ORIGINAL

Hydroxyapatite formation in synthetic medium *in vitro*

— Possible application of Ca- β -glycerophosphate
to direct pulp capping —

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Abstract: Basic experiments were carried out to explore whether an organic phosphate, Calcium β -glycerophosphate (Ca-BGP), could be used as the medicament for direct pulp capping. Synthetic incubation medium nearly matched the exposed surface of the dental pulp. Crystal formation in this medium by supplementation of serum and Ca-BGP was ultrastructurally evaluated. After 3 hours of incubation, numerous globular structures, consisting of small granules were formed. These globular structures were identified as amorphous calcium phosphate (ACP) by electron diffraction and IR analyses. With time, globular structures decreased and needle-like crystals increased. Needle-like crystals were identified as hydroxyapatite (HAP) by electron diffraction. Alkaline phosphatase (ALP) activity in the medium with Ca-BGP decreased for up to 12 hours of incubation, and after that time, equilibrium occurred. These findings suggest that supplemented Ca-BGP is hydrolyzed by serum ALP in the synthetic incubation medium. Also the ACP is preferentially produced in high Ca X inorganic phosphate (Pi) ion-product medium and finally ACP converts to thermodynamically stable HAP. Then, Ca-BGP could be used as new agent for direct pulp capping.

Introduction

Direct pulp capping is defined as the protection of a pulp exposed by traumatic fracture or in the course of excavating deep dentinal caries. Protection is provided by placing a medicated material in direct contact with the pulp tissue to promote a reparative reaction¹⁾. Calcium hydroxide is the most commonly used agent in capping. It produces a necrotic layer on exposed surface of the pulp because of its high alkalinity and causes defects in the pulp²⁾.

Matrix vesicles containing high ALP activity were isolated from apical pulp and induced crystal formation *in vitro*, when incu-

bated in medium supplemented with Na-BGP³⁾. ALP is widely distributed in mammalian tissues, and hydrolyzes a wide variety of phosphate esters. ALPs are classified into three main types: kidney/liver/bone type, intestinal type, and placental type⁴⁻⁶⁾. Serum contains all types of ALPs. It has been suggested that this enzyme influences the mineralization process by: 1) hydrolyzing organic phosphate esters and increasing local Pi concentration, 2) hydrolyzing the physiological crystal growth inhibitors such as pyrophosphate⁷⁻¹¹⁾ and ATP¹²⁾, 3) acting as a Pi-binding or transport protein¹³⁻¹⁶⁾.

I conducted basic experiments to explore whether Ca-BGP could be used as the medic-

ament for direct pulp capping. The synthetic incubation medium was supplemented with Ca-BGP and serum. Temporal changes associated with crystal deposition by serum ALP were examined.

Materials and Methods

Male wistar rats (weight about 150 g) were anesthetized with ether, and blood was directly withdrawn from the heart into a syringe. The collected blood, transferred into a tube, was immediately centrifuged at $3,000\times g$ for 5 minutes twice, and then rat serum was obtained. Ca concentration in the serum was determined by an atomic absorption spectrometer, Shimazu AA-640, an Pi concentration was determined by the method of Fiske and Subba Row, using potassium dihydrogen phosphate as standard¹⁷⁾. ALP activity in the serum was assayed at pH 10.3 using p-nitrophenylphosphate as substrate.

The electrolyte concentrations of the incubation medium were applied to a standardized calcifying medium of S. Murphree et al, containing near physiologic levels of Ca^{2+} and $\text{Pi}^{18)}$. The standardized calcifying medium, supplemented with 10 % rat serum, approximately consistent with ALP activity in matrix vesicles isolated from apical pulp of rat incisors³⁾, contained 1.35 mM Ca, 1.97 mM PO_4 , 85 mM NaCl, 15 mM KCl, 50 mM TES (pH 7.40), 10 mM NaHCO_3 . This Ca-Pi medium containing the serum was incubated with 5 mM Ca-BGP in a total volume of 4 ml, and then placed in a humidified incubator at 37 °C in an atmosphere of 5 % CO_2 for up to 24 hours. In some controls the Ca-Pi medium containing rat serum without Ca-BGP, and the serum-free Ca-Pi medium with Ca-BGP were incubated up for 3 days, in the same manner.

