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Effects of Calcium Hydroxide and Tumor Growth Factor-β on Collagen Synthesis in Subcultures I and V of Osteoblasts

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Collagen protein synthesis by osteoblasts is influenced by transforming growth factor-β (TGF-β1) and is essential to bone formation. The effectiveness of TGF-β1 depends on efficient delivery of the growth factor to target cells, adequate binding to cell surface receptors, and an optimum environment for promotion of collagen synthesis. The effects of calcium hydroxide ($Ca(OH)_2$), TGF-β1, and $Ca(OH)_2$/TGF-β1 co-administration on total protein, collagen protein, and noncollagen protein synthesis by early (subculture I) and late (subculture V) osteoblast cultures were tested. TGF-β1 significantly increased all protein synthesis in subculture I osteoblasts ($p = 0.001$; $p < 0.001$; $p = 0.019$). $Ca(OH)_2$/TGF-β1 co-administration significantly increased total protein and collagen protein levels in subculture I osteoblasts as well ($p = 0.048$; $p = 0.012$). TGF-β1 increased total protein and collagen protein synthesis significantly in subculture V cells ($p = 0.025$; $p = 0.01$). These data indicate that co-administration of $Ca(OH)_2$ and TGF-β1 enhances collagen synthesis by osteoblasts and may have implications for the clinical setting.

Calcium hydroxide ($Ca(OH)_2$) has been widely used for a variety of applications in dentistry due to its antimicrobial effects (1) and its capability of inducing hard tissue formation (2). A number of theories have been postulated as to the mechanism of $Ca(OH)_2$ activity in the mineralization process. Studies suggest that a rise in pH by $Ca(OH)_2$ may initiate the mineralization process. The high pH combined with the availability of Ca^{2+} and OH^- ions have an effect on enzymatic pathways and hence mineralization (3). $Ca(OH)_2$ has also been shown to enhance bone metabolism processes, including collagen synthesis, glycolysis, and protein synthesis (4). Whereas $Ca(OH)_2$ can neutralize bacterial lipopolysaccharides (5), destroy bacteria (1), and initiate tissue dissolution (6), the mechanism by which it permits or induces bone formation is relatively unclear. Likewise its interactive role with cytokines in the stimulation of a productive osseous response is also unknown.

Osteogenesis may be summarized as the osteoblastic production of an organic matrix (type I collagen and noncollagen proteins) that is capable of accepting a mineral component, with a prerequisite of both an environment rich in alkaline phosphatase and a generous vascular supply. Type I collagen is the most abundant extracellular protein of bone and is essential for bone tensile strength. Its synthesis by osteoblasts can be modified by different cytokines, hormones, vitamins and growth factors. For example, growth factors act as modulators of cellular activities. One of the major growth factors responsible for these activities is transforming growth factor-β (TGF-β) (7).

TGF-β is synthesized, with only a few exceptions, by virtually all cells (7). It is a multifunctional protein that regulates cell proliferation, differentiation, and extracellular matrix production (8). TGF-β has been shown to regulate the synthesis of collagen by growth plate chondrocytes, increasing the synthesis of type I collagen compared with type II (9). However, bone is the largest reservoir for TGF-β1 (10). TGF-β is secreted in a biologically inactive latent precursor form (11). This latent form can be activated by physical and chemical conditions such as transient alkalization to about pH 12, acidification (pH 2), heat, and chaotropic agents (12). The mechanism of this activation, however, is still unclear. In cell culture TGF-β1 stimulates osteoblast-like cells to proliferate and synthesize collagen. Its maximal effect on cell proliferation has been observed at a concentration of 10.0 ng/ml (13).

An in vivo study by Mustoe et al. (14) demonstrated that a suitable carrier is needed for the growth factor. When applied alone, TGF-β diffuses from the site of application without exerting any appreciable influences. Therefore a more effective method is needed for delivering TGF-β1 to the site of osseous defect that will maintain the cytokine in association with the regenerating osteoblasts. $Ca(OH)_2$ is available in the form of a nonsetting paste that can diffuse through dentin (15). This would be an ideal carrier to combine with TGF-β if the anabolic potential for this cytokine could be maintained.

