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BIOCHEMICAL CHARACTERIZATION OF EMBRYONIC CHICK CALVARIAL CELLS

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Chicken calvarial bone is known to contain various cell types, but their exact composition is unknown. By characterizing the chicken calvarial bone biochemically, it can be used to study biochemical, histochemical actions of bone cells in general.

Calvaria of 18-day-old white leg horn embryo was aseptically dissected and bone cell populations were isolated by sequential enzymatic digestion.

Histochemical study for osteoclast-like bone cell population was performed with tartrate resistant acid phosphatase(TRAP) stain and for osteoblast-like bone cell population, alkaline phosphatase(ALP) stain was performed.

Biochemical study for osteoblast-like bone cell population was performed using alkaline phosphatase(ALP) assay.

Following conclusions were obtained from this study.

1. TRAP positive multi and mononuclear cells were mostly observed in group I and II, indicating that osteoclast-like bone cell population is mostly found in these groups.
2. All the cultured groups showed almost equal ALP activities and were positive for ALP stain, indicating that osteoblast-like bone cell population is evenly dispersed in all culture groups.
3. Experimental group treated with $1,25(OH)_2D_3$ showed increase in ALP activity in contrast to the control group, confirming previous studies that $1,25(OH)_2D_3$ increases ALP activities in *in vitro* bone cultures.
4. Results from von Kossa's stain indicated that *in vitro* bone formation had occured after 3 weeks of culture with beta-glycero phosphate.

Key Words : biochemical characterization, osteoblast, osteoclast

Orthodontic tooth movement is possible by means of resultant interactions between the applied force, the periodontal ligament and the alveolar bone. Biochemical, histochemical actions regarding alveolar bone must be clarified for better understanding of biophysical principles of orthodontic tooth movement[11].

Biochemical researches in orthodontics have been performed partly on animals and partly on human subjects using cell culture techniques.

Cell culture techniques were developed for the study of behavior of animal cells free of systemic variations that might arise in the animal both during normal homeostasis and under the stress of any experiment[10]. The use of isolated bone cells, free from their matrix and grown in culture has become an important tool in skeletal research[6].

Various methods for the isolation of periosteal cells from rat[2], mouse[15], and chicken[28] calvaria have been described. Among these, the sequential enzymatic digestion procedure of mouse calvaria[15] was developed with the aim of separating osteoblasts from osteoclasts. Such separation of the two types of bone cells with opposite activities in the bone metabolism has important meaning in cell biological studies[26].

The method devised for chicken calvaria[28] aimed at enriching one cell population designated osteogenic cells as "OB" and another population of primarily

periosteal fibroblasts as "PF".

Characterization can be defined as a process of discriminating two distinct bone cell populations by assessing biochemical and histochemical properties. Luben et. al[4] measured six metabolic and enzymatic activities, including acid and alkaline phosphatase, hyaluronate synthesis, citrate decarboxylation, prolyl hydroxylase and general protein synthesis. Cohn et. al[9] measured morphology, adherance to polymeric surfaces, hormone stimulated accumulation of cAMP, acid and alkaline phosphatase, synthesis of hyaluronic acid, decarboxylation of citrate and collagen prolyl hydroxylase for the characterization of separated bone cells.

In the present report, using isolated bone cells from 18-day-old embryonic white leg horn calvaria, histochemical study with tartrate resistant acid phosphatase(TRAP) stain was done to verify osteoclast-like bone cell group(OC group). For the characterization of osteoblast-like cell group(OB group), histochemical study with alkaline phosphatase(ALP) stain and von Kossa's in situ stain were performed and biochemical study with alkaline phosphatase assay was carried out.

Primary cultures of cells growing out from bone chips obtained by sequential enzymatic digestion[27] of tissue were shown to undergo in vitro an ordered pattern of differentiating events, if kept unpassaged in medium containing ascorbic acid and beta glycero - phosphate[1,19]. Von Kossa's in situ stain can identify calcium phosphate deposition, which indicates differentiation of the bone matrix.

