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Hydroxyapatite-induced alkaline phosphatase activity of human pulp fibroblasts

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Hydroxyapatite is widely used clinically in the field of dentistry, however, the modulating action of hydroxyapatite with respect to the cells has not been investigated. In this study, it is described that one of the cytokines released by fibroblasts can cause increasing alkaline phosphatase (ALPase) activity of fibroblasts *in vitro*. Human pulp fibroblasts (HPFs) were cultured with hydroxyapatite (30–480 $\mu g\ cm^{-2}$) in serum-supplemented medium for 6 to 96 h, then the serum-free conditioned medium was obtained and added to sub-confluent HPF cultures. Significantly enhanced ALPase activity was detected when HPFs were incubated with the conditioned medium. This HPF-derived activity which induces increasing ALPase activity appeared in the conditioned medium with a 12 h culture period, and the appearance of this mediator was completely inhibited in the presence of cycloheximide. Enhanced ALPase activity is detected in lesions in which calcification is occurring. The results of this experiment suggest that HPFs in contact with hydroxyapatite serve as a mediator in cell-to-cell interaction, and are related to the functional differentiation of cells for tissue repair including pulpal calcification.

1. Introduction

Calcium phosphate biomaterials, based mainly on hydroxyapatite, are widely used clinically in the field of dentistry, as an artificial root implant, as a replacement filling material for bone defects due to periodontal disease, as a direct pulp capping material, and as material for root canal filling [1–5]. Several reasons can be given for using hydroxyapatite clinically. These are

(i) it is not recognized as a foreign substance, so immune reactions do not occur;
(ii) it has superior biocompatibility; and
(iii) it is a bioactive material which can induce the formation of calcified tissues.

It has been clearly shown, based on animal experiments and clinical observation, that hydroxyapatite has remarkable biocompatibility compared to other materials [1–7]. When hydroxyapatite is applied *in vivo*, the newly developed capillaries and increased osteoblasts penetrate into the hydroxyapatite and the hydroxyapatite is totally incorporated into the newly formed calcified tissues [6, 7]. Also, when hydroxyapatite is applied to dental pulp tissue, addition of minerals occurs, with the hydroxyapatite as the core, and secondary dentin forms [4, 5].

However, there is no adequate basic study examining the interaction between hydroxyapatite and cell functions. Thus it is not clear whether the capability to induce osteoconduction exists in the hydroxyapatite, in other words whether or not the hydroxyapatite which is applied in the tissue affects the cells in the surrounding area, nor whether it can differentiate among the cells which have the ability to form calcified tissues.

In this study, using human pulp fibroblasts (HPFs) obtained from permanent and deciduous teeth, showing stable growth and high alkaline phosphatase (ALPase) activity sensitive to 1, $25(OH)_2D_3$ (vitamin D_3) [8], we examined the functional changes in HPFs which were cultured with hydroxyapatite crystals.

2. Experimental procedure

2.1. Medium and culture conditions

Human pulp tissues and pulp fibroblasts were cultured in Dulbecco's modified Eagle medium (DME, containing 100 IU ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin and 2 mM glutamine, Grand Island Biological Co., USA) supplemented with 10% fetal calf serum (FCS, Whittaker M.A. Bioproducts, USA) (complete DME) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C [9].

2.2. Isolation of human pulp fibroblasts

HPFs were obtained as follows. Human permanent and deciduous teeth were extracted for the correction of malocclusions under acupuncture anaesthesia. The pulp was carefully removed, rinsed with DME, placed in 25 cm^2 tissue culture flasks (Corning, USA) and grown in complete DME [8].

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The outgrowing fibroblasts were trypsinized (0.01% trypsin in Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+}, containing 0.2% glucose and 0.1 mM EDTA, pH 7.4), and subcultured at the rate of 1×10^6 cells/10 ml complete DME in 75 cm^2 tissue culture flasks every 5 days.

