Calcitonin Stimulates Bone Formation When Administered prior to Initiation of Osteogenesis

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ABSTRACT The influence of calcitonin (CT) on various stages of bone formation was investigated. A demineralized collagenous bone matrix-induced bone forming system in rats was used to temporally segregate chondrogenesis and osteogenesis. Administration of CT (15 Medical Research Council Units [MRCU]) daily) at the initiation of matrix-induced bone formation (BF) resulted in a 76% stimulation of BF as measured by ^{45}Ca incorporation and alkaline phosphatase activity. This increase was due, in part, to a stimulation of cartilage and bone precursor cell proliferation monitored by the rate of [^{3}H]thymidine incorporation and ornithine decarboxylase activity. Chondrogenesis on day 7 as measured by $^{35}SO_4$ incorporation was increased by 52% with CT treatment. To rule out the possibility of a secondary response due to parathyroid hormone, similar studies were done in parathyroidectomized animals and CT stimulation of BF was still observed. However, when CT injections were started after cartilage formation (day 8) there was no stimulation of BF but a significant decrease in ^{45}Ca incorporation was observed. These results indicate CT has two actions: (*a*) when CT is administered during the initial phases of bone formation, it increases BF due to a stimulation of proliferation of cartilage and bone precursor cells; and (*b*) when CT is administered after bone formation has been initiated, subsequent bone formation is suppressed.

INTRODUCTION

It has been well documented that calcitonin (CT)[1] can inhibit osteoclastic bone resorption (1, 2) and perhaps osteocytic osteolysis (3–5). However, the role of CT in osteoblastic bone formation is controversial. Some investigators have found that pharmacologic doses of CT are without effect on bone formation (6), while others have found it is stimulatory (7–9) or inhibitory (10). To determine the influence of CT on developing cartilage, bone and bone marrow, a demineralized collagenous bone matrix-induced endochondral bone-forming system was used (11, 12). This permitted the temporal segregation of the various stages of bone formation and allowed us to examine the influence of CT on each of the phases.

METHODS

Demineralized bone matrix prepared from rat diaphyses was implanted subcutaneously in the thoracic region into 21–23 d-old male rats, Long Evans strain (75–85 g). The day of implantation was designated as day 0. The present study investigated the influence of salmon calcitonin, 15 MRCU subcutaneous injections daily, (Calcimar, Armour Pharmaceuticals, Scottsdale, Ariz.) on four distinct phases of matrix-induced bone formation (11) in treated and untreated control rats: (*a*) proliferation of mesenchymal cells 3 d after implantation by [^{3}H]thymidine incorporation (13) and ornithine decarboxylase (ODC) activity (14, 15); (*b*) chondrogenesis on day 7, monitored by $^{35}SO_4$ incorporation into proteoglycans (11, 12); (*c*) osteogenesis on day 14 monitored by ^{45}Ca incorporation (11, 12); and (*d*) bone marrow formation was monitored by ^{59}Fe incorporation on day 21, (11, 12). The tissue activities of alkaline and acid phosphatases (11) were determined because of their close association with bone formation and resorption, respectively. Beta-glucuronidase was also measured in the developing tissues as a marker for lysosomal enzyme release (15). Two plaques (implants) from each rat were assayed in duplicate, and there were four rats in each experimental group and control group. Epiphyseal growth plates were also assayed in the CT-treated and untreated control rats. The data reported are an average of three separate experiments. The statistical significance of the experimental data was evaluated by means of the Student's *t* test.

The vehicle of calcimar was an isotonic aqueous solution

Received for publication 8 May 1981 and in revised form 26 June 1981.

[1] *Abbreviations used in this paper*: CT, calcitonin; ODC, ornithine decarboxylase; PTX, parathyroidectomized.

of sodium chloride, sodium acetate, and acetic acid, pH 7.4. Phenol, 0.5% was used as a preservative. The vehicle was without effect on any of the parameters measured.

