

Effects of Endothelial Cell Growth Factor on Bone Remodelling In Vitro

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Abstract

Endothelial cell growth factor (ECGF) α was studied for its effects on bone formation in cultured fetal rat calvariae and on bone resorption in cultured fetal rat long bones.

ECGF at 0.1–100 ng/ml stimulated [³H]thymidine incorporation into DNA, an effect enhanced by heparin. Treatment with ECGF for 24 h decreased the incorporation of [³H]proline into collagen but treatment for 48–96 h increased collagen and noncollagen protein synthesis, an effect that was concomitant with an increase in DNA content. ECGF did not alter collagen degradation in calvariae or ⁴⁵Ca release from long bones, which indicated it had no effect on bone resorption. Although ECGF increased prostaglandin E₂ concentrations, its effect on DNA synthesis was not prostaglandin-mediated.

In conclusion, ECGF stimulates calvarial DNA synthesis, which is an effect that results in a generalized increase in protein synthesis, but ECGF has no effect on matrix degradation or bone resorption.

Introduction

Bone formation is a complex process regulated by systemic hormones and by systemic and local growth factors. The effects of systemic hormones on bone formation have been studied extensively (1, 2) but only recently we have started to learn about the regulation of bone formation by systemic and local growth factors (2, 3).

Local regulators of skeletal growth are factors, synthesized and released by bone or bone-related cells, that affect skeletal growth directly without the use of the circulatory system. Growth factors have been isolated from bone cultures and bone matrix and it is likely that a number of them are synthesized by bone or bone-related cells and act as autocrine or paracrine regulators of skeletal growth (4–6). Local growth factors play an essential role in the regulation of tissue growth and this role may be at least as important as that played by the systemic hormones (7). Furthermore, some of the effects of the systemic hormones on skeletal and nonskeletal tissue function are likely mediated by local factors, and hormones could act by modifying the synthesis or effects of a given local growth factor.

Endothelial cell growth factor (ECGF)¹ is a mitogen for human endothelial cells isolated from bovine brain and is a member of a family of polypeptides that includes acidic fibroblast growth factor (FGF) and eye-derived growth factor II (8–10). These factors have similar biological activities and cross-react in both immunological and radioreceptor assays (10). Two forms of ECGF have been described: (a) α -ECGF, with an M_r of 17,000, and (b) β -ECGF, with an M_r of 20,000. The two forms have the same biological properties and significant amino acid homology and α -ECGF is identical to acidic FGF-2 (11, 12, and Burgess, W. H., and T. Maciag, unpublished observations). Although the source of this family of mitogens has been primarily the central nervous system, similar factors have been isolated from a variety of tissues (13). A significant proportion of the growth factor activity present in bovine bone matrix is immunoprecipitated by antibodies to ECGF/acidic FGF (14). This finding is important because it indicates that ECGF is either preferentially trapped by the bone matrix or is synthesized by skeletal cells. It was, therefore, critical to determine if ECGF had effects on bone remodelling and establish whether it is a local regulator of skeletal growth. A growth factor with effects on bone formation, in addition to effects on endothelial cell replication and neovascularization, could play a significant role in bone remodelling and particularly in bone healing.

The present studies were undertaken to characterize the role of ECGF in bone formation and bone resorption. We assessed the direct effects of α -ECGF on bone DNA, collagen and noncollagen protein synthesis in rat calvarial cultures, and on ⁴⁵Ca release in rat long bones.

Methods

Bone formation

The method used to study effects on bone formation has been described in detail previously. One or two half calvariae from 21-d-old fetal rats were cultured in sterile 25-ml flasks containing 2 ml of Biggers Gwatkin medium modified by Fitton Jackson (BGJ medium) supplemented with bovine serum albumin (4 mg/ml; Reheis Chemical Co., Phoenix, AZ), 1 mM unlabeled proline (except for collagen degradation experiments), and 0.1 mM thymidine but no serum (15). The flasks were gassed with 5% CO₂ in air, sealed, and placed in a continuously shaking water bath at 37°C for periods of 24–96 h.

α -ECGF was purified from bovine brain to homogeneity by the sequential use of ammonium sulfate fractionation, heparin sepharose chromatography, and reverse-phase high performance liquid chromatography (HPLC) as previously described (9). α -ECGF was stored frozen in a solution of 33% acetonitrile and 0.1% trifluoroacetic acid (TFA) and

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1. Abbreviations used in this paper: BGJ medium, Biggers Gwatkin medium modified by Fitton Jackson; CDP, collagenase-digestible protein; ECGF, endothelial cell growth factor; NCP, noncollagenase-digestible protein; Pg, prostaglandin.

diluted 1:650 or greater in BGJ medium. An equal amount of organic solvent was added to control calvariae. All experiments were performed with homogeneously pure α -ECGF except for one, presented in Table I, in which the preparation used contained a small amount (<20%) of contaminant β -ECGF, which is known to have the same biological effects as α -ECGF (9). Hydroxyurea (Sigma Chemical Co., St. Louis, MO) and heparin (Ries Biologicals Inc, Irvine, CA) were added directly to BGJ medium, and indomethacin (Sigma Chemical Co.) was dissolved in absolute alcohol and diluted 1:10,000 in BGJ medium; an equal amount of alcohol was added to control cultures. Calvariae were continuously exposed to the agents studied unless specified otherwise.

DNA SYNTHESIS

DNA synthesis was studied by examining effects on the incorporation of [3 H]thymidine and on total DNA content.

