Effects of Rat Heart Endothelial Cell-Conditioned Medium on the Proliferation and Differentiation of Osteoblasts

In-Seok Lee, Kyu-Won Choi, Jeong-Hwa Baek and Gwan-Shik Kim

*Department of Pharmacology and Dental Therapeutics,
College of Dentistry, Seoul National University*

Bone development and remodeling depend on complex interactions between bone-forming osteoblasts, bone-degrading osteoclasts, and other cells present within the bone microenvironment. Especially, bone vascular endothelial cells may play a pivotal role in these interactions via linking circulatory and local signals with cells of the bone microenvironment and actively contributing itself to the regulation of bone cell physiology. Therefore, the understanding of the role of endothelial cells in bone remodeling and their interactions with stromal and hematopoietic systems of bone tissue could be very important.

In this paper, rat heart endothelial cells (RHEC) were isolated and endothelial cell-conditioned medium (ECCM) was collected from RHEC culture. The effects of ECCM on the proliferation and differentiation of osteoblast were investigated using osteoblastic cell lines and fetal calvarial cells. And the mitogenic activities were identified in partially purified ECCM.

Isolated rat heart endothelial cells had a relatively uniform morphology and exhibited a cobblestone-like appearance, and were positively stained with Dil-Ac-LDL. ECCM stimulated the proliferation of MG 63 and MC3T3-E1 cells, but inhibited the proliferation of ROS 17/2.8 cells. ECCM did not show any stimulatory or inhibitory effects on the alkaline phosphatase activity of MG 63 and MC3T3-E1 cells. However, it demonstrated a significant stimulatory effect on the alkaline phosphatase activity of ROS 17/2.8 cells. And ECCM inhibited in vitro mineralized nodule formation by fetal rat calvarial cells. On MG 63 cells, mitogenic activities were evident in the fraction eluting close to the void volume of the column(>60 KDa) and in fractions corresponding to the molecular weight of 27, 24, 19 and 16 KDa. On MC3T3-E1 cells, mitogenic activity was evident only in the fraction close to the void volume. However, on ROS 17/2.8 cells, growth-inhibiting activity appeared in the fraction corresponding to >60 KDa.

The demonstration that osteoblastic cell lines respond to ECCM may prove to be an important contribution with respect to establishing a direct link between initiation of bone formation and vascularization.

Key words: Endothelial cell, Osteoblastic cell, Mitogenic activity, Alkaline phosphatase activity

Introduction

The bone has a well-organized architecture with various cells and extracellular matrices. Also the bone is a dynamic tissue which is constantly remodeled by subsequent cycles of bone resorption and formation (Nijweide et al., 1986). Both phases of the remodeling process are controlled by systemic and local factors that affect the proliferation and/or differentiation of osteoblast and osteoclast (Kahn and Partridge, 1987; Vaes, 1988; Canalis et al., 1989a, b). But there is little understanding of the role of other types of cell in bone tissue. Especially, bone vasculature appears to play a major role and the understanding of the role of bone endothelial cells in bone formation and their interactions with stromal and hematopoietic systems

of bone tissue could be very important (Streeten and Brandi, 1990).

Both intramembranous and endochondral ossification occur in association with blood capillaries. Intramembranous ossification is characterized by capillary ingrowth into a mesenchymal region of the embryonic connective tissue, followed by mesenchymal cell differentiation into osteogenic cells that secrete collagen and mineral to produce bone (Collin-Osdoby, 1994). In early endochondral ossification, the cartilage may produce an antiangiogenic factor inhibiting vascular penetration. After vascular invasion, the hypertrophied cartilage core is degraded and replaced by bone marrow and later by bone. Only cells which are situated near capillaries give rise to bone tissue (Trueta and Amato, 1960; Eisenstein et al., 1975; Diaz-Flores et al., 1992). Therefore the vasculature in bone tissue is important in skeletal de-

velopment and repair, and may direct new bone formation by serving as a scaffolding for osteoblasts (Streeten and Brandi, 1990).

The vascular endothelial cells have been traditionally regarded important solely for its role in maintaining vascular integrity. But now the endothelial cells are recognized to have a variety of synthetic and metabolic capabilities. Many reports showed that cultured endothelial cells synthesize and secrete polypeptides into their growth medium which have mitogenic effects upon several connective tissue cells, such as smooth muscle cells isolated from large vessel, Swiss 3T3 and BALB/c-3T3 cells (Gajdusek *et al.*, 1980), and human bone marrow fibroblasts (Scarra *et al.*, 1985). Partial purification of these mitogenic factors revealed heterogeneity in its composition with molecular weight ranging from 10,000 to greater than 100,000 Da. Immunological and biochemical studies have shown that the endothelial cell-derived growth factors are distinct and different from fibroblast growth factor, epidermal growth factor and platelet-derived growth factor or the somatomedins (DiCorleto *et al.*, 1983). Other studies reported that endothelial cells synthesize and secrete fibroblast growth factor, interleukin-1 and -6, colony stimulating factors and endothelin-1 (Canalis *et al.*, 1987; Norioka *et al.*, 1988; Yanagisawa *et al.*, 1988; May *et al.*, 1989; Zaidi *et al.*, 1993a, b). These factors also control the recruitment, proliferation, differentiation, function, and survival of various cells including osteoblasts and osteoclasts (Zaidi *et al.*, 1993a, b; Favus, 1993). Recently, aortic endothelial cells have been shown to synthesize bone cell active mitogen(s) and enhance bone formation when implanted subcutaneously in diffusion chambers with calvarial cells (Guenther *et al.*, 1986; Villanueva and Nimni, 1990). These reports represent that the endothelial cells could be an important element in the process of bone formation.