Expression of alkaline phosphatase(ALP) in embryonic rat calvaria, in embryonic chick tibia and in adult human osteoblasts is an early event in the osteogenic differentiation[20]. Although the function of skeletal ALP *in vivo* is unknown, the enzyme is thought to be involved in the bone formation[30,31,22], and skeletal ALP is localized in the plasma membranes of osteoblasts. *In vitro* studies have shown that the amount of ALP activity in fetal rat calvaria is proportional to the rate of collagen production[7],and *in vivo* studies with normal young mice have shown a correlation between serum ALP activity and osteoblast number[21]. And finally, the amount of skeletal ALP activity in human serum is known to be proportional to the rate of bone formation, at least when both are elevated, as in young children[8] and patients with Paget's disease[13].

Previous studies with rat osteogenic sarcoma cells have shown an increase in ALP activity after treatment with 1,25 dihydroxyvitamin D_3 [1,25 $(OH)_2D_3$], and that receptors were necessary in cell lines which demonstrated this response[25]. In this report, bone cells from 18 day old embryonic white leg horn calvaria were treated with 1,25$(OH)_2D_3$, to see whether it had similar effect on ALP activity as in previous experiments using different species.

The purpose of this study can be summarized as follows. : chicken calvarial bone is known to possess various cell types but their biochemical characterization has not been made. By characterizing the chick calvarial bone cells, this study can be used as a basis for the future studies regarding biochemical, histochemical actions of bone cells in general.

MATERIALS AND METHODS

1. Bone Cell Isolation and Culture

A modification of sequential enzymatic digestion of Wong and Cohn[32] was used to obtain population of bone cells. Calvaria of 18-day-old white leg horn embryo were aseptically removed, cleaned of adherant connective tissues and transferred to Reactivial (Pierce, Rockford) containing 1.5ml of enzymztic solution of 0.01% collagenase, 0.05% trypsin, and 0.5mM EDTA. The calvaria was stirred slowly for 15 minutes at room temperature and digestion fluid containing released cells was transferred to 15ml centrifuge tube. 3.5ml of Hank's balanced salt solution(HBSS,Gibco) was added to dilute the enzymatic solution and the cells were harvested by centrifugation at 4° C for 10 minutes at 200g.

Supernatant fluid was decanted and cell pellets were resuspended in culture medium of minimum essential medium(MEM,Gibco) + 10% fetal bovine serum(FBS, Gibco) by vortexing slightly. Suspended, isolated cells were placed in 60 mm culture dish containing 3ml of culture medium and incubated at 37 ° C, in 95% humidity with 95% air and 5% Co_2. Fresh enzyme solution was added to the calvaria remains and the procedures above were repeated until 5 groups of isolated cells were collected.

Culture medium was initially changed overnight and every 48 hours subsequently. All groups of isolated

cells were cultured for 6 - 7 days before any study was performed.

2. Histochemical Study

A. Histochemical Study for OC-like bone cell population.

Tartrate resistant acid phosphatase(TRAP) stain was performed using tartrate resistant acid phosphatase kit(Sigma). A fragment of isolated cells from each group was transferred to multi-well tissue culture dish(Falcon), and placed in 37° C incubator for 60 minutes. Attached cells were washed with Delbecco's phosphate buffer saline(D-PBS, pH 7, Gibco) for 30 seconds with citrate-acetone-formaldehyde fixatives. Fixtures were rinsed with prewarmed distilled diluted water (DDW).

Naphthol AS - BI phosphate solution and fast garnet GBC base+acetate buffer were used as substrate and dye mixture, respectively. The mixtures were incubated at 37° C for 1 hour and counterstained with hematoxylin solution. After staining dishes were sealed and observed at 100×, 200× magnifications by light microscope, TRAP positive multi-, mononuclear cells were counted for each of the dishes and photographs were taken.