DNA labeling. [Methyl- 3 H]thymidine (5 μ Ci/ml; specific activity 60–80 Ci/mmol; New England Nuclear, Boston, MA) was added during the last 60 min of the culture period. Calvariae were extracted with cold 5% TCA, acetone, and ether; the bones were then dried and weighed and the incorporated radioactivity was determined after digesting them with NCS tissue solubilizer (Amersham Corp., Arlington Heights, IL). The acid-extractable pool was determined by measuring the radioactive counts in the TCA wash. Data are expressed as disintegrations per minute per microgram dry weight or as percent of control after dry weight correction.

DNA content. To measure total DNA content, calvariae were homogenized, extracted with 5% TCA and 10 mM potassium acetate in absolute ethanol, and dried. DNA was measured according to a modification of the fluorometric method of Kissane and Robbins (16). Data are expressed as microgram DNA per half calvaria or as percent of control.

HISTOLOGY AND MITOTIC INDEX

To study effects on cell mitosis, calvariae were cultured and *N*-desacetyl-*N*-methyl colchicine (colcemid, 4×10^{-6} M; Gibco, Grand Island, NY) was added for the last 3 h of the culture period (17). Calvariae were fixed in neutral formalin, embedded in paraffin, stained with hematoxylin-eosin, and sections examined at a magnification of 250–400. Metaphase-arrested cells were counted in at least two random cross sections taken from each one of five control and treated bone explants. Data are expressed as number of mitoses per section of half calvaria.

COLLAGEN AND NONCOLLAGEN (NCP) PROTEIN SYNTHESIS

Effects on newly synthesized collagen were determined by studying the incorporation of [2,3- 3 H]proline (5 μ Ci/ml; specific activity 25–40 Ci/mmol; New England Nuclear) into total collagen and into type I collagen. Labeled proline was added for the last 2 h of the culture period, calvariae were extracted as described for DNA labeling and homogenized in 0.5 M acetic acid.

Total collagen synthesis. An aliquot of the homogenized calvaria was incubated with repurified bacterial collagenase (Worthington Biochemical Corp., Freehold, NJ) and the labeled proline incorporated into collagenase-digestible protein (CDP) and NCP was measured according to the method of Peterkofsky and Diegelmann (18). Data are expressed as disintegrations per minute per microgram dry weight and as percent of control after dry weight correction. Percent collagen synthesis was calculated after multiplying NCP by 5.4 to correct for the relative abundance of proline in CDP and NCP (19).

Type I collagen synthesis. A pool of four homogenized half calvariae was digested with pepsin (1 mg/ml; Sigma Chemical Co.) in the presence of 1 mg of nonradioactive type I collagen over a 12–16-h period at 4°C; the collagen was precipitated with 2 M NaCl, dialyzed against 0.5 M acetic acid, and freeze-dried. An aliquot representing one calvaria and a radioactive type I collagen standard were dissolved in 0.5 M acetic acid, diluted in sample buffer, and the collagen chains were separated on a 6% polyacrylamide gel, according to a modification of the method described by Sykes et al. (20). 2-Mercaptoethanol was applied to half of the gels 60 min after the electrophoresis was started (reduced collagen), while the other half was not treated (unreduced collagen). The gels were fixed and the protein profiles were displayed by fluorography.

COLLAGEN DEGRADATION

To study effects on collagen degradation, calvariae were cultured in control medium in the absence of unlabeled proline and treated with 5 μ Ci/ml [3 H]proline for a 24-h period. Bones were washed with BGJ medium containing 10 mM proline for 1 h and transferred to either control or ECGF (30 ng/ml)-containing medium for a 3-, 6-, or 24-h “chase period”. Calvariae were extracted (with TCA, acetone, and ether) and homogenized in 0.5 M acetic acid; an aliquot of this homogenate and of the respective culture medium was hydrolyzed in 6 N HCl at 107°C for 24 h. The samples were dried, derivatized with phenylisothiocyanate, and the [3 H]proline (used for the labeling) and [3 H]hydroxyproline (representing newly labeled collagen) were separated by reverse-phase HPLC using an amino acid analysis pico tag column (Waters Associates, Millipore Corp., Milford, MA) and an acetonitrile gradient. [3 H]Hydroxyproline and [3 H]proline eluted respectively with 19 and 26% of acetonitrile 3 and 11 min after the chromatography was started and their separation was complete under the conditions used. The amount of [3 H]proline eluting as hydroxyproline was 1% or less of the total radioactivity applied to the column. This was corrected by constructing a standard curve of [3 H]proline “spillover” into the [3 H]hydroxyproline peak by using increasing amounts of [3 H]proline. Recovery of radioactively labeled and of unlabeled added hydroxyproline was 88–100%. Data are expressed as picomoles of total and TCA-precipitable [3 H]hydroxyproline present in the medium, and as percent of [3 H]hydroxyproline released from bone to the culture medium.

The proportion of newly synthesized macromolecular collagen released from the bone into the medium during the pulse was determined in aliquots of the medium obtained during the 2-h [3 H]proline pulse used in studies of collagen synthesis. The medium was exhaustively dialyzed in Spectrapor 3 dialysis tubing (Spectrum Medical, Los Angeles, CA; assigned molecular weight cut-off of 3,500) against 0.5 M acetic acid and analyzed for labeled CDP. Data are expressed as percent of CDP released to the medium.

PROSTAGLANDIN (Pg) E₂ ASSAY

PgE₂ was measured in the culture medium in triplicate using a specific radioimmunoassay (New England Nuclear). The cross-reactivity of the antibody was 3.7% with PgE₁ and <0.4% with other known prostaglandins or related compounds. PgE₂ content was calculated from the linear portion of the binding curve. Data are expressed in nanomolar concentrations and as nanograms or picograms of PgE₂ per half calvaria.