In order to rule out the possibility that increased bone formation was due to a secondary response of parathyroid hormone to CT-induced hypocalcemia, similar studies were done in parathyroidectomized (PTX) animals. Animals were parathyroidectomized by cauterization and implanted with bone matrix particles 2 wk later. Half of the PTX animals were given the regular diet (1.0 g Ca/kg) and the other half were given a diet containing an additional 100 mg/kg Ca. Animals were given CT 2 h before killing and sampling of blood was done for Ca, P analyses.

RESULTS AND DISCUSSION

In the control animals there were two peaks of cell proliferation as determined by [^{3}H]thymidine incorporation and ODC activity (Fig. 1). One peak occurred on day 3, corresponding to the proliferation of chondroprogenitor cells and another peak on day 9, corresponding to the proliferation of osteoprogenitor cells. In those animals treated daily with CT there was a two- and fourfold increase of [^{3}H]thymidine and ODC activity, respectively, on day 3. Chondrogenesis as measured by the rate of $^{35}SO_4$ into proteoglycans was increased by 52% in those animals treated daily with injections of CT. There was a 55% increase in the rate of $^{35}SO_4$ incorporation in the epiphyseal growth plate. The rate of ^{45}Ca incorporation was markedly increased in the CT-treated animals (Fig. 2a). Tissue alkaline phosphatase (Fig. 2b) was also increased in activity in those animals injected with CT. A 76% increase in activity was observed on day 14. Acid phosphatase (Fig. 2c) and beta-glucuronidase (Fig. 2d) were both inhibited by CT treatment during the initial 11 d. These enzymes, which are found in osteoclasts, are markers of bone resorption. The findings of inhibited activity of acid phosphatase and beta-glucuronidase agrees with the acknowledged inhibitory effect of CT on osteoclastic resorption. The monitoring of bone marrow formation by the rate of incorporation of ^{59}Fe revealed there was no stimulation or inhibition of marrow formation with daily injections.

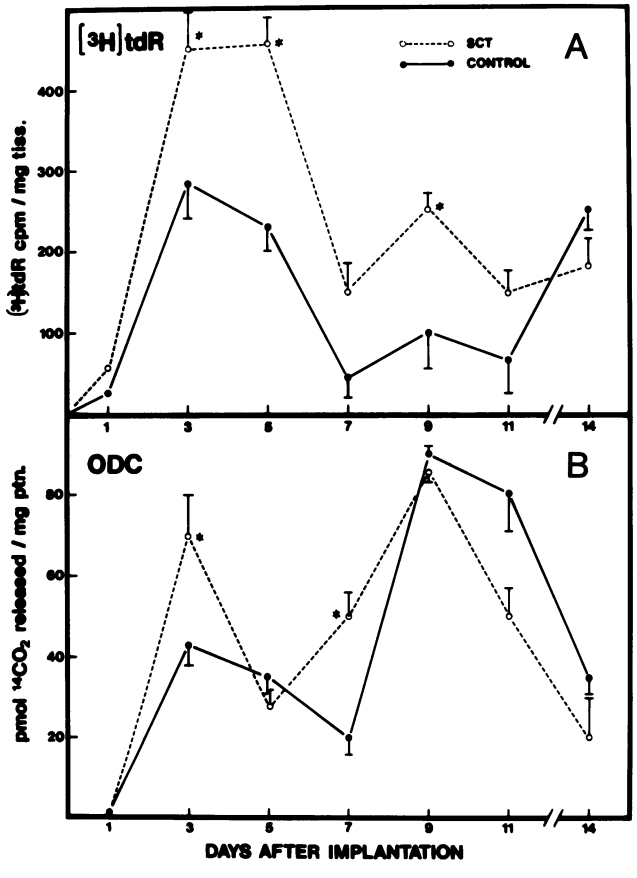

FIGURE 1 Influence of daily CT treatment (15 MRCU) on cell proliferation assessed by [^{3}H]thymidine (tdR) incorporation (A) and ODC (B). Abscissa: days after implantation. Upper ordinate: counts per minute [^{3}H]thymidine per milligram tissue. Lower ordinate: picomoles of $^{14}CO_2$ released per hour per milligram protein. *, $P < 0.01$, $n = 8$ animals. Tiss, tissue.

