TGF-β1 alone and in combination with calcium hydroxide is synergistic to TGF-β1 production by osteoblasts *in vitro*

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Abstract

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Aim To examine the effects of calcium hydroxide ($Ca(OH)_2$), transforming growth factor-beta (TGF-β1), and $Ca(OH)_2$/TGF-β1 coadministration on TGF-β1 and interleukin-6 (IL-6) synthesis by early (subculture 1) and late (subculture 5) osteoblast cultures.

Methodology Early and late cultures were established using bone cells harvested from 21-day-old fetal rat calvaria. Cell cultures of both early and late osteoblasts were divided into four groups: group 1, control; group 2, cells challenged with $Ca(OH)_2$; group 3, cells challenged with TGF-β1; and group 4, cells challenged with $Ca(OH)_2$ and TGF-β1 in combination. TGF-β1 and IL-6 levels for all groups were determined using ELISA methodology.

Results ANOVA and Tukey HS analyses revealed that osteoblasts of groups 3 and 4 significantly increased ($P < 0.001$) TGF-β1 synthesis in both early and late cultures of osteoblasts. IL-6 was not detected in any of the groups considered in this study.

Conclusions Exogenous TGF-β1 has an autocrine effect on cell cultures of osteoblasts. Administration of TGF-β1 alone or in combination with $Ca(OH)_2$ increases the synthesis of TGF-β1 in osteoblast cultures. $Ca(OH)_2$ and TGF-β1 are compatible when placed in a culture of osteoblasts. $Ca(OH)_2$ provides a favourable environment for the anabolic effects of TGF-β1.

Keywords: calcium hydroxide, cell culture, IL-6, osteoblasts, TGF-β1.

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Introduction

Bone remodelling involves dynamic mechanisms that maintain the functional integrity of normal bone. The constant resorption and deposition of bone is not only necessary for normal growth and calcium balance, but also for bone homeostasis. At the heart of the resorptive processes are large multinucleated osteoclasts capable of migrating to sites that require the removal of bony matrices. Endocrinological studies clearly demonstrate that certain systemic hormones, such as parathyroid hormone, 1, 25-dihydroxyvitamin D_3 and calcitonin, regulate calcium homeostasis and modulate bone formation and resorption (McSheehy & Chambers 1986). Similarly, pro-inflammatory cytokines also play a role in maintaining the bony environment by stimulating osteoclast formation and thereby contributing to bone resorptive events.

Growth factor-beta (TGF-β) is a ubiquitous member of the TGF growth factor family that is synthesized by virtually all cells (Lawrence 1996). Although synthesized in a biologically inactive latent precursor form (Gleizes *et al.* 1997), this relatively stable factor can be activated by transient alkalization of about pH 12, acidification of pH 2, heat, and many chaotropic agents (Miyazono & Heldin 1989). TGF-β, as well as other growth factors such as insulin-like growth factor and fibroblast growth

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factor, is vital to normal bone growth and development (Price *et al.* 1994).

One of the largest reservoirs for TGF-β is bone (Seyedin *et al.* 1986). *In vitro* studies of TGF-β reveal that this growth factor stimulates osteoblast-like cells to proliferate and synthesize collagen (Centrella *et al.* 1986). This extracellular matrix sets the stage for the mineralization process. Noda & Camilliere (1989) have studied TGF-β *in vivo* and showed that, when applied adjacent to the periosteum, bone thickness is increased.

Tissue engineering studies suggest that a suitable carrier is needed to ensure the greatest effect of TGF-β during bone repair. For instance, Mustoe *et al.* (1987) have shown that TGF-β, when applied alone, diffuses from the site of application without exerting any appreciable influences. In another study, injection of TGF-β adsorbed on resorbable microcrystals of hydroxyapatite induced large amounts of new bone in dogs compared to growth factor alone (Strates *et al.* 1992). Therefore, an effective vehicle for delivering TGF-β to sites of osseous defects may have direct positive influence on successful reparative outcome in bone.

