研　　究

Effects of elcatonin on matrix calcification of Meckel's cartilage *in vitro*

Kiyoto Ishizeki, Tadayoshi Kagiya, Naoki Fujiwara, and Hidemistu Harada
Department of Oral Anatomy, School of Dentistry, Iwate Medical University
(Chief : Prof. Hidemistu Harada)

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Abstract : The effects of elcatonin (ELC) on Meckel's cartilage obtained from 17-day embryonic mice were investigated using cell and organ cultures. Specimens for cell and organ cultures were cultured for 4 weeks in different media as follows: Control medium, alpha-modified Eagle's medium (α -MEM); β -Gly medium, α -MEM plus β -glycerophosphate (β -Gly); ELC medium, α -MEM plus ELC; and β -Gly & ELC medium, α -MEM plus β -Gly plus ELC. Specimens were analyzed by histological and histochemical methods, including immunohistochemistry for alkaline phosphatase (ALPase) activity and bromodeoxyuridine (BrdU) incorporation. Von Kossa staining revealed that the ELC medium induced effective calcification similar to β -Gly medium, while BrdU incorporation showed that there was no significant difference in the rates of cell proliferation between Control medium and ELC medium. Histochemical and immunofluorescence staining indicated that ALPase was activated prior to matrix calcification in cell and organ cultures. β -Gly & ELC medium induced synergistically the highest level of matrix calcification and ALPase activity. The present results suggest the possibility that ELC accelerates activity of ALPase in Meckel's chondrocytes and subsequently induces matrix calcification.

Key words : Meckel's cartilage, Chondrocyte, Elcatonin, Calcification, Cell and organ culture

Introduction

Elcatonin (ELC) is a calcitonin analogue that differs from the native peptide hormone by replacement of the disulfide bond with an ethylene bridge and deletion of the N-terminal amino group[1]. To date, in addition to synthetic derivatives of eel calcitonin (Asu[1, 7])[1), 2)] and salmon calcitonin (sCT) [3], a novel derivative, SB205614, has been reported[4]. ELC possesses a high degree of stability and the full biological activities of eel calcitonin[5], and has been

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reported to inhibit bone resorption in osteoclasts. Due to its effects on osteoclasts, ELC treatment has been used successfully for hypercalcemia[6] and osteoporosis[5, 7, 8]. Although the cytological effects of ELC have been widely accepted to involve inhibition of the formation of cellular elements, such as actin rings and ruffled borders in osteoclasts[9, 10], its effects on chondrocytes, in particular, are not yet sufficiently understood.

Meckel' s cartilage undergoes site-specific matrix formation[11~14]. Specifically, distal portions, such as the anterior part of the midportion and proximal portion, undergo endochondral ossification[15, 16], while the posterior part of the midportion gives rise to the sphenomandibular ligament in the absence of obvious matrix calcification[17~19]. In addition to our previous reports describing the presence of alkaline phosphatase (ALPase) in Meckel's cartilage under *in vivo* and *in vitro* conditions[20~23], Miyake et al[24] provided a detailed description of the stage-specific expression of ALPase in Meckel's cartilage of inbred C57BL/6 mice. Even though the midportion in Meckel' s cartilage contains ALPase, sphenomandibular ligaments are formed in the absence of calcified matrix deposition. Therefore, it remains unknown whether calcification induction in Meckel' s cartilage is regulated by any factors. The potential for matrix calcification in Meckel' s cartilage has been shown to depend on site-specific elements, and we are especially interested in the involvement of calcification-inducing factors such as ELC.

In the present study, we analyzed the effects of ELC under cell and organ culture conditions without the cellular influences of osteoclasts and osteoblasts, and indicated that ELC accelerated matrix calcification through induction of high level of ALPase in Meckel's chondrocytes.

Materials and Methods

1. Animals

Meckel' s cartilage dissected from 17-day embryonic mice (ddY strain, vaginal plug = day 0) was used in the present study. The mandible with Meckel's cartilage was removed from the embryonic mice and placed in phosphate-buffered saline (PBS) containing 0.2% glucose (PBS-G) and kanamycin (60 μg/ml; Meiji Seika Co., Tokyo, Japan). After treatment with 10 mM di-sodium dihydrogen ethylenediamine tetraacetate dihydrate (EDTA), Meckel' s cartilage was extracted from the mandibular arch, transferred to fresh PBS-G and used for cell and organ cultures. All animal experiments were performed according to the protocols for the humane treatment of animals of Iwate Medical University.