B. Histochemical Study for OB like bone cell population

Alkaline phosphatase stain was performed using alkaline phosphatase kit(Sigma). Isolated bone cells were cultured to confluency and their culture medium was removed. After washing with DPBS, cells were fixed with citrate-acetone-formaldehyde fixative. Napthol AS-BI phosphate (Sigma) and fast blue BB base (Sigma) were used as a substrate and alkaline dye mixture respectively. The mixtures were incubated at room temperature for 15 minutes and counterstained with neutral red solution. After staining, dishes were sealed and observed at 100×, 200× magnifications using light microscope.

C. von Kossa's stain for in vitro bone formation.

Von Kossa's in situ staining was performed to verify calcium phosphate deposition by OB-like bone cell population. 1.1ml of ascorbic acid and 1mM of beta-glycerophosphate were added to normal medium of MEM + 10% FBS. The cells were cultured for minimum of 3 weeks before staining was performed.

3% silver nitrate solution was used as a dye mixture and dishes were incubated by direct sun light for 30 minutes. Counterstaining with toluidine blue was performed. After staining, dishes were sealed and observed at 100×, 200× magnifications by light microscope. Cells were counted and photographs were taken.

3. Biochemical Study

Alkaline phosphatase(ALP) assay was performed to determine ALP activity. After the cells were cultured to confluency, medium was removed and cells were treated with DPBS after incubating at 37° C , for 2 hours. After cells were collected by centrifugation at 220g for 10 minutes and 0.5ml of triton X-100/saline was added. Cells were lysed by sonication at 30% output for 10 seconds, using ultrasonic dismembranator(Fisher) and 0.2ml of cell lysate was used for ALP assay and 0.2ml of cell lysate was used for protein determination. For ALP assay, cell lysates were mixed with 0.1 N glycine-NaOH buffer containing 15mM *p*-nitro phenyl phosphate(PNPP). The samples were incubated at 37° C for 30 minutes, placed in ice, and the reaction was terminated by dilution with 2.5ml of 0.1N NaOH. Absorbance was read at 410 nm by spectrophotometer.

Protein was determined by modified Lowry's method[14], using 1.3mg/ml of bovine serum albumin (fraction V, Sigma) as standard solution. Absorbance was read at 540nm by spectrophotometer.

RESULT

The results for histochemical study for osteoclast-like bone cell population using tartrate resistant acid phosphatase(TRAP) staining, are summarized in Table 1 and Figure 1. TRAP positive multi-, mononuclear cells, which could be designated as osteoclast- like bone cell, were observed primarily in group I and II.

The results of biochemical study for osteoblast-like cell population using alkaline phosphatase(ALP) assay are summarized in Table 2 and Figure 2. Almost equal amount of ALP activities were observed

Table 1. Results of histochemical study for OC like bone cell population(TRAP stain)

	I	II	III	IV	V
MEAN	62	28	10	9	4
S.E.	0.015	0.011	0.02	0.013	0.019

mean number of TRAP-positive osteoclast-like cells

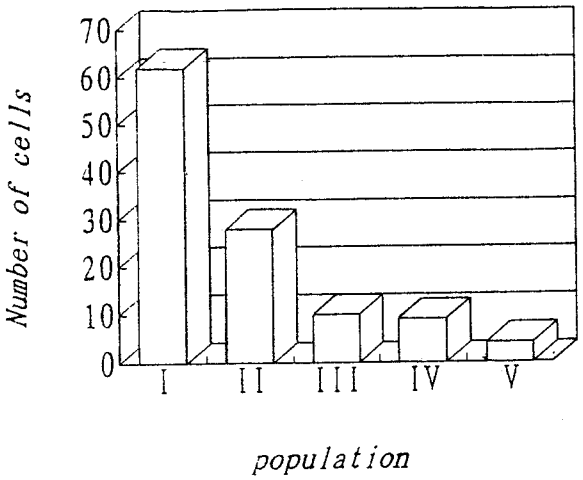


Fig. 1. Results of histochemical study for OC-like bone cell population(TRAP stain)

Table 2. Results of biochemical study for OB like bone cell population(Cleared PNPumol /hr/mg prot.)

