

# Systemic Administration of Transforming Growth Factor- $\beta$ 2 Prevents the Impaired Bone Formation and Osteopenia Induced by Unloading in Rats

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## Abstract

We investigated the effect of recombinant human transforming growth factor  $\beta$ 2 (rhTGF- $\beta$ 2) administration on trabecular bone loss induced by unloading in rats. Hind limb suspension for 14 d inhibited bone formation and induced osteopenia as shown by decreased bone volume, calcium and protein contents in long bone metaphysis. Systemic infusion of rhTGF- $\beta$ 2 (2  $\mu$ g/kg per day) maintained normal bone formation rate, and prevented the decrease in bone volume, bone mineral content, trabecular thickness and number induced by unloading. In vitro analysis of tibial marrow stromal cells showed that rhTGF- $\beta$ 2 infusion in unloaded rats increased the proliferation of osteoblast precursor cells, but did not affect alkaline phosphatase activity or osteocalcin production. Northern blot analysis of RNA extracted from the femoral metaphysis showed that rhTGF- $\beta$ 2 infusion in unloaded rats increased steady-state levels of type I collagen mRNA but not alkaline phosphatase mRNA levels. rhTGF- $\beta$ 2 infusion at the dose used had no effect on metaphyseal bone volume and formation, osteoblast proliferation or collagen expression in control rats. The results show that systemic administration of rhTGF- $\beta$ 2 enhances osteoblast precursor cell proliferation and type I collagen expression by osteoblasts, and prevents the impaired bone formation and osteopenia induced by unloading. (*J. Clin. Invest.* 1995; 96:1245–1253.) Key words: osteoblasts • TGF- $\beta$  • osteopenia • bone formation • unloading

## Introduction

The maintenance and renewal of the skeletal tissue are controlled by mechanical forces exerted by loading and by systemic and local factors affecting bone cell recruitment and activity. Experimental and clinical observations indicate that skeletal unloading is associated with inhibition of bone formation (1, 2). The cellular and molecular mechanisms involved in the decreased bone formation induced by skeletal unloading are, however, poorly understood. We previously reported that skeletal unloading induced by removal of weight bearing in rats is associated with impaired proliferation of osteoblast precursor cells in the marrow stroma (3). Unloading was also found to

reduce the osteogenic capacity of osteoblast precursor cells in rat bone marrow (4). A decreased production of mRNA for bone matrix proteins was also found in disuse osteopenia in rats (5), suggesting that the decreased bone formation induced by removal of mechanical forces on the skeleton involves alteration of osteoblast progenitor cell recruitment and/or defective function of differentiated osteoblasts.

Local factors acting on the recruitment and function of cells of the osteoblast lineage are likely to play a central role in the induction of osteogenesis by loading. Experimental studies suggest that prostaglandins may be involved in the strain-induced osteogenesis in vitro and in vivo (6–8). Other local factors, however, may be implicated since prostaglandins stimulate the production of insulin-like growth factor-I (IGF-I) by osteoblasts (9). Recently, we have reported that IGF-I stimulates bone formation, increases trabecular bone mass and marrow-derived osteoblastic cell proliferation in non-weight-bearing rat bones. However, IGF-I treatment does not totally prevent the decreased bone formation and osteopenia in unloaded rats (10). We, therefore, postulated that other factors which promote osteoblastic cell growth and differentiation might prevent the inhibitory effect of unloading on bone formation.

Transforming growth factor- $\beta$  (TGF- $\beta$ s) are multifunctional polypeptides that affect many cell types (11, 12). TGF- $\beta$ s appear to play a major role in the regulation of bone formation. TGF- $\beta$ s are produced by osteoblasts (13), stored in large amount in the bone matrix (14), and are potent factors affecting the growth and differentiation of osteoblastic cells (15). However, the effects of TGF- $\beta$ s may differ depending on TGF- $\beta$  concentration, cell density, origin and maturational stage of osteoblastic cells (15). TGF- $\beta$ s have chemotactic (16) and mitogenic effects on normal rodent and human osteoblastic cells (13,17–20). In addition, TGF- $\beta$ s modulate gene expression and production of noncollagenous bone matrix proteins (21, 22), enhance steady state levels of collagen mRNA and collagen synthesis (18) and stimulate bone formation in vitro (23). In vivo, local injections of TGF- $\beta$ 1 or TGF- $\beta$ 2 in rodent calvaria (24–28) or femur periosteum (29) accelerate bone formation and induce closure of skull defects (30), indicating that TGF- $\beta$ s are potent stimulators of periosteal bone formation. However, few studies have tested the effects of TGF- $\beta$  on endosteal bone in vivo. Intermittent administration of TGF- $\beta$ 2 was found to stimulate endosteal bone formation in rats (31) and to inhibit bone resorption in ovariectomized rats (32). However, TGF- $\beta$ 2 has no effect on trabecular bone loss induced by ovariectomy (32), perhaps due to the short half life of TGF- $\beta$  in vivo (33). The effect of continuous administration of TGF- $\beta$  on endosteal bone in osteopenic animals has not been evaluated.

In the present study, we have evaluated whether systemic continuous administration of recombinant human TGF- $\beta$ 2 (rhTGF- $\beta$ 2) can prevent the inhibition of endosteal bone formation and osteopenia induced by non-weight-bearing in unloaded rats. We report here that rhTGF- $\beta$ 2 infusion in unloaded

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rats stimulates the proliferation of osteoblast precursor cells in the marrow stroma, increases type I collagen mRNA expression by metaphyseal osteoblasts, and prevents the impaired bone formation and osteopenia induced by unloading.

## Methods

**Animals and treatment.** 40 adult 4-wk-old Wistar male rats weighing 120 grams (Iffa-Credo, France) were randomly assigned to four groups in two separate experiments. In each experiment, two groups (controls) were not suspended and two groups were suspended by the tail as previously described (1, 3), after the study was approved by a local Review Board. Briefly, the base of the tail was attached via a clip to the top of a specially designed Plexiglass cage (CERMA-Biomeca, France) in order to have hindlimbs non weight bearing. This method of hindlimb elevation causes minimal transient stress, is well tolerated and allows normal physical activity by the animals which had free movement in the cage by using their forelimbs (3). In each experiment, one group of suspended rats, and one group of control rats were treated for 14 d with rhTGF- $\beta$ 2 (generously provided by Ciba-Geigy Ltd., Basel, Switzerland). The stock solution was made by solubilizing the compound in 10 mM HCl/10% ethanol with 1 mg/ml BSA. rhTGF- $\beta$ 2 was then diluted in saline with 1 mg/ml BSA, and administered by continuous infusion using osmotic minipumps (Alza Corp., Palo Alto, CA) at the dose of 2  $\mu$ g/kg body weight per day. The administration of rhTGF- $\beta$ 2 for 14 d did not induce noticeable side effects, except a thin layer of fibrotic tissue localized around the implanted minipumps in half of treated rats. The content of each pump was checked at death to ensure that all rhTGF- $\beta$ 2 was delivered to the animals. The rats were maintained on a 12 h light/12 h dark cycle and body weight was recorded every 2 d. The animals were fed a standard chow containing 1% calcium and 0.8% phosphorus (UAR, Epinay s/Orge, France). The rats were pair-fed by adjusting the food intake of the untreated control group to that of the suspended untreated rats. All animals were given two doses of calcein (10 mg/kg body weight) at 6 and 2 d before death, in order to label the sites of mineralization (3). After 14 d of treatment, animals were anesthetized, the tibias and femurs were removed, weighed, and bone lengths were measured. The right tibias were processed for histomorphometric analysis and the right femurs were used for analysis of bone mineral content. The left tibias and femurs were used for the isolation of marrow stromal cells and extraction of total RNA, respectively.

