

# The effects of age on the response of rabbit periosteal osteoprogenitor cells to exogenous transforming growth factor- $\beta$ 2

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## SUMMARY

Additional bone and cartilage are formed if transforming growth factor- $\beta$  is injected into the periosteum of calvariae or long bones. To investigate this further, transforming growth factor- $\beta$ 2 was injected into the periosteum of the tibia of 3-day-old, 3-month-old and 2-year-old rabbits. In all instances, there was an increase in proliferation of the cells of the cambial layer of the periosteum, that is, the osteoprogenitor cells, and breakdown of the fibrous layer. Oedema was induced in the surrounding connective tissues.

Over the experimental period the normal neonatal tibia is undergoing rapid growth; there is periosteal bone formation and endosteal resorption. In the experimental neonatal tibiae, an increase in periosteal bone formation is seen after three injections of 20 ng of transforming growth factor- $\beta$ 2, which is accompanied by cartilage after five injections; the amounts of induced bone and cartilage increase with the number of injections. The chondrocytes hypertrophy after 4 days and the cartilage is replaced by bone endochondrally.

In contrast, after seven injections of 20 ng transforming

growth factor- $\beta$ 2, there is only a small amount of new bone on the 3-month-old tibia and none on the 2-year-old tibia. One day after seven injections of 200 ng transforming growth factor- $\beta$ 2, there is a small amount of bone formation, while seven days after cartilage is found as small discrete nodules on the 3-month-old tibia, but as small areas within the bone on the 2-year-old tibia.

It is concluded that the primary effect of transforming growth factor- $\beta$ 2 in this experimental model is to increase the proliferative rate of the osteoprogenitor cells in the periosteum. It is argued that transforming growth factor- $\beta$ 2 does not initiate osteoblastic or chondrocytic differentiation of osteoprogenitor cells. It is suggested that their differentiation is controlled by the local environment, in particular, the vascularity and locally circulating growth factors.

Key words: periosteum, rabbit, transforming growth factor- $\beta$ , bone, cartilage

## INTRODUCTION

The transforming growth factor- $\beta$ s (TGF- $\beta$ ) are thought to act both as immunoregulatory cytokines and as morphogenetic growth factors during foetal and neonatal development (Shull et al., 1992). They may also have an important role in the control of bone formation and remodelling (Bonewald and Mundy, 1990). Five TGF- $\beta$ s have been identified and they are members of a large family of growth factors that includes the bone morphogenetic proteins (BMPs), activins and inhibins (Hoffman, 1991; Wozney et al., 1990). The effects of exogenous TGF- $\beta$ 2 on normally developing neonatal and adult skeletal tissues are described in this paper.

TGF- $\beta$ s 1 and 2 are the most abundant and most widely distributed of the five TGF- $\beta$ s in vertebrate tissues. In vivo, based on the expression of mRNAs and immunolocalization of the protein, TGF- $\beta$ s are synthesized by cells of the skeletal tissues and most other organs (Bonewald and Mundy, 1990; Flanders et al., 1989; Gatherer et al., 1990; Heine et al., 1987; Thompson

et al., 1989). Osteoblasts synthesize TGF- $\beta$ s 1 and 2 in vitro (Centrella and Canalis, 1985; Robey et al., 1987).

Observations such as these prompted studies of the effects of TGF- $\beta$  on osteoblasts and chondrocytes both in vitro and in vivo. Cultured fetal and neonatal osteoblasts and periosteal cells from various sources respond to TGF- $\beta$  with an increase in proliferation (Ten Dijke et al., 1990; Hock et al., 1990; Rosier et al., 1989; Robey et al., 1987), whereas osteoblastic and osteosarcoma cell lines, that is, transformed cells, exhibit reduced proliferative rates (Pfeilschifter et al., 1987; Noda and Rodan, 1986). TGF- $\beta$  increases the synthesis and secretion of some matrix components, for example, the production of type I collagen (Ber et al., 1991; Strong et al., 1991) and of cartilage proteoglycans (Morales, 1991).

The effects of TGF- $\beta$ s on bone in vivo were investigated by administering daily injections of the growth factor onto the surface of neonatal mouse and rat calvariae or femora (Joyce et al., 1990; Mackie and Trechsel, 1990; Marcelli et al., 1990; Noda and Camilliere, 1989); in all instances bone formation

increased and in the femora de novo cartilage formation was induced. The interpretation of these results is that TGF- $\beta$  both increases the proliferative rate of osteoprogenitor cells and stimulates their differentiation into osteoblasts and chondrocytes. When older rats were used there was no increase in bone matrix production (Mackie and Trechsel, 1990). This latter result agrees with the response of rabbit and rat fractures to TGF- $\beta$  injected onto the callus during the first week of healing; both bone and cartilage formation may be inhibited (M. A. Critchlow and D. E. Ashhurst, unpublished observations; Terek et al., 1989).

The dichotomy of the responses of the progenitor cells in the periosteum of neonatal and older animals to TGF- $\beta$  in vivo requires further investigation. TGF- $\beta$ 2 was, therefore, injected into the periosteum of the tibiae of neonatal, 3-month-old and 2-year-old rabbits. TGF- $\beta$ 2 was chosen because Joyce et al. (1990) found that it stimulated a greater response than TGF- $\beta$ 1 in the rat. The results confirm that the response to exogenous TGF- $\beta$ 2 is age-dependent and suggest that the primary effect is to increase the number of osteoprogenitor cells.

**MATERIALS AND METHODS**

**Administration of TGF- $\beta$ 2**

New Zealand White rabbits 3 days old and 3 months old (2.5 to 3.0 kg) of either sex, and 2-year-old multiparous females, were used.

TGF- $\beta$ 2 (purified porcine, R & D Systems, Inc., Minneapolis,

USA) was injected in a carrier solution containing 4 mM HCl and 1 mg/ml bovine serum albumin (fatty acid-free). Samples (5, 20 or 200 ng) of TGF- $\beta$ 2 in 20  $\mu$ l carrier solution were injected from the lateral side into the periosteum of the antero-lateral, mid-diaphyseal region of the left tibia (the mid-point between the tibial plateau and medial malleolus) using a Hamilton syringe with a 26 gauge needle; the right tibia was injected in the same position with carrier only and served as the control. After two injections, a discrete swelling develops on the tibia that enables subsequent injections to be sited precisely. No determinations were made of the half-life of TGF- $\beta$ 2 in vivo. Previous studies of TGF- $\beta$  implanted subcutaneously in a collagen gel showed that the half-life of the initial rapid clearance phase was 22 hours (Sprugel et al., 1987).

There were ten experimental groups. The animals in groups 1 to 4 were 3 days old at the start of the experiments; those in groups 5 to 7 were 3 months and in groups 8 to 10 were 2 years old. The protocols were as follows and are summarized in Tables 1 and 2.

Group 1: daily injections of 20 ng TGF- $\beta$ 2 for 3, 5, 7, 10 or 14 days, killed 1 day after the final injection.

Group 2: daily injections of 5 ng TGF- $\beta$ 2 for 7 or 10 days, killed 1 day later.

Group 3: daily injections of 20 ng TGF- $\beta$ 2 for 7, 10 or 14 days, killed 7 days later.

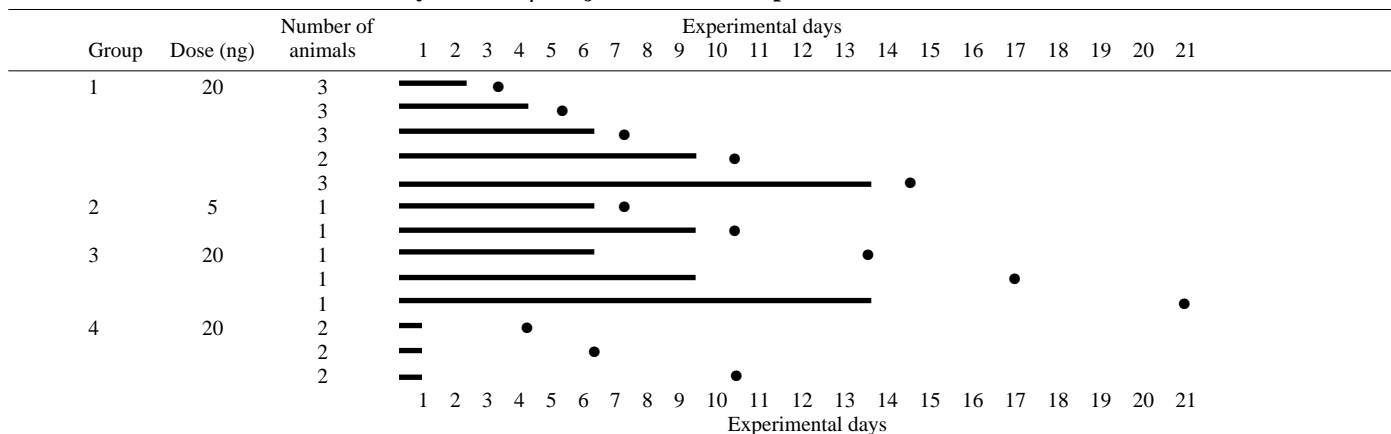
Group 4: one injection of 20 ng TGF- $\beta$ 2, killed 4, 6 or 10 days later.

Groups 5 and 8: seven daily injections of 20 ng TGF- $\beta$ 2, killed 1 day later.

Groups 6 and 9: seven daily injections of 200 ng TGF- $\beta$ 2, killed 1 day later.

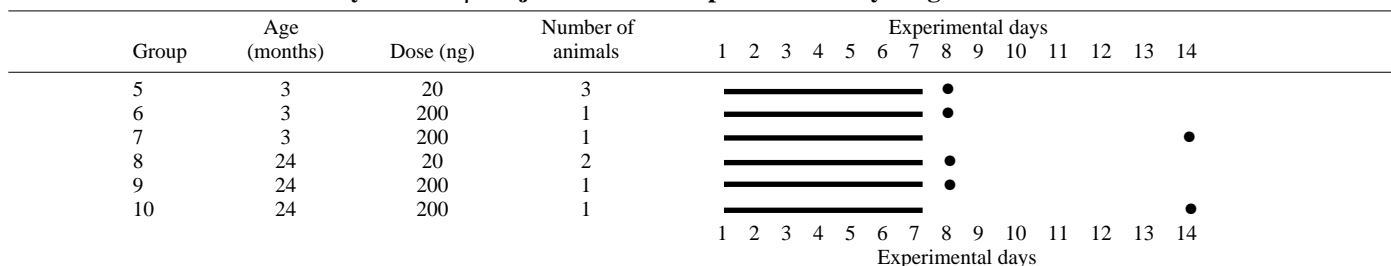
Groups 7 and 10: seven daily injections of 200 ng TGF- $\beta$ 2, killed 7 days later.