	I	II	III	IV	V
MEAN	0.221	0.208	0.2	0.193	0.198
S.E.	0.016	0.009	0.02	0.007	0.014

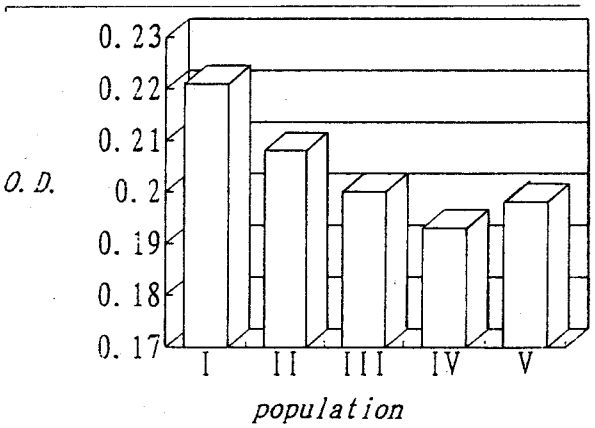


Fig. 2. Results of biochemical study for OB-like bone cell population(Cleared PNPumol/hr /mg prot.)

Table 3. Comparison of biochemical study for OB like bone cell population between 1,25(OH)2D3 treated and control.(Cleared PNPumol/hr/mr prot.)

	I	II	III	IV	V
Vit.D-	.412±.033	.327±.046	.245±.017	.192±.001	.162±.036
Vit.D+	1.47±.42	.217±.01	.417±.045	.352±.035	.274±.012

Datas are mean±standard error

in all the groups, indicating that OB-like bone cells were evenly distributed in all the groups.

Comparison of ALP activity between $1,23(OH)_2D_3$ treated group and control group is shown in Table 3 and Figure 3. All of the treated groups showed increse in ALP activity although statistical differences were not found.

Figure 4 and 5 are photographs of ALP staining, taken in 100 X magnification. Cells exhibiting ALP activities are stained in violet color, and they are arranged in clusters.

Figure 6 and 7 are photographs for von Kossa's *in situ* staining, taken in 100X magnification. Those stained in dark brown are deposited calcium phosphates, which indicate bone nodule formation by osteoblast-like cell population in vitro.

DISCUSSION

In this study, bone cells from the calvaria of 18-day -old embryonic white leg horn were isolated. Isolation and culture of bone cells has been previously described by others[29]. For the present study, however, calvariae of fetal chicks were used instead of those from fetal mice or rats. Fetal chicks have the advantages of being cheaper and easier to handle. Moreover, the periosteum is much easier to remove from a fetal chick calvarium than from a fetal mouse

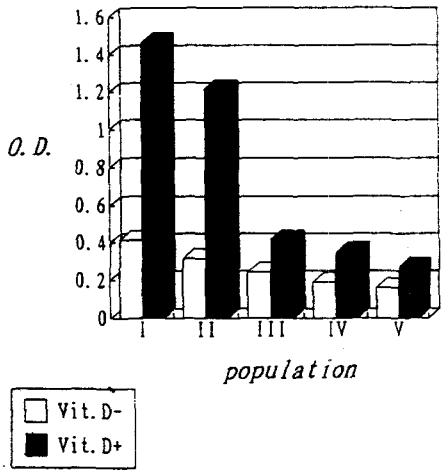
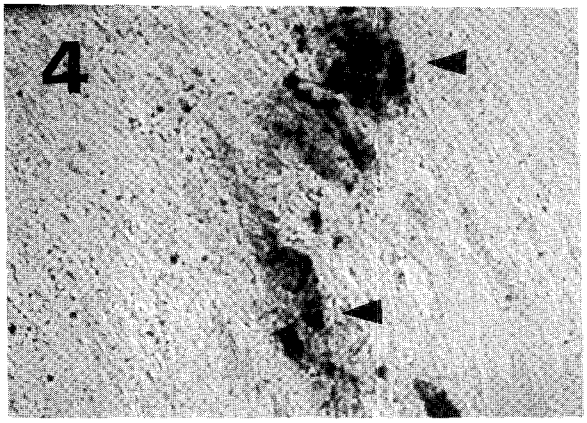


Fig. 3. Comparison of biochemical study for OB-like bone cell population between 1,25(OH)2D3 treated and control.(Cleared PNPumol/hr/mr prot.)

or a fetal rat calvarium, which means less damage to the underlying layer of osteoblasts.

