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COLLAGENOLYTIC ACTIVITY DURING TOOTH MOVEMENT IN RABBITS

BY

Takemasa OZAKI*1

ABSTRACT

An attempt was made to investigate the effect of mechanical force on the collagenolytic activity of the periodontal membrane of rabbits during experimental tooth movement, by means of sterile incubation of the excised tissues on the reconstituted collagen gel plate *in vitro*.

An orthodontic elastic band was applied between lower incisor and first molar so as to exert about 100 g reciprocal force upon the intermediate tissues for 1, 3, 5, 7 and 21 day periods respectively.

It was demonstrated that the periodontal membrane of first molar subjected to compressive force had the ability of collagenolysis, and that the appearance of collagenolytic activity was dependent upon the duration of force application.

The results suggest that the incidence of collagenolytic activity may be closely related to the deposition of fresh granulation tissue accompanied by vascularity and osteoclasts.

INTRODUCTION

It is well recognized that orthodontic tooth movement causes bone resorption on the side of pressure and bone apposition on the side of tension. In the course of tooth movement, it is also associated with disappearance and reconstruction of the periodontal ligaments.

When the tissues are in a steady state, tissue remodeling requires that biosynthesis and degradation of structural elements should be precisely synchronized in time and space. In this sense, a series of periodontal tissue alteration incident to tooth movement can be considered as an accelerated remodeling process caused by the application of mechanical force.

Recently, utilizing the autoradiographic method, Crumeley[1] reported that the formation of collagen decreased initially in stressed periodontium but was followed by an elevation above the control level. Baumrind *et al.*[2] also reported that the rate of cell replication and general metabolic activity increased while the rate of collagen synthesis decreased in both pressure

*1 尾崎武正: Department of Orthodontics (Chief: Prof. F. MIURA), School of Dentistry, Tokyo Medical and Dental University (Tokyo Ika Shika Daigaku).
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and tension regions.

On the other hand, from the viewpoint of collagen breakdown, the changes in supporting tissues affected by orthodontic force, namely hyalinization and disappearance of the periodontal ligament accompanying alveolar bone resorption, can be regarded as tissue collagen degradation, since the major organic constituent of periodontal tissues is known to be collagen. These processes must require enzymatic breakdown of the tissue collagen by specific proteolytic enzymes.

Recently, a tissue culturing method has been devised to allow the detection and measurement of tissue collagenolytic activity in tissue culture fluid of metamorphosing tadpoles *in vitro* by Gross and Lapiere[3]. Subsequently, similar kinds of activity of collagenolytic enzymes have been observed in the tissue culture fluids of human skin, human and goat bone, human gingiva, postpartum uterus, healing wound and the granulation tissues during root resorption of bovine deciduous tooth (Eisen *et al.*[4], Fullmer and Lazarus[5], Fullmer *et al.*[6], Morrione *et al.*[7], Grillo and Gross[8], Bennick and Hunt[9], Morita *et al.*[10]).

The present study was undertaken in an attempt to provide some information with regard to the effect of mechanical force on the collagenolytic activity of periodontal membrane in rabbits. X-ray examination was performed to record the features of tooth movement, and histological examination was also undertaken for the interpretation of the relationship between the collagenolytic activity and histological changes during tooth movement with time lapse experiments.

Materials and Methods

Animals: Fifty-seven male adult laboratory rabbits weighing approximately 2.7–2.9 kg were used.

Application of mechanical force: The animals were anaesthetized by intravenous injection of 1% Nembutal (50 mg per kg of body weight). An orthodontic elastic band of 0.25 inch inside diameter (Heavy 1/4, Unitek Co. Ltd.) was applied unilaterally between lower incisor and first molar so as to exert about 100 g reciprocal force upon the intermediate tissues (Fig. 1). The molar on the opposite side was used as a control.

Assay of collagenolytic activity: The detection and estimation of collagenolytic activity was accomplished by sterile incubation on the reconstituted collagen gel in Tyrode medium enriched with amino-acids, vitamines and antibiotics according to the method of Gross and Lapiere[3].