At the end of incubation, the reaction mixture was centrifuged at $300,000\times g$ for 10 minutes. The resulting precipitate was fixed *in situ* in centrifuge tubes by addition of 2 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for 2 hours, and post-fixed in 2 % osmium tetroxide in the same buffer for 1 hour, and then dehydrated in increasing concentrations of ethanol and embedded in epoxy resin. Ultrathin

sections were cut in a apatite saturated solution and mounted on Formvar-coated 150-mesh copper grids, and examined without staining in a Hitachi H-7000 electron microscope at 100 kV.

Furthermore, unstained semithin ($0.5\text{ }\mu\text{m}$) sections of reaction mixture were used for selected area (about $2.2\text{ }\mu\text{m}$ in diameter) electron diffraction to reveal newly formed mineral phase. Electron diffraction was carried out using a Hitachi H-9000 electron microscope at 300 kV. D-spacings of each sample were calibrated from those of gold obtained with completely same diffraction conditions.

The precipitates after 3, 6, 24 hours incubation in the presence of Ca-BGP were dehydrated in centrifuge tubes in increasing concentrations of ethanol and then air-dried. The dried material from each timed incubation and synthetically prepared mineral standard (about 1 mg) was incorporated into KBr (100–150 mg) pellets under pressure. The KBr pellets were used for infrared (IR) absorption spectroscopy analyses over a range of $4000\text{--}400\text{ cm}^{-1}$ on a Hitachi IR 260-30 spectrometer. Hydroxyapatite (HAP)¹⁹⁾ was used as standard for calcium phosphate mineral.

In order to investigate whether serum ALP hydrolyzes organic phosphate added to Ca-Pi medium, ALP activity in the reaction mixture with Ca-BGP and without Ca-BGP was assayed for up to 3 days.

Results

The pellets obtained after 3 hours of exposure to the Ca-BGP-containing medium showed numerous globular structures, which revealed a variation in shape and size ($0.3\text{--}1.7\text{ }\mu\text{m}$ in diameter) (Fig. 1). These globular structures consisted of the aggregates of small electron-dense granular ones. Short linear densities were occasionally observed in association with globular structures. The pellets obtained after 6 hours of incubation with Ca-BGP showed an increase in the number of linear crystalline densities (Fig. 2). These linear densities were in contact with the granular materials. There was a decrease in the number of granular structures and numerous needle-like crystals were formed after 12 hours of exposure to the reaction mixture

with Ca-BGP (Fig. 3). After 24 hours of incubation with Ca-BGP, a marked increase in the number of needle-like crystals were observed (Fig. 4). The reaction mixture which did not contain Ca-BGP and the serum-free incubation with Ca-BGP failed to sediment any pellets even after 24 hours.

Selected area electron diffraction patterns from sections were observed from after 6 hours of exposure to the Ca-BGP-containing medium. They revealed a pattern of concentric inner double rings, and the pattern after 24 hours of incubation was clearer than that after 6 hours (Figs. 5 and 6). These inner double rings had d-spacings (about 3.45 Å and 2.79 Å, respectively) consistent with HAP (3.44 Å and 2.819 Å).

The IR spectra of pellets incubated with Ca-BGP collected at different time intervals were shown in Fig. 7. These spectra were dominated by the two orthophosphate (P-O) absorption bands at 1130-1030 cm^{-1} and 600-560 cm^{-1} . The IR spectra of pellets after 3 hours incubation with Ca-BGP showed two broad symmetrical singlet absorption bands centered at 1035 cm^{-1} and 550 cm^{-1} . The first clear indication of a separate mineral phase was seen in the spectrum taken at 6 hours: well-resolved doublet in the 600-560 cm^{-1} , central peak at 1035 cm^{-1} and shoulders at 1110 cm^{-1} and 1130 cm^{-1} in the 1130-1030 cm^{-1} . With time, the 600-560 cm^{-1} bands intensified and the shoulder at 1110 cm^{-1} still appeared, but the shoulder at 1130 cm^{-1} tended to obscure.