**Bone mineral content.** Bone mineral content in the proximal femur metaphysis was determined as described (34). After dehydration in ethanol, the femurs were cut in half, the dry metaphyseal bone was carefully collected by scraping, weighed and dissolved in 5% trichloroacetic acid (TCA) for 24 h. The total calcium (kit BioMerieux, France) and protein contents (35) in the TCA extract were then determined.

**Histomorphometric analysis.** The right tibia was fixed in 10% phosphate-buffered formaldehyde, dehydrated in ethanol and embedded in methylmethacrylate. Three to five longitudinal sections (5–7  $\mu$ m thick) were stained with Goldner trichrome, and 10–15- $\mu$ m-thick sections were unstained for visualization of calcein labels under fluorescent microscopy. Histomorphometric indices of bone formation and resorption were measured as previously described (3) using a semiautomatic image analyzer coupled to a digitizing table (VideoPlan, Kontron). The following indices (36) were measured in the proximal metaphyseal area of the tibia in a standardized zone (3.6 mm<sup>2</sup>) at distance (500  $\mu$ m) from the growth plate: the trabecular bone volume (percent bone tissue composed of calcified and uncalcified matrix), the trabecular thickness (average of all trabecular thickness,  $\mu$ m), the trabecular density (number of trabeculae per mm<sup>2</sup>), the osteoid surface (percent trabecular bone surface covered with uncalcified bone matrix), the osteoblast surface (percent trabecular surface covered with osteoblasts), the resorption surface (percent trabecular surface covered with eroded lacunae and osteoclasts), and the number of osteoclasts per mm of trabecular bone surface. The mineral apposition rate (mean distance between the double fluorescent labels divided by the interval labeling time, and the double-

labeled surface (percent of the bone surface with double fluorescent markers) were also measured. The bone formation rate at the tissue level was calculated by multiplying the mineral apposition rate by the double fluorescent-labeled surface (36). The mean cortical thickness was measured along 500  $\mu$ m of the two cortices in the tibial diaphysis at distance (4 mm) of the growthplate.

**Proliferation of marrow stroma-derived cell cultures.** To determine the effects of rhTGF- $\beta$ 2 on osteoblast precursor cells, marrow stromal cells were isolated from the left tibia of each treated or untreated rat, as previously described (37). The tibia was cleaned of soft tissues and aseptically dissected in sterile phosphate-buffered saline (PBS). After removal of the epiphyseal area, the tibias were cut transversally, the marrow was flushed out with PBS and collected. After centrifugation, the bone marrow cell pellets were suspended in DME supplemented with antibiotics (100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin). The cells were then dispersed by passage through a fine metal mesh, counted and plated in 25-cm<sup>2</sup> flasks at the same initial density. After 12 d of culture in DME with 10% FCS, confluent cells were detached with 0.05% trypsin, and counted. To determine the proliferation rate of marrow stromal cells, the cells were plated at 5,000 cells/cm<sup>2</sup> in multiwell chambers (LabTek, Nunc) and cultured in DME with 10% FCS. 1 d after plating, the medium was replaced and the cells were cultured for five days. At day 1, 3, and 5 of culture, the medium was removed, the cells were washed with PBS, fixed in 70% ethanol at 4°C and stained cytochemically for alkaline phosphatase activity (ALP) to identify osteoblast precursors in the marrow stroma (38). The total number of marrow stromal cells and ALP-positive (ALP+) cells was counted in the total surface (1 cm<sup>2</sup>) area of four wells per rat per time-point using a Zeiss II eyepiece integrator mounted on a microscope ocular (3).

**Alkaline phosphatase activity and osteocalcin production.** ALP activity and osteocalcin production, two parameters of osteoblast differentiation (39), were determined in confluent marrow stromal cells isolated from treated and untreated rats with rhTGF- $\beta$ 2. Bone marrow stromal cells were plated at 5,000 cells/cm<sup>2</sup> in six-well plates (Falcon), cultured until confluence in DME with 10% FCS, and then cultured for 2 d in serum-free medium. The medium was removed and frozen for osteocalcin determination. The cells were rinsed with cold PBS, scraped in distilled water, sonicated and ALP activity in the cell lysate was determined by a colorimetric method (BioMerieux, France) and corrected for cell protein content (BioRad). Osteocalcin levels released in the medium were measured by a specific radioimmunoassay using a specific rabbit rat osteocalcin antibody (40). The intra- and inter-variabilities of the assay are 6 and 7%, respectively (40).

**RNA extraction and Northern blot analysis.** The left femur was removed from each treated and untreated rat and the metaphysis was rapidly dissected under sterile conditions and washed in PBS to remove marrow cells. Total RNA was extracted using the guanidinium thiocyanate-phenol-chloroform method (41) with some modifications. Briefly, samples were homogenized in a denaturing solution containing 4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl, 0.1 M  $\beta$ -mercaptoethanol and 1 mM EDTA. RNA was extracted with water saturated phenol and purified by isopropanol precipitations. 20  $\mu$ g total RNA per bone was separated on 1% formaldehyde/agarose gels, blotted on GeneScreen membranes (New England Nuclear, Boston, MA) and hybridized under stringent conditions. Blots were probed with a 520-bp EcoRI restriction fragment encoding the rat ALP gene (42), a 850-bp XhoI restriction fragment of mouse type I collagen (43) and a 1975-bp SalI-EcoRI restriction fragment of mouse 18S (44) as a control for the amount of RNA loaded. Inserts were purified by electrophoresis and labeled with (<sup>32</sup>P)-dCTP (specific activity: 2–4. 10<sup>8</sup> cpm/mg) by random priming methods using the manufacturer procedures (Promega Corp., Madison, WI). After hybridization, filters were washed and the signal was detected by autoradiography at –80°C using x-ray film (Amersham Corp., Madison, WI) with intensifying screens. The filters were re-probed for 18 S signal to take in account the variations in RNA loading. The Northern blots were quantitated by densitometric analysis of autoradiographs using a scanning densitometer (Transidyne