In this paper, three osteoblastic cell lines were used to investigate the direct effect of endothelial cell-conditioned medium (ECCM) on the proliferation of osteoblast, and alkaline phosphatase activity was assayed for the effect of it on the osteoblastic activity. To further examine the effect of ECCM on the differentiation of osteoblast, the effect of it on the *in vitro* mineralized nodule formation was evaluated using fetal rat calvarial cells. And the mitogenic activities were identified in par-

tially purified ECCM.

Materials and Methods

Materials

The cell culture medium, fetal bovine serum (FBS) and other culture reagents were obtained from GIBCO/BRL (Grand Island, NY, USA) and plastic culture wares from Corning (Corning, NY, USA). Crude bacterial collagenase and deoxyribonuclease I (DNAse I) were purchased from Sigma (St. Louis, MO, USA) and [methyl-^{3}H]thymidine (25 Ci/mmol) from Amersham (Amersham, UK), and Bio-Gel P-60 from Bio-Rad (Richmond, CA, USA). Minitan ultrafiltration system and filter plates were obtained from Millipore (Bedford, MA, USA). Dil-Ac-LDL (acetylated low-density lipoprotein labeled with 1.1'-dioctadecyl-1-3,3,3',3'-tetramethyl- indocarbocyanine perchlorate) was obtained from Biomedical Technologies (Stoughton, MA, USA). FR cell - fetal rat skin fibroblast - was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

Isolation and culture of rat heart endothelial cell (RHEC)

The differential adhesion technique by Kasten (1973) was used to obtain endothelial cells of heart from rats (Kasten, 1973). Ventricles were removed from five 4-day-old male rats (Sprague-Dawley) after cervical dislocation. The tissues were washed three times, and finely minced with No. 15 blades in Dulbecco's modified Eagle's medium (DMEM) containing 100 U penicillin-100 μg streptomycin per ml. These were then subjected to two successive enzyme treatment [0.2% collagenase and 0.02% DNAse I in Ca^{2+}- and Mg^{2+}- free Hank's balanced salt solution (HBSS)] at 37°C for 30 min with stirring in reaction vials. After enzyme treatment, the cells were collected with centrifugation at 200×g, resuspended in 10 mM HEPES-buffered DMEM (H-DMEM) with 20% FBS, and plated in 0.1% gelatin-coated 100-mm tissue culture dishes for 90 min. The culture medium was then removed by aspiration and the culture dishes were washed three times with HBSS to remove nonadherent (mostly myocardial) cells. Thereafter, H-DMEM with 20% FBS was added to the re-

maining adherent cells. Since the myocardial cells require a much greater time (approximately 24 h) to adhere to a culture surface, the adherent cells were used in this experiments as endothelial cell (RHEC) (Richards et al., 1986). In order to confirm the type of cell, RHEC were plated in 35-mm tissue culture dishes and 10 μg/ml Dil-Ac-LDL, specific marker of endothelial cell, was loaded for 4 h at 37°C (Voyta et al., 1984; Nishida et al., 1993). The dishes were washed, fixed with 10% formalin, and then examined for fluorescence using a fluorescence microscope (Olympus, BHS). Rat skin fibroblasts (FR cells) were also processed as the same manner as RHEC for negative control.

Culture of osteoblastic cell lines

Three osteoblastic cell lines were used for these experiments. MG 63 cells, a human osteosarcoma cell line, were obtained from ATCC and maintained in minimun essential medium (MEM) with nonessential amino acid containing 10% FBS plus antibiotics. ROS 17/2.8 cells, a rat osteosarcoma cell line, were maintained in F12 containing 5% FBS, and MC3T3-E1 cells, a osteogenic non-transformed murine cell line, were maintained in α-MEM containing 10% FBS. ROS 17/2.8 and MC 3T3-E1 cells were kindly provided by Dr. Hauschka at Harvard School of Dental Medicine (Boston, MA, USA).

Isolation and culture of fetal rat calvarial cells

Calvaria from fetal rat (Sprague-Dawley) at 19 days of gestation were dissected and subjected to sequential enzyme digestion protocol as previously described (An et al., 1989; Kim et al., 1990). Briefly, fetal frontal and parietal bone were digested consecutively five times with enzyme mixture containing 0.1% collagenase, 0.05% trypsin and 0.5 mM EDTA for predetermined periods. Immediately after digestion, isolated cells were washed with Hank's balanced salt solution two times and viable cells were counted with trypan blue exclusion method. Calvarial cells, released in later stages, which exhibit osteoblastic characteristics, were pooled, and maintained in MEM with nonessential amino acid containing 10% FBS.

Preparation of endothelial cell-conditioned medium (ECCM)

RHEC cultures were established by seeding 1× 10^6 cells/dish in 100-mm tissue culture dishes with H-DMEM containing 20% FBS. After 2 days, media were removed and cells were rinsed with HBSS, and serum-free H-DMEM was added. After one more day, fresh serum-free H-DMEM was replaced. Then the conditioned media were collected two times successively at 2-day interval. Control conditioned medium (CCM) was prepared the same way without RHEC. After removing floating cells by centrifugation at 400×g for 10 min, the media were subjected to ultrafiltration with a Minitan ultrafiltration system equipped with Minitan filter plates (molecular weight cutoff 10,000). Ten-fold concentrates were dialyzed against PBS for 24 h at 4°C using a Spectrapor No. 3 tubing (molecular weight cutoff 3,500). To partially purify ECCM, 20-fold concentrates were prepared and extensively dialyzed against several changes of 0.1 M acetic acid at 4°C, using a Spectrapor No. 3 tubing. After dialysis, acetic acid-insoluble fraction was removed by centrifugation at 1000×g for 10 min.