Analysis of osteogenesis in parathyroidectomized rats showed that CT stimulated bone formation to the same degree as in nonparathyroidectomized control rats (Table I). Therefore, one can rule out the possibility that observed stimulation of bone formation is due to increased parathyroid hormone secretion.

The stage of tissue development determined the nature of the tissue response to CT (Fig. 3). If CT was administered daily at the onset of bone induction, BF was stimulated as we have described above. However, when daily CT administration was not begun until day 8 following implantation, subsequently there was an inhibition (32%) of bone formation. This indicates that if CT is given after bone formation has been initiated the stimulatory effect was abolished. On the other hand if CT was given at the time of implantation and then stopped prior to bone differentiation (day 8), there was no observed stimulation or inhibition of bone formation (Fig. 3).

The observation that CT can stimulate bone formation when administered at the initiation of bone formation suggests that this hormone may play a role in the developmental processes of the skeleton. The suppression of bone formation when CT is given after the initiation of osteogenesis is similar to the observed clinical patterns of decreased bone formation when the hormone is administered to patients with various bone diseases. While there is much controversy in the literature concerning the utility of CT in enhancing the rate of fracture repair, our results with the bone matrix-induced bone forming model

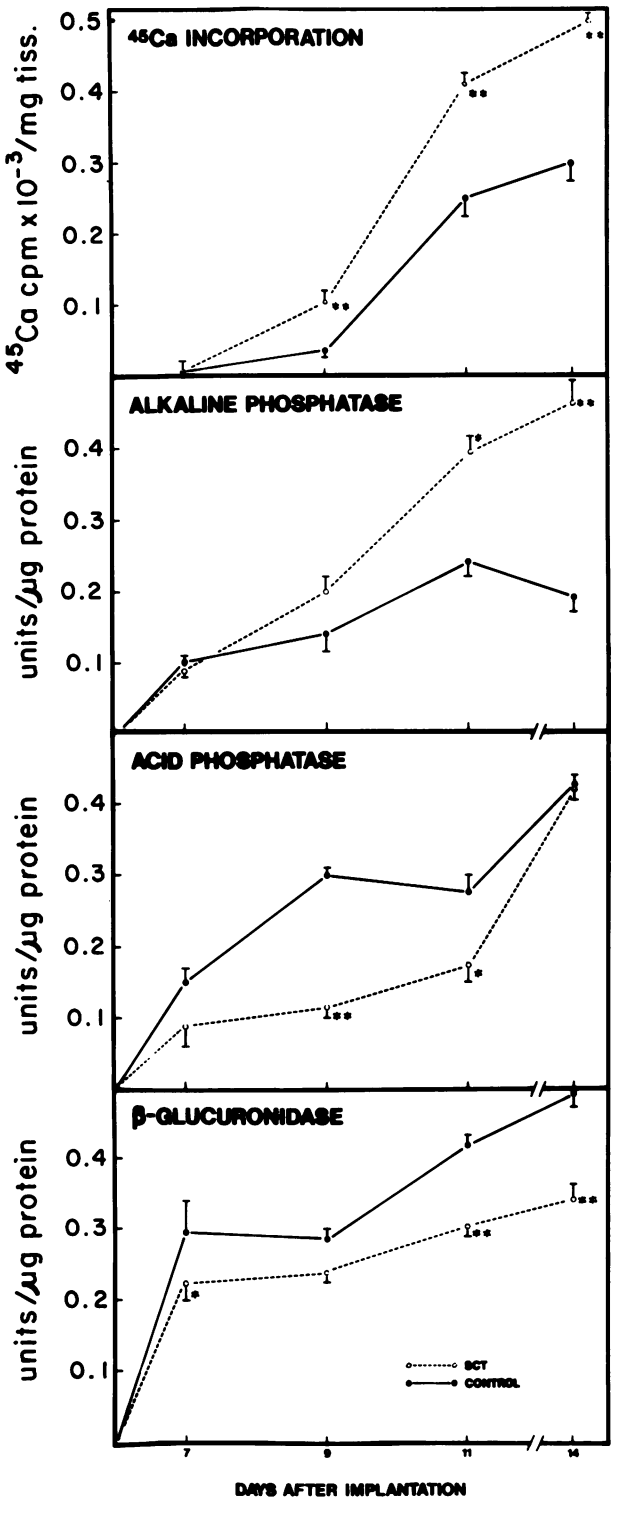

FIGURE 2 Influence of daily CT treatment (15 MRCU) on ^{45}Ca incorporation, alkaline and acid phosphatase activities, and beta-glucuronidase activity. Abscissa: days after implantation. *, $P < 0.05$; **, $P < 0.01$. $n = 8$ animals.

TABLE I

Influence of Daily CT Treatment (15 MRCU) and PTX on Serum Calcium and Phosphorus and ^{45}Ca Incorporation into Day 14 Plaques

	Ca	P	^{45}Ca
	mg/dl	*mg/dl*	*cpm/mg tissue*
Control	9.1±0.8	5.90±0.45	310±25
Control + CT*	6.9±0.2‡	5.33±0.80	515±32‡
