Release of lysosomal hydrolases from bone explants affected by dental plaque

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Lysosomal hydrolases as indicators of plaque-induced bone resorption in tissue culture were studied. Fetal rat bones cultured in a synthetic medium containing sonicated and filtrated human dental plaque were used as bones to be resorbed. Acid phosphatase and ß-glucuronidase were found to be the most suitable enzymes for studying the degradation rate of bone cell lysosomes. When dental plaque is used as a resorbing agent, special attention has to be paid to the fact that plaque extract contains similar lysosomal hydrolase activity. Plaque hydrolases in the present study were quite stable in culture medium. No significant adsorption of enzymes by fetal rat bones occurred while remarkable adsorption by synthetic hydroxyapatite was found.

The present results indicate that dental plaque is able to release lysosomal hydrolases from bone explants. This release corresponds to the degree of resorption measured by ^{45}Ca release from bones.

Key-words: Tissue culture; bone resorption; lysosomal enzymes; parathyroid hormone; ^{45}Ca release

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Lysosomal hydrolases have been previously used as indicators of bone resorption in tissue culture (1, 9, 15–20). A clear correlation between lysosomal hydrolase activity and bone resorption has been observed (1). Several studies have shown the importance of lysosomal enzymes in bone resorption (13–16) and the presence of these enzymes in bone celles has been shown (2). Morphological studies on the progress of bone resorption correspond closely with the increasing activity of hydrolases (1, 10). The inhibitors of bone resorption block lysosomal enzyme release at the same time that calcium release decreases (3).

None of the earlier studies have dealt in detail with the problems concerning the reliability of enzyme assays as indicators of bone resorption. However, several problems arise when using enzymes as indicators, e.g. their origin, stability in the medium, possible adsorption by inorganic components etc. The aims of the present study were to measure the release of lysosomal hydrolases from bone explants affected by dental plaque and to compare this release to calcium release, which is frequently used as an indicator of bone resorption.

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MATERIAL AND METHODS

Bone culture

Female Long Evans rats, 4 to 8 months old and 19–21-day pregnant, were used as donor animals. The fetal radii and ulnae were prepared under sterile conditions and explanted as pairs (left radius and right radius) into bone culture. The culture technique was in principle the same as that of Saxen (12). The culture used contained 3.5 ml of synthetic protein free BGJb medium supplemented with streptomycin (50 μg/ml) and penicillin (50 I.U./ml). The antibiotics were diluted with a small amount of medium and sterilized by the aid of a Millipore filter (pore size 0.45 μm).

The cultures were performed in an automatic incubator (Assab Medicin AB, Stockholm, Sweden) at +37°C, 5% CO_2/air-atmosphere and 80% humidity. The medium was changed and a resorbing agent added after 24 hours preincubation. Sterilized 0.9% NaCl-solution was used in control cultures instead of the resorbing agent. The incubation time varied from 4 hours to 6 days.

Collection and treatment of dental plaque

Plaque was collected from adult patients visiting the Institute of Dentistry, University of Turku. After the tooth surfaces had been dried, the plaque was collected with an excavator and suspended in 1.0 ml of saline in an ice bath. Care was taken not to mix plaque with saliva or blood. Each plaque pool was collected from at least five persons. Immediately after the collection the suspension was frozen.

Before use the plaque was thawed, sonicated in a MSE Ultrasonic Desintegrator (100 W Model, end diameter of the probe 9 mm) for 10 min and centrifuged in a Sorvall Superspeed RC-2B centrifuge at 23500x*g* for 10 min at +4°C. The supernatant fluid was sterilized by passing it through a Millipore® filter (pore size 0.45 μm). The clear solution was used as the resorbing agent in bone culture. In some of the experiments parathyroid hormone at a concentration of 0.86 μg/ml was used as a reference.

Enzyme and protein assays

The enzyme assays were performed on the following samples: (1) culture medium at various stages of incubation (2) bones after treatment with a Teflon homogenizer (60 sec) in 2.0 ml of 0.5% Triton X-100 solution followed by centrifugation (23500 xg, 10 min) (3) saline solution in which the bones were stored prior to homogenization and (4) plaque samples after sonication and centrifugation.

Because of high enzyme activity in plaque samples (μmole/ml x 10^4–10^5 with different enzymes) the true activity released from the bones was calculated according to the following formula

$$A_r = \frac{A_t - A_p,}{n}$$

where A_r is the released activity from bones, A_t the activity measured in the medium, A_p the activity of plaque suspended in medium and *n* the number of bones in culture. The incubation times of A_t and A_p were identical, the amount of medium was also identical in all the cultivations.

