IN VITRO STUDIES OF BONE RESORPTION BY THE ROOT-
RESORBING TISSUE FROM THE BOVINE
DECIDUOUS TOOTH*1

BY

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ABSTRACT

Granulation tissue which is responsible for root resorption of deciduous tooth lies between root of the deciduous tooth and its permanent tooth germ. This tissue is called “root-resorbing tissue”. Its bone-resorbing activity was investigated in vitro.

Bovine root-resorbing tissue was cultured in close contact with 45Ca-labeled dead calvaria of rats. Bone-resorbing activity was determined by measuring 45Ca released from labeled calvaria during culture. It was found that only the root-resorbing tissue which was rich in odontoclasts and had a good blood supply in its surface layer had bone-resorbing ability, and that bone resorption occurred only when it was placed in close contact with calvarium. The root-resorbing tissue which was poor in odontoclasts and blood vessels failed to stimulate bone resorption. Bone resorption by the root-resorbing tissue was enhanced markedly by 25-hydroxy-vitamin D3 or heparin, but not by larger amounts of parathyroid hormone, vitamin D3, and dihydrotachysterol when added to the culture.

INTRODUCTION

Resorption of the roots of deciduous teeth is a physiological phenomenon, and results in the shedding of these teeth. The resorptive process is not continuous, but alternates with periods of repair1,2).

Many investigators have been interested in the causes of abnormal tooth resorption rather than those related to the physiological process of shedding of the deciduous teeth and subsequent eruption of their permanent teeth. For instance, trauma is an important factor in abnormal resorption which may be caused by occlusal interferences3), orthodontic appliances4,5) or chemical agents used in restoring teeth6,7,8). Prolonged effects of inflammation are also believed to be important. Cahn9) and Scopp10) demonstrated that idiopathic resorption was due to an increase in the pulpal blood supply which enhanced the resorption of calcium salts. Others suggested that some hormonal factors are involved in this process, such as hyperthyroidism suggested by Beck11) and by Henry and Weinmann12), or hyperparathyroidism which is believed to be the cause of tooth resorption in experimental animals13). There may be, therefore, many causes influencing tooth resorption, but none of them is thought to explain the physiological process involved.

*1 This paper was partly reported at the 12th General Meeting of the Japanese Society for Pedodontics in 1974.
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*3 Abbreviations used: PTH = parathyroid hormone, 25-OH-D3 = 25-hydroxycholecalciferol, 1,25-(OH)2-D2 = 1,25-dihydroxycholecalciferol, and DHT3 = dihydrotachysterol.
in the loss of deciduous teeth.

At the end of the 19th and the beginning of the 20th century, it was believed that physiological resorption of deciduous teeth was solely dependent on the eruption of the permanent teeth, whose pressure may cause root resorption. At that time, some investigators presumed the existence of a special resorptive organ. It has been established that no such organ exists and that the roots of deciduous teeth are resorbed by granulation tissue containing odontoclasts. Hopewell-Smith investigated the granulation tissue of man and cat during root resorption by light microscopy and found that the granulation tissue resorbed and invaginated the root of the deciduous tooth. Sognnaes also examined mechanism of root resorption and found that the surrounding granulation tissue rich in blood supply was essential for this process.

A number of histological and histochemical studies concerning deciduous tooth resorption have been reported. However, no biochemical data are available concerning this process.

Morita et al. demonstrated collagenolytic activity in bovine granulation tissues which is necessary for the root resorption of deciduous teeth. He also showed that the resorption took place when a thin section of bovine dentin was incubated in close contact with the layer that had a good blood supply and was rich in odontoclasts.

The present report deals with bone-resorbing activity of the granulation tissues by determining quantitatively $^{45}$Ca released from prelabeled calvaria. The granulation tissues which are rich in odontoclasts and blood vessels had the bone-resorbing activity, while the root-resorbing tissues which were poor in odontoclasts and blood vessels failed to stimulate bone resorption. Effects of some stimulating factors for bone resorption were also investigated in this system.