2. Cell culture

Chondrocytes for cell culture were isolated with a mixture of enzymes, namely 0. 1% hyaluronidase (type I-S; Sigma Chemical Co., St. Louis, MO, USA) and 0.15% collagenase (type II; Worthington Biochemical Corp., Freehold, NJ, USA), as described previously[16),25] and inoculated at an initial density of 1 x 10^4 cells/Penicylinder cup (0. 28cm^2; Top Labo-ware, Osaka, Japan).

In the present study, four kinds of medium were prepared as follows: Control medium, alpha-modified Eagle's medium (α-MEM; Flow Laboratories, Irvine, Scotland) supplemented with 10% fetal bovine serum (FBS; ICN Biomedicals Inc., Aurora, OH, USA), 0.03 mg/ml *L*-ascorbic acid phosphate magnesium salt *n*-hydrate (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 60 μg/ml kanamycin;

β-Gly medium, α-MEM plus 2 mM β-glycerophosphate (β-Gly; Nacalai Tesque, Inc., Kyoto, Japan); ELC medium, α-MEM plus 100 ng/ml ELC (Biogenesis, Poole, UK); β-Gly & ELC medium, α-MEM plus β-Gly plus ELC. Cultures were maintained for up to 4 weeks in a humidified atmosphere of 5% CO^2 in air and the media were changed every other day.

3. Organ culture

Meckel's cartilage was cultured using our original organ culture system[16]. Briefly, Meckel's cartilage explants of approximately 3 mm in length prepared as described above were carefully placed across a pit-hole that was open on the inner chamber membrane (Chemotaxicell; Kurabo, Osaka, Japan). These inner chambers with Meckel's cartilage were set within glass culture dishes. Respective culture medium consisting of four kinds of medium was added gently to the culture dishes and the explants were cultured under the same conditions as the above-described cell cultures for 4 weeks. Specimens were used for alizarin red staining and immunostaining for ALPase.

4. Alizarin red staining

Frozen section had been mounted on silan-coated slides and rinsed with PBS, they were stained with 1% alizarin red S (Kanto Chemical Co., Tokyo, Japan) as our previous report[23].

5. Statistical analysis of cell proliferation

At 2 days after cell seeding, chondrocytes cultured in Control medium or ELC medium were exposed to 0.05 mg/ml bromodeoxyuridine (BrdU) for 30 min, followed by fixation with 4% paraformaldehyde for 15 min and immunohistochemical processing using a BrdU-detection kit (Oncogene Research Products, Cambridge, MA, USA). Using a hemocytometer (Nitirin, Tokyo, Japan), the numbers of BrdU-positive cells/mm^2 were counted in 10 different areas in 3 dishes, and the findings were expressed as the mean ± SD.

6. Von Kossa staining

Von Kossa staining for calcium deposition was performed in specimens cultured for 3 weeks. Briefly, the specimens were thoroughly rinsed in PBS and incubated in the reaction medium for 20 min at room temperature [22].

7. Alkaline phosphatase (ALPase) assay

Meckel's chondrocytes were cultured for up to 4 weeks in the different media. The media were exchanged for fresh media at 1-week intervals and the cells were cultured for a further 2 days. The media were then collected for measurement of the ALPase activity and analyzed rapidly by a commercial analysis company (SRL Co. Ltd., Tokyo, Japan).

8. Staining of ALPase activity

After 2 weeks of cell culture, the cultures were fixed with 4% paraformaldehyde for 30 min and then incubated in Gomori's reaction medium[26] for 20 min at room temperature. Control specimens were incubated in the absence of the substrate under the same conditions. After the incubation, the specimens were thoroughly washed in distilled water and lightly counterstained with hematoxylin.

9. Statistical analysis of the calcified matrix

Specimens cultured for 3 weeks in the respective media were compared for their matrix calcification. Cultures were fixed with 4% paraformaldehyde and treated with von Kossa reaction medium, before the percentage of calcified area/0.28-cm^2 Penicylinder cup was

calculated.