The standard reaction mixture of 0.6 ml contained 15 μmoles of acetate buffer, pH 4.0, 0.1 μmoles of substrate (p-nitrophenyl-derivatives) and enzyme solution (0.1 ml). The incubation time at +30°C was 16 to 20 hours. The reactions were stopped by adding 1.0 ml of 0.2 M NaOH solution after placing the tubes in an iced water bath. The colour intensity was read at 410 nm on a Hitachi Perkin-Elmer

UV-VIS Spectrophotometer, Model 139. The substrates used were p-nitrophenyl-ß-D-glucuronide, p-nitrophenyl-ß-D-galactopyranoside, p-nitrophenyl-N-acetyl-ß-D-glucosaminide and p-nitrophenylphosphate. In case of alkaline and acid phosphatase the buffers used were 0.05 M glycine-NaOH, pH 9.7 and 0.05 M ßß-dimethylglutarate buffer, pH 4.5, respectively. The enzyme activities were expressed in terms of µmoles substrate hydrolyzed/min x bone.

The possible adsorption of the enzymes to hydroxyapatite and bones was studied as follows: 50 mg of synthetic hydroxyapatite was suspended in 3.0 ml of medium containing various lysosomal hydrolases derived from bones. After shaking for 5 min and centrifugation (24000 xg, 10 min), the sediment was washed two times with distilled water and treated with 3.0 ml of 0.5 M phosphate buffer, pH 7.0, to demonstrate possible desorption. Enzyme assays were performed on the medium before and after treatment with apatite and on the phosphate buffer. For bones the procedure was the same except that 2 to 6 bones were added to the medium instead of hydroxyapatite.

The protein content was analysed according to Lowry et al. (11) using bovine serum albumin as a standard.

Bone resorption assay with ^{45}Ca

Pregnant rats were injected with 330 µCi of $^{45}CaCl_2$ two days prior to preparation of radii and ulnae. ^{45}Ca release to the medium was measured by the aid of an LKB 1210 Ultrobeta Liquid Scintillation counter. The efficiency of the method was 90 %. In these experiments the medium was supplemented with 5 % heat-inactivated fetal calf serum and bovine serum albumin fraction V (1 mg/ml). The results are expressed as the treated/control ratio with ratios greater than 1.0 representing increased resorption.

Chemicals

BGJb medium was a product of Gibco Biocult, Paisley, U.K., parathyroid hormone of Calbiochem, San Diego, Ca, USA and ^{45}Calcium chloride, 1.47 mCi/ml, of the Radiochemical Centre, Amersham, England. p-Nitrophenyl substrates were products of Sigma Chemical Company (St. Louis, MO, USA) and streptomycin and penicillin were purchased from Hoechst AG (Frankfurt, FRG). Synthetic hydroxyapatite was prepared under CO_2-free conditions (5). All chemicals were of highest available purity and, unless otherwise mentioned, obtained from E. Merck AG (Darmstadt, FRG). The water used was distilled and deionised with specific resistance of approximately 1ΩM – cm.

RESULTS

In a total of 14 experiments the activities of acid phosphatase and ß-glucuronidase increased as a function of time in plaque-treated culture medium (Fig 1). Plaque induced an immediate increase of acid phosphatase activity in culture medium during the first 24 hours, and another increase occurred after 48 hours. ß-Glucuronidase was also found to increase remarkably in culture medium after 24 hours in the presence of plaque. The activities of ß-galactosidase and N-acetylglucosaminidase were 50–80 % lower than those of the other enzymes tested. However, their activities changed in a manner similar to that of ß-glucuronidase. The release of lysosomal enzymes increased slightly when the plaque concentration was increased; the plaque concentrations in various exsperiments