Calcium hydroxide ($Ca(OH)_2$) is a basic salt that has been shown to induce the formation of fibrous tissue and immature bone when placed in direct contact with host tissue (Seltzer & Bender 1984). Because of the high alkaline properties (pH 11) of $Ca(OH)_2$, mineralization events may benefit from this basic environment. In addition, $Ca(OH)_2$ may also neutralize the lactic acid secreted by osteoclasts and buffer the acidic by-products of the inflammatory process, which may help to prevent further destruction of mineralized tissue (Heithersay 1975). Murakami *et al.* (1997) have shown that $Ca(OH)_2$ enhances bone metabolic processes, including collagen synthesis, glycolysis and protein synthesis. Accordingly, the effectiveness of $Ca(OH)_2$ in the dental setting is well documented (King *et al.* 1965, Tagger & Tagger 1989, Tronstad 1992) in its application to pulp tissue and its use in apexification.

Interleukin-6 (IL-6) is a cytokine that has been extensively studied and shown to influence bone resorption (Suda *et al.* 1995). Specifically, osteoblasts secrete IL-6, which in turn upregulates the *de novo* synthesis of osteoclasts (Löwik *et al.* 1989, Roodman 1992, Manolagas & Jilka 1995). Of interest is the report by Al-Humidan *et al.* (1991) that the influence of IL-6 on pre-existing osteoclasts is essentially negligible. Also, human studies have implicated IL-6 in tumour growth and bone destructive processes (Mills & Frausto 1997). For example, levels of IL-6 are significantly increased in multiple myeloma states as well as Gorham-Stout disease, or disappearing bone syndrome (Pelliniemi *et al.* 1995, Devlin *et al.* 1996). An increase in IL-6 status would be an appropriate indicator of osteoclast activity and bone resorption.

Experimental studies focusing on the impact of clinically relevant materials such as $Ca(OH)_2$ on the function of growth factors in bone are lacking. Accordingly, examination of the effect of experimental challenges of $Ca(OH)_2$ and specific anabolic and catabolic cytokines on bone cells is appropriate. The purpose of this study was to evaluate the effects of exogenous TGF-β1 on the synthesis of endogenous TGF-β1 and IL-6 in osteoblast cell cultures.

Materials and methods

Preparation of cell cultures

Bone cells were harvested from 21-day-old fetal rat calvaria (Williams *et al.* 1980). The calvaria were dissected using a light microscope, removing the periosteum, dura and suture. The bone was cut into small pieces, harvested and placed in a tube containing phosphate-buffered saline (PBS). The tube was kept on ice before transferring cut sections of the calvaria into 4.0 mL of digestion solution. Digestion solution was prepared using 20.0 mL of PBS, 1.0 mL of collagenase (20.0 mg mL^{-1}) and 0.4 mL of $10 \times 2.5\%$ trypsin. After stirring for 15 min, the supernatant was discarded and 5.0 mL of digestion solution added. This step was repeated four times and in each case the supernatant fraction was collected and stored on ice. The four fractions were combined and resuspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS) and an antibiotic mixture of penicillin (76 U mL^{-1}) and streptomycin (76 μg mL^{-1}). The cells were counted with a haemacytometer and plated in 60 mm dishes at 3×10^5 cells per plate. Cells were grown in an atmosphere of 5% CO_2 at 37 °C. Culture medium was changed every 3 days. After reaching confluence, the cells were subcultured and again plated at 3×10^5 cells per plate. A growth curve for all cells was established over a 15-day period by counting the cells with a haemacytometer and by determining DNA content for each of the six plates at the following time points: 3, 5, 7, 9, 11, 13, and 15 days. The growth curve was established in order to determine a point on the logarithmic growth phase at which the experiments would be performed. Growth curves were established for both a first subculture population of cells and a fifth subculture population of cells. For each of the first and fifth populations, the cell

cultures of osteoblasts were divided into four groups: group 1, control; group 2, cells challenged with $Ca(OH)_2$; group 3, cells challenged with recombinant human TGF-β1 (rhTGF-β1); and group 4, cells challenged with combination of $Ca(OH)_2$ and rhTGF-β1.

DNA synthesis assay

Cells from each culture dish were harvested by scraping with a rubber policeman and transported to glass test tubes. Eight DNA standards ranging in concentration from 0 to 15 μg mL^{-1} were prepared from a stock solution of Salmon Sperm DNA (10.0 mg mL^{-1}). Cell precipitates contained in glass tubes were solubilized with 1.0 mL of 1% solution of Triton-X and incubated for 3 h. For 1.0 mL of sample or standards; 175 μL of 70% perchloric acid was added and heated at 74 °C for 20 min in water bath. Two grams of diphenylamine were dissolved in 50.0 mL of glacial acetic acid and 1.0 mL was added to each tube. Then 500 μL of acetaldehyde was added to 250.0 mL of distilled water and 50 μL of this dilution was added to each tube and capped tightly with a stopper. The samples were covered with foil and the colour was allowed to develop overnight at room temperature. Samples were centrifuged at 3000 r.p.m. for 5 min and the optical density (OD) of this supernatant was read at 596 nm.