PTX	7.2±0.2‡	8.72±0.23	280±19
PTX + Ca-enriched diet§	8.2±0.1	6.68±0.68	340±18
PTX + CT*	4.4±0.7‡	7.26±0.92	499±40‡
PTX + CT + Ca-enriched diet§	8.3±0.4	6.52±0.41	543±22‡

* Calcitonin given 2 h before blood sampling.
‡ Significant difference from the control group, $P \leq 0.01$.
§ A diet containing 100 mg/g of additional calcium by coating the rat chow with calcium lactate using corn oil as a wetting agent.
$n = 8$ rats/group.

(which is a reasonable model for fracture repair) would indicate that CT may prove to be beneficial, provided therapy is started immediately.

We have no data concerning the mechanisms of the

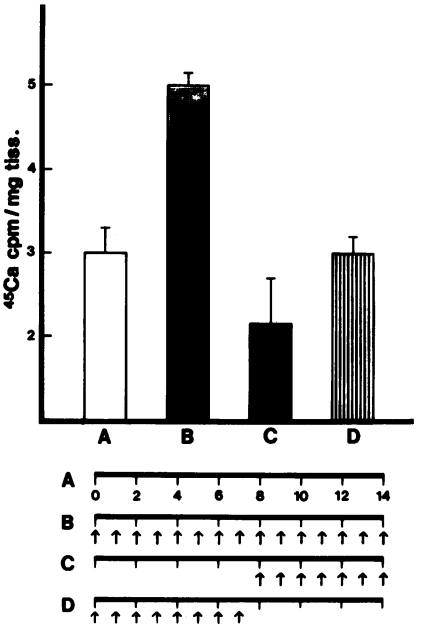

FIGURE 3 The influence of various CT treatment protocols on matrix induced bone formation 14 d after implantation. Ordinate: counts per minute ^{45}Ca/milligram tissue. Bar A: control, no CT injected; Bar B: daily injections of CT; Bar C: CT injected on days 8–14; and Bar D: CT injected on days 0–7. $n = 8$ animals/group.

paradoxical effects of CT on matrix-induced endochondral bone formation. There are several possible explanations for the stimulatory effects of CT on osteogenesis. Osteoblast precursor cells may have a large number of CT receptors or higher affinity receptors compared to differentiated osteoblasts. Alternatively, the receptors may be similar in both osteoblast precursors and differentiated osteoblasts, but it is possible that differences in postreceptor events may account for the paradoxical effects. In addition one cannot rule out the possibility that CT treatment may stimulate bone formation indirectly via stimulation of 1α,25-dihydroxyvitamin D (16). Finally, it is well known that bone homeostasis is maintained by a balance between resorption and formation and that these two processes are closely coupled (10). Therefore the apparent inhibitory effect of CT on differentiated osteoblasts may not arise as a direct effect of the hormone. CT is known to inhibit the activity of osteoclasts which may result in a concommitant inhibition of bone formation through the regulation of local factors (17).

