In this study, we investigated the effect of bisphosphonate on the osteoblastic differentiation of human dental stem cells (hDPSCs). In the first experiment, we evaluated the effect of bisphosphonate on the differentiation of hDPSCs into osteoblasts by alkaline phosphatase staining after culturing hDPSCs. As a result, on day 13, the osteogenic differentiation of hDPSC was suppressed at 5 μM in clodronate and 2 μM in zolendronate. In NBP, osteogenic differentiation is more suppressed. In second experiment, cytotoxicity and proliferation test, the cell proliferation (examined by MTT assay) was more suppressed as the concentrations of zolendronate were larger than those of alendronate and clodronate. Western blotting, a third experiment, was found that AKT phosphorylation was inhibited in cell signaling proteins involved in cell proliferation inhibition and death by bisphosphonate concentration. In human dental stem cells, bisphosphonates inhibit osteoblast differentiation, and this phenomenon is clearly observed in NBPs (zolendronate), and it has been found that it is related to AKT phosphorylation of cell signaling proteins.

Key words: Bisphosphonate, Dental pulp stem cell, Osteoblast differentiation

I. INTRODUCTION

Bisphosphonates are relatively safe drugs that have been used in the treatment of osteoporosis, malignant tumors, and bone metastasis of prostate and breast cancer inhibiting excessive bone resorption by inducing apoptotic osteoclast cell death and decreasing activity. Since their introduction in 1995, they have been widely used for more 10 years in treating osteoporosis with their ability to prevent bone loss and fractures proved in various large-scale clinical studies. A known mechanism of bisphosphonate is that simple bisphosphonates such as clodronate enter the osteoclasts and bind to AMP, blocking many cellular enzymes to inhibit cell function, leading to apoptotic cell death. In the case of more powerful bisphosphonates containing nitrogen such as zolendronate, they are known to inhibit the synthesis of sterols through the mevalonate pathway and induce apoptosis.
of osteoclasts. The inhibition of bone resorption suppresses the bisphosphonates containing nitrogen (NBPs) more strongly than those not containing nitrogen (non-NBPs).

In vitro experiments with various cells showed that NBPs directly inhibited cell growth and endothelial angiogenesis. Without any difference in concentration (10μM, 100μM), Non-NBPs prevent cytotoxicity in periodontal cells and other cells like oral keratinocyte, gingival fibroblast in the in vitro experiment, whereas NBP was found to be toxic to cause inflammation and soft tissue necrosis. Recent experiments on human mesenchymal stem cell (hMSC) related titanium surface have shown that bone mineralization is increased in low-concentration bisphosphonates at nM concentrations regardless of non-NBPs and NBPs. The effect of bisphosphonate on the cells including MSCs varies depending on concentration and more studies are needed.

Among human mesenchymal stem cells, particularly human dental pulp stem cells (hDPSCs) which are derived from various tissue such as dental follicle, extracted deciduous teeth and periodontal ligament are convenient to obtain and can be used in stem cell therapy. These hDPSCs have a high capacity to proliferate and differentiate into osteoblast, odontoblast, chondrocyte and tissue for repair process. However, the effects of bisphosphonates on human dental pulp stem cells have not been clearly elucidated yet. Once the effect of bisphosphonates on hDPSCs is found, it could improve our knowledge to treat such injury of jaw bone or gastrointestinal tissue. In this study, we investigate the effect of bisphosphonate on osteoblast differentiation and mineralization through hDPSCs.

II. MATERIALS and METHODS

1. Reagents and cell culture

In order to differentiate human dental pulp stem cell (hDPSC, Lonza product) into osteoblast cells, the cells were cultured for 2 days in Dulbecco’s modified Eagle medium (DMEM), β-glycerophosphonate (10 mM) and Ascorbic acid (50ug/ml) for up to 13 days. During the osteogenic induction of hDPSC, N-containing bisphosphonate (Zolendronate), non-containing bisphosphonate (Clodronate) were added to the culture medium, cells were cultured for 6 and 13 days with indicated doses of bisphosphonates.

2. Alkaline phosphatase staining

Alkaline phosphatase (ALP) staining was performed to determine the osteogenic differentiation of hDPSC according to manufacturer’s instructions (Leukocyte Alkaline Phosphatase Kit, Sigma-Aldrich). After discarding culture medium, cells were fixed with Fixation Solution at room temperature for two minutes. The Staining Solution by Mixing Solution A and Solution B at a 1:1 ratio was prepared. The plate with aluminum foil in the dark for approximately 15 min was covered. It was observed color change.

3. Cytotoxicity Measurement and Cell Proliferation Assay

The cells were cultured using 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) solution for cytotoxicity and cell proliferation. Alendronate, clodronate, and zolendronate were divided into 0μM, 1.25μM, 2.5μM, 5μM, 10μM and 20μM for 3 days respectively. The blue formazan product was measured at a wavelength of 570nm.