Preparation of the $Ca(OH)_2$ test solution

The $Ca(OH)_2$ solution was prepared by dissolving 0.0121 g of $Ca(OH)_2$ in 10.0 mL of distilled water at 23 °C. This solution was stirred and centrifuged, filtered and titrated to make sure that the pH of culture media (pH = 7.76) would not be significantly altered. A total of 0.015 mL of the $Ca(OH)_2$ solution was added to the culture medium (Torneck *et al.* 1983).

TGF-β test solution

Lyophilized rhTGF-β1 was purchased commercially (R & D Systems, Minneapolis, MN, USA). The rhTGF-β1 was reconstituted with sterile 4 mM HCl containing 1.0 mg mL^{-1} bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO, USA) to a concentration of 1.0 μg mL^{-1}. A concentration of 5.8 ng mL^{-1} of TGF-β1 was used in this study. $Ca(OH)_2$ in combination with rhTGF-β1 was added to cultures of osteoblasts. TGF-β1 concentration in the media and IL-6 production by osteoblasts were determined for each of four groups at day 5 of the logarithmic growth curve.

ELISA assay for IL-6

Standards and samples of a monoclonal antibody, specific for IL-6 (R & D Systems, Minneapolis, MN, USA) were pipetted into the wells and any IL-6 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-6 was added to the wells. After a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. Following incubation time, an amplifier solution was added to the wells and colour developed in proportion to the amount of IL-6 bound in the initial step. The colour development was stopped and the intensity of the colour was measured at wavelengths of 450 nm and 540 nm.

ELISA assay for TGF-β

Standards and samples of TGF-β soluble receptor Type II, which binds TGF-β1 (R & D Systems, Minneapolis, MN, USA) were pipetted into the wells and any TGF-β1 present was bound by the immobilized receptor. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TGF-β1 was added to the wells to sandwich the TGF-β1 immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of TGF-β1 bound in the initial step. The colour development was stopped and the intensity of the colour was measured at wavelengths of 450 nm and 540 nm.

Statistical analysis

The amount of rhTGF-β1 added to culture media was subtracted from the total amount of TGF-β1 detected. Statistical analysis was performed using a one-way analysis of variance (ANOVA) to determine significance amongst the groups for each population. This was followed by a Tukey HS to determine the location of the significant values. A *P*-value of less than or equal to 0.05 was considered significant at the 95% confidence limits.

Results

Early culture of osteoblasts (subculture 1)

Figure 1 illustrates the growth curve for cultures of osteoblasts derived from fetal rat calvaria. Day 5 was

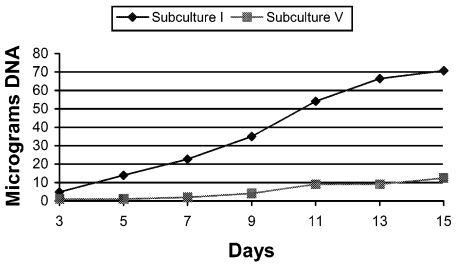

Figure 1 Growth curves of early and late subcultures of osteoblasts.

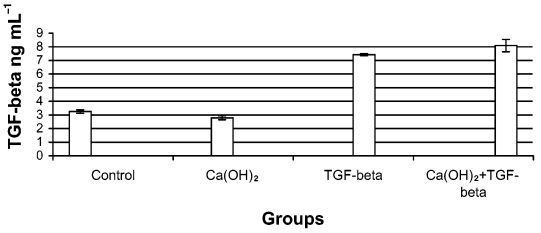

Figure 2 TGF-β1 levels in early subcultures (subculture 1) of osteoblasts (ng mL^{-1}).

