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Recombinant Human Parathyroid Hormone Related Peptide (1-34) Stimulates Osteoclastic Bone Resorption in Both Rodent and Avian Disaggregated Osteoclast Culture

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Recombinant human parathyriod hormone related peptide (1-34) (rhPTHrP) has been known to stimulate bone resorption in intact bone tissue culture system. Osteoclast has been known as a primary responsible cell for bone resorption. To examine the effect of rhPTHrP on this cell, we employed disaggregated rat osteoclast culture. As a result, we found that rhPTHrP significantly elevates both the number and total area of resorbed pits in this culture. On the other hand, the conflicting results between disaggregated rat osteoclast culture and Ca^{2+}-deficient hen osteoclast culture system have been a big obstacle for the progress of bone research. To verify the differences between rat and chick osteoclast system, we performed the same experiment using chick embryonic osteoclast. Since the similar results were obtained from the disaggregated chick osteoclast culture, the discrepancy between chick and rat osteoclast culture study seemed to be due to the difference in culture method, rather due to the species-difference.

KEY WORDS: bone resorption, osteoclast, parathyroid hormone, chick osteoclast culture

The bone remodeling involves two distinct processes, bone formation and bone resorption. Until recently, the information about the effects of various factors on the bone metabolism, specifically on the osteoclastic bone resorption, was obtained from tissue culture study (Sabatini *et al.*, 1988; Boyce *et al.*, 1989). Because there are various kinds of cells in the bone tissue, it was impossible to show the direct effects or the synergic effects of given factors on specific cell type. Osteoclast is the cell that resorbs bone. Because the resorption usually occurs before the bone formation, the regulation of osteoclast activity may be the crucial step in the regulation of bone remodeling (Mundy and Roodman, 1987; Vaes, 1988; William and Frolik, 1991). Osteoclast is very unique cell with its characteristic and highly specialized ultrastructure and functional responses (Baron *et al.* 1985). This unique cell has been notoriously hard to study, mainly because of its relative inaccessibility and its fragility in culture (Baron, 1989; Mundy, 1991). The absence of a useful cell line is another obstacle for the progress in understanding the cell biology and physiology of osteoclasts. To circumvent this difficulty, a variety of model system has been developed for studying isolated osteoclast. Among them, the disaggregated rat osteoclast culture (Chambers *et al.*, 1985; Kanehisa and Heersche, 1988; Lee *et al.*, 1990) and the Ca^{2+}-deficient hen osteoclast

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culture (Zambonin-Zallone *et al.*, 1982; de Vernejoul *et al.*, 1988) were frequently used in measuring the resorption activity of osteoclasts. In the former method, the cells were detached from the long bone of neonatal rat and loaded on bone or dentine slices. In this culture, the cells resorbed the bone slices to form the pits on its surface, i.e. resorption lacunae. The resorption activity of osteoclast can be accessed by measuring the area or the volume of pits. In the Ca^{2+}-deficient hen osteoclast culture, the cells were obtained from femora of the laying hen fed with the low Ca^{2+} diet. Arabinosyl cytosine (ara-C) was treated to remove the replicating non-osteoclastic cells. The resorption activity of osteoclast can be measured by culturing the cell with ^{3}H-proline pre-labeled bone particles and monitoring the released radioactivity in the media. Although these two model systems were developed for the same purpose, the interpretation of data is very difficult owing to the discrepancy between two assay systems (Vaes, 1988; Mundy, 1991). For example, Murrills *et al.* (1990) reported that PTH, a well known stmulator for bone resorption in both *in vivo* and in organ culture system, enhances the bone resorption in the disaggregated rat osteoclast culture. De Vernejoul *et al.* (1988) reported, however, that PTH (1-34) had no effect in the Ca^{2+}-deficient hen osteoclast culture. The similar discrepancy was observed with retinoic acid, transforming growth factor-β and various conditioned media (McSheely and Chambers, 1986; Oreffo *et al.*, 1988; O'Neill *et al.*, 1992).