The most important reason for choosing the chick calvarium was because the biochemical characterization of the chick calvarium has not been reported. Despite all the advantages cited above, chick calvarium has not been used as experiment materials extensively. By investigating basic biochemical and histochemical properties regarding the chick calvarium, it is hoped that this study provides a basis for future studies regarding properties of bone cells in general.

In the previous studies, osteoclast-like bone cell population was postulated on the basis of their biochemical properties, i.e., their responsiveness to calcitonin and parathyroid hormones and their capacity to resorb dead bones when stimulated by



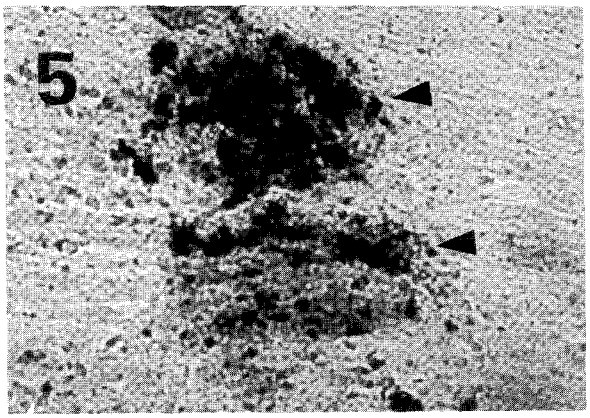
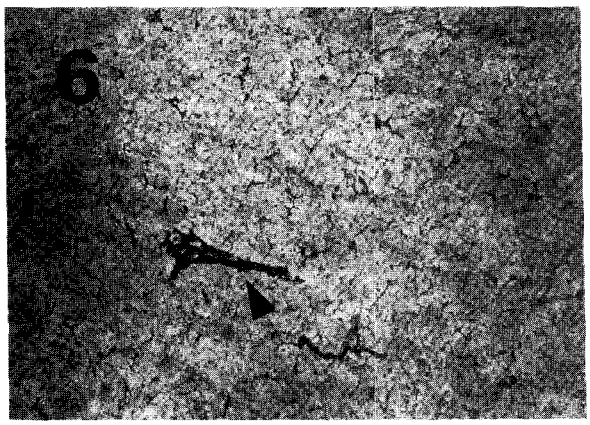


Fig. 4, 5. Alkaline phosphatase stain for OB-like bone cell population(100X magnification)



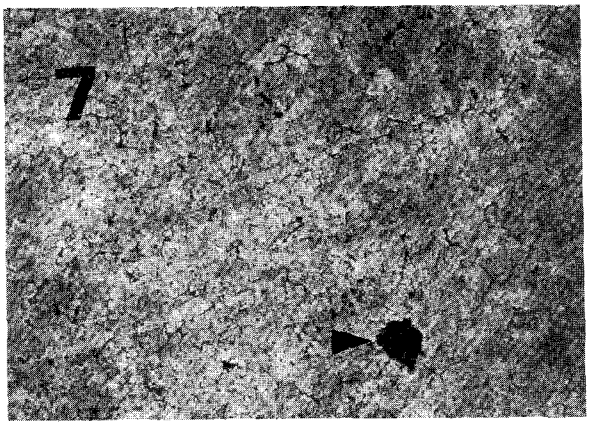


Fig. 6, 7. Von Kossa's stain for in vitro bone formation by OB-like bone cell population(100X magnification)

parathyroid hormones[16]. However, histochemical identification of the cells was not presented. In the chicken procedure no evidence for osteoclasts or their precursors was found at all[5].

But in this study, the presence of osteoclast-like bone cell population was postulated by counting TRAP positive multi-, and mononuclear cells according to previous study which denominated TRAP as a histochemical marker for osteoclasts[23].

