ABSTRACT

The objective of these investigations was to determine if the receptor-dependent effects of 1,25-dihydroxyvitamin D were essential for normal skeletal growth. Mice with targeted ablation of the vitamin D receptor were engineered, and the skeletal consequences of vitamin D receptor ablation were studied in the presence of normal and abnormal mineral ion homeostasis. Prevention of abnormal mineral ion homeostasis resulted in the development of a normal skeleton in the absence of a functional vitamin D receptor. The metabolic cause of rickets was found to be hypophosphatemia. The major receptor-dependent actions of 1,25-dihydroxyvitamin D on skeletal development are indirect and are a reflection of the role of this hormone on intestinal calcium absorption.
THE ACTIVE METABOLITE of vitamin D, 1,25-dihydroxyvitamin D, is thought to exert its effects by binding to the vitamin D receptor (VDR), a member of the nuclear receptor family of ligand-dependent transcription factors. The receptor-dependent actions of 1,25-dihydroxyvitamin D have been shown to promote intestinal calcium absorption, suppress PTH gene transcription, regulate the expression of bone-matrix proteins, and promote osteoclast differentiation by inducing the expression of RANK (receptor activator of nuclear factor κB) ligand. Although these studies have demonstrated that 1,25-dihydroxyvitamin D has several actions that contribute to the regulation of skeletal and mineral ion homeostasis, questions remained as to whether the actions of 1,25-dihydroxyvitamin D were essential and whether the in vivo consequences of vitamin D deficiency were a direct result of impaired hormone-dependent receptor actions.

To address these issues, studies were performed in VDR-null mice. These mice, which have no detectable receptor protein, are a phenocopy of the human disorder hereditary vitamin D-resistant rickets. They are phenotypically normal at birth but develop secondary hyperparathyroidism the third week of life. Osteomalacia is also seen, which is characterized by a dramatic expansion of the growth plate and hypomineralized flared metaphyses, is observed by the fourth week of life. Osteomalacia is also seen, which leads to impaired biomechanical properties of the skeleton.

To dissect which of these skeletal manifestations of VDR ablation were a direct consequence of impaired hormone-dependent receptor actions versus the resultant abnormalities in mineral ion homeostasis, VDR-null mice were placed on a diet enriched in calcium, phosphorus, and lactose before the development of second mites. We therefore undertook investigations to clarify the basis for the growth-plate abnormality in the VDR-null mice. Within 2 days of the development of hyperparathyroidism, we observed an expansion in the hypertrophic chondrocyte layer of the VDR-null mice. On the basis of the work of other investigators, which demonstrated that extracellular calcium promotes expression of markers of terminal chondrocyte differentiation, we addressed whether MGP played a pathophysiological role in expansion of the late hypertrophic chondrocyte layer by making the VDR-knockout mice null for MGP as well. This did not normalize the growth-plate phenotype. Because the cellular basis for the rachitic changes involved expansion of the terminally differentiated, osteopontin-expressing late hypertrophic chondrocytes, we evaluated apoptosis, the final stage in differentiation of these cells. Histologic analyses demonstrated a marked decrease in apoptosis of the late hypertrophic chondrocytes in the rachitic VDR-null mice, thus clarifying the cellular basis for this abnormality.

Although these studies were critical in identifying the cellular basis for the rachitic changes, they did not address the underlying pathophysiology that led to this abnormality. Therefore, we performed studies to demonstrate that normalizing mineral ion homeostasis in the VDR-null mice, which is associated with normal growth-plate histology, normalizes apoptosis. These results raised the question of whether the impaired apoptosis was secondary to hypocalemia, hyperparathyroidism, or hypophosphatemia. To address this issue, studies were performed to characterize the growth-plate phenotype in 2 additional murine models: diet-induced hypophosphatemia/hypercalcemia and the hyp mouse (which has a mutation in the PHEX gene and is the murine model for the human disease X-linked hypophosphatemia). The mice with hypophosphatemia in the presence of hypercalcemia (and suppressed PTH levels) and the hyp mice (normal calcium and PTH levels) both demonstrated expansion of the late hypertrophic chondrocyte layer associated with impaired apoptosis of these cells, which points to hypophosphatemia as the common etiologic factor.
Interpretation of studies in the hyp mice are not straightforward because of the possibility that mutation of the PHEX gene may lead to an intrinsic chondrocyte defect, analogous to the osteoblast defect that has been reported. Therefore, to address whether hypophosphatemia, as opposed to an intrinsic chondrocyte defect, was the primary cause of the rachitic changes in the hyp mice, the growth plate of hyp mice with normal mineral ion homeostasis was examined. Because the serum phosphate of the hyp fetuses is indistinguishable from that of their wild-type littermates, examining the growth plate before and after birth permitted investigations directed at correlating the development of rickets with that of hypophosphatemia. It is interesting to note that at 18.5 days of embryonic life, correlating with a normal phosphorus level, the growth-plate phenotype of the hyp mice was normal both histologically and by terminal deoxynucleotidyltransferase-mediated 2'-deoxyuridine 5'-diphosphate nick end labeling (TUNEL) evaluation of apoptotic cells. The development of rachitic changes in this model paralleled the development of impaired chondrocyte apoptosis and hypophosphatemia, which lends further credence to the hypothesis that hypophosphatemia leads to rickets by impairing apoptosis of late hypertrophic chondrocytes. It is interesting to note that in the models examined, impaired apoptosis was observed at a time when there was still considerable mineralized matrix surrounding the late hypertrophic chondrocytes, which suggests that circulating, rather than locally deposited, phosphate is the critical determinant of apoptosis. Supporting this hypothesis is the observation that tissue-nonspecific alkaline phosphatase–knockout mice (a model for the human disease hypophosphatasia) have normal circulating phosphate levels, have markedly impaired matrix mineralization, and do not develop rickets.

Because hypophosphatemia impairs hypertrophic chondrocyte apoptosis in vivo, which leads to rickets, the pathway by which phosphate mediates chondrocyte apoptosis was examined. Studies by other investigators in an avian chondrocyte culture system demonstrated that phosphate induces chondrocyte apoptosis in a dose-dependent manner. A primary murine chondrocyte culture model was used to determine if phosphate-mediated chondrocyte apoptosis involved the extrinsic (membrane) or intrinsic (mitochondrial) apoptotic pathway. These studies demonstrated that phosphate treatment of hypertrophic chondrocytes led to activation of caspase-9, a mediator of the mitochondrial apoptotic pathway. Furthermore, inhibition of mitochondrial permeability transition (an initial step in activation of the mitochondrial apoptotic pathway that involves the inner-membrane pathway) with cyclosporin A inhibited caspase-9 activation. It is interesting to note that activation of caspase-9 by phosphate was cell type– and differentiation stage–specific in that phosphate did not activate caspase-9 in 3T3 fibroblasts or in primary chondrocytes that had not undergone hypertrophic differentiation. To demonstrate that activation of the mitochondrial apoptotic pathway was critical for hypertrophic chondrocyte apoptosis in vivo, wild-type mice were treated with caspase inhibitors for 6 days. These studies demonstrated that inhibition of caspase-9 in vivo leads to expansion of the late hypertrophic chondrocytes of wild-type mice, demonstrating a role for the mitochondrial apoptotic pathway in growth-plate maturation in vivo.

Although calcium has been shown to promote chondrocyte differentiation, terminal differentiation of hypertrophic chondrocytes leading to apoptosis depends on adequate levels of circulating phosphate. Thus, the receptor-dependent actions of 1,25-dihydroxyvitamin D are critical for optimal intestinal calcium absorption, which provides an optimal metabolic environment for skeletal mineralization.

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