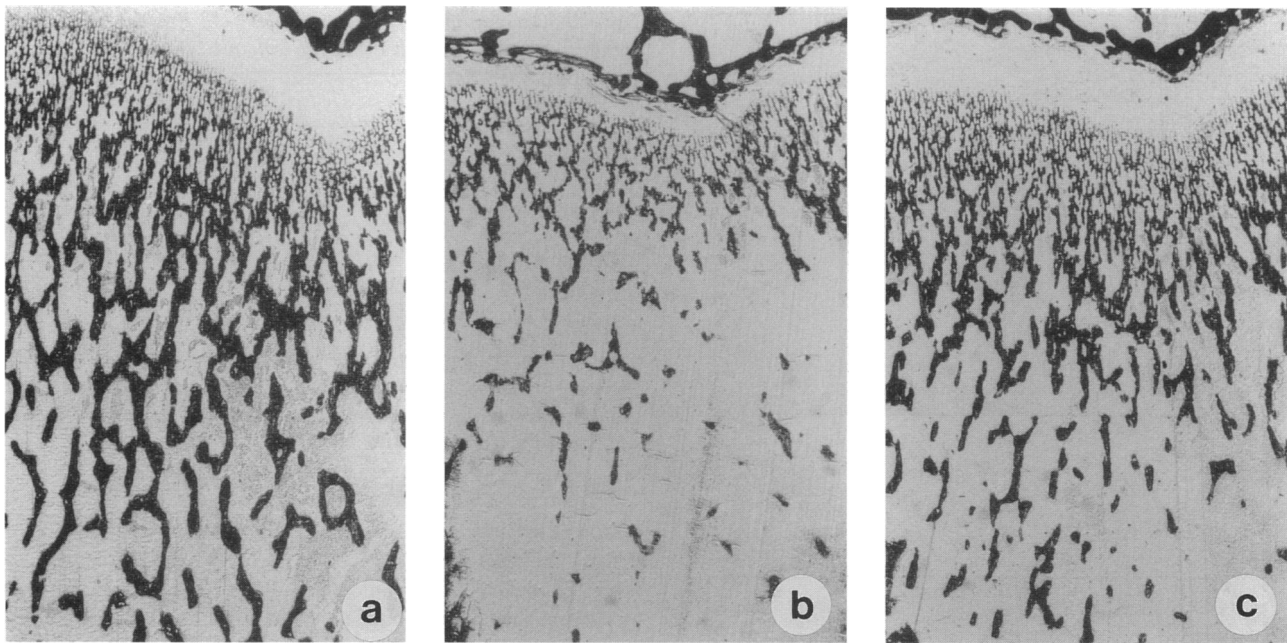


Figure 1. Photomicrographs of the tibial metaphysis in a control rat (a), unloaded rat (b), and unloaded rat treated with rhTGF- $\beta$ 2 (2  $\mu$ g/kg per day) for 14 d (c), showing that unloading induced metaphyseal bone loss (b) and that the bone volume and trabecular orientation were maintained after rhTGF- $\beta$ 2 infusion (c).

2500; General Corp., Ann Arbor, MI) and the integrated area under the curve for each signal was standardized against the signal for 18 S rRNA.

**Statistics.** All data are expressed as mean  $\pm$  SE. The results obtained from the two separate experiments were found to be not statistically different. The data were pooled and analyzed by two-factor analysis of variance (ANOVA) using the statistical package super-ANOVA (Macintosh, Abacus Concepts, Inc., Berkeley, CA). When significant interactions were indicated, differences between control and unloaded rats, and between untreated and TGF- $\beta$  treated rats, were evaluated by a multiple comparison procedure (Fisher's test) with a minimal significance of  $P < 0.05$ .

## Results

**Systemic rhTGF- $\beta$ 2 prevents osteopenia in unloaded rats.** Skeletal unloading for 14 d induced a marked decrease in metaphyseal bone volume in tibia of suspended rats compared to controls (Figs. 1 and 2 A). This metaphyseal osteopenia was associated with a marked reduction in the trabecular thickness and number in unloaded rats compared to controls (Fig. 2, B and C). Osteopenia induced by unloading was also demonstrated by low trabecular bone weight, and decreased bone calcium and protein contents in the femoral metaphysis compared to that of controls (Table I).

The infusion of rhTGF- $\beta$ 2 in unloaded rats prevented the decrease in metaphyseal bone volume induced by unloading (Figs. 1 and 2 A). The amount of bone matrix and orientation of trabeculae were similar in unloaded-treated rats and in controls (Fig. 1). Prevention of metaphyseal bone loss resulted from maintenance of normal trabecular thickness and number, the latter being increased above control values in unloaded rats treated with rhTGF- $\beta$ 2 (Fig. 2, B and C). rhTGF- $\beta$ 2 infusion completely prevented the decrease of metaphyseal bone weight, bone calcium content and bone protein content induced by unloading in femur (Table I). In contrast, systemic rhTGF- $\beta$ 2 administration in control rats had no effect on bone volume,

number, and thickness of trabeculae in the tibial metaphysis (Fig. 2), and had no effect on metaphyseal bone calcium and protein contents in femur (Table I).

The preventing effect of rhTGF- $\beta$ 2 on metaphyseal bone loss in unloaded rats was not associated with change in skeletal growth. The administration of rhTGF- $\beta$ 2 had no significant effect on body weight, tibia length and cortical thickness in unloaded rats (Table II), indicating that rhTGF- $\beta$ 2 administration at the dose used had no stimulatory effect on body or longitudinal bone growth. However, systemic administration of rhTGF- $\beta$ 2 induced a modest (6%,  $P < 0.05$ ) increase in the diaphyseal cortical thickness in control rats, suggesting a slight stimulation of periosteal bone formation in this group (Table II). These results indicate that the preventive effect of systemic administration of rhTGF- $\beta$ 2 on metaphyseal bone loss in unloaded rats did not result from change in bone growth.