Table 1 Effects of TGF-β1 and $Ca(OH)_2$ on TGF-β1 synthesis in early subcultures (subculture 1) of osteoblasts (ng mL^{-1})

Group (*n*)	Mean	SD	SE of mean
Control (8)	3.25	0.38	0.13
$Ca(OH)_2$ (8)	2.79	0.39	0.14
TGF-b (8)	7.42[a,b]	0.24	8.44E-02
$Ca(OH)_2$ + TGF-b (8)	8.09[c,d]	1.28	0.45
Total (32)	5.39	2.51	0.44

[a]SD, standard deviation; SE, standard error.
[b]Tukey HS test: a = III > I, $P < 0.001$; b = III > II, $P < 0.001$; c = IV > I, $P < 0.001$; d = IV > II, $P < 0.001$.

shown to be on the logarithmic phase for both early and late subcultures. This time-point was chosen for subsequent experimental challenges. Figure 2 summarizes the effects of TGF-β1 and $Ca(OH)_2$ on early subcultures of osteoblasts. Recombinant human TGF-β1 increased TGF-β1 synthesis in early osteoblast subcultures (Table 1). After 24 h of challenge, TGF-β1 alone (group 3) significantly stimulated ($P < 0.001$) TGF-β1 synthesis (7.42 ± 0.08 ng mL^{-1}) relative to controls (3.25 ± 0.13 ng mL^{-1}). The combination of TGF-β1 with $Ca(OH)_2$ (group 4) also significantly increased ($P < 0.001$) TGF-β1 synthesis (8.09 ± 0.45 ng mL^{-1}) compared to controls. Group 3 was significantly greater ($P < 0.001$) than group 2 (2.79 ± 0.14 ng mL^{-1}). Also group 4 was significantly greater ($P < 0.001$) than group 2. There was no significant difference between group 2 and the control group. There also was no significant difference between group 3 and group 4, although there seemed to be a trend toward higher levels of TGF-β1 in group 4 than in group 3.

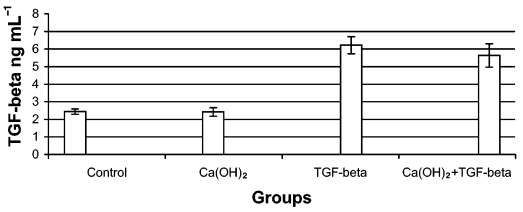

Figure 3 TGF-β1 levels in late subcultures (subculture 5) of osteoblasts (ng mL^{-1}).

Table 2 Effects of TGF-β1 and $Ca(OH)_2$ on TGF-β1 synthesis in late subcultures (subculture 5) of osteoblasts (ng mL^{-1})

Group (*n*)	Mean	SD	SE of mean
Control (8)	2.44	0.43	0.15
$Ca(OH)_2$ (8)	2.42	0.67	0.24
TGF-b (8)	6.22[a,b]	1.37	0.49
$Ca(OH)_2$ + TGF-b (8)	5.63[c,d]	1.88	0.67
Total (32)	4.18	2.14	0.38

[a]SD, standard deviation; SE, standard error.
[b]Tukey HS test: a = III > I, $P < 0.001$; b = III > II, $P < 0.001$; c = IV > I, $P < 0.001$; d = IV > II, $P < 0.001$.

Late culture of osteoblasts (subculture 5)

Figure 3 summarizes the effects of TGF-β1 and $Ca(OH)_2$ on late subcultures of osteoblasts. Table 2 outlines TGF-β1 synthesis for late subcultures of osteoblasts. Group 3 significantly increased ($P < 0.001$) TGF-β1 synthesis (6.22 ± 0.49 ng mL^{-1}) relative to group 1 (2.44 ± 0.15 ng mL^{-1}) and group 2 (2.42 ± 0.24 ng mL^{-1}). TGF-β1 synthesis in group 4 (5.63 ± 0.67 ng mL^{-1}) was significantly greater ($P < 0.001$) than group 1 and also significantly greater ($P < 0.001$) than group 2. There was no significant difference in TGF-β1 synthesis between group 1 and group 2 or between group 3 and group 4.

Interleukin-6 (IL-6) was not detected in any of the four groups that were considered in either early cultures (subculture 1) or late cultures (subculture 5) of osteoblasts.