After induction of tooth movement for periods of 1, 3, 5, 7 and 21

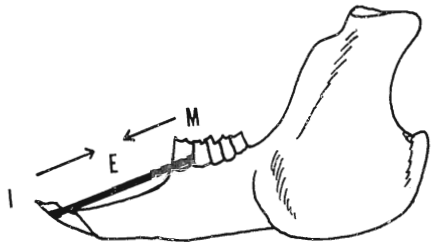

Fig. 1. Diagram of elastic band applied between lower incisor and first molar, so as to exert about 100 g reciprocal force between them. I: lower incisor, M: lower first molar, E: elastic band.

days, the animals were sacrificed. Mandibles were dissected immediately, and muscles and connective tissues around the crest area were exposed carefully from medial and distal sides of first molars. They were then cut to approximately 2.0×2.5 mm^2 pieces at 1 mm below the crest of the alveolar bone in order to eliminate the inclusion of gingival epithelium and to avoid the influence of inflammatory gingival disturvances. The speciments were placed on the reconstituted collagen gel plates prepared in 10 ml glass centrifuge tubes. Finally, 50 μl Tyrode medium was added to each.

The acid-extracted rat skin collagen used to make the reconstituted collagen gel was prepared by the method of Glimcher *et al.*[11]. Aliquot samples of freeze-dried collagens were dissolved as 0.1–0.2% solution in sodium phosphate buffer (pH 7.6) at 2°C and clarified by high centrifugation at 15000 g for 1 hour. The rigid opalescent reconstituted collagen gel was composed of 1 to 3 ratio of rat skin collagen solution and mammalian Tyrode medium. The mammalian Tyrode medium for collagen gel was composed of 92.5 ml of mammalian Tyrode solution, 1.5 ml each of 100 times concentrated Vitamine mixture, 100 times concentrated amino-acid mixture, and 200 mM L-glutamine and 3 ml of antibiotics containing 100 units each of penicillin, streptomycin and mycostatin. The collagen gel thus prepared was tested by bovine gingival epithelium which had demonstrated lysis of collagen gel.

Cultures were incubated at 37°C for 5 days in a moist atmosphere containing 95% O_2 and 5% CO_2 and were observed daily for lytic activity. Photographs were taken daily during incubation from a direction of the bottom of glass centrifuge tubes. Visible lysis was recorded by means of scores ranging from 1+ to 3+: 1+ indicating minimal lysis and 3+ indicating complete lysis of the collagen gel (Fig. 2). The score of 3+ was evaluated as "lysis +". Simultaneously, repeatedly frozen and thawed specimens were cultured under same circumstances for testing the necessity of tissue

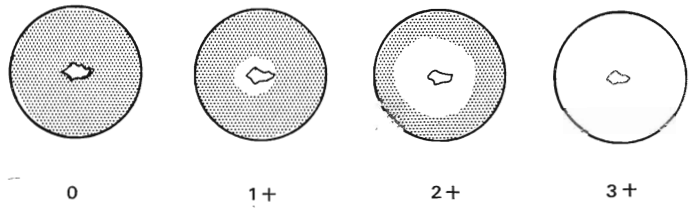

Fig. 2. Diagram of score of visible lysis. O indicates a gel unaffected by the explant on the gel and 3+ indicates complete lysis.

viability for the appearance of collagenolytic activity.

At the end of incubation period, all of the tissue fragments of periodontal tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Serial sections of 5 μ in thickness were made and stained with haematoxylin-eosin and Azan-Mallory staining for subsequent histological evaluation and interpretation.

X-ray and histological examination: Two animals each in the experimental group were sacrificed at time intervals of 1, 3, 5, 7, 9, 14, 21, 35 and 42 days after the application of elastic bands. Each mandible was removed immediately after sacrifice; cut into two pieces at the symphysis and fixed in 10% neutral buffered formalin. Prior to decalcification, each half of the mandible was placed on Soft X-ray film and exposed to 27 kV X-ray for 10 seconds (Softex J Type, Nihon Softex Co. Ltd., Japan). After taking the X-ray photographs, the specimens were decalcified in 5% formic acid, dehydrated, and embedded in paraffin. They were then serially sectioned parasagittally at a thickness of 6μ. All sections were stained with haematoxylin and eosin.

Results

Findings of tooth movement:

Fig. 3 illustrates the representative lateral roentgenograms at varying intervals after induction of tooth movement.

Macroscopic observation based on these roentgenograms revealed that, by the application of elastic force, the first molar showed medial tipping movement with the fulcrum near the root apex.

The gross amount of tooth movement was approximately 0.7 mm after 7 days, 1.5 mm after 21 days and one tooth width (3.0 mm) after 42 days based on the increase of the interdental space between first and second molars.