**Systemic rhTGF- $\beta$ 2 prevents the impaired bone formation and reduces indices of bone resorption in unloaded rats.** Unloading induced a marked inhibition of bone formation in the tibial metaphysis. This was demonstrated by the decrease in the extent of trabecular bone surface with osteoblasts and the decrease in the extent of double calcein labeled surface compared to control rats (Fig. 3). The bone formation rate was 39% lower in unloaded than in control rats, and this resulted in a marked reduction of osteoid surface in unloaded rats compared to controls (Table III). The systemic administration of rhTGF- $\beta$ 2 in unloaded rats completely prevented the decrease in the amount of osteoid induced by unloading (Table III). This resulted from the enhancing effect of rhTGF- $\beta$ 2 on the osteoblast surface (+67%) and on the double-labeled surface (+89%) which remained normal, or were increased above control values (Fig. 3). However, rhTGF- $\beta$ 2 had no significant effect on the mineral apposition rate or osteoid thickness (Table III). Systemic administration of rhTGF- $\beta$ 2 in unloaded rats increased the bone formation rate by 92.5% compared with

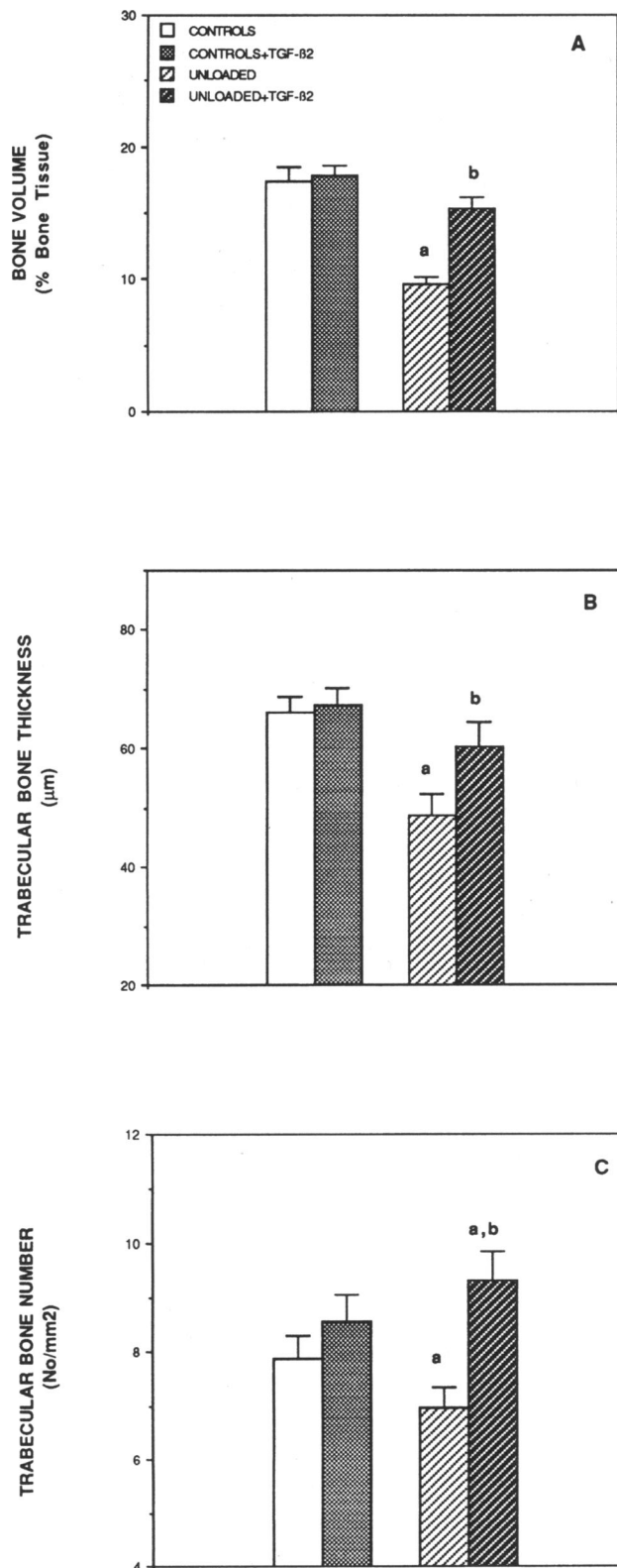


Figure 2. Systemic administration of rhTGF-β2 at the dose of 2 μg/kg per day prevents the bone loss induced by unloading in tibial metaphysis. Skeletal unloading for 14 d induced a marked decrease in bone volume (A), trabecular bone thickness (B), and trabecular bone number (C) in the proximal tibia. rhTGF-β2 administration maintained bone volume and trabecular bone thickness to values similar to controls, and increased the trabecular number above control values. Data are the mean±SE (n = 10 per group). a and b indicate a significant difference with controls and unloaded rats, respectively.

Table I. Systemic Administration of rhTGF-β2 Prevents the Metaphyseal Bone Loss in Femur of Unloaded Rats

Group	Metaphyseal bone weight	Metaphyseal bone calcium content	Metaphyseal bone protein content
	mg	mg	μg
Unloaded	7.58±0.56*	442.5±56.4*	1088±125*
Unloaded + rhTGF-β2	10.07±0.62†	662.0±94.8†	1647±146†
Controls	9.30±0.58	685.8±72.5	1499±128
Controls + rhTGF-β2	8.47±0.39	603.8±95.0	1332±104

Data are the mean±SE of values obtained from 10 rats per group. \* A significant difference with controls (P < 0.05). † A significant difference with unloaded rats (P < 0.05).

untreated unloaded rats, and this parameter was increased above control values (Fig. 3). In contrast to this marked anabolic effect of rhTGF-β2 in unloaded rats, rhTGF-β2 infusion in control rats had no effect on static and dynamic histomorphometric indices of bone formation in the tibial metaphysis (Table III, Fig. 3). These results indicate that rhTGF-β2 administration prevented the impaired trabecular bone formation induced by unloading, and that the anabolic effect of rhTGF-β2 was mediated mainly by an increased number of bone-forming sites.

