Osteoblast Cultures: A Valuable Tool for the Study of Hypophosphatemic Vitamin D-Resistant Rickets

Brigitte Ecarot and Francis H. Glorieux

Genetics Unit, Shriners Hospital for Crippled Children, 1529 Cedar Avenue, Montreal, Quebec, Canada H3G 1A6

Bone matrix formation and mineralization is a complex process which is under the control of the osteoblasts and nearby osteoid osteocytes. Impaired bone mineralization (osteomalacia) can result from alterations in the bone cell environment and/or from abnormalities in the function of the bone-forming cells. Because of the methods available, most identified forms of osteomalacia have been related to disturbances in mineral or vitamin D metabolism. However, with the recent advances in cell biology, methods have been developed for osteoblast isolation. The isolated cells, provided they retain their differentiated phenotype in culture, offer a powerful tool for investigating the properties and functional responsiveness of osteoblasts and therefore for assessing intrinsic defects of the osteoblast function associated with genetic bone diseases. In addition, the availability of animal models closely resembling the human disorders has allowed more direct investigation of the pathophysiology of these disorders.

Studies will be described in this chapter that illustrate the suitability of an animal model, the hypophosphatemic (Hyp) mouse and the usefulness of osteoblast cultures for investigating the pathogenesis of human vitamin D-resistant rickets (VDRR). Data will be presented which support the view that the osteoblast is an important target for the Hyp (and VDRR) mutation. Conceivably, methods will be developed in the future for isolating homogenous populations of osteoblasts from human origin that will allow direct evaluation of the function of osteoblasts from VDRR patients.

HUMAN HYPOPHOSPHATEMIC VITAMIN D-RESISTANT RICKETS

Hypophosphatemic vitamin D-resistant rickets also termed familial X-linked hypophosphatemia was first recognized in 1937 by Albright (1). The term describes a hereditary condition of hypophosphatemic rickets and osteomalacia unresponsive to physiological doses of vitamin D. This disorder is transmitted as an X-linked dominant trait and is characterized by hypophosphatemia, normocalcemia, and normal

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plasma levels of 1,25-dihydroxyvitamin D[1,25(OH)_{2}D]. The patients have defective mineralization of the cartilaginous epiphyseal plate (rickets) and of the endosteal bone (osteomalacia) with consequent slow growth and skeletal deformities. The pathogenesis of this condition remains obscure (see chapter by F. H. Glorieux et al.). A defect in renal tubular phosphate reabsorption has been well established (2). Impaired regulation of 1,25(OH)_{2}D synthesis which was first suggested by the paradoxical occurrence of normal plasma concentrations of 1,25(OH)_{2}D (3,4) in the face of hypophosphatemia (5,6) has been recently documented (2). However, these defects do not account for all the abnormalities in this disorder, nor for the response to phosphate therapy. Significant hypophosphatemia without apparent bone involvement has been reported in relatives of VDRR patients (8) suggesting that factors other than hypophosphatemia are involved in the etiology of the bone disease. Indeed, several observations point towards a specific disorder of the bone-forming cells. Treatment of VDRR patients with phosphate alone or combined with pharmacologic doses of vitamin D heals rickets but not osteomalacia (9). Pharmacologic doses of 1,25(OH)_{2}D in conjunction with phosphate supplements are required to improve and heal the bone lesions (10,11), despite the fact that circulating levels of 1,25(OH)_{2}D in untreated patients are similar to those of age- and growth rate-matched normal subjects (3,4). The mechanism by which 1,25(OH)_{2}D promotes bone mineralization in VDRR is unsettled. 1,25(OH)_{2}D may exert its effect indirectly by providing adequate extracellular levels of phosphorus (12,13) or directly through an osteoblast action. Indeed, histomorphometric studies have shown that calcitriol promotes mineralization only at osteoid surfaces covered by osteoblasts (14). The reported failure of phosphate and vitamin D therapy to improve cortical bone mineralization in VDRR patients despite normalization of serum phosphate levels (15) further supports a direct effect of 1,25(OH)_{2}D on bone cells. Thus, the need for supraphysiologic amounts of 1,25(OH)_{2}D in order to heal the osteomalacia may reflect a failure of the osteoblast to respond to normal circulating levels of 1,25(OH)_{2}D.