EFFECTS ON PERIOSTEUM AND PERIOSTEUM-FREE CALVARIA

These experiments were performed to compare effects on periosteal tissue, rich in fibroblasts and precursor cells, with those on the central calvaria, which is rich in osteoblasts (21, 22). Intact bones were cultured and [3 H]thymidine or [3 H]proline was added for the last 60 or 120 min of the incubation period, respectively. Calvariae were washed with saline (9 g/liter NaCl) and the periosteum was removed from the superior and inferior surfaces of the bone with a scalpel blade. The incorporation of [3 H]thymidine into DNA and of [3 H]proline into CDP and NCP were independently determined in the periosteum and in the central bone or periosteum-free calvaria. Data are expressed as disintegrations per minute per half calvaria.

Bone resorption

Bone resorption was measured by the release of 45 Ca from fetal rat long bones in culture as previously described (23). Pregnant rats were injected with 0.2 mCi of 45 Ca on the 18th day of pregnancy and sacrificed the following day. Radii and ulnae were dissected free of their cartilaginous ends and cultured in BGJ medium for 24 h. The bones were transferred to fresh medium and cultured in the continuous presence of α -ECGF (stored in 33% acetonitrile and 0.1% TFA and diluted in BGJ medium 1:650 or greater) or control medium, containing an equal amount of organic solvent, for a 5-d period. At the completion of the culture period, the bones were dissolved in 5% TCA and 45 Ca was quantitated in bone and medium. Bone resorption is expressed as percent of total radioactivity present in the medium over the culture period.

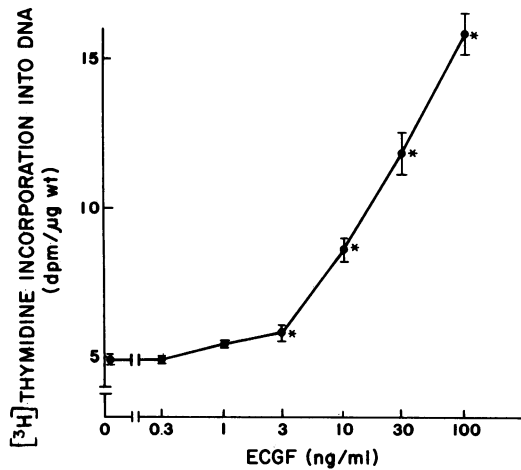


Figure 1. Effect of ECGF on [³H]thymidine incorporation into acid-precipitable material (DNA) in fetal rat calvariae. Symbols and vertical lines represent mean values±SE for five to six half calvariae cultured for 24 h in the continuous presence of ECGF. * Significantly different from control, $P < 0.05$.

Statistical methods

Data are expressed as mean±SEM. Statistical differences were analyzed using *t* test.

Results

Bone Formation

DNA synthesis and cell replication. ECGF caused a dose-dependent stimulation on the incorporation of [³H]thymidine into acid-precipitable material (DNA) in calvariae treated for 24 h (Fig. 1). The effect was observed consistently at concentrations of 3 ng/ml, whereas at 1 ng/ml it was seen in about half of the experiments; at 100 ng/ml, the highest concentration tested, ECGF increased DNA labeling threefold. ECGF had no effect on the uptake of [³H]thymidine into the acid-extractable pool (data not shown) and ECGF at 1–100 ng/ml increased the number of metaphase-arrested cells after colcemid treatment (Table I). The time course of the ECGF effect on DNA labeling was tested by adding 30 ng/ml ECGF at various times before the end of a 24-h culture period; treatment with ECGF for 3 or 6 h was not effective but 12 h of exposure to ECGF resulted in a significant stimulation of [³H]thymidine incorporation into DNA (Fig. 2). After 96 h of continuous treatment, ECGF stimulated

Table I. Effect of ECGF on Mitotic Index in Fetal Rat Calvariae

Treatment	ng/ml	Mitotic index
Control		2.9±0.3
ECGF	1	5.2±0.5*
	10	10.0±1.7*
	100	16.3±1.7*

Values are means±SE for five half calvariae cultured for 24 h in the continuous presence or absence of ECGF and treated with colcemid for the last 3 h of the incubation. Each calvaria was processed as described in Methods and the number of cells arrested in metaphase counted in two or more random sections for each bone. Data are expressed as number of cells arrested in metaphase per calvarial section. * Significantly different from control, $P < 0.05$.

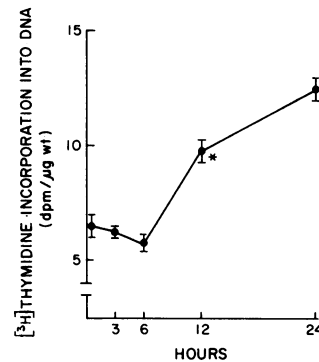


Figure 2. Time course for the effect of ECGF on [³H]thymidine incorporation into acid-precipitable material (DNA). Symbols and vertical lines represent mean values±SE for six half calvariae. Control and treated calvariae were cultured for 24 h and 30 ng/ml ECGF was added 3, 6, 12, or 24 h before the end of the experiment. * Significantly different from control, $P < 0.05$.

[³H]thymidine incorporation into DNA at doses as low as 0.1 ng/ml and the effect was maximal at 10 ng/ml (Table II). Whereas treatment with ECGF for 24 h did not change total DNA content, continuous treatment with ECGF for 96 h at 1–30 ng/ml resulted in a significant increase in calvarial DNA (Table II).

The effect of ECGF on DNA labeling was enhanced in the presence of heparin (Fig. 3). Heparin by itself did not modify the incorporation of [³H]thymidine into DNA but it enhanced the effect of ECGF about twofold. Further, in the presence of heparin, 24-h treatment with ECGF caused a stimulation of DNA labeling at doses (0.1–0.3 ng/ml) that were ineffective in the absence of heparin.

