European Journal of Orthodontics 18 (1996) 237–243

The influence of transforming growth factor β1 on the development of embryonic mouse long bones

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SUMMARY Transforming growth factor β (TGF-β) is an important regulator of bone metabolism, and is found in large quantities in embryonic and adult bone tissue. The influence of TGF-β1 on chondro-osteogenesis was studied. In organ cultures of developing long bone rudiments of embryonic mice, growth and development of the various cartilaginous and osseous compartments were investigated by morphometric analysis and autoradiography after [^{3}H]-thymidine labelling.

TGF-β1 (l ng/ml) inhibited both chondrogenesis and osteogenesis, and also inhibited matrix calcification. The effect was greatest in cell populations with the highest proliferation rate. It was noticed that the bone collar formation was inhibited. This may be due to an inhibition of osteoblast proliferation or differentiation, but it seems more likely to be an inhibition of the manufacture of matrix substance. These data suggest that TGF-β1 may be an important regulator of embryonic bone development.

Introduction

Bone remodelling processes are influenced by orthodontic treatment. Mechanical forces used to achieve the desired movement of teeth are distributed over the periodontium and bone tissues, which respond by resorption and apposition (Reitan, 1951). The regulation of bone remodelling is still not fully understood. Both environmental factors (such as nutrition, mechanical loading, chemical agents) and internal factors (such as the central nervous system, genetic influences, age, function) are involved. They act on the bone cells, via complex interactions with systemic and local regulators, i.e. hormones and growth factors (Canalis, 1983).

Hormones, synthesized by glands, are distributed in tissues by the circulation of the blood (endocrine factors) (D'Ercole and Underwood, 1985). Growth factors are synthesized by many different cell types, and act on neighbouring cells in the same tissue (paracrine factors), or on their producer cells (autocrine factors) (D'Ercole and Underwood, 1985).

In the past 10 years many growth factors have been described (Canalis *et al.*, 1988). Several of them have an effect on the bone remodelling process and are synthesized by the skeletal cells found in the bone matrix (Linkhart *et al.*, 1987; Centrella *et al.*, 1988; Canalis *et al.*, 1989).

During orthodontic treatment, interaction seems to occur between applied mechanical stimuli and local growth factors. Local input of a mechanical stimulus seems to activate cells of the periodontium and the alveolar bone (van de Velde *et al.*, 1988). It is speculated that these cells translate pressure and tension into secretion or binding of growth factors that activate osteoclasts and osteoblasts, resulting in bone resorption and bone apposition. Yamauchi *et al.* (1992) found a relationship between intermittent compressive force, and the synthesis of a growth factor in cultured bone cells.

Growth factors can cause both inhibition and stimulation of cell proliferation, as well as other reactions, such as the secretion of cell products (Centrella *et al.*, 1988). Growth factors which have been isolated from, and act on, bone and cartilage are: insulin-like growth factor (IGF I and II), fibroblast growth factor (FGF), platelet derived growth factor (PDGF) and the transforming growth factor-β (TGF-β) family

(Linkhart *et al.*, 1987; Centrella *et al.*, 1988; Canalis *et al.*, 1989). The latter comprises a family of peptides, including among others, TGF-β1, TGF-β2 and TGF-β3 and the subfamily of bone morphogenetic proteins (BMP).

TGF-βs are important regulators of bone and cartilage tissue *in vivo* (Wozney, 1989; Noda and Camilliere, 1989; Joyce *et al.*, 1990). TGF-β has been shown to stimulate chondrogenesis and osteogenenesis after *in vivo* injection into periosteal skeletal tissue (Noda and Camilliere, 1989; Joyce *et al.*, 1990). This growth factor has been described as a multifunctional peptide that controls cellular activity, such as proliferation, differentiation and other functions in many cell types (Massagué, 1987). Many cells, including osteoblasts, synthesize TGF-β and most of them have specific receptors for this peptide (Sporn *et al.*, 1986; 1987). Seyedin *et al.* (1986) found high concentrations of TGF-β1 in bone indicating a possible role during skeletal development. In the developing mouse embryo, TGF-β is conspicuous in craniofacial tissues derived from neural crest mesenchyme, such as the palate, larynx, facial mesenchyme, nasal sinuses, meninges and teeth (Heine *et al.*, 1987).

The aim of this experimental study was to investigate the influence of TGF-β1 on chondro-osteogenesis in long bones. Rudiments derived from embryonic mice were used, and were treated with TGF-β1 during organ culture.

