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Effect of Gallium Nitrate In Vitro and in Normal Rats

L.G. Jenis, C.E. Waud, G.S. Stein, J.B. Lian, and D.T. Baran

Departments of Orthopedics (L.G.J., D.T.B.), Endocrinology (C.E.W., D.T.B.), and Cell Biology (G.S.S., J.B.L.), University of Massachusetts Medical Center, Worcester, Massachusetts 01655

Abstract Gallium nitrate (GN) is an inhibitor of bone resorption and thereby may result in a change in coupled bone formation. In the present investigation the effects of GN on bone formation were studied in the rat osteosarcoma (ROS) 17/2.8 cell line and normal diploid rat osteoblasts (ROB) in vitro and the femur of rats treated in vivo, measuring mRNA levels for two osteoblast parameters, type I collagen, a marker of matrix formation, and osteocalcin, a bone specific protein and also histone H_4, a marker of cell proliferation. GN, at 50 μM for 3 h, increased type I collagen mRNA levels by 132% in ROS 17/2.8 cells and by 122% in proliferating ROB cells. Osteocalcin (OC) mRNA levels were decreased by 61% in ROS 17/2.8 cells and by 97% in differentiated ROB cells. These changes occurred in the absence of any effects on cell proliferation. Seventy-day-old female rats were then treated with GN, 0.5 mg/kg/day, for 3 weeks. As previously reported, GN decreased serum calcium levels, but had no effect on lumbar or femoral bone density. In contrast to the in vitro effects, GN had no effect on type I collagen steady-state mRNA levels in the femur; however, it decreased OC steady-state mRNA levels in the femur by 58%. These results suggest that GN has similar in vitro effects in transformed and normal osteoblasts, while the collagen-stimulatory effects observed in vitro cannot be extrapolated to in vivo models. The consistent inhibition of osteocalcin in vitro and in vivo suggests a more specific target for GN that may relate to its effects in inhibiting bone resorption in normal rats.

Key words: gallium nitrate, bone mineral density, osteocalcin, collagen, tartrate-resistant acid phosphatase

Gallium nitrate (GN) is effective in the treatment of hypercalcemia of malignancy [Warrell et al., 1984, 1987] and high bone turnover states such as Paget's disease [Warrell, 1990]. Studies suggest that GN is incorporated into bone after short term administration and inhibits osteoclast function [Donnelly, 1991; Hall and Choimbeus, 1990]. In addition, in vitro studies suggest that GN has effects on osteoblasts [Bockman et al., 1987; Guidon and Bockman, 1990; Lakatos et al., 1991]. This combination of osteoclastic and osteoblastic activity suggests that GN may be a potential therapeutic agent in the treatment of low bone density states such as osteoporosis.

The purpose of this study was to investigate the effects of GN on osteoblast activity initially in vitro in normal rat osteoblast cultures which produce a bone-like mineralized matrix and in the osteoblast-like rat osteosarcoma cell line (ROS) 17/2.8 and to compare these in vitro activities to the effects of GN in vivo in normal female rats. We measured cellular levels of osteoblast related mRNAs, type I collagen, a gene expressed early for bone formation, and osteocalcin, a gene expressed later reflecting active matrix mineralization [Owen, 1990]. Additionally, in vivo, bone density measurements were carried out. The results demonstrate that GN increases collagen mRNA in normal and transformed rat osteoblasts in vitro but does not appear to exert similar effects in vivo. In contrast, GN decreased osteocalcin mRNA levels both in vitro and in vivo. Bone density after 3 weeks of therapy was not changed in the normal rats, suggesting that the collagen-stimulatory effects of GN in vitro cannot be extrapolated to in vivo models. The consistent inhibition of osteocalcin in vitro and in vivo suggests a specific target for GN that may relate to its effects in inhibiting bone resorption.