Collagen and noncollagen protein synthesis. Treatment with ECGF for a 24-h period did not increase the incorporation of [³H]proline into CDP or NCP and at 30–100 ng/ml ECGF caused a small but consistent inhibition on the labeling of CDP (Table III, Fig. 4). In contrast, treatment with ECGF for 48–96 h resulted in an increase on the incorporation of [³H]proline into CDP and NCP (Fig. 4). This effect was time dependent and concomitant with an increase in DNA content that started to appear after 48 h of treatment and was significant after 72 h of exposure to ECGF. The effect of ECGF on protein synthesis was generalized and not specific for collagen and the percent of collagen synthesized was decreased by ECGF in about half of the experiments, the control and ECGF-treated values calculated from the data presented in Fig. 4 were (mean±SE; $n = 6$) 11.8±0.4 and 7.3±0.5% respectively, $P < 0.05$. However, the collagen stimulated after 96 h of ECGF exposure was type I and there was no detectable type III collagen in treated calvariae (data not shown).

Table II. Effect of ECGF on [³H]Thymidine Incorporation into DNA and on DNA Content in Calvariae Cultured for 96 h

Treatment	ng/ml	DNA synthesis (dpm/μg wt)	DNA content (μg/half calvaria)
Control		6.6±0.7	7.8±0.9
ECGF	0.1	8.8±0.5*	N.D.
	1	10.8±0.3*	12.3±0.7*
	10	15.5±0.2*	11.3±0.7*
	30	16.6±1.2*	15.2±1.3*

Values represent the ECGF effect on the 1 h incorporation of [³H]thymidine into acid-precipitable material and on DNA content in calvariae cultured and continuously treated for 96 h. Values are means±SE for 6–12 half calvariae. Data for DNA synthesis are pooled from two experiments. N.D., not determined.

* Significantly different from control $P < 0.05$.

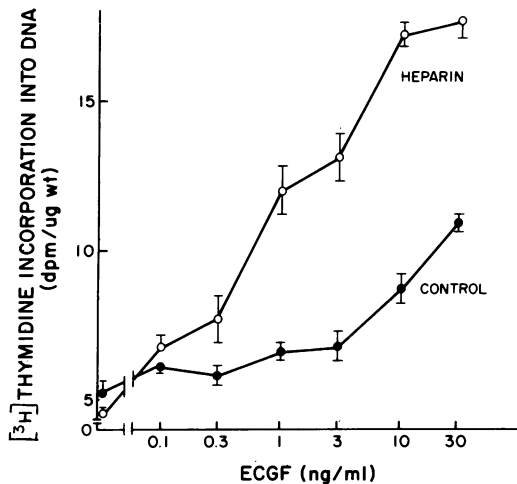


Figure 3. Effect of ECGF in the presence (open circles) and absence (filled circles) of 50 $\mu\text{g/ml}$ heparin, on $[^3\text{H}]$ thymidine incorporation into acid-precipitable material (DNA) in fetal rat calvariae. Symbols and vertical lines represent mean values \pm SE five half calvariae cultured for 24 h in the continuous presence of ECGF.

ECGF at 30 ng/ml did not affect collagen degradation. The amount of TCA-precipitable $[^3\text{H}]$ hydroxyproline present in the medium, representing newly synthesized macromolecular collagen, was small ($<10\%$ of the total $[^3\text{H}]$ hydroxyproline counts in the medium) and not different in control and ECGF-treated bones (Fig. 5). Therefore, most of the total $[^3\text{H}]$ hydroxyproline present in the medium represented fully degraded newly synthesized collagen, and it was the same in control and treated calvariae during a 3–24-h chase period; over the 24-h period $\sim 20\%$ of the newly labeled collagen was released into the medium of control and ECGF-treated bones. To confirm that ECGF did not alter macromolecular collagen release, we examined the labeled CDP present in the medium during the 2-h $[^3\text{H}]$ proline pulse used to determine effects on collagen synthesis. After a 24-h culture, $5.7 \pm 1.1\%$ (mean \pm SE) of the total newly synthesized macromolecular collagen was released to the culture medium in control cultures and $6.2 \pm 0.5\%$ in ECGF (100 ng/ml)-treated cultures. After a 96-h culture the respective values for control and ECGF (30 ng/ml)-treated bones were 7.6 ± 1.5 and $8.7 \pm 1.5\%$.

Effects on periosteum and periosteum-free calvaria. The incorporation of $[^3\text{H}]$ thymidine into DNA and the incorporation

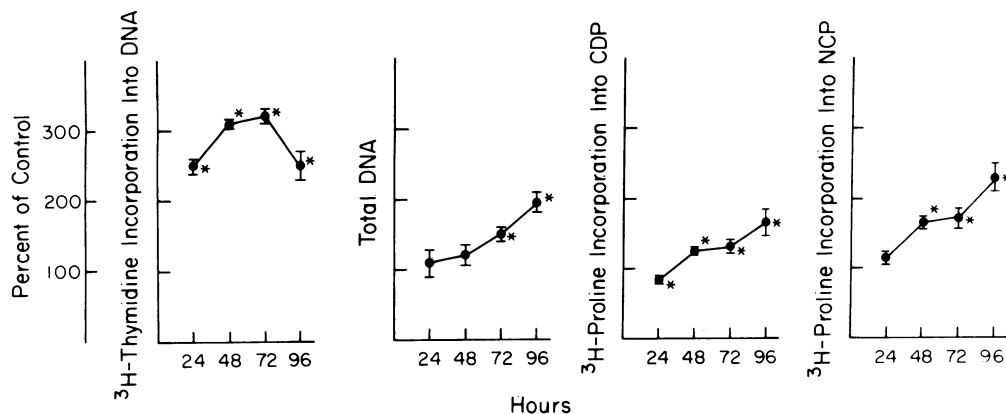


Figure 4. Effect of ECGF on $[^3\text{H}]$ thymidine incorporation into acid-precipitable material (DNA), DNA content, and $[^3\text{H}]$ proline incorporation into CDP and NCP. Calvariae were cultured in the continuous presence of 30 ng/ml ECGF for the indicated periods of time. Data for the labeling of DNA, CDP, and NCP were corrected per bone dry weight (disintegrations per minute per weight); all data are expressed as percent of control. Symbols and vertical lines represent mean values \pm SE for six half calvariae. * Significantly different from control, $P < 0.05$.