Cell proliferation

As indexes of cell proliferation, the rate of DNA synthesis was evaluated by measuring the incorporation of [methyl-^{3}H]thymidine into trichloroacetic acid (TCA)-insoluble fraction and cell number was counted with a cell counter (Coulter, Model ZM). Osteoblastic cell lines were seeded at density of 5×10^4 cells/well in 24-well plates or 1× 10^5 cells/35-mm tissue culture dish. After incubation for 2 days, the media were removed and the cells were equilibrated with serum-free media for 1 day. After equilibration, the media were changed with fresh serum-free media containing ECCM or CCM at various concentrations. In case of [^{3}H]thymidine incorporation assay, incubation was carried for an additional 24 h, and the cells were pulsed with 2.5 μCi/well of [methyl-^{3}H]thymidine for final 2 h. After aspirating the medium, the cells were fixed for 15 min at 4°C and washed 3 times with ice-cold 5% TCA. Precipitate was solubilized in 0.5 M NaOH and radioactivity was measured with liquid scintillation counter (Beckman, LS 5000 TA). To determine the number of cells, the cells were incubated for another 2 days with ECCM or CCM, suspended with Isoton II (Coulter, Luton, England), and counted.
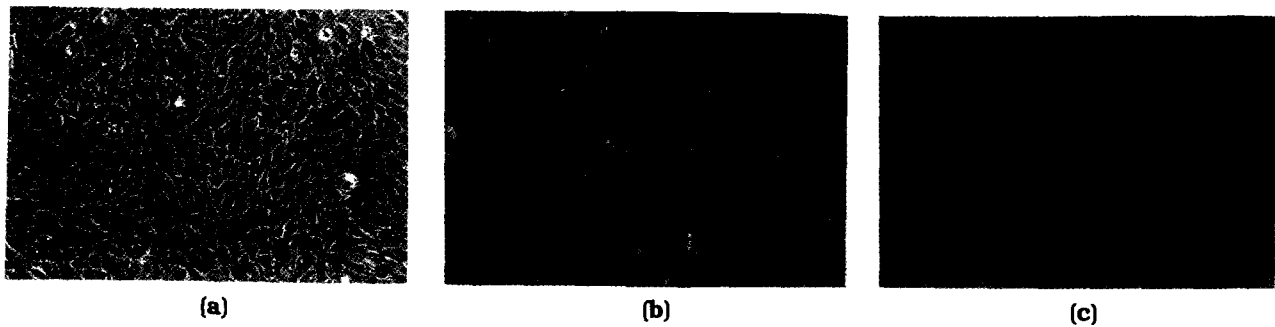
Fig. 1. Characterization of rat heat endothelial cells (RHEC). Primary isolates of RHEC reached confluency at 5 days, demonstrating a cobblestone-like appearance characteristic of endothelial cell (a) ($\times 100$). RHEC and rat skin fibroblasts (FR cells) were loaded with Dil-Ac-LDL for 4 h at 37°C. RHEC exhibited granular cytoplasmic staining (b) ($\times 200$), but FR cells showed negative staining (c) ($\times 200$).

Alkaline phosphatase activity

Alkaline phosphatase activity was measured by spectrophotometry using p-nitrophenyl phosphate (15 mM) as a substrate. After equilibration, the osteoblastic cell lines were cultured with serum-free media containing 10-fold concentrated ECCM or CCM at 50, 100 and 200 µl/ml for 48 h. The cells were collected and sonicated in 0.5 ml distilled water. Aliquots of the cell homogenates were incubated at 37°C for 30 min in 0.1 M glycine-NaOH buffer (pH 10.3). The optical density of p-nitrophenol, a reaction product, was read at 410 nm. Protein content in cell homogenate was determined by BCA (Pierce, Rockford, IL, USA) assay with bovine serum albumin as a standard.

Mineralized nodule formation *in vitro*

Fetal rat calvarial cells were plated at a density of 2×10^4 cells/well in 4-well dishes, and cultured with α-MEM supplemented with 10% FBS, 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate. Media were changed every second or third days and cultures were maintained up to 19 days in the presence of 100 µl/ml ECCM or CCM. At the end of cultures, cell layer was fixed with neutral buffered formalin and stained *in situ* by the von Kossa technique for mineral deposits. Numbers of mineralized nodules were counted at 40X magnification using a microscope.

Gel filtration chromatography

Five ml of acetic acid-soluble fraction of 20-fold concentrated ECCM was acidified to 0.5 M with acetic acid and then applied onto a Bio-Gel P-60 column (2.5×60 cm) which was equilibrated with

0.5 M acetic acid. The protein samples were eluted from the column with 0.5 M acetic acid at room temperature, at a flow rate of 30 ml/h. Fractions (2.5 ml) were lyophilized with Speedvac (Savant, A 160) and then reconstituted with 1.8 ml PBS and filtered with 0.22 µm membrane filter for the measurement of mitogenic activity.

For the estimation of the apparent molecular weight of the active fraction, the column was calibrated with proteins of known molecular weight.

Results

Isolation and characterization of RHEC

Isolated RHEC had a relatively uniform morphology, and within 5 days, the RHEC grew to confluence and exhibited a cobblestone-like appearance as viewed by phase contrast microscopy (Fig. 1a).

Primary cells of the second passage were characterized by examining Dil-Ac-LDL uptake. RHEC exhibited granular cytoplasmic appearance positively stained with Dil-Ac-LDL (Fig. 1b), but FR cells showed negative staining (Fig. 1c).