ANIMAL AND TISSUE CULTURE PREPARATION

Cell Cultures

Rat osteosarcoma cell (ROS 17/2.8) cultures were grown and maintained at the cell culture facility at the University of Massachusetts Med-

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Address reprint requests to Dr. Daniel T. Baran, Department of Orthopedics, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01655.

ical Center (Worcester, MA). Cells were grown in culture medium consisting of Dulbecco Minimum Essential Medium (DMEM) and F12 (50:50) and 5% fetal calf serum. Normal diploid rat osteoblasts (ROB) were isolated from fetal rat calvariae of 20 days gestation. Calvariae were subjected to sequential digestions of 20, 20, and 90 min at 37°C in 1 mg/ml collagenase P (Boehringer-Mannheim, Indianapolis, IN) /0.25% trypsin (Gibco, Grand Island, NY). The cells of the first two digests were discarded, and those of the third digestion were plated in MEM and 10% fetal calf serum as previously described [Owen, 1990].

ROS 17/2.8 and normal rat proliferating (day 3) and differentiated osteoblasts (day 28) (1.0×10^7 cells/ml) were treated at 37°C for 3 h in HEPES-balanced salt solution (HEPES-BSS) containing 0.15 g albumin as previously described [Baran et al., 1991] with either 1) $1\alpha,25(OH)_2$ D_3, 20 nM, 2) gallium nitrate, 50 μM, 3) a combination of $1\alpha,25(OH)_2D_3$ and gallium nitrate, or 4) alcohol (vehicle), 10 μl. At the conclusion of the treatment the cells were washed with HEPES-BSS without albumin, pelleted, and collected.

Total cellular RNA was isolated by the commercial RNAZOL method (Cinna/Biotex Labs, Friendswood, TX). The RNA was resuspended in diethylpyrocarbonate-treated water and quantitated by absorbance at 260 nm. The intactness of the RNA was determined by electrophoresis on a 1% agarose gel with ethidium bromide staining. 4 μg of each RNA sample was immobilized onto Zetaprobe membranes (Biorad) and cross-linked by exposure to ultraviolet light for 1 min.

Animals

Twenty female rats (70-day-old, 200 g, Charles River Lab, Wilmington, MA) were randomly divided into two groups. The animals were housed four to a cage and fed standard lab chow and had free access to water. Rats in group one were treated with (GN) (Fujisawa, Deerfield, IL), 0.5 mg/kg/day, subcutaneously for 21 days. Rats in group two received an equivalent dose of normal saline subcutaneously for 21 days. After 21 days of GN treatment, the animals were sacrificed and serum obtained for osteocalcin, TRAP, albumin, creatinine, phosphorus, and calcium. Each femur consisting of the diaphysis and proximal and distal metaphyses were immediately removed, stripped of soft tissue and periosteal attachments, and immediately frozen in liquid nitrogen and stored at −70°C until further analysis was performed. Total cellular RNA was isolated as previously described [Baran et al., 1991; Shalhoub et al., 1991] from each bone by a modification of the Chirgwin procedure [Chirgwin et al., 1979]. Briefly, each frozen bone was pulverized into a powder with a Bessman Tissue Pulverizer (Fisher) precooled in liquid nitrogen, placed in guanidium isothiocyanate, and RNA collected after centrifugation in a 5.7 M cesium chloride gradient (36k rev/min for 18 h at 20°C). After ethanol precipitation, the RNA was treated with DNAase and an ethidium bromide gel showed sharp 28S and 18S bands reflecting absence of DNA. RNA was quantitated by measuring the absorbance at 260 nm and the 260/280 nm ratio was measured to ensure the absence of protein contamination. RNA was handled similarly to that obtained from in vitro samples from this point on. Ten micrograms of each sample was immobilized onto Zetaprobe membranes (Biorad) using northern and slot blot analysis. Probes were labeled by the random primer method [Feinberg and Vogelstein, 1983] using $\alpha(^{32}P)$ dCTP and included overnight hybridizations of mRNA to osteocalcin (pOC 3.4) [Lian et al., 1989], type I collagen (α1R1) [Genovese et al., 1984], histone H4 (pPs2) [Grimes et al., 1987], osteopontin [Oldberg et al., 1986], alkaline phosphatase (pRAP54) [Noda et al., 1987], tartrate-resistant acid phosphatase (TRAP) [Kotcham et al., 1989], and ribosomal RNA (28s) (LS6) [Wilson et al., 1976]. The resultant autoradiographs were quantitated by scanning laser densitometry (LKB 2400 gelscan ×1) and mRNA values normalized to ribosomal RNA.