**Materials and Methods**

Fresh mandibles of about one-year-old calves were obtained from a local slaughter house. Granulation tissues lying between the root of the lower first or the second deciduous incisor which was undergoing resorption and its permanent tooth germ were obtained. The granulation tissue was treated with Ca- and Mg-free phosphate buffer solution (CMF-PBS, pH 7.4) containing 500 units/ml of penicillin and 500 mg/ml of streptomycin.

Male weanling Wistar rats (60 g) were intraperitoneally injected $60\mu$Ci of $^{45}$Ca dissolved in 0.06 ml of 0.9% NaCl solution. Seven days thereafter the rats were sacrificed, and their heads were frozen and stored for several days before use. The calvaria were dissected and freed of adhering soft tissues. Each calvarium was divided into 16 equal parts (about 40 mg in fresh weight) in sterilized CMF-PBS containing 50 units/ml of penicillin and 50 mg/ml of streptomycin. Fourteen fragments were washed with the sterilized CMF-PBS for 2 days under continuous stirring and pre-cultured for 1 day to eliminate weakly bound $^{45}$Ca. The other two fragments of the calvarium were dissolved in 1 ml of HNO$_3$, and each radioactivity was determined by means of a liquid scintillation counter.

The root-resorbing tissues were cut into approximately $3\times3\times1.5$ mm³ pieces and each fragment was placed in close contact with a labeled fragment of rat calvarium in a Petri dish, as shown in Fig. 1. Seven Petri dishes (diameter 22 mm) were placed in a small glass desiccator in 95% O$_2$-5% CO$_2$ (Fig. 1, a). Rat calvaria were packed by two sheets of bovine resorbing tissues and cultured on a stainless steel mesh (Fig. 1, b).
The medium consisted of 89% synthetic medium Ham F12 (Nissui, Tokyo), 10% calf serum, and 1% antibiotic solution (50 units/ml of penicillin and 50 mg/ml of streptomycin). Each Petri dish was filled with 1 ml of the medium. The culture was maintained at 37°C for 4 days. The tissue culture media were changed every day, at which time the cultures were freshly gassed. All media were adjusted to pH 7.52 before gassing. Calvaria were cultured with (experimental group) or without (control group) granulation tissues. After the cultivation, 45Ca in the media of the experimental as well as the control groups was measured in a mixture of 5.5 ml of Insta Gel (Packard) and 3.5 ml distilled water using a liquid scintillation counter (Packard Model 3385). Resorption was examined in terms of release of 45Ca from bones into media. This release was expressed as the ratio of experimental/control group. The t-test was used to determine the significance of the difference in the observed ratios.

Some possible stimulating substances of bone resorption were added to the medium in order to test whether the 45Ca release by the granulation tissues was enhanced or not.

The substances tested were as follows:

Parathyroid extract (PTE, Lilly Co.): PTE dissolved in a solution of 1.6% glycerol, 0.2% phenol, and 0.9% NaCl was added to the medium at the concentration of 0.5–1.0 U/ml. In the control dish, only the vehicle was added to the medium.

Heparin (Novo heparin powder, Novo Industri A/S): Heparin was added to the medium at the concentration of 10 U/ml.

Vitamin D and its analogs: Various con-
centrations of synthetic 25-hydroxycholecalciferol (25-OH-D₃ kindly provided by Dr. T. Suda), dihydrotachysterol (DHT₂), or vitamin D₃ was added in 95% ethanol solution.

Microscopic observation: The resorbing tissues were fixed in 10% neutralized Formalin, dehydrated in graded ethanol, embedded in paraffin, sectioned, and stained with haematoxylin and eosin.

Results

Histological examination showed that the inner layer of the root-resorbing tissues was composed of connective tissues and exhibited many collagen fibres, while the surface layer mainly consisted of granulation tissues which contained many osteoclasts. These observations were identical to those reported previously by Morita et al.¹⁷. The root-resorbing tissues consisted of two types of granulation tissue; one was red, whose surface layer contained a number of osteoclasts and blood vessels, and the other was white, whose surface layer had very little blood supply (Fig. 2).