Cell proliferation

ECCM stimulated the proliferation of MG 63 and MC3T3-E1 cells as demonstrated by [³H]thymidine incorporation assay. At all concentrations, ECCM showed statistically significant stimulatory effects on the proliferation of MG 63 cells (Fig. 2a) and at concentrations of 100 and 200 µl/ml of media, ECCM showed statistically significant stimulatory effects on the proliferation of MC3T3-E1 cells (Fig. 2b). But ECCM significantly inhibited the proliferation of ROS 17/2.8 cells at all
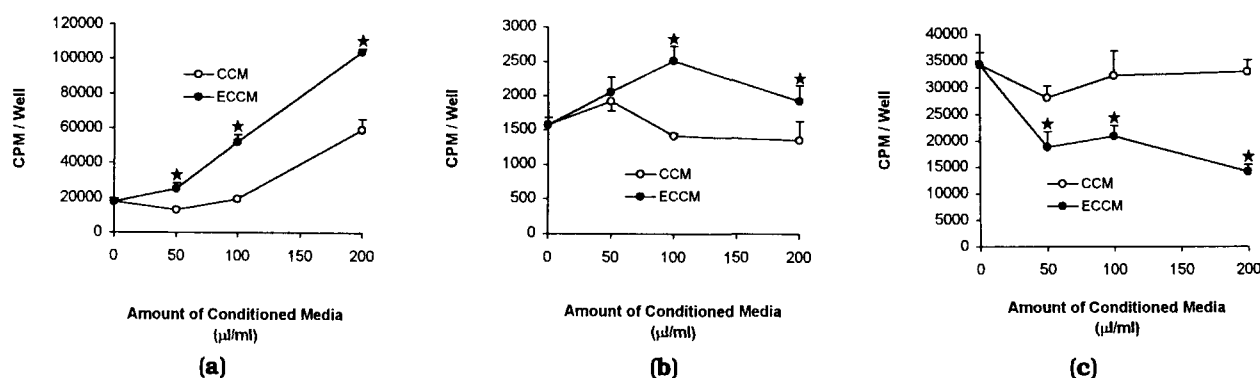
Fig. 2. Effect of ECCM on the incorporation of [^{3}H]thymidine into DNA by cultures of osteoblastic cell lines. Cultures maintained at a density of 5×10^4 cells/well in 24-well plates were treated with ECCM or CCM for 24 h, pulsed with 2.5 μCi of [^{3}H]thymidine during the final 2 h and incorporated radioactivities into TCA-insoluble fraction were counted. ECCM stimulated the proliferation of MG 63 (a) and MC3T3-E1 cells (b), but inhibited the proliferation of ROS 17/2.8 cells (c). Values are mean ± SE (n=3). ★: p < 0.05 (Compared with paired control).

concentrations (Fig. 2c). These results were confirmed by the cell counting. ECCM increased the number of MG 63 and MC3T3-E1 cells significantly. However, ECCM significantly decreased the number of ROS 17/2.8 cells when compared with paired control (Table 1).

Alkaline phosphatase activity

ECCM did not show any significant effect on the alkaline phosphatase activities of MG 63 and MC 3T3-E1 cells when compared with paired control (Fig. 3a,b). However, ECCM demonstrated a significant stimulatory effect on the alkaline phosphatase activity of ROS 17/2.8 cells (Fig. 3c).

Mineralized nodule formation in vitro

At the beginning of the culture, fetal rat calvarial cells exhibited spindle-shaped appearance but became polygonal, and multilayered, three dimensional nodular structure was observed around day 7. When calvarial cells were cultured with ascorbic acid and β-glycerophosphate, considerable number of mineralized nodules was developed aft-

er 19 days. The number of mineralized nodules was not changed significantly by the continuous presence of CCM during the whole culture period. But ECCM inhibited completely the formation of mineralized nodule (Fig. 4).

Gel filtration chromatography of ECCM

The mitogenic activities of fractions collected from the Bio-Gel P-60 column were determined. The protein elution profile demonstrated that most proteins were eluted in void volume fractions (Fig. 5a). On MG 63 cells, mitogenic activities were evident in the fraction eluting close to the void volume, and then in the fractions corresponding to the molecular weight of 27, 24, 19 and 16 KDa (Fig. 5b). On MC3T3-E1 cells, mitogenic activity was evident only in the fractions close to the void volume (Fig. 5c). However, on ROS 17/2.8 cells, growth-inhibiting activity appeared in the fraction corresponding to >60 KDa (Fig. 5d).

Discussion

Many reports described that whether osteogenic

Table 1. Effect of ECCM on the growth of osteoblastic cell lines

	Number of cells × 10⁻³		
	MG 63	ROS 17/2.8	MC3T3-E1
No Treatment	299.6 ± 13.93	669.9 ± 20.58	440.0 ± 12.35
CCM	310.1 ± 4.28	594.3 ± 31.72	415.3 ± 6.01
ECCM	459.6 ± 16.85**	527.3 ± 11.00*	573.4 ± 4.22**

Cultures maintained at a density of 1×10^5 cells/dish in 35-mm tissue culture dishes were treated with ECCM or CCM at 100 μl/ml for 2 days. The cells were suspended with Isoton II, and counted. Coulter counter was setted as follows: Current=100 mA, Full scale=1, Polarity= +, Attenuation=4, Preset gain=1, Aperture diameter=100 μm, Lower threshold=15.0, Upper threshold=99.0 Data represent mean ± SE of 4 replicates. * : p<0.05 (Compared with paired control) ** : p<0.01 (Compared with paired control)