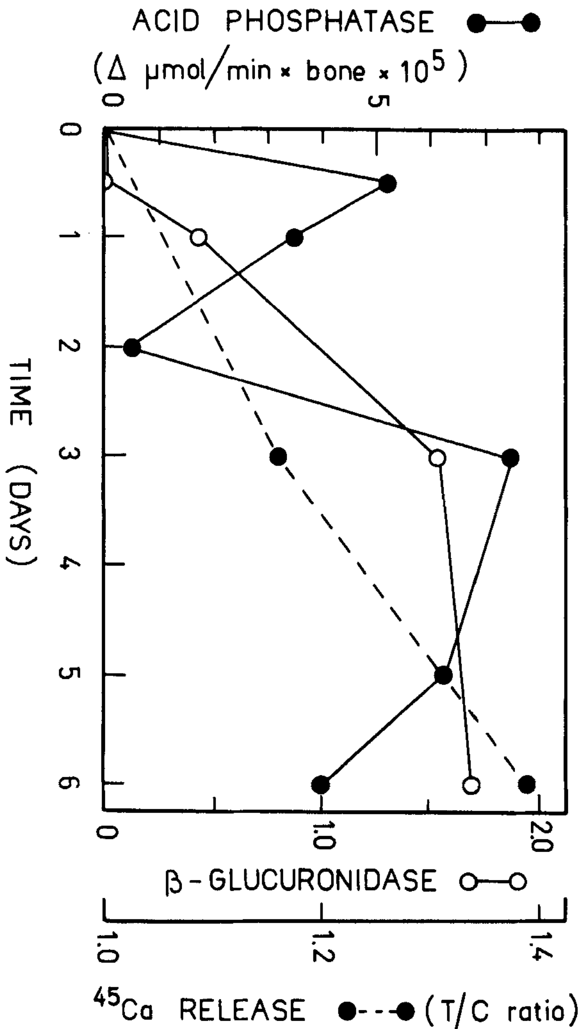

Fig. 1. The effect of dental plaque (protein concentration 30 µg/ml) on the activities of acid phosphatase (• – •) and ß-glucuronidase (◦ – ◦) in bone culture medium as a function of time. The curves represent activity changes (Δµmol/min x bone x 10^5) in the test culture compared to the control culture, which contained saline instead of plaque (n = 14). ^{45}Ca release is expressed as a treated/control (T/C) ratio. Because it was impossible to get bones of the same age and identical plaque in all the cultivations, no means and standard deviations could be calculated.

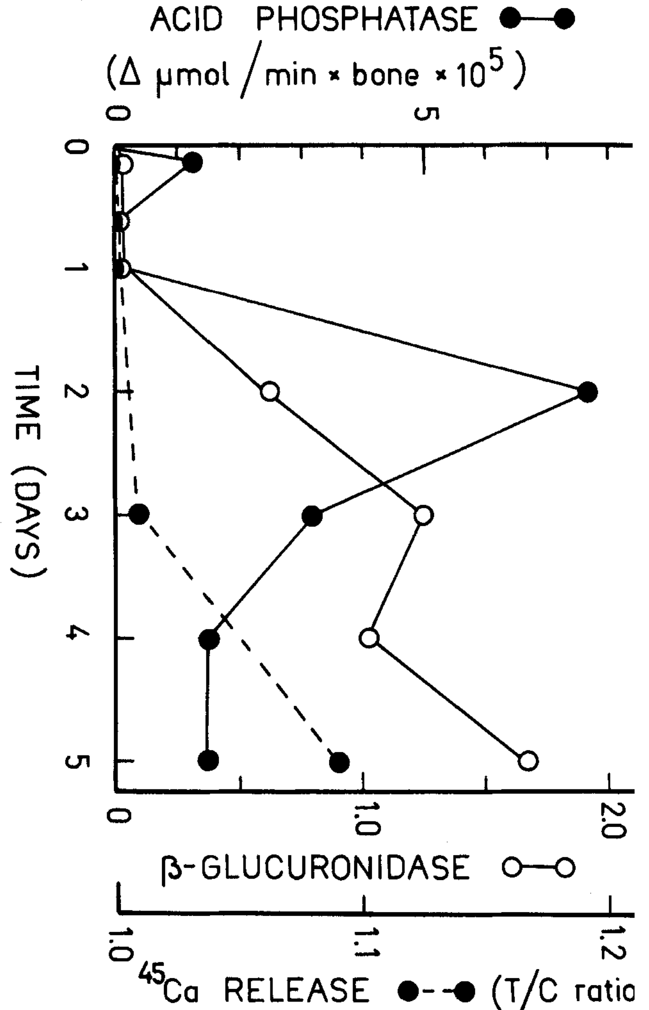

Fig. 2. The effect of parathyroid hormone (0.86 µg/ml) on the activities of ß-glucuronidase (◦ – ◦) and acid phosphatase (• – •) in bone culture medium as a function of time (n = 3). The activities are expressed as changes in the test culture compared to the control culture (saline instead of plaque). ^{45}Ca release is expressed as a treated/control (T/C) ratio.

ranged from 12 to 237 µg protein/ml. Parathyroid hormone extract (0.86 µg/ml) caused enzymatic changes in bone culture medium similar to dental plaque (Fig. 2).

The enzymatic activites were generally much higher (as much as 10^3 times) in Triton X-100 treated and homogenized bones. It was therefore considered more appropriate to evaluate the relatively small changes in activity from measurements of the culture medium. With time, the activities in bones tended to decrease. The saline solutions where the bones

Table 1. *Decrease in enzyme activity derived from dental plaque and incubated for 18 hours in culture medium (without bones) at +30°C. The reaction mixture contained 3.0 ml of medium and 100 µl of filtered plaque extract (the protein concentration in the reaction mixture was 61 µg/ml)*

Enzyme	Decrease in activity (Δµmole/min x ml x 10^4)	Decrease–%
ß-glucuronidase	0.0	0
ß-galactosidase	0.0	0
N-acetyl-ß-D-glucosaminidase	0.2	34
acid phosphatase	1.8	37
alkaline phosphatase	5.9	46

Table 2. *Adsorption and desorption of acid and alkaline phosphatase and ß-glucoronidase by hydroxyapatite and by fetal rat bones incubated in culture medium at +2°C*

	Medium +OH-apatite (pH 8.1)		Medium +bones (pH 8.3)	
	% adsorption	desorption*	% adsorption	desorption*
ß-glucuronidase	58	81	9	50
acid phosphatase	41	0	1	0
alkaline phosphatase	96	4	0	0

*per cent of the adsorbed activity liberated by 0.5 M phosphate buffer, pH 7.0

were stored had very low activities indicating no noticeable loss of enzyme.