Fig. 3 shows time course of the cumulative ⁴⁵Ca released from rat calvaria by bovine red or white granulation tissues. The amount of the cumulative ⁴⁵Ca released from rat calvaria either by red or white granulation tissues increased linearly for 4 days of cultivation. The red granulation tissues always enhanced the ⁴⁵Ca release more effectively than the white tissues did. Therefore, the treated/control ratio of the cumulative ⁴⁵Ca release after a 4-day cultivation was used for the evaluation of bone-resorbing activity in the following experiments.

Table 1 indicates the effect of bovine red and white resorbing tissues on the ⁴⁵Ca release from rat calvaria during a 4-day cultivation. The red granulation tissue released ⁴⁵Ca three times more effectively than the white one did (Table 1). The release of ⁴⁵Ca did not increase during the 4-day cultivation when the inner layer of the resorbing tissue was placed in contact with rat calvaria, nor did it increase when the red tissue was placed apart from the calvaria (Table 1). Any stimulation of the release of ⁴⁵Ca was not observed during cultures when the surface layer of the red

<table>
<thead>
<tr>
<th>Bovine root-resorbing tissues*</th>
<th>Contact layer with rat calvaria</th>
<th>Cumulative ⁴⁵Ca release during 4-day cultivation</th>
<th>Treated/control Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red tissues</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fresh</td>
<td>surface layer</td>
<td>1.67±0.13 (30)**</td>
<td></td>
</tr>
<tr>
<td>fresh</td>
<td>inner layer</td>
<td>1.03±0.02 (20)</td>
<td></td>
</tr>
<tr>
<td>fresh</td>
<td>surface layer</td>
<td>1.01±0.04 (30)</td>
<td></td>
</tr>
<tr>
<td>fresh</td>
<td>placed apart from calvaria</td>
<td>1.03±0.03 (20)</td>
<td></td>
</tr>
<tr>
<td>White tissues</td>
<td>fresh</td>
<td>1.21±0.11 (20)***</td>
<td></td>
</tr>
</tbody>
</table>

* Each layer of bovine red or white root-resorbing tissues was placed in contact with (or apart from) rat calvaria. Some red tissue was frozen at -20°C for 1 day before use. In the control group, rat calvaria were cultured without resorbing tissues. Data are expressed as the mean value ± standard deviation (number of experiments).

** significantly different from 1.0 (p<0.01)

*** significantly different from 1.0 (p<0.05)
resorbing tissues had been kept frozen at 
-20°C for 1 day before cultivation, sug-
gestng that bone-resorbing activity had been lost upon freezing (Table 1). Other 
tissues tested, such as rat liver, spleen, and peritoneum did not stimulate the release of 
45Ca from the bone at all (Table 2). From these results, only the surface layer of the

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**Fig. 2.** (a): Micrograph of bovine red root-resorbing tissue. A number of odontoclasts (OCL) and blood vessels (BV) are observed on the sur-
face layer of the tissue. Haematoxylin and eosin staining. ×500

(b): Micrograph of bovine white root-resorbing tissue. Although few odontoclasts are seen on the surface layer, blood vessels are seldom present. Haematoxylin and eosin staining. ×500
fresh red resorbing tissues seemed to have the bone-resorbing ability.

Bone resorption was also stimulated by the cultivation with other bovine tissues, such as gingival epithelium and dental sac (Table 2). Stimulation of bone resorption by these tissues was less pronounced than that by the red root-resorbing tissues. Stimulation of bone resorption by gingival epithelium was higher than that by dental sac. Gingival tissues released $^{45}$Ca two times more effectively compared to dental sac. Gingival tissues release $^{45}$Ca two times more effectively compared to dental sac. This confirmed the results reported previously by Jacobsen and Goldhaber$^{18}$, who reported that human and mouse skin epithelia have some bone-resorbing activity.