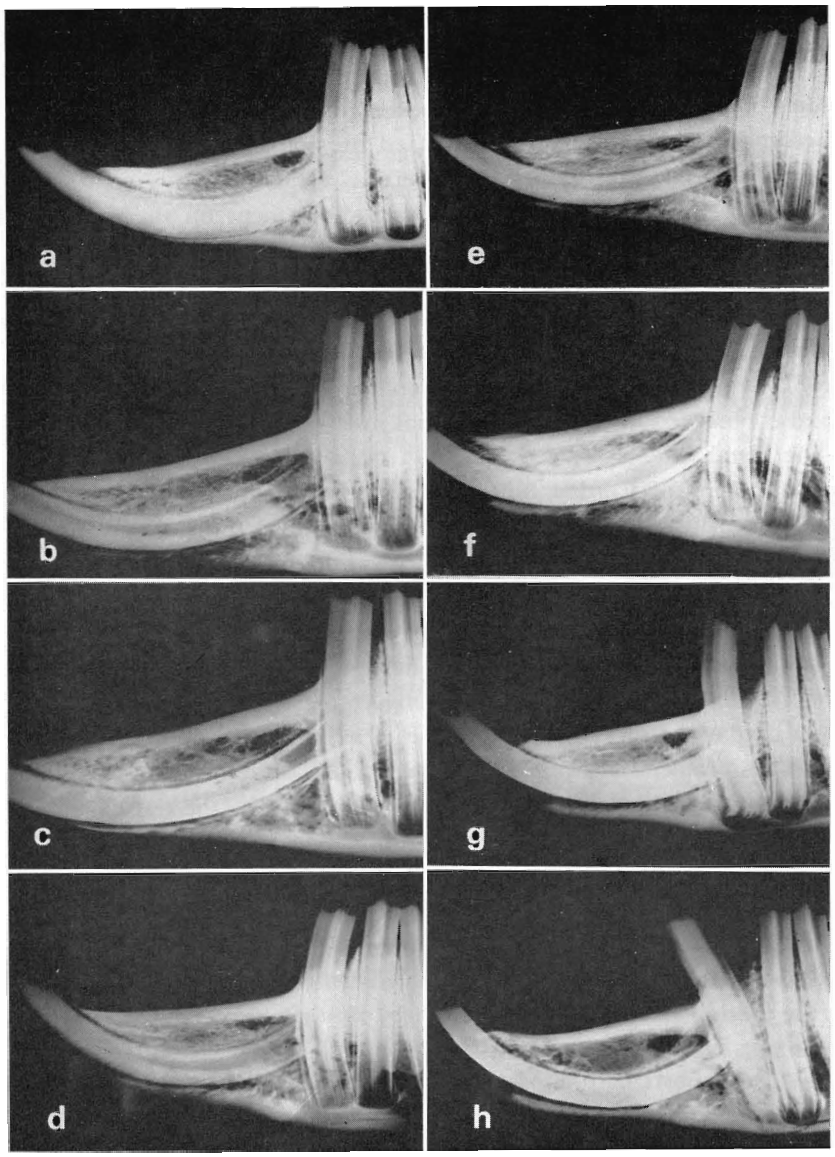

Fig. 3. Lateral roentgenograms of the mandibles of rabbits after induction of tooth movement. a; control, b; after 1 day, c; after 3 days, d; after 5 days, e; after 7 days, f; after 14 days, g; after 21 days, h; after 42 days.

Histological findings:

The histological observations of the periodontal tissue changes around the first molar in response to the applied force in the present study were in accordance with the findings reported previously by many other investigators. In brief, the main points were as follows:

At the end of 1st day, the periodontal ligament on the pressure side was compressed to half of its original width against the alveolar bone (Fig. 4, 5). The nuclei of fibroblasts became pyknotic and the cellularity of the periodontal membrane decreased. On the tension side, the periodontal ligaments around the crest area were stretched and the nuclei of fibroblasts were elongated. Infiltration by a small number of round cells was also observed around the marginal area of the periodontal membrane on the distal side.

At the end of 3rd day, on the pressure side, the periodontal membrane was reduced to one third of its original width and transformed into a disorganized mass of tissue devoid of cell structure (Fig. 6). Many osteoclasts had appeared already and direct bone resorption was observed just below these hyalinized tissues.

At the end of 7th day, the undermining bone resorption was pronounced and the proliferation of young connective tissue accompanied by new vascularity were evident in the proximity of the resorption area. Numerous osteoclasts lining along the surface of resorption front as well as in bone marrow spaces were observed (Fig. 7). On the tension side, new bone apposition was found in addition to the formation of new capillaries.