10. Immunohistochemical staining

For immunofluorescence staining, we used an antibody against ALPase (Nordic Immunological Laboratories, Tilburg, Netherlands) as an initial marker of matrix calcification. Explants were harvested after 2 weeks of organ culture, and fixed with ethanol-acetic acid solution (99:1, v/v) for 30 min at room temperature. After washing with PBS, the explants were embedded in Tissue-Tek Compound 4583 (Sakura Finetechnical Co., Tokyo, Japan) prior to cutting with a cryostat. Cryosections of 6-μm thickness were obtained from the frozen samples at -20℃, washed thoroughly with PBS and then processed for ALPase immunofluorescence staining. Briefly, the cryosections were incubated with a polyclonal rabbit antibody against ALPase (diluted 1:50) for 1 h at 37℃, washed three times with PBS and incubated with a fluorescein isothiocyanate (FITC)-conjugated antibody for 1 h at 37℃. Control sections were treated with the FITC-labeled secondary antibody alone. After thorough washing with PBS, the specimens were mounted in a mixture of glycerol and PBS (9:1, v/v), and observed using a fluorescence confocal laser microscope (LSM-GB 200; Olympus, Tokyo, Japan).

Results

1. Cell culture

(1) Cell proliferation

The proliferation rates were compared between cultures in Control medium and ELC medium. The number of BrdU-positive cells in Control medium was 50.4 cells/mm^2, while that in ELC medium was 55.3 cells/mm^2. There was no significant difference ($p = 0.6757$) between these cultures (Fig. 1).

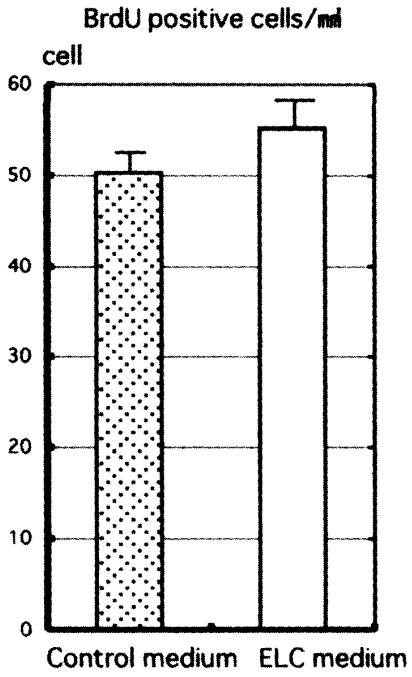

Fig. 1. Comparison of the proliferation rates between Control medium and ELC medium. The number of BrdU-positive cells in Control medium is 50.4 cells/mm^2, while that in ELC medium is 55. 3 cells/mm^2. There is no significant difference ($p > 0.05$) between these media.

(2) ALPase staining

Meckel's chondrocytes cultured for 2 weeks in Control medium revealed weak ALPase activity (Fig. 2a), whereas those cultured in ELC medium showed intense expression of ALPase activity along the extracellular matrix of cartilage nodules consisting of mature chondrocytes (Fig. 2b). In particular, ALPase activity was intensely positive in the areas that subsequently underwent matrix calcification.

(3) ALPase activity assay

In general, the level of ALPase activity peaked during weeks 2-3 of cell culture, and then decreased slightly with increasing duration of the culture (Fig. 3). In particular, cultures in β-Gly & ELC medium showed high levels of ALPase activity throughout the entire culture period. In contrast, cultures in β-Gly medium and ELC medium showed a similar

