# Transforming Growth Factor-β and the Initiation of Chondrogenesis and Osteogenesis in the Rat Femur

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*Abstract.* We have investigated the ability of exogenous transforming growth factor-β (TGF-β) to induce osteogenesis and chondrogenesis, critical events in both bone formation and fracture healing. Daily injections of TGF-β1 or 2 into the subperiosteal region of newborn rat femurs resulted in localized intramembranous bone formation and chondrogenesis. After cessation of the injections, endochondral ossification occurred, resulting in replacement of cartilage with bone. Gene expression of type II collagen and immunolocalization of types I and II collagen were detected within the TGF-β-induced cartilage and bone. Moreover, injection of TGF-β2 stimulated synthesis of TGF-β1 in chondrocytes and osteoblasts within the newly induced bone and cartilage, suggesting positive autoregulation of TGF-β. TGF-β2 was more active in vivo than TGF-β1, stimulating formation of a mass that was on the average 375% larger at a comparable dose ($p < 0.001$). With either TGF-β isoform, the dose of the growth factor determined which type of tissue formed, so that the ratio of cartilage formation to intramembranous bone formation decreased as the dose was lowered. For TGF-β1, reducing the daily dose from 200 to 20 ng decreased the cartilage/intramembranous bone formation ratio from 3.57 to zero ($p < 0.001$). With TGF-β2, the same dose change decreased the ratio from 3.71 to 0.28 ($p < 0.001$). These data demonstrate that mesenchymal precursor cells in the periosteum are stimulated by TGF-β to proliferate and differentiate, as occurs in embryologic bone formation and early fracture healing.

THE most abundant sources of the 25,000-mol wt homodimeric peptide, transforming growth factor-β (TGF-β)[1], are bone and platelets (1, 45, 48). This multifunctional peptide has a broad range of cellular activities including control of the proliferation and expression of the differentiated phenotype of several cell types specific to bone, among them mesenchymal precursor cells, chondrocytes, osteoblasts, and osteoclasts (1, 8, 9, 45, 46, 47, 48). TGF-β exists in several distinct homodimeric forms, two of which, TGF-βs 1 and 2, have been isolated from bone in approximately a 4:1 ratio (10, 11, 25, 45, 46, 47, 48). In vitro studies have suggested that both TGF-βs 1 and 2 may regulate osteogenesis and chondrogenesis by affecting replication, gene expression, and structural protein synthesis in nearly all the cell types involved in bone formation (4, 7, 35, 45). Moreover, in vivo studies based on both immunohistochemical staining and on in situ hybridization have demonstrated synthesis of TGF-β by both chondrocytes and osteoblasts (15, 21, 41) and accumulation of TGF-β in a model of endochondral ossification (5).

TGF-β plays a central role in regulating the complex cascade of cellular events involved in healing soft tissues, a process initiated by release of the peptide after platelet degranulation (1, 2). In addition, exogenous application of TGF-β stimulates formation of granulation tissue typical of a healing response (34) and strengthens incisional wounds (32). The present study was designed to determine if TGF-β might play a similar role in stimulating the formation of new cartilage and bone, as occurs during the healing response after a bone fracture. We have investigated whether TGF-β, when injected into uninjured bone, can initiate chondrogenesis and osteogenesis, analogous to the induction of granulation tissue observed after subcutaneous injection of the peptide into uninjured soft tissue sites (34). Our results show that either TGF-β1 or 2, injected subperiosteally into growing bone, initiates a self-propagated tissue response leading to formation of new bone, similar to the response seen in fracture healing. TGF-β alone initiated a cellular cascade of events that included the stimulation of proliferation, differentiation, and extracellular matrix synthesis in target periosteal mesenchymal cells, chondrocytes, and osteoblasts, as occurs during physiological chondrogenesis and osteogenesis.

1. *Abbreviations used in this paper*: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TGF, transforming growth factor.

## Materials and Methods

### Animal Procedures

A total of 88 newborn Long Evans rats, ages 2–4 d, were divided into 6

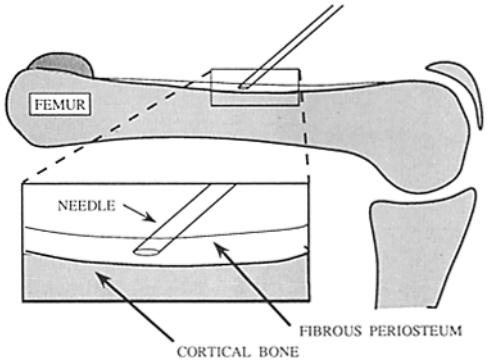



*Figure 1.* Subperiosteal injection technique. Using a dull 27-gauge needle, microinjections were directed into the subperiosteal region of the anterior surface of the rat femur. The technique was perfected by injecting dye until injections could be consistently reproduced. Verification of growth factor application to the subperiosteal region was confirmed with immunohistologic localization by anti-TGF-β1 antibodies (data not shown).

*Table I. Experimental Design*

| Group | Growth factor injected | Dose | Days injected | Days harvested | Number of animals |
|---|---|---|---|---|---|
| I | TGF-β1 | 200 ng | 14 | 3, 5, 8, 15, 21, 35 | 18 |
| II | TGF-β1 | 20 ng | 14 | 3, 5, 8, 15, 21, 35 | 18 |
| III | TGF-β2 | 200 ng | 14 | 3, 5, 8, 15, 21, 35 | 18 |
| IV | TGF-β2 | 20 ng | 14 | 3, 5, 8, 15, 21, 35 | 18 |
| V | BSA | 100 ug | 14 | 8, 15 | 6 |
| VI | TGF-β2 | 200 ng | 14 | 15 | 10 |

experimental groups (Table I). Animals in groups I, II, III, and IV received daily 10-μl injections of 200 ng TGF-β1, 20 ng TGF-β1, 200 ng TGF-β2, or 20 ng TGF-β2, respectively, into the subperiosteum of the anterior, mid-diaphyseal portion of their uninjured right femur for 14 consecutive days (Fig. 1); the TGF-β was dissolved in PBS, pH 7.4. The left femur was injected in a similar fashion with PBS alone, allowing each animal to serve as its own control. Animals were harvested for histology either during the injection phase of the experiment, or at different time points after receiving 14 injections. Animals in group V received seven daily injections of 1% BSA. Animals in group VI received 14 daily injections of 200 ng TGF-β2; on day 15, both legs were used for radiographic and histologic analysis. The porcine TGF-β1 and TGF-β2 used in this study were purchased from R&D Systems Inc. (Minneapolis, MN).

## Histological Analysis

Tissues were fixed for 2–3 d in 10% neutral buffered formalin, 2 d in Bouin's solution, followed by decalcification in a 10% acetic acid, 0.85% NaCl, 10% formalin solution (AFS) (26). This protocol resulted in adequate decalcification, without loss of antigenic sites for immunohistologic studies. Sagittal and cross sections were obtained in groups I, II, III, and IV, while the specimens in the remaining groups were all sectioned sagittally. Paraffin-embedded specimens were stained with hematoxylin and eosin, or Masson's Trichrome (American Histology Labs, Frederick, MD).

## Quantitative Analysis and Statistics

Photomicrographs and radiographs were quantified using a computerized image analysis program. Photomicrographs, at a 10× magnification, were taken of the histologic sections described above and scanned on an Apple digital scanner ($n$ = 87). In a similar fashion, radiographs of femurs that received TGF-β injections, were also scanned ($n$ = 10). The computerized images were analyzed for cross-sectional area on a Macintosh IIx computer using an image analysis program (Image Version 1.20, Wayne Rasband, National Institutes of Health, Bethesda MD). Areas of induced mass, intramembranous bone, cartilage, and endochondral bone were measured in triplicate and averaged for each histological image. For quantification, the induced mass was defined as the area of periosteal proliferation, intramembranous bone, endochondral bone, cartilage, and remodeled trabecular bone, but not fibrous tissue. For the determination of "percent cartilage formation" the areas of cartilage and endochondral bone were added together. This calculation is valid since endochondral bone must form from cartilaginous matrix, in contrast to intramembranous bone which forms without a cartilaginous matrix.

In determining the effect of TGF-β1 and 2 on total mass formation, significance was calculated using the SAS general linear model for an unbalanced, three-way ANOVA in which the three factors were growth factor, dose, and day ($n$ = 87) (43). The analysis was done on transposed data to stabilize the variance (43). In determining the effect of the dose of TGF-β1 or 2 on the stimulation of either cartilage or intramembranous bone formation, significance was determined as above, with a three-way ANOVA, and confirmed with a two-tail $t$ test ($n$ = 72).

## Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded sections using an avidin-biotin peroxidase detection system (Vector Laboratories, Inc., Burlingame, CA), as described by Heine et al. (15). Two polyclonal antibodies to TGF-β1 (anti-LC and anti-CC) were made in rabbits to the same synthetic peptide corresponding to the amino-terminal 30 amino acids of TGF-β1 (12, 13). These antibodies are specific for TGF-β1 and do not recognize TGF-β2 or 3. The anti-LC antibody recognizes intracellular TGF-β, while the anti-CC antibody recognizes extracellular peptide (13, 50). Serial sections of the treated tissue were stained with a polyclonal antibody against type I collagen (3) and a monoclonal antibody against type II collagen (44) after limited hyaluronidase digestion. Sections were counterstained with May Grunwald and Giemsa stains. Serial sections developed after omission of

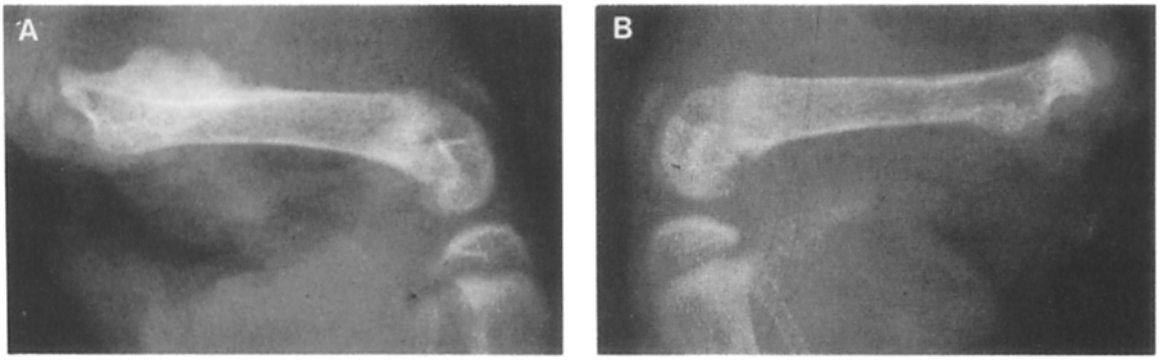



*Figure 2.* Radiographic analysis. Limbs were radiographed after receiving 14 daily injections of either TGF-β2 (200 ng) or PBS. (*A*) Radiographic appearance of a large calcified mass located on the anterior aspect of the right femur injected with TGF-β2; (*B*) normal appearance of a control limb injected with PBS.

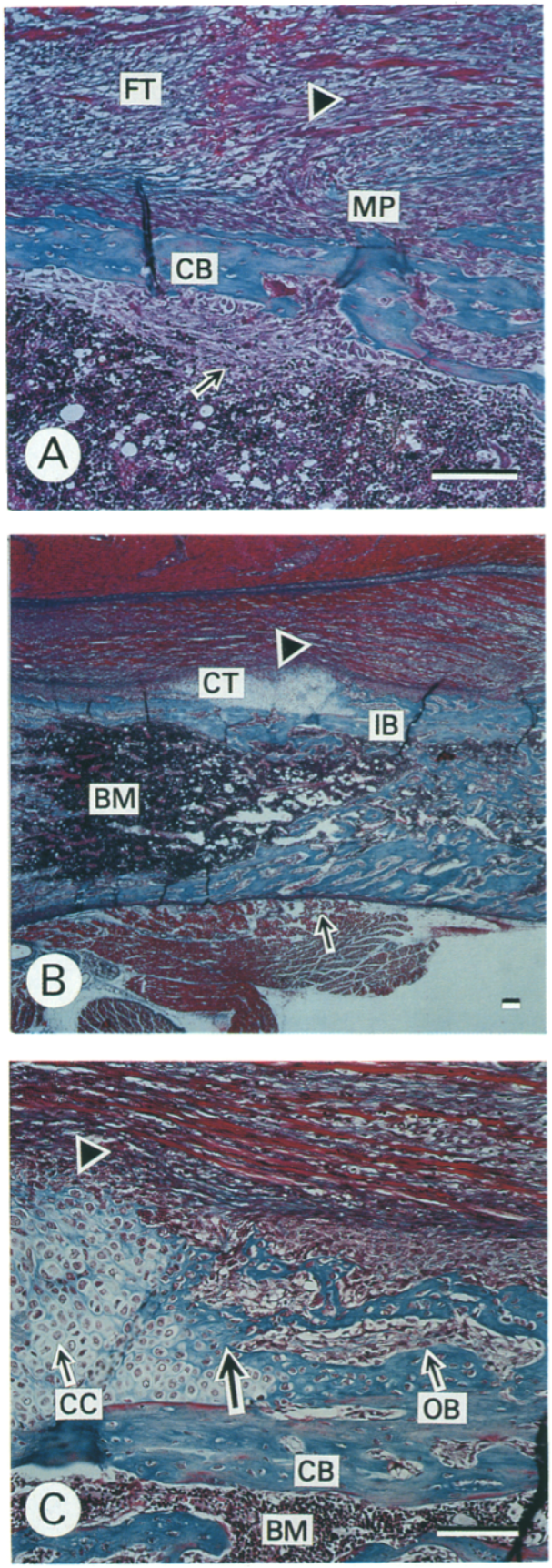



*Figure 3.* Histology of femurs after two and four daily subperiosteal injections of TGF-β2. Daily injections of 200 ng of TGF-β2 were given. (*A*) Rat killed on day 3, after two TGF-β2 injections; mesenchymal cell proliferation (*MP*) was evident both within the periosteum above cortical bone (*CB*) and along the endosteal surface (*arrow*); also noted is the fibrous tissue (*FT*) penetrating the muscle fibers at the site of the injection. (*B*) Rat killed on day 5, after four 200-ng TGF-β2 injections; at the injection site a newly formed cartilaginous matrix (*CT*) is flanked by regions of intramembranous bone (*IB*) while the opposing cortex (*arrow*) appears unaffected; (*C*) on higher magnification, chondrocytes (*CC*) can be identified within the cartilage, as can osteoblasts lining bone spicules (*OB*). A well-demarcated border separates these regions of chondrogenesis and osteogenesis (*large arrow*). (*BM*) bone marrow; (*arrowhead*) injection site. Bar, 100 μm.

either the primary or secondary antibody controlled for nonspecific staining. Negative controls consisted of parallel sections incubated with comparable dilutions of rabbit, mouse, or goat IgG preimmune serum.

### *RNA Extraction and Northern Blotting*

15 newborn Long Evans rats received daily injections of 200 ng TGF-β1 as described above. After two, four, and seven injections, five animals were killed, and the region of the femur and overlying soft tissue that had been exposed to the TGF-β was dissected and pooled. RNA was extracted by a technique modified for bone and cartilage (27), denatured, and electrophoresed in agarose gels. Northern hybridization was carried out using standard techniques (27) with cDNA probes for alpha-1-(II)procollagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (23, 52). Autoradiographs were made by exposing nylon membranes (Hybond-N) to radiographic film.

## *Results*

### *Development of a Model for Subperiosteal Injection of Long Bones*

To determine the in vivo effects of TGF-β on skeletal tissue, we developed a model in which exogenous growth factors are delivered into the subperiosteal region of newborn rat femurs (Fig. 1). The periosteum is comprised of two tissue layers: an outer fibroblast layer and an inner region of undifferentiated mesenchymal cells (14, 30). Extensive studies of fracture healing have demonstrated that this mesenchymal cell population is the likely source of chondrocyte and osteoblast precursor cells (51). Exposure of this population of cells to exogenous TGF-β provides an ideal model in which the effects of TGF-β on cell proliferation, differentiation, and extracellular matrix synthesis can be studied in vivo.

### *Radiographic Detection of Bone Formation Resulting From Subperiosteal Injection of TGF-β2*

To survey the net effect of TGF-β2 on new bone formation, 10 newborn Long Evans rats received 14 daily 200-ng injections of TGF-β2 into the subperiosteal aspect of their right femur. The animals were killed on day 15 for radiographic analysis (Fig. 2). In all 10 animals, a large calcified mass was detected at the site of injection of TGF-β2 (Fig. 2 *A*), with no apparent deviation from the normal on the PBS-injected side (Fig. 2 *B*). Although the injections were initially directed at the mid-diaphaseal region of the bone, disproportional elongation of the growing femur distally results in the proximal position of the mass by the time of x-ray. The size of the induced calcified mass was measured using the integral density of its radiographic image and then reported as