At the end of 21st day, the histological picture was essencially similar to that of 7th day except that bone resorption on the pressure side and apposition on the tension side was proportionally more extensive. By this time, one fifth of the alveolar bone had been resorved from the crest area. On the tension side, appositional changes of new bone were correspondingly prominent.

Collagenolytic activity:

The frequency of the appearance of collagenolytic activity in the tissue fragments of periodontal membrane obtained from both experimental animals and control animals during tooth movement is shown in Table 1.

Collagen gel, incubated with the tissue fragments of normal periodontal membrane obtained from the control sides of the experimental animals and from the control animals, did not show any collagenolysis. Histologically, the tissues maintained their original structure (Fig. 8).

In contrast, on the pressure side, after 7 days of pressure application

Table 1, Effect of mechanical force on collagenolytic activity of periodontal membrane in rabbits

Duration (days)	Number of animals	Tissues obtained from Pressure side		Tension side	
		Experiment (lysis/fr.)	Control (lysis/fr.)	Experiment (lysis/fr.)	Control (lysis/fr.)
0	5		0/10		0/10
1	3	1/6	0/6	0/3	0/3
3	3	1/5	0/5	0/3	0/3
5	3	5/6	0/6	1/3	0/3
7	5	10/10	0/10	1/5	0/5
21	1	2/2	0/2	1/2	0/2

Lysis/fr. indicates total number of "lysis+"/total number of fragment cultures

all of the tissue fragments showed collagenolytic activity. It must be pointed out that these tissue fragments were histologically free of gingival epithelium. They were composed of granulation-like connective tissue rich in vascularity and in osteoclasts lining the periosteal surface (Fig. 9). From the histological sections taken after incubation, most of the osteoclasts, blood vessel cells and connective tissue cells were degenerated and connective tissue fibers were degraded (Fig. 10), while the tissue fragments obtained from the tension side, consisting of connective tissue with fewer cell components, showed collagenolytic activity to a lesser extent. Little alteration of these tissues after incubation was seen except for a diminution in cell number.

The relationship between collagenolytic activity and the duration of force application is also indicated in Table 1. On the pressure side, collagenolysis occured with only one of six specimens exposed to pressure for 1 day and one of five specimens exposed to pressure for 3 days. However, after 5 days pressure application, five of six specimens showed lysis of the collagen gel. At the end of 7th day the frequency of collagenolysis had reached its peak. Histologically, the samples obtained from the pressure side after 3 days of force application consisted of compressed and partially hyalinized connective tissue (Fig. 11). Furthermore, the number of osteoclasts in the tissue fragments increased in number from 3rd day to 7th day, while hyalinized connective tissue decreased and was replaced with granulation tissue; that is, newly formed mesenchymal connective tissue.

On this occasion it was also found that rabbit epithelium was equally effective in lysing the collagen gel, which is consistent with the findings reported by Fullmer *et al.*[6] and Bennick and Hunt[9]. Repeatedly frozen and thawed specimens failed to lyse collagen gel.

The area of lysis was not directly proportional to the size of explants, according to the pictures taken at varying intervals of incubation. This is probably due to the varying amount of altered periodontal membrane in