**Table 1. Summary of TGF- $\beta$ 2 injections into the periosteum of neonatal rabbit tibiae**

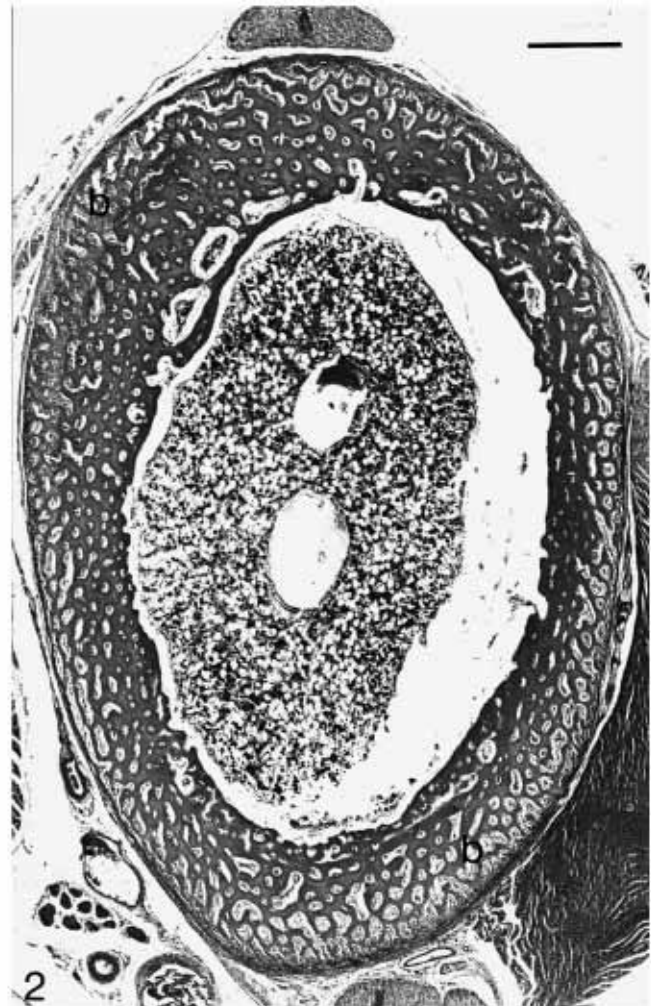


All rabbits were 3 days old on experimental day 1. The period of injections is indicated by a bar and the day of killing by a circle.

**Table 2. Summary of TGF- $\beta$ 2 injections into the periosteum of young and old adult rabbit tibiae**



The period of injections is indicated by a bar and the day of killing by a circle.



**Fig. 1.** Photomicrograph of the tibia of a 3-day-old rabbit. The developing bone (b) consists of thin trabeculae. Bar, 0.5 mm.

**Fig. 2.** Photomicrograph of the tibia of an 18-day-old rabbit. The inner regions of the developing bone (b) are becoming haversian, while the outer regions are still composed of thin trabeculae. Comparison of Figs 1 and 2 illustrates the rapid growth over the 15-day period. Bar, 0.5 mm.

#### Periosteal removal

Six 17-day-old rabbits were anaesthetized with Halothane. The mid-diaphyseal region of the left tibia was exposed and the periosteum was removed with a scalpel blade. The incision was sutured. They were killed 4, 6 or 10 days later.

#### Uninjected neonatal control animals

The tibiae of two neonatal rabbits aged 3, 7, 11, 18 and 24 days were taken to establish the size and structure of untreated, developing tibiae.

#### Preparation for microscopy

The mid-diaphyseal region of each tibia was fixed in 4% paraformaldehyde in 0.05 M Tris-HCl buffer, pH 7.4, overnight at room temperature. After washing in buffer, the bone was decalcified in 14.3% aqueous EDTA, pH 7.0. The tissue was dehydrated in graded ethanols, cleared in methyl salicylate and embedded in paraffin wax. Transverse serial sections were cut at 7  $\mu$ m. They were stained with 0.5% Alcian Blue, pH 2.6, for 30 minutes, followed by Haematal 8 and Biebrich's Scarlet, or Ehrlich's haematoxylin and eosin.

#### Tartrate-resistant acid phosphatase (TRAP) reaction to localize osteoclasts

Tartaric acid at a concentration of 0.75% (w/v) was added to the following acid phosphatase substrate: 5 mg naphthol AS-BI phosphate was dissolved in 1 drop of dimethyl formamide and added to 10 ml of a solution containing 5 mg Fast Red TR in 0.1 M sodium acetate buffer, pH 5.0. The sections were incubated at 37°C for 2 to 4 hours and mounted in glycerine jelly.

#### Quantitative analysis

This was confined to the tibiae in group 1. A section at the injection site and the 20th section proximal and distal to it in the experimental tibiae were chosen for analysis. Sections in the same region of the control tibiae were similarly chosen. A drawing was made of each section using a drawing tube. The relative areas of the whole tibia, medullary cavity, bone and cartilage were obtained using a point counting method. The results are presented as histograms (see Fig. 19).

#### Estimation of rates of bone growth

##### Normal bone growth of neonatal animals

The medial-lateral and anterior-posterior axes of similar regions of

the tibiae of 3-, 7-, 11-, 18- and 24-day-old rabbits were measured. The approximate rates of periosteal bone formation in micrometers per day were calculated from these measurements (Table 3).

**Experimental bone and cartilage formation**

The drawings of corresponding experimental and control sections, i.e. three pairs per animal, used for the quantitative analysis were superimposed and the maximal width of the additional tissue, that is, bone and cartilage, on the antero-lateral surface was measured. The rate of formation of the additional bone and cartilage in micrometers per day was calculated. The values in Table 3 are the maximum and minimum calculations and indicate the range of rates between animals. It must be stressed that the rates of tissue formation estimated in this way are approximate.

**RESULTS**

The first series of experiments was designed to determine whether TGF-β2 injected into the periosteum induces bone and cartilage formation in all rabbits, irrespective of age. The second series was designed to determine whether the effect of periosteal disruption in neonatal rabbits is similar to that of exogenous TGF-β2; periosteal disruption in skeletally mature rabbits causes localized bone formation.

**Growing neonatal tibiae**

Over the experimental period there is very rapid growth and reorganization in the tibia. At 3 and 7 days, the tibial cortex consists of a network of thin bony trabeculae surrounding large spaces that are lined by osteoblasts (Fig. 1) and the cambial layer of the periosteum contains several layers of osteoprogenitor cells. The irregular endosteal surface is being resorbed

by osteoclasts (Fig. 3), while thin trabeculae of bone are growing out along the periosteal surface. By 18 and 24 days the tibia is much larger (Fig. 2). The bone is more compact and haversian systems are beginning to form. Figs 1 and 2, which are of approximately the same region of the 3- and 18-day tibiae, respectively, illustrate that the bone enlarges, especially in the medial-lateral axis, during this period of growth. Periosteal appositional growth rates of up to 150 μm per day are achieved between 7 and 10 days postnatal, while between 18 and 24 days the rate is less than 10 μm per day (Table 3). The effects of exogenous TGF-β2 are, therefore, superimposed upon rapid, but variable, normal growth rates in the neonatal animal.

**Neonatal rabbits, groups 1, 2, 3 and 4 (Table 1)**

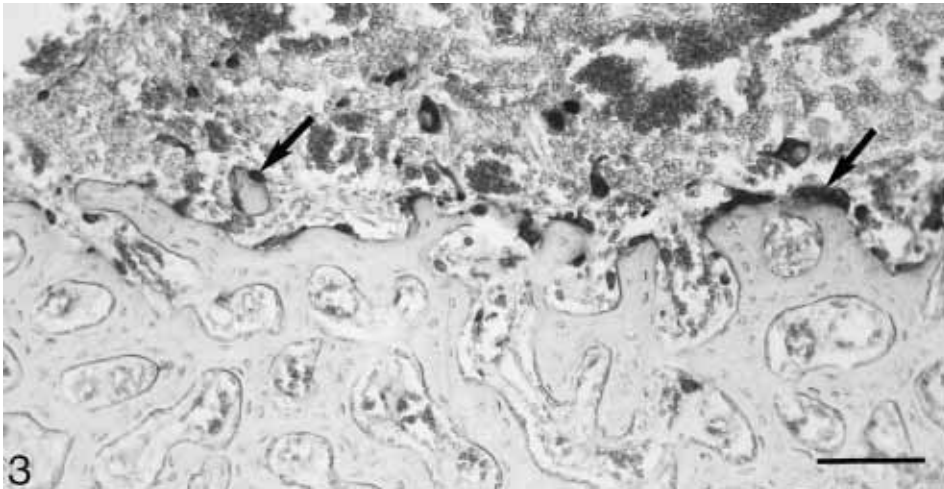
All the neonatal rabbits were 3 days old at the time of the first injection. The gross response to the injections is an overall enlargement of the leg around the injection site that is already apparent after three injections (Figs 4,5). This is caused both by oedema and by an increase in the amount of fibrous tissue under the skin, around the periosteum and between the muscles, and both continue to increase with the number of injections. After 14 injections, the leg at the level of the injection site is about twice the size of the contralateral control leg.

The rabbits in group 1 were killed 1 day after receiving the last daily injection of 20 ng TGF-β2. After three injections, the fibrous layer of the periosteum at the injection site on the antero-lateral cortex has dispersed and cannot be distinguished from the oedematous fibrous tissue. The cambial layer is thicker than that around the medial and posterior cortices, and also that around the same region of the control contralateral