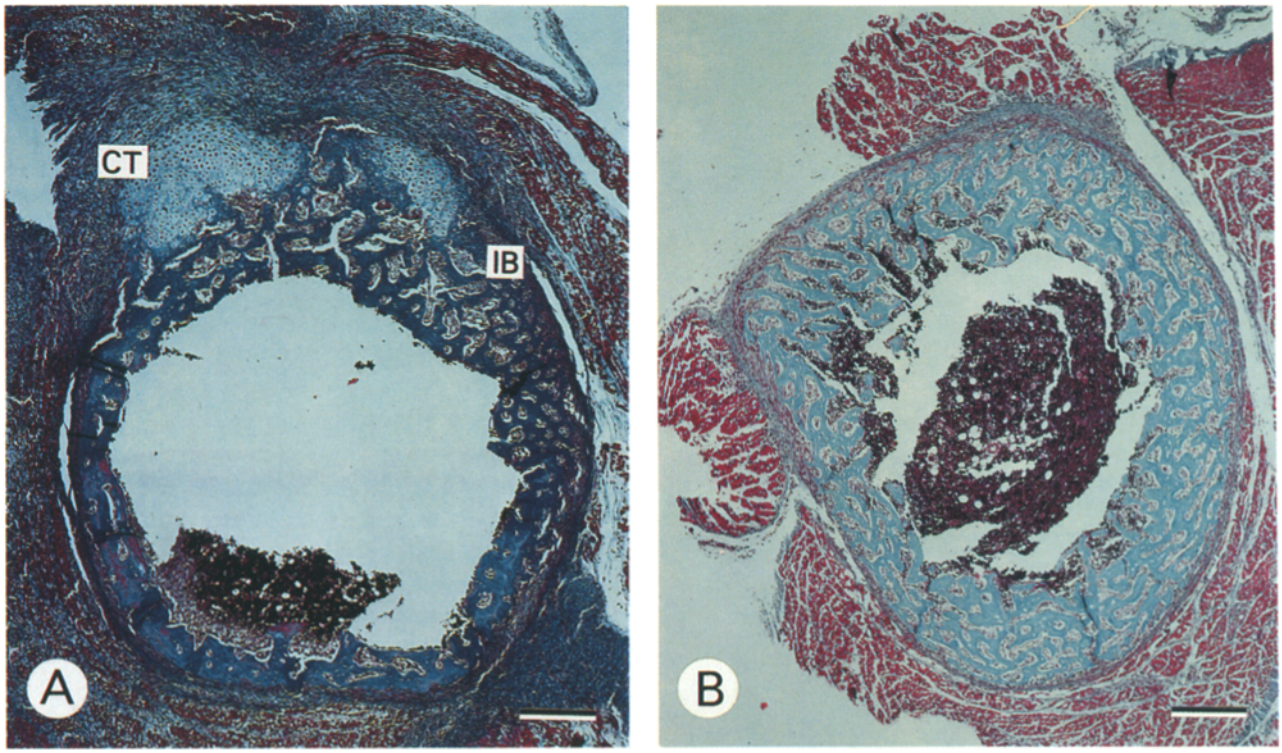



*Figure 4.* Cross-sectional histology of femurs after four TGF-β2 subperiosteal injections. Daily 200-ng injections were given. Rats were killed on day 5, after 4 injections; (*A*) cross section, at the site of injection, of a femur injected with TGF-β2, demonstrating the localized extent of cartilage (*CT*) and intramembranous bone formation (*IB*) at the site of injection, with the opposing cortex appearing unaffected; (*B*) cross section at the site of injection of a control femur that received PBS, demonstrating a normal histological appearance. Bar, 250 μm.

a fraction of the whole femur. The mean induced mass was determined to comprise 24.7% of the femur (SD = 6.2%).

### *TGF-β Initiates Chondrogenesis and Osteogenesis in Injected Limbs*

Having demonstrated a potent effect of TGF-β on the induction of new bone formation in this model system, we undertook a complete study of the dose and time-dependence of the response to TGF-β, based on detailed examination of the histology of the induced tissue. Daily subperiosteal injections of either TGF-β1 or TGF-β2 into the rat femur resulted in a complex and reproducible pattern of tissue formation and differentiation that included subperiosteal proliferation, intramembranous bone formation, and chondrogenesis. In contrast, either 7 or 14 daily injections of 10 μg/μl BSA or 14 injections of PBS resulted in only minimal periosteal proliferation at the site of injection in 4 of 88 control sections examined (Fig. 4 *B*). Since the most profound tissue response was seen with the 200-ng TGF-β2 dose, this experiment (Table I, group III) has been used to illustrate the temporal sequence of events induced by injection of TGF-β.

***Periosteal Proliferation.*** Mesenchymal cell proliferation within the inner cambrial layer of the periosteum was seen after two 200-ng injections of TGF-β2, resulting in expansion from its resting, three cell layers, to >8-cell layers thickness (Fig. 3 *A*, *MP*). The outer fibrous layer of the periosteum remained intact except for mechanical disruption at the injection site. As evidence of proliferation, mitotic figures were seen in a small number of the cells within the enlarging tissue mass. The distribution of responding tissue was specific. The most intense proliferation was at the injection site, with a minimal response laterally, and no effect on the opposing cortex. Periosteal proliferation remained evident throughout the 14 d of TGF-β2 injections and returned to its resting state within 1 wk of discontinuing the TGF-β.

***Chondrogenesis.*** Chondrocytes were identified within a cartilaginous matrix after four injections of 200 ng TGF-β2 (Figs. 3 *B* and 4 *A*). Cartilage, lacking structural organization, formed at the site of injection and enlarged with 7 injections. At this time, chondrocytes appeared immature with a compact cytoplasm and small lacuna. Cartilage not only formed above the cortex, but also appeared to replace underlying cortical bone. Numerous osteoclasts were identified within regions where cortical bone was being resorbed, possibly facilitating the continued invasion of cartilage into the underlying cortex. After 14 injections, the size of the cartilaginous mass had increased several fold and an organizational pattern had emerged (Fig. 5). Hypertrophic chondrocytes were seen near the cortical border of the mass, while smaller immature chondrocytes were seen in the vicinity of the proliferating mesenchymal cells nearer the periosteum (Figs. 5, *B* and *C*, *CT* and *CC*). Throughout the period of TGF-β injections, the cartilaginous mass was surrounded by areas of intramembranous bone formation.

***Intramembranous Bone Formation.*** New bone was detected in the region lateral to the induced cartilage after four injections of TGF-β2 (Figs. 3, *B* and *C*, and 4 *A*). Without evidence for a prior cartilage matrix, it must be assumed that