The enzyme activities from bones were quite variable during the preincubation period. This was considered to result from adaptation of bones to the experimental conditions. Incubation of plaque and medium (without bones) caused a noticeable decrease in the activities of acid and alkaline phosphatase and N-acetylglucosaminidase (Table 1). The method of calculating the amount of enzyme released from the bones has been described under methods in this paper.

No remarkable changes were found in the activity of alkaline phosphatase in any of the cultivations. The activity tended to increase somewhat in the medium during prolonged incubation, but there were no significant differences between the test and the control.

Slight differences were found in the adsorption of various enzymes to hydroxyapatite (Table 2). The adsorption by apatite was remarkable but the ability of fetal rat bones to adsorb the same enzymes under identical conditions was very low.

Determination of ^{45}Ca from culture medium confirmed that with the increase of hydrolase activities, higher amounts of inorganic material were at the same time dissolved into the medium (Figs. 1 and 2).

DISCUSSION

Although lysosomal hydrolases have been widely used as indicators of bone resorption, no detailed experiments concerning their suitability have been made. The present results show acid phosphatase and ß-glucuronidase to be suitable indicators, since these activities are high and constant enough even during prolonged incubation periods. Of all the lysosomal enzymes, acid phosphatase has been most widely studied and although

present in all bone cells it is particularly active in osteoclasts, which are the main cells involved in bone resorption. Measurements indicating bone resorption can be carried out after 1–2 days of cultivation, the time needed for sufficient lysosomal activity to be liberated from the bone cells. The results are well in accordance with those presented by Vaes (18), Eilon & Raisz (3) and Lenaers-Claeys & Vaes (10), who have used parathyroid hormone extract as an agent to induce resorption. Acid phosphatase is released somewhat faster than ß-glucuronidase under the influence of parathyroid hormone (17). The same phenomenon is seen in the present study both with dental plaque and parathyroid hormone.

Because many proteins, e.g. enzymes, are adsorbed by hydroxyapatite (8) it can be expected that enzymes present or liberated into culture medium are also immediately adsorbed by bone inorganic material. The present results show that the enzymes studied were indeed effectively adsorbed to synthetic hydroxyapatite, but the adsorption to fetal rat bones was very low and did not affect the results. The difference can result e.g. from a low mineral content of 19 to 21 days old rat bones and/or from a smaller surface area of bones compared to apatite powder. During adsorption the enzymes may gradually loose their activities. This can subsequently affect the phosphate-liberated activities.

Alkaline phosphatase served as an anabolic reference. The slight increase during incubation may indicate that proliferative processes occurred because the activity of alkaline phosphatase reflects the activity of calcification and the rate of the formation of organic matrix in mineralizing tissues (4). Moreover Vaes (18) did not find any effect of parathyroid hormone on alkaline phosphatase activity in bone cultures.

The role of lysosomal hydrolases in bone resorption has largely been studied by Vaes (15–20). Vaes has shown that bone resorption occurring in tissue culture due to the action of parathyroid hormone or other agents is accompanied by an increased synthesis and excretion of lysosomal acid hydrolases. Vaes has suggested that these enzymes, together with acids (particularly lactic and citric acid) which are simultaneously released by the explants, play an active role in the destruction of the bone matrix in the resorption lacunae. Calcitonin inhibits both the resorption process and the excretion of lysosomal hydrolases thus supporting this hypothesis (20). Eilon & Raisz (3) suggested that lysosomal enzymes may have a primary role in initiating resorption perhaps by acting on non-collagenous matrix or tissue components before mineral removal and collagen degradation. The present results also suggest that the increase in lysosomal enzyme release occurred earlier than the increase in ^{45}Ca release.

Lysosomal hydrolases of dental plaque may also have a function in bone resorption, e.g. by acting as soluble factors destroying alveolar bone through non-cellular mechanisms. This could result in dissolution of bone mineral and hydrolysis of organic matrix (6). However, the process of alveolar bone loss in human periodontal disease is multifactorial. Hydrolysis by plaque components is not necessarily the most important one. In fact, soluble extracts of plaque from adults with periodontitis stimulate bone resorption in tissue culture by a mechanism associated with the formation of osteoclasts (7). The present results show that lysosomal hydrolases may be involved in this process.

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