A defect at the osteoblast level is further supported by the presence of characteristic hypomineralized periosteocytic lesions in cortical bone of VDRR patients (16–18). These lesions are not seen in patients with vitamin D-deficiency or dependency and with tumor-induced osteomalacia who display chronic hypophosphatemic osteomalacia (19,20). Moreover, these lesions were still found in growing osteons despite normalization of bone mineralization in VDRR patients by phosphate and calcitriol [1,25(OH)_{2}D_{3}] therapy (19). Thus, several clinical observations point to an abnormal osteoblast/osteocyte function in VDRR.

THE HYPOPHOSPHATEMIC MOUSE

Human VDRR has been difficult to study in part because of the lack of a suitable animal model. In 1976, an hypophosphatemic mutant in the laboratory mouse was described (21) which exhibits most of the biochemical and clinical features of VDRR patients (22–25) and comparable response to treatments (26–28). The hypominer-
alized periosteocytic lesions are also seen in cortical bone of Hyp mice (29). However, unlike VDRR patients, Hyp mice display slight hypocalcemia associated with a mild secondary hyperparathyroidism (30). Despite these differences, this mutant mouse is considered to be a good model for the study of human VDRR as evolution in the mammals is characterized by conservation of genes located in the X chromosome and because these genes code for comparable products in different species.

Over the past several years, we have conducted studies using this mutant mouse for exploring the hypothesis of an osteoblast dysfunction in VDRR. We have isolated osteoblasts from normal and mutant mice, grown them in culture and transplanted them into animals of both genotypes for evaluating their function in different environments. We have also recently assessed their response to $1,25(OH)_2D_3$ in vitro.

OSTEOBLAST CULTURES

Osteoblasts were isolated from newborn mouse calvaria by a non-enzymatic method using their ability to migrate from bone onto glass fragments (31). Our culture technique has been previously described (32,33). Briefly, glass fragments were laid upon the osteoblast layer on the endocranial surface of the calvaria which had been exposed upon removal of the periosteum. After 5–6 days in culture in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal calf serum and ascorbate (50 μg/ml), cell multilayers covering the glass were scraped and transferred into dishes for subsequent culture. The cultured cells produced several osteoblast-associated products such as type I collagen (95% of total collagen), high levels of alkaline phosphatase (32), and small proteoglycans similar to those extracted from bone (34). They expressed some characteristic hormone responses such as $1,25(OH)_2D_3$ stimulation of alkaline phosphatase and inhibition of collagen synthesis (43). These parameters however, do not establish conclusively the osteoblastic nature of the cells since they have not been identified as specific for osteoblasts. Since the principal and specific function of osteoblasts is to elaborate a mineralized matrix with the histologic characteristics of bone, demonstration of the osteogenic capacity of the cultured cells provides the best evidence of the osteoblastic phenotype of these cells. Indeed, we have shown that the cultured cells were able to produce in vitro a mineralized matrix with the ultrastructural and chemical properties of woven bone (32,35). Mineral deposits that stained positively using von Kossa’s method were observed within cell clusters of cultures supplemented with an exogenous source of phosphate: 10 mM β-glycerophosphate or 3 mM inorganic phosphate (corresponding to the serum levels of normal mice). To ensure that mineral deposition in culture represents “bone” formation rather than physicochemical precipitation of calcium phosphate or dystrophic calcification associated with cell debris, cultures were examined by electron microscopy (35). In areas of early calcification, the extracellular matrix showed mineral nodules and crystallites closely associated with collagen fibrils (Fig. 1). We have shown previously (35) that the underlying organic matrix of the mineralized nodules visualized after decalcification resembled structures pre-
FIG. 1. Electron micrograph of a 10-day-old osteoblast culture from normal mice grown in the presence of β-glycerophosphate. The extracellular matrix adjacent to an osteoblast (OB) shows mineralized nodules and crystallites clearly associated with collagen fibrils. Bar, 1 μm (×16,800).