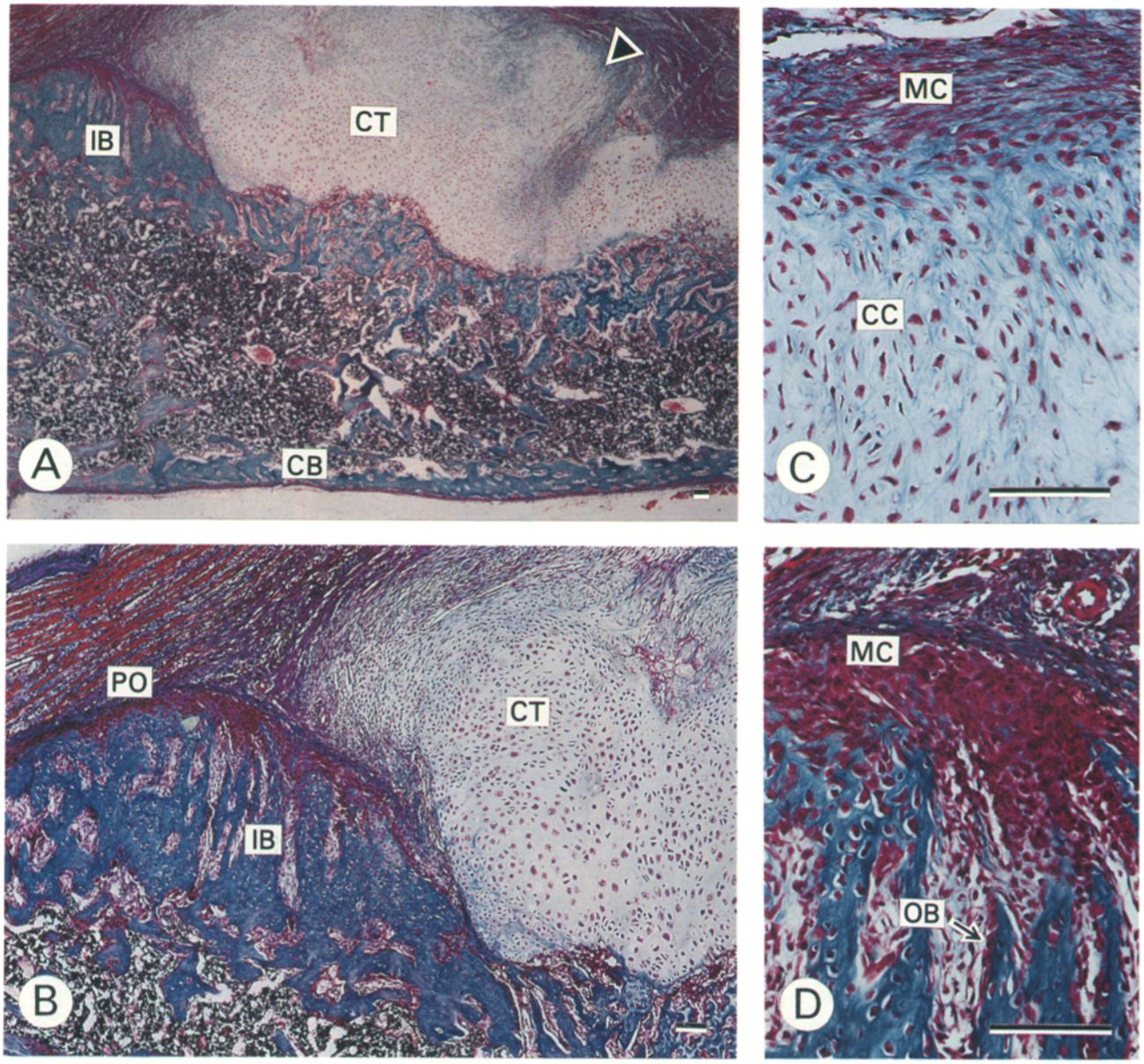



*Figure 5.* Histology of femurs after 14 daily subperiosteal injections of TGF-β2. Rat killed on day 15, after 14 daily injections of 200 ng TGF-β2; (*A*) low power photomicrograph demonstrating a large cartilaginous mass (*CT*) at the site of injection that is flanked by intramembranous bone (*IB*). The response is localized to the injected cortex, with a normal appearing opposite cortex (*CB*). (*B*) Enlargement of *A* emphasizing the sharp demarcation between active chondrogenesis (*CT*) and osteogenesis (*IB*). (*C* and *D*) High power photomicrographs demonstrating a maturation progression of cells from a primitive mesenchymal appearance (*MC*) to a chondrocytic phenotype (*CC*) in a cartilaginous matrix or a maturational progression of cells from a mesenchymal appearance (*MC*) to an osteoblastic phenotype (*OB*) lining newly formed bone spicules. (*Arrowhead*) Injection site; *PO*, intact periosteum. Bar, 100 μm.

these newly formed bony spicules, lined with osteoblasts, arose directly from the underlying cortical bone by the process of intramembranous bone formation (Fig. 3 *C*, *OB*). The mass of intramembranous bone increased in size throughout the 14 d of TGF-β2 injections (Fig. 5 *B*, *IB*). Early in this bone formation process, osteoblasts had a rounded appearance, indicative of active matrix synthesis. As injections continued, cellular synthetic activity decreased, as evidenced by a flattening of their cytoplasm. Throughout this region, mature osteoblasts were seen near the bony cortex, while immature osteoblasts could be identified closer to the proliferating mesenchymal cells of the periosteum (Fig. 5 *D*, *MC* and *OB*).

### *Cessation of TGF-β Injections Results in Endochondral Ossification and New Bone Remodeling*

To determine the fate of the newly formed cartilage induced following the TGF-β2 injections, animals were killed 1 and 3 wk after TGF-β injections had been stopped. Histologic analysis demonstrated that the cartilage was first replaced with bone by the process of endochondral ossification, and that the bone formed by intramembranous and endochondral ossification was then remodeled into thickened cortical bone.

***Endochondral Ossification.*** The large cartilaginous mass that formed as a result of TGF-β injection underwent en-

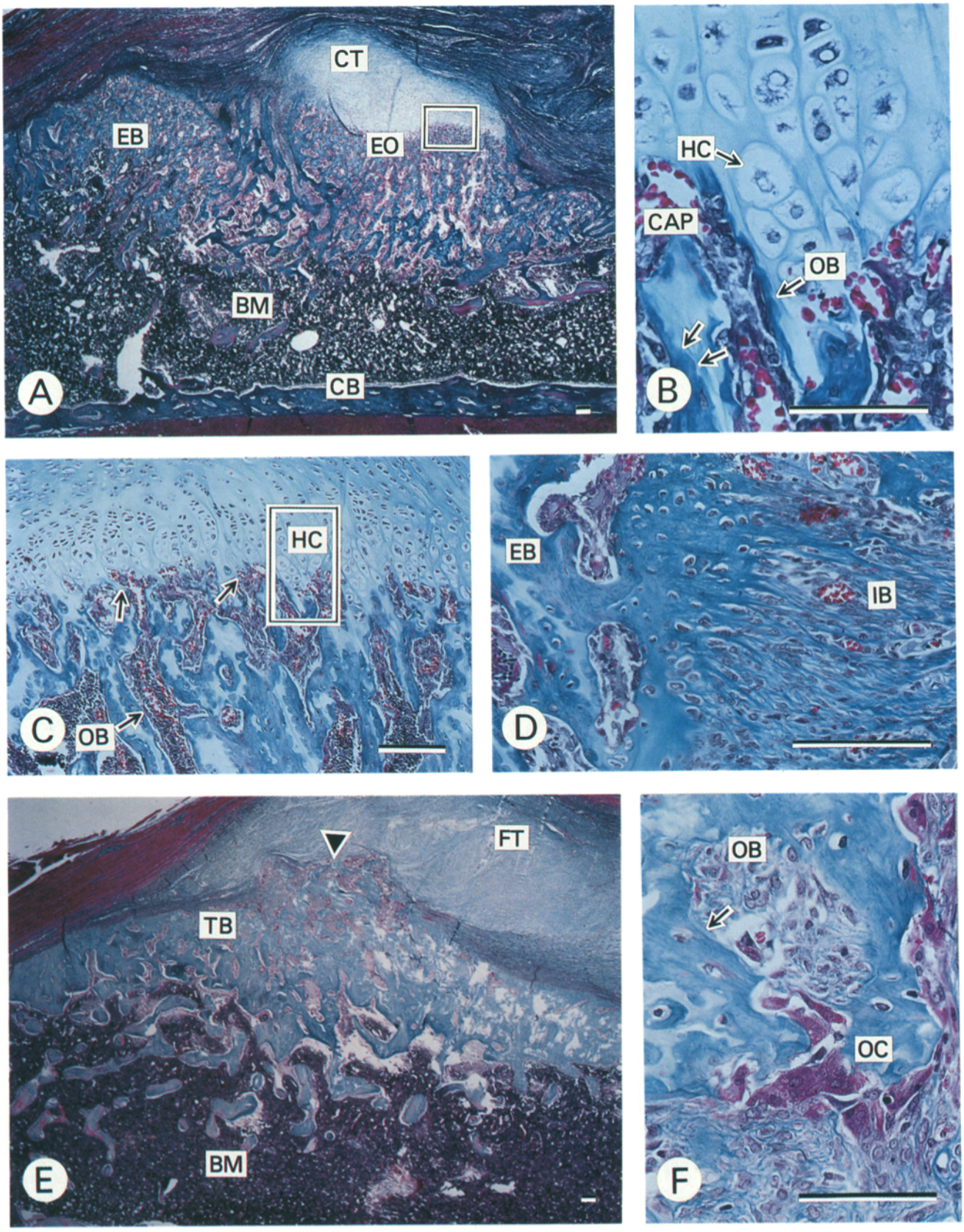



*Figure 6.* Histology of femurs after cessation of TGF-β2 injections. Femurs were analyzed 1 and 3 wk after the completion of the series of 14 daily injections of 200 ng TGF-β2. (*A*) Low power photomicrograph of a femur injected with TGF-β2, on day 21, demonstrating the development of an endochondral ossification front (*EO*) where cartilage (*CT*) is replaced with endochondral bone (*EB*). Box is enlarged in *C*. (*B* and *C*) High power views of the endochondral ossification front demonstrating hypertrophic chondrocytes (*HC*), osteoblasts (*OB*) newly synthesized osteoid (*arrows*), and vascular invasion (*CAP*). Box in *C* is enlarged in *B*. (*D*) Photomicrograph demonstrating bone