ACKNOWLEDGMENTS

We thank Steven Dux, Chuck Williams, and Sharon Sikora for technical assistance.

This work was supported by a grant from the Marian and Pinkas Hapt Fund and grants from the National Institutes of Health (AM10358) and Orthopaedic Hospital (Kaiser fund).

REFERENCES

1. Holtrop, M. E., L. G. Raisz, and H. A. Simmons. 1974. The effects of parathyroid hormone, colchicine, and calcitonin on the ultrastructure and the activity of osteoclasts in organ culture. *J. Cell Biol.* **60:** 346–355.
2. Kallio, D. M., P. Garrant, and C. J. Minkin. 1972. Ultrastructural effects of calcitonin on osteoclasts in tissue culture. *J. Ultrastruct. Res.* **39:** 205–216.
3. Whalen, J. P., L. Krook, I. MacIntyre, and E. A. Nunez. 1975. Calcitonin, parathyroidectomy and modelling of bone in the growing rat. *J. Endocrinol.* **66:** 207–212.
4. Nunez, E. A., M. Horwith, L. Krook, and J. P. Whalen. 1979. An electron microscope investigation of human familial bone dysplasia. *Am. J. Pathol.* **94:** 1–18.
5. Matthews, J. L., R. V. Talmage, and R. Dopplelt. 1980. Response of the osteocyte lining cell complex the bone cell unit to calcitonin. *Metab. Bone Dis. Rel. Res.* **2:** 113–122.
6. Sing, M., and J. Jowsey. 1970. Failure of calcitonin to prevent disuse osteopenia: An experimental study in rabbits. *J. Endocrinol.* **87:** 183–186.
7. Ziegler, R., and G. Delling. 1972. Effect of calcitonin on the regeneration of a circumscribed bone defect (bored hole in the rat tibia). *Acta Endocrinol.* **69:** 497–506.
8. Foster, G. V., and F. H. Doyle. 1966. Effect of thyrocalcitonin on bone. *Lancet.* **II:** 1428–1431.
9. McWhinnie, D. J. 1975. In vivo effects of mammalian thyrocalcitonin on bone growth and alkaline phosphatase activity in the chick embryo. *Comp. Biochem. Physiol.* **50:** 169–175.
10. Rasmussen, H., and P. Bordier. 1974. The physiological and cellular basis of metabolic bone disease. Williams and Wilkins Co., Baltimore. pp. 364.
11. Reddi, A. H., and C. B. Huggins. 1972. Biochemical sequences in the transformation of normal fibroblasts in adolescent rats. *Proc. Natl. Acad. Sci. U. S. A.* **69:** 1601–1605.
12. Reddi, A. H., and W. A. Anderson. 1976. Collagenous bone matrix induced endochondral ossification and hemopoiesis. *J. Cell Biol.* **69:** 557–572.
13. Sigma Chemical Company. 1978. Technical bulletin 325. St. Louis, Mo.
14. Rath, N. C., and A. H. Reddi. 1978. Changes in ornithine decarboxylase activity during matrix induced cartilage, bone and bone marrow. *Biochem. Biophys. Res. Commun.* **82:** 106–113.
15. Jänne, J., and H. G. Williams-Ashman. 1976. On the purification of L-ornithine decarboxylase from rat prostate and effect of thiol compounds on the enzyme. *J. Biol. Chem.* **246:** 1725–1731.
16. Galante, L., K. W. Colston, S. J. MacAuley, and I. MacIntyre. 1972. Effects of calcitonin in vit. D metabolism. *Nature* (Lond.). **238:** 271–273.
17. Puzas, J. E., R. H. Drivahl, G. A. Howard, and D. J. Baylink. 1981. Endogenous inhibitors of bone cell proliferation. *Proc. Soc. Exp. Biol. Med.* **166:** 112–122.