2.3. Conditioned medium

HPFs were cultured at the rate of 7.5×10^5 cells/3 ml complete DME in 25 cm^2 tissue culture flasks for 16 h. 30 to 480 μg cm^{-2} of hydroxyapatite (45–63 μm diameter, Mitsui Toatsu Chemical Co., Tokyo) or calcium fluoride (Sigma, St Louis, USA), was added to the fibroblast cultures, which were then incubated for 6 to 96 h. The cultures were washed with serum-free medium and incubated for 24 h in the absence of serum. The cell-free supernatants, used as the conditioned medium, were obtained for each experiment [9].

2.4. Determination of ALPase activity

HPFs were cultured at 3×10^4 cells cm^{-2} in 96 well tissue culture plates for 4 days in complete DME. The cultures were washed with serum-free medium and incubated for 16 h in the absence of serum. After aspirating this medium, diluted serum-free fibroblast supernatants were added to the fibroblast cultures for 4 days. The fibroblasts were then dissovled with 250 mM sucrose solution buffered by 10 mM tris-HCl (pH 7.4) containing 1% Triton X-100 and 2.5 mM $MgCl_2$ at 4 °C for 10 min. The determination of the ALPase activity was based on the Kind–King method [10]. The substrate was 6.18 mM phenylphosphate with 50 mM sodium bicarbonate buffer (pH 10.0), and reaction was at 37 °C for 15 min. Measurements were made at 492 nm. Activity was expressed as nmol of substrate utilized per μg protein per 15 min. The protein contents of the cultures were measured by the Bradford method [11].

3. Results

3.1. Mediation of increasing ALPase activity

HPFs were cultured with or without hydroxyapatite for 48 h *in vitro*. The conditioned media obtained from the HPF cultures were diluted to final concentrations of 1/32 to 1/4 with DME and tested for their ability to induce ALPase activity of HPF.

Significantly higher levels of ALPase activity were detected when HPFs were incubated with the conditioned medium of HPFs in contact with hydroxyapatite, compared to that of which had not been in contact with hydroxyapatite (Fig. 1). HPFs were incubated with hydroxyapatite with a range of from 30 to 480 μg cm^{-2}. The appearance of this activity increased depending on the volume of hydroxyapatite (Fig. 2). However, when over 960 μg cm^{-2} of hydroxyapatite was added, the activity in the conditioned medium significantly decreased (data not shown).

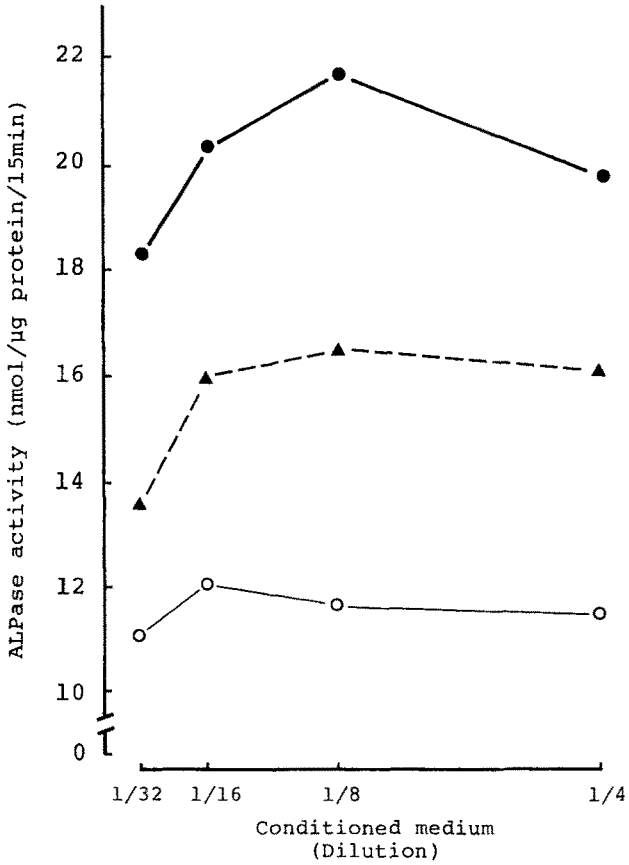

Figure 1 Significantly enhanced ALPase activity was detected when HPFs were incubated with the conditioned medium of HPF in contact with hydroxyapatite. HPFs were cultured with or without hydroxyapatite for 48 h *in vitro*. The conditioned media obtained from the HPF cultures were diluted to final concentrations of 1/32 to 1/4 with DME and tested for their ability to induce ALPase activity of HPFs. (●) HAp, (▲) CaF_2, (○) control.