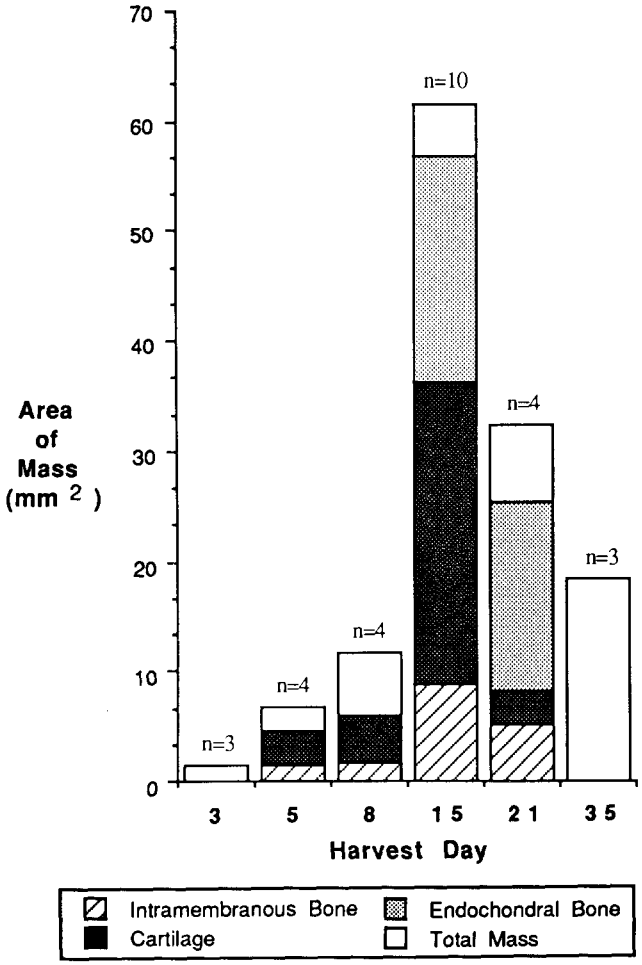



*Figure 7.* Quantitation of cartilage and bone formation resulting from subperiosteal injection of 200 ng TGF-β2. TGF-β2 (200 ng) was injected daily. Specimens were harvested for histologic analysis as indicated. Sections were photographed and subsequently digitized on an Apple scanner. The resulting digital images were quantified for (*a*) size of total induced tissue mass (not including fibrous tissue); (*b*) area of intramembranous bone formation; (*c*) area of cartilage formation; and (*d*) area of endochondral bone formation. On harvest day 35, the mass was comprised of induced trabecular bone.

dochondral ossification once the TGF-β treatment was discontinued (Fig. 6, *A–D*). Within 1 wk of stopping TGF-β injections (day 21 of the experiment) 50% of the cartilaginous mass had been replaced with bone (Fig. 6 *B*). Histologic analysis demonstrated that during this process the chondrocytes developed an organized appearance similar to the growth plate with resting above proliferating above hypertrophic chondrocytes (Fig. 6, *C* and *D*). Columns of these chondrocytes extended perpendicular to the long axis of the bone. Mineralization of the longitudinal septa between columns of cells coincided with chondrocyte hypertrophy (Fig. 6 *B, HC*). Newly formed endochondral bone had the classic mixed spicule appearance that resulted from the deposition of osteoid (new bone matrix) onto the calcified cartilaginous matrix (Fig. 6, *B* and *C, EB*). Endochondral ossification continued and 3 wk after the last TGF-β2 injection (day 35 of the experiment) the cartilaginous mass was completely replaced with bone (Fig. 6 *E, TB*).

***Remodeling.*** New bone, arising by either endochondral or intramembranous ossification, and existing cortical bone, was continually being remodeled (Fig. 6 *E, TB*). Histological examination suggested active remodeling as evidenced by the presence of multinucleated osteoclasts resorbing bone within the irregular surfaces of excavation sites along trabecular bone (Fig. 6 *F, OC*). This process was followed by the appearance of osteoid-synthesizing osteoblasts (Fig. 6 *F, OB*), resulting finally in the formation of thickened cortical bone.

Histological sections were analyzed quantitatively throughout the course of the TGF-β2 injection study. Areas of total tissue formation and specific types of tissue formation (intramembranous bone, cartilage, and endochondral bone) were measured. The results of this analysis are summarized in Fig. 7. The total area of tissue formation increased to 61.7 $mm^2$ during the injection phase of the experiment, and then decreased to 18.6 $mm^2$ 3 wk after the last injection. The composition of the tissue that comprised this mass was also found to vary over the course of the experiment.

### *Comparison of the Effects of TGF-β2 and TGF-β1 on Bone Formation In Vivo*

TGF-β1 was found to induce the formation of cartilage and bone in a similar, yet not identical, sequence to that just described for TGF-β2 (Fig. 8). Comparison of TGF-β1 to TGF-β2, at either the 200-ng/d or the 20-ng/d dose, demonstrated a difference in the amount of induced tissue formed (Fig. 9 *a*). TGF-β2 was found to be more active than TGF-β1, stimulating formation of a mass that was on the average 375% larger at the comparable dose ($p < 0.001$; $n = 87$). Although much larger masses of bone and cartilage were formed following TGF-β2 injections, the ratio of cartilage to intramembranous bone formation within those masses was not significantly different (Fig. 9 *b*). At the 200-ng/d dose, this ratio was 3.57 for TGF-β1 and 3.71 for TGF-β2 ($p = 0.046$), while at the 20-ng/d dose, the ratio was 0.0 for TGF-β1 and 0.28 for TGF-β2 ($p = 0.049$).

The formation of cartilage, at the immediate site of injection, and intramembranous bone, lateral to the site of injection, was a consistent finding (Fig. 8). This spatial relationship could be explained by assuming that TGF-β diffused from the injection site with a decreasing concentration gradient, and that the concentration of TGF-β, and not the isoform, determined the pathway of tissue formation into either cartilage or bone. This hypothesis was tested by repeating the injection sequence for both TGF-β1 and TGF-β2 at a lower,

formation by two different processes, endochondral ossification (*EB*) and intramembranous ossification (*IB*), and the well-demarcated border between these two types of bone. (*E*) Low power photomicrograph of a femur on day 35, 3 wk after completion of 14 TGF-β2 injections, demonstrating remodeling of both newly formed bone and existing cortical bone into thickened trabecular bone (*TB*); (*F*) high power view of regions of osteoclastic bone resorption (*OC*) and osteoid deposition (*arrow*) by osteoblasts (*OB*) on day 35. (*BM*), bone marrow; (*arrowhead*) injection site; (*PO*) intact periosteum; (*FT*) fibrous tissue. Bar, (*B* and *F*) 50 μm; (*A, C, D,* and *E*) 100 μm.