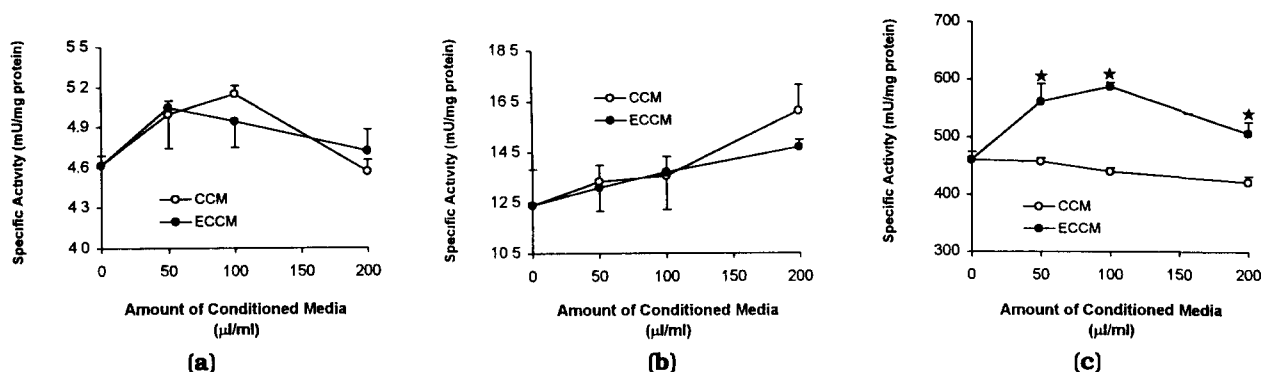**Fig. 3.** Effect of ECCM on the alkaline phosphatase activity of osteoblastic cell lines. Cultures maintained at a density of 5×10^5 cells/dish in 35-mm tissue culture dishes were treated with ECCM or CCM for 48 h. One unit of the enzyme activity was defined as that amount of enzyme which catalyzes the transformation of 1 μmole of substrate per min at 37°C. ECCM did not show stimulatory or inhibitory effect on MG-63 (a) and MC3T3-E1 cells (b), but demonstrated stimulatory effect on ROS 17/2.8 cells (c). Values are mean ± SE (n=4). ★: P<0.05 (Compared with paired control).
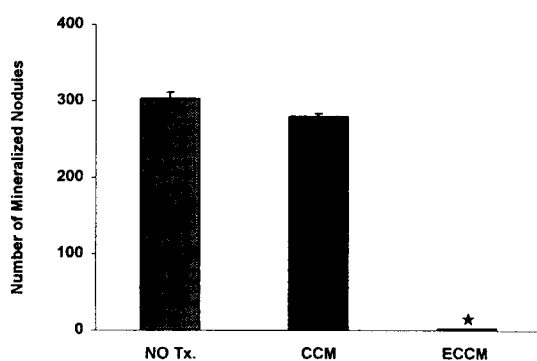

Fig. 4. Effect of ECCM on the *in vitro* mineralized nodule formation by fetal rat calvarial cells. Cells were seeded at a density of 2×10^4 cells/well in 4-well plate and cultured in the presence of 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate for 19 days. During culture periods, the cells were continuously treated with ECCM or CCM at a concentration of 100 μl/ml of media. Compared with CCM, ECCM inhibited completely the formation of mineralized nodule. Data represent mean ± SE of 4 replicates. ★: p<0. 001 (Compared with paired control).

cells of the periosteum differentiated to form bone or cartilage depended on the presence or absence of vascular capillaries (Trueta and Amato, 1960; Schenk *et al.*, 1967). And other studies showed that initiation sites of bone formation, so-called centers of osteogenesis, always reside in a vascular environment of intramembranous and endochondral ossification as well as bone fracture healing (Schenk *et al.*, 1967; Buring, 1975). Therefore the importance of vascular endothelium in bone formation and resorption was postulated.

To obtain pure population of endothelial cells, RHEC were isolated and cultured using differential adhesion technique in 0.1% gelatin-coated dishes. These cells have been well characterized on a mor-

phologic and cytochemical basis (Wenzel *et al.*, 1970; Kasten, 1973; Richards *et al.*, 1986). In this study, isolated RHEC had a relatively uniform morphology with a cobblestone-like appearance. Although high concentration of FBS (20%) was generally necessary for endothelial cell proliferation and viability in monolayer culture, RHEC remained viable in serum-free media. Subcultured RHEC after 3-4 passages began to lose their polygonal morphology and cobblestone-like appearance at confluency. Thus only the second passage of RHEC was used for the preparation of ECCM.

To exclude the indirect effect of endothelial cells on bone cells through the fibroblasts, three osteoblastic cell lines were used. It is well known that MG 63, ROS 17/2.8 and MC3T3-E1 cells have numerous osteoblastic traits (Haussler *et al.*, 1980; Majeska and Rodan, 1982; Chen *et al.*, 1983; Sudo *et al.*, 1983; Nakatani *et al.*, 1984; Franceschi *et al.*, 1985; Kurihara *et al.*, 1986). Fetal rat calvarial cells were used for the investigation of the effect of ECCM on the *in vitro* mineralized nodule formation, because the cells stably formed mineralized nodule *in vitro* and bone *in vivo* (Bellows *et al.*, 1986).