Bone mineral densities were measured of the left femur and L4–L5 vertebral body segments using dual energy X-ray absorptiometry (Hologic QDR 1000/W, Waltham, MA). The animals were anesthetized with 0.3 cc IP injection of Ketamine (70%) and Rompum (30%). Measurements were made at baseline and every week until the completion of the study. Bone mineral densities were measured on one rat five times over a 2 day period. The coefficient of variation for the spine and femur were 3.39% and 0.92%, respectively.

Serum creatinine, calcium, phosphorus, and albumin were measured using a Beckman CX-4. Osteocalcin was measured using a commercial radioimmunoassay kit for rat osteocalcin (Biomedical Technologies Inc., Stoughton, MA). Tar-

TABLE I. Cellular Representation of Type I Collagen mRNA Levels In Vitro After 3 H of Treatment*

Cell type	Treatment			
	CON	Gallium (50 μM)	D_3 (20 nM)	D^3 + gallium
ROS 17/2.8	1.14 ± 0.06	2.64 ± 0.60 $P < 0.002$	0.37 ± 0.12 $P < 0.007$	1 22 ± 0.25 P = NS
ROB (proliferating)	0 703 ± 0.08	1.56 ± 0.02 $P < 0.009$	0.60 ± 0.06 P = NS	1.13 ± 0.16 P = NS
ROB (differentiated)	—	0.749 ± 0.224 $P < 0.001$	0 047 ± 0.081 P = NS	0.393 ± 0.25 $P < 0.005$

*D_3, 1α,25-$(OH)_2D_3$, ROB, rat osteoblast, ROS, rat osteosarcoma Values represent the mean in densitometry units ± standard deviation of four observations in each group Statistical significance was determined by a two factor analysis of variance for each treatment compared to control

TABLE II. Cellular Representation of Osteocalcin mRNA Levels In Vitro After 3 H of Treatment*

Cell type	Treatment			
	CON	Gallium (50 μM)	D_3 (20 nM)	D^3 + gallium
ROS 17/2 8	0.87 ± 0.07	0.34 ± 0.04 $P < 0.001$	2.11 ± 0.38 $P < 0.001$	1.68 ± 0.54 P = NS
ROB (proliferating)	—	—	—	—
ROB (differentiated)	1 21 ± 0.37	0.04 ± 0.04 $P < 0.001$	2.02 ± 0.15 $P < 0.001$	0.00 $P < 0.002$

*D_3, 1α,25-$(OH)_2D_3$, ROB, rat osteoblast, ROS, rat osteosarcoma Values represent the mean in densitometry units ± standard deviation of four observations in each group Statistical significance was determined by a two factor analysis of variance for each treatment compared to control

TABLE III. Cellular Representation of Histone H4 mRNA Levels In Vitro After 3 H of Treatment*

Cell type	Treatment			
	CON	Gallium (50 μM)	D_3 (20 nM)	D^3 + gallium
ROS 17/2.8	0.77 ± 0.12	0.86 ± 0.33	0.79 ± 0.15	0.85 ± 0.10
ROB (proliferating)	0 78 ± 0.14	1.04 ± 0.29	0.80 ± 0.19	0.73 ± 0.26
ROB (differentiated)	0.32 ± 0.18	0.37 ± 0.12	0.37 ± 0.06	0.36 ± 0.14

*D_3, 1α,25-$(OH)_2D_3$, ROB, rat osteoblast, ROS, rat osteosarcoma Values represent the mean in densitometry units ± standard deviation of four observations in each group Statistical significance was determined by a two factor analysis of variance for each treatment compared to control

trate-resistant acid phosphatase was measured using an Acid Phosphatase Assay (Sigma Diagnostics, St. Louis, MO).

The data from the in vitro studies was subjected to a two-factor analysis of variance and represent the mean ± standard deviation of at least four trials, while the in vivo data was analyzed by the paired *t*-test.