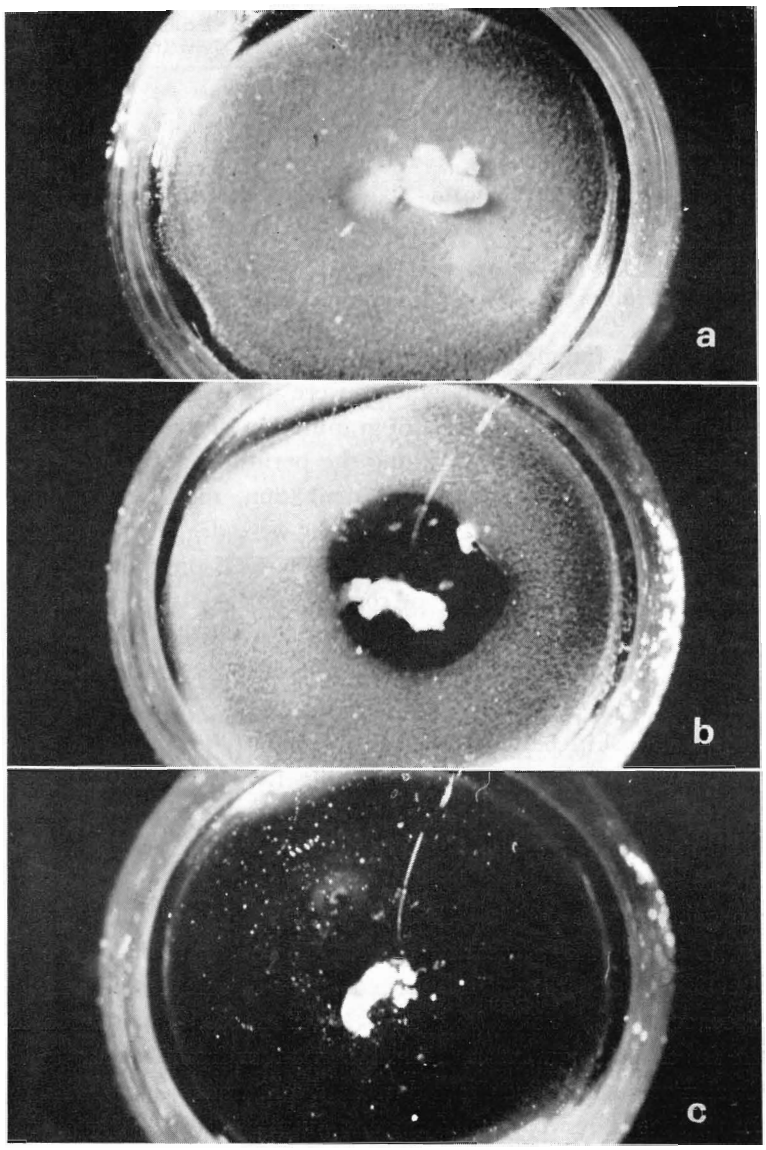

Fig. 12. Fragments of periodontal membrane resting on the reconstituted collagen gel. a) before incubation, b) after 48 hours incubation, showing lysis of adjacent gel, c) after 5 days incubation, showing much more extensive and complete lysis of collagen gel.

the specimens at the time of excision. Comparing the size of explants at the beginning and at the end of incubation, it was noted that the tissues which showed collagenolysis were somewhat reduced in size, whereas the tissues which did not show collagenolysis hardly changed.

Since the method used to detect collagenolytic activity in this experiment is not as sensitive as the method using a radioactive collagen substrate (Nagai *et al.*[12]), a negative result does not necessarily mean that there is no collagenolytic activity in these tissues. Whenever the tissue fragments showed collagenolytic activity, the lysis of reconstituted collagen gel usually started after 24–36 hours incubation and the gel was completely lysed within 5 days incubation (Fig. 12). The pH of the reaction mixture checked at the end of incubation remained within the range of neutrality (pH 7–8).

Discussion

It was demonstrated that the periodontal membrane of rabbits first molars subjected to compressive force for varying intervals developed collagenolytic activity *in vitro,* and that the appearance of this activity was dependent upon the duration of force application. The histological changes accompanying these forces applied to rabbits teeth continuously erupting agreed, in general, with the findings previously reported by other investigators (Oppenheim[13], Schwarz[14], Gottlieb[15], Macapanpan[16], Reitan[17]). On the pressure side the periodontal membrane was disorganized and transformed into hyalinized tissue after 3 days application of mechanical force, and between the 5th and 7th day the proliferation of granulation tissue; rich in vascularity with many osteoclasts lining the periosteal surface of the tissues, was replacing the degenerated periodontal membrane. Although the cellular origin of collagenolytic activity is still unknown, the results suggested that the appearance of such activity might be closely related to the proliferation of young mesenchymal connective tissues.

According to the kinetic study on the collagen metabolism of such tissues, as skin, bone and tendon, the turnover of collagen in mature animals is extremely slow. However, it is well recognized that the biosynthesis and degradation of collagen increase during the active remodeling of the tissues, in healing wounds, carrageenin granuloma, postpartum uterus and growing bone (Grillo and Gross[8], Jackson[18], Morrione and Seifter[7], Shimizu *et al.*[19]). Some of the enzymes, which can attack native collagen under physiological conditions, have been isolated recently and characterized (Evanson *et al.*[20], Eisen *et al.*[4], Nagai *et al.*[12]). Most of these collagenolytic en-

zymes have been shown to cleave the collagen molecule into two fragments comprizing three quarters and one quarter of the molecule (Nagai *et al.*[12], Fullmer *et al.*[11]).