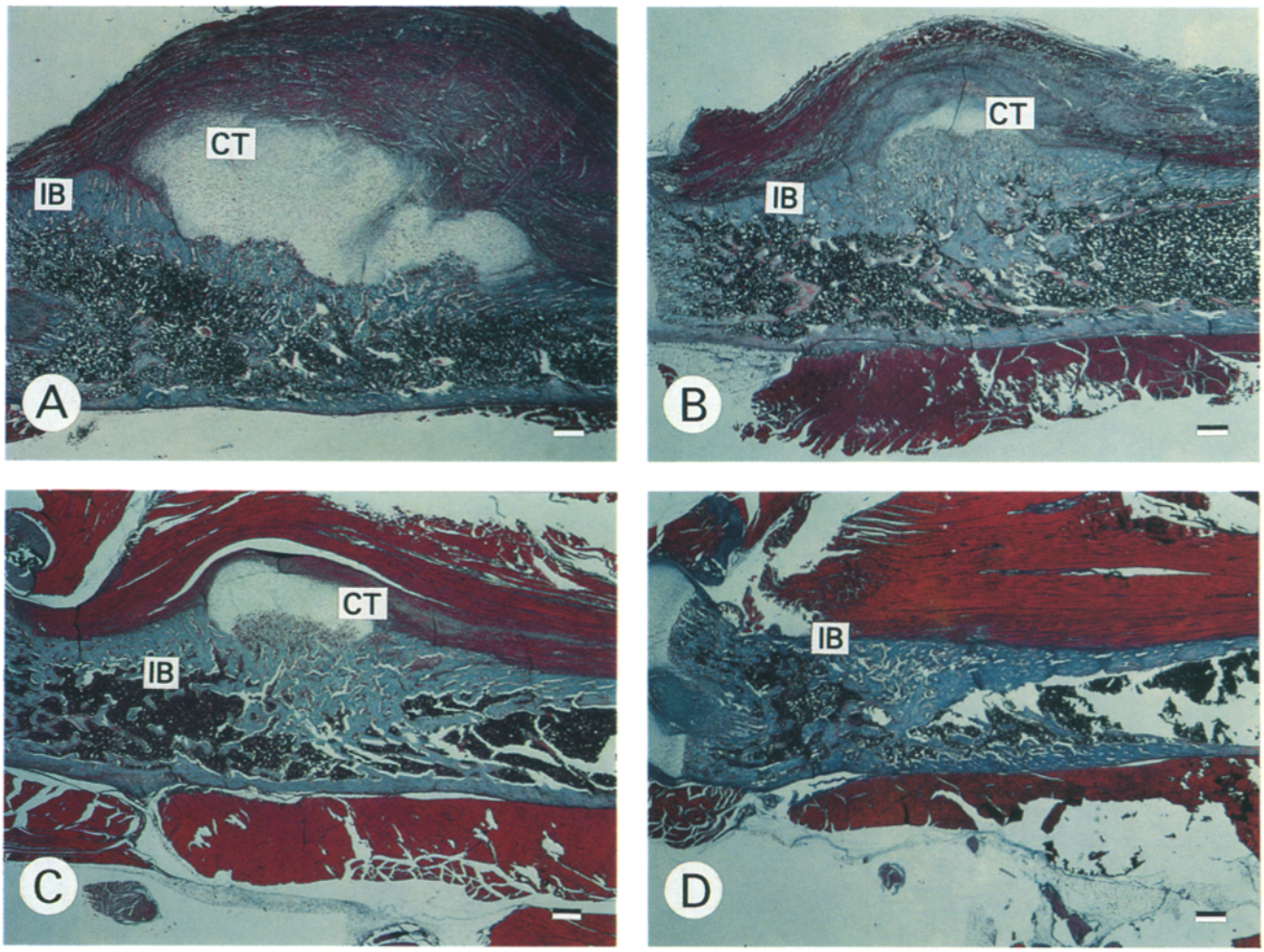



*Figure 8.* Histological comparison of high and low dose subperiosteal injections of TGF-β2 and TGF-β1. Animals received 14 daily injections of either TGF-β1 or TGF-β2 at two doses (200 or 20 ng) and were killed 1 wk later. (*A*) TGF-β2, 200-ng dose, a large cartilaginous mass (*CT*) at the site of injection, is flanked by a small region of intramembranous bone formation (*IB*). In contrast is (*B*) TGF-β2, 20-ng dose. Although a smaller mass of cartilage (*CT*) is evident at the injection site, it is flanked by an increased area of intramembranous bone formation (*IB*). (*C*) TGF-β1, 200-ng dose, a relatively large area of cartilage (*CT*), at the injection site is flanked by a region of intramembranous bone (*IB*). (*D*) TGF-β1, 20 ng dose, a small area of intramembranous bone (*IB*) is seen at the injection site. There is no detectable cartilage. Bar, 250 μm.

20-ng/d, dose (Fig. 9). For both isoforms there was a dose dependent increase in the amount of tissue formed (Fig. 9 *a*; $p < 0.001$, $n = 87$). Whereas, the relative proportion of intramembranous bone formation compared to cartilage formation increased as the dose was lowered (Fig. 9 *b*). For TGF-β1, decreasing the daily dose from 200 to 20 ng decreased cartilage formation from 48.2% to zero ($p < 0.001$; $n = 72$) and increased intramembranous bone formation from 13.5% to 47.0% ($p < 0.001$, $n = 72$); with TGF-β2, the same dose change decreased cartilage formation from 61.7% to 14.5% ($p < 0.001$, $n = 72$) and increased intramembranous bone formation from 16.6% to 51.4% ($p < 0.001$, $n = 72$).

### *TGF-β2–induced Matrix Contains both Type I and Type II Collagen*

To further define the composition of the matrix induced by injection of TGF-β2, we stained sections throughout the study with antitype I and antitype II collagen antibodies (Fig. 10). Antitype II collagen antibodies selectively stained the matrix surrounding proliferative chondrocytes, but failed to stain the matrix surrounding the hypertrophic chondrocytes (Fig. 10 *A*, *PC*). Type I collagen was localized throughout the entire cartilaginous mass; however, staining was most striking around the hypertrophic chondrocytes (Fig. 10 *B*, *HC*). In addition, type I collagen antibodies stained osteoid within the newly formed bone spicules, in areas of both intramembranous and endochondral ossification (Fig. 10 *B*, *arrow*). There was no collagen type II staining in areas of bone formation.

TGF-β1 treatment increased expression of mRNA for type II collagen, compared to the control limbs, after two 200-ng injections. This increase in type II collagen expression was not sustained, however, as message levels were identical to that of control limbs after both four and seven daily injections of TGF-β1 (Fig. 11). The expression of GAPDH was not affected by the TGF-β1 injections.

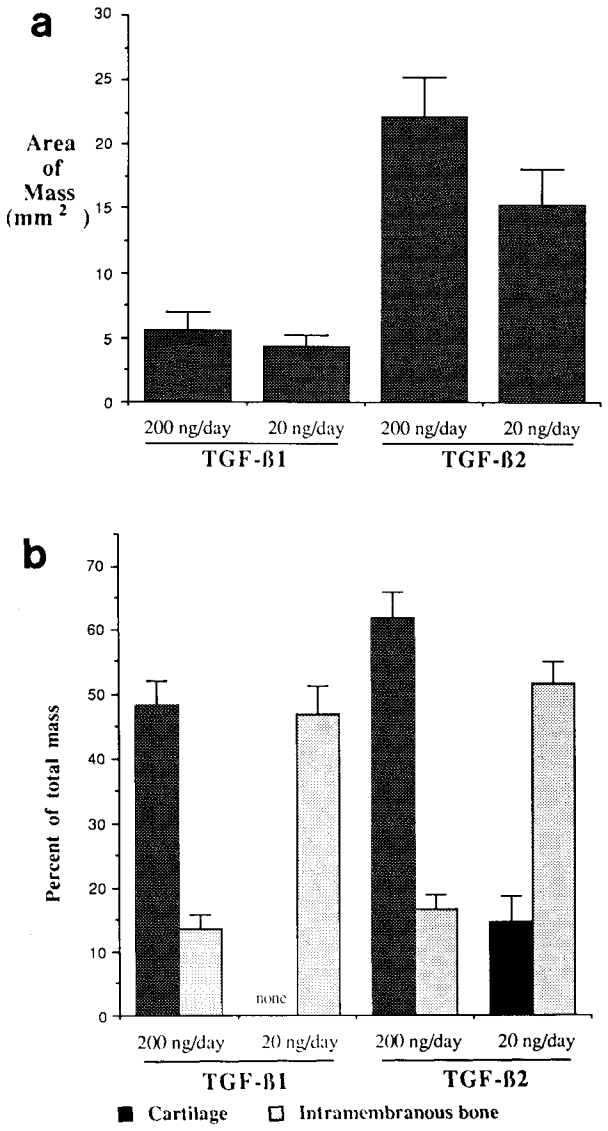



*Figure 9.* Quantitative comparison of TGF-β1 and TGF-β2 at two doses. (*a*) Rat femurs were treated with either TGF-β1 or TGF-β2 at both 200 and 20 ng daily dose. Animals were killed, sagittal histology was performed, and the area (in $mm^2$) of induced tissue formation was quantified as described. Both TGF-β1 ($p < 0.001$) and TGF-β2 ($p < 0.001$) stimulated tissue mass formation (defined as periosteum, cartilage, intramembranous bone, endochondral bone, and new trabecular bone, but not fibrous tissue) in a dose dependent fashion. Compared to TGF-β1, TGF-β2 stimulated the formation of a larger mass at both the 200-ng dose and the 20 ng doses ($p < 0.001$, $n = 87$). (*b*) Decreasing the TGF-β1 dose from 200 to 20 ng/d resulted in a decreased percent of cartilage formation ($p < 0.001$) and an increased percent of intramembranous bone formation ($p < 0.001$). Likewise, for TGF-β2 decreasing the dose from 200 to 20 ng/d resulted in a decrease in cartilage formation ($p < 0.001$) and an increase in intramembranous bone formation ($p < 0.001$). At either the 200- or 20-ng dose, TGF-β2 stimulated a larger percent of cartilage formation ($p = 0.016$, $p = 0.010$, respectively), but no significant difference was found in the percent of intramembranous bone formation. (For the determination of "percent cartilage formation," the areas of cartilage and endochondral bone were added together. This calculation is valid since endochondral bone must form from cartilaginous matrix, in contrast to intramembranous bone that forms without a cartilaginous matrix.)

### *Induction of TGF-β1 Synthesis by Treatment with TGF-β2*

Since autocrine induction of TGF-β synthesis had been demonstrated in an osteoblastic cell line in vitro (35, 52), we investigated whether synthesis of TGF-β might be induced within the tissue as a result of the TGF-β injections (Fig. 12). Using anti-TGF-β1 (*LC*) and anti-TGF-β1 (*CC*) antibodies to stain the bone and cartilage formed as a result of TGF-β2 injections, we found intracellular staining of proliferating chondrocytes and osteoblasts with the LC antibodies and extracellular staining for TGF-β1 within the matrix surrounding hypertrophic chondrocytes using the CC antibodies. This pattern is identical to that observed in growth plate chondrocytes of young rats (20) and in developing vertebrae of a 15-d-old mouse embryo (13). Western blot analysis has demonstrated no cross-reactivity between the TGF-β1 antibodies used in this study and the native TGF-β2 peptide injected into these animals (12, 13). Since the animals had been injected with TGF-β2, the observed staining cannot represent the exogenous TGF-β2, but rather demonstrates induction of TGF-β1 synthesis by TGF-β2 in both chondrocytes and osteoblasts in vivo.

## *Discussion*

In this study, we have shown for the first time that TGF-β injected subperiosteally into the femur of young rats can initiate a complex series of events resulting ultimately in new bone formation. Our results suggest that not only can TGF-β induce the differentiation of periosteal mesenchymal cells into osteoblasts and chondrocytes but that it can also stimulate these cells to proliferate and synthesize the extracellular matrix proteins characteristic of bone and cartilage. The mechanism by which TGF-β promotes chondrogenesis and osteogenesis is unknown; however, it is likely to involve the possibilities that TGF-β may be chemotactic for cells involved in these processes, or even less directly, that TGF-β may induce cells to secrete other peptides with these activities (22, 33, 54). Moreover, the demonstrated autostimulatory effect of TGF-β injections is certainly an important aspect of its action (Fig. 12).

