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Transforming Growth Factor-Beta Stimulates Osteoclastic Bone Resorption *in vitro*

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Osteoclast has been known as a primary responsible cell for the bone resorption. The activation of osteoclast, therefore, may be the key event in the regulation of bone growth and remodeling. Various factors were reported to have influence on the resorbing activity of osteoclast in organ culture. Among those factors, transforming growth factor-β (TGF-β) has been known to have a profound effect on bone metabolism. Since a large amount of TGF-β presents in bone tissue, it may be important for the understanding the regulatory mechanism of bone resorption to elucidate the effect of TGF-β on the osteoclast.

We have reported the disaggregated chick embryonic osteoclast culture as an useful assay method for determining the resorption activity of osteoclast. In this culture, we found that TGF-β significantly enhaced the osteoclastic bone resorption activity. We also found that the stimulatory effect seemed to be an indirect one that is mediated by other cells. As nordihydroguaiaretic acid significantly inhibited the TGF-β1-induced osteoclastic bone resorption, we suggest that the lipoxygenase derivative of arachidonic acid may participate in the action of TGF-β as a paracrine or an autocrine mediator.

KEY WORDS: Transforming Growth Factor-β, Osteoclast, Eicosanoid, Nordihydroguaiaretic Acid, Bone Resorption

Osteoclast is a highly specialized multinucleated cell to resorb the bone (for review; Williams and Frolik, 1991; Mundy, 1993). During the resorption process, osteoclast forms special adhesion ring-like zone with the well developed membrane structure called ruffled border. The notorious diffculties in culturing the osteoclast has been a big obstacle in studying the bone remodeling process. Osteoclast attaches to the bone surface so tightly that the detachment from the bone surface during the isolation of osteoclast obligatorily causes significant damage on the cell. In addition, since the precise cell lineage has not been elucidated, the collecting the precursor cells nor the immotarization of the osteoclastic cell have never been successful. In spite of those difficulties, since the osteoclast is the major and possibly the sole bone resorbing cell, there were tremendous efforts in establishing the osteoclastic cell line (Gattei *et al.*, 1992) and in primary culture of osteoclasts (Zmabinin-Zallon *et al.*, 1982). As a result several primary culture methods have been

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developed to access the bone resorption activity of osteoclast. By the histochemical research, it has been reported that osteoclasts are surrounded by various kinds of osteogenic and non-osteogenic cells (Huffer, 1988; Vaes, 1988). The hormonal effects on osteoclastic bone resorption, therefore, would be very complicated process in which other cells were involved. For example, parathyroid hormone (PTH) stimulates osteoclastic bone resorption in fetal rat long bone assay, however, the mature osteoclast seemed not to have the receptor for PTH (Rouleu *et al.*, 1988). McSheerly and Chambers (1986) have suggested osteoblast as a primary target for PTH and that osteoblast secrets unknown factor which acts directly on osteoclast, and there were many attempts to characterize the mediating factors (Nanes *et al.*, 1993). As a results, several local mediators were suggested as a direct stimulator for osteoclast. For example, 5-hydroxyeicosatetraenoic acid and related compounds have been suggested as an primary effector molecule for the stimulation of osteoclastic bone resorption in giant tumor patient (Gallwitz *et al.*, 1993). More recently, the production of nitric oxide (NO) by human osteoblastic cells was reported and they inferred NO may mediate the action of interleukin-1, tumor necrosis factor α, and interferon γ (Ralston *et al.*, 1994). However, the mediator for the major regulator of bone metabolism in normal condition, such as PTH, 1,25-dihydroxyvitamin D_3 ($1,25\text{-}(OH)_2D_3$), thyroid hormone, and transforming growth factor-β (TGF-β) is remaining to be elucidated (Britto *et al.*, 1994).

TGF-β is the mutifunctional cytokine presents in a large amount in bone tissue in inactive latent form. Although the precise functional role of TGF-β in bone tissue is not clearly revealed yet (Centrella *et al.*, 1994), the effect of TGF-β on the osteoclastic bone resorption has been examined in several organ culture system (Pfeilshifter *et al.*, 1988; Chenu *et al.*, 1988). However, the results were confused and inconsistant according to the employed systems. For example, in the mouse calvarial culture study, TGF-β has been reported to stimulate the prostaglandin production which in turn leads to the stimulation of osteoclastic bone resorption (Tashjian *et al.*, 1985). By contrast, at higher concentration, TGF-β were reported to inhibit the osteoclastic activity (Pfeilshifter *et al.*, 1988). The conflicting data on the effect of TGF-β in the organ culture necessitated the cell culture study to clarify further the effect of this peptide on osteoclasts.

