A Bone Resorptive Agent Extracted from Orthodontically-Treated Tissues of the Rat

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A bone resorptive agent extracted from the tissues surrounding orthodontically-treated teeth is purified using ultrafiltration and isoelectric focusing. A two-fold increase in release of ^{45}Ca could be achieved with as little as 0.6 μg/ml of this material.

KEY WORDS: • ELECTROPHORESIS • RESORPTION •

An extractable bone resorptive agent has been previously demonstrated in the tissues surrounding orthodontically-treated teeth (KING AND THIEMS 1979, KING AND FISCHLSCHWEIGER 1982). Peaks have been found during that part of the orthodontic cycle when tooth movement is delayed due to tissue damage from "hyalinization" (KING AND THIEMS 1979), and this correlates well with the magnitude of force applied and the total area of cemental cratering (KING AND FISCHLSCHWEIGER 1982).

The purpose of the present study is to partially purify the molecules containing this bone resorptive factor and to perform preliminary characterizations, including molecular weight, isoelectric pH, and presence of subunits.

— Material and Methods —

Appliances: A mesial tipping orthodontic force averaging 60gm was applied to the maxillary first molars of 300gm male Sprague-Dawley rats for seven days, using an appliance previously described by HELLER AND NANDA (1979).

Bone Resorption Assay: The assay for bone resorptive activity was based on the release of preincorporated ^{45}Ca from cultured fetal radii and ulnae. Briefly, the percentage of ^{45}Ca released from pairs of cultured fetal bones can be compared by calculating a series of treated/control ratios. Resorptive activity is indicated by a ratio higher than a treated/control ratio of 1.00 by a statistically significant

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amount. Alternatively, release data as percentages from several treatments can be compared using analysis of variance and the unpaired Student's t-test.

In these experiments, all samples were tested in the bone resorption assay at various doses as determined by protein concentration using an albumin standard curve (LOWRY AND ROSEBROUGH 1951).

Sample Preparation: Orthodontically-treated tissues were dissected as previously described (KING AND FISCHLSCHWEIGER 1982).

For the first series of experiments, designed to test the relative bone resorption activity of 100,000D filtrates and retentates, the following procedures were performed. The dissected tissues were first homogenized at 0°C in 6M quanidine-HCl. These were then extracted for one hour at 0°C in the same buffer and cleared by centrifugation. This extract was diafiltered against five volumes of phosphate-buffered saline (pH 7.4) at 4°C using an Amicon YM-100 membrane. Both retentate and filtrate were cleared, dialyzed against double-distilled H_20, and lyophilized.

Similar preparations were made from treated and untreated animals. These latter extracts were chosen as controls because previous studies had indicated that cultured fetal bones treated with like doses of lyophilized extracts from untreated animals and those treated with unactivated appliances showed no difference in ^{45}Ca release (KING AND FISCHLSCHWEIGER 1982).

Since subsequent experiments clearly demonstrated substantial bone resorptive activity in extracts from treated tissues using cold phosphate-buffered saline (PBS) at pH 7.4, further purification studies using the UM-100 retentate were performed exclusively on preparations obtained from this less harsh procedure. However, PBS extraction did not yield a sufficient quantity of YM-100 filtrate from untreated animals, necessitating the continued use of 6M quanidine-HCl for the first series of experiments.

Biologically active retentate was next mixed with a continuous sucrose density gradient containing ampholine at pH 5.0-8.0. Preparative isoelectric focusing was carried out using an LKB electrofocusing column until current was minimal. The column was then fractionated, collecting the aliquots of 1.5ml.

The pH and protein concentrations (LOWRY AND ROSEBROUGH 1951) of alternate fractions were measured. Peaks were then pooled into four distinct fractions which were also stored in liquid nitrogen for use in the bone resorption assay. Medium controls were considered appropriate for this comparison, as no statistical differences in ^{45}Ca release could be demonstrated between medium and extracts prepared from the YM-100 retentate of sham-treated tissues (dosage=3μg/ml; T/C=1.08; SEM=0.09; n=7).

Electrophoresis: Polyacrylamide gradient gel electrophoresis (PGGE) was performed on a vertical slab apparatus using 5-25% acrylamide in the presence of sodium dodecylsulfate (SDS). Using appropriate standards, approximate molecular weight for each of the bands in the fraction demonstrating bone resorptive activity were calculated.