Previously described in fetal and woven bone (36,37). Matrix vesicles were seen occasionally in the mineralized matrix of the cultures but were particularly abundant in the matrix of uncalcified cultures. Highly calcified areas of the cultures showed osteocyte-like cells completely embedded within the mineralized matrix but still separated from the mineral by an osteoid-like layer (Fig. 2). The mineral deposited in culture was found to be poorly crystalline hydroxyapatite by x-ray diffraction analysis (35). Thus, the mineralization pattern observed in culture resembled that seen in woven bone and demonstrates the osteoblastic nature of the cultured cells. No difference in the mineralization pattern in normal and Hyp osteoblast cultures was apparent at the light and electron microscopic levels.

IN VIVO TRANSPLANTATION OF OSTEOBLAST CULTURES

Primary osteoblast cultures from normal and Hyp mice were transplanted intramuscularly into 22–28-day-old normal and mutant mice to evaluate their respective behavior in different environments. Histologic examination of transplants maintained two weeks in mice revealed bone nodules surrounded by an osteoid layer covered with osteoblasts (Fig. 3). Bone formation in transplants was assessed by measuring the osteoid thickness (O. Th) at the periphery of the bone nodules and the osteoid volume (percentage of total bone tissue composed of unmineralized matrix, OV/BV) in undecalcified sections stained with von Kossa’s method and toluidine blue.

Impaired bone formation was evidenced in transplants of Hyp osteoblasts into
Hyp mice by excessive osteoid thickness and volume compared with transplants of normal osteoblasts into normal animals (Figs. 4 and 5). When normal cells were transplanted into mutant mice, a dramatic increase in osteoid thickness and volume was observed up to values not significantly different from those observed in transplants of Hyp osteoblasts. This observation stresses the critical role of the extracellular environment on bone formation. However, when Hyp osteoblasts were transplanted into normal mice, improvement but not normalization of the osteoid thickness and volume was seen. This impaired bone formation does not reflect a hypophosphatemia-induced defect of the osteoblast prior to transplantation since cells isolated from phosphate-deficient normal mice produce normal bone when transplanted into normal animals (38). The inability of Hyp osteoblasts to produce normal bone when transplanted into a normal environment is consistent with an intrinsic osteoblast defect in the Hyp mouse. In accordance with a genetic defect of the osteoblast, we have also demonstrated impaired bone formation by transplanted Hyp osteoblast precursor cells (periostea) into normal animals (39). Further support for an osteoblast dysfunction comes from recent studies showing that abnormal bone formation induced by implantation of an osteosarcoma-derived substance in Hyp mice (40) was not fully corrected when serum phosphate levels were normalized in mice fed a high phosphate diet (41).
RESPONSE OF HYP OSTEOBLASTS TO 1,25(OH)$_2$D$_3$

As discussed earlier, the requirement for supranormal amounts of 1,25(OH)$_2$D in combination with phosphate to induce healing of osteomalacia in VDRR patients and Hyp mice suggests an altered responsiveness of bone to 1,25(OH)$_2$D.

To explore this possibility, we have recently assessed the response of normal and
Hyp osteoblasts to a physiologic dose of $1,25(OH)_2D_3$ ($10^{-10}$M) at different medium phosphate concentrations with respect to alkaline phosphatase activity and cell proliferation (42). At phosphate concentrations corresponding to the serum levels in normal mice, $1,25(OH)_2D_3$ stimulated alkaline phosphatase activity and inhibited cell proliferation in normal osteoblast cultures but had no effect on Hyp osteoblasts which required a higher medium phosphate concentration for responding to the vitamin D metabolite. The cause for this unresponsiveness is not immediately apparent. We have recently shown that total $1,25(OH)_2D_3$ receptors in Hyp and normal osteoblasts cultured in Dulbecco’s Modified Eagle’s Medium were not different in terms of affinity and number and that receptor numbers were not affected by changes in medium phosphate concentration (43). However, it is possible that the abnormal response of Hyp osteoblast to $1,25(OH)_2D_3$ may reflect alterations in the nuclear uptake of $1,25(OH)_2D_3$, a defect which has been demonstrated in duodenal mucosal cells of the Hyp mouse (44). Alternatively, a phosphate transport defect, akin to the renal cell defect may account for the abnormal modulation of $1,25(OH)_2D_3$ action by phosphate in Hyp osteoblasts. Further studies will be necessary to determine the precise mechanism underlying the osteoblast resistance to $1,25(OH)_2D_3$.