It has been shown previously that TGF-β is capable of influencing the proliferation and phenotypic expression of osteoblasts, chondrocytes, and primitive mesenchymal cells in vitro (4, 35, 37, 38, 45). Immunohistochemical staining and in situ hybridization have demonstrated that TGF-β is synthesized by chondrocytes and osteoblasts during embryogenesis (15, 41), fracture healing (21), and in an in vivo model of endochondral ossification (5). Our histochemical analysis of the effects of TGF-β on periosteal mesenchymal cells in vivo corroborate in vitro observations demonstrating both that TGF-β induces differentiation of mesenchymal cells into a chondrocytic phenotype (37, 45) and that TGF-β stimulates osteoblast proliferation and synthesis of type I collagen (6, 7, 35). The present study demonstrates that continued injections of TGF-β result in increasing amounts of cartilage formation; however, it is unclear whether this results from the proliferation of chondrocytes, or the differentiation (or recruitment) of mesenchymal cells. Nevertheless, we found that when TGF-β injections were discontinued, the cartilaginous mass no longer enlarged and was replaced by bone.

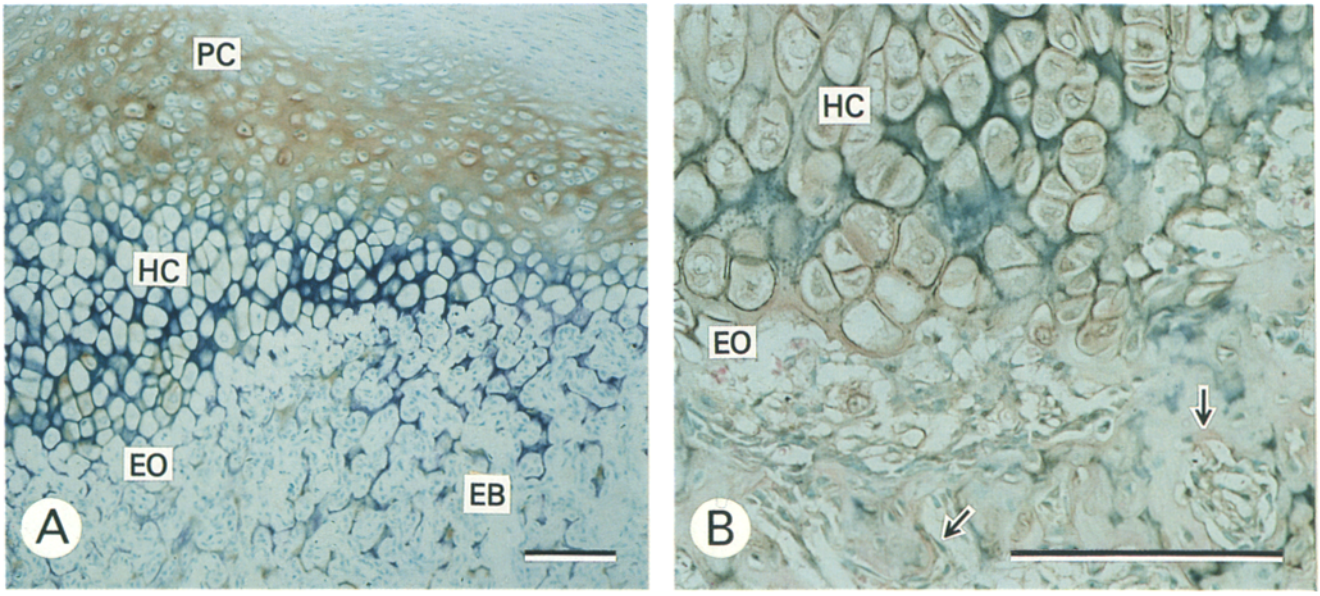
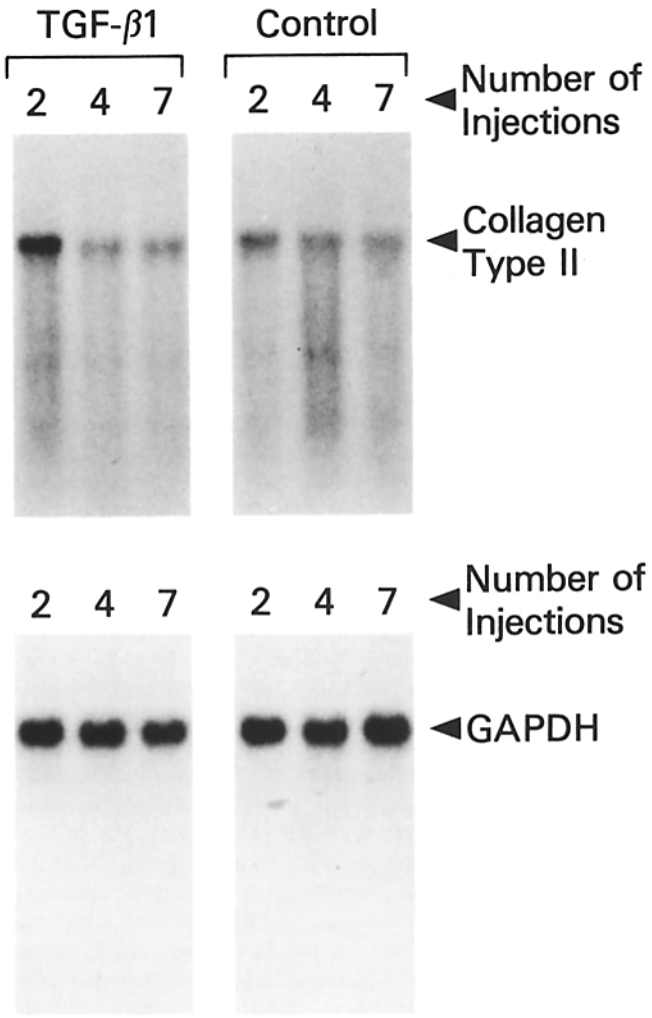



*Figure 10.* Immunohistology with antitypes I and II collagen antibodies. Femurs received 14 daily injections of 200-ng TGF-β2, and rats were killed 1 wk later. (*A*) Antibodies to type II collagen stain the extracellular matrix surrounding proliferating chondrocytes (*PC*) within the cartilage. Antibodies fail to detect type II collagen surrounding hypertrophic chondrocytes (*HC*), and endochondral bone (*EB*). (*B*) Antibodies to type I collagen stain hypertrophic chondrocytes (*HC*) and their surrounding extracellular matrix. Type I collagen is also present within newly synthesized osteoid (*arrow*) in the region of the endochondral ossification front (*EO*). Bars, 100 μm.




With either isoform of TGF-β, the dose of the growth factor determined which type of tissue formed, such that the ratio of cartilage formation to intramembranous bone formation decreased as the dose was lowered. At high doses (200 ng), and at the immediate injection site, differentiation proceeded in the direction of chondrogenesis; at the lower dose (20 ng), and at the higher dose in regions further from the immediate injection site (i.e., where there would be a lower concentration of the peptide), there was relatively less chondrogenesis and more osteoblast differentiation resulting in intramembranous bone formation (Figs. 8 and 9). These data suggest that the local concentration of TGF-β may determine the path of differentiation of the periosteal mesenchymal cells into either chondrocytes or osteoblasts.

Although a much larger mass of bone and cartilage was formed in response to injection of TGF-β2 (at both the 200-ng and 20-ng doses), the ratio of bone to cartilage within that mass was not significantly different than in the smaller mass stimulated by TGF-β1. These data suggest that TGF-β1 and 2 stimulate periosteal mesenchymal cell differentiation through a similar mechanism, while affecting the ultimate amount of tissue formation through a different mechanism. The possibility that TGF-β1 and TGF-β2 act similarly to affect mesenchymal differentiation pathways is supported by in vitro data

*Figure 11.* Northern blot analysis for expression of type II procollagen mRNA. The right leg of newborn rats received daily injections of 200-ng TGF-β1 and the left leg control injections of PBS. Five animals were killed on days 3, 5, or 8, after two, four or seven injections, respectively, and the injection sites were dissected and pooled for RNA extraction. 5 μg of total RNA was resolved on a 1.5% agarose-formaldehyde gel, transferred to nylon membranes, and probed for alpha-1-(II)procollagen and GAPDH as described in Materials and Methods.