Previously we suggested the disaggregated chick osteoclast culture as one of model system to determine the resorption activity of osteoclast (Yang *et al.*, 1994). In this assay condition, monocytes and macrophages, that have been shown to degrade devitalized bone in the long term culture, were inable to form the resorption lacunae on the bone surface. Therefore, this pit assay would be appropriate to represent exculsively the resorption activity of osteoclast. Using that resorption pit assay, we attemp to clarify the effect of TGF-β on the resorption activity of osteoclast.

Materials and Methods

Fetal Bovine Serum (FBS) and Dulbecco's Modified Eagle's Medium (DME) and Medium 199 were purchased from Gibco Lab (Charlin Falls, OH). Transforming growth factor-β1 was purchased from Boehringer Manheim(Germany). Most of the other chemicals were purchased from Sigma (St.Louse, MO).

Isolation of osteoclasts

Osteoclasts were obtained from 16-day-old chick embryo as described in Yang *et al.* (1994). Some modifications have been applied to reduce the variation among each experiment. Ten or more chick embryos were sacrificed by decapitation and the femora, humor, and tibiae were dissected out. Following the removal of adherent soft tissues and the cartilaginous portion, the remaining shafts were minced and chopped in a 35 mm Falcon culture dish containing 1.5 ml of ice-cold Medium 199. The minced bone tissues were transferred into 15 ml capped test tube. After the addition of 3 ml of the same medium, osteoclasts were detached from the bone by vortexing at maximum speed for 1 min. After allowing the large bone particles to settle down,

the resulting cell suspension were loaded on a dentine slice which had been incubated in Medium 199 (with 1.3 g of sodium bicarbonate) containing 10% FBS. The cells were allowed to sediment on the top of the dentine slices in CO_2 incubator for 1 hour, then the non-adherent cells were removed by transferring the slice into the culture medium(Medium 199 containing 2% FBS). The culture was performed using 96 well plate in 5% CO_2 incubator for 48 hours. In the vigorous washing osteoclast culture, the slice was incubated for 30 min and non-adherent or weakely adherent cells were washed out by agitating the slice in Medium 199 with forceps before transferring into the culture medium.

Preparation of dentine slice

Ivory was cross sectioned using a diamond blade slow-wheel sawing machine. The slice of 6 mm × 6 mm in size and 200 μm in thickness were prepared by longitudinal cutting. The surface of slices was polished using fine-polished glass-plates. The resulting slices were washed by sonication in DW for over 30 min. After drying in air, the slices were sterilized by UV illumination, and stored under UV light. At one day before the usage, the dried dentine slices were rehydrated by dipping into Medium 199 containing 10% FBS in CO_2 incubator.

Factor treatment

The metabolic inhibitor of arachidonic acid were dissolved in absolute ethanol just before the use. The inhibitors were pre-treated for 30 min before the factor addition. The final concentration of solvent was never exceeded 0.1% in the culture medium. For the identification of osteoclast, the cells were stained for tartrate-resistant acid phosphatase and photographed as described in Yang *et al.* (1994).

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 was performed using the image analysis system (image-pro) Student's t-test was used for the statistical analysis.

Results

As shown in Fig.1A, we were able to identify the multinucleated tartrate-resistant acid phophatase (TRAP)-positive cells in this culture condition. After 48 hours cultivation, those cells were capable of forming several resorption lacuna as shown in Fig.1B.

The disaggregated osteoclast culture method was initially invented by Chambers and Magnus (1982). During the last 15 years, the culture

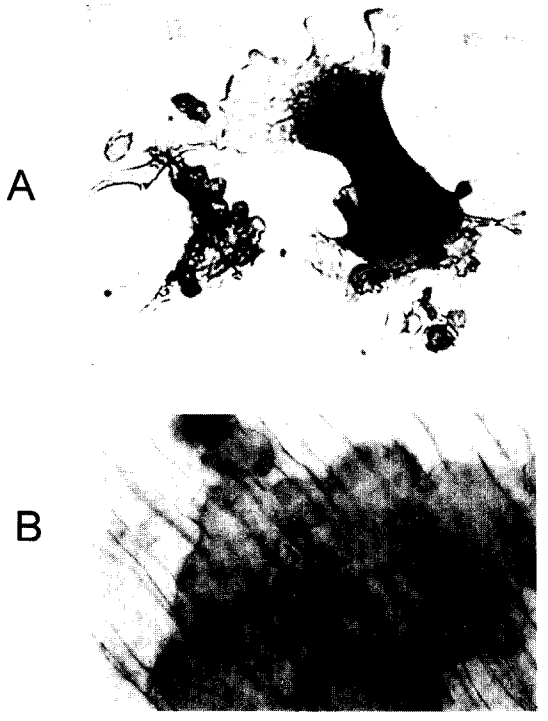

Fig. 1. Microphotographs of chick osteoclast (A) and the resorption lacuna (B) which were formed by osteoclasts during culture. The cell was staind by tartrate-resistant acid phosphatse staining, and the pit was stained with 1% toluidine blue.