**SUMMARY AND CONCLUSION**

Osteoblasts isolated from the Hyp mouse, the animal model for human VDRR have been useful for testing the hypothesis of a defective osteoblast function in this disorder and for evaluating their hormonal responsiveness. By transplanting isolated bone cells from normal and mutant mice into animals of both genotypes, we have evaluated their function and defined the role of the extracellular environment. Our data suggest that alterations in bone cell environment do not fully account for the bone lesions in the Hyp mouse but that an intrinsic defect of the osteoblast contributes to the etiology of the bone disease. This ectopic bone formation system will be valuable for examining the effect of different factors and hormones in the process of bone formation by normal and Hyp cells. In further studies, we intend to evaluate how treatment of Hyp recipient mice with phosphate alone or combined with $1,25(OH)_2D_3$ affects bone formation by transplanted normal and Hyp cells.
Since the isolated osteoblasts maintain their differentiated phenotype in culture, such cultures also provide an experimental system for elucidating the response of the cells to defined changes in levels of ions and hormones. Preliminary in vitro studies have indicated an abnormal response of the Hyp osteoblast to 1,25(OH)$_2$D$_3$ as demonstrated by the failure of 1,25(OH)$_2$D$_3$ to stimulate alkaline phosphatase and to inhibit cell proliferation at physiologic phosphate concentration. This unresponsiveness may account for the "resistance" of bone to 1,25(OH)$_2$D$_3$ observed in VDRR patients and Hyp mice and may be related to an intrinsic defect of osteoblast function.

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REFERENCES

OSTEOBLAST CULTURES


DISCUSSION

Dr. Glorieux: Let me first comment on the system just presented to you. It represents a tremendous amount of work, and is really the type of model that we have been looking for in order to understand better, at the cellular level, the questions unanswered at the clinical level. This model is one of the very first which, because of the methodology utilized, is able to maintain the phenotypic differentiation of the cultured cells as osteoblasts, and demonstrate bone formation in vitro. It no doubt has potential for studying the hypophosphatemic mutation because a model exists in the mouse. It certainly could be extended to other diseases when similar models will be available.

Dr. Holick: You have shown convincingly that it is not dystrophic calcification that we are seeing in the osteoblast-like cultures but truly physiologic mineralization with calcium hydroxyapatite crystal formation. Have you tried other cells in the same system and shown that there is dystrophic crystal formation or not?

Dr. Ecarot: Yes, we have shown that primary multilayer cultures of fibroblasts grown in the presence of 10 mM β-glycerophosphate failed to exhibit mineral deposition.

Dr. Holick: What about chondrocytes?

Dr. Ecarot: We have not used chondrocytes, but other investigators have reported mineralization in chondrocyte cultures supplemented or not with β-glycerophosphate.

Dr. Arnaud: What is the difference between injecting these cells in vivo versus looking at them in vitro?

Dr. Ecarot: The cells in vivo produce bone with its characteristic organization: the osteoblasts are sitting on the osteoid seam at the periphery of the calcified matrix. In vitro, the cells lose their polarity and become embedded in the matrix they secrete. Therefore, histomorphometric analysis of the bone nodules produced in vitro is not possible.

Dr. Pettifor: You have shown matrix vesicles in normal cell cultures; were those present in your Hyp cultures?

Dr. Ecarot: Yes, we see similar matrix vesicles in both normal and Hyp cultures.