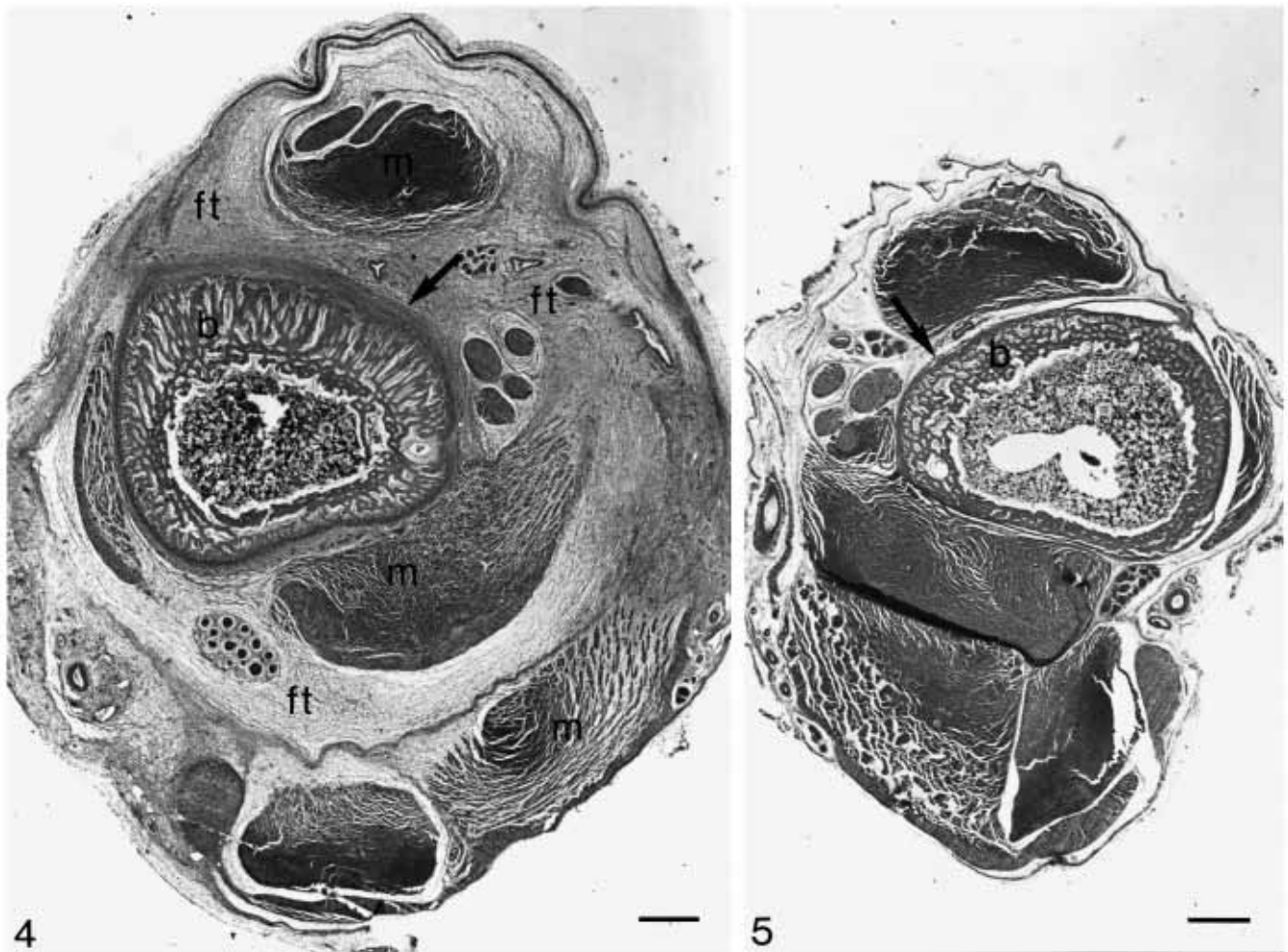
**Table 3. Estimated rates of periosteal bone or cartilage formation per day in response to exogenous TGF-β2 over the different experimental time periods, and in normally growing neonatal rabbits**

Actual age (days)	Injection days	Periosteal bone or cartilage formation per day (μm)			
		Experimental animals	Control animals		Anterior-posterior
			Medial-lateral	Anterior-posterior	
3	1				
4	2				
5	3				
6	4	86-194			
7	5		80	20	80
8	6	161-213			20
9	7				
10	8	207-300			
11	9		115	44	150
12	10				
13	11	123-177			
14	12				
15	13				
16	14				
17		152-177			
18			75	43	26
19					
20					
21					
22					
23					
24			53	33	<10

The period over which each calculation has been made is indicated by the lines.

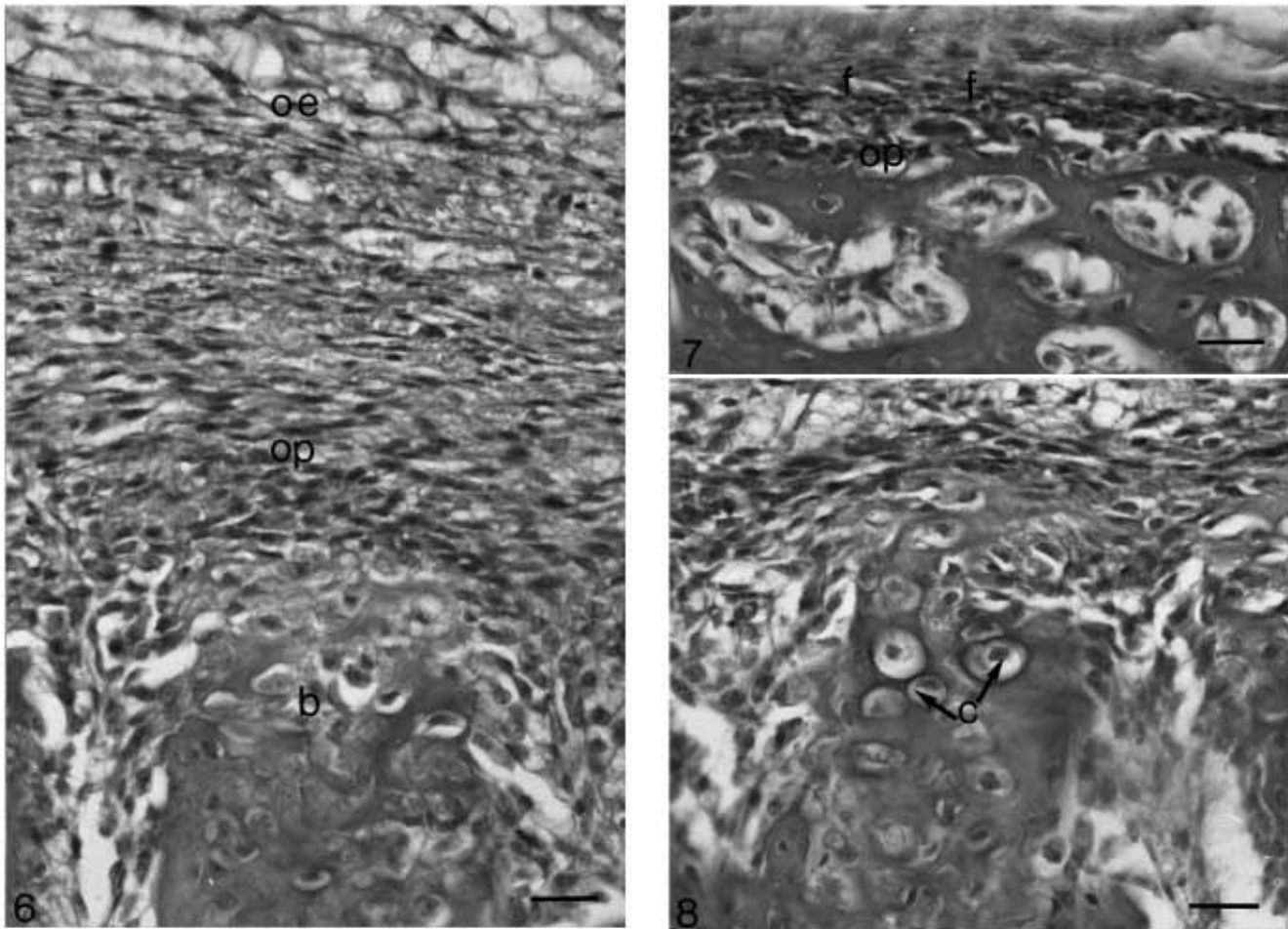


**Fig. 3.** Photomicrograph to show TRAP-positive osteoclasts (arrows) along the endosteal surface of the tibia of a 7-day-old rabbit. Bar, 100  $\mu$ m.



**Fig. 4.** Photomicrograph of a transverse section of the leg of a neonatal rabbit that had received 3 daily injections of 20 ng TGF- $\beta$ 2 (group 1; 6 days old). Oedema is present throughout the fibrous tissues (ft) so that the muscles (m) are pushed apart. The cortical bone is thicker than that of the contralateral control tibia (Fig. 5), and the antero-lateral cortex is thicker than the other cortices. It consists entirely of bone (b). The injection site is indicated by the arrow. Bar, 0.5 mm.

**Fig. 5.** Photomicrograph of the contralateral control tibia of that shown in Fig. 4 (group 1; 6 days old). The injection site is indicated by the arrow. The bone (b) is unaffected. Bar, 0.5 mm.



**Fig. 6.** Photomicrograph to show the thickened periosteum on the antero-lateral cortex of the tibia shown in Fig. 4 near the injection site. The outer fibrous layer has dispersed, while the inner, cambial layer consists of many layers of osteoprogenitor cells (op) adjacent to the developing trabeculae of bone (b). Above the osteoprogenitor cells, the tissue is oedematous (oe). Bar, 25  $\mu$ m.

**Fig. 7.** Photomicrograph of the same region of the periosteum of the control tibia shown in Fig 5. The periosteum consists of the outer fibrous layer (f) and the inner cambial layer that consists of 2 or 3 layers of osteoprogenitor cells (op). Bar, 25  $\mu$ m.

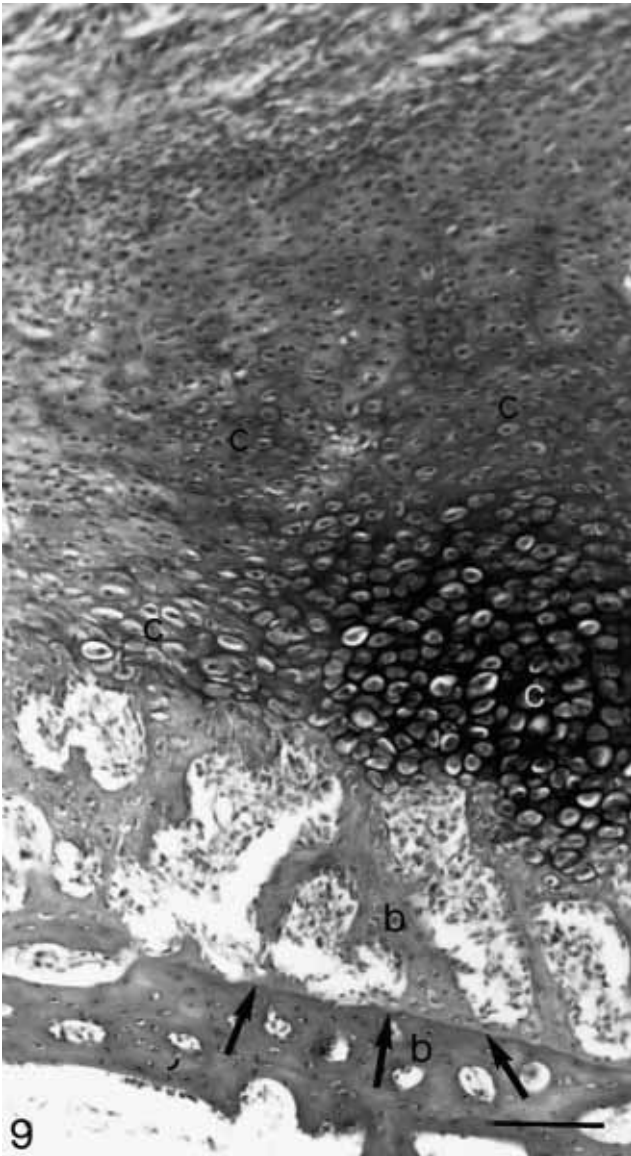
**Fig. 8.** Photomicrograph of the outer region of a developing trabeculum of bone in which there are isolated chondrocytes (c). The tibia had received 3 injections of 20 ng TGF- $\beta$ 2. (group 1; 6 days old). Bar, 25  $\mu$ m.

cortex (Figs 6,7). There is increased periosteal bone formation all round the tibiae, but at the injection site the cortex is now almost twice the thickness of the opposite cortex and that of the control contralateral tibia (Figs 4,5). The new bone forms as long trabeculae that are very thin peripherally. Small clusters of chondrocytes (about 5 to 10 cells) surrounded by an Alcian Blue-staining matrix were seen within a few trabeculae near the injection site of one tibia (Fig. 8).