3.2. Kinetics of HPF-mediated activation of increasing ALPase activity

HPFs were cultured with or without hydroxyapatite over various time periods. The conditioned media of the HPF cultures were obtained and tested for their ability to induce ALPase activity of HPF.

No significant inducing activity was detected in the conditioned medium of HPFs which had been in contact with hydroxyapatite within the first 6 h after the application of hydroxyapatite. However, by 12 h, HPF had elaborated enhanced levels of this activity that continued to increase throughout the 48 to 96 h HPF culture periods (Fig. 3).

3.3. Inhibition of HPF-mediated activity by cycloheximide

HPFs were cultured in complete DME for 16 h. Hydroxyapatite (480 μg cm^{-2}) was applied to the cultures, which were then incubated for 48 h. The cultures were washed with serum-free medium and incubated with cycloheximide (1 μg ml^{-1}) for 24 h in the absence of serum. Elaboration of the activity inducing ALPase activity in the conditioned medium required protein synthesis, since its appearance was completely inhibited by the addition of cycloheximide to the HPF cultures (Fig. 4). This inhibition was not

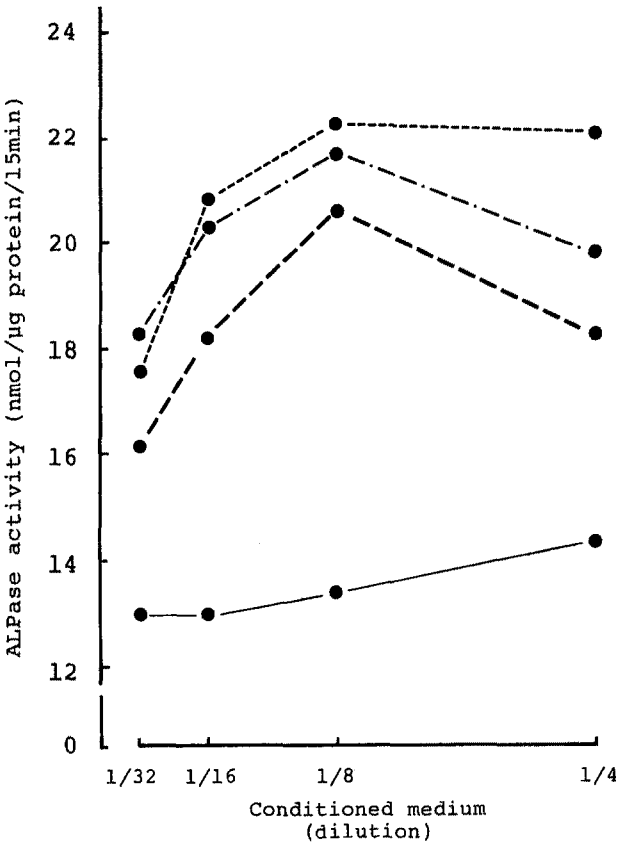

Figure 2 Dose-dependent increasing ALPase activity with conditioned medium. HPFs were incubated with hydroxyapatite (30–480 μg cm^{-2}). The serum-free supernatants were diluted 1/32 to 1/4, and tested for their ability to induce ALPase activity of HPFs. (——) 30, (—·—) 30×4, (– – –) 30×4^2 μg cm^{-2}; (——) control.