The time-dependent decrease in ALP activity was observed during incubation with Ca-BGP (Fig. 8). Maximal decrease rate

occurred during 3 hours of incubation, and apparent equilibrium occurred after 12 hours. While ALP activity after 24 hours of incubation without Ca-BGP was about 93 % of the original activity, that with Ca-BGP was about 38 %.

Discussion

Na-BGP has been used as a substrate for ALP to provide a source of inorganic phosphate for mineralization *in vitro*^{9,20-23}. Osteogenic cells cultured with Na-BGP produce a mineralized tissue²⁴⁻²⁷. As no pellets were obtained after 3 hours of incubation with Na-BGP, Ca-BGP was used in the present study. The concentration of Ca-BGP was determined at 5 mM as the addition of over 5 mM Ca-BGP to the medium did not produce clear solution.

Present study showed that maximal decrease rate of ALP activity occurred during initial 3 hours, and that equilibrium occurred after 12 hours of incubation. Several possible reasons for the decrease of ALP activity were indicated^{28,29}; 1) the mineral phase might bind to the enzyme and block the active site, 2) metal ions (Zn^{2+} and Mg^{2+}) might be lost from the active site of the enzyme, 3) irreversible denaturation of the enzyme might occur during mineralization, 4) free GP in the medium might cause conformational change of ALP, and 5) ALP activity might decrease in dependence on increases in phosphate accumulation. In the present study, with Ca-BGP, ALP activity was decreased during 12 hours of incubation, whereas without Ca-BGP, ALP activity was preserved for over 12 hours of incubation. And in Ca-BGP-containing medium, amorphous cal-

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- Fig. 1 Pellets after 3 hours of incubation. Numerous globular structures are evident. Tiny linear densities are occasionally observed in association with globulars (arrows). Undemineralized and unstained section. $\times 90,000$.
- Fig. 2 Pellets after 6 hours of incubation. The linear crystalline densities are in contact with the granular structures (arrows). Undemineralized and unstained section. $\times 90,000$.
- Fig. 3 Pellets after 12 hours of incubation. Note needle-like crystals associated with granular structures (arrows). Undemineralized and unstained section. $\times 90,000$.
- Fig. 4 Pellets after 24 hours of incubation. Note numerous needle-like crystals. Granular structures seen at early stage are hardly identified. Undemineralized and unstained section. $\times 90,000$.
- Fig. 5 Electron diffraction pattern of a section of the reaction mixture after 6 hours of incubation. Note the faint concentric inner double rings characteristic of apatite.
- Fig. 6 Electron diffraction pattern of a section of the reaction mixture after 24 hours of incubation. Note the concentric inner double rings characteristic of apatite.

Fig. 7 IR spectra of the reaction mixture obtained by ultracentrifugation after 3 (A), 6 (B), and 24 (C) hours of incubation, and HAP standard (D). The first clearly discernible mineral phase was observed at 6 hours. Note the progressive increase in intensity of spectra with time.

Fig. 8 Time-dependent decrease in ALP activity during incubation with Ca-BGP (filled circles) and without Ca-BGP (open circles).

cium phosphate (ACP) formed after 3 hours of incubation and HAP formation was ultrastructurally observed after 6 hours of incuba-

tion. These findings indicate that Ca-BGP may be hydrolyzed by ALP and that Ca X Pi ion product in the medium may increase. These data strongly suggest that phosphate accumulation and the formation of calcium phosphate deposits in the medium inhibits ALP activity.