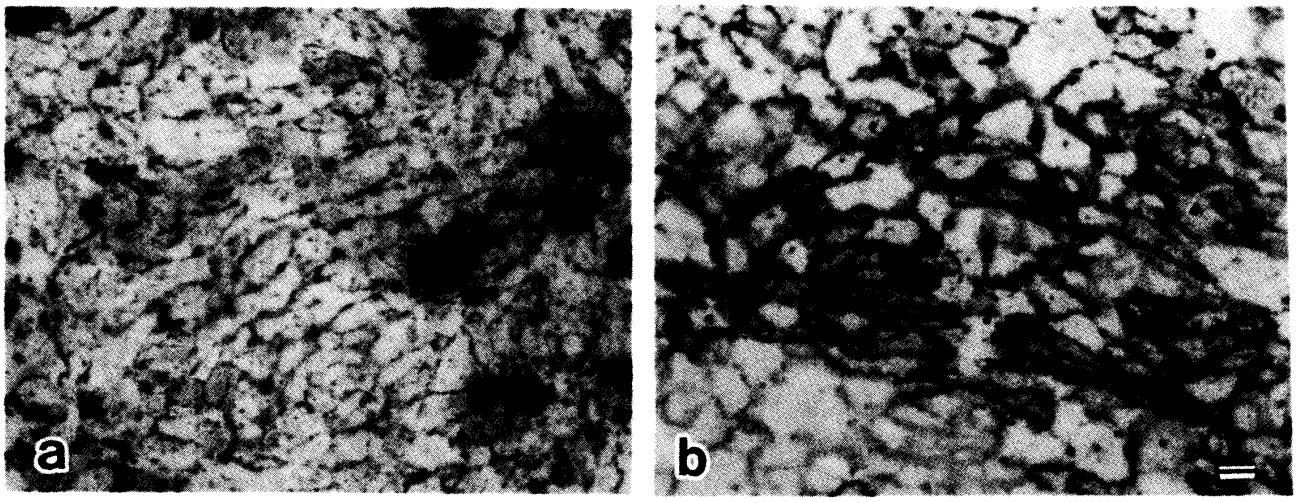

Fig. 2. Photomicrographs of ALPase activity after 2 weeks in culture. (a) The chondrocytes in Control medium show a low level of ALPase activity. (b) The chondrocytes in ELC medium express intense ALPase activity along the extracellular matrix of cartilage nodules containing mature chondrocytes. Scale bars = 10 μm (a, b)

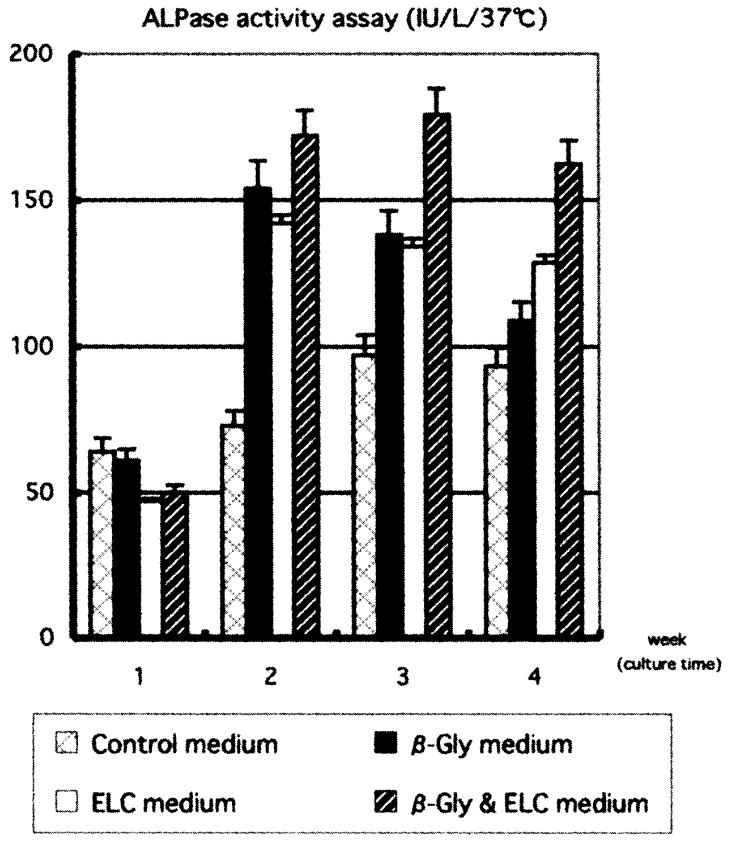

Fig. 3. Changes in ALPase activity during 4 weeks of culture. The ALPase activity in β-Gly medium and ELC medium increases to a peak after 2 weeks of cell culture and then decreases slowly until 4 weeks of culture. The ALPase activity in β-Gly & ELC medium synergistically increased throughout the 4 weeks of culture. There is statistical significant difference ($p < 0.05$) between Control medium and β-Gly & ELC medium at 4 weeks in culture.