4. Western blotting

After incubation, cells were cultured in RIPA lysis buffer (50mM Tris, pH 8.0, 150mM NaCl, 0.5% sodium deoxycholate, 1.5mM MgCl2, 1mM EGTA, and 10mM sodium phosphate buffer).
buffered saline) 1% Triton X-100, 10 mM NaF and complete protease inhibitor cocktail), Cell lysates were denatured with detergent-compatible protein assay kit (Bio-Rad Laboratories, CA, USA) and electrophoresed on 8-10% SDS-polyacrylamide gel. The results were then measured by chemiluminescence assay on protein antibodies associated with cell.

### III. RESULTS

#### 1. Alkaline phosphatase staining

ALP staining showed that osteogenic differentiation of hDPSC was reduced at 5μM clodronate on the 13th day and mineralization was suppressed at 2μM, 5μM concentration of zolendronate. Zolendronate inhibited mineralization at lower concentrations than clodronate. (Fig. 1)

#### 2. Cytotoxicity Measurement and Cell Proliferation Assay

In the cytotoxicity and proliferation test, hDPSCs proliferation (examined by MTT assay) was more suppressed as the concentrations of 0μM, 1.25 μM, 2.5μM, 5μM, 10μM and 20μM in the zoledronate were larger than those of alendronate and clodronate. (Fig. 2.C) There was no significant inhibition pattern in alendronate and clodronate, (Fig. 2.A,B)

#### 3. Western blotting

Since the phosphorylated AKT band was hardly visible in zoledronate (Fig. 3), AKT phosphorylation was inhibited in cell signaling proteins involved in cell proliferation inhibition and cell death by bisphosphonate concentration.

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**Fig. 1.** AP stained nodules were less visible at 2μM zolendronate media on the 13th day. These Data suggest that Zolendronate inhibited mineralization at lower concentrations than clodronate,
IV. DISCUSSION

In this study, our research found that bisphosphonates inhibit osteoblastic differentiation and cell proliferation of hDPSCs, which phenomenon clearly observed in N-containing bisphosphonates (NBPs, zolendronate), and found that it is related to AKT phosphorylation of cell signaling proteins. The osteogenic differentiation of hDPSC was suppressed at 5 μM in clodronate and 2μM in zolendronate. The cell proliferation (examined by MTT assay) was more suppressed as the concentrations of zolendronate were larger than those of alendronate and clodronate. Western blotting was found that AKT phosphorylation was inhibited in cell signaling proteins involved in cell proliferation inhibition and apoptosis by bisphosphonate concentration.

There are several other studies on the differentiation and proliferation of stem cells depending on the concentration of bisphosphonate. Dileep et al. showed that high bisphosphate concentration (>5μM) has a inhibitory effect on placental mesenchymal stem cells (pMSC) proliferation. Regardless of the type of bisphosphonate, cell viability was suppressed...
after 5 days. The minimal concentration that inhibits pMSCs proliferation was at 2 μM clodronate, 1 μM alendronate, 0.5 μM Zolendronate. This result is similar to the inhibitory effect of hDPSCs in the zolendronate which we studied. N.R. Alqhtani et al showed that much low bisphosphonate concentration (100 nM & 10 nM, alendronate, pamidronate) promotes mineralization of human mesenchymal stem cells (hMSCs), which further increases cell proliferation and migration on titanium surface.19) With the aid of titanium surface, low concentrations (nM) of bisphosphonates seem to enable hMSCs migration and titanium adhesion. After all the analysis, NBPs over a certain concentration (>0.5 μM) may be expected to inhibit stem cell differentiation and proliferation.

The associated signaling proteins are not yet known but appear to be associated with AKT phosphorylation. AKT/PKB (protein kinase B) is involved in the PI3K signaling pathway. Phosphoinositide 3-kinase (PI3K) signaling pathway plays an important role in Mesenchymal stem cells (MSCs) proliferation and cell apoptosis inhibition. Phosphorylated AKT inhibits apoptosis and enhances survival and inactivation of target protein including tumor suppressor P53, proapoptotic BAD, a regulator of bcl-2.20)-22) Our results show that the zolendronate-treated cells inhibit AKT phosphorylation. Several recent studies have shown that AKT-related proteins are hyperactivated in a variety of cancers such as colon cancer and breast cancer cell allowing for continuous cell growth.23)-25) This associated gene expression and signaling pathways also need to be more studied.

Recently, many treatments for stem cell-based therapies are being studied, among which MSCs take the center stage. As mesenchymal stem cell (MSCs) are derived from various adult tissues, blood fluid, bone marrow, umbilical cord, liver, dental pulp, there is not required ethical issue to use stem cell. DPSCs are in the pulp chamber and during dental development, interact with epithelial cells to differentiate into ameloblasts, odontoblasts. They protect dental pulp through reparative dentin formation during tooth injury. Dental pulp stem cells (DPSCs) are most convenient to extract and can highly differentiate into osteoblast, chondrocyte, neuron, cells which stimulate vasculogenesis /angiogenesis under different condition. The effects of bisphosphonates depending on concentration on hDPSCs confirmed in this study may be useful for other factors affecting stem cell research, and cellular response signaling pathway research in hDPSCs.

REFERENCES