The aim of tissue engineering is to develop biological substitutes that will maintain, restore, or improve tissue function when they are delivered to the appropriate site. The specific impact of clinically relevant materials such as $Ca(OH)_2$ in combination with

anabolic growth factors such as TGF-β has not been studied to our knowledge. The purpose of this study was to determine the compatability of $Ca(OH)_2$ with recombinant human (rh) TGF-β. In this regard, the effects of $Ca(OH)_2$ alone and in combination with rhTGF-β on the early stages of bone formation collagen synthesis were studied in a culture system of early and late osteoblasts derived from fetal rat calvaria.

MATERIALS AND METHODS

Preparation of Cell Cultures

Bone cells were harvested from 21-day-old fetal rat calvaria (16). 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. 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 phosphate-buffered saline, 1.0 ml of collagenase (20.0 mg/ml), 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 containing 10% fetal calf serum, and an antibiotic mixture of penicillin (76 units/ml) and streptomycin (76 μg/ml). The cells were counted with a hemacytometer 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 media were 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 hemacytometer and by determining DNA content for each of the six plates at the following times: 3, 5, 7, 9, 11, 13, and 15 days. The growth curve was established 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 I—control; group II—cells challenged with $Ca(OH)_2$; group III—cells challenged with rhTGF-β; and group IV—cells challenged with a 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 were prepared from a stock solution of salmon sperm DNA (10.0 mg/ml). 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 were 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 were 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 color was allowed to develop overnight at room temperature. Samples were centrifuged at 3000 rpm for 5 min, and the optical density 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 (17).

TGF-β Test Solution

Lyophilized rhTGF-β1 was purchased commercially (R & D Systems, Minneapolis, MN) and reconstituted with sterile 4 mM HCl containing 1.0 mg/ml bovine serum albumin (Sigma Chemical Co., St. Louis, MO) to a concentration of 1.0 μg/ml. A concentration of 5.8 ng/ml of TGF-β1 was used in this study. $Ca(OH)_2$ in combination with rhTGF-β1 was added to cultures of osteoblasts. Collagen and noncollagen protein synthesis were determined for each of four groups at day 5 of the logarithmic growth curve.

Collagen and Noncollagen Protein Synthesis Assay

Collagen and noncollagen protein levels were determined using the specific enzyme degradation assay described by Peterkofsky and Diegelmann (18), and later modified by Sodek et al. (19). For the purpose of the collagen protein assay, Dulbecco's Modified Eagle's Medium without fetal calf serum was used. This media was supplemented with ascorbic acid (50 μg/ml) and 3.5 mM of L-proline (8.0 mg/20.0 ml). On day 5 [2,3-^{3}H]proline (New England Nuclear, Boston, MA; 5 μCi/ml) was added to the culture medium of each dish and allowed to incubate for 24 h. Samples were collected, placed in dialysis bags, and dialyzed against 1% glacial acetic acid to remove the unincorporated radiolabeled proline from collagen-incorporated proline. The samples were then placed in glass test tubes, lyophylized, and reconstituted with 1.0 ml of 50 mM Tris buffer. Reconstituted samples then received 25 μl of purified bacterial collagenase and 50 μl of *N*-ethylmalamide to give a final concentration of 0.025 mM. The samples were incubated at 37°C in a water bath for 1 h. After incubation, 85 μl of 100% trichloracetic acid (TCA) were added, and the samples were centrifuged for 5 min at 3000 rpm. Two 100-μl samples of the supernatant were taken and added to 7.0 ml of aquasol in scintillation vials to measure the uptake and hydroxylation of [^{3}H]proline by collagen. The precipitate was washed twice with 6% TCA and centrifuged for 5 min to precipitate noncollagen protein. To dissolve the precipitate, 200 μl of 70% formic acid were added, and 200 μl of this sample were taken for scintillation counting. Collagen in the cell layer was also assayed by adding 2.0 ml of 6% TCA to each dish and subsequently scraping and transferring the cells to glass tubes. After centrifugation for 5 min, the supernatant was removed and discarded. Cells were washed twice with 1.0 ml of 6% TCA. After a final wash, 800 μl of 6% TCA were added to the cell pellet, and the tubes were heated to 90°C for 15 min in a water bath. Subsequently the tubes were cooled on ice and centrifuged for 5 min. Two 100-μl samples of the supernatant were taken and added to 7.0 ml of aquasol in scintillation vials and counted. The remaining 500 μl of supernatant were taken for DNA