Similar gels were also run on material which had been pretreated with 5% beta-mercaptoethanol in 2% SDS buffer (62.5 5mM Tris; 10% glycerol; pH 6.8) at 100°C for two minutes. Gels were fixed in 10% trichloracetic acid, stained with Coomassie Brilliant Blue R-250 and destained in a solution of ethanol and acetic acid.

— Results —

The YM-100 retentate demonstrated significant bone resorptive activity when compared to equal concentrations of similarly prepared samples from sham-treated animals, indicating that it is either a large molecule or complexed in the crude state. The YM-100 filtrate demonstrated a dose-dependent inhibition of bone resorption when a similar comparison was made between orthodontically-treated and sham-treated animals (Fig. 1).

The elution profile from the isoelectric focusing column showed five major peaks across the pH gradient (Fig. 2). This profile was divided into four fractions for further testing. Because of limited sample in the pH range 5.5-7.0, these peaks were pooled. The isoelectric focusing data indicated that fraction II contained at least two major components (Fig. 2). This was confirmed by SDS-PGGE of Fraction II (Fig. 3).

Table 1 presents a comparison of the bone resorptive activities found in the various fractions at two different doses. The data clearly indicate that there is a significant bone resorptive factor present in Fraction II at both doses. Fraction III also demonstrated some activity which was substantially less than fraction II, but a statistically significant difference from medium controls could not be shown. Fractions I and IV had no detectable bone resorptive factor.

Figure 4 presents dose-response data comparing doses of Fraction II and release of preincorporated ^{45}Ca from cultured fetal bones. A two-fold stimulation of ^{45}CA release as compared to medium alone could at first be achieved at 0.6 μg/ml, followed by a plateauing of the effect toward 3 μg/ml, where it became constant until at least 6 μg/ml.

Polyacrylamide gradient gels in sodium dodecylsulfate are shown in Fig. 3. Note that fraction II has two bands. The molecular weights of these were determined to be 234,000D and 263,000D by plotting electrophoretic mobility as a function of the logarithm of molecular weight, utilizing the standards shown on the left. PGGE gels of 2,mercaptoethanol-treated fraction II and similar standards are demonstrated in Fig. 5, indicating one major band with a molecule weight of 67,000D.

— Discussion —

These data clearly establish that a large molecule, either 234,000D or 263,000D, which has the ability to stimulate bone resorption in organ culture in a dosage-dependent fashion, can be extracted and purified from orthodontically-treated tissues. Previous studies using this model demonstrate that the amount of bone resorptive activity correlates well within the time when greatest tissue damage occurs in the tooth movement cycle, as assessed by cemental cratering (King and Fischlschweiger 1982).

It would be reasonable to expect increased tissue protease activity in these tissues (Morland and Kaplan 1977). It may also be reasonable to suggest that these enzymes have the ability to enhance bone resorption in culture, as they have been clearly established to be present in culture media where bone resorption has been stimulated (Vaes 1968).

Of the known mediators of bone resorption, the various systemic factors including parathyroid hormone, metabolites of vitamin D, thyroid hormone, and epidermal growth factor, can probably be eliminated as possible sources of the activity in this context because of molecular size. However, some of the bone resorptive agents found in serum (Stern and Raisz 1967, Stern and Miller 1978) could conceivably be at work in the orthodontic context if one hypothesizes a mechanism whereby hemostasis and