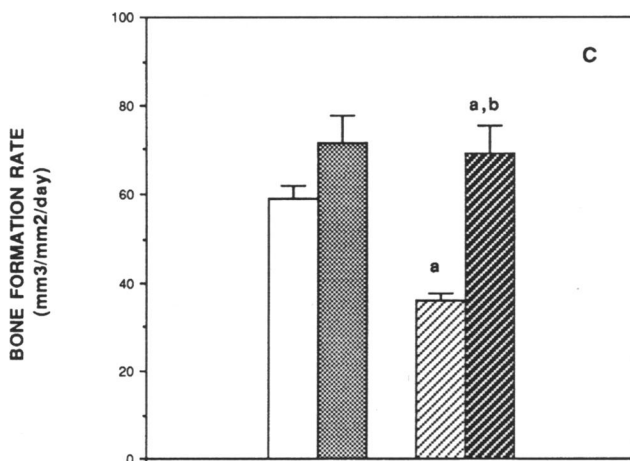
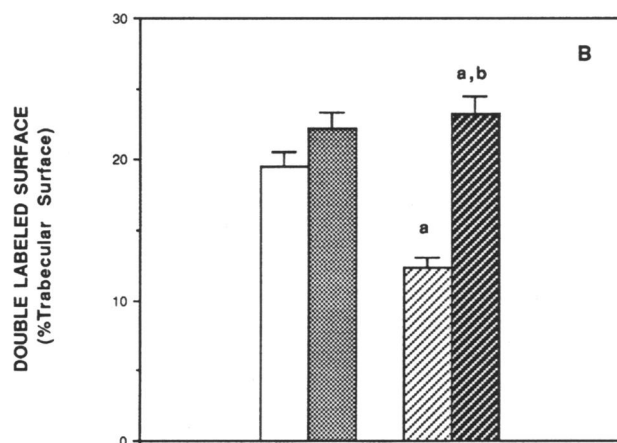
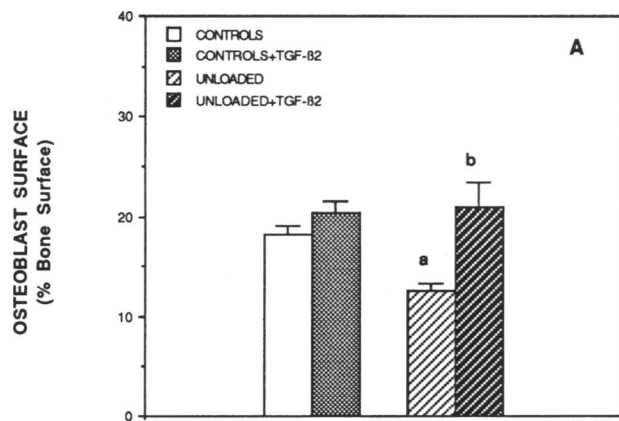
In addition to stimulation of bone formation, systemic administration of rhTGF-β2 decreased histomorphometric indices of bone resorption in the tibial metaphysis. rhTGF-β2 administration decreased both the resorption surface and osteoclast number in unloaded rats and in controls (Fig. 4). These data suggest that the metaphyseal bone resorption was decreased by systemic rhTGF-β2 administration, an effect that may have contributed partly to prevent the metaphyseal bone loss in unloaded rats.

rhTGF-β2 stimulates the proliferation of marrow stroma-derived osteoblast precursor cells. We then evaluated whether the stimulatory effect of rhTGF-β2 on bone formation resulted in part from changes in the proliferation of osteoblast precursor cells derived from the marrow stroma. A fraction of cultured marrow stroma cells showed ALP staining in vitro, a marker of marrow-derived osteoblast precursor cells (38). The effect of systemic administration of rhTGF-β2 on total and ALP+ stromal cells was determined in vitro during the period of active cell growth (3 d) and at pre-confluence (5 d). At one day of culture, the total number of marrow stroma-derived cells was similar in control and unloaded rats, treated or not with rhTGF-

Table II. Systemic rhTGF-β2 Administration Does not Affect Body Weight and Longitudinal Bone Growth in Unloaded Rats

	Final body weight	Tibial weight	Tibial length	Diaphyseal cortical thickness
	g	mg	mm	μm
Unloaded	177.5±4.8*	371.2±10.4*	33.3±0.2*	350.9±5.1*
Unloaded + rhTGF-β2	184.5±3.6*	395.3±8.7*	33.4±0.2*	354.1±5.1*
Controls	220.8±3.4	490.3±6.6	34.9±0.1	382.6±7.5
Controls + rhTGF-β2	220.3±3.9	475.9±9.0	34.4±0.2	405.2±9.4*

Data are the mean±SE of 10 rats. \* A significant difference with controls (P < 0.05).



**Figure 3.** Systemic administration of rhTGF- $\beta$ 2 prevents the inhibition of bone formation induced by unloading in the tibial metaphysis. Unloading induced a 31–39% decrease in the osteoblast surface (A), double calcein labeled surface (B) and bone formation rate (C). Systemic rhTGF- $\beta$ 2 administration maintained these parameters to values similar, or above control values. Data are the mean  $\pm$  SE. *a* and *b* indicate a significant difference with controls and unloaded rats, respectively.

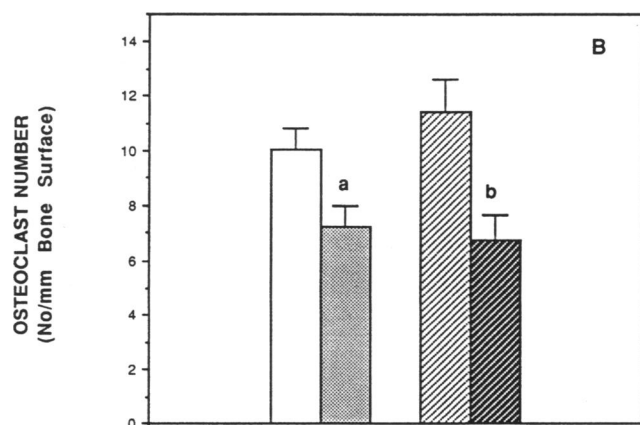
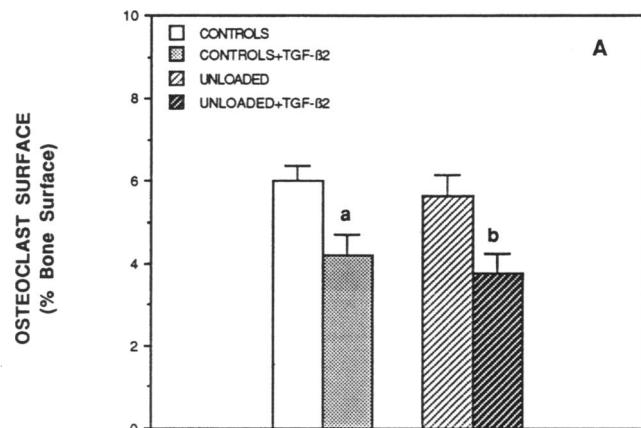
$\beta$ 2, reflecting similar plating efficiency of the isolated cells in all groups (Fig. 5 A). We found that rhTGF- $\beta$ 2 infusion in unloaded rats increased the total number of marrow derived-

**Table III.** Effect of rhTGF- $\beta$ 2 on Histomorphometric Indices on Bone Formation in Unloaded and Control Rats

Group	Osteoid surface (Percent Bone Surface)	Osteoid thickness	Mineral apposition rate
		$\mu\text{m}$	$\mu\text{m/d}$
Unloaded	9.6 $\pm$ 0.7*	4.44 $\pm$ 0.50	3.05 $\pm$ 0.17
Unloaded + rhTGF- $\beta$ 2	13.2 $\pm$ 1.1 <sup>†</sup>	5.20 $\pm$ 0.57	2.89 $\pm$ 0.19
Controls	14.0 $\pm$ 0.7	5.30 $\pm$ 0.47	3.12 $\pm$ 0.07
Controls + rhTGF- $\beta$ 2	14.8 $\pm$ 0.6	5.30 $\pm$ 0.60	3.11 $\pm$ 0.14

Data are the mean  $\pm$  SE of values obtained from 10 rats per group. \* A significant difference with controls ( $P < 0.05$ ). <sup>†</sup> A significant difference with unloaded rats ( $P < 0.05$ ).

stromal cells at 5 days of culture (Fig. 5 A) and induced a 2.5-fold increase in the number of ALP+ marrow stromal cells compared with untreated rats at 1, 3, and 5 d of culture (Fig. 5 B). At each time-point, ALP+ marrow stromal cell number in rhTGF- $\beta$ 2-treated unloaded rats was increased above values in control rats, indicating that systemic rhTGF- $\beta$ 2 administration increased the proliferation of osteoblast precursor cells in the marrow stroma in unloaded rats. In contrast, rhTGF- $\beta$ 2



**Figure 4.** Systemic administration of rhTGF- $\beta$ 2 decreases the histomorphometric indices of bone resorption in the tibial metaphysis of unloaded and control rats. Both osteoclast surface (A) and number (B) were decreased by rhTGF- $\beta$ 2. Data are the mean  $\pm$  SE. *a* and *b* indicate a significant difference with controls and unloaded rats, respectively.