The results demonstrate that cell populations obtained by sequential enzymatic digestion of chick calvarium can be further fractioned into osteoclast-like and osteoblast-like cell populations, after histochemical and biochemical studies. Analysis of these populations demonstrates that OC-like bone cell populations were dispersed in all groups, although their numbers, determined by counting TRAP positive multi-, mononuclear cells, declined drastically after group II. All groups showed high levels of alkaline phosphatase activity and were positive for ALP stain. The activity of ALP together with the ability to form type I collagen, has been used as a indicator for osteoblast- or osteoblast-like bone cell population[24]. In this study, only the ALP activity was measured because only the evidence for presence, not the quantity, of osteoclasts was needed for the characterization of the chick calvarium.

Controlled sequential digestion of fetal chick calvaria provides relatively pure populations of OB-like bone cell population except in early released bone cell groups in which contaminations of osteoclast-like cells are seen(group I and II).

On the basis of von Kossa's staining for calcium phosphate deposition or the presence of electron dense areas in electron micrographs, several previous studies have demonstrated that beta-glycerophosphate promotes the calcification of the extracellular matrix produced by osteoblasts[3]. The results from this study confirm this finding. Extracellular matrix or bone nodules formed by OB-like bone cell populations were observed in all the experimental groups.

This study showed that $1,25(OH)_2D_3$ treatment resulted in an increase in the specific acivity of ALP in the chick calvarium. Radioimmunoassay from published studies reported that the absolute amount of bone isoenzyme protein was significantly higher in cells which had been treated with $1,25(OH)_2D_3$. This could result from increased synthesis or decreased degradation of ALP protein, and demonstrates that the increase in phosphatase.

Specific activity is not merely due to a direct activation by $1,25(OH)_2D_3$ of preexisting phosphatase molecules[25]. The mechanism by which $1,25(OH)_2D_3$ increases the activity of the bone type alkaline phosphatase isoenzyme appears to be a specific activation, which requires the presence of the $1,25(OH)_2D_3$ receptor.

Recently it was shown that in cell lines that do not possess the specific receptors for $1,25(OH)_2D_3$, ALP secretion was not stimulated, indicating a direct involvment of $1.25(OH)_2D_3$ in bone mineralization[12]. However, it was demonstrated on mouse calvaria osteoblastic cells that analogues of vitamin D_3, dose-dependently, induced ALP activity *in vitro*, requiring effective concentrations of 100 or 1000 times the $1,25(OH)_2D_3$ to produce equivalent amount of stimulation of the enzyme[18]. Moreover, increased ALP activity in rat osteogenic sarcoma cells cultured in the presence of $1,25(OH)_2D_3$ is mediated through the specific $1,25(OH)_2D_3$ receptor and involves RNA transcription and new protein synthesis, indicating that $1,25(OH)_2D_3$ is closely linked to the differentiation of osteoblasts in vitro.

CONCLUSION

Following conclusions were obtained after histochemical and biochemical studies for OC- and OB-like. bone cell populations. TRAP stain was performed to verify OC-like bone cell population and for OB-like bone cell population, ALP stain and ALP assay were performed.

Von Kossa's stain was employed to see whether *in vitro* bone formation is possible by OB-like bone cell population. And finally, the effects of $1,25(OH)_2D_3$ on ALP activity was assessed.

1. TRAP positive multi- and mononuclear cells were mostly observed in group I and II, indicating that OC-like bone cell population is mostly found in these groups.
2. All the cultured groups showed almost equal ALP activity and were positive for ALP stain, indicating that OB-like bone cell population is evenly dispersed in all culture groups.

3. Experimental group treated with $1,25(OH)_2D_3$ showed increase in ALP activity in contrast to the control group, confirming previous study that $1,25(OH)_2D_3$ increases ALP activity in *in vitro* bone cultures.
4. Results from von Kossa's stain indicated that in vitro bone formation had occured after 3 weeks of culture with beta-glycero phosphate.

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