1,25-Dihydroxyvitamin D₃ Increases Synthesis of the Vitamin K-dependent Bone Protein by Osteosarcoma Cells*

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Rat osteosarcoma cells respond to 1,25-dihydroxyvitamin D₃ with a 6-fold increase in intracellular and secreted levels of the vitamin K-dependent protein of bone (BGP). The rise in intracellular BGP levels is half-maximal at 6.6 h and precedes the rise in medium BGP levels by 6 h, a time course which is consistent with the postulated steroid hormone action of 1,25-dihydroxyvitamin D₃. This effect is achieved by physiological levels of 1,25-dihydroxyvitamin D₃ with half of the maximal response at a vitamin concentration of 0.04 ng/ml. The specificity of this effect for BGP is demonstrated by the absence of a 1,25-dihydroxyvitamin D₃ effect on total protein synthesis by these cells.

To our knowledge, BGP is the first example of a bone protein whose rate of synthesis is dramatically and specifically increased by physiological levels of 1,25-dihydroxyvitamin D₃. The possible functions of BGP in the biological actions of 1,25-dihydroxyvitamin D₃ on bone are discussed.

The active metabolite of vitamin D, 1,25-dihydroxyvitamin D₃, stimulates bone mineral mobilization (1) in a process which requires parathyroid hormone (2). This response is blocked by prior administration of actinomycin D (3) suggesting that protein synthesis is required for the action of 1,25(OH)₂D₃ on bone. The initial event in 1,25(OH)₂D₃-induced bone calcium mobilization is probably the association of 1,25(OH)₂D₃ with the cytosolic receptor of bone cells (4-7). The probable subsequent action of the receptor complex with 1,25(OH)₂D₃ in bone cells is best illustrated by the action of 1,25(OH)₂D₃ on intestinal cells. Here the cytoplasmic receptor plus 1,25(OH)₂D₃ moves to the nucleus and saturates chromatin receptor binding sites (8) which then alters transcriptional events (9-12) and leads to de novo synthesis of the vitamin D-dependent cytosolic calcium binding protein (13, 14). This evidence suggests that the 1,25(OH)₂D₃-induced bone calcium mobilization response is mediated by a protein or proteins whose synthesis rate is increased by the action of 1,25(OH)₂D₃ on bone cells. The putative proteins synthesized by bone cells in response to 1,25(OH)₂D₃ have yet to be identified, however, and the mechanism by which such proteins might mobilize bone calcium is not understood.

In the present study, we have examined the effect of 1,25(OH)₂D₃ on the synthesis of the vitamin K-dependent bone protein. This 49-residue protein of known structure (15-17) contains γ-carboxyglutamic acid and has been termed bone Gla protein or BGP. BGP is one of the most abundant noncollagenous proteins in the extracellular bone matrix (18, 19). It is synthesized in bone (20) and binds strongly to bone mineral in an association which is mediated by γ-carboxyglutamylate residues (21, 22). BGP is also found in plasma (23-25), and clinical studies demonstrate that plasma BGP levels are elevated in patients with metabolic bone diseases characterized by increased rates of bone turnover (23, 26). Although the function of BGP is presently unknown, there is some evidence to suggest that it may play a role in calcium or skeletal homeostasis (22, 26).

The clonal osteosarcoma cell line used for these studies (ROS 17/2) secretes BGP into culture medium (27) and has receptors for 1,25(OH)₂D₃ (28). It also displays in cell culture such features of the osteoblastic phenotype as high parathyroid hormone responsiveness and alkaline phosphatase activity (29) and forms a mineralized osteosarcoma when implanted into a rat (29).

EXPERIMENTAL PROCEDURES

Materials—Bone Gla protein was purified from the proteins released by demineralization of rat bone by gel filtration over Sephadex G-100 and subsequent gradient elution from DEAE-Sephadex A-25 as described previously (19). [4,5-³H]Leucine (110 Ci/mol) and [³¹P]phosphate (4 × 10⁶ cpm/mol) were purchased from Amersham. Coons F12, fetal calf serum, and antibiotic-antimycotic solution were purchased from Irvine Scientific. Rat osteosarcoma cells ROS 2/3, ROS 17/2, ROS 25/1, ROS 24/1, and ROS 24/1 subclone were generously provided by Dr. Gideon Rodan. Ultrapure guanidine HCl was purchased from Bethesda Research. 1,25-Dihydroxyvitamin D₃ was a generous gift from Hoffman-La Roche and vitamin D₃ was obtained from Sigma.