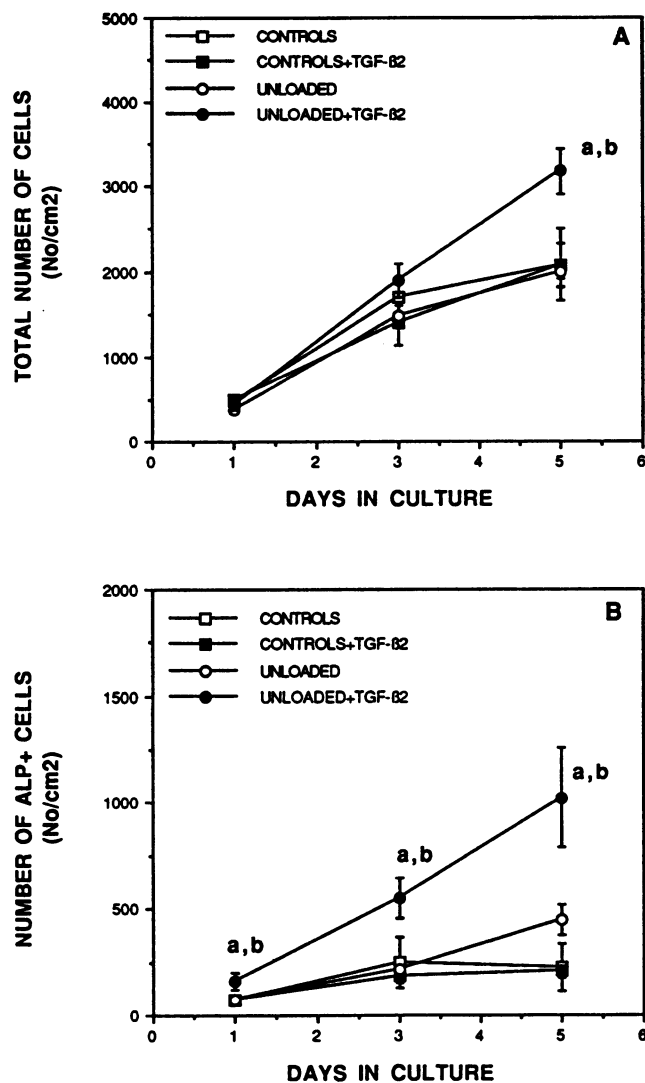


Figure 5. Systemic administration of rhTGF- $\beta$ 2 increases the proliferation of marrow stroma-derived osteoblast precursor cells in tibias of unloaded rats. Treatment with rhTGF- $\beta$ 2 in unloaded rats increased the total number of marrow stromal cells cultured for 5 d (A) and increased the number of phosphatase alkaline positive (ALP+) stromal cells at 1, 3, and 5 d of culture (B). Data are the mean  $\pm$  SE of separate cultures obtained from 3–5 individual rats. Each culture was performed in triplicate. *a* and *b* indicate a significant difference with controls and unloaded rats, respectively.

infusion in control rats did not affect the number of marrow stromal cells or the number of ALP+ cells (Fig. 5 B), indicating that rhTGF- $\beta$ 2 at the dose used had no effect on osteoblast precursor cell proliferation in control rats.

We then evaluated whether rhTGF- $\beta$ 2 administration affected alkaline phosphatase activity and osteocalcin production in marrow stromal cells derived from unloaded and control rats. rhTGF- $\beta$ 2 administration induced a non significant ( $P = 0.26$ ) decrease in ALP activity in control rats. The mean alkaline phosphatase activity and osteocalcin production by bone marrow-derived cells in vitro were not significantly different in unloaded rats and rhTGF- $\beta$ 2-treated unloaded rats (Table IV), indicating that the increased proliferation of ALP+ cells induced by rhTGF- $\beta$ 2 in unloaded rats was not associated with changes in ALP activity or osteocalcin production per cell.

Table IV. Lack of Effect of Systemic Administration of rhTGF- $\beta$ 2 on ALP Activity and osteocalcin Production by Bone Marrow Stromal Cells Isolated from Control and Unloaded Rats

	ALP activity	Osteocalcin production
	nmol PNP/mg protein/min	ng/mg protein
Controls	21.2 $\pm$ 6.7	115.5 $\pm$ 15.1
Controls + rhTGF- $\beta$ 2	11.9 $\pm$ 1.7	164.9 $\pm$ 20.8
Unloaded	24.9 $\pm$ 4.4	151.6 $\pm$ 16.9
Unloaded + rhTGF- $\beta$ 2	20.2 $\pm$ 3.0	132.7 $\pm$ 15.7