Materials and methods

Organ culture

The middle three long bone rudiments from the metatarsus of 15- and 16-day-old Swiss albino mouse embryos [15 or 16 days post conception (p.c.)] were used.

The long bones were dissected using a standard procedure without removal of the cartilaginous ends, and care was taken not to damage the perichondrium. They were cultured in α-minimal essential medium (MEM) without-nucleosides (Gibco, UK) supplemented with 10% rat serum (HSD, Zeist, The Netherlands) and 1 mM Naβ-glycerophosphate (complete medium), with or without 1 ng/ml TGF-β1.

The long bones were cultured in 48-well tissue culture plates (Greiner, Alphen a/d Rijn, The Netherlands) in 150 μl medium, incubated in 95% air plus 5% CO_2 at 37°C and 98 per cent humidity. Culture time was 2 or 4 days and the medium was renewed on the 2 second day. Human TGF-β1 (R&D Systems, Minneapolis, USA) was added to the experimental cultures at a concentration of 1 ng/ml, throughout the culture period.

The length of the whole long bone (Fig. 1, zone A) and of the calcified bone centre (Fig. 1, zone Ca) were measured at days 2 and 4 of the culture, using an inverted microscope.

Histomorphometry

Cultured and non-cultured bones were fixed in 4% formalin (Merck, Darmstadt, Germany) containing 0.1% $CaCl_2$ in dextran 70000 (Macrodex, dextran poviet 70, Organon Teknika, Oss, The Netherlands) for 2 hours at 4°C, dehydrated in a graded series of ethanol and embedded in hydroxyethylmethacrylate (BDH, Poole, UK). Sections (3 μm) were cut with glass knives on a microtome (Reichert-Jung, Heidelberg, Germany). They were stained with toluidine blue and counterstained with Mayer's haematoxylin (both from Merck, Darmstadt, Germany).

Histomorphometry was performed on the cultured rudiments. For each rudiment, 10 evenly spaced longitudinal sections were selected for evaluation. Measurements were performed with a linear ocular scale, mounted on a microscope at $\times 40$ magnification. The length of the following zones was scored (Fig. 1):

(B) the zone of hypertrophic chondrocytes;
(C1) he distal zone of flattened chondrocytes;
(D1) the proximal zone of flattened chondrocytes;(sum of B+C1+D1) the osteogenetic area;
(F) the bone collar.

Statistical evaluation of the data was performed using Student's *t*-test.

Autoradiography

Proliferating cells in the long bones were detected by radiolabelling with [^{3}H]-thymidine (Amersham International, Amersham, UK). The bones were exposed during the last 16 hours of the 2 day culture to 18.5 μCi/ml [^{3}H]-thymidine (specific activity 25 Ci/mmol) in complete medium, with or without TGF-β1. Subsequently, the bones were fixed in 4% formalin and metamethacrylate (BDH, Poole, UK) embedded as described above. From each bone, one longitudinal section was used for measurements.

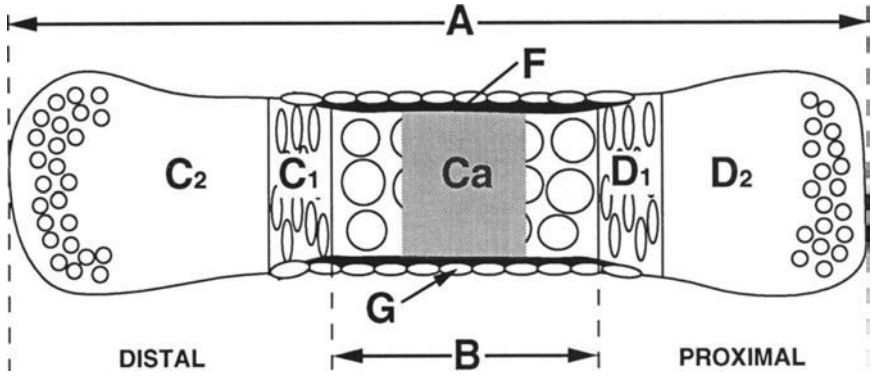

Figure 1 Schematic drawing of different zones of long bone rudiments after culture. The whole long bone (total length: A), consists of a distal area of the epiphyseal cartilage (C2), a distal zone of flattened chondrocytes (C1), a zone of hypertrophic chondrocytes (B), a bone collar (F) with an osteoblast seam (G), the proximal zone of flattened chondrocytes (D1) and a proximal area of the epiphyseal cartilage (D2). In the bone centre, mineralization (Ca) has occurred in hypertrophic cartilage and bone collar.