RESULTS

In Vitro Effects of GN on Osteoblast Growth and Differentiation

The effects in each cell type of GN on basal and 1α,25$(OH)_2D_3$ -modulated synthesis of type I collagen, osteocalcin, and H_4 mRNA levels are shown in Tables I–III, respectively. In ROS 17/2.8 cells, GN significantly increased type I collagen and decreased osteocalcin mRNA levels compared to controls. Vitamin D is well known to modulate these transcription parameters [Spiess et al., 1986; Lian and Stein, 1992], inhibiting type I collagen and stimulating osteocalcin mRNA as shown in Tables I and II. GN blunted both the 1α,25$(OH)_2D_3$-induced rise in osteocalcin and decrease in type I collagen mRNA levels when the two agents were administered in combination (Tables I and II). No change in histone H_4 mRNA was observed indicating these effects

TABLE IV. Blood Chemistry Values of Gallium-Treated Nonovariectomized Rats*

	Gallium	Control	Probability of difference
Osteocalcin (ng/dl)	0 110 ± 0 022	0 105 ± 0 017	P = NS
TRAP (U/L)	1 12 ± 1 78	0 84 ± 0 39	P = NS
Calcium (ng/dl)	10 20 ± 0 10	10 7 ± 0 20	$P < 0$ 001
Phosphorus (ng/dl)	8 40 ± 0 50	8 0 ± 0 30	P = NS
Albumin (g/dl)	1 5 ± 0 10	1 50 ± 0 10	P = NS
Creatinine (g/dl)	0 50 ± 0 10	0 50 ± 0 10	P = NS

*Values represent the mean ± standard deviation of at least nine observations in each group Significance was determined by a paired t test

TABLE V. Bone Mineral Density of L_4 and L_5 in Normal Rats Treated With Gallium or Normal Saline*

	Pretreatment	Week 1	Week 2	Week 3
Gallium-treated rats	0 199 ± 0 008 n = 10	0 219 ± 0 020 n = 10	0 223 ± 0 011 n = 9	0 226 ± 0 011 n = 9
Normal saline-treated rats	0 196 ± 0 010 n = 10	0 210 ± 0 009 n = 10	0 220 ± 0 010 n = 10	0 226 ± 0 012 n = 10
Probability of difference	P = NS	P = NS	P = NS	P = NS

*Values represent mean (g/cm^2) ± standard deviation at each time point Statistical significance was determined by a paired t test

were not related to changes in cell proliferation (Table III)

To ensure that the effect seen was not limited to a transformed cell line (ROS 17/2 8), we treated normal and differentiated rat osteoblasts in a similar manner Proliferating ROB cells expressed high levels of type I collagen mRNA (Table I) which was stimulated two-fold by GN similar to ROS cells There was no detectable osteocalcin mRNA in proliferating osteoblasts (Table II) as would be predicted at this stage in the developmental sequence [Owen, 1990, Spiess et al , 1986], as well as barely detectable collagen mRNA in the mature differentiated ROB cells harvested from cultures with extensive mineralized matrix (Table III) GN resulted in a significant increase in type I collagen mRNA in differentiated cells In the differentiated cells, vitamin D resulted in detectable levels of collagen mRNA, consistent with previously reported biphasic effects of the hormone [Lian and Stein, 1992] GN blunted the $1\alpha,25(OH)_2D_3$-reduced mRNA levels of type I collagen in proliferating ROB similar to ROS 17/2 8 cells In the differentiated cells, collagen levels remained elevated from control in the presence of $1\alpha,25(OH)_2D_3$ and GN but were reduced 50% compared to GN-treated cells

In Vivo Effects of GN on Normal Female Rat Bone Molecular Parameters

After 3 weeks of GN, these 13-week-old rats showed a moderate, but significantly lowered, serum calcium without significant affects on serum creatinine, phosphorus, albumin, osteocalcin, or TRAP levels (Table IV) At the start of the study both groups of rats had similar weights and bone densities At the completion of the study the GN-treated rats were significantly heavier (292 ± 18 g (n = 10) vs 270 ± 25 g (n = 9), $P < 0$ 05) but GN had no effect on either final bone mineral density (BMD) or change in BMD from baseline GN affected neither lumbar nor femoral BMD (Tables V and VI, respectively) Despite its lack of effect on serum osteocalcin, measured after 3 weeks exposure to GN, it decreased bone osteocalcin steady-state mRNA levels in the femur (Table VII) similar to its effects on ROS and differentiated ROB cells in vitro (Table II) However, in contrast to its in vitro effects to increase type I collagen mRNA (Table I), GN had no significant effect on type I collagen mRNA levels in the femur (Table VII) Histone H_4 mRNA (which is coupled to DNA synthesis) was quantitated to reflect changes in