With five injections, periosteal thickening continues. The amount of bone formation increases, but adjacent to the injection site areas of cartilage are developing between the cortical bone and the periosteum (Fig. 9). There may be one large area but, more frequently, there are two or more areas with smaller isolated groups of chondrocytes within developing bone trabeculae. The larger areas of cartilage are heterogeneous; there are a few hypertrophic chondrocytes near the bone, while next to the periosteum, chondrocytes are differentiating. After seven injections, the cartilage is buttressed by

new bone (Fig. 10). Away from the injection site bone is beginning to form between the cartilage and periosteum; this is appositional growth from the bone buttressing the cartilage. At the junction between the bone and cartilage endochondral ossification has started (Fig. 11). Because the chondrocytes are randomly arranged, ossification is disorganized compared with that in a growth plate. The presence of differentiating chondrocytes indicates that cartilage formation is continuing immediately under the periosteum near the injection site (Fig. 12). The periosteum is re-forming; the fibrous layer is present over most of the antero-lateral surface.

These processes continue so that after 10 and 14 injections the amount of new bone has increased and a greater area of the tibia is affected. The cartilage away from the injection site is now separated from the periosteum by a layer of bone (Figs 13,14). The areas of endochondral bone and of active endochondral ossification are larger (Figs 13,14). In regions away from the injection site the cartilage is entirely hypertrophic, but



**Fig. 9.** Photomicrograph of part of the tissue developing on the antero-lateral surface of a tibia that has received 5 daily injections of 20 ng TGF- $\beta$ 2 (group 1; 8 days old). The border between the original bone (b) and that produced after the injections is indicated by the arrows. Cartilage (c) is now developing on the periosteal surface of the bone. Bar, 100  $\mu$ m.

at the injection site there may still be a few differentiating chondrocytes (Fig. 16). After 14 injections, the cortical bone of the medial and posterior cortices is more compact and this contrasts with the long thin trabeculae under the injection site (Fig. 14). The smaller control contralateral tibia is shown in Fig. 15.

Throughout the experimental period, the changes induced by TGF- $\beta$ 2 are superimposed on rapid growth (see above). The medullary cavities of the experimental tibiae enlarge to approximately the same extent as those of the contralateral control tibiae (compare Figs 4,5, and 13,14,15). To facilitate this remodelling, many osteoclasts are present on the endosteal surface of the bone and in regions of endochondral ossification

(Figs 17,18). None of the bone present at the time of the first injection in the 3-day-old tibia remains after 14 injections.

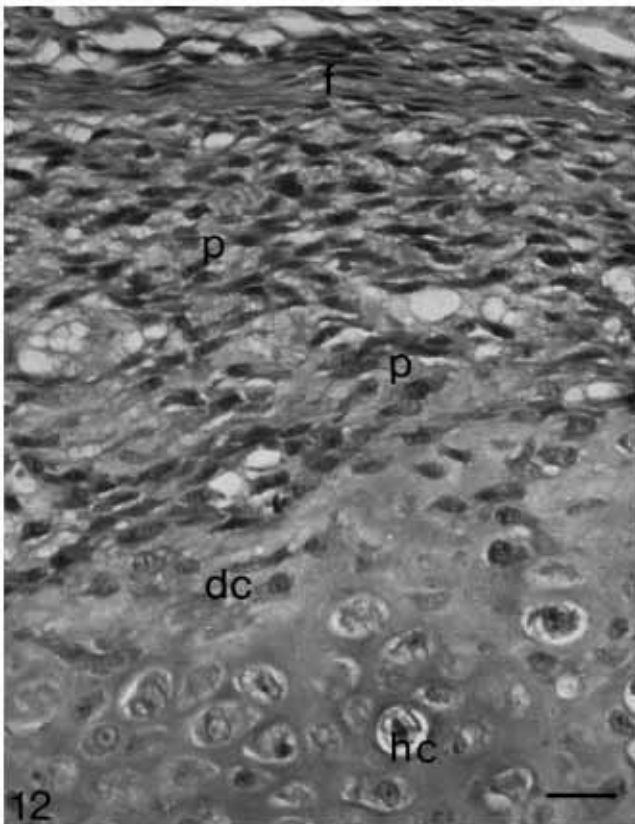
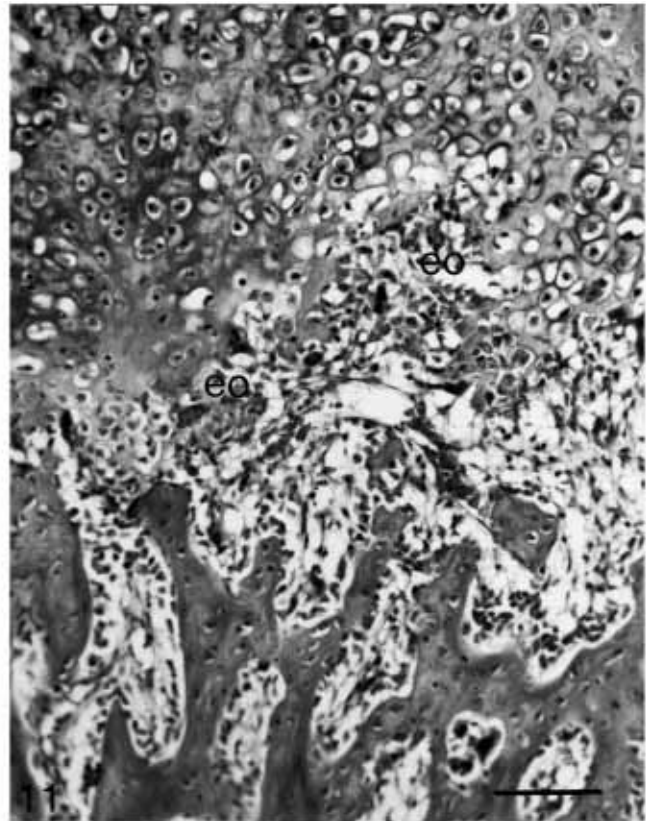
The relative areas of bone and cartilage induced by the injections of TGF- $\beta$ 2 were quantified and compared with the area of bone of the contralateral tibia. The results are shown in the histograms (Fig. 19). There is a gradual increase in the total area of the whole tibia, including bone and cartilage with increasing numbers of injections. The values calculated for the relative areas of bone, including endochondral bone, and cartilage around the injection site show progressive increases in these tissues in response to TGF- $\beta$ 2. After seven injections, the cross-sectional area of the tibia has increased by 2.5 times, after 14 injections by 3 times.

Calculations of the approximate amount of new bone or cartilage produced per day over the experimental period (see Table 3), indicate that in the experimental tibiae the rate of new matrix formation increases with the number of injections in animals up to 10 days old; the maximal rate is 300  $\mu$ m per day in animals that received seven injections of TGF- $\beta$ 2. Thereafter, despite increasing numbers of injections, the rate is below 177  $\mu$ m per day. These rates are between 2 and 3 times those of the uninjected control tibiae. In common with the uninjected control tibiae, the rate peaks when the rabbits are between 6 and 10 days old and declines thereafter.

A further series of 3-day-old rabbits (Table 1, group 2) was given daily injections of 5 ng TGF- $\beta$ 2 for 7 or 10 days and killed 1 day later. The effect is similar, but its development is slower than that following injections of 20 ng TGF- $\beta$ 2. The overall size of the tibiae is smaller than after the 20 ng dose and thus the amounts of additional bone and of cartilage are less (Fig. 20). Lateral buttressing of the cartilage by bone occurs after 10 injections, but at this stage the bone has not grown over the cartilage to separate it from the periosteum. The areas of cartilage are smaller than after injections of 20 ng TGF- $\beta$ 2. There are very few hypertrophic chondrocytes and endochondral ossification has not started.

The third group of 3-day-old rabbits (Table 1, group 3) was given 7, 10 or 14 daily injections of 20 ng TGF- $\beta$ 2, but they were not killed until 7 days after the final injection. At the time of killing, the legs were thinner than those of groups 1 and 2 because the oedema had resolved. The amount of fibrous tissue between the muscles is similar to that of the contralateral control leg (Figs 21,22). The periosteum is of approximately equal thickness around all aspects of the tibiae. In contrast, the bone on the injection side of the cortex is thicker than that of rabbits killed 1 day after the final injection. Most of this is endochondral bone because remnants of cartilage are present within the trabeculae. A small mass of cartilage with only hypertrophic chondrocytes remains under a layer of bone and the periosteum (Fig. 23). The overall size of the bone and remaining cartilage is greater than that of the tibiae 1 day after a series of 14 injections (compare Fig. 14).

The final group of neonatal rabbits (Table 2, group 4) were given one injection of 20 ng TGF- $\beta$ 2 and killed 4, 6 or 10 days later. The effects evident after 4 days are minimal. The periosteum is slightly thicker around the injection site and there may be a little extra bone formation in this region (Fig. 24), but after 6 or 10 days the tibiae (Fig. 25) are indistinguishable from the contralateral control tibiae.



**Fig. 10.** Photomicrograph of a tibia that has received 7 injections of 20 ng TGF- $\beta$ 2; the injection site is indicated by the arrow. One large region of cartilage (c) is present and it is buttressed by bone (arrowheads). Endochondral ossification is beginning (large arrow). Bar, 0.5 mm.

**Fig. 11.** Photomicrograph to show the region of endochondral ossification (eo) in the tibia shown in Fig. 10. Bar, 100  $\mu$ m.