system has been developed as a measuring system for the activity of osteoclast in resorbing bone (Chambers *et al.*, 1985; Lee *et al.*, 1990). We did put some modifications, such as the simultaneous processing of collected long bones and the processing in ice-cold medium, to improve the constancy of results from each experiments and to get a larger number of cells from the bone. According to the analysis of their multinucleated morphology and the presence of TRAP-activity, the number of osteoclasts which were obtained from a chick embryo was estimated to be over 500 cells which is comparable with other reports (Chambers *et al.*, 1985). As mentioned in earlier reports, non-osteoclastic mononucleated cell is also present in this culture condition.

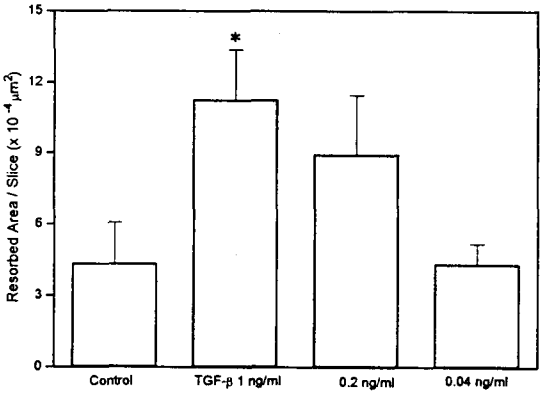

Fig. 2. Effects of TGF-β1 on the pit formation activity of chick embryonic osteoclasts. The cells were cultured on the calcified matrices (ivorys) for 48 hours in 199 medium containing 2% FBS. The pits were visualized by toluidin blue staining and observed under the microscope. The results were expressed as the mean ± SE (n=8 dentine slices per treatment). *, $p < 0.05$.

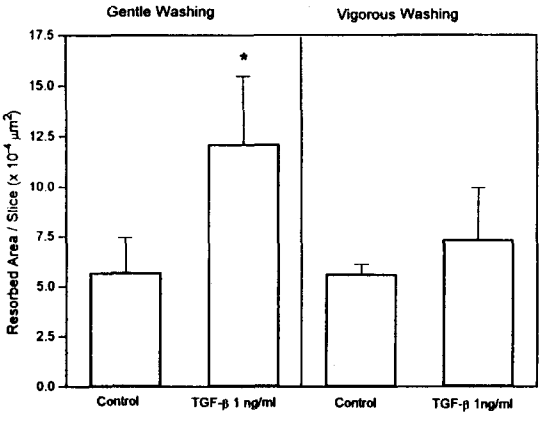

Fig. 3. The differece of the stimulatory effect of TGF-β1 in the gentle-washing vs in the vigorous washing culture porcedure. After loading the cells on the ivory slices, the slices were washed with media to remove the non-adherent or weakly bound cells. The vigorous washing was able to reduce the number of the non-osteoclastic cells without significant reduction in the resorbed area in the control. The results were expressed as the mean ± SE (n=4 dentine slices per treatment). *, $p < 0.05$.

The resorption activity of osteoclast was expressed by the number of pits formed during culture, and the resorbed area on the surface of dentine slice. As shown in Fig. 2, the significant increase in both number and the area were observed by the treatment of TGF-β1. Previous report indicated the extreme sensitivity of rat osteoclast culture (O'Neil *et al.*, 1992), and same was true in our culture system because the stimulation was elicited at as low as 0.2 ng/ml concentration. Considering the reports suggesting that non-osteoclastic cell mediates the effect of various cytokines, the resorption activity of osteoclast would be influenced by the number of non-osteoclastic cell as well as the number of osteoclast. To develop the assay system that is

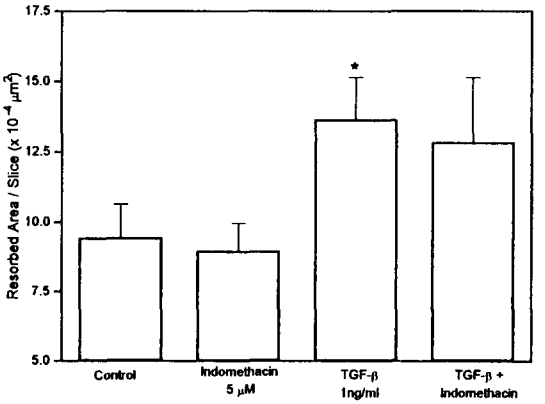

Fig. 4. Indomethacin had little effect on the stimulatory activity of TGF-β1 in the osteoclast pit formation assay. The cells were preincubated with indomethacin for 1 hour prior to the factor addition. The results were expressed as the mean ± SE (n=4 dentine slices per treatment). *, $p < 0.05$.