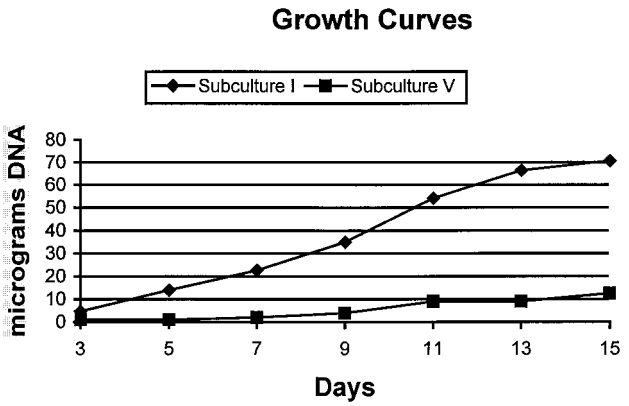

FIG 1. Growth curves of early (subculture I) and late (subculture V) subcultures of osteoblasts.

assay. The precipitate was washed twice with 6% TCA to eliminate any labeled collagen fraction. After a final wash, 200 μl of 70% formic acid were added to dissolve the residual protein, and the 200 μl were transferred to aquasol in vials, and the samples were analyzed in a beta counter.

Statistical Analysis

Statistical analysis was performed using a one-way ANOVA to determine significance among the groups for each population. This was followed by a Tukey Honestly Significant Difference test to determine the location of the significant values. A p value of ≤ 0.05 was considered significant at the 95% confidence limits.

RESULTS

The growth curves for both early and late subcultures of osteoblasts are shown in Fig. 1. Day 5 was shown to be on the logarithmic phase for both early and late subcultures. This timepoint was chosen for subsequent experimental challenge with $Ca(OH)_2$ alone, rhTGF-β1 alone, and rhTGF-β1 in combination with $Ca(OH)_2$.

Early Culture of Osteoblasts (Subculture I)

TOTAL PROTEIN SYNTHESIS

Figure 2 summarizes the effects of rhTGF-β1 and $Ca(OH)_2$ on protein synthesis in early subcultures of osteoblasts. rhTGF-β1 increased total protein synthesis in early osteoblast subcultures (Table 1). After 24 h of challenge, rhTGF-β1 alone (group III) significantly stimulated ($p = 0.001$) total protein synthesis ($1.48 \pm 0.11 \times 10^5$ dpm/μg DNA) relative to controls ($0.79 \pm 0.06 \times 10^5$ dpm/μg DNA). The combination of rhTGF-β1 with $Ca(OH)_2$ (group IV) also significantly increased ($p = 0.048$) total protein synthesis ($1.2 \pm 0.18 \times 10^5$ dpm/μg DNA) relative to the control group. Group III was significantly greater than group II ($0.66 \pm 0.04 \times 10^5$; $p < 0.001$). Also group IV was significantly greater than group II ($p = 0.007$). There was no significant difference between group II and the control group. There also was no significant difference between group III and group IV.

COLLAGEN SYNTHESIS

Table 2 outlines the effects of rhTGF-β1 and $Ca(OH)_2$ on collagen protein synthesis. Group III alone significantly increased ($p < 0.001$) collagen protein synthesis ($0.83 \pm 0.06 \times 10^5$ dpm/μg DNA) relative to the control group ($0.45 \pm 0.03 \times 10^5$ dpm/μg DNA) and was also significantly greater ($p < 0.001$) than group II ($0.39 \pm 0.02 \times 10^5$ dpm/μg DNA). Collagen synthesis in group IV ($0.68 \pm 0.07 \times 10^5$ dpm/μg DNA) was significantly greater ($p = 0.012$) than in group I and also significantly greater ($p = 0.001$) than in group II. There was no significant difference in collagen protein synthesis between group I and group II or between group III and group IV.