The present study has provided data which indicate that osteoblastic cells respond markedly to a factor synthesized and released by RHEC in vitro. However, the osteoblastic cell lines used in this study responded to ECCM in different manner. ECCM had a stimulatory effect on the proliferation of MG 63 and MC3T3-E1 cells, but inhibitory effect on ROS 17/2.8 cells. While ECCM had a stimulatory effect on the alkaline phosphatase ac-
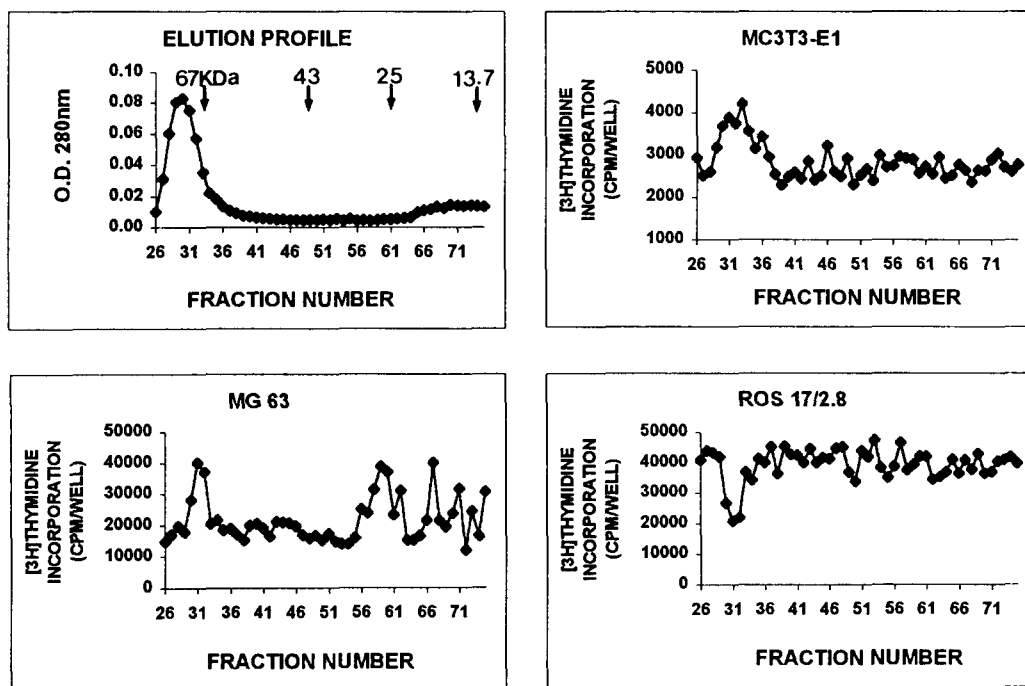
Fig. 5. Partial purification of ECCM. Mitogenic activity was determined as outlined in "Materials and Methods". The protein elution profile was determined at 280 nm and mitogenic activity of partially purified ECCM was tested on MG 63, MC3T3-E1 and ROS 17/2.8 cells at a concentration of 100 µl fraction volume/ml of culture media. Molecular weight markers were used; Vo=BSA, 67,000, ovalbumin, 43,000, chymotrypsinogen A, 25,000, and ribonuclease, 13,700 Da. On MG 63 cells, mitogenic activities were evident in fraction eluting close to the void volume of the column (>60 KDa), and in fractions corresponding to the molecular weght of 27, 24, 19 and 16 KDa. And on MC3T3-E1 cells, mitogenic activity was evident only in fraction close to the void volume. However, on ROS 17/2.8 cells, growth-inhibiting activity appeared in fraction corresponding to >60 KDa.

tivity of ROS 17/2.8 cells, it had no effect on MG 63 and MC3T3-E1 cells. These findings may reflect species differences, differences in the mechanism of cell line immortalization, or differences in the degree of osteoblastic differentiation among the osteoblastic cell lines. In previous studies, multifunctional growth factors such as transforming growth factor-β or interleukin-1 modulate the proliferation and differentiation of bone cells in different way according to the degree of differentiation (Centrella et al., 1987). Therefore, our results possibly reflects either that ECCM may contain multifunctional growth factors or that three osteoblastic cell lines may be different in the degree of differentiation (Schwartz et al., 1992). And also, it seems that because ECCM is collected from rat endothelial cells, the responses by ROS 17/2.8 cells whose origin is rat osteosarcoma differ from those of other cell lines of human or mouse origin. Although this study showed different results according to the type of osteoblastic cell lines, our results indicated that ECCM has an important role in the initiation of bone formation

because ECCM stimulates the cell proliferation or alkaline phosphatase activity which are closely associated with bone formation.

In our experiment, ECCM inhibited completely the formation of mineralized nodule by fetal rat calvarial cells. The result means that ECCM may initiate the bone formation by stimulating the proliferation of osteoblasts but inhibit the maturation or mineralization to bone tissue. It is supported by the phenomenon that mineralization do not occur at a area adjacent to blood vessel in bone remodeling site. Whether that ECCM indirectly inhibit the mineralization by continuous stimulation of proliferation of osteoblasts or that it directly inhibit the mineralization is unclear. Villanueva and Nimni reported that endothelial cells promoted calvarial cell osteogenesis when diffusion chambers containing two above cells, alone or combination, were implanted subcutaneously in the backs of rats. Compared with this report, our result means that other systemic and local factor or cell-to-cell interactions may be necessary to form the mineralized bone rather than endothelial cell alone.

As to the mitogenic nature of ECCM, it appeared to be heterogenous being eluted in several fractions from a Bio-Gel P-60 column. The spread of mitogenic activity over a relatively broad molecular weight range is consistent with previous reports (Gajdusek *et al.*, 1980; DiCorleto *et al.*, 1983; Scarra *et al.*, 1985). Guenther *et al.* reported that mitogenic activities on calvarial cells have approximate molecular weight of 30,000 - 43,000 Da in bovine aortic endothelial cell-conditioned medium. But ECCM used in this study had not shown mitogenic activity in the fraction of this range. On the other hand, Gajdusek *et al.* and DiCorleto *et al.* demonstrated that mitogenic activities on fibroblasts in bovine aortic endothelial cell-conditioned medium have apparent molecular weight of 10,000 - 30,000 Da. Present results in MG 63 cells are consistent with those reports (Gajdusek *et al.*, 1980; DiCorleto *et al.*, 1983), but not in ROS 17/2.8 and MC3T3-E1 cells. Mitogenic activity in the fraction of >60 KDa corresponds to the effect of ECCM on the proliferation of three osteoblastic cell lines. But its apparent molecular weight is greater than those of other growth factors that affect the proliferation of osteoblasts, suggesting that either this mitogen associates nonspecifically with contaminating proteins in the conditioned medium, or it possesses a binding protein of high molecular weight. Further purification under various conditions will be necessary to identify the nature of mitogenic activity in this fraction.

The fact that osteoblastic cell lines are the target cells of ECCM may suggest a possible dependency of these cells on bone tissue-invading endothelial cells. Because ECCM stimulated alkaline phosphatase activity as well as cell growth in two osteoblastic cell lines, ECCM might function as more than mere mitogen. It is probable that ECCM may contain multiple growth factors, and further purification and identification of growth factors will be necessary to look into the role of endothelial cell in bone development, remodeling, and repair.