Radioimmunoassay—The procedures used for the radioimmunoassay of rat BGP in osteosarcoma cell culture media and in guanidine HCl extracts of osteosarcoma cells have been described (27).

Osteosarcoma Cell Culture—The procedures used to maintain osteosarcoma cell lines in culture have been described (27). For measurement of the effect of 1,25(OH)₂D₃ on BGP secretion, each cell type was grown to confluency in 60-mm culture plates (in 8 per 60-mm culture pellet and assayed for BGP.

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†The abbreviations used are: 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; Gla, γ-carboxyglutamic acid; bone Gla protein (BGP), γ-carboxyglutamic acid-containing protein of bone.

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above the control level of 15 ng of BGP per 10⁶ cells. Some increase in intracellular BGP levels is seen at 1 h, and half of the total increase is achieved by 6.6 h. 1,25(OH)₂D₃ also dramatically elevates the level of BGP in culture media (Fig. 1). The increase in media BGP level occurs about 6 h after the increase in intracellular levels.

Fig. 2 demonstrates that the kinetics of BGP appearance in media is identical for cultures pretreated with 1,25(OH)₂D₃ for 24 and 48 h. Thus, the response of rat osteosarcoma cells to 1,25(OH)₂D₃ is complete by 24 h.

The dependence of BGP secretion on the concentration of 1,25(OH)₂D₃ was determined after pretreatment with the vitamin at the test concentration to ensure that the response was maximal. Table I shows that the dose response is essentially the same in media sampled at various times after a 24- or 48-h pretreatment. The average increase in media BGP at each 1,25(OH)₂D₃ concentration is graphed in Fig. 3 to illustrate that the response is centered at physiological levels of 1,25(OH)₂D₃ in plasma.

The response of ROS 17/2 cells to vitamin D₃ was also evaluated using the protocol described in Table I. Cells treated with vitamin D₃ at concentrations from 0.1 to 100 ng/ml of culture medium did not increase BGP secretion above control after either a 24- or 48-h pretreatment with vitamin D₃. Higher vitamin D₃ levels did stimulate BGP secretion and 40% of the maximal 6-fold increase in BGP secretion was achieved at a vitamin D₃ concentration of 1 μg/ml. This vitamin D₃ concentration is over 10⁴-fold greater than the concentration of 1,25(OH)₂D₃ which gives the same level of BGP response.

The effect of 1,25(OH)₂D₃ on total protein synthesis by ROS 17/2 osteosarcoma cells was evaluated by comparing the levels of leucine label incorporated into protein at different 1,25(OH)₂D₃ concentrations. As can be seen in Table II, at no concentration of 1,25(OH)₂D₃ is there a significant increase in net protein synthesis. This result is compatible with a 6-fold stimulation in BGP synthesis since BGP synthesis accounts for only about 0.2% of total protein synthesis by osteosarcoma cells (27).

Several other clonal rat osteosarcoma cell lines were also tested for an effect of 1,25(OH)₂D₃ on BGP synthesis and secretion. The ROS 2/3 cell line, a low BGP producer (27), increased intracellular and media BGP levels about 8-fold after a 24-h pretreatment with 1 ng/ml of 1,25(OH)₂D₃. This result is incompatible with the previous report that the 2/3 cell line lacks receptors for 1,25(OH)₂D₃ (28).