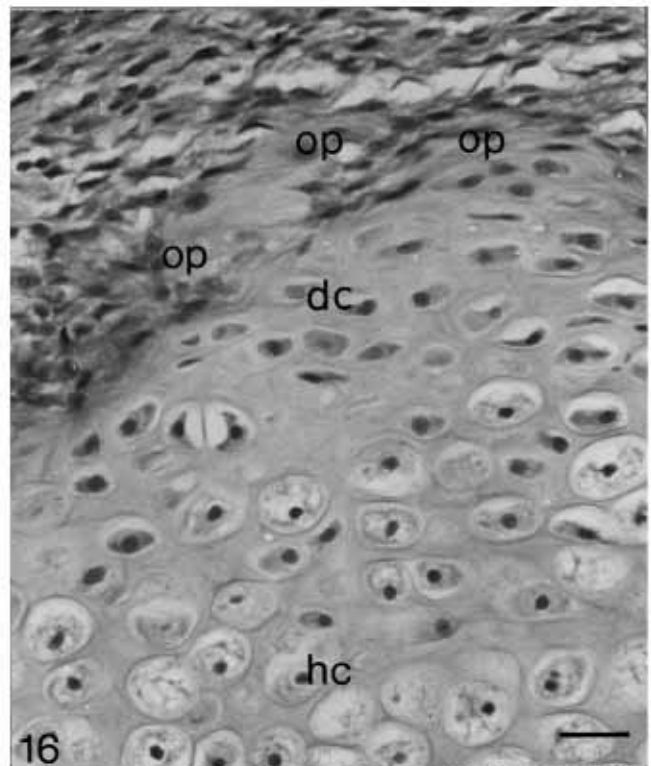
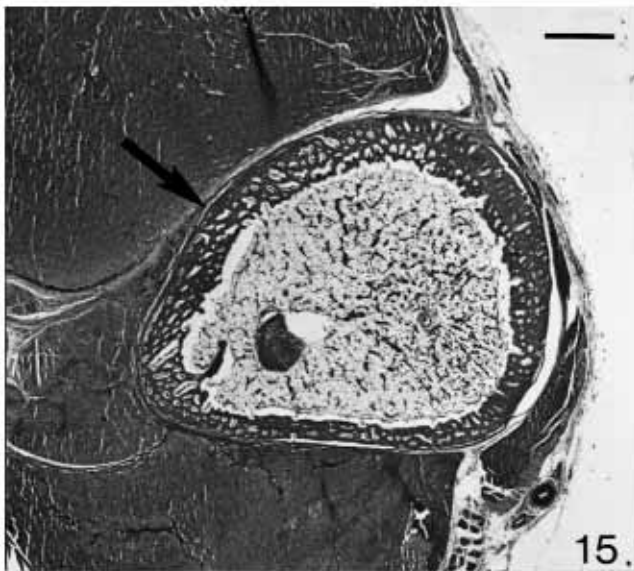
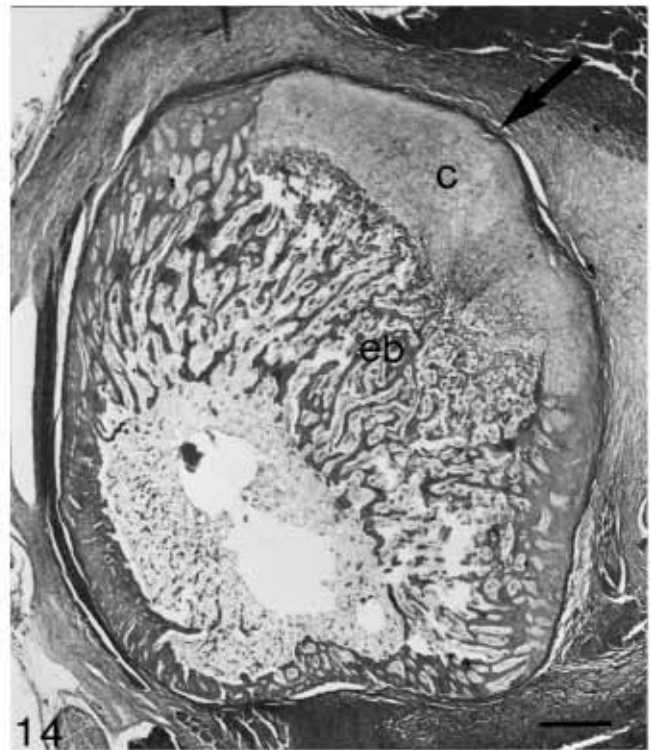
**Fig. 12.** Photomicrograph to show the superficial regions and periosteum of the tibia shown in Fig. 10 near the injection site. The cambial layer contains many layers of osteoprogenitor cells (p). Under this are layers of differentiating chondrocytes (dc), but very close to these the chondrocytes are becoming hypertrophic (hc). The fibrous layer (f) is re-forming. Bar, 25  $\mu$ m.

### Young adult rabbits, groups 5 to 7 (Table 2)

The bone in the mid-diaphyseal region of the tibiae in these animals is haversian. The rabbits received daily injections of 20 ng (group 5) or 200 ng (groups 6 and 7) TGF- $\beta$ 2 for 7 days and the tibiae were fixed 1 or 7 days later. There is some

oedema and an increase in the amount of fibrous tissue at the injection site and between the muscles. The layer of osteoprogenitor cells is thicker and overlies short trabeculae of new bone that have grown from the periosteal surface of the compact bone 1 day after the injections cease, irrespective of

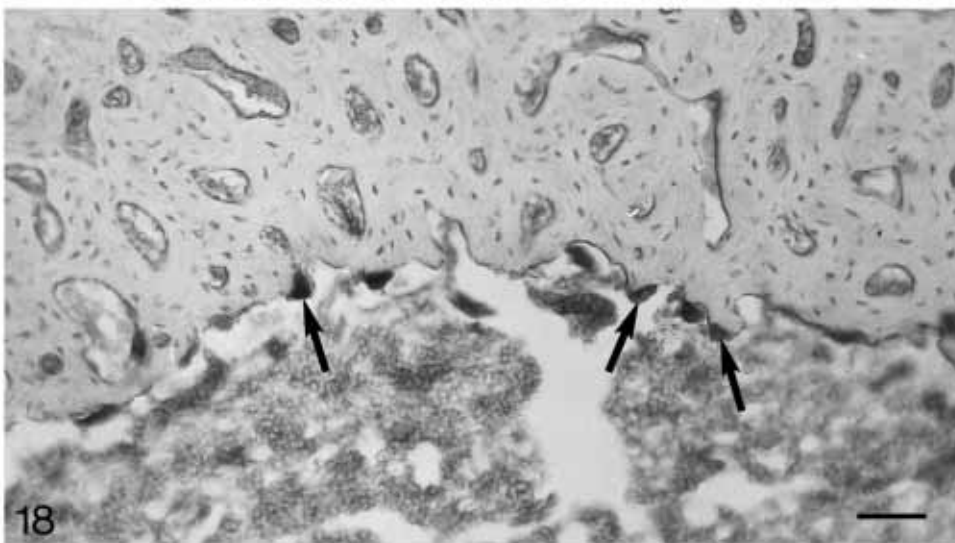
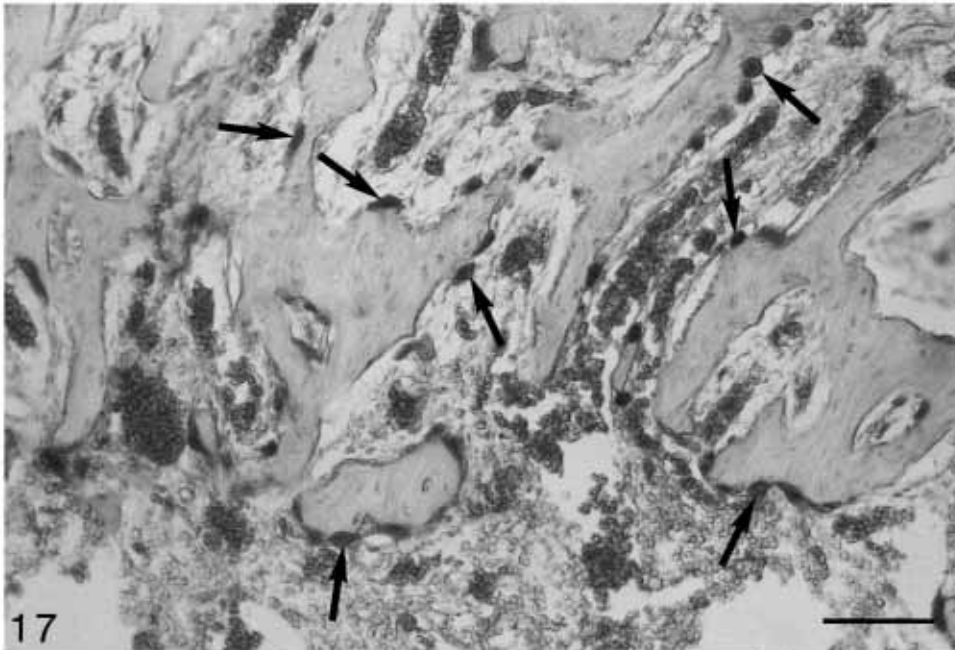




**Figs 13 and 14.** Photomicrograph of a tibia that has received 14 injections of 20 ng TGF- $\beta$ 2 (group 1; 17 days old). The tibia is now very large compared to the contralateral control tibia (compare Fig. 15). The area of induced bone is very large and much of this is endochondral bone (eb). The area of cartilage (c) is relatively smaller. In Fig. 13 it is covered by a layer of bone (small arrows), but in Fig. 14, which is at the injection site (large arrow), it is covered only by the periosteum. Bar, 0.5 mm.

**Fig. 15.** Photomicrograph of the control contralateral tibia of the 14 injection tibia shown in Figs 13 and 14 (group 1; 17 days old). The injection site is indicated by the arrow. Bar, 0.5 mm.

**Fig. 16.** Photomicrograph to show the periosteal region of the tibia in Fig. 14 in which the osteoprogenitor cells (op) overlie a few differentiating chondrocytes (dc) and the hypertrophic chondrocytes (hc) are immediately beneath them. Bar, 25  $\mu$ m.



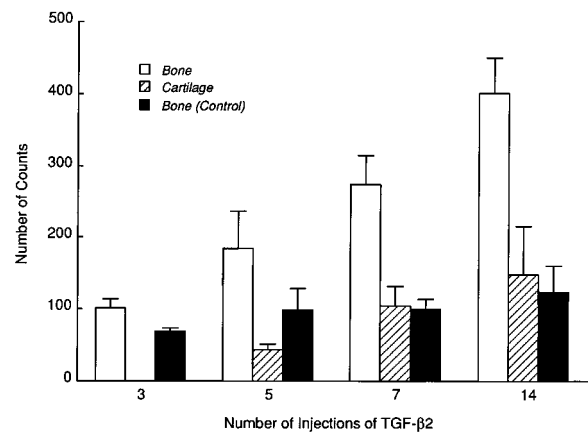
**Fig. 17.** Photomicrograph to show TRAP-positive osteoclasts (arrows) on the endosteal surface and along the trabeculae in the region under the injection site of the tibia shown in Figs 13 and 14 (group 1; 17 days old). Bar, 100  $\mu$ m.