Autoradiography was performed according to standard procedures. The slides were dipped in Kodak NTB-2 nuclear track emulsion (Kodak, San Diego, USA) and diluted 1 : 1 with 600 mM NH_4Ac at 43°C. After dipping, the slides were placed on a cold metal plate for 30–60 min, dried at high humidity at room temperature for 60 min, and kept at 4°C in light-tight boxes containing desiccant for 10 per cent days. They were developed in Ilford Phenisol developer for 4 min at 15°C, stopped with 10 per cent acetic acid (10 seconds), and fixed for 5 min in 25 per cent sodium thiosulphate. The sections were counterstained with Mayer's haematoxylin. All chemicals were supplied by Baker, Deventer, The Netherlands.

In the autoradiograph the percentage of labelled cells was determined in the following zones (Fig. 1):

(C2) the distal area of epiphyseal cardlage;
(C1) the distal zone of aflattened chondrocytes;
(B) the zone of hypertrophic chondrocytes;
(D1) the proximal zone of flattened chondrocytes;
(D2) the proximal area of epiphyseal cartilage;
(G) the osteoblast seam.

In the midsagittal sections the total number of osteoblasts along the longest side of the bone collar was counted. The selected osteoblast seam was representative for the situation of each bone. Statistical evaluation of the data was performed by using Student's *t*-test.

Results

Before culture the explants consisted of hyaline cartilage surrounded by a primitive perichondrium. In these primitive rudiments (15 days p.c.) the formation of a bone collar, chondrocytic hypertrophy and matrix mineralization in the centre had not yet started. During the 4 day culture, the whole long bone rudiment increased some 60 per cent in length, a bone collar developed, chondrocytes in the centre hypertrophied and calcification of cartilage and bone collar started. As a result after 4 days, the organ cultures had obtained a stage of development comparable to an *in vivo* developed bone of about 16.5 days p.c.

TGF-β1 (1 ng/ml) inhibited longitudinal growth of the whole long bone significantly after 4 days of culture (Fig. 2A). Calcification, monitored as the length of the calcified area (Fig. 1, Ca) was also inhibited, after 2 and 4 days (Fig. 2B).

Histomorphometry of the cultured rudiments showed that TGF-β1 inhibited the growth in length of all zones except the hypertrophic area (Fig. 3A,B). After 2 days the length of the bone collar and the osteogenic area (B+C1+D1) differed significantly (Fig. 3A). In the 4 day culture group the differences in the individual zones of flattened cells were also significant (Fig. 3B).

To further examine the effects of TGF-β1 on growth, cell proliferation was studied in the different zones of the long bone rudiments after 2 days of culture, using autoradiography after

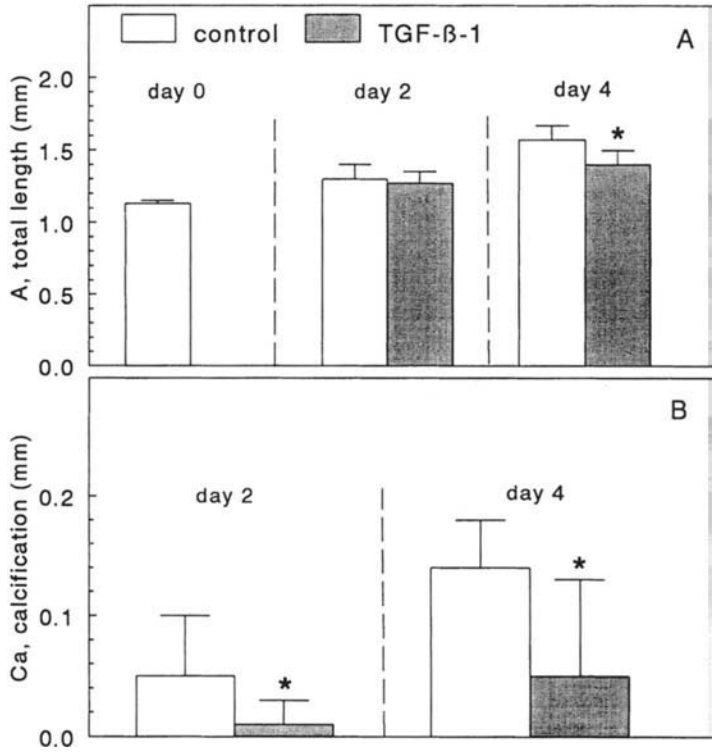

Figure 2 Effects of Transforming Growth Factor (TGF)-β1 (1 ng/ml) on (A) growth in the length of the whole long bone and (B) the appearance of a calcified centre during 2 and 4 days culture in metatarsal long bone rudiments. Values are $x \pm$SEM, $n = 7$; $^*P \leqslant 0.05$, Student's t-test, TGF-β1 treated versus controls.