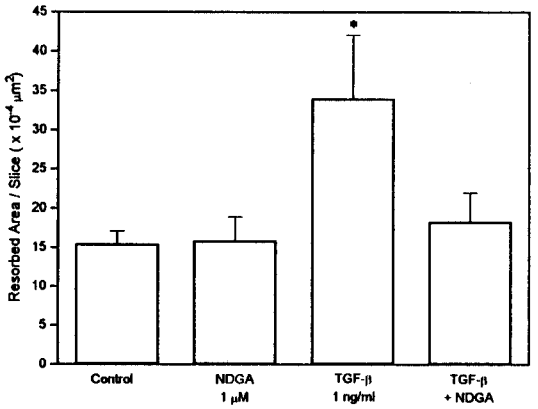

Fig. 5. Nordihydroguaiaretic acid significantly inhibited the stimulatory activity of TGF-β1 in the osteoclast pit formation assay. The cells were preincubated with the inhibitor for 1 hour prior to the factor addition. The results were expressed as the mean ± SE (n=4 dentine slices per treatment). *, $p < 0.05$.

able to distinguish the indirect effect on osteoclast from the direct one, we attempted to get the cell population that is more predominant with osteoclast. The shortening of incubation time and the vigor washing resulted in a reduced cell numbers on the dentine slices, but it did not cause the significant reduction in the resorbed area in control (Fig.3). Comparing with the conventional culture, the effect of TGF-β was apparently reduced, implying that osteoclast itself is not the target cell for TGF-β, and the presence of non-osteoclastic contaminant cell is necessary to reveal the stimulatory effects of TGF-β.

To get the information of mediating molecule, we examined the effect of TGF-β1 in the presence of metabolic inhibitors of arachidonate. As shown in Fig. 4, indomethacin that inhibits both cyclooxygenase-I and II had little influence on the TGF-β1 effect. However, nordihydroguaiaretic acid (NDGA), that inhibits lipoxygenase pathway, significantly reduced the TGF-β-induced increase in osteoclastic bone resorption (Fig.5).

Discussion

Osteoclast is the primary responsible cell for the resorption of the bone matrix. Since only a limited number of osteoclasts were available from intact bone, the biochemical analysis of this cell would be impossible before establishment of osteoclastic cell lines. Although there were continuing efforts to establish the osteoclast culture system, it seemed never successful to get the reliable osteoclastic cell lines. Some cell lines were successful in eliciting TRAP-positive multinucleated feature (Gattei *et al.*, 1992). However, the cells were not able to resorb the bone to form pits on its surface in culture. Therefore, the differentiation condition and the differentiated phenotypes of osteoclast should be clarified before applying the data from those cell lines in understanding the physiology of osteoclast. In the bone resorption study, the bone organ culture such as fetal rat long bone assay and neonatal mouse calvaria assay have been widely used for determining the effects of various cytokines including TGF-β on osteoclastic bone resorption (Pfeilschifter *et al.*, 1988; Bertolini and Strassmann, 1991). Although those studies provided intriguing results, the presence of various cells in intact bone gives confusion in interpreting the data from tissue culture study. In the intact bone, various kinds of cells, including osteoclast, osteoblast, monocytic cells, stromal cells, may produce a lot of local factors which affect bone metabolism. For example, Martin *et al.* (1992) reported the presence of interleukin-6 (IL-6) producing cells in the bone tissue, and it is well known that IL-6 stimulates osteoclastic bone resorption (Poli *et al.*, 1994). In addition, many reports showed the predominance of hematopoetic and stromal cells to osteoclast and osteoblast. Therefore, to elucidate the influence of a cytokine on osteoclastic bone resorption, it would be necessary to examine it in the cell culture system of osteoclast (McSheely and Chambers, 1986; Zambonil-Zalone, 1982).