**Fig. 18.** Photomicrograph to show TRAP-positive osteoclasts (arrows) along the endosteal surface of the control tibia shown in Fig. 16. Bar, 100  $\mu$ m.

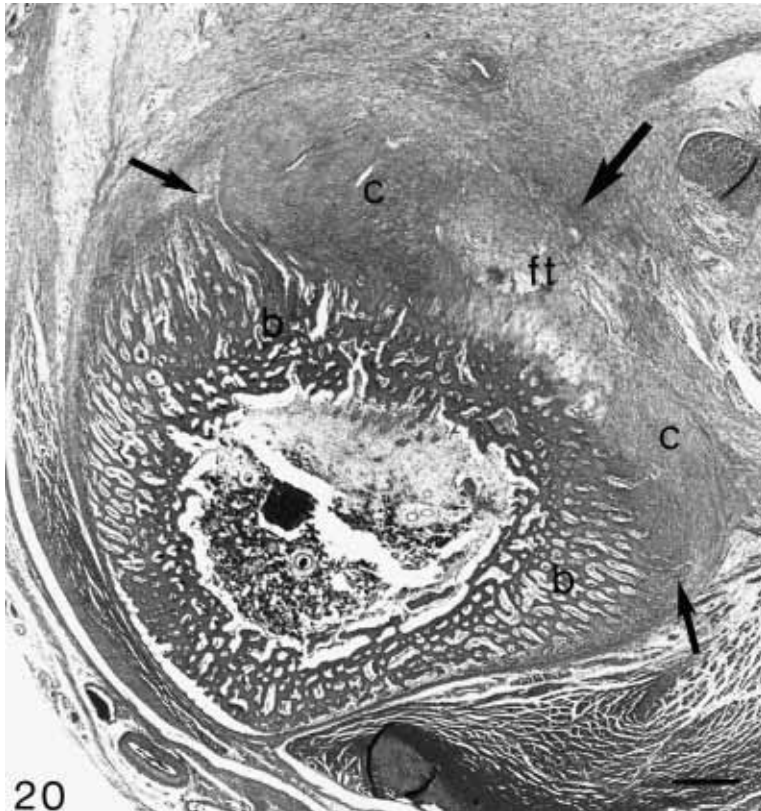
dosage (groups 5 and 6) (Fig. 26). Seven days after the injections of 200 ng TGF- $\beta$ 2 cease (group 7), small isolated nodules of cartilage are present on the surface of the newly formed bone (Fig. 27). Most chondrocytes are hypertrophic and endochondral ossification is taking place.

**Mature adult rabbits, groups 8 to 10 (Table 2)**

One day after seven daily injections of 20 ng (group 8) or 200 ng TGF- $\beta$ 2 (group 9), the periosteum around the injection site is disrupted. There is proliferation of the osteoprogenitor cells and oedema in the surrounding tissues. Thick strands of extracellular material are present in the fibrous tissue near the site after injections of 20 ng TGF- $\beta$ 2 (Fig. 28), while after the 200 ng injections, a thin layer of bone is found. Seven days after seven injections of 200 ng TGF- $\beta$ 2 (group 10), a thicker layer of cancellous bone has formed around the injection site (Fig. 29) and this spreads as a thin layer of new matrix over the



**Fig. 19.** Histograms to show the relative amounts of bone, cartilage and endochondral bone formed in response to injections of 20 ng TGF- $\beta$ 2 into the periosteum of 3-day-old neonatal rabbits.



**Fig. 20.** Photomicrograph of a tibia that received 7 injections of 5 ng TGF- $\beta$ 2 (group 2; 10 days old). The amount of bone (b) and cartilage (c) induced by the injections is smaller than that induced by the larger doses (compare Fig. 10). Two small areas of cartilage are separated by fibrous tissue (ft) at the injection site (large arrow). Bone buttresses the sides of the cartilage (small arrows). Endochondral ossification has not started. Bar, 0.5 mm.

entire periosteal surface. Groups of chondrocytes are present within some bony trabeculae; the cells are not hypertrophic.

### Periosteal removal

These experiments were carried out on 17-day-old rabbits. Four and six days after disruption, there is thickening of the periosteum and surrounding fibrous tissues, and a few small trabeculae of new bone are present in this tissue (not shown). At 10 days, the bone looks normal, except for a slight thickening of the cortical bone at the removal site.

### DISCUSSION

The experiments described here demonstrate that injections of TGF- $\beta$ 2 into the periosteum of rabbit tibiae of different ages stimulate the proliferation of osteoprogenitor cells. Subsequently, depending on the age of the animal and the injection regime, extra bone and also cartilage may be formed. These findings are similar to those of Joyce et al. (1990), who examined the effects of TGF- $\beta$ s 1 and 2 on neonatal rat femora.

The bone of the tibia of neonatal rabbits is rapidly turning over. That none of the bone present in the 3-day-old remains in the 11-day-old rabbit must be taken into consideration when interpreting the results of the experiments on neonatal rabbits.

The effects of TGF- $\beta$ 2 are not confined to the cambial layer of the periosteum. There may be an increase in the number of fibroblasts both in the fibrous layer of the periosteum, which is dispersed around the injection site, and in the fibrous tissue between the developing muscles, but the muscle fibres appear unaffected. The major part of the swelling of the experimental

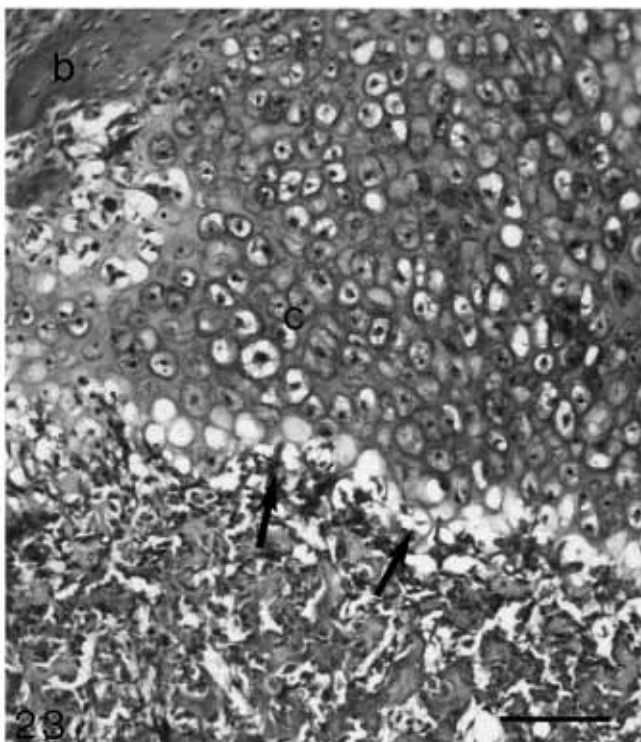
leg is caused by oedema, which diminishes rapidly when injections cease.

In neonatal animals the rapid proliferation of osteoprogenitor cells in response to daily injections of 20 ng TGF- $\beta$ 2 leads initially to rapid new bone formation, so that after three injections the cortex under the injection site has doubled in thickness. Cartilage proper is not seen until five injections have been given, that is, 6 days after injections have started. This contrasts with neonatal rat femora in which a large area of cartilage is present after four injections. This may be partly a dosage effect because the rat femora received injections of 200 ng TGF- $\beta$ 2. Joyce et al. (1990) note that lower doses appear to favour bone formation. Dose dependence was observed in neonatal rabbit tibiae; after seven injections of 20 ng TGF- $\beta$ 2 the area of cartilage at the injection site is much larger than that formed after seven injections of 5 ng TGF- $\beta$ 2.

The effect of a single injection of TGF- $\beta$ 2 is transient. The increased thickness of the cortex 4 days after the injection suggests that cell proliferation is enhanced, but by 6 days, the experimental tibiae cannot be distinguished from the controls.

Disruption of the periosteum in young adult rabbits leads to periosteal bone formation (D. E. Ashhurst, unpublished observation), but in the neonatal rabbits, although there was periosteal proliferation, bone formation was restricted to a few small trabeculae. This reaction is comparable to that after a single injection of TGF- $\beta$ 2 and could be masked by the rapid turnover of the neonatal bone.

These results indicate that the primary effect of TGF- $\beta$ 2 is to increase the number of osteoprogenitor cells. Their differentiation into osteoblasts and the formation of bone follows.



**Fig. 21.** Photomicrograph of a tibia 7 days after 10 injection of 20 ng TGF- $\beta$ 2 (group 3; 19 days old). The injection site is indicated by the arrow. Only a small amount of cartilage (c) remains, but there is a very large area of endochondral bone (eb). The region indicated by the arrowhead is shown at higher magnification in Fig. 23. Bar, 0.5 mm.

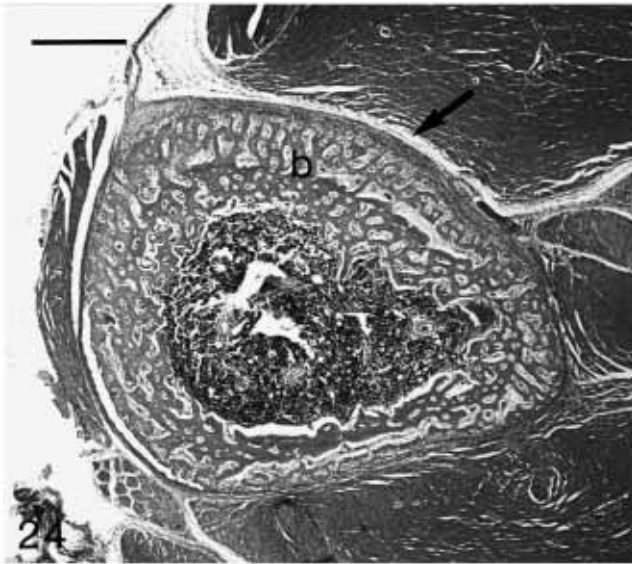
**Fig. 22.** Photomicrograph of the control, contralateral tibia of the injected tibia shown in Fig. 21. The injection site is shown by the arrow. Bar, 0.5 mm.

**Fig. 23.** Photomicrograph of part of the cartilage remaining on the tibia shown in Fig. 21. It is covered by bone (b). All the chondrocytes (c) are hypertrophic and endochondral ossification is underway (arrows). Bar, 100  $\mu$ m.