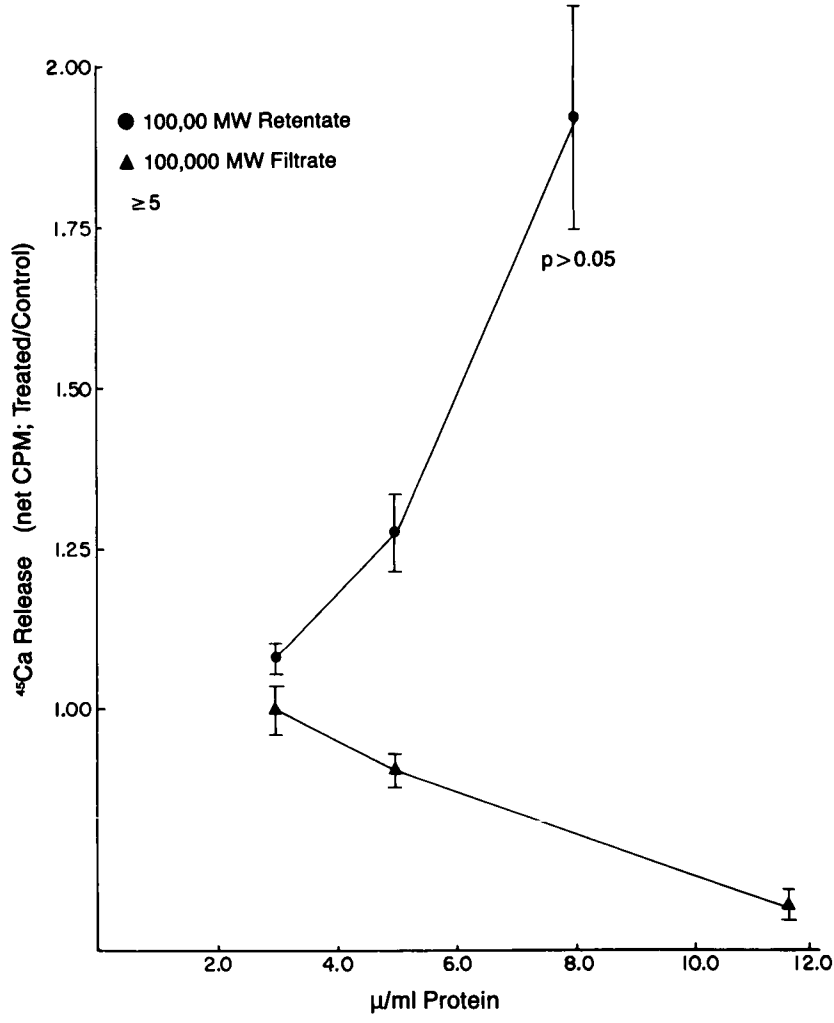

Fig. 1 ^{45}Ca release from cultured fetal bones treated with various doses of YM100 retentate and filtrate. Treated wells were dosed with the indicated concentration of extract from orthodontically-treated animals. Doses were based on protein concentration compared to an albumin standard curve. Control cultures contained comparable concentrations of extract from untreated animals. Percent ^{45}Ca released into the media were determined, and treated/control was calculated for each matched culture pair. Each point represents data from at least five culture pairs, with the vertical bars representing the standard error of the mean. The highest dose in each curve is statistically significant from T/C = 1.00 ($p < .05$).

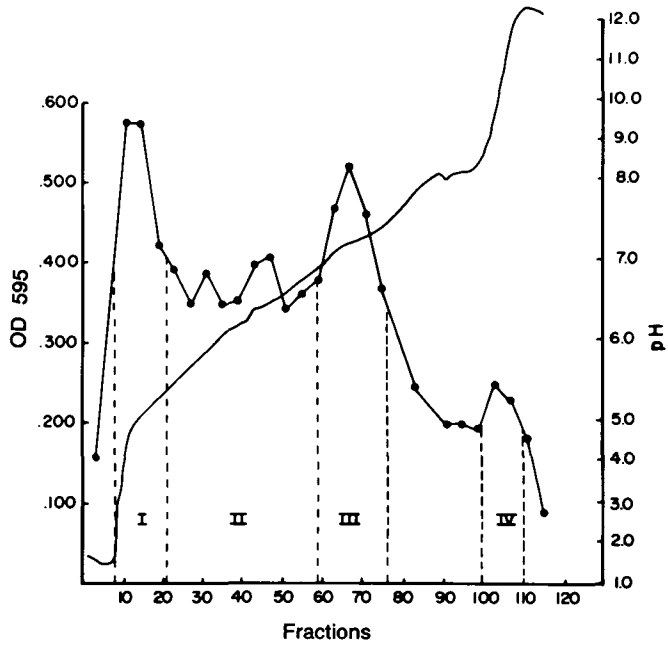

Fig. 2 Elution profile from isoelectric focusing column of YM100 retentate. The solid line represents the pH gradient and the dotted line is the OD 595 reading of alternate 1.5ml fractions.

extravasation result in increases of serum in orthodontically-treated tissues.

The various mediators of localized bone resorption, including prostaglandins (KLEIN AND RAISZ 1970) and osteoclast activating factor (RAISZ, LUBEN, MUNDY, HORTON AND TRUMMEL 1975) show some promise as mediators of orthodontic bone resorption.