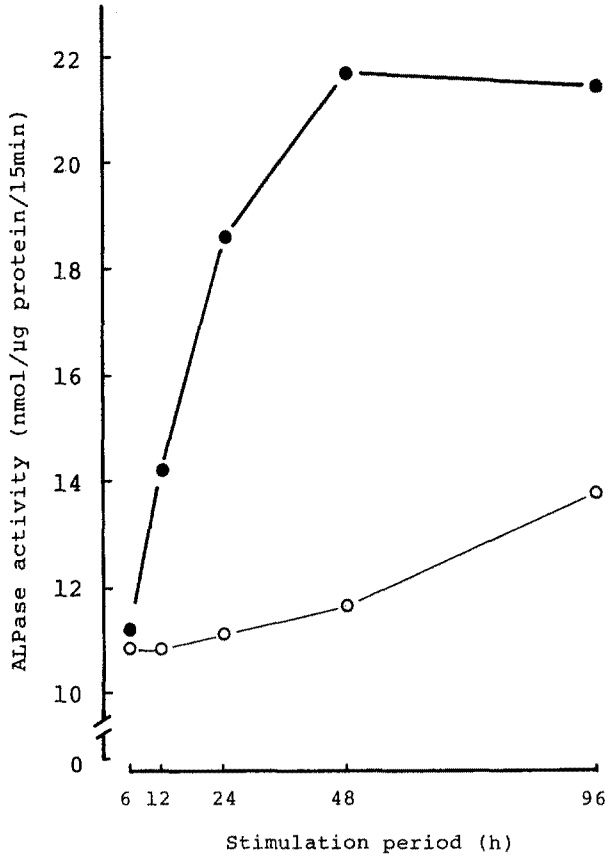

Figure 3 Kinetics of the production of HPF-derived ALPase-inducing activity. HPFs were cultured with or without hydroxyapatite (480 μg cm^{-2}) over varying time periods (6 to 96 h), and the conditioned media collected, diluted (1/4) and tested for their ability to induce ALPase activity of HPFs. (●) HAp, (○) control.

attributed to toxicity since HPFs remained viable as determined by the exclusion of trypan blue in the presence of cycloheximide. Also, when cycloheximide was added to the conditioned medium of HPFs which had been cultured without the addition of cycloheximide, no significant decrease was detected on the ALPase activity of HPFs.

4. Discussion

Since Robison proposed the ALPase theory [12, 13], the focal point for cell biologists is that ALPase is an enzyme which might be related to calcification. Because a high ALPase activity is detected in the matrix vesicles which appear in the early stage of calcification, it is believed that the calcification process begins at this location. Also, when ALPase activity is inhibited, calcification does not occur [14]. Furthermore, the ALPase activity of osteoblastic cells reacts to 1, $25(OH)_2D_3$ and increases [15–18]. Thus, the increase in ALPase activity is thought of as one of the markers showing that the phenomenon of calcification is occurring.

However, the fact that in several non-calcified tissues and organs ALPase activity is also detected indicates that ALPase activity is not just a marker of the calcification process. ALPase activity is markedly high in such organs as the kidney, small intestines and placenta, while it is very low in skin tissues [19].

It has been reported that cells in pulpal connective tissue have the potential to differentiate into cells which have the ability to calcify [20]. However, it is not clear whether the pulp cells differentiate directly into cells which have the ability to form calcified tissues or whether the pulp cells, after de-differentiating once, differentiate into cells having the ability to form calcified tissues. Also, one of the elemental questions deals with the source of the new differentiated odontoblasts in these reports.

In this study, HPFs were cultured with hydroxyapatite in serum-supplemented medium for 6 to 96 h, then the serum-free conditioned medium was harvested and added to sub-confluent HPF cultures to determine the activity which induces the increasing ALPase activity. Significantly enhanced ALPase activity was detected when HPFs were incubated with the conditioned medium. This HPF-derived activity appeared in the conditioned medium between 6 and 12 h and continued to increase throughout the 48 to 96 h culture periods. Additionally, the production of this mediator required protein synthesis since its appearance was completely inhibited in the presence of cycloheximide. Based on these results, it became clear that the HPFs cultured with hydroxyapatite produced a proteinaceous biological mediator which induced their increasing ALPase activity. Furthermore, it is suggested that the fibroblasts which are in contact with hydroxyapatite differentiate into cells which produce such a mediator, and may induce functional