Table III. Transient Inhibitory Effect of ECGF on Collagen Synthesis

Treatment	CDP	NCP	Collagen synthesis	
	ng/ml	dpm/ μg weight	%	
Control		27 ± 2	37 ± 3	12.0 ± 0.7
ECGF	1	27 ± 1	40 ± 1	11.2 ± 0.4
	10	25 ± 1	38 ± 2	10.6 ± 0.1
	100	$20 \pm 1^*$	35 ± 4	10.0 ± 0.6

Values represent the ECGF effect on the 2-h incorporation of $[^3\text{H}]$ proline into CDP and NCP and on the percent of collagen synthesized in calvariae cultured and treated for 24 h. Values are means \pm SE for five half calvariae.

* Significantly different from control, $P < 0.05$.

of $[^3\text{H}]$ proline into NCP were higher in the periosteum than in the periosteum-free bone of control and treated calvariae (Table IV), whereas the labeling of CDP and the percentage of collagen synthesized were greater in the periosteum-free bone. The stimulatory effect of ECGF on the incorporation of $[^3\text{H}]$ thymidine and on $[^3\text{H}]$ proline incorporation into CDP and NCP occurred in the periosteum and periosteum-free bone but the effect on DNA labeling was more pronounced in the periosteal tissue.

Prostaglandin synthesis. ECGF also increased the PgE_2 content in the medium of cultured calvariae. This effect was dose dependent and observed as early as 3 h after ECGF (30 ng/ml) treatment (Fig. 6). The PgE_2 concentration in control bones cultured for 24 h was (mean \pm SE; $n = 6$) 0.7 ± 0.2 nM or 249 ± 81 pg/half calvaria; in the presence of ECGF at 10–100 ng/ml for a 6-h period the PGE_2 concentration increased from 2.8 ± 0.4 to 11.7 ± 2.4 nM or from 973 ± 128 to $4,107 \pm 837$ pg/half calvaria respectively (Fig. 6B). The stimulatory effect of ECGF on PgE_2 synthesis was sustained for 96 h and it became more pronounced as calvariae were treated for longer periods of time. When PgE_2 concentrations were measured at 24-h intervals in the medium of calvariae cultured for 96 h in control or in the continuous presence of 30 ng/ml ECGF, the agent increased PgE_2 levels about twofold in the first 24-h period and about ninefold in the last 24-h period.

Although the stimulatory effect of ECGF on PgE_2 synthesis appeared before the effect on DNA synthesis, the two effects did

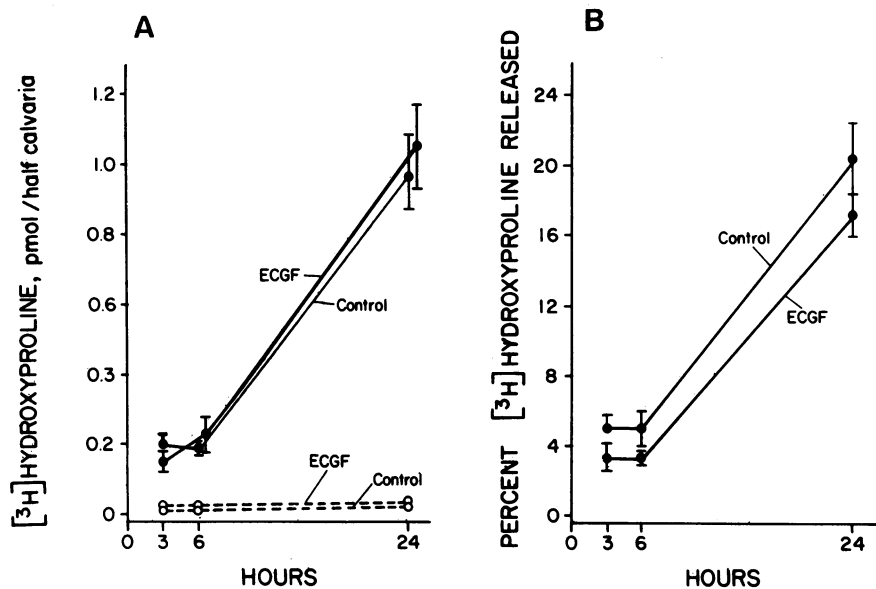


Figure 5. Effect of ECGF on collagen degradation. Calvariae were cultured in control medium for 24 h in the absence of unlabeled proline in the presence of [³H]proline (5 μCi/ml), rinsed, and transferred to medium containing 10 mM proline and “chased” in the presence or absence of 30 ng/ml ECGF for the indicated periods of time. Symbols and vertical lines represent means±SE for four half calvariae. Data for acid-precipitable [³H]hydroxyproline was obtained from a pool of four half calvariae. Data are expressed as picomoles of total and acid precipitable [³H]hydroxyproline released to the culture medium (A) or as the percent of the newly synthesized [³H]hydroxyproline released to the culture medium (B). None of the treated values was significantly different from control.

not appear related because ECGF stimulated DNA synthesis to a similar extent in the presence and absence of indomethacin at 1 μM (Table V).