Table 3 shows the effect of PTE, vitamin $\mathrm{D}_3$, DHT$_2$, 25-OH-$\mathrm{D}_3$, and heparin on the cumulative $^{45}$Ca release from rat calvaria cultured for 4 days in contact with the red resorbing tissues. When PTE was added to the medium cultured with the red resorbing tissues there was no further enhancement of bone resorption (Table 3), suggesting that PTE does not stimulate bone-resorbing activities of the red granulation tissues. When vitamin $\mathrm{D}_3$ and DHT$_2$ were added at the concentration of 1$\mu$g/ml, bone resorption was not enhanced either (Table 3). Vitamin $\mathrm{D}_3$ did not stimulate bone-resorbing activity of the red granulation tissues even at the concentration of 8$\mu$g/ml (Table 3).

### Table 2. Cumulative $^{45}$Ca release from rat calvaria, when various tissues were cultured for 4 days in close contact with the bone

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissues</th>
<th>Cumulative $^{45}$Ca release during 4-day cultivation Treated/control Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine</td>
<td>Red resorbing tissue</td>
<td>1.70±0.11 (21)*</td>
</tr>
<tr>
<td>Rat</td>
<td>Liver</td>
<td>1.00±0.01 (19)</td>
</tr>
<tr>
<td>Rat</td>
<td>Spleen</td>
<td>1.00±0.03 (19)</td>
</tr>
<tr>
<td>Rat</td>
<td>Peritoneum</td>
<td>1.02±0.02 (18)</td>
</tr>
<tr>
<td>Bovine</td>
<td>Gingival epithelium</td>
<td>1.40±0.10 (24)**</td>
</tr>
<tr>
<td>Bovine</td>
<td>Dental sac</td>
<td>1.21±0.09 (24)**</td>
</tr>
</tbody>
</table>

In the control group, rat calvaria were cultured alone.

Data are expressed as the mean value ± standard deviation (number of experiments).

* significantly different from 1.0 ($p<0.01$)

** significantly different from 1.0 ($p<0.05$)
Table 3. Effects of PTE, Vitamin D₃, DHT₂, 25-OH-D₃, and heparin on the cumulative ⁴⁰Ca release from rat calvaria cultured for 4 days in contact with the red resorbing tissues

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentrations</th>
<th>Cumulative ⁴⁰Ca release during 4-day cultivation Treated/control Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>1.65±0.11 (36)</td>
</tr>
<tr>
<td>PTE</td>
<td>0.5 U/ml</td>
<td>1.67±0.12 (21)</td>
</tr>
<tr>
<td></td>
<td>1.0 U/ml</td>
<td>1.67±0.09 (21)</td>
</tr>
<tr>
<td>Vitamin D₃</td>
<td>1.0 μg/ml</td>
<td>1.64±0.09 (18)</td>
</tr>
<tr>
<td></td>
<td>8.0 μg/ml</td>
<td>1.66±0.08 (18)</td>
</tr>
<tr>
<td>25-OH-D₃</td>
<td>0.1 μg/ml</td>
<td>1.90±0.10 (18)*</td>
</tr>
<tr>
<td></td>
<td>1.0 μg/ml</td>
<td>2.21±0.13 (18)*</td>
</tr>
<tr>
<td>DHT₂</td>
<td>1.0 μg/ml</td>
<td>1.65±0.09 (18)</td>
</tr>
<tr>
<td></td>
<td>10.0 μg/ml</td>
<td>1.64±0.07 (18)</td>
</tr>
<tr>
<td>Heparin</td>
<td>10.0 U/ml</td>
<td>2.09±0.08 (20)*</td>
</tr>
</tbody>
</table>

Data are expressed as the mean value ± standard deviation (number of experiments). * significantly different from 1.65 (in the case of no addition) (p<0.05)

On the other hand, release of ⁴⁰Ca into the medium by bovine red resorbing tissues increased markedly when 25-OH-D₃ was added at the concentrations of 0.1 and 1.0 μg/ml. At the concentration of 0.1 μg/ml, bone resorption by the red resorbing tissues was increased by 23% compared with the no addition group. In the case of 1.0 μg/ml, the rate of the increase was 54% (Table 3). Heparin also enhanced bone resorption by bovine red resorbing tissues at the concentration of 10 units/ml. The rate of the increase was 42% (Table 3).