[^{3}H]-thymidine labelling. A high cell proliferation, as indicated by the percentage of labelled cells, was found in the zones of flattened chondrocytes (C1, D1) but almost none in the zone of hypertrophic chondrocytes (B) (Fig. 4). Treatment with TGF-β1 inhibited cell proliferation in every zone. The proliferation of the proximal area of the epiphyseal cartilage (D2) was significantly decreased (Fig. 4). The control group of this area showed significantly more cell proliferation than the distal area of epiphyseal cartilage (C2) ($P < 0.05$, Student's t-test). However, in the osteoblastic seam TGF-β1 had no effect. In addition, we found no significant difference between the total number of osteoblasts (labelled and unlabelled) in the control group (27.8 ± 2.5) compared with the TGF-β1 treated group (35.0 ± 3.4; $P = 0.2$, Student's t-test).

Discussion

The present study shows that TGF-β1 not only reduces the growth in length of embryonic bone in organ cultures but also inhibits osteogenesis. Organ cultures of embryonic mouse long bones are an appropriate model to study chondro-osteogenesis at the cellular level. Development *in vitro* was slower than *in vivo* but proceeded in a similar manner. The developmental stage of a 15-day embryonic metatarsal long bone cultured for 4 days was comparable to an *in vivo* developed bone of about 16.5 days p.c. This applies to the area of calcification, the size of the hypertrophic area and the length of the bone collar.

TGF-β inhibited growth in the length of the long bone rudiment as a result of inhibition of chondrocyte proliferation. In cultures of isolated chondrocytes, TGF-β has been applied with contradictory results. In isolated cells of chick growth plate cartilage, TGF-β1 markedly stimulated DNA synthesis, with greatest effect on proliferating and early hypertrophic cells (Rosier *et al.*, 1989). However, in isolated chondrocytes from rabbit articular cartilage, TGF-β inhibited cell proliferation after 24 hours expo-

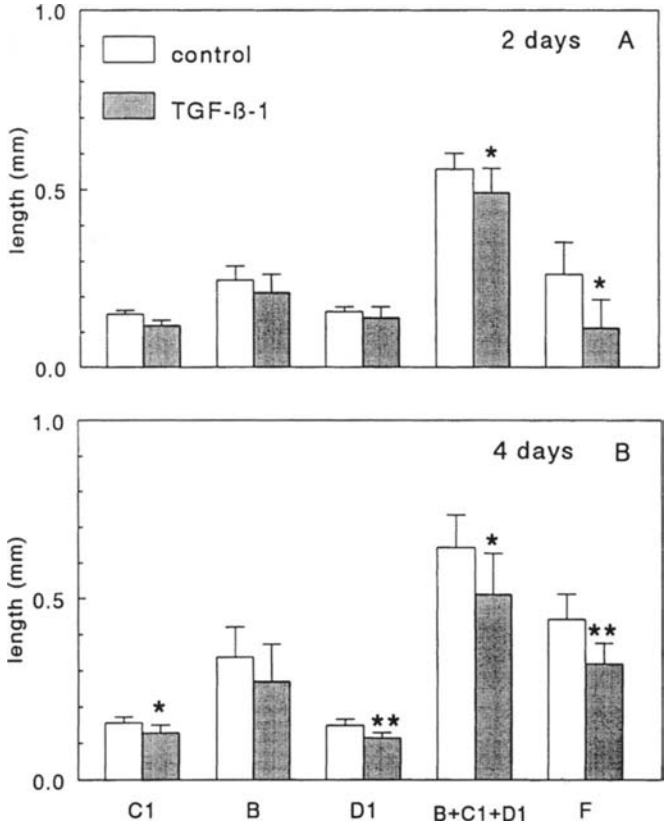

Figure 3 Effects of Transforming Growth Factor (TGF)-β1 (1 ng/ml) after (A) 2 and (B) 4 days of culture on the length of the distal zone of flattened chondrocytes (C1), the hypertrophic zone (B), the proximal zone of flattened chondrocytes (D1), the osteogenic centre (B+C1+D1), and the osteoid seam (F). Values are $x \pm$SEM, $n = 4$–6; $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$, Student's t-test, TGF-β1 treated versus controls.