Conclusion

Vascular endothelial cells actively synthesize and secrete many soluble mediators either constitutively or in response to induction stimuli. To investigate how endothelial cells function in bone tissue, the effects of ECCM on the proliferation and differentiation of osteoblast were investigated using osteoblastic cell lines and fetal rat calvarial cells. And the mitogenic activities were identified in partially purified ECCM.

As indexes of cell proliferation, the rate of DNA synthesis was evaluated by measuring the incorporation of [methyl-^{3}H]thymidine into trichloroacetic acid (TCA)-insoluble fraction and cell number was counted with Coulter counter. Alkaline phosphatase activity was measured by spectrophotometry using p-nitrophenyl phosphate as a substrate. The effect on *in vitro* mineralized nodule formation was assessed by histochemical methods. To partially purify ECCM, acetic acid-soluble fraction of ECCM was acidified to 0.5 M with acetic acid and then applied onto a Bio-Gel P-60 column.

Isolated RHEC had a relatively uniform morphology, and the RHEC grew to confluence and exhibited a cobblestone-like appearance. RHEC exhibited granular cytoplasmic appearance positively stained with Dil-Ac-LDL but FR cells showed negative staining.

ECCM stimulated the proliferation of MG 63 and MC3T3-E1 cells, but inhibited the proliferation of ROS 17/2.8 cells. ECCM did not show any significant effect on the alkaline phosphatase activities of MG 63 and MC3T3-E1 cells, however, ECCM demonstrated a significant stimulatory effect on the alkaline phosphatase activity of ROS 17/2.8 cells. And ECCM inhibited *in vitro* mineralized nodule formation by fetal rat calvarial cells.

On MG 63 cells, mitogenic activities were evident in the fractions eluting close to the void volume of the column, and then in the fractions corresponding to the molecular weight of 27, 24, 19 and 16 KDa. On MC3T3-E1 cells, mitogenic activity was evident only in the fraction close to the void volume. However, on ROS 17/2.8 cells, growth-inhibiting activity appeared in the fraction corresponding to >60 KDa.

The demonstration that osteoblastic cell lines respond to ECCM may prove to be an important contribution with respect to establishing a direct link between initiation of bone formation and vascularization.

References

An, J.-J., Kim G.-S. and Cheong, D.-K.: Osteogenic capacity of rat calvarial cell populations. *Kor. J. Oral Biol.* **13**: 105, 1989.

Bellows, C.G., Aubin, J.E., Heersche, J.N.M. and An

tosz, M.E.: Mineralized bone nodules formed *in vitro* from enzymatically released rat calvaria cell populations. *Calcif. Tissue Int.* **38**: 143, 1986.

Buring, K: On the origin of cells in heterotropic bone formation. *Clin. Orthop. Relat. Res.* **110**: 293, 1975

Canalis, E., Lorenzo, J., Burgess, W. and Maciag, T.: Effects of endothelial cell growth factor on bone remodelling *in vitro*. *J. Clin. Invest.* **79**: 52, 1987.

Canalis, E., McCarthy, T.L. and Centrella, M.: The regulation of bone formation by local growth factors. In bone and mineral research, Vol. 6, edited by Peck, W.A., pp. 27-56, Elsevier Science Publishers B.V., Amsterdam, 1989a.

Canalis, E., McCarthy, T.L. and Centrella, M.: The role of growth factors in skeletal remodeling. *Endocrin. Metabol. Clin. N. Am.* **18**: 903, 1989b.

Centrella, M., McCarthy, T.L. and Canalis, E.: Transforming growth factor β is a bifunctional regulator of replication and collagen synthesis in osteoblast-enriched cell cultures from fetal rat bone. *J. Biol. Chem.* **262**: 2869, 1987.

Chen, T.L., Cone, C.M., Morey-Holton, E. and Feldman, D.: 1-alpha, 25-dihydroxyvitamin D_3 receptors in cultured rat osteoblast-like cells. Glucocorticoid treatment increases receptor content. *J. Biol. Chem.* **258**: 4350, 1983.

Collin-Osdoby, P.: Role of vascular endothelial cells in bone biology. *J. Cell. Biochem.* **55**: 304, 1994.

Diaz-Flores, L., Gutierrez, R., Lopez-Alonso, A., Gonzalez, R. and Varela, H.: Pericytes as a supplementary source of osteoblasts in periosteal osteogenesis. *Clin. Orthop. Relat. Res.* **275**: 280, 1992.

DiCorleto P.E., Gajdusek C.M., Schwartz S.M. and Ross R.: Biochemical properties of endothelium derived growth factor: comparison to other growth factors. *J. Cell. Physiol.* **114**: 339, 1983.

Eisenstein, R., Kuettner, K.E., Neapolitan, C., Lawrence, B.S., Soble, W. and Sorgente, N.: The resistance of certain tissues to invasion. *Am. J. Pathol.* **81**: 337, 1975.

Favus, M.: Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. New York: Raven Press, 1993.

Franceschi, R.T., James, W.M. and Zerlauth, G.: 1-alpha, 25-dihydroxyvitamin D_3 specific regulation of growth, morphology, and fibronectin in a human osteosarcoma cell line. *J. Cell. Physiol.* **123**: 401, 1985.

Gajdusek C., DiCorleto P., Ross R. and Schwartz S.M.: An endothelial cell-derived growth factor. *J. Cell Biol.* **85**: 467, 1980.

Guenther H.L., Fleisch H. and Sorgente N.: Endothelial cells in culture synthesize a potent bone cell active mitogen. *Endocrinology* **119**(1): 193, 1986.

Haussler, M.R., Manolagas, S.C. and Deftos, L.J.: Glucocorticoid receptor in clonal osteosarcoma cell lines:

a novel system for investigating bone active hormones. *Biochem. Biophys. Res. Commun.* **94**: 373, 1980.