NONCOLLAGEN PROTEIN SYNTHESIS

Table 3 outlines noncollagen protein synthesis for early subcultures of osteoblasts. Group III significantly increased ($p = 0.019$) noncollagen protein synthesis ($0.64 \pm 0.07 \times 10^5$ dpm/μg DNA) relative to group I ($0.33 \pm 0.03 \times 10^5$ dpm/μg DNA) and group II ($0.27 \pm 0.03 \times 10^5$ dpm/μg DNA; $p = 0.004$). There was no significant difference between group IV ($0.53 \pm 0.11 \times 10^5$ dpm/μg DNA) and any of the other groups. Early subculture osteoblasts synthesized more collagen than noncollagen protein with a ratio in the range of 1.30 to 1.45. Neither rhTGF-β1 nor $Ca(OH)_2$ had an effect on this ratio.

Late Culture of Osteoblasts (Subculture V)

TOTAL PROTEIN SYNTHESIS

Figure 3 summarizes the effects of rhTGF-β1 and $Ca(OH)_2$ on protein synthesis in late subcultures of osteoblasts. Late subculture osteoblasts did not have as robust a response to rhTGF-β1 challenge as early subculture osteoblasts after 24 h (Fig. 2). As outlined in Table 4, rhTGF-β1 alone (group III) significantly increased ($p = 0.025$) total protein synthesis ($1.15 \pm 0.04 \times 10^5$ dpm/μg DNA) when compared with group I ($0.89 \pm 0.05 \times 10^5$ dpm/μg DNA), but it was not significant between group II ($0.94 \pm 0.08 \times 10^5$ dpm/μg DNA) and group IV ($0.98 \pm 0.08 \times 10^5$ dpm/μg DNA). There was no significant difference among group I, group II, or group IV.

COLLAGEN SYNTHESIS

Table 5 outlines the effects of rhTGF-β1 and $Ca(OH)_2$ on collagen protein synthesis. Group III alone significantly increased ($p = 0.01$) collagen protein synthesis ($0.39 \pm 0.03 \times 10^5$ dpm/μg DNA) relative to the control group ($0.27 \pm 0.02 \times 10^5$ dpm/μg DNA). There was no statistically significant difference in collagen protein synthesis between group III and group II ($0.32 \pm 0.03 \times 10^5$ dpm/μg DNA), and group III and group IV ($0.32 \pm 0.03 \times 10^5$ dpm/μg DNA). There was no significant difference in collagen protein synthesis among group I, group II, or group IV.

NONCOLLAGEN PROTEIN SYNTHESIS

Table 6 outlines noncollagen protein synthesis for late subcultures of osteoblasts. There was no statistically significant differ-