Dr. Pettifor: The reason I asked this question is the following: Do you believe that the defect you observed is in the osteoblast itself or perhaps in the matrix vesicle or matrix vesicle number?

Dr. Ecarot: We don't know what osteoblast component is affected by the Hyp mutation and is responsible for the osteoblast dysfunction.

Dr. Marx: Your histologic data emphasize that the in vivo environment has a striking effect on normal cells. The effect of hypophosphatemia seemed to be much greater quantitatively than the effect of the abnormal osteoblast function per se. The osteoblast dysfunction could be interpreted as a manifestation of the HYP gene within the osteoblast, but there is still the alternative that this represents an expression of the in vivo plasma composition of the
X-linked hypophosphatemic mouse that has been maintained through the tissue culture system. Unfortunately, it must be difficult or impossible to maintain a passage of these cells outside the animal for several generations.

**Dr. Ecarot:** Cells in secondary culture lose their ability to produce bone in vivo and in vitro, unless they went through a very short primary culture. As you pointed out, the abnormal bone formation by transplanted Hyp cells could be a consequence of prior exposure to an hypophosphatemic environment. To test this possibility, we have transplanted cells from normal mice made hypophosphatemic into normal animals and we have found that normal bone was produced.

**Dr. Marx:** How do you make the gene assignment in those young mice?

**Dr. Ecarot:** Identification on the genotype of the newborns is based on serum phosphate concentration. In case of doubt, the animals are classified after histomorphometric analysis of caudal vertebrae. We are also preparing a colony of other mutant mice, the Gyro mice. The Gy mutation also affects renal phosphate transport and is located close to, but clearly distinct from the Hyp gene. This new model of hypophosphatemia should generate interesting insights into osteoblast metabolism.

**Dr. Senterre:** We know from the textbooks that 1,25(OH)\_\_D, in synergy with PTH, is involved in bone resorption. Today we have heard that PTH is only acting on osteoblasts and you have now shown that in normal osteoblasts, 1,25(OH)\_\_D stimulates alkaline phosphatase activity. I would therefore like to have your view about the activity of 1,25(OH)\_\_D on bone formation on one side, and how it is cast as a bone resorbing agent on the other side.

**Dr. Ecarot:** The role of 1,25(OH)\_\_D in bone formation is not clear. Underwood and DeLuca (12) have shown that, in vitamin D-deficient rats, adequate levels of calcium and phosphate were only required for normal bone development. On the other hand, Meunier and collaborators (14) have reported that in osteomalacic patients treated with 1,25(OH)\_\_D stimulation of bone mineralization occurred only at bone surfaces covered by osteoblasts. The question is still open whether 1,25(OH)\_\_D promotes bone mineralization directly through an osteoblast action or indirectly through an increase in serum levels of calcium and phosphorus following stimulation of intestinal absorption of these ions. Conflicting data has also been reported on the effect of 1,25(OH)\_\_D on bone matrix formation in vivo and in vitro. With respect to bone resorption, there is convincing evidence that stimulation of osteoclastic bone resorption by 1,25(OH)\_\_D (and PTH) is mediated by the osteoblasts. Specific receptors for these bone-resorbing hormones have been demonstrated in osteoblasts and not in osteoclasts. Presumably, the osteoblast in response to 1,25(OH)\_\_D produce a soluble factor which acts on the osteoclast to modulate its activity. A direct action of 1,25(OH)\_\_D on the osteoblast is supported by in vitro studies showing an effect of 1,25(OH)\_\_D on alkaline phosphatase activity, and on the synthesis of type I collagen, osteocalcin, and bone sialoprotein in osteoblast cultures.

**Dr. Arnaud:** In one experiment, we compared bone biopsies, obtained from osteomalacic subjects, 24 hours after either calcium infusion or administration of 25(OH)D. When calcium infusions were given, there was diffused mineralization of the osteoid, suggesting that calcium went to the matrix but did not appear specifically at the mineralization front. Whereas with 25(OH)D, the mineral appeared at the mineralization front, implying that 25(OH)D had a quite different effect than calcium infusions through a probable specific cellular effect.