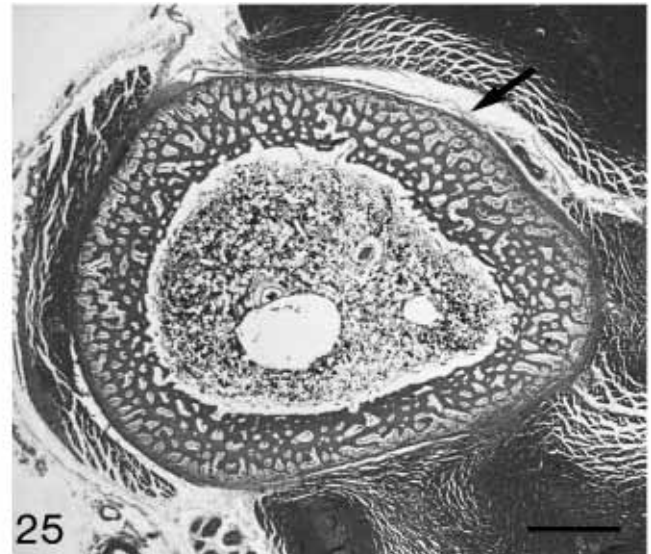
Chondrocytic differentiation and cartilage formation is a later event confined to the area near the injection site where the periosteal response is maximal.

The time of onset of endochondral ossification is earlier in rabbits than in rats. Hypertrophic chondrocytes are seen after five injections of 20 ng TGF- $\beta$ 2 and endochondral ossification

has started after seven injections. In the rat femur, hypertrophic chondrocytes are present only after a series of 14 injections of 200 ng TGF- $\beta$ 2, and Joyce et al. (1990) emphasize that endochondral ossification is not seen until 7 days later, that is, only after TGF- $\beta$  is withdrawn. In the rabbit experiment, chondrocytic differentiation to hypertrophy takes about



**Fig. 24.** Photomicrograph of a tibia 4 days after a single injection (arrow) of 20 ng TGF- $\beta$ 2 (group 4; 7 days old). The bone (b) of the antero-lateral cortex is thicker than that of the other cortices. Bar, 0.5 mm.



**Fig. 25.** Photomicrograph of a tibia 6 days after a single injection (arrow) of 20 ng TGF- $\beta$ 2 (group 4; 9 days old). The tibia appears normal, there is no indication of additional bone formation. Bar, 0.5 mm.

2 days and endochondral ossification starts within the next 2 days. These times parallel those in the rat growth plate (Farnum and Wilsman, 1993) and in rabbit fracture callus (Ashhurst, 1986). TGF- $\beta$  inhibits chondrocyte hypertrophy in vitro (Ballock et al., 1993a; Tschan et al., 1993), but if TGF- $\beta$  has the same effect in vivo, then in the neonatal rabbit, TGF- $\beta$ 2 must not penetrate from the injection site to the region of hypertrophy.

The response of the mature haversian bone to TGF- $\beta$ 2 is very different. Around the injection site, there is proliferation of the periosteal progenitor cells and local oedema. Seven injections of 20 ng TGF- $\beta$ 2 induce a thin layer of bone in 3-month-old rabbits and none in the 2-year-old rabbits. Increasing the amount of TGF- $\beta$ 2 administered to 200 ng raised the amount of bone produced and 7 days after the last injection small isolated areas of cartilage with hypertrophic chondrocytes were present in the 3-month-old rabbit, but in the 2-year-old rabbit there were only scattered groups of chondrocytes within the bony trabeculae.

In other in vivo investigations, TGF- $\beta$  was injected onto the calvaria of mice or rats. It induced an increase in the number of periosteal osteoprogenitor cells and subsequently a 50 to 100% increase in the thickness of the calvarial bone in the region around the injection site (Harashima et al., 1992; Mackie and Trechsel, 1990; Marcelli et al., 1990; Noda and Camilliere, 1989). Similar results were obtained with animals up to 6 weeks old. In old rats, Mackie and Trechsel (1990) found no bone formation, but an increase in the percentage of extracranial bone surfaces covered by osteoblasts. The injections of TGF- $\beta$  were of 1 to 5  $\mu$ g, that is, up to 250 times the dose used in the present experiments. In contrast, Harashima et al. (1992) reported that in 12-week-old rat calvariae, TGF- $\beta$  induces the proliferation of chondrocytes and cartilage formation.

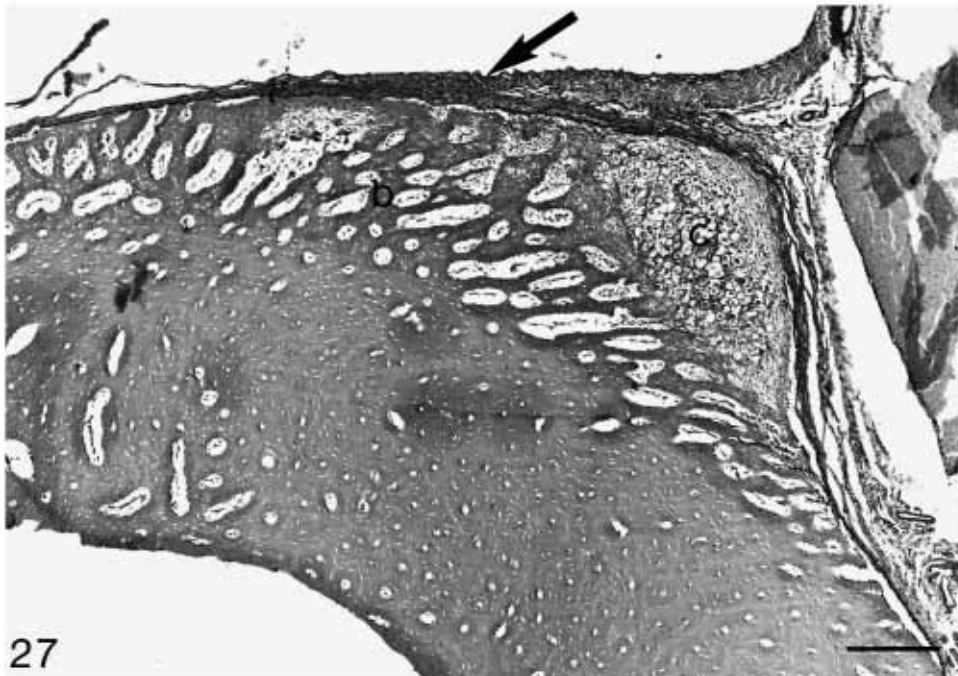
TGF- $\beta$ s increase collagen, osteocalcin and osteopontin

syntheses and alkaline phosphatase activity in osteoblastic cultures, while in chondrocytic cultures, it increases synthesis, size and sulphation of proteoglycans (Ber et al., 1991; D'Angelo and Green, 1991; Morales, 1991; Redini et al., 1991; Strong et al., 1991; Wrana et al., 1991). This raises the possibility that TGF- $\beta$ 2 produces the observed increase in matrix synthesis in the experimental tibiae by increasing the rate of synthesis per cell. This is considered unlikely because the ratio of cells to matrix appears to be higher in the TGF- $\beta$ 2-induced matrices than in the normally developing matrices.

### Proliferation of osteoprogenitor cells

The periosteal surface of growing bones is covered by three layers of cells: fibroblasts in the fibrous layer, preosteoblasts or osteoprogenitor cells (defined as those cells not on the bone surface) in the cambial layer, and a layer of osteoblasts on the bone. Owen (1963) from a detailed study of cell population kinetics in growing femora of 14-day-old Dutch rabbits concluded that the osteoblast population is enlarged mainly by division of osteoprogenitor cells and that the majority of the osteoblasts do not divide again (Owen and Macpherson, 1963). This conclusion was also reached by Kember (1960), Tonna (1961) and Young (1962) from kinetic experiments using young rat and mouse tibiae.

Normal growth rates in the rabbit decrease with age. This may be correlated with a decrease in both the rate of cell division and the number of cells capable of division with age. Reduction in the number of osteoprogenitor cells that are dividing and increases in cell cycle times with age in rodents have been reported (Owen, 1963; Tonna, 1961; Tonna and Cronkite, 1962; Young, 1962). Other indications of these changes are that very few cells released from the cortical bone of 2-year-old rats retain the capacity to proliferate.



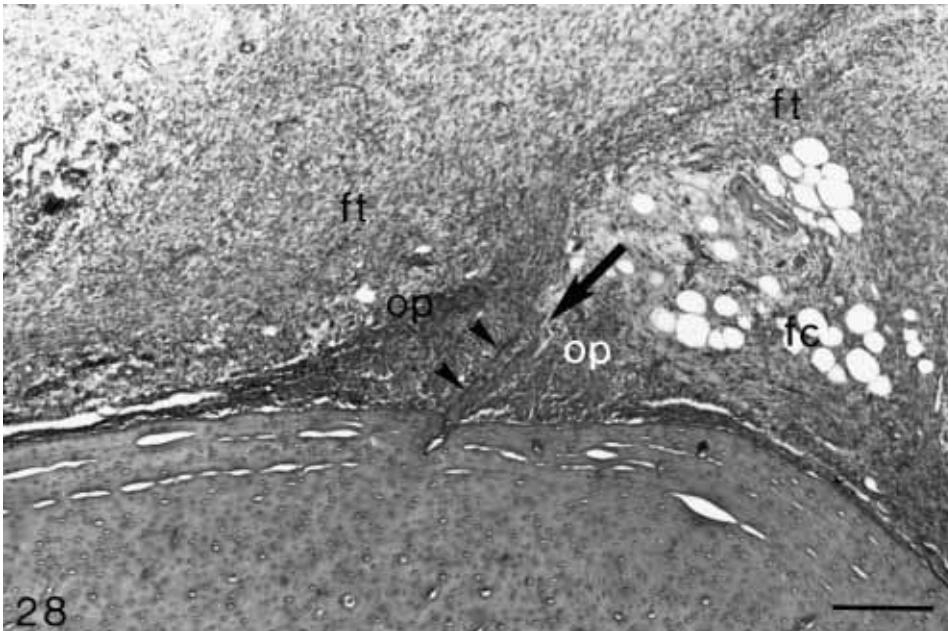
**Fig. 26.** Photomicrograph of a tibia of a 3-month-old rabbit 1 day after 7 daily injections of 20 ng TGF- $\beta$ 2 (group 5). A thin layer of bone (b) has formed along the antero-lateral cortex; there is no cartilage. The injection site is indicated the arrow. Bar, 0.25 mm.

**Fig. 27.** Photomicrograph of a tibia of a 3-month-old rabbit 7 days after 7 injections of 200 ng TGF- $\beta$ 2 (group 7). A thin layer of bone (b) has formed and an isolated nodule of cartilage (c) is present. The arrow indicates the injection site. Bar, 0.25 mm

erate in vitro (Mabrey et al., 1993), and that human osteoblasts in vitro show a decreased response to mitogenic growth factors with age (Pfeilschifter et al., 1993). A decrease in matrix synthesis with age is illustrated by reductions in the mRNAs for the bone proteins (Kuhn et al., 1993; Turner and Spelsberg, 1991) and the absence of periosteal new bone formation in turkey ulnae in response to physical stimulae at 3 years old (Rubin et al., 1992). These observa-

tions may explain, in part, the reduced response of the osteoprogenitor cells of the skeletally mature rabbit to exogenous TGF- $\beta$ 2.