Three possible explanations were suggested for the reason of this discrepancy. 1) The species-specificity may cause the difference. 2) The purity of cell population may be the reason, since many factors were suggested to act through other cell types. 3) The difference in the cell type or the degree of maturation of osteoclast may be the reason. In rat osteoclast culture, the osteoclasts began to resorb bone within few hours after plating. In contrast, the bone resorption was negligilbe until 3-days after plating in the chick osteoclast culture. Therefore, the isolated cells in these two culture methods were apperently not in the same state, and the 3-days duration in chick osteoclast culture may represents the time needed for the further differentiation or any kind of change to become a bone resorbing cells. The reason for the discrepancy between two assay systems should be clarified before interpreting the data from osteoclast cell culture study.

In this study, rhPTHrP (1-34) that is a well-known regulatory hormone in bone metabolism were tested in disaggregated rat osteoclast system to verify the stimulatory effect of PTH on osteoclastic bone resorption. Furthermore, we attempted to culture the chick osteoclast using the same method with disaggregated rat osteoclast culture, and test the effect of rhPTHrP (1-34) again. The result showed stimulatory effect of rhPTHrP on both rat and chick osteoclast system. Therefore, the discrepancy between disaggregated rat osteoclast and Ca^{2+}-deficient hen osteoclast system might be due to the difference in the culture system not due to the species-difference.

Materials and Methods

Materials

Fertilized eggs were purchased from local farm, and incubated at 37°C with 60% relative humidity. Fetal Bovine Serum (FBS) and culture media were purchased from Gibco Lab (Charlin Falls, OH). Human recombinant parathyroid hormone related protein (1-34) [hrPTHrP (1-34)] was the product of Peninsula Lab (Belmont, CA). Most of the other chemicals were purchased from Sigma (St.Louse, MO).

Osteoclast Culture

Rat osteoclast cultures were performed as previously described (Gallwitz *et al.*, 1993). Briefly, 2-day-old rat pups were sacrificed and the humeri, femora, and tibiae were dissected out and put into the ice-cold medium 199. Following the removal of adherent soft tissues and cartilagious portions, the long bones were chopped and agitated vigorously to detach the osteoclasts from them. After waiting for few seconds to settle down the bone debris, the osteoclast suspension was loaded on the dentine slices which were placed in 96 well plate. After 90 min incubation in CO_2 incubator, the non-adherent cells were removed by

agitating the dentine slice in 199 medium. The remaining cells on the slice were cultured in medium 199 (with 1.8g/L of sodium bicarbonate) containing 2% FBS for 48 hr in 5% CO_2 concentration. For disaggregated chick osteoclast culture, essentially the same procedures were employed except that the femora and tibiae of 16-day-old chick embryos were used. The dentine slice was prepared by cutting elephant dentine with low-speed sawing machine, and the slices (6 mm × 6 mm × 0.2 mm) were sterilized under UV illumination. They were re-hydrated with 199 medium containing 10% FBS for 1hr before use.

Tartrate-Resistant Acid Phosphatase Staining

The number of osteoclast from various source was determined by tartrate-resistant acid phosphatase (TRAP) staining. The staining was performed according to Sigma TRAP staining protocol except counter staining. The cell suspension was added into the 96 well plate, and incubate for 90 min. The unattached cells were removed by rinsing with medium 199, and the remaining cells were fixed by cold citrate/acetone fixative (60% acetone, 7.6 mM sodium citrate, pH 5.4) for 30 sec. After three times washing with distilled water (D.W.), cells were air dried for staining. The TRAP reaction mixture was prepared by mixing 1 ml of 2.5 M acetic acid, 1 ml of 12.5 mg/ml naphthol AS-BI phosphoric acid in N,N′-dimethyl formamide (Merck), 1 ml of 0.67 M sodium tartrate, pH 5.2, and 22 ml of 37°C pre-warmed D.W., then finally 7 mg of fast garnet GBC sulfate salt was added. After removing the insoluble dye by filtration, the reaction mixture was added into the specimens, and incubated for 60 min at 37°C in the dark. The reaction was ceased by rinsing out the reaction mixture with D. W. The stained samples were air dried for observation. TRAP-positive cells revealed reddish pink color.