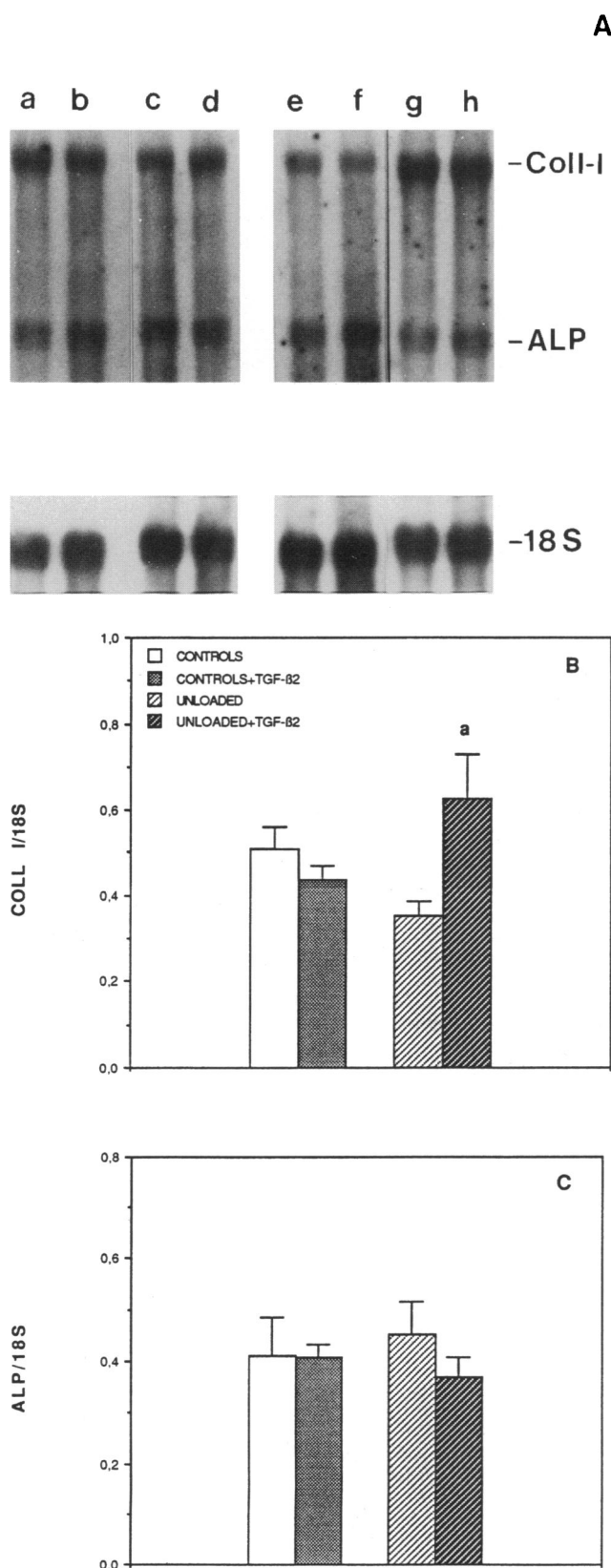
Data are given as mean  $\pm$  SE of five values. Each value is the average of triplicate cultures. ALP activity and osteocalcin production were determined in confluent cells derived from the marrow stroma in control rats and in rats treated with rhTGF- $\beta$ 2 for 14 d.

rhTGF- $\beta$ 2 increases collagen type I mRNA levels in metaphyseal bone. We then determined whether systemic administration of rhTGF- $\beta$ 2 affected the osteoblast function in the femoral metaphysis. To do that, RNA were isolated from femur metaphysis in each rat, and changes in type I collagen expression, a marker of differentiated osteoblasts, and alkaline phosphatase activity, an early marker of the osteoblast phenotype (39) were determined (Fig. 6 A). The steady-state levels of type I collagen mRNA corrected for RNA loading were 30% lower in unloaded rats compared to controls. rhTGF- $\beta$ 2 infusion induced a twofold increase in the mean levels of steady-state type I collagen mRNA levels (Fig. 6, A and B). In contrast, rhTGF- $\beta$ 2 administration did not change ALP mRNA levels in unloaded rats (Fig. 6, A and C). In addition, rhTGF- $\beta$ 2 infusion in control rats had no effect on type I collagen or ALP mRNA levels (Fig. 6). These results indicate that the systemic administration of rhTGF- $\beta$ 2 increased the expression of type I collagen by osteoblasts in the femoral metaphysis of unloaded rats.

## Discussion

To our knowledge, this is the first report showing that the endosteal bone loss in an experimental model of osteopenia can be prevented by the systemic administration of a growth factor which stimulates trabecular bone formation, increases osteoblast recruitment and enhances collagen expression by osteoblasts in vivo. Since the in vivo half-life of TGF- $\beta$  is very short (33), we used a continuous administration of rhTGF- $\beta$ 2 to ensure constant delivery of the compound. The dose of rhTGF- $\beta$ 2 administered in the present study is lower than the amount of TGF- $\beta$  reported to stimulate endosteal bone formation by multiple local injections in rats (31, 32). It is noteworthy that, at the low dose used, rhTGF- $\beta$ 2 infusion was effective in maintaining normal trabecular bone formation and bone mass in unloaded rats without affecting longitudinal bone growth.

The systemic administration of rhTGF- $\beta$ 2 in unloaded rats led to prevent the trabecular bone loss induced by unloading. This was demonstrated by the metaphyseal bone weight, bone mineral and protein contents in tibia and femur which remained similar to normal values. The prevention of unloading-induced osteopenia by rhTGF- $\beta$ 2 was related to maintenance of normal number and thickness of trabeculae, an effect resulting from the marked stimulatory effect of rhTGF- $\beta$ 2 on metaphyseal bone formation. Indeed, rhTGF- $\beta$ 2 infusion in unloaded rats increased the number of bone-forming cells, as shown by the



**Figure 6.** Systemic administration of rhTGF- $\beta$ 2 increases the expression of type I collagen in the femoral metaphysis of unloaded rats. A shows a representative Northern blot analysis of total RNA isolated from femoral metaphysis in two control rats (lanes a and b), two control rats treated with 2  $\mu$ g/kg per day of rhTGF- $\beta$ 2 for 14 d (lanes c and d), two unloaded rats (lanes e and f), and two unloaded rats treated with TGF- $\beta$ 2 (lanes g and h). The femoral metaphysis of each rat was

dissected, RNA was extracted and 20  $\mu$ g of total RNA was electrophoresed, transferred to nylon membranes and probed with the  $\alpha$ 1-chain of type I collagen (Coll I) and alkaline phosphatase (ALP) and the filters were reprobbed for 18 S rRNA for calibration, as described in Methods. The mRNA levels were measured by densitometric analysis of the bands and were normalized to 18 S rRNA. rhTGF- $\beta$ 2 infusion in unloaded rats increased type I collagen mRNA (B) but not ALP mRNA levels (C). The data represent the mean  $\pm$  SE of densitometric determinations of separate preparations of RNA obtained from 3–5 individual rats. a indicates a significant difference with control rats.

increase in osteoblast surface and double-labeled surface, which resulted in an increased amount of osteoid along the trabeculae. The lack of change in the appositional rate suggests that rhTGF- $\beta$ 2 acted mainly by increasing the number of bone forming sites, in accordance with the effect of intermittent injections of rhTGF- $\beta$ 2 in adult normal rats (31). Interestingly, the systemic administration of rhTGF- $\beta$ 2 in unloaded rats was more effective than IGF-I infusion since this factor does not completely correct bone formation in unloaded rats (10). These in vivo results are in accordance with in vitro data showing that TGF- $\beta$  is more potent than IGF-I in stimulating bone formation (45). These growth factors may however have complementary effects to initiate, promote and maintain the process of bone formation and, as such, may be involved with other agents acting locally (6–8, 46) as local mediators of loading on bone formation.