Recent reports indicated that the regulatory events between osteoclast and osteoblast might be crucial event in the regulation of bone growth and remodeling process (Nanes *et al.*, 1990). During the bone growth and remodeling, the resorption of calcified marix is tightly coupled with the bone formation. It is the balance between these two events that determines skeletal mass and shape. As it has been proved that the latent-TGF-β was released as an active form during the bone

resorption process (Pfeilshifter and Mundy, 1987), the concentration of active TGF-β would be proportionally regulated by the bone resorbing activity of osteoclast. Therefore, TGF-β has been thought as a coupling factor between the bone resorption and the bone formation. The effects of TGF-β on osteoblast and osteoclast are rather confusing, since it has a biphasic effect on these cells. For the bone resorption, it seems clear that TGF-β inhibits the differentiation of osteoclastic cell lineage (Chenu *et al.*, 1988). However, on the bone resorbing activity of osteoclastic, it showed contadictory results according to the employed assay system (Oreffo *et al.*, 1990; Chambers *et al.*, 1985; Tashjian *et al.*, 1985). In this study, we found that TGF-β strongly stimulated the osteoclastic bone resorption, in addition, it seemed to be an indirect one. It was demonstrated that the bone resorption stimulating hormones, PTH and 1,25-(OH)2D3, thyroxin, and several cytokines act indirectly, via osteoblasts or stromal cells, to activate osteoclast. Although the cellular component of the indirect action pathway were suggested to be osteoblast (McSheely and Chambers, 1986), the involved molecule which is produced in response to osteotropic hormones, and stimulates osteoclast directly to resorb bone was not elucidated yet. For the action of TGF-β, osteoblast seems a primary target for TGF-β in bone tissue. However, it is not known whether ostoblast mediates the action of TGF-β to modulate the osteoclastic bone resorption. The inhibitor study gave a intriguing information of the molecule that is mediating the TGF-β action in this culture condition. Indomethacin, the inhibitor of cyclooxygenase I and II, had no effect on the TGF-β activity, instead NDGA, the inhibitor of lipoxygenase and monooxygenase (Agarwal *et al.*, 1991), significantly reduced the TGF-β-induced stimulation in osteoclastic bone resorption activity. This results strongly suggests that the effect of TGF-β is mediated by a arachidonic acid metabolite, especially the lipoxygenase product. Previous organ culture study showed the involvement of prostagladins in the action of thyroid hormone and TGF-β on bone resorption (Klaushofer *et al.*, 1989). However, subsequent studies showed that indomethacin did not block the thyroid hormone-stimulated bone resorption in fetal rat long bone assay and neonatal rat osteoclast culture (De Vernejoul *et al.*, 1988). Furthermore, prostaglandins had no effect or inhibitory effect on osteoclastic bone resorption in rat osteoclast culture (Chambers *et al.*, 1985). Considering that TGF-β may act as a coupling reagent between bone formation and resorption and that the coupling of these two distinct process is the key mechanism for proper bone growth and remodeling, the further identification of the mediating molecule of TGFβ action would be critical in understanding the regulatory mechanism of bone metabolism.

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파골세포에 대한 Transforming Growth Factor-β의 활성화 작용

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파골세포는 골조직을 분해하는 세포로 알려져 있다. 따라서, 파골세포의 활성조절은 골조직의 성장과 재조합의 조절에 있어 매우 중요한 의미를 갖는다. 기관배양을 통해 파골세포의 활성을 조절하는 여러가지 인자들이 알려져 있다. 그 중에서 transforming growth factor-β(TGF-β)는 골조직의 대사에 중요한 영향을 미치는 것이 알려있고, 또한 골조직내에 다량 존재하고 있기 때문에, TGF-β의 파골세포에 대한 효과를 알아 보는 것은 전체 파골작용의 조절기작을 알아보는데 있어 중요한 의미를 갖는다.

본 연구인들은 계배를 이용한 파골세포의 배양법을 개발하였고, 이를 파골세포의 활성을 측정하는데 사용하였다. 이 방법을 통해, TGF-β1이 파골세포의 골분해 활성을 증가시킨다는 사실을 알 수 있었다. 또한, 이러한 활성화작용은 TGF-β의 파골세포에 대한 직접적인 효과라기 보다는 다른 세포를 통한 간접적인 효과일 가능성이 높다는 사실을 알 수 있었다. 이에 더하여, TGF-β에 의한 파골세포의 활성화는 nordihydroguaiaretic acid에 의해 현저하게 저해된 반면, indomethacin에 의해서는 저해되지 않았다. 이러한 실험결과들은 TGF-β가 arachidonic acid의 lipoxygenase 유도체를 통해 파골세포에 영향을 미칠 가능성을 제시하고 있다.