_2$D in fracture repair. The *Cyp24a1*-deficient mouse strain represents an invaluable tool to examine the putative role of 24,25$(OH)_2$D in mammalian fracture repair. We have compared fracture repair between *Cyp24a1*$^{-/-}$ mice and wild-type controls. We have observed a delay in the mineralization of the cartilaginous matrix of the soft callus in *Cyp24a1*$^{-/-}$ mutant animals, accompanied by reduced expression of chondrocyte marker genes. These results show that *Cyp24a1* deficiency delays fracture repair and strongly suggest that vitamin D metabolites hydroxylated at position 24, such as 24,25$(OH)_2D_3$, play an important role in the mechanisms leading to normal fracture healing.

1. Introduction

Vitamin D must be metabolized twice to be activated and function as a key regulator of mineral ion homeostasis [1]. In the liver, it is hydroxylated at position 25, and in the kidney, through the action of the CYP27B1 enzyme, it is hydroxylated at position 1 to produce 1,25$(OH)_2$D, the hormonal form of vitamin D [2]. Upon reaching its target tissues, 1,25$(OH)_2$D binds to its specific receptor, the vitamin D receptor (VDR), to regulate the transcription of vitamin D target genes responsible for carrying out the physiological actions of 1,25$(OH)_2$D: mineral homeostasis, skeletal homeostasis, and cellular differentiation [3]. Amongst several target genes, the 1,25$(OH)_2$D hormone induces in target cells the expression of the gene encoding the key effector of its catabolic breakdown: 25-hydroxyvitamin D-24-hydroxylase (*Cyp24a1*) [4,5]. This insures attenuation of the 1,25$(OH)_2$D biological signal inside target cells and helps regulate vitamin D homeostasis.

CYP24A1 catalyzes the addition of an hydroxyl group on carbon 24 of vitamin D. This initiates the 24-oxidation pathway that leads to 1,25$(OH)_2$D metabolite inactivation [4]. This pathway comprises five enzymatic steps involving successive hydroxylation/oxidation reactions at carbons 24 and 23 followed by cleavage of the seco-osteroid at the C-23/C-24 bond and subsequent oxidation of the cleaved product to calcitroic acid [4]. In cell culture systems, the role of the CYP24A1 enzyme in the catabolism of 1,25$(OH)_2$D has been examined utilizing cytochrome P450 inhibitors: blocking P450 activity by treatment with ketoconazole inhibits catabolism and results in increased specific accumulation of 1,25$(OH)_2$D [6]. The function of the CYP24A1 protein as an effector of 1,25$(OH)_2$D breakdown has also been confirmed *in vivo*. Indeed, we have previously shown that mice deficient for the *Cyp24a1* gene cannot effectively clear 1,25$(OH)_2$D from their circulation [7].

The 25(OH)D metabolite can also serve as the substrate for the CYP24A1 enzyme, leading to the production of 24,25$(OH)_2$D. The putative bioactivity of 24,25$(OH)_2$D remains controversial. An extensive literature demonstrates that *Cyp24a1* is expressed in growth plate chondrocytes and that cells from the growth plate respond to 24,25$(OH)_2$D in a cell maturation-dependent manner [8]. Most of these studies were performed using the *in vitro* rat cos-

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tochondral primary culture system. Dissection of the tissue allows to culture cells from different regions of the growth plate. Each region represents a different maturation stage along the chondrocytic differentiation pathway. In this model system, the less differentiated cells of the resting zone, also called the reserve zone, appear to respond to 24,25$(OH)_2$D [9]. The more mature cells of the growth zone, comprising the prehypertrophic and hypertrophic compartments, respond primarily to 1,25$(OH)_2$D [10]. Interestingly, treatment of resting zone chondrocytes with 24,25$(OH)_2$D induces a change in maturation state [11], supporting the hypothesis that 24,25$(OH)_2$D plays a role in cartilage development. The maturation stage-dependent responses of chondrocytes to the vitamin D metabolites include both genomic and non-genomic effects [12].

Contrasting with the tissue culture results is the observation that the growth plates from *Cyp24a1*$^{-/-}$ mice do not show major defects [7,13]. These observations suggest that the absence of CYP24A1 activity does not affect growth plate development and that 24,25$(OH)_2$D is not required for chondrocyte maturation *in vivo* [7]. It remains possible, however, that a redundant endocrine system is able to compensate for the function of 24,25$(OH)_2$D in animals.

2. Fracture repair

It has also been proposed that 24,25$(OH)_2$D might play a role in fracture repair, but there is limited information available on this putative function of the metabolite. Fracture repair is a complex multistep process that involves response to injury, intramembranous bone formation, chondrogenesis, endochondral bone formation, and bone remodelling. The immediate response to the fracture trauma results in the infiltration of inflammatory cells, macrophages, and platelets during formation of an initial hematoma [14]. Soon after the fracture event, nearby bone marrow cells reorganize into regions of high and low cellular density. Within a day of the fracture event, cells in the high cellular density regions undergo differentiation along the osteoblastic lineage [14]. Together with the osteoblasts that were lining the cortical bone, these differentiating osteoblasts lay down new bone via an intramembranous pathway to form the 'hard' callus of woven bone adjacent to the fracture site. In mice, this takes place as early as 3 days post-fracture and continues until day 14 post-fracture, with proliferation peaking between days 7 and 10. Mesenchymal cells proliferate for several days, then differentiate into chondrocytes, leading to the formation of the cartilaginous, 'soft' callus that bridges the fracture site. Proliferation of these new chondrocytes continues from day 7 to day 21 post-fracture. The soft callus provides the initial stabilization at the fracture site. The mineralization of the soft callus begins at the interface between the maturing cartilage (hypertrophic chondrocytes) and the newly formed woven bone of the hard callus. Angiogenesis occurs closely after hypertrophic chondrocyte mineralization of the matrix, mimicking endochondral bone formation at the growth plate. The hypertrophic chondrocytes undergo apoptosis, and the mineralized cartilage matrix is replaced by woven bone laid down by the osteoblasts that accompanied the infiltrating new vascular structures. The new bone repairing the fracture site will be subsequently remodelled by cooperative osteoblast/osteoclast activity, producing bone that is indistinguishable from the original intact bone [15].