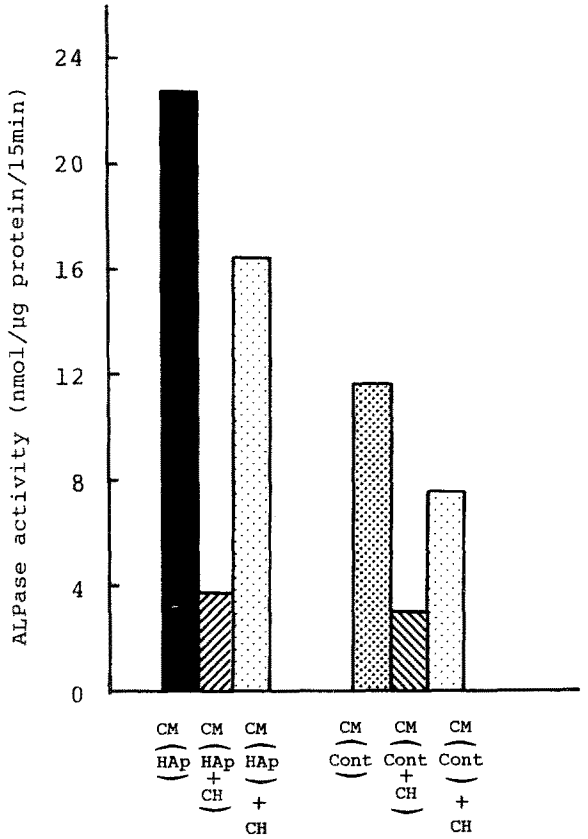

Figure 4 Elaboration of the activity inducing increasing ALPase activity in the conditioned medium required protein synthesis, since its appearance was completely inhibited by the addition of cycloheximide to the HPF cultures. HPFs cultured in complete DME for 16 h. Hydroxyapatite (480 $\mu g\ cm^{-2}$) was added to the cultures, which were then incubated for 48 h. The cultures were washed with serum-free medium and incubated with cycloheximide (1 $\mu g\ ml^{-1}$) for 24 h in the absence of serum. The conditioned media were collected and used for the assay.

differentiation in the various surrounding cells through this mediator.

The production of this mediator was enhanced depending on the volume of hydroxyapatite in the cultures. As a result of this, it is possible that only the cells which had been in contact with the hydroxyapatite actually produce this mediator. Also, in both cases in which HPFs were in contact with calcium fluoride or the volume of hydroxyapatite exceeded 960 $\mu g\ cm^{-2}$, the activity or the amount of the mediator decreased. These results indicate that the production of the mediator which induces increasing ALPase activity not only occurs just simply by some particles or crystals contacting with cells, but also that HPFs recognize the hydroxyapatite crystals among the other particles and produce the mediator.

When hydroxyapatite is applied to pulp tissues, it becomes the core of newly formed dentine and adds minerals to the surface. However, there are a number of reports which are rather negative as to whether or not the hydroxyapatite itself causes undifferentiated mesenchymal cells to have the ability to form dentine, and this contributes in a positive way towards osteoconduction [4–7]. As for the material which induces osteoconduction, the non-collagenous protein in the bone matrix, the bone morphogenetic protein (BMP), is well known, whereas hydroxyapatite has become the subject of study as a carrier of BMP, along with atelocollagen, fibrin and calcium phosphate [21].

In the present study, it became clear that fibroblasts cultured with hydroxyapatite produce a mediator which itself induces the increasing ALPase activity. Whether the ALPase activity is a marker for calcification or not, hydroxyapatite does not simply just increase the size of the crystal as a core of calcification, or supplement the addition of minerals, but rather affects the fibroblasts and induces the production of mediators for cell-to-cell interactions. These functions of the fibroblasts, in pulp healing, may be related to the extensive fibroblast infiltration and dentine bridge formation. Moreover, such biological mediators induce the steady differentiation of the surrounding cells, a phenomenon resembling bone formation. This means that the fibroblasts of pulp tissues are capable of inducing this phenomenon resembling osteoconduction.

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