Obviously, prostaglandins are much too small to be directly involved in this activity, but the suggestion has been made that several substances can stimulate bone resorption by a prostaglandin-activating mechanism (RAISZ ET AL. 1974 AND TASHJIAN ET AL. 1978). This mechanism also shows promise in light of histologic data demonstrating that osteoclast numbers vary in orthodontically-treated tissues co-treated with various drugs known to enhance or inhibit the biochemical chain of events in prostaglandin activation (YAMASAKI ET AL. 1980 AND YAMASAKI 1983). Inhibition studies are underway in our laboratories to determine whether the activity reported here is similarly affected.

The lymphokine osteoclast activating factor can probably be safely eliminated, as it has a molecular weight reported to be between 25,000D and 12,5000D (MUNDY AND RAISZ 1977). Also, it should be

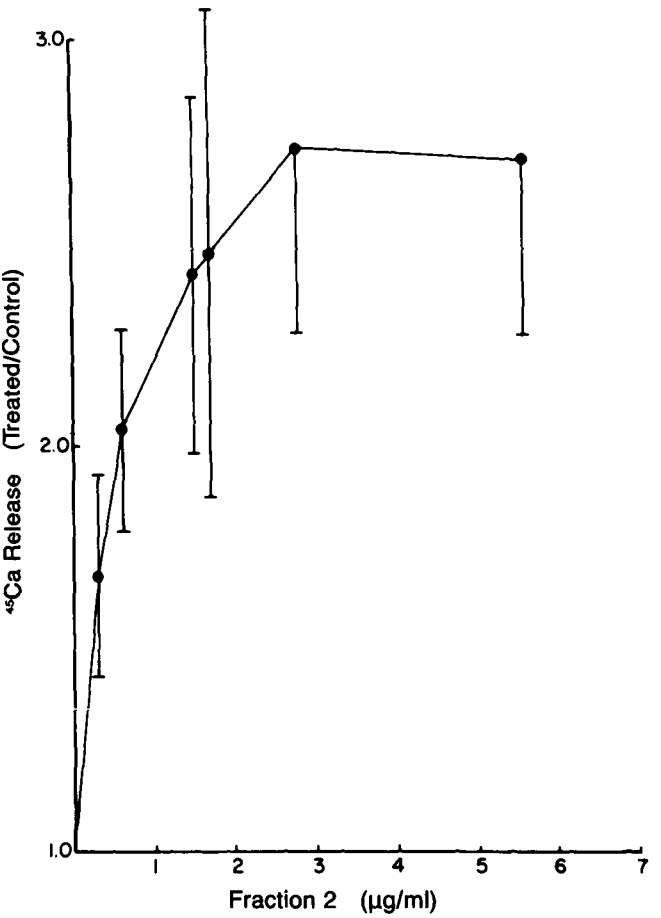

Fig. 3 SDS-Polyacrylamide gradient gels demonstrating the presence of two bands in fraction II on the right. Molecular weights of appropriate standards appear on the left. Gels were stained with Coomassie Brilliant Blue.

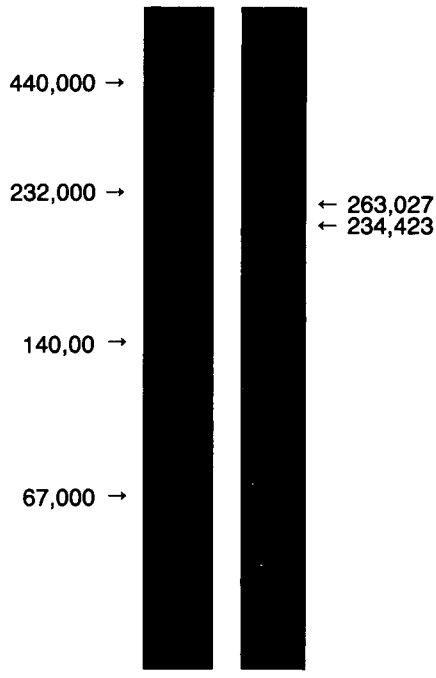

Fig. 4 **^{45}Ca release from cultured fetal bones treated with various doses of fraction II. The control bones in each pair were in medium, and each point represents at least five culture pairs with the vertical bars representing the standard error of the mean. All points representing concentrations equal to or greater than 0.6μg/ml were significantly greater than 1.00 ($p < .05$).**

noted that early infiltration of lymphocytes in tooth movement is not characteristic (REITAN 1975).