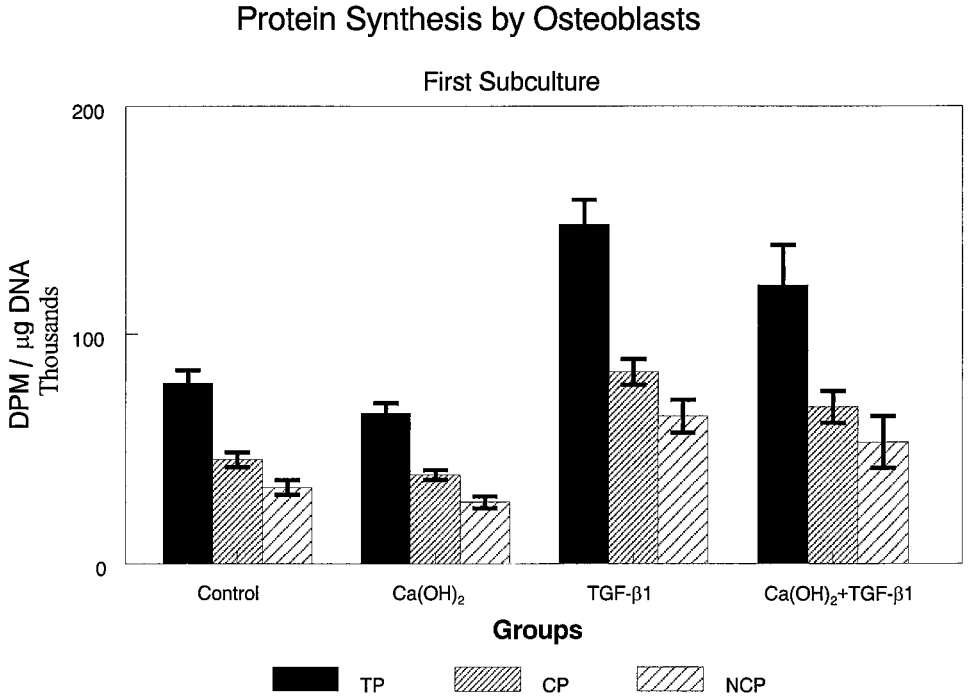

FIG 2. Protein synthesis by early subculture of osteoblasts (*TP* = total protein; *CP* = collagen protein; *NCP* = noncollagen protein).

TABLE 1. Effects of TGF-β1 and $Ca(OH)_2$ on total protein synthesis in early subcultures (subculture I) of osteoblasts (1×10^5 dpm/μg DNA)

Group (*n*)	Mean	Standard Deviation	Standard Error of the Mean
Control (8)	0.79	0.16	0.06
$Ca(OH)_2$ (8)	0.66	0.13	0.04
TGF-β (8)	1.48[a,b]	0.31	0.11
$Ca(OH)_2$ + TGF-β (8)	1.2[c,d]	0.5	0.18
Total (32)	1.03	0.45	0.08

Tukey Honestly Significant Difference test: a = III > I, p = 0.001; b = III > II, p < 0.001; c = IV > I, p = 0.048; d = IV > II, p = 0.007.

TABLE 2. Effects of TGF-β1 and $Ca(OH)_2$ on collagen synthesis in early subcultures (subculture I) of osteoblasts (1×10^5 dpm/μg DNA)

Group (*n*)	Mean	Standard Deviation	Standard Error of the Mean
Control (8)	0.45	0.09	0.03
$Ca(OH)_2$ (8)	0.39	0.06	0.02
TGF-β (8)	0.84[a,b]	0.16	0.06
$Ca(OH)_2$ + TGF-β (8)	0.68[c,d]	0.2	0.07
Total (32)	0.59	0.22	0.04

Tukey Honestly Significant Difference test: a = III > I, p < 0.001; b = III > II, p < 0.001; c = IV > I, p = 0.012; d = IV > II, p = 0.001.

ence among the groups. In late subcultures of osteoblasts, $Ca(OH)_2$ exhibited a slight stimulatory effect on protein synthesis when compared with the control, but tended to have an inhibitory effect when compared with rhTGF-β1 alone (Fig. 2). The ratio of collagen protein to noncollagen protein for subculture V osteoblasts ranged from 0.45 to 0.52. rhTGF-β1 and $Ca(OH)_2$ alone or in combination did not affect this ratio.

TABLE 3. Effects of TGF-β1 and $Ca(OH)_2$ on noncollagen protein synthesis in early subcultures (subculture I) of osteoblasts (1×10^5 dpm/μg DNA)

Group (*n*)	Mean	Standard Deviation	Standard Error of the Mean
Control (8)	0.33	0.09	0.03
$Ca(OH)_2$ (8)	0.27	0.07	0.03
TGF-β (8)	0.64[a,b]	0.2	0.07
$Ca(OH)_2$ + TGF-β (8)	0.53	0.32	0.11
Total (32)	0.44	0.24	0.04

Tukey Honestly Significant Difference test: a = III > I, p = 0.019; b = III > II, p = 0.002.