**DISCUSSION**

There are two types of the root-resorbing tissues. One is the red, and the other is the white. The red granulation tissue has a number of multinucleated odontoclasts aligned along its surface layer with a good blood supply in its inner layer, while the white granulation tissue is extremely poor in blood supply though few odontoclasts can be seen in its surface layer. Histological as well as biochemical data clearly indicate that the granulation tissue responsible for the root resorption of deciduous teeth is the red one. The red granulation tissue seems to convert to the white one. This conversion appears to be reversible and related to the discontinuous resorptive process of the roots of deciduous teeth. Multinucleated giant cells (odontoclasts) seen in the surface layer of the red granulation tissue appear to have similar morphological as well as functional features to odontoclasts. Furseth has demonstrated by light and electron microscopic studies that the odontoclasts have (1) a number of nuclei, (2) a complex edge of resorbing microvilli or ruffled border, and (3) a large number of mitochondria and free ribosomes. These giant cells appear to play a central role on the root resorption of deciduous teeth. Ohno also reported similar results to Furseth’s observations.

Cultivation of red granulation tissues with prelabeled dead calvaria made it possible to examine quantitatively bone-resorbing activity by the root-resorbing tissues. Bone-resorbing activity by the red granulation tissues was enhanced only when the surface layer of the red tissues was placed in close contact with the dead bone in culture. Frozen red tissue or fresh white tissues failed to stimulate bone resorption.
These results strongly suggest that odontoclasts play an essential role in the root resorptive process of deciduous teeth.

Liver, spleen, and peritoneum from a rat did not show any bone-resorbing activities in this system, while bovine gingival epithelium and dental sac stimulated 45Ca release from the dead calvaria. Goldhaber also reported that human gingival epithelium has collagenolytic as well as bone-resorbing activities. Data reported here are consistent with his observation.

It is interesting that PTE did not stimulate bone-resorbing activity of the red granulation tissues when PTE was added to the medium at the concentrations of 0.5 and 1.0 U/ml. Freilich has reported that histological features of odontoclasts were essentially unchanged after the administration of PTE. Strock also found that root resorption of deciduous teeth did not occur at all in patients of hyperparathyroidism, in which prominent bone resorption was seen in the maxillary bone. These data together with the results report here suggest that parathyroid hormone may not play a central role in the physiological process of the deciduous root resorption.

Vitamin D₃ and DHT₂ did not show any stimulatory effect on bone-resorbing activity by the red granulation tissues at the concentrations of 1 and 8 µg/ml, while 0.1 µg/ml of 25-OH-D₃ enhanced it significantly (Table 3). Raisz and Trummel have reported that vitamin D₂ or D₃ itself added to the cultures was totally ineffective. Trummel et al. found that DHT₂ did not produce any significant stimulation of bone resorption in tissue culture at the concentration of 150 µg/ml, while 25-OH-D₃ produced prominent stimulation of bone resorption at the concentration of 0.03 to 3.0 µg/ml. Although the final active metabolite of vitamin D₃, 1,25-dihydroxycholecalciferol (1,25-(OH)₂-D₃), has not been tested yet in this system, vitamin D or its active metabolite(s) may play some regulatory role on the process of deciduous root resorption.

These results suggest that odontoclasts in the red granulation tissues play an essentially important role in the deciduous root resorption, and that some systemic factors such as metabolite(s) of vitamin D₃ might have some regulatory effects on its process.

Acknowledgement

I wish to express my gratitude to Prof. H. Yamashita, Department of Pedodontics, and Prof. S. Sasaki, Department of Biochemistry, for their valuable advice and guidance. My deep gratitude is also expressed to Associate Prof. T. Suda, Department of Biochemistry, and Prof. G. L. Mechanic, University of North Carolina, for their valuable advice and kind help in preparing the manuscript. I am also grateful to all the staff of the Department of Pedodontics and the Department of Biochemistry.

References