The first crucial step in the development of periosteal bone or cartilage is the proliferation of the osteoprogenitor cells. Thus the primary effect of exogenous TGF- $\beta$ 2 must be to raise the proliferative rate of these cells. The estimations of the rate of new bone formation in control rabbits from 3 to 24 days



**Fig. 28.** Photomicrograph of part of the tibia of a 2-year-old rabbit 1 day after 7 daily injections of 20 ng TGF- $\beta$ 2 (group 8). There is oedema and an increase of fibrous tissue (ft); some fat cells (fc) are present. The osteoprogenitor cells (op) near the injection site (arrow) have proliferated. There is no obvious bone formation, but there are some strands of amorphous material radiating outwards from the bone (arrowheads). The arrow indicates the injection site. Bar, 0.25 mm.

old ranges from 20 to 150  $\mu$ m per day, which is in general agreement with Owen's (1963) value of 78  $\mu$ m per day in Dutch rabbits between 14 and 19 days old. To achieve the rates of 86 to 300  $\mu$ m per day seen after TGF- $\beta$ 2 injections, the proliferative rate of the osteoprogenitor cells must be increased by 2 to 3 times. A similar increase in their proliferative rate was deduced by Hock et al. (1990) in foetal rat calvariae treated with TGF- $\beta$  in organ culture. The effect of TGF- $\beta$ 2 on osteoprogenitor cells of older rabbits with a low proliferative rate may in fact be comparable to that on the neonatal rate, but it results in a very small increase in total cells.

The conclusion that the primary effect of TGF- $\beta$ 2 is to increase the proliferative rate of osteoprogenitor cells may appear to conflict with the evidence that TGF- $\beta$  inhibits proliferation of osteoblastic cell lines (Noda and Rodan, 1986; Pfeilschifter et al., 1987), but these are transformed, differentiated osteoblasts and great emphasis is placed on their osteoblastic properties. In vivo differentiated osteoblasts rarely, if ever, divide (Kember, 1960; Owen, 1963; Tonna, 1961; Young, 1962). The findings that TGF- $\beta$  may increase DNA synthesis and proliferation in calvarial cells (Centrella et al., 1986; Ten Dijke et al., 1990) are in general agreement with the present observations because the population of cells that migrate from calvariae includes osteoprogenitor cells as well as osteoblasts.

### Differentiation of osteoprogenitor cells

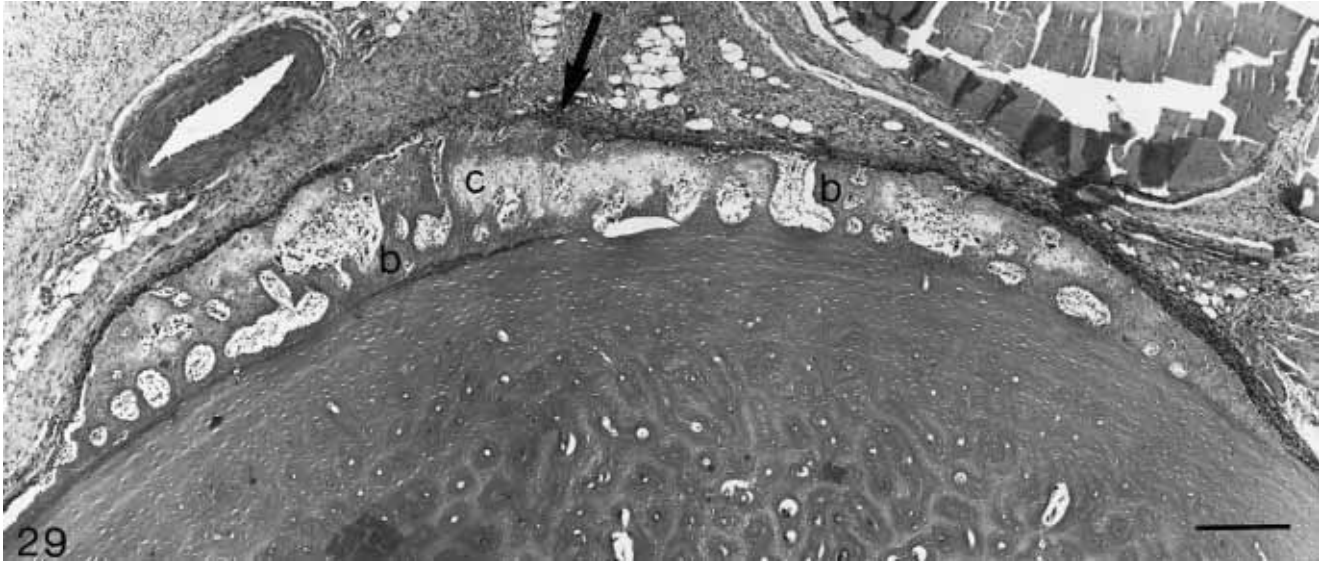
The mechanism that initiates the differentiation of osteoprogenitor cells into osteoblasts or chondrocytes is not understood. There is a precise spatial arrangement of bone and cartilage in the experimental tibiae. Because the additional bone forms before cartilage, the environmental and other factors that control differentiation must initially favour differentiation of osteoblasts. It is pertinent to consider how these factors may alter and then stimulate chondrocytic differentiation.

The blood supply is one possible environmental factor. Bone formation occurs only in well vascularized regions. In contrast, cartilage develops in regions of sparse blood supply. Examples of this correlation between cartilage formation and the blood supply are seen in the callus of mechanically unstable fractures and developing limb buds (Ashhurst, 1986; Caplan and Koutropas, 1973; Hallman et al., 1987; Wilson, 1986). Furthermore, chondrocytic differentiation in culture is favoured by reduced oxygen tensions (Bassett and Herrman, 1961; Pawelek, 1969) and they are sensitive to toxic oxygen-derived substances in the medium (Tschan et al., 1990).

In the neonatal tibia, the blood supply to the diaphyseal cortical bone is endosteal and the capillaries must grow continually outwards to supply the newly formed periosteal bone. Any increase in the rate of bone formation must be matched by a corresponding increase in the rate of capillary growth to supply the extra bone. It is suggested that over the injection site the rate of capillary growth is insufficient to keep pace with increased osteoprogenitor cell proliferation and the resulting poorly vascularized region favours differentiation into chondrocytes. Where proliferation is slower away from the injection site, cartilage formation is less, or absent.

Joyce et al. (1990) suggest that the differentiation of osteoprogenitor cells might be influenced by the concentration of exogenous TGF- $\beta$ 2, so that where it is high, for example around the injection site, chondrocytic differentiation occurs. The results in the rabbit do not support this suggestion, because the initial response around the injection site is increased bone formation.

The results presented here do not support the hypothesis that TGF- $\beta$ s initiate osteoblastic or chondrocytic differentiation directly. The suggested evidence for this hypothesis is that osteoblasts and chondrocytes express the mRNAs for and synthesize the TGF- $\beta$  proteins (Joyce et al., 1990). TGF- $\beta$ , however, up-regulates the expression of mRNAs for, and the secretion of types I and II collagens, by periosteal cells that have differentiated into osteoblasts and chondrocytes in vitro



**Fig. 29.** Photomicrograph of part of the tibia of a 2-year-old rabbit 7 days after 7 injections of 200 ng TGF- $\beta$ 2 (group 10). The arrow indicates the injection site. There is a layer of bone (b) along the periosteal surface. Cartilage (c) is confined to small areas within the bone (b). Bar, 0.25 mm.

(Ballock et al., 1993b; Izumi et al., 1992; Miura and O'Driscoll, 1993), which suggests that it acts as an autocrine regulator of matrix synthesis. Other factors must be responsible for initiating differentiation and the subsequent synthesis of molecules such as TGF- $\beta$ s.

Several other growth factors have been suggested as candidate differentiative agents. These include the bone morphogenetic proteins (BMPs) of which eight are known (Wozney et al., 1990) and which, in ectopic sites, induce cartilage, followed by endochondral bone formation (Reddi, 1981). In contrast, TGF- $\beta$ 1 (=CIF-A) injected or implanted on a carrier subcutaneously stimulates only fibrosis and collagen synthesis (Bentz et al., 1987; Roberts et al., 1986). Many other growth factors, for example, acidic and basic fibroblast growth factors, insulin-like growth factor and platelet-derived growth factor, are present in developing and healing skeletal tissues and could act as differentiative agents.

Periosteal osteoprogenitor cells can differentiate into osteoblasts or chondrocytes *in vivo*. Because cultures derived from periosteal cells contain both osteoblast-like and chondrocyte-like cells, it has been postulated that there are committed osteoblast- and chondrocyte-progenitor cells (Izumi et al., 1992; Nakahara et al., 1990, 1991; Nakase et al., 1993). This contradicts the *in vivo* experimental evidence presented here. If the osteoprogenitor cells are committed, it follows: firstly, that unless the bone is injured, the prechondrocytes are redundant; and secondly, that after injury, or experimental stimulation, selective migrations of prechondrocytes and preosteoblasts must take place to produce discrete areas of bone and cartilage.

It is most probable that differentiation in this experimental model is controlled by both the environmental factors, such as the blood supply, and a combination of endogenous growth factors. TGF- $\beta$ 2 induces oedema; the lymphocytes, macrophages and other cells associated with the oedema are potential sources of these growth factors.

## Conclusions

Exogenous TGF- $\beta$ 2 injected into the periosteum increases the proliferation of osteoprogenitor cells; this response is age-dependent. It is most probable that under normal conditions *in vivo*, proliferation is controlled by many factors of which TGF- $\beta$ 2 may be one. It is noteworthy that the proliferation of osteoprogenitor cells necessary to form a fracture callus in a mature animal is considerably greater than that induced by TGF- $\beta$ 2 in the most rapidly growing rabbits.

It is argued here that there is no conclusive evidence that TGF- $\beta$  is a differentiative factor for osteoprogenitor cells. It is concluded that local environmental factors, as well as growth factors, some not yet identified, are responsible for the differentiation of osteoprogenitor cells.

It is obvious from these experiments and those on the rat and mouse calvariae and femora (Joyce et al., 1990; Mackie and Trechsel, 1990; Marcelli et al., 1991; Noda and Camilliere, 1989), that TGF- $\beta$ s induce proliferation of osteoprogenitor cells. Conversely, TGF- $\beta$ s inhibit proliferation of other cells, including those of tumours (Shull et al., 1992). This anomaly has yet to be resolved.

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