Discussion

The results of this study showed that $Ca(OH)_2$ and TGF-β1 are biologically compatible in a culture of osteoblasts. The administration of TGF-β1 alone, or in combination with $Ca(OH)_2$, significantly increased TGF-β1 synthesis in both osteoblast cultures. In a typical biological system, levels of certain substances are maintained at very precise levels to ensure homeostasis. One way that this is accomplished is via negative feedback mechanisms. If TGF-β1 is maintained at constant levels by negative feedback loops, any increase in growth factor levels would down-regulate the synthesis of this protein. We observed that TGF-β1 synthesis was actually increased by administration of TGF-β1. It is possible that TGF-β1 may have an autocrine effect on osteoblasts in cell cultures. This effect, although manifest in cell cultures, may not be obvious in organ cultures or *in vivo*, where other inhibitory mechanisms are present. TGF-β1 is known to increase cell proliferation (Centrella *et al.* 1987). The increase in TGF-β1 found in the cell culture system might also be due to an increase in osteoblast numbers. However, in our study there was an increase in TGF-β1 beyond the increased cell numbers.

$Ca(OH)_2$ may act as an effective carrier for delivery of TGF-β1 to sites of osseous remodelling. The high alkaline environment created by the presence of $Ca(OH)_2$ would be ideal for the activation of inactive TGF-β1. Initially present in an inactive form, this growth factor becomes activated during events that manifest extreme fluctuations in pH (either acidic or alkaline). Activated TGF-β1 will increase collagen protein synthesis. An increase in collagen synthesis can serve as a marker for the early stages of bone formation, since this protein becomes up-regulated during bone forming events.

Interleukin-6 was not detected in any of the groups of subculture 1 and subculture 5 of osteoblasts. Pfeilschifter *et al.* (1988) found that TGF-β1 inhibits bone resorption. Hock *et al.* (1990) demonstrated that TGF-β1 administration in organ culture of rat calvaria reduced the number of osteoclasts on bone. This inhibition may be due to down-regulation of IL-6 production. It is possible that elevations of TGF-β1 could secondarily result in decreased synthesis of IL-6. No appreciable amount of IL-6 was detected in any of the osteoblast groups, including controls. However, this could be explained by the inhibitory effects of TGF-β1 in all of the groups. As previously shown, the combination of $Ca(OH)_2$ and TGF-β1 is anabolic in cultures of osteoblasts (Jaunberzins *et al.* 2000).

This present study suggests that this combination may amplify the synthesis of TGF-β1 in the host population of bone cells. Although care must be taken in the extrapolation of results from *in vitro* studies, there are a number of factors that may have direct clinical relevance from this present study. The first issue is the finding that $Ca(OH)_2$ and TGF-β1 are compatible. In fact, the alkaline environment set up by the $Ca(OH)_2$ is conducive to the anabolic effects of the TGF-β1. Secondly, extracellular matrix and collagen in particular are necessary in the early stages of bone healing. Our previous findings have demonstrated an increase in collagen synthesis and suggest that this combination of reagents can provide the foundation for bone healing. Certainly, this *in vitro* study is preliminary and *in vivo* experiments are necessary to study the effects of $Ca(OH)_2$ plus TGF-β1 on the later stages of bone mineralization. It is not expected that the use of TGF-β1 or other growth factors will supplant the highly successful use of $Ca(OH)_2$ alone for routine endodontic procedures. However, in cases involving critical size defects various growth factors, including TGF-β1 may be beneficial not only in increasing the rate of bone healing, but also for enhancement of the quantity of bone necessary for regeneration and repair. *In vivo* experiments are underway in this regard. This would be especially desirable in the clinical setting. Critical size defects, when present in periradicular bone, are slow to heal following endodontic treatment. Since TGF-β is important to bone formation, administration of a substance that would enhance growth factor synthesis would be appropriate. The further increase of this anabolic cytokine in the healing environment would enhance bone formation, therefore reducing the complications associated with critical size defects.

In conclusion, administration of TGF-β1 alone or in combination with $Ca(OH)_2$ increases the synthesis of TGF-β1 in osteoblast cultures, suggesting a synergistic effect of TGF-β1 administration on TGF-β1 synthesis. This study also demonstrates that $Ca(OH)_2$ and TGF-β1 are compatible when placed in a culture of osteoblasts, confirming that $Ca(OH)_2$ provides a favourable environment for the anabolic effects of TGF-β1. Further studies, both *in vitro* and *in vivo*, are underway to examine the effects of various growth factors in combination with $Ca(OH)_2$ on bone formation.

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