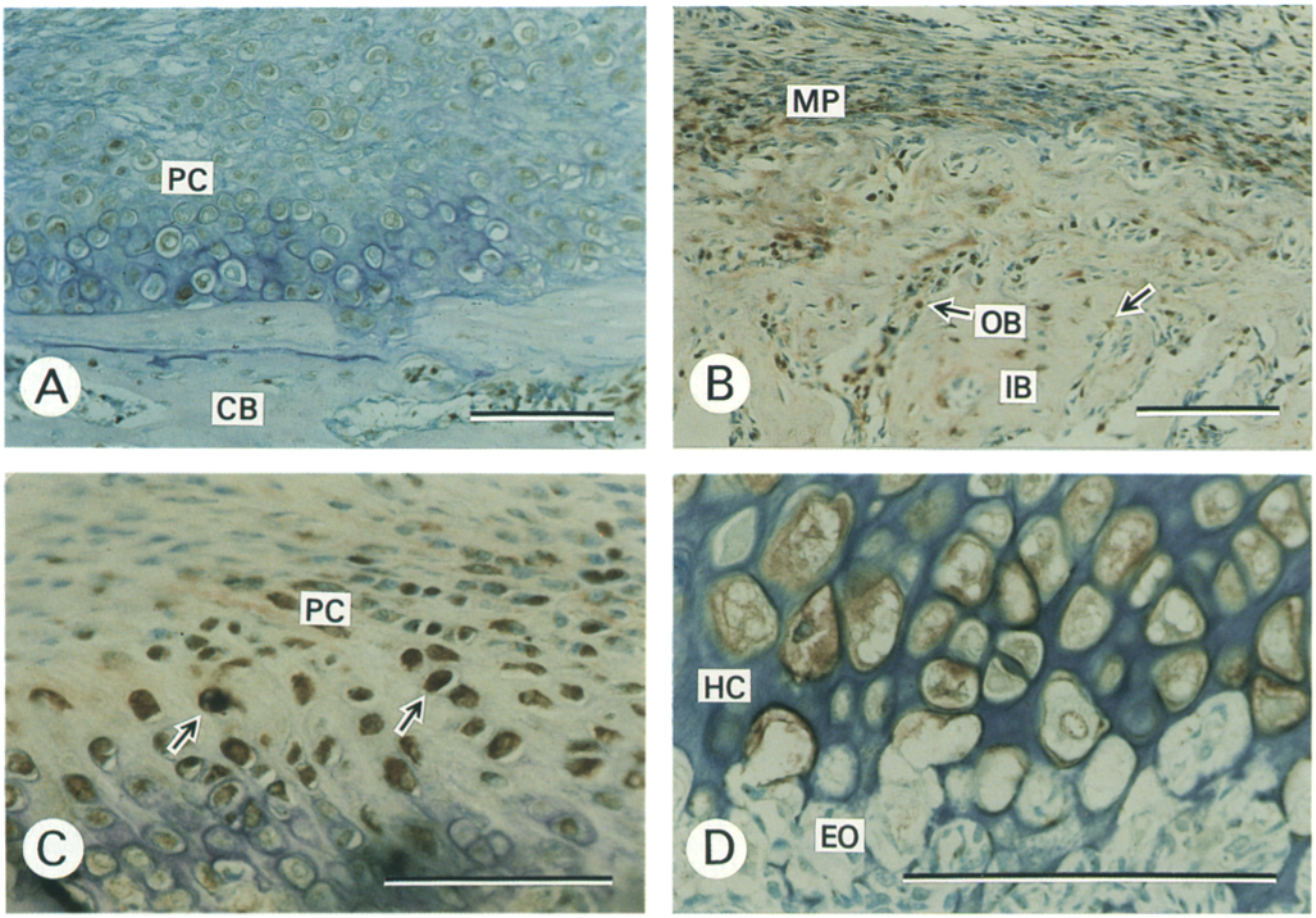



*Figure 12.* Immunohistology with anti-TGF-β1(*LC*) and (*CC*) antibodies. Femurs received 14 daily injections of 200 ng TGF-β2 and were harvested at 8, 15, or 21 d after the initial injection. (*A*) Photomicrograph of an early cartilaginous mass, overlying cortical bone (*CB*), induced by TGF-β2 injections (day 8). Anti-TGF-β1(1-30)-LC antibody stained TGF-β1 in proliferating chondrocytes (*PC*). (*B*) Photomicrograph of the mass induced by TGF-β2 (day 15) in the region of periosteal mesenchymal cell proliferation (*MP*) and underlying intramembranous bone formation (*IB*). Anti-TGF-β1(1-30)-LC antibody stained TGF-β1 in mesenchymal cells and osteoblasts (*OB, arrows*) throughout the induced tissue mass. (*C* and *D*) Photomicrographs on day 21, the cartilaginous region in *C* contains proliferating chondrocytes (*PC*) that stained intensely with the anti-TGF-β1(1-30)-LC antibody (*arrow*). At the endochondral ossification front (*EO*) hypertrophic chondrocytes (*HC*) are stained with the anti-TGF-β1(1-30)-CC antibody. Bars, 100 μm.

(45). Although these two isoforms of TGF-β are only 72% identical, they are interchangeable in most in vitro assays, including several with bone cells (7, 35). In addition, Noda et al. demonstrated that both TGF-β1 and 2 act similarly by inducing formation of periosteal woven bone in newborn rat parietal bones (28). In contrast, a selective response pattern has been observed in certain systems. These include the selective action of TGF-β1 in inhibition of growth of endothelial cells (18) and the selective action of TGF-β2 in induction of mesoderm in explants of amphibian ectoderm (36). The present data suggest that not only is TGF-β2 a more active stimulator of osteogenesis and chondrogenesis than TGF-β1 in vivo, but that both isoforms of TGF-β share the ability to influence the pathway of tissue differentiation into bone or cartilage by a dose dependent action.

Type I and II collagen were localized to specific regions of the newly formed bone and cartilage induced by the injections of TGF-β2. Type I collagen is the principal extracellular matrix protein of bone, and has been immunolocalized around hypertrophic chondrocytes and in mineralized cartilage (16, 53). In addition, it is found in areas of new bone formation at the growth plate and in the fracture callus (21*a*, 42). Type II collagen, the major extracellular matrix protein in cartilage, is synthesized in the growth plate in regions of proliferating chondrocytes, but not hypertrophic chondrocytes (24, 40). From the perspective of the localization of these major matrix proteins, it appears that the mechanism of bone and cartilage formation stimulated by TGF-β closely parallels the normal physiologic process.

There are many published studies of the effects of TGF-β on chondrocytes, osteoblasts, and other mesenchymal cells in vitro. Although these studies have shed light on many functions of TGF-β, they are at times conflicting and confusing. For example, TGF-β has been shown to be both a stimulator and inhibitor of osteoblast proliferation (7, 35), and to be able to either increase or decrease type II collagen synthesis in chondrocytes (39). These contradictions, in many cases, can be explained by differences in serum concentration, source and age of the cells, and the precise phenotypic description of the cells under study. In addition, the apparent function of TGF-β can be altered by other growth factors acting in combination with it (17, 45, 48), or by systemic hor-

mones (29, 31). In fact, the well documented multifunctionality of TGF-β action (48) precludes prediction of its in vivo activities based solely on in vitro experiments.

These data add to the growing body of evidence on the in vivo activity of TGF-β. The ability of TGF-β, in nanogram quantities, to stimulate formation of granulation tissue, accelerate the accumulation of protein, collagen, and DNA, and increase wound strength has been demonstrated in numerous rat subcutaneous tissue models (32, 34, 49). In these studies, TGF-β did not stimulate bone or cartilage formation. This would support the crucial role that the subperiosteal mesenchymal cell population plays in our experiments where the predominant response to TGF-β is the induction of bone and cartilage. TGF-β1 or TGF-β2 injected onto the periosteum of newborn rat parietal bones results in the formation of woven bone, without evidence of cartilage formation (28). In light of the data presented here, where cartilage formation was stimulated by both TGF-β1 and 2, it would appear that the mesenchymal cell populations in calvaria and long bones respond differently to TGF-β.

The present study on the effects of TGF-β on bone and cartilage formation adds increasing support for a role for the peptide in fracture healing. TGF-β is released by platelets (1, 2) at the time of fracture and is synthesized by both osteoblasts and chondrocytes at specific times, in specific regions, during the fracture healing process (21). Injection of TGF-β into the subperiosteum of an uninjured bone mimics the platelet release of TGF-β in the same region, when a bone is fractured. The data presented here show that the effect of TGF-β on the periosteum is first to stimulate the proliferation of mesenchymal cells that then differentiate into chondrocytes and osteoblasts. The net result of continued TGF-β injection was the formation of a callus with central cartilage and lateral intramembranous bone; the endochondral replacement of the cartilage, and the remodeling into cortical bone after cessation of the TGF-β treatments. These processes correlate with those described in normal fracture healing (19). The ability to initiate such a response by exogenous application of TGF-β suggests many important therapeutic uses for the peptide in fracture repair and bone grafting.

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