Endotoxin, another stimulator of localized bone resorption (HAUSMANN AND RAISZ 1970), could be disregarded in this context because there are few reasonable mechanisms which could be postulated where endotoxin would be regularly found in orthodontically-treated tissues.

Alterations in ion concentrations (calcium, magnesium, phosphates and hydrogen) have been implicated in bone resorption, and these also may play an indirect role in the mechanism described here (RAISZ AND NIEMANN, 1969, MINKIN AND JENNINGS 1972). However, a sole and direct role in this context would be unlikely, as these ions would be easily dialyzed away from the sample unless bound to a larger molecule.

If our active agent were functioning as a chelator or other molecule with a high likelihood of retaining charge, this would probably be reflected in a very acidic isoelectric pH. Since the bone resorptive potential is localized in a fraction with a pH range between 5.5 and 7.0, it may be possible that the molecule is causing bone resorption by significantly lowering the

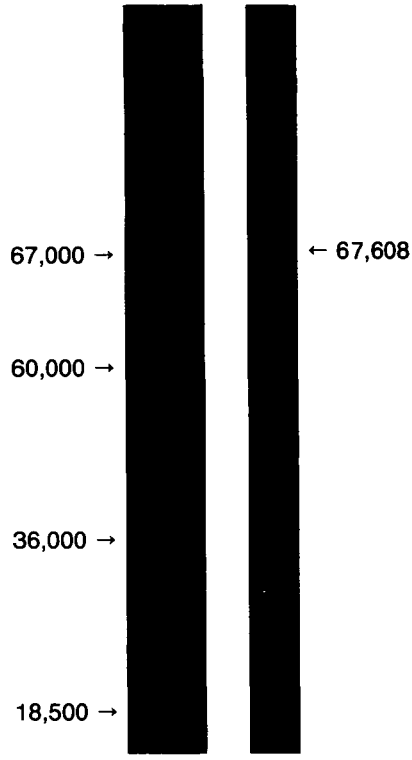

Fig. 5 SDS-polyacrylamide gradient gels of 2-mercaptoethanol treated standards (left) and fraction II (right). Gels were stained with Coomassie Brilliant Blue.

pH in the microenvironment (DOMINQUEZ AND RAISZ, IN PRESS) or by chelation of calcium ions. However, if such a mechanism were active, one might reasonably expect to find the most acidic fraction (fraction I) to demonstrate the greatest bone resorptive activity, and this is not supported by the data (Table 1).

An appealing line of reasoning explaining this bone resorptive mediator in tooth movement holds a role for the infiltration of macrophages to the sites of greatest pressure in the periodontal ligament. Such infiltration has been clearly demonstrated histologically (REITAN 1975). Moreover, activation of macrophages results in a series of specialized responses, some of which are central to bone resorption. These include enhanced lysosomal enzyme activity (MORLAND AND KAPLAN 1977), production of neutral proteinases including collagenase (WAHL ET AL. 1974), and elastase (WEBB AND GORDON 1975) and prostaglandins (HUMES ET AL. 1977).

Indeed, collagenase has been implicated in orthodontic tooth movement (OSAKI ET AL. 1971), and a collagenase inhibitor has been shown to inhibit bone resorption (HORTON ET AL. 1978). The latter may be similar to the dose-dependent

Table 1

Comparison of ^{45}Ca from Fractions Eluted from Isoelectric Focusing Column

Expt	Medium Control	Test Conc. (μg/ml)	Test Fractions I	II	III	IV
1	28.0 (4.6)	2.0	–	42.0 (4.4)	33.7 (3.2)	27.8 (5.4)
2	24.2 (1.6)	5.0	29.8 (3.1)	–	–	–
3	22.5 (3.2)	10.0	17.3 (2.4)	–	–	–
4	24.2 (1.8)	20.0		37.5 (2.2)	39.4 (14.8)	22.5 (2.0)

p<.05 p<.01

Each experiment consisted of at least four cultures per group.
Numbers in parentheses are standard errors of the mean.
All cultures were for five days.

inhibition seen in the filtrate of the extracts of orthodontically-treated tissues.

Future studies should be designed to clarify this resorptive mechanism, with particular attention to the macrophage chemotaxis to orthodontically-treated tissues and the role that it plays in this process. Localization of the bone resorptive factor in these tissues as a function of time, and studies on the inhibition seen in the ultrafiltrate, could also prove valuable.

This investigation was supported by USPHS Research Grant DE-04844 from the National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20205.

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