When calcium hydroxide is applied to exposed pulp, its high pH produces superficial necrosis in the exposed pulp and this is followed by migration and proliferation of mesenchymal cells. It is suggested that matrix vesicles-induced mineralization occurs following direct pulp capping and pulpotomy^{30,31}. In these cases, the origin of calcium during the mineralization is not the calcium hydroxide as a medicament but pulp fluids, since calcium hydroxide applied to exposed pulp reacts with carbon dioxide in the tissue and calcium carbonate is formed³². Although Ca X Pi ion product and pH in the present original medium were physiological levels, supplemented Ca-BGP, which is not present in biological system, was hydrolyzed by added serum ALP and high Ca X Pi ion-product was rapidly formed in the medium. Therefore crystal formation in the present study would be thought to differ from that found following direct pulp capping *in vivo*.

The most widely accepted hypothesis to account for maturational changes in the X-ray diffraction characteristics of bone mineral has been an ACP theory, which postulates that an initial ACP solid phase is deposited that gradually converts to poorly crystalline HAP³³. Recent studies using X-ray radial distribution function analysis³⁴ and P-31 nuclear magnetic resonance³⁵ have demonstrated that a solid phase of ACP does not exist in bone in any significant amount, and the presence of acid phosphate groups in a brushite-like configuration. Chemical modeling studies have shown that in metastable aqueous solution, HAP formation is preceded by the formation of one or more intermediate calcium phosphate mineral phase. The most frequently implicated HAP precursors are ACP, dicalcium phosphate dihydrate, or octacalcium phosphate, depending on the conditions present in the solution during the precipitation reaction³⁶. It has been demonstrated that

alkaline pH stabilizes ACP, and that solution-mediated dissolution of ACP precedes nucleation of HAP^{37,38}. Furthermore, ⁴⁵Ca-accumulation study under various electrolyte conditions using matrix vesicles *in vitro* has shown that at constant Ca/Pi of 1.3 and Ca X Pi of 2.63 mM², maximal uptake rate occurred at pH 7.6–7.8 over a pH range of 7.0–8.0, with significant uptake being supported over the narrow range of pH 7.4–7.8³⁹.

In the present study, needle-like structures in contact with granular ones were observed in the section after 6 hours of incubation with Ca-BGP. Furthermore, granular structures were demonstrated as ACP by electron diffraction and IR analyses. These findings suggest that at physiological pH (7.4) and Ca X Pi of 2.66 mM² in the present original incubation medium, supplemented 5 mM Ca-BGP is rapidly hydrolyzed by serum ALP,

and that ACP is preferentially produced in high Ca X Pi ion-product reaction medium and that ACP phase is stabilized in alkaline pH condition. Finally, once solution-mediated dissolution of ACP occurs³⁷, thermodynamically stable HAP phase appears.

In conclusion, crystal formation occurs by supplementation of serum and Ca-BGP in the synthetic incubation medium nearly matched the exposed surface of dental pulp. Ca-BGP dose not seem to impair crystal formation. Then, Ca-BGP could be used as new agent for direct pulp capping to induce rapid apatitic mineral formation.

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抄録: 直接覆髄時, 歯髄創傷面へ有機リン酸塩である Ca-β glycerophosphate (Ca-BGP) を応用することが可能か否かを検討するための基礎実験を行った。歯髄創面の環境に近づけた合成培養液中に、血清および Ca-BGP を添加した場合の結晶形成過程を超微形態的に検討した。培養3時間後、顆粒状物質の集合した球形構造物が形成され、これらを電子線回折, IR 分析したところ, amorphous calcium phosphate (ACP) と同定された。時間経過とともに球形構造物は減少し、針状様結晶構造物が増加した。針状様結晶構造物を電子線回折したところ, hydroxyapatite (HAP) であることが確認された。Ca-BGP 添加培養液の alkaline phosphatase (ALP) 活性は培養12時間までは減少し、その後、平衡に達した。以上の結果から、今回用いた培養液中で、添加した血清中の ALP によって Ca-BGP が加水分解され、カルシウム・リンの高イオン積となった培養液中で、ACP が選択的に生じ、ACP は、最終的に、熱力学的に安定なアパタイトに変換されたものと思われる。これらのことから、Ca-BGP を、直接覆髄時に応用するのは可能と思われる。

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