Table I

Dependence of BGP secretion on the concentration of 1,25(OH)₂D₃

| Time media sampled | 24-h pretreated | 48-h pretreated |
|--------------------|----------------|----------------|
| A                  | BGP in media (1,25(OH)₂D₃-treated/control) |
|                   | 0.01" 0.1" 1.0" | 0.01" 0.1" 1.0" |
| 1                  | 1.79 5.92 7.46 | 2.00 5.91 7.55 |
| 2                  | 1.41 4.52 5.85 | 1.82 4.00 5.42 |
| 3                  | 1.64 3.51 4.68 | 2.13 4.00 4.85 |
| 4                  | 2.32 5.10 5.65 | 2.15 4.95 5.58 |
| 6                  | 2.50 4.40 4.76 | 2.50 4.40 4.76 |
| 8                  | 1.95 4.35 5.00 | 1.95 4.35 5.00 |
| Average            | 1.82 4.68 5.73 | 2.12 4.82 5.90 |

" Nanogram/milliliter concentrations of 1,25(OH)₂D₃.
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FIG. 3. Dose dependence of BGP secretion on the concentration of 1,25(OH)₂D₃. The average experimental to control ratios from Table I are plotted for 24-h pretreated (○) and 48-h pretreated (□) cultures.

Table II
Effect of 1,25(OH)₂D₃ on protein synthesis

| 1,25(OH)₂D₃ ng/ml | Intracellular cpm × 10⁴ (X ± SD) | Secreted cpm × 10⁻³ (X ± SD) |
|-------------------|---------------------------------|-------------------------------|
| Control           | 187.1 ± 37.1                    | 0.6 ± 3.8                     |
| 0.01              | 183 ± 20.6                      | 10.5 ± 0.9                    |
| 0.1               | 174 ± 32.1                      | 13.1 ± 2.2                    |
| 1.0               | 165.8 ± 6.8                     | 7.3 ± 2.8                     |

DISCUSSION

The present study demonstrates that physiological 1,25(OH)₂D₃ concentrations dramatically and specifically increase the rate of biosynthesis of bone Gla protein by osteosarcoma cells. The kinetics of this effect suggest that 1,25(OH)₂D₃ may act by activating the gene for BGP in the manner postulated for the 1,25(OH)₂D₃-dependent regulation of the cytosolic calcium binding protein of intestine. To our knowledge, BGP is the first bone protein whose synthesis rate increases in response to 1,25(OH)₂D₃. Given the novelty of this regulation, it seems justified to review what is known about the bone Gla protein and to discuss the roles it may play in mediating the action of 1,25(OH)₂D₃ on bone metabolism.

Although the bone Gla protein is a numerically abundant constituent of the extracellular bone matrix, the evidence to date indicates that it has no function in the formation of this matrix. Bones from warfarin-treated rabbits have less than 5% of the normal level of BGP yet are indistinguishable from bones of control animals in morphology, in mineral and protein content, and in strength (22). In addition, developmental studies show that BGP is virtually absent from the bones of neonatal rats (25) and so cannot play a role in initial bone formation. The present evidence that 1,25(OH)₂D₃ increases BGP synthesis supports the conclusion that BGP does not function in bone matrix formation since 1,25(OH)₂D₃ inhibits rather than stimulates the synthesis of matrix proteins (30).

Several lines of evidence support an informational function for BGP. The protein is only 49 residues long at the point of secretion from cells (27), a size compatible with hormones. It also has sequence features (15) such as two pairs of basic residues, which are proteolytic cleavage sites in the activation of hormones such as proinsulin (31), and a Pro-Lys unit, which is an effector site in many informational proteins (32). Finally, BGP is present in plasma as well as bone and has a uniquely high affinity for hydroxyapatite which could direct it to specific bone domains.

It seems to us most likely that the active informational species is plasma BGP and that its site of action is the exposed mineral surface of bone. Developmental studies indicate that plasma BGP may arise from new synthesis rather than from release of matrix BGP by resorption (25). In addition, calculations based on the affinity of BGP for hydroxyapatite show that the BGP found in bone could come from equilibrium binding of plasma BGP to bone hydroxyapatite (24). If BGP is synthesized and secreted into plasma in response to 1,25(OH)₂D₃, what kind of biological response is likely? One clue is provided by the results of clinical studies which demonstrate that BGP is elevated in plasma from patients with metabolic bone diseases characterized by increased rates of bone turnover, such as Paget's disease of bone and primary and secondary hyperparathyroidism. The elevated plasma BGP levels may target exposed mineral surfaces for the increased levels of osteoblastic and osteoclastic activity observed in those metabolic bone diseases. Experiments are in progress to test these and other possible roles for BGP as mediator of the action of vitamin D on bone.

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