Kahn, A.J. and Partridge, N.C.: New concepts in bone remodeling: An expanding role for the osteoblast. *Am. J. Otolaryngol.* **8**: 258, 1987.

Kasten, F.H.: Mammalian myocardial cells. In Tissue Culture Methods and Applications, pp. 72-81, Academic Press, New York. 1973.

Kim, J.-G., Ko, S.-H., Kim H.-R., Kim, G.-S. and Cheong, D.-K.: Effects of some growth factors on the bone nodule formation in rat calvarial cells. *J. Dent. College SNU* **14**: 93, 1990.

Kurihara, N., Ishizuka, S., Kiyoki, M., Haketa, Y., Ikeda, K. and Kumegawa, M.: Effects of 1,25-dihydroxyvitamin D3 on osteoblastic MC-3T3-E1 cells. *Endocrinology* **118**: 940, 1986.

Majeska, R.J. and Rodan, G.A.: The effects of 1,25-$(OH)_2D_3$ on alkaline phosphatase in osteoblastic osteosarcoma cells. *J. Biol. Chem.* **257**: 3362, 1982

May, L.T., Gabriella, T., Cozzolino, F., Ray, A., Tatter, S.B., Santhanam, U., Sehgal, P.B., and Stern, D.: Interleukin-6 gene expression in human endothelial cells: RNA start sites, multiple IL-6 proteins and inhibition of proliferation. *Biochem. Biophys. Res. Commun.* **159**: 991, 1989.

Nakatani, Y., Tsunoi, M., Hakeda, Y., Kurihara, N., Fujita, K. and Kumegawa, M.: Effects of parathyroid hormone on cAMP production and alkaline phosphatase activity in osteoblastic clone MC-3T3-E1 cells. *Biochem. Biophys. Res. Commun.* **123**: 894, 1984.

Nijweide, P.J., Burger, E.H. and Feyen, J.H.M.: Cells of bone : Proliferation, Differentiation, and Hormonal Regulation. *Physiol. Rev.* **66**: 855, 1986.

Nishida. M., Carley, W.W., Gerritsen, M.E., Ellingsen, ♀., Kelly, R.A. and Smith, T.W.: Isolation and characterization of human and rat cardiac microvascular endothelial cell. *Am. J. Physiol.* **264**: H 639, 1993.

Norioka K., Hara, M., Harigai, M., Kitani, A., Hirose T., Suzuki, K., Kawakami, M., Tabata, H., Kawagoe, M., and Nakamura, H.: Production of B cell stimulatory factor-2/interleukin-6 activity by human endothelial cells. *Biochem. Biophys. Res. Commun.* **153**: 1045, 1988.

Richards, P.S., Saba, T.M., Del Vecchio, P.J., Vincent, P.A. and Gray, V.C.: Matrix fibronectin disruption in association with altered endothelial cell adhesion induced by activated polymorphonuclear leukocytes. *Exp. Mol. Pathol.* **45**: 1, 1986.

Scarra G.B., Fiorentini P., Garbarino G., Bellone G., Sessarego M. and Ajmar F.: Effect of endothelial cell conditioned medium on the growth of human bone marrow fibroblasts. *J. Cell. Physiol.* **123**: 343, 1985.

Schenk, R.K., Spiro, D. and Wiener, J.: Cartilage resorption in the tibial epiphysial plate of growing rats. *J. Cell Biol.* **34**: 275, 1967.

Schwartz, Z., Dennis, R., Bonewald, L., Swain, L., Gomez, R. and Boyan, B.D.: Differential regulation of prostaglandin E_2 synthesis and phospholipase A_2 activity by 1,25-$(OH)_2D_3$ in three osteoblast-like cell lines (MC-3T3-E1, ROS 17/2.8, and MG-63). *Bone* **13**: 51, 1992.

Streeten E.A. and Brandi M.L.: Biology of bone endothelial cells. *Bone Min.* **10**: 85, 1990.

Sudo, H., Kodama, H.A., Amagai, Y., Yamamoto, S. and Kasai, S.: In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. *J. Cell. Biol.* **96**: 191, 1983.

Trueta, J. and Amato, V.P.: The vascular contribution to osteogenesis. *J. Bone Joint Surg.* **42**: 571, 1960.

Vaes, G.: Cellular biology and biochemical mechanism of bone resorption: A review of recent developments on the formation, activation, and mode of action of osteoclasts. *Clin. Orthop. Relat. Res.* **231**: 239, 1988.

Villanueva J.E. and Nimni M.E.: Promotion of calvarial cell osteogenesis by endothelial cells. *J. Bone. Min.*

Res. **5**(7): 733, 1990.

Voyta, J.C., Via, D.P., Butterfield, C.E. and Zetter, B.R.: Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. *J. Cell Biol.* **99**: 2034, 1984.

Wenzel, D.G., Wheatley, J.W. and Byrd, G.D.: Effect of nicotine on cultured rat heart cells. *Toxicol. Appl. Pharmacol.* **17**: 774, 1970.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T.: A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332**: 411, 1988.

Zaidi, M., Alam, A.S.M.T., Bax,B.E., Shankar, V.S., Bax, C.M.R., Gill, J.S., Pazianas, M., Huang. C. L.-H., Sahinoglu, T.S., Moonga, B.S., Stevens, C.S. and Blake, D.R.: Role of the endothelial cell in osteoclast control: New prospectives. *Bone* **14**: 97, 1993a.

Zaidi, M., Pazianas, M., Shankar, V.S., Bax, B.E., Bax, C.M.R., Bevis, P.J.R., Stevens, C.S., Huang. C. L.-H., Blake, D.R., Moonga, B.S. and Alam, A.S.M.T.: Osteoclast function and its control. *Exp. Physiol.* **78**: 721, 1993b.