TABLE VI. Bone Mineral Density of Left Femur in Normal Rats Treated With Gallium or Normal Saline*

	Pretreatment	Week 1	Week 2	Week 3
Gallium-treated rats	0.241 ± 0.009	0.257 ± 0.018	0.272 ± 0.007	0.280 ± 0.009
	n = 10	n = 10	n = 9	n = 9
Normal saline-treated rats	0.236 ± 0.012	0.260 ± 0.010	0.274 ± 0.010	0.281 ± 0.010
	n = 10	n = 10	n = 10	n = 10
Probability of difference	P = NS	P = NS	P = NS	P = NS

*Values represent mean (g/cm^2) ± standard deviation at each time point. Statistical significance was determined by a paired t-test.

TABLE VII. Steady-State mRNA Levels In Vivo in Gallium Treated and Control Rats*

	Osteocalcin	Type I collagen	Histone H4
Gallium (n = 6)	0.62 ± 0.03	2.94 ± 1.10	0.900 ± 0.49
Normal saline (n = 5)	1.48 ± 0.83	3.87 ± 1.25	1.24 ± 0.85
Probability of difference	$P < 0.05$	P = NS	P = NS

*Values represent mean in densitometry units ± standard deviation at each time point. Statistical significance was determined by a paired t-test.

cell growth, but significant differences were not observed (Table VII).

DISCUSSION

The two markers used in this study to analyze the selective effects of GN were type I collagen, an early gene expressed in the rat osteoblast developmental sequence, which is correlated with bone formation rate [Turner and Spelsberg, 1991], and osteocalcin, a marker of the mature osteoblast in a mineralized matrix, which is correlated with deposition of hydroxyapatite in vitro and in vivo [Owen, 1990]. In addition, osteocalcin may play a role in osteoclastic bone resorption [Glowacki and Lian, 1987; Glowacki et al., 1991]. The consistent inhibition of osteocalcin found in this study suggests a possible mechanism by which GN inhibits bone resorption via a direct effect on osteoblasts. We have shown that GN increases type I collagen mRNA levels in transformed rat osteoblast-like osteosarcoma cells, while modifying the reduction which occurs secondary to $1\alpha,25(OH)_2D_3$ stimulation. GN was also shown to diminish osteocalcin mRNA levels at both basal and $1\alpha,25(OH)_2D_3$ induced rates of production. The effect of GN is not limited to transformed cell lines. We have also shown that with respect to osteocalcin and type I collagen mRNA, normal and transformed rat osteoblasts respond similarly to GN.

Studies have demonstrated the ability of GN to exhibit preferential uptake in metabolically active bone, in particular, metaphyseal areas [Bockman and Guidon, 1990; Bockman et al., 1986; Repo et al., 1988; Blumenthal et al., 1989]. GN resulted in increased calcium and phosphorous content and more perfect hydroxyapatite crystal formation [Bockman et al., 1986; Blumenthal et al., 1989], suggesting that GN may enhance bone mass. Our in vitro observations encouraged in vivo studies on the effects of GN on osteoblast activity in normal rats. After 21 days of treatment, the data indicate that in vivo, while GN did not increase bone density, it does reduce osteocalcin steady-state mRNA levels. This effect on osteocalcin in vivo was similar to our in vitro findings (above). However, GN in vivo did not have a stimulatory effect on type I collagen steady-state mRNA levels that we observed in vitro. An explanation for the differences we observed between in vitro and in vivo investigations of the effects of GN with respect to collagen synthesis may stem from the dosage administered. Given a starting weight of 237 ± 13 g in treated rats and that GN is absorbed and distributed throughout total body water, then the circulating concentration used during this study was approximately 1,670 μM. The half-life of GN after a single injection is 1.0–1.5 h [Todd and Fitton, 1991], therefore, circulating levels of GN would be 50 μM or less after five half-lives in 5–7 h. It is possible that a single daily dose of GN given subcutaneously may not achieve adequate steady-state levels of the drug to have an effect on bone cell activity. This is an unlikely explanation in that the dosage utilized was the therapeutic dosage which is administered in the treatment of Paget's disease [Bock-