Bone resorption

Treatment with ECGF at 1–100 ng/ml for a 5-d period did not increase ⁴⁵Ca release from fetal rat long bones (Table VI), indicating lack of an ECGF effect on bone resorption in this system.

Discussion

The present studies were undertaken to examine the effects of ECGF on bone formation and bone resorption in vitro. ECGF stimulated DNA synthesis in cultures of fetal rat calvariae because it increased the incorporation of [³H]thymidine into acid-precipitable material, the bone DNA content, and the number of metaphase-arrested cells after colcemid arrest. Recent data have shown that heparin increases the affinity of ECGF to its

cell surface receptor and enhances the mitogenic effect of ECGF on endothelial cells (24). Similarly, heparin enhanced the ECGF effect on bone DNA synthesis. The mechanism of the interaction between heparin and ECGF is unclear but it appears related to conformational changes that may stabilize the peptide. This would result in an enhancement of the ECGF effect that is not selective for endothelial cells and would explain the results observed in calvarial cultures. ECGF increased the concentration of PgE₂ in the culture medium; however, the significance of this effect remains to be determined because indomethacin, a known inhibitor of prostaglandin synthesis (25), did not abolish the ECGF effect on DNA synthesis.

ECGF decreased bone collagen synthesis in 24-h cultures, which indicated a direct inhibition of osteoblastic function. However, ECGF increased collagen and noncollagen protein synthesis in long-term cultures, an effect that was concomitant with, and likely secondary to, an increase in DNA content and cell number. The stimulatory effect of ECGF was not specific

Table IV. Effect of ECGF on DNA, Collagen, and Noncollagen Protein Synthesis in Periosteum and Periosteum-free Calvaria

Treatment	DNA labeling <i>dpm/bone</i>	CDP <i>dpm/bone</i>	NCP <i>dpm/bone</i>	Collagen synthesis %
Periosteum				
Control	1,400±140	2,470±260	9,850±800	4.4±0.5
ECGF	3,430±290*	4,070±680*	16,980±1,900*	4.9±0.3
Periosteum-free calvaria				
Control	640±44	6,180±730	4,950±590	20.5±1.0
ECGF	870±80*	11,170±1,120*	7,150±940	20.0±1.6

Values represent the effect of 30 ng/ml ECGF on [³H]thymidine into acid-insoluble residues (DNA) in calvariae cultured and treated for 24 h and the effect of ECGF, 10 ng/ml, on [³H]proline into CDP and NCP and the percent of collagen synthesized in calvariae treated for 96 h. The periosteum was removed by dissection after the incubation was completed. Values are means±SE for 6–8 half calvariae.

* Significantly different from control, *P* < 0.05.

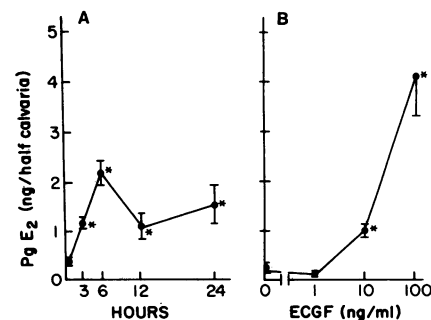


Figure 6. Effect of ECGF on PgE₂ production by fetal rat calvariae. Bones were cultured for 24 h and PgE₂ levels were measured by radioimmunoassay in the culture medium. A, time course of the ECGF effect; calvariae were cultured for 24 h and treated with 30 ng/ml ECGF for 3, 6, 12, or 24 h before the end of the culture. B, dose-response curve for ECGF; calvariae were cultured for 24 h and treated with ECGF for the last 6 h at the indicated doses. Symbols and vertical lines represent mean values±SE for five half calvariae. * Significantly different from control, *P* < 0.05.

Table V. Effect of ECGF and Indomethacin on DNA Synthesis in Fetal Rat Calvariae

Treatment	DNA	PgE ₂
	dpm/ μ g wt	Pg/half calvaria
Control	5.2 \pm 0.4	199 \pm 51
ECGF	10.9 \pm 0.3*	575 \pm 95*
Indomethacin	4.9 \pm 0.3	25 \pm 2
Indomethacin + ECGF	9.2 \pm 0.4*	24 \pm 3

Values represent the effect of 30 ng/ml ECGF and 1 μ M indomethacin on the 1-h incorporation of [³H]thymidine into acid-precipitable material (DNA) and on PgE₂ content in the medium of calvariae cultured for 24 h. Values are means \pm SE for five half calvariae.

* Significantly different from control, $P < 0.05$.

for collagen, in fact, a larger effect was observed on noncollagen than on collagen protein synthesis, but the collagen synthesized was type I, indicating that ECGF stimulated the replication of cells, which included those of the osteoblastic lineage. This was supported by the data demonstrating that the stimulatory effect of ECGF on collagen synthesis was observed in the central non-periosteal bone, which is rich in osteoblasts (21, 22). Collagen synthesis in the periosteal layers was probably stimulated because the dissection of the calvaria did not remove all the osteoblasts from the periosteum. The effect on DNA synthesis was primarily observed in the periosteum, which is rich in fibroblasts and progenitor cells, and it is likely that ECGF affected both cell types (21, 22, 24).