DISCUSSION

The results of this study indicate that $Ca(OH)_2$ and rhTGF-β1 are compatible when added to cultures of osteoblasts. In this environment the combination of carrier and cytokine increased the synthesis of collagen protein. The most robust response to the experimental treatments was observed in the early subculture population (subculture I) of osteoblasts when compared with the late subculture population of osteoblasts (subculture V). Previous studies have demonstrated that exogenous administration of TGF-β to in vitro models of bone formative systems results in an enhanced synthesis of collagen (11). This study demonstrates that the co-administration of $Ca(OH)_2$ with rhTGF-β1 also upregulates collagen synthesis. In fact, the co-administration of $Ca(OH)_2$ with TGF-β1 may provide a more favorable pH for activation of the TGF-β within the local environment, because inactive TGF-β may become activated at a pH of ~12.

In the early subculture population of osteoblasts (subculture I), $Ca(OH)_2$ alone tended to have an inhibitory effect on all protein synthesis (total, collagen, and noncollagen) when compared with the controls, rhTGF-β1 alone, or co-administration with rhTGF-β1. Co-administration of $Ca(OH)_2$ and rhTGF-β1 was stimulatory to total

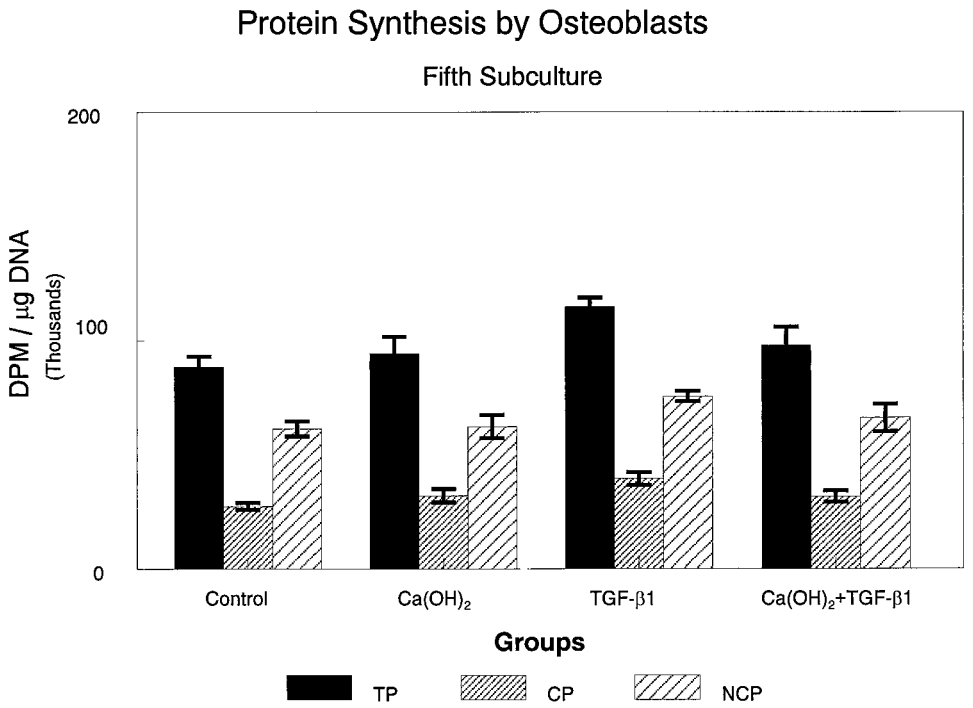

FIG 3. Protein synthesis by the late subculture of osteoblasts (*TP* = total protein; *CP* = collagen protein; *NCP* = noncollagen protein).

TABLE 4. Effects of TGF-β1 and $Ca(OH)_2$ on total protein synthesis in late subcultures (subculture V) of osteoblasts (1×10^5 dpm/μg DNA)

Group (*n*)	Mean	Standard Deviation	Standard Error of the Mean
Control (8)	0.89	0.13	0.05
$Ca(OH)_2$ (7)	0.94	0.2	0.08
TGF-β (8)	1.15[a]	0.11	0.04
$Ca(OH)_2$ + TGF-β (8)	0.98	0.23	0.08
Total (31)	0.99	0.19	0.03

Tukey Honestly Significant Difference test: a = III > I, p = 0.025.