Measurement of Resorbed Area

After 48 hr of cultivation, the cultures were rinsed with phosphate buffered saline (PBS), and fixed by 4% formaldehyde in PBS for 15 min. After fixation, the cultures were rinsed with PBS and stained with 1% toluidine blue-o in PBS for 30 sec. For the pit observation, the cells were removed by rubbing each slice with fingers and tissue paper. The slices were restained with toluidine blue-o for 5 min and air dried. The analysis of the pits were performed using the image analysis system personally provided by Hyon-Wook Song (Gold-Star Electric Company). Student's t-test was used for the statistical analysis.

Results

As shown in Fig. 1, the disaggregate osteoclast culture revealed a large number of multinucleated cells with characteristic well-developed membrane structure. To identify the cell more specifically, the presence of tartrate-resistant acid phosphatase (TRAP) was visualized by TRAP staining. Because TRAP has been regarded as a specific marker of osteoclast, the presence of this enzyme, together with multinucleated morphology, was virtually used as the identification marker for osteoclast. Since 16-day-old chick embryo gave the maximum yield of TRAP-posotive multinucleated cell, the 16-day-old chick embryonic osteoclasts were used for the further experiments (Table 1). In this disaggregated chick embryonic osteoclast culture, typical resorption lacuna were observed. The osteoclast actively resorbed dentine surface in the

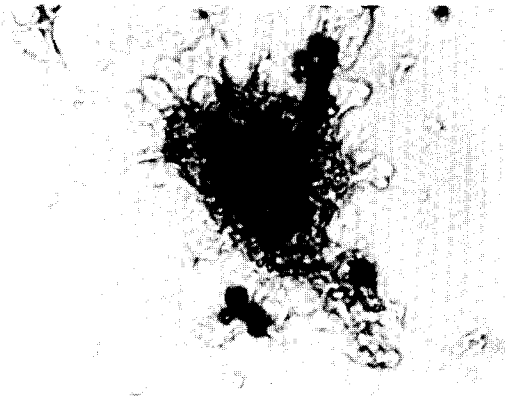

Fig. 1. Microphotographs of chick embryonic osteoclasts. The cells were obtained from 16-day-old chick embryonic long bone, and osteoclasts were specifically visualized by TRAP staining.

disaggregated osteoclast culture compared with Ca^{2+}-deficient hen osteoclast culture judging by their size and the numbers of pits (Fig. 2). As observed by previous investigators (Kanhehisa and Heersche, 1988), the resorption lacunae were located mainly underneath the cells clumps which may contain other cell types and osteoclast as well. TRAP-positive multinucleated cells were frequently found under the cell clumps, and they seemed to be responsible for the pit formation (Fig. 2).

In this study, we expressed the resorption activity of osteoclast by the number and total area of resorption lacunae. Some investigator argued that osteoclast resorption activity would not proportionally relate with the resorbed area or the pit number (O'Neill *et al.* 1992). The previous observation revealed that approximately half of the osteoclasts can not resorb any bone, therefore, it is likely that the increase in the number of resorption pit is closely linked to the activation of osteoclasts. We found significant increase in both the number and total area of resorbed pits by rhPTHrP (1-34) treated rat osteoclast culture (Fig. 3). Because only few hundred cells were available at each experiment, we were unable to test the effect of rhPTHrP (1-34) at the various concentrations simutaneously. In other experiment, however, rhPTHrP (1-34) was effective at as low as 10^{-9}M concentration (data not shown). This result is in good agreement with the previous report which showed stimulatory effects of bovine PTH, PTHrP in both number and total area of resorption lacunae (Murrills *et al.*, 1990).

Table 1. The yields of TRAP-positive multinucleated cells from chick embryos with various incubation time. The cells were TRAP stained and observed under the light-microscope.