About 20% of the newly synthesized collagen was released to the culture medium over a 24-h period and most of it was in fully degraded form. Only a small proportion of macromolecular collagen appeared in the culture medium during the [³H]proline pulse or during a 24-h chase period, indicating that most of the newly synthesized collagen is laid on the bone matrix and the jettison of the intact molecule does not occur in this culture model to any significant extent. ECGF did not increase bone collagen degradation in cultured calvariae or the release of ⁴⁵Ca from fetal rat long bones, which indicated that it has no effect on matrix degradation or bone resorption in these models. However, the stimulatory effect of ECGF on PgE₂ synthesis could result in a stimulation of bone resorption in neonatal mouse calvariae, because in this model prostaglandins frequently mediate growth factor-stimulated bone resorption (26).

Table VI. Effect of ECGF on ⁴⁵Ca Release from Fetal Rat Long Bones

Dose of ECGF tested ng/ml	Release of ⁴⁵ Ca	
	Control	ECGF
	%	%
1	31 \pm 2	30 \pm 1
10	29 \pm 1	29 \pm 1
100	25 \pm 3	29 \pm 3

Values are means \pm SE for six cultures of ECGF-treated and paired control bones cultured for 5 d.

The effects of ECGF on bone remodelling in vitro are different from those of other known growth factors including epidermal growth factor, platelet-derived growth factor, insulin-like growth factor I/Somatomedin-C and bone-derived growth factor (15, 27–30). Interleukin-1, which has limited amino acid sequence homology with acidic FGF (31), stimulates bone resorption and has stimulatory effects on collagen synthesis in 24-h cultures, whereas in 96 h cultures it is inhibitory and these effects are temporarily opposite to those of ECGF (32, 33). Transforming growth factor- β is the only growth factor that has effects resembling those of ECGF on bone formation, but it has been shown to stimulate bone resorption in mouse calvariae, an effect not reported in fetal rat long bones (26, 34).

The stimulatory effects of ECGF on endothelial cell replication and neovascularization in correlation with those on bone cell replication may be important for bone repair, particularly after fractures. Moreover, the mitogenic effect of ECGF on endothelial and bone cells is enhanced by heparin, and the fracture callus is rich in heparin-containing mast cells (35). Heparin increases the binding of ECGF to its receptor and this could have significant physiological implications in the process of bone repair, particularly if mast cell degranulation and heparin release occur at the fracture site. ECGF may also be important in osteoinduction, observed with matrix-derived factors (5), and its effects on cell replication and neovascularization may be essential for this process.

ECGF/acidic FGF is present in bone matrix (14), but synthesis by bone cells has not as yet been demonstrated. Studies to address this question are currently being performed in our laboratory and they will be necessary before we can conclude that ECGF is a true local regulator of skeletal growth. Whether ECGF is trapped by bone matrix or is synthesized by bone cells, its significant concentrations in this tissue combined with its effects on bone cell replication make it an important regulator of skeletal remodelling. Moreover, ECGF may be essential for normal bone repair.

In conclusion, ECGF stimulates bone DNA synthesis, an effect that results in an increased number of cells capable of synthesizing bone matrix proteins but ECGF has no effect on bone matrix degradation or bone resorption.

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References

1. Canalis, E. 1983. The hormonal and local regulation of bone formation. *Endocr. Rev.* 4:62–77.
2. Canalis, E. 1985. Effects of growth factors on bone cell replication and differentiation. *Clin. Orthop. Related Res.* 193:246–263.
3. Centrella, M., and E. Canalis. 1985. Local regulators of skeletal growth, a perspective. *Endocr. Rev.* 6:544–551.
4. Canalis, E., W. A. Peck, and L. G. Raisz. 1980. Stimulation of DNA and collagen synthesis by autologous growth factor in cultured fetal rat calvaria. *Science (Wash. DC)*. 201:1021–1023.