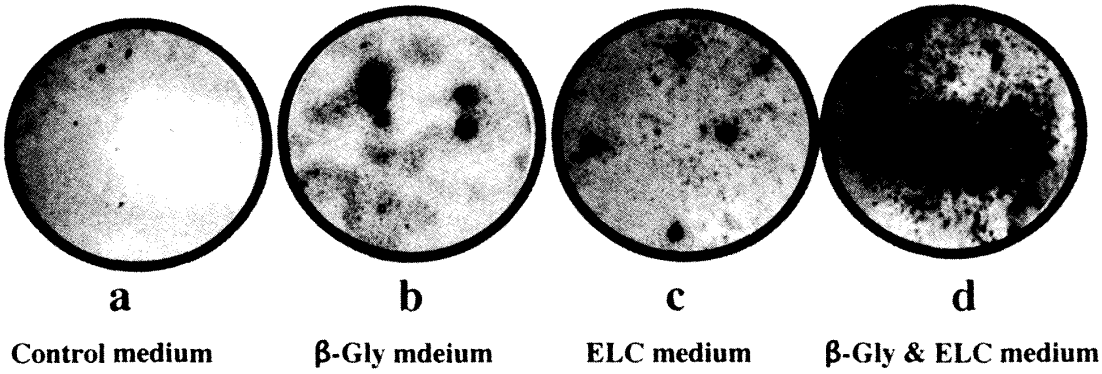

Fig. 4. Von Kossa staining of cultures in the respective media at 3 weeks of cell culture. (a) Cultures in Control medium show a small amount of matrix calcification. (b), (c) Specimens cultured in β-Gly medium (b) and ELC medium (c) reveal similar degrees of calcium deposition. (d) Note that β-Gly & ELC medium induces intense matrix calcification. x 0.7

inductive effect for ALPase activity after 2-3 weeks in culture ($p > 0.05$ between β-Gly medium and ELC medium but at a lower level than that in β-Gly & ELC medium.

(4) Von Kossa staining

Calcium deposition was evaluated by von Kossa staining. When von Kossa staining was carried out after 3 weeks in culture, matrix calcification in Control medium was detected as small spot-like deposits (Fig. 4a). The matrix calcification in β-Gly medium (Fig. 4b) appeared to be similar distributed with ELC medium (Fig. 4c). However, the calcified matrix in β-Gly & ELC medium had expanded most extensively along the nodular structures (Fig. 4d).

(5) Statistical analysis of the calcified areas

When the calcification areas were compared among cultures that had been grown for 3 weeks in the respective media (Fig. 5), there was no significant difference ($p = 0.1012$) between β-Gly medium and ELC medium. In β-Gly & ELC medium, the von Kossa-positive areas indicating calcification occupied approximately 18% of the area and evaluation by Student's *t*-test revealed a considerable statistically significant difference ($p = 0.0003$) between Control medium and β-Gly & ELC medium.

2. Organ culture

(1) Immunostaining for ALPase

Immunofluorescence staining for ALPase in Meckel's cartilage cultured in Control medium for 2 weeks revealed slight ALPase activity (Fig. 6a). In contrast, intense intracellular ALPase activity was detected in cartilage cultured in ELC medium (Fig. 6b).

(2) Alizarin red staining

When alizarin red staining was performed to reveal calcium deposition in specimens cultured for 3 weeks, no significantly calcified matrix was observed in explants cultured in Control medium (Fig. 7a). Specimens grown in β-Gly medium showed calcification to the same degree as those grown in ELC medium (Figs. 7b and c), while the highest level of matrix calcification occurred in β-Gly & ELC medium (Fig. 7d). These findings for calcification deposition were in agreement with the light-opaque structures observed by phase-contrast microscopy.