Incubation Days of Chick Embryo	No. of TRAP-positive multinucleated cells/field[a]
16-day	13.2 ± 0.41
17-day	3.5 ± 0.29
18-day	7.5 ± 1.04
19-day	5.3 ± 0.85
20-day	9.1 ± 1.08

[a]Data are shown as mean ± SEM.

The same experiments were performed using 16-day-old chick embryo, and we found significant increase in total resorbed area by the treatment of rhPTHrP (1-34)(Fig. 4). However, the stimulatory effects of rhPTHrP on the number of pit were not significantly different from that of control. Comparing with the data of rat osteoclast culture, chick embryonic osteoclast showed much higher resorbing activity even in the control. Therefore, the elevated resorption in control might be why the stimulation was fragile in chick osteoclast system.

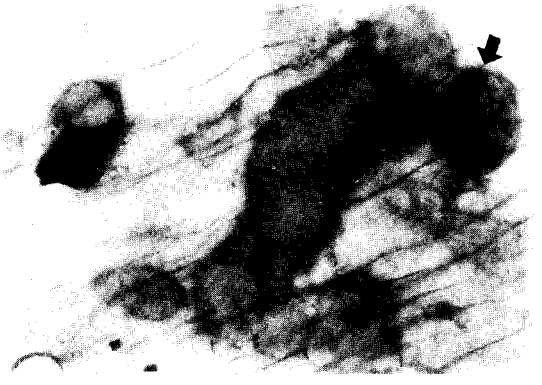

Fig. 2. The resorption lacunae which were formed during chick embryonic osteoclast culture. After 48 hr of cultivation, the culture was fixed, and stained with 1% toluidine blue. Arrow indicates the multinucleated cells within pits.

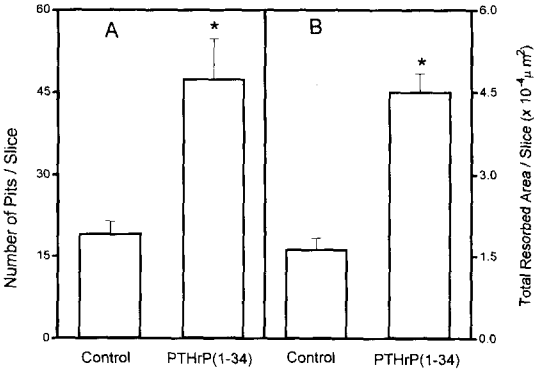

Fig. 3. The stimulatory effect of recombinant human PTHrP (1-34) at 10 nM on disaggregated rat osteoclast culture. Values are the mean ± SEM for 5 slices, significantly different from control: *$p<0.02$(A), *$p<0.01$ (B).

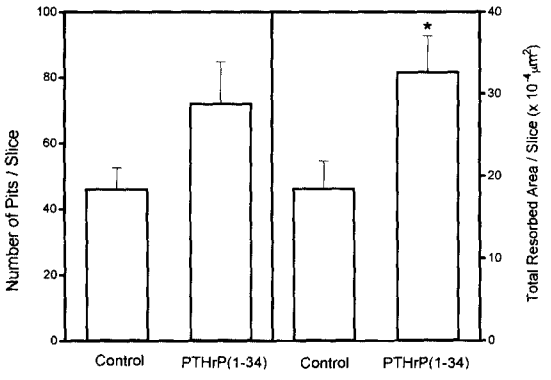

Fig. 4. The effects of recombinant human PTHrP (1-34) on disaggregated chick osteoclast culture. Values are the mean ± SEM for 5 slices, significantly different from control: *$p<0.05$.

Discussion

We are the first to report that rhPTHrP (1-34) stimulates osteoclastic bone resorption in chick osteoclast culture. This result suggests that chick has the similar mechanism with that of rat in the regulation of osteoclastic bone resorption.