5. Urist, M. R., R. J. DeLange, and G. A. M. Finerman. 1983. Bone cell differentiation and growth factors. *Science (Wash. DC)*. 220:680-686.
6. Seyedin, S. M., A. Y. Thompson, H. Bentz, D. M. Rosen, J. M. McPherson, A. Conti, N. R. Siegel, G. R. Galluppi, and K. A. Piez. 1986. Cartilage inducing factor. Apparent identity to transforming growth factor- β . *J. Biol. Chem.* 261:5693-5695.
7. Nilsson, A., J. Isgaard, A. Lindahl, A. Dahlstrom, A. Skottner, and O. G. P. Isaksson. 1986. Regulation by growth hormone of number of chondrocytes containing IGF-I in rat growth plate. *Science (Wash. DC)*. 233:571-574.
8. Maciag, T., T. Mehlman, R. Friesel, and A. B. Schreiber. 1984. Heparin binds endothelial cell growth factor, the principal endothelial cell mitogen in bovine brain. *Science (Wash. DC)*. 225:932-935.
9. Burgess, W. H., T. Mehlman, R. Friesel, W. V. Johnson, and T. Maciag. 1985. Multiple forms of endothelial cell growth factor. *J. Biol. Chem.* 260:11389-11392.
10. Schreiber, A. B., J. Kenney, J. Kowalski, K. A. Thomas, G. Gimenez-Gallego, M. Rios-Candelore, J. DiSalvo, D. Barritault, J. Courty, Y. Courtois, M. Moenner, C. Loret, W. H. Burgess, T. Mehlman, R. Friesel, W. Johnson, and T. Maciag. 1985. A unique family of endothelial cell polypeptide mitogens: The antigenic and receptor cross-reactivity of bovine endothelial cell growth factor, brain-derived acidic fibroblast growth factor, and eye-derived growth factor-II. *J. Cell Biol.* 101:1623-1626.
11. Esch, F., N. Ueno, A. Baird, F. Hill, L. Denoroy, N. Ling, D. Gospodarowicz, and R. Guillemin. 1985. Primary structure of bovine brain acidic fibroblast growth factor (FGF). *Biochem. Biophys. Res. Commun.* 133:554-562.
12. Gimenez-Gallego, G., J. Rodkey, C. Bennett, M. Rios-Candelore, J. DiSalvo, and K. Thomas. 1985. Brain-derived acidic fibroblast growth factor. Complete amino acid sequence and homologues. *Science (Wash. DC)*. 230:1385-1388.
13. Lobb, R., J. Sasse, R. Sullivan, Y. Shing, P. D'Amore, J. Jacobs, and M. Klagsbrun. 1986. Purification and characterization of heparin-binding endothelial cell growth factors. *J. Biol. Chem.* 261:1924-1928.
14. Hauschka, P., S. Dolman, M. Iafrazi, J. Sassae, and M. Klagsbrun. 1986. Characterization of growth factors derived from bone matrix. *J. Cell. Biochem. (Suppl. 10B)*:111. (Abstr.)
15. Canalis, E. 1980. Effect of insulin-like growth factor I on DNA and protein synthesis in cultured rat calvaria. *J. Clin. Invest.* 66:709-719.
16. Kissane, J. M., and E. Robbins. 1958. The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. *J. Biol. Chem.* 233:184-188.
17. Canalis, E. 1983. Effect of glucocorticoids on type I collagen synthesis, alkaline phosphatase activity and deoxyribonucleic acid content in cultured rat calvariae. *Endocrinology*. 112:931-939.
18. Peterkofsky, B., and R. Diegelmann. 1971. Use of a mixture of proteinase-free collagenases for the specific assay of radioactive collagen in the presence of other proteins. *Biochemistry*. 10:988-993.
19. Peterkofsky, B. 1972. The effect of ascorbic acid on collagen polypeptide synthesis and proline hydroxylation during the growth of cultured fibroblasts. *Arch. Biochem. Biophys.* 152:318-328.
20. Sykes, B., B. Puddle, M. Francis, and R. Smith. 1976. The estimate of two collagens from human dermis by interrupted gel electrophoresis. *Biochem. Biophys. Res. Commun.* 72:1472-1480.
21. Peck, W. A., S. J. Birge, and S. A. Fedak. 1964. Bone cells: biochemical and biological studies after enzymatic isolation. *Science (Wash. DC)*. 146:1476-1477.
22. Wong, G. L., and D. V. Cohn. 1975. Target cells in bone for parathormone and calcitonin are different. Enrichment for each cell type by sequential digestion of mouse calvaria and selective adhesion to polymeric surfaces. *Proc. Natl. Acad. Sci. USA*. 72:3167-3171.
23. Raisz, L. G., and J. Niemann. 1969. Effect of phosphate, calcium and magnesium on bone resorption and hormonal responses in tissue culture. *Endocrinology*. 85:446-452.
24. Schreiber, A. B., J. Kenney, W. J. Kowalski, R. Friesel, T. Mehlman, and T. Maciag. 1985. Interaction of endothelial cell growth factor with heparin: characterization by receptor and antibody recognition. *Proc. Natl. Acad. Sci. USA*. 82:6138-6142.
25. Levine, L. 1972. Prostaglandin production by mouse fibrosarcoma cells in culture: inhibition by indomethacin and aspirin. *Biochem. Biophys. Res. Commun.* 47:888-896.
26. Tashjian, A. H., E. F. Voelker, M. Lazzaro, F. R. Singer, A. B. Roberts, R. Derynck, M. E. Winkler, and L. Levine. 1985. α and β human transforming growth factors stimulate prostaglandin production and bone resorption in cultured mouse calvaria. *Proc. Natl. Acad. Sci. USA*. 82:4535-4538.
27. Canalis, E., and L. G. Raisz. 1979. Effect of epidermal growth factor on bone formation in vitro. *Endocrinology*. 104:862-869.
28. Raisz, L. G., M. A. Simmons, A. L. Sandberg, and E. Canalis. 1980. Direct stimulation of bone resorption by epidermal growth factor. *Endocrinology*. 107:270-273.
29. Canalis, E. 1981. Effect of platelet-derived growth factor on DNA and protein synthesis in cultured rat calvaria. *Metabolism*. 30:970-975.
30. Canalis, E., and M. Centrella. 1986. Isolation of a nontransforming bone-derived growth factor from medium conditioned by fetal rat calvariae. *Endocrinology*. 118:2002-2008.
31. Thomas, K. A., M. Rios-Candelore, G. Gimenez-Gallego, J. DiSalvo, C. Bennett, J. Rodkey, and S. Fitzpatrick. 1985. Pure brain-derived acidic fibroblast growth factor is a potent angiogenic vascular endothelial cell mitogen with sequence homology to interleukin 1. *Biochemistry*. 82:6409-6413.
32. Gowen, M., D. D. Wood, E. J. Ibrrie, M. K. B. McGuire, and R. G. G. Russell. 1983. An interleukin 1-like factor stimulates bone resorption in vitro. *Nature (Lond.)*. 306:378-380.
33. Canalis, E. 1986. Interleukin-1 has independent effects on DNA and collagen synthesis in cultures of rat calvariae. *Endocrinology*. 118:74-81.
34. Centrella, M., J. Massague, and E. Canalis. 1986. Human platelet-derived transforming growth factor beta stimulates parameters of bone growth in fetal rat calvariae. *Endocrinology*. 119:2306-2312.
35. Lindholm, R., S. Lindholm, P. Liukkop, J. Paasimaki, S. Iso-kaanta, R. Rossi, E. Autio, and E. Taminen. 1969. The mast cell as a component of callous in healing fractures. *J. Bone J. Surg. Am. Vol.* 51B:148-155.