(3) Comparison of the calcification capacities in the respective media

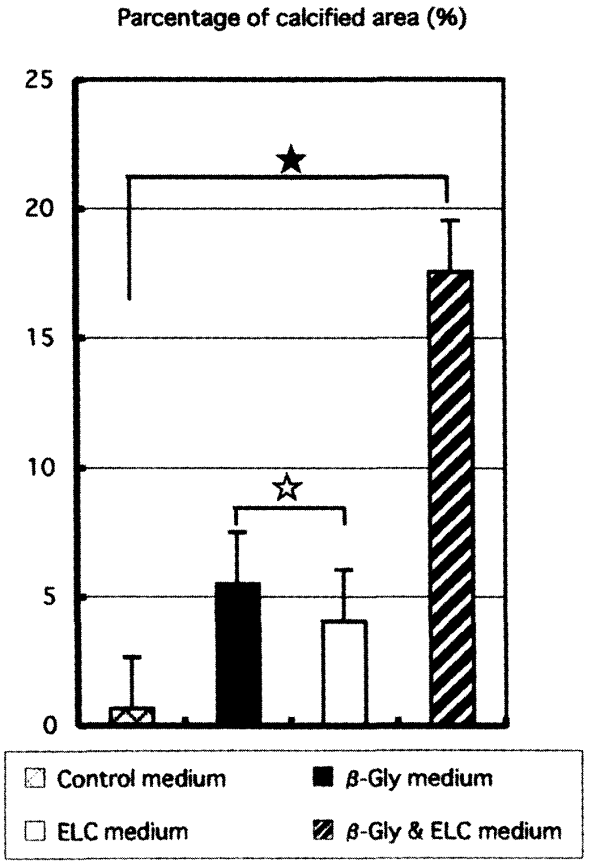

Fig. 5. Comparison of the calcification areas among cultures grown for 3 weeks in the respective media. There is no significant difference (☆ $p < 0.05$) between β-Gly medium and ELC medium, although β-Gly medium appears to induce more calcification than ELC medium. In β-Gly & ELC medium, the calcified matrix occupies approximately 18% of the area, and evaluation by Student's *t*-test indicates a considerable statistically significant difference (★ $p < 0.05$) between Control medium and β-Gly & ELC medium.

The degrees of calcification deposition in the respective media at 1-week intervals are shown in Table 1. Calcification was induced in 10% (2/8) of explants in Control medium during 4 weeks in culture. Although calcification was induced in 62.5% (5/8) of explants cultured in ELC medium for 4 weeks, it was induced slowly than that in β-Gly medium. However, β-Gly & ELC medium induced matrix calcification in 100% (8/8) of explants at an early period of the culture. Therefore, although ELC induced a significant increase in calcification, the coexistence of β-Gly and ELC induced the most effective matrix calcification.

Discussion

In the present study, we examined the effects of ELC on matrix calcification in Meckel's cartilage using cell and organ culture techniques, and indicated that ELC induced ALPase activity of high level in Meckel's chondrocytes and accelerated subsequent matrix calcification.

Meckel's cartilage has been reported to undergo site-specific matrix calcification that is unrelated to the expression of ALPase[20)~23)]. This observation suggests that matrix calcification in Meckel's cartilage may be associated with local environmental factors, and led us to study the calcification effects of exogenous factors, such as ELC. ELC synthesized from eel calcitonin has been reported to have inhibitory effects on bone resorption by osteoclasts[1)]. Ikegame et al[10)] proposed that, since ELC binds to the plasma membrane of osteoclasts and becomes incorporated into the Golgi apparatus, the synthesis of lysosomal enzymes for bone resorption may decreases. Although Okubo et al[27)] examined the effects of ELC on osteoinduction and revealed an anabolic effect on osteoblasts and an antiresorptive effect, little is known about the effects of ELC on chondrocytes.

In the present study, small amounts of matrix calcification occurred in both cell and organ cultures in Control medium in the absence of β-Gly and ELC, and thereby confirming that Meckel's cartilage has an intrinsic capacity for calcification induction. Meckel's chondrocytes cultured in β-Gly medium induced more effective calcification, indicating that the