Several possible mechanisms whereby PTH might stimulate osteoclastic resorption were suggested. McSheely and Chambers (1986) demonstrated that PTH alone was incapable of stimulating osteoclast, but the hormone stimulated resorption if osteoblastic cells are added to the cultures. Perry *et al.* (1987) also found that osteoblast-like cells cultured with PTH release a macromolecular stimulator of bone resorption. They argued, because the Ca^{2+}-deficient hen osteoclast culture contains relatively pure population of osteoclast that PTH which has been suggested as an indirect stimulator could not be functional in this system. It does not give the answer, however, to the ill-responsiveness of chick osteoclast to the osteoblast-conditioned medium, which is supposed to contain the direct stimulator for resorption (Thomson *et al.*, 1986). Therefore, the ill-responsiveness of Ca^{2+}-deficient hen osteoclast to PTH might not be simply due to the purity of cultured cells.

Retinoic acid has been reported as a unique stimulator for osteoclastic bone resorption in the Ca^{2+}-deficient hen osteoclast system (Oreffo *et al.*, 1988). It enhanced the tartrate-resistant acid phosphatase (TRAP) expression in culture osteoclasts as well as the release of ^{3}H-proline from pre-labelled bone particles by the osteoclasts. However, O'Neill *et al.* (1992) recently showed the inhibitory effect of retinoic acid in disaggregated chick osteoclast system, which is the same with the disaggregated rat osteoclast culture. Furthermore, retinoic acid has never been regarded as stimulatory factor in rat osteoclast culture system and in the bone organ culture as well. According to these results, the conflicts between disaggregated rat osteoclast culture and Ca^{2+}-deficient hen osteoclast culture may be due to the difference in the cell culture method, but not due to the species-difference. However, it is still not clear whether the conflict is due to the difference in the purity of cell population or due to the difference of the osteoclast itself.

The cells in the bone marrow include macrophages and monocytes, and they are somehow capable of bone resorption (Scheven *et al.* 1986). Macrophges were suggested to be in the same cell lineage with osteoclast, and they are somehow capable of bone resorption, futhermore, they were stimulated by retinoic acid (Scheven *et al.* 1986; Mundy, 1990). Those results made a suggestion that the cells in Ca^{2+}-deficient chick osteoclast culture were in the various stages of differentiation from macrophage-like preosteoclast to mature osteoclast.

The osteoclast culture system might be a useful model system for the investigation of bone metabolism. In this culture, however, only few percent of cultured cells were identified as osteoclasts based on TRAP staining. The complexity of cell population in bone tissue has been an obstacle to interpreting the data (Huffer, 1988). Therefore, the further development of pure osteoclast culture would be necessary for understanding the precise mechanism of bone remodeling (Lee *et al.* 1990).

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파골세포배양에서 나타난 부갑상선호르몬의 설치류 및 조류 파골세포에 대한 촉진 효과

양대석 · 김일찬 · 남궁용 · 이창호(강릉대학교 자연과학대학 생물학과)

부갑상선호르몬은 동물의 조직배양에서 골조직의 분해과정을 촉진하는 것으로 알려져 있다. 우리는 이 호르몬이 골조직의 분해에 깊이 관여하는 파골세포(osteoclast)에 미치는 영향을 알아 보기위하여, 설치류의 파골세포배양을 시도하였으며, 여기에 부갑상선 호르몬을 처리하여 파골세포의 활성이 어떻게 변하는지 알아보았다. 이 실험에서, 우리는 이 호르몬이 파골세포 배양에서도 확연히 파골세포에 의한 골분해과정을 활성화 시키는 것을 알 수 있었다. 한편, 그동안 골대사과정을 연구하는데 혼란을 가중시켜왔던, 설치류와 조류간의 차이점을 알아 보기위하여, 이와 같은 실험을 계배 파골세포를 사용하여 실시하였다. 그 결과, 설치류에서와 비슷한 실험 결과를 얻었고, 이는 그동안 두 동물을 이용한 실험들이 서로 다른 결과를 보여주었던 것이, 단순히 종이 다르기 때문이 아니라, 세포배양의 방법적인 차이에 의한 것이라는 사실을 암시하는 것으로 사료되었다.