Although rhTGF- $\beta$ 2 prevented the impaired bone formation in unloaded rats, the continuous administration of rhTGF- $\beta$ 2 had no effect on metaphyseal bone formation in control rats, most likely because we used a low dose of rhTGF- $\beta$ 2. Endosteal (31) and periosteal (24–28) bone formation in normal rats are increased by subcutaneous injections of TGF- $\beta$ 1 or TGF- $\beta$ 2 at higher doses than those used in the present study. We found, however, that infusion of rhTGF- $\beta$ 2 slightly increased the cortical thickness in control rats, in accordance with the reported effect of local injections of TGF- $\beta$  on periosteal bone formation (24–28). Higher doses of rhTGF- $\beta$ 2 may however be required to increase the cortical thickness in unloaded rats. In contrast to its effects on bone formation, we found that rhTGF- $\beta$ 2 infusion decreased the indices of bone resorption in both normal and unloaded rats. This is in accordance with in vitro studies showing that TGF- $\beta$  inhibits the formation of multinucleated osteoclast-like cells in marrow cultures (47, 48). Although TGF- $\beta$  was reported to enhance the activity of osteoclasts in vitro (48), it inhibits bone resorption in organ culture (49) and in ovariectomized rats (32). The inhibitory effect of rhTGF- $\beta$ 2 on bone resorption in unloaded rats may have contributed partly to maintain a normal bone mass in unloaded rats.

The cellular mechanisms whereby rhTGF- $\beta$ 2 stimulated bone formation in unloaded rats may involve an increase in osteoblast number and/or function. In experimental models of osteopenia including unloading (3, 37), the abnormal bone formation is associated with alterations in the proliferation of osteoblast precursor cells in the marrow stroma. We thus investigated the effects of rhTGF- $\beta$ 2 on parameters of proliferation and differentiation of osteoblastic cells derived from the marrow stroma. We found that systemic administration of rhTGF- $\beta$ 2 in unloaded rats markedly increased the number of ALP+ cells, indicating that rhTGF- $\beta$ 2 increased the growth of osteoblast precursors in the marrow stroma. This stimulatory effect of rhTGF- $\beta$ 2 on the proliferation of osteoblast precursor cells was

dissected, RNA was extracted and 20  $\mu$ g of total RNA was electrophoresed, transferred to nylon membranes and probed with the  $\alpha$ 1-chain of type I collagen (Coll I) and alkaline phosphatase (ALP) and the filters were reprobbed for 18 S rRNA for calibration, as described in Methods. The mRNA levels were measured by densitometric analysis of the bands and were normalized to 18 S rRNA. rhTGF- $\beta$ 2 infusion in unloaded rats increased type I collagen mRNA (B) but not ALP mRNA levels (C). The data represent the mean  $\pm$  SE of densitometric determinations of separate preparations of RNA obtained from 3–5 individual rats. a indicates a significant difference with control rats.

not associated with significant changes in alkaline phosphatase activity and osteocalcin production in vitro. Although rhTGF- $\beta$ 2 did not affect ALP activity per cell, the number of marrow stromal ALP+ cells was increased in unloaded treated rats, suggesting that rhTGF- $\beta$ 2 affected the number of ALP+ cells rather than their ALP activity. This is in contrast with the effect of rhIGF-I administration in unloaded rats which stimulates both parameters of cell growth and differentiation in marrow stromal cells (10). The finding that the proliferation of ALP+ marrow stromal cells and the double labeled surface were increased above control values in rhTGF- $\beta$ 2 treated unloaded rats suggests that the increased number of bone forming sites resulted from the increased growth of osteoblast precursor cells. This indicates that the stimulatory effect of rhTGF- $\beta$ 2 infusion on bone formation was related mainly to an increased growth of osteoblast precursor cells in the marrow stroma. This is in accordance with in vitro data showing that TGF- $\beta$  induces the replication of normal osteoblastic cells (13,17–20), and with in vitro studies indicating that the stimulation of bone formation by TGF- $\beta$  are caused by effects on osteoblast precursor cells (50–53). The cellular mechanisms implicated in the mitogenic effects of TGF- $\beta$  on osteoblastic cells may involve an increased production of PDGF (54). In addition, TGF- $\beta$  may have direct effects on osteoblastic cell growth through induction of the expression of *c-fos* (55) and *c-jun* proto-oncogenes (51).

We also found that rhTGF- $\beta$ 2 administration in unloaded rats increased type I collagen mRNA levels in the metaphysis. The increased collagen expression found in rhTGF- $\beta$ 2-treated unloaded rats is in accordance with the stimulatory effect of TGF- $\beta$  on type I collagen mRNA levels in fibroblasts (56) and osteoblastic cell cultures (18, 57). Although TGF- $\beta$  was found to inhibit collagen expression in long-term developing rat calvaria cell cultures, this appears to occur secondarily to induction of cell growth (50, 51). The increased collagen expression induced by rhTGF- $\beta$ 2 infusion may have resulted from multiple transcriptional, posttranscriptional and posttranslational effects (57) since TGF- $\beta$  increases procollagen transcription (58) and procollagen mRNA stability (59, 60). This increased collagen expression may have in part contributed to the anabolic effect of rhTGF- $\beta$ 2 on metaphyseal bone formation in unloaded rats. However, the lack of significant change in the appositional rate suggests that the increased bone formation induced by rhTGF- $\beta$ 2 was mainly related to the increased number of osteoblasts in the metaphysis. In contrast to type I collagen, ALP mRNA levels were not changed in the metaphysis of rhTGF- $\beta$ 2-treated unloaded rats. Previous studies indicate that ALP mRNA levels do not correlate with bone matrix deposition whereas steady-state mRNA levels for collagen often reflect osteoblast activity (61). On the other hand, rhTGF- $\beta$ 2 infusion may have increased the expression of bone matrix proteins (22, 60, 62), induced chemotactic attraction of osteoblast precursors (16), inhibited the degradation of the extracellular matrix (63) and autoinduced TGF- $\beta$  expression by osteoblasts (64), which may have contributed to the observed stimulatory effect of rhTGF- $\beta$ 2 on bone formation in unloaded rats.

Although the cellular and molecular effects of TGF- $\beta$  on bone cells in vivo are likely to be complex, our findings indicate that the prevention of the impaired bone formation and trabecular bone loss by rhTGF- $\beta$ 2 infusion in unloaded rats is associated with stimulation of osteoblast precursor cell proliferation and increased expression of type I collagen by metaphyseal osteoblasts. These results are consistent with previous indications showing that TGF- $\beta$  is an important mediator of soft and hard

tissue repair (30, 65, 66). The finding that the systemic administration of a growth factor which exerts anabolic effects on bone cells, can prevent the trabecular bone loss in an animal model of osteopenia may be of significant clinical importance. Further experimental studies will determine whether the systemic administration of TGF- $\beta$  alone, or in combination with other osteogenic factors, or the single injection of TGF- $\beta$  (65), may be of therapeutic interest to enhance the endosteal bone formation and to maintain bone integrity in osteopenic disorders.

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