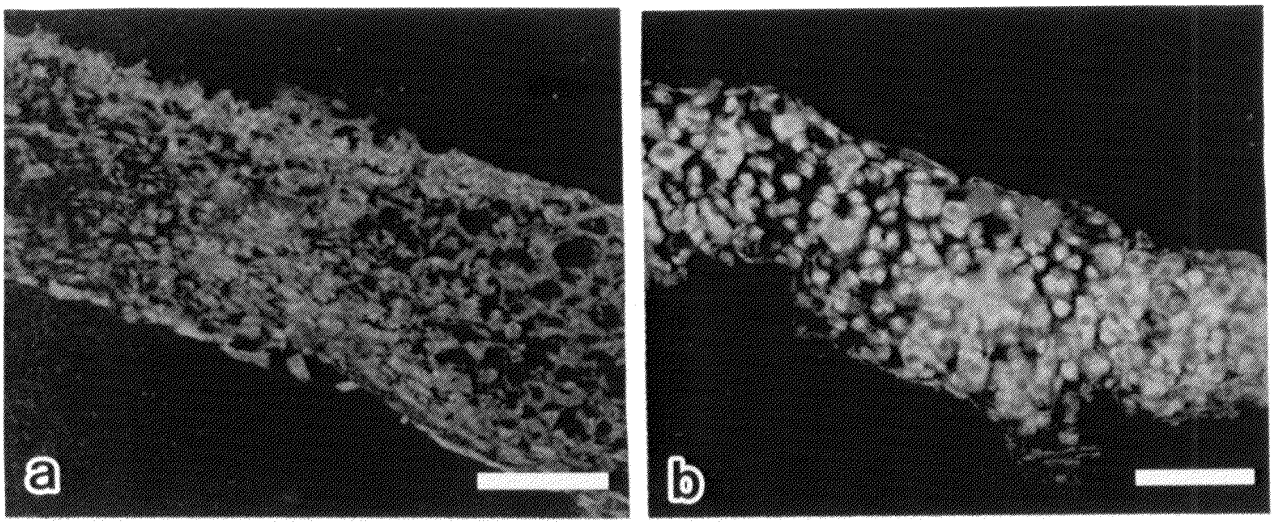

Fig. 6. (a), (b) Immunofluorescence staining for ALPase activity after 2 weeks of organ culture in Control medium (a) and ELC medium (b). Intense immunoreactivity is observed in the explants cultured in ELC medium. Scale bars = 300 μm (a, b)

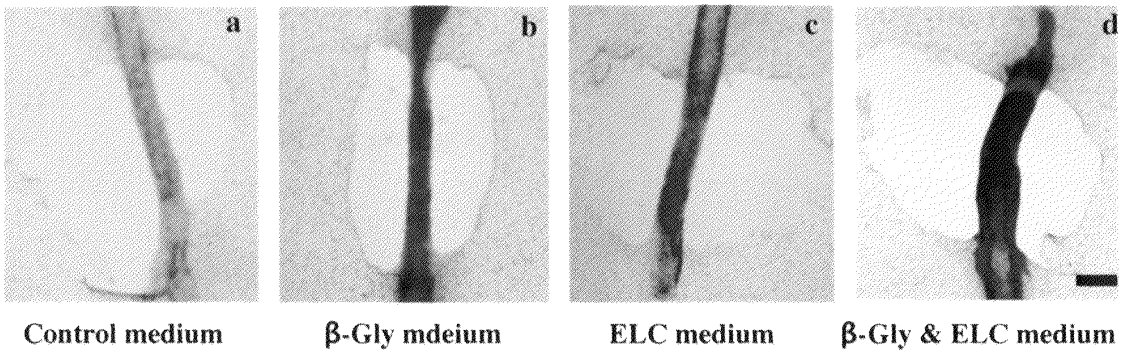

Fig. 7. Alizarin red staining for calcification of the extracellular matrix after 3 weeks of organ culture in the respective media. (a) In Control medium, no significantly calcified matrix is seen. (b), (c) Specimens grown in β-Gly medium (b) and ELC medium (c) show the same degrees of calcification. (d) Explant cultured in β-Gly & ELC medium shows the highest level of calcification. Scale bars = 500 μm (a, b, c)

Table 1. Effects of elcatonin (ELC) on calcification during organ culture of Meckel's cartilage

Medium	Cultured period (weeks)			
(n = 8)	1	2	3	4
Control medium	-	-	-	+ (2/8)
β-Gly medium	-	+ (2/8)	++ (5/8)	+++ (8/8)
ELC medium	-	-	++ (3/8)	++ (5/8)
β-Gly & ELC medium	-	++ (3/8)	+++(8/8)	+++ (8/8)

Explants were cultured for up to 4 weeks in four different media, and then observed by phase-contrast microscopy for their calcified deposits. The presence of calcification was evaluated as follows: - no calcification, + one-third calcification, ++ two-thirds calcification, +++ complete calcification. The calcification induced by ELC alone after 3 weeks in culture is inferior than that induced by β-Gly, while the medium containing both β-Gly and ELC induces the most effective matrix calcification.