sure but stimulated after 48 hours (Vivien *et al.*, 1990). These studies were performed with monolayers of isolated cells, devoid of their extracellular matrix. In the present study whole organ cultures were used, such that the extracellular matrix remained intact. The cartilage matrix binds TGF-β produced by chondrocytes (Morales *et al.*, 1991) and TGF-β1 is an endogenous product of these embryonic mouse rudiments. Therefore, use of intact bone organs in an *in vitro* experiment may better imitate the *in vivo* conditions than monolayers of isolated cells.

Because TGF-β activity is produced by the bone rudiments, both *in vivo* and during organ culture (Heine *et al.*, 1987), the present study may not demonstrate the actual endogenous role this factor plays. Rather, it may show what happens if an overproduction of this factor occurs, which may however give clues to its local physiological role. We noticed a remarkable difference between the distal and proximal epiphysis. Although both consist of small-celled hyaline cartilage, TGF-β1 inhibited the cell proliferation in the proximal epiphysis but not in the distal end of the rudiment. However, the control group of proximal epiphysis showed a higher rate of proliferation than the control group of distal epiphysis. Our data suggest that cell populations with the highest cell proliferation rate were most sensitive to TGF-β1.

In contrast to its inhibiting effects on chondrocyte proliferation, TGF-β1 did not affect DNA synthesis in the osteoblastic cell seam, nor did it change the total number of osteoblasts. However, the length of the bone collar around the cartilage anlage was significantly reduced. This indicates that the reduction of

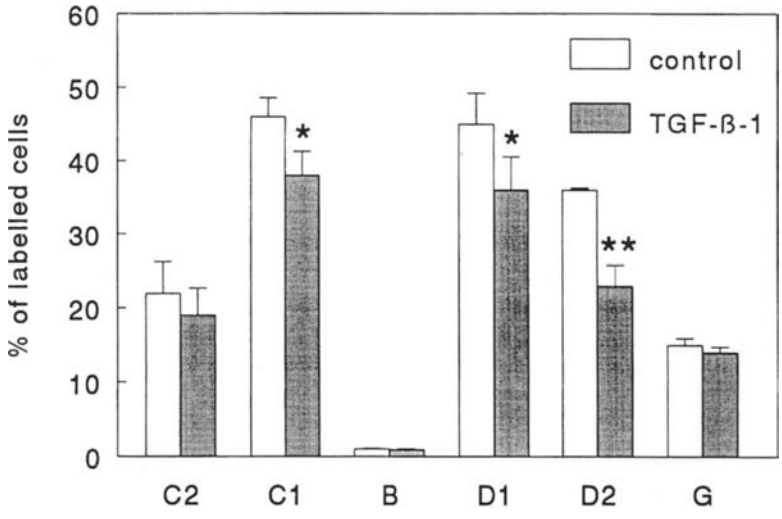

Figure 4 Effects of Transforming Growth Factor (TGF)-β1 (1 ng/ml) on the percentage labelled cells in the distal area of the epiphyseal cartilage (C2), the distal zone of flattened chondrocytes (C1), the hypertrophic chondrocytes (B), the proximal zone of flattened chondrocytes (D1), the proximal area of the epiphyseal cartilage (D2) and the osteoblast seam (G). Values are $x \pm$SEM, $n = 8$; $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$, Student's t-test, TGF-β1 treated versus controls.

the length of the osteoid seam could not be explained by a reduction of cell differentiations into osteoblasts. In fact, the synthesis of bone matrix by the osteoblasts was probably reduced by TGF-β, which also inhibited mineralization of the bone matrix as well as the hypertrophic cartilage. This suggests that in mature, mineralizing skeletal cells TGF-β regulates the production and mineralization of extracellular matrix.

A similar inhibiting effect of TGF-β on cartilage mineralization has been described in cell aggregates of rabbit rib cartilage by Kato *et al.* (1988). The conclusion from the present study is that TGF-β1 inhibits ossification in developing bone rudiments. It also inhibits cell proliferation in highly proliferative cartilage zones. As TGF-β is an endogenous product of developing bones (Heine *et al.*, 1987), these data suggest that TGF-B is an important local regulator of bone growth.

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Acknowledgements

The authors wish to thank Dr. A. Sandham for proof reading the manuscript.

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