TABLE 5. Effects of TGF-β1 and $Ca(OH)_2$ on collagen synthesis in late subcultures (subculture V) of osteoblasts (1×10^5 dpm/μg DNA)

Group (*n*)	Mean	Standard Deviation	Standard Error of the Mean
Control (8)	0.27	0.04	0.02
$Ca(OH)_2$ (7)	0.32	0.08	0.03
TGF-β (8)	0.39[a]	0.08	0.03
$Ca(OH)_2$ + TGF-β (8)	0.32	0.07	0.03
Total (31)	0.33	0.08	0.01

Tukey Honestly Significant Difference test: a = III > I, p = 0.01.

TABLE 6. Effects of TGF-β1 and $Ca(OH)_2$ on noncollagen protein synthesis in late subcultures (subculture V) of osteoblasts (1×10^5 dpm/μg DNA)

Group (*n*)	Mean	Standard Deviation	Standard Error of the Mean
Control (8)	0.61	0.09	0.03
$Ca(OH)_2$ (7)	0.62	0.14	0.05
TGF-β (8)	0.76	0.06	0.02
$Ca(OH)_2$ + TGF-β (8)	0.66	0.17	0.06
Total (31)	0.66	0.13	0.02

protein and collagen protein synthesis, although there was no statistically significant difference when compared with rhTGF-β1 alone. There was no statistically significant difference on noncollagen protein synthesis when compared with all other groups. Addition of rhTGF-β1 alone seemed to have a more beneficial effect on all protein synthesis than when administered together with $Ca(OH)_2$, although this observation was not necessarily always statistically significant. In the late subculture population of osteoblasts (subculture V), there was a tendency of $Ca(OH)_2$ to increase total protein (collagen protein as well as noncollagen protein synthesis) when administered alone or with rhTGF-β1, although the differences were not statistically significant. Administration of rhTGF-β1 by itself had the most vigorous effect on total protein and collagen protein synthesis, compared with controls. rhTGF-β1, however, had no statistically significant impact on any protein synthesis when compared with $Ca(OH)_2$ administration alone, or when applied in combination with $Ca(OH)_2$.

Murakami et al. (4) have recently (1997) observed that $Ca(OH)_2$ enhances collagen synthesis, glycolysis, and protein synthesis. Our observations are not in agreement with this report. In fact $Ca(OH)_2$ seemed to have an inhibitory effect on all protein synthesis in early subcultures. Although $Ca(OH)_2$ had a tendency to increase protein synthesis in late subcultures, these data were not statistically significant. One possible reason for this discrepancy might be that Murakami's group made their observations in organ cultures. Possibly some of the regulatory mechanisms that are present in organ cultures may be missing in cell cultures. Another variable that may have contributed to the contradiction in results is the actual incubation time of each experimental challenge. Murakami et al. subjected their groups to 3 days of incubation in experimental media, but in this study incubation for 24 h only occurred. It is possible

that the observations that Murakami reported are merely time-dependent phenomena.

Studies of this nature are necessary for determining if the efficacy of growth factor therapy may be improved when an appropriate vehicle is used. $Ca(OH)_2$ was chosen as the vehicle in this study because of its biochemical properties and universal acceptance for use in dental procedures (1–6). This study suggests that co-administration of rhTGF-β1 with $Ca(OH)_2$ may be beneficial to those treatments whose success depends on bone healing (e.g. chronic periradicular periodontitis). These in vitro results, however, demonstrate that newly differentiated osteoblasts will respond more favorably to TGF-β/$Ca(OH)_2$ treatment than older, well-differentiated osteoblasts within the bone.

Mustoe et al. (14) have shown that TGF-β administration alone rapidly diffuses from incisional wounds of rats without any appreciable influences. Strates et al. (20) showed that injection of TGF-β1 adsorbed on resorbable microcrystals of hydroxyapatite induced the formation of large amounts of new bone, compared with growth factor alone in dogs. A review of the literature reveals that no studies exist that consider the effects of TGF-β combined with $Ca(OH)_2$ in a living host. Further studies are indicated to examine the effects of $Ca(OH)_2$ in combination with various growth factors on bone formation both in vitro and in vivo.

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