Thus fracture healing involves a sequential series of cellular and biochemical events proceeding from inflammation through intramembranous bone formation, chondrogenesis, endochondral bone formation, and finally remodeling. Several studies have described a complex pattern of gene expression that occurs during the course of these events [16–19]. Extracellular matrix components are differentially expressed during the different stages of fracture repair. In mice, osteocalcin gene expression is induced and reaches a maximum around day 15 [14,15]. Collagen type II and aggrecan are expressed initially but are turned off by 9 days post-fracture. This is followed by type X collagen expression when the chondrocytes hypertrophy [20]. The chondrocytes also express alkaline phosphatase, whose expression peaks around days 17–18 post-fracture [21]. Taken together, results from gene expression monitoring during bone repair suggest that the molecular regulation of fracture healing is complex but recapitulates some aspects of embryonic skeletal formation [22,23].

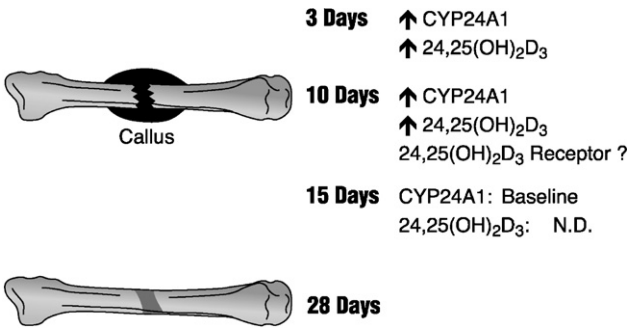

Fig. 1. CYP24A1 activity and 24,25$(OH)_2D_3$ levels during fracture healing in chicks. The changes in CYP24A1 activity and circulating 24,25$(OH)_2D_3$ concentrations are listed besides the temporal sequence of fracture healing: ↑, increase; N.D., not determined. The putative expression of a receptor/binding protein for 24,25$(OH)_2D_3$ at day 10 post-fracture is expressed by a question mark. Based on the data described in Refs. [24–27].

3. 24,25$(OH)_2$D and fracture repair

The circulating levels of 24,25$(OH)_2$D increase during fracture repair in chickens due to an increase in CYP24A1 activity [24] (Fig. 1). When the effect of various vitamin D metabolites on the mechanical properties of healed bones was tested, treatment with 1,25$(OH)_2D_3$ alone resulted in poor healing [25]. However, the strength of healed bones in animals fed 24,25$(OH)_2D_3$ in combination with 1,25$(OH)_2D_3$ was equivalent to that measured in a control population fed 25-hydroxyvitamin D_3 [25]. These results support a role for 24,25$(OH)_2$D as being an essential vitamin D metabolite important for fracture repair. It is likely that 24,25$(OH)_2$D would act through receptor-mediated signaling (as do other vitamin D metabolites and hormones), and circumstancial evidence suggests the presence of a non-nuclear membrane receptor for 24,25$(OH)_2$D in the chick tibial fracture-healing callus [26,27]. Cell fractionation to isolate a membrane fraction followed by ligand binding studies using hydroxylapatite to separate bound and free ligands described a receptor/binding protein for 24,25$(OH)_2D_3$ in the fracture-healing callus membrane fraction from vitamin D-depleted chicks [27]. These observations were never followed through and to date, no molecular entity corresponding to this binding activity has ever been cloned and characterized.

The *Cyp24a1*-deficient mouse strain that we have engineered [7] represents an invaluable tool to examine the putative role of 24,25$(OH)_2$D in mammalian fracture repair. *Cyp24a1* mutant animals that survive past weaning appear to use an alternative pathway of 1,25$(OH)_2$D catabolism to regulate circulating levels of the hormone [28] and are normocalcemic and normophosphatemic when fed regular rodent chow. This has allowed us to study bone healing in these animals. We have compared fracture repair between *Cyp24a1*$^{-/-}$ mice and wild-type controls. We have observed a delay in the mineralization of the cartilaginous matrix of the soft callus in *Cyp24a1*$^{-/-}$ mutant animals, accompanied by

reduced expression of chondrocyte marker genes (data not shown). The repair delay and the aberrant pattern of gene expression could be rescued by treatment with $24,25(OH)_2D_3$. Our results strongly support a role for $24,25(OH)_2D$ in mammalian fracture repair.

These studies could open up exciting perspectives for treatment following fracture or orthopaedic surgery using 24-hydroxylated vitamin D metabolites or analogs. It can be argued that $24,25(OH)_2D_3$ is an abundant circulating vitamin D metabolite and that it is present in sufficient amounts to efficiently promote bone healing without the need for additional supplementation. However, it is now recognized that a sizeable proportion of the population suffers from vitamin D insufficiency [29–31], which may have deleterious effects for optimized fracture repair. Thus fracture healing could benefit from supplementation with $24,25(OH)_2D_3$ or a suitable analog.

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