man et al., 1989] and greater than the concentration used our in vitro investigations (50 μM). Therefore, one would expect to detect increased or stimulated bone formation parameters at the conclusion of the treatment if they were to occur. Indeed, since osteocalcin mRNA was decreased in vivo, at least this parameter of osteoblast activity is affected. The dosage used in the present study is 10% of the effective dose used to treat hypercalcemia of malignancy [Bockman et al., 1989] and it is unlikely that higher dosages would be well tolerated in normocalcemic humans or rats with the intent of increasing bone formation.

Preliminary reports have described the effects of GN in vitro and in vivo. GN, in concentrations varying from 0 to 200 μM for 48 h, caused a dose-dependent increase in procollagen mRNA levels in ROS 17/2.8 and normal osteoblast cell lines [Bockman et al., 1987; Guidon and Bockman, 1990]. Our results at 50 μM, demonstrating an increase after 3 hours of GN exposure, are consistent with these reports [Bockman et al., 1987; Guidon and Bockman, 1990] but further identify a possible role for GN in bone formation through its rapid increase in gene expression of collagen. Another study under different conditions does not show increases in collagen. In mouse calvarial organ cultures, preincubation for 24 h with 7.2 μM GN followed by PTH or $1\alpha,25(OH)_2D_3$ was demonstrated not only to be a powerful inhibitor of bone resorption, but also to decrease collagen synthesis [Lakatos et al., 1991]. There are several possible explanations for the differing effects seen in each study, including the present investigation, which include different concentrations of GN and different systems utilized, along with varying lengths of treatment. A biphasic effect on type I collagen synthesis by GN has been proposed by several authors [Lakatos et al., 1991].

It has been reported that GN causes mild hypocalcemia in two thirds of patients that receive it for hypercalcemia of malignancy [Warrell et al., 1984]. Recently, it has also been shown to significantly reduce serum calcium levels in rats [Wakley et al., 1992]. Our results agree with these findings. We found a significantly lower serum calcium level in the GN-treated rats than in the rats treated with normal saline. This difference could not be explained by a difference in the serum albumin levels in the saline-treated animals. Similarly, GN, 0.9 mg/kg/d, for 21 days has been shown to inhibit endosteal formation and apposition rate in rats [Wakley et al., 1992]. This histomorphometric study supports our findings of unaltered expression of type I collagen mRNA in the femur of rats treated with GN for 21 days. Thus, although GN appears to increase collagen production in vitro [Bockman et al., 1987; Guidon and Bockman, 1990] [Table I], at doses that could be tolerated by normocalcemic subjects, it has no effect on type I collagen mRNA levels in vivo (Table VII) and inhibits bone formation [Wakley et al., 1992].

In summary, we have shown that GN increases basal type I collagen mRNA levels, reduces basal osteocalcin mRNA levels, and modifies the effects of $1\alpha,25(OH)_2D_3$ in transformed and normal rat osteoblasts in vitro. We have also shown that GN reduces osteocalcin steady-state mRNA levels, while having no effect on type I collagen mRNA levels or bone mineral density in vivo. This lack of effect in vivo suggests that the activity of GN in vitro may not be extrapolated to in vivo treatments. Thus, the in vitro effects of GN and other agents must be analyzed cautiously with respect to in vivo investigation. However, the consistent inhibition of osteocalcin both in vivo and in vitro suggests a more specific target for GN that may relate to its effects in inhibiting bone resorption. Osteocalcin function has been implicated in mediating bone resorption by promoting recruitment and differentiation of osteoclast progenitors [Glowacki and Lian, 1987; Glowacki et al., 1989]. Thus, GN may have therapeutic potential specifically in a high bone turnover osteoporotic state similar to its usefulness in hypercalcemia of malignancy and Paget's disease [Warrell et al., 1984, 1987; Warrell, 1990]. Further in vivo investigations in different model systems relevant to osteoporosis will be necessary for clinical decisions to be made regarding the use of gallium nitrate.

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