calcification capacity was deeply involved with the absence or presence of β-Gly. Many investigators have reported that addition of 10 mM β-Gly to medium is required for high calcification efficiency. Furthermore, β-Gly has been suggested to act as a primary physicochemical factor for hydroxyapatite deposition[28),29)], at least *in vitro*[29),30)]. In our present investigation, β-Gly medium contained a physiological concentration of β-Gly (2mM), and the mechanism of matrix calcification seemed to differ from those described for cultures in the presence of 10 mM β-Gly. The results for ALPase activities and calcification rates in ELC medium resembled those for cultures in β-Gly medium, and furthermore, cells cultured in β-Gly & ELC medium induced the most intense calcification during the 4-week culture period. When the amounts of ALPase were compared between β-Gly medium and ELC medium, ELC medium induced ALPase activity at the similar level with β-Gly medium at 3 weeks of culture. Thus it is likely that β-Gly and ELC synergistically activate the expression of ALPase and subsequently induce matrix calcification.

Several investigators have reported the effect of calcitonin on the osteoblasts and chondrocytes. Ito et al[31)] and Yamaguchi et al[2)] reported significant induction of ALPase activity in the presence of calcitonin in cultures of an osteoblastic clonal cell line, MC3T3-E1, and rat calvaria, while Rao et al[32)] suggested specific binding sites for calcitonin in osteoblasts. Regarding the effects of calcitonin on cartilages, Kato et al[33)] reported that calcitonin did not accelerate cell proliferation, but elevated glycosaminoglycan synthesis in cultures of costal chondrocytes from rabbits and rats. Burch[34)] and Burch and Corda[35)] revealed that calcitonin stimulates growth and maturation during cartilage development. Although calcitonin binds to calcitonin receptor (CT-R) on the plasma membrane of osteoclasts and is well known to directly inhibit bone resorption[36)~38)], it is unclear whether such roles are associated with CT-R on chondrocytes. In the present study, since we found that ALPase activity was accelerated in ELC-containing medium, we propose that ELC may be involved directly in synthesis of ALPase.

In conclusion, we investigated the effects of ELC on matrix calcification in Meckel's chondrocytes *in vitro*. The present results suggest that ELC may act directly to induce matrix calcification via expression of ALPase activity in Meckel's cartilage.

Acknowledgements

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培養メッケル軟骨の石灰化に対するエルカトニンの効果

石関　清人，鍵谷　忠慶，藤原　尚樹，原田　英光
岩手医科大学歯学部口腔解剖学第二講座
（主任：原田　英光　教授）

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抄録：ウナギ由来のエルカトニン（ELC）は優れた薬理学的効能から実験的ないし臨床試験的改善薬として骨粗鬆症患者に投与され良好な成績が得られている。本実験では，胎生17日マウスメッケル軟骨に対するELCの効果を細胞培養と器官培養を用いて解析した。細胞培養と器官培養のためのメッケル軟骨は以下の4種類の培養液で最長4週間まで培養した。培養液として，培養液1；イーグル培地（α-MEM），培養液2;α-MEM + β-グリセロリン酸（β-Gly），培養液3; α-MEM + ELCと培養液4;α-MEM + β-Gly + ELCを用いた。試料はアルカリフォスファターゼ（ALPase）とbromodeoxyuridine（BrdU）の取り込みを含む組織学，組織化学的方法によって解析した。ELCを含む培養液は，より効果的に石灰化を誘導することがホン・コッサ染色によって示された。BrdUの取り込みによる細胞増殖率は，培養液1と3との間では有意差が認められなかった。ALPase活性は，基質の石灰化に先駆けて促進されることが細胞培養と器官培養での組織化学と蛍光免疫染色によって確認された。ELCはβ-Glyとほぼ同等量のALPaseを発現し，さらにELCとβ-Glyを含む培養液4では相乗的にALPase活性と石灰化を誘導した。本研究の結果から，ELCはメッケル軟骨細胞のALPase活性を促進し，その後の石灰化の誘導因子として作用する可能性が示唆された。

キーワード：メッケル軟骨，軟骨細胞，エルカトニン，石灰化，細胞培養と器官培養