It has been also shown that the direct measurement of proteolytic activity in remodeling tissues such as tadpole skin, bone, postpartum uterus and carrageenin granuloma indicated that tissue resorption was accompanied by an increase in the amount of acid hydrolases. These enzymes are required for the removal of different tissue proteins and may also be involved in the complete lysis of the collagen molecule to dialysable peptides and free amino acids following the limited activity of specific collagenase. It has been established, however, that collagen fibrils are resistant to the attack by any known digestive proteolytic enzymes such as cathepsin, trypsin and chymotrypsin at physiologic pH and temperature (under the condition of this experiment).

Lapiere and Gross[21] stated that the accretion and diminution of structural elements such as collagen is probably accomplished by altering the balance between the rate of synthesis and rate of degradation. In the instance of tooth movement, therefore, it can be considered that the balance between synthesis and degradation of tissue collagen is lost and there occurs an increase in degradative activity, even though synthetic activity remains constant. As mentioned before, the rate of collagen synthesis decreased in the initial stage of tooth movement (Crumeley[1], Baumrind and Buch[2]). Stallard[22] also investigated the alteration in cellular dynamics within the periodontium as a result of compressive forces and concluded that any compressive force placed upon a tooth will result in a decrease in cellular division and a drop in fibroblast cell density within the periodontal membrane. Tayer *et al.*[23] observed the proliferative response to an applied force within the supporting tissue cells at the end of a 28 day experiment on dogs, by means of autoradiographic analysis, and suggested that these proliferative responses might represent repair processes, similar to the findings of Macapanpan[16].

Here, the present results of frequency of collagenolysis showed considerable correspondence generally and reasonably with the histological alterations in the periodontal membrane. When comparing lytic activity and the corresponding tissue changes, the data suggest that the time the collagenolytic enzyme took part in the tissue collagen resorption and reached its peak of lytic activity did not correspond to the stage of hyalinization but to that of proliferation of granulation tissues. Grillo[8] observed the close relationship between collagenolytic activity and the appearance of granulation tissues in the process of mammalian wound healing. Lazarus[24] reported on collagenolytic activity in the synovium of rheumatoid

arthritis and suggested that such activity was not related to the immunological competent cells but rather associated with the deposition of granulation tissues, especially with the development of new blood vessels.

The fact that periodontal tissues during tooth movement have the ability of collagenolysis *in vitro* suggests that the appearance of granulation tissues might be responsible for the collagenolytic activity as reported by Grillo[8] and Lazarus[24]. Moreover, this fact confirmed the assumption, based on the histological findings heretofore, that a series of histological alterations during tooth movement might be a kind of regenerating or wound healing process in the supporting tissues.

The most conspicuous histological differences between experimental and control tissue fragments prepared before as well as after incubation revealed the increased vascularity and osteoclasts in the former.

It should be noted that osteoclasts increased in number in the tissue fragments from 3rd day to 7th day with the increased frequency of collagenolysis. Because of the intimate structure between osteoclasts and resorption zone in the bone, it is considered that osteoclasts are responsible for bone resorption. Moreover, osteoclasts are known to contain a wide range of enzymes (Hancox and Boothroid[25]). Walker *et al.*[26] found collagenolytic activity in the tissue culture media of parathyroid-hormone-treated-bone actively undergoing bone resorption. They suggested that the increased collagenolytic activity is related to progressive tissue transformation and perhaps to the accumulation of osteoclasts.

The relationship between increased vascularity and bone resorption has been described by many authors. It is believed that bone resorption is always accompanied by circulatory increase. Gianelly[27] and Nakamura[28] also observed the force-induced alteration of vascularity of the periodontal membrane and pointed out that patent vessels would appear to be essential for resorptive activity. Hancox[29] observed the well known fact that osteoclasts or multinucleated giant cells responsible for bone resorption appeared in the proximity of the enlarged capillaries and termed "the vascular resorption". Morita *et al.*[10] also demonstrated collagenolytic activity in a similar kind of granulation tissue during root resorption of bovine deciduous tooth.

The characteristic feature of osteoclasts is a large number of mitochondria, vacuoles and free ribosomes, and a deficiency in endoplasmic reticulum. These structures might indicate increased metabolic activity. Goldhaber[30] stated that the oxygen tension in the gas phase of tissue culture of young mouse calvaria played a major role in determining the rate and extent of response to various bone resorption stimuli, such as parathy-

roid extracts, and high concentrations of vitamin A and D. Preliminary experiments showed that the tissue fragments obtained from periodontal membrane during tooth movement need a high oxygen tension to demonstrate collagenolytic activity under these experimental conditions. From these results it can be speculated that the high vascularity of these tissues may provide oxygen and nutrient to cells with high metabolic activity *in vivo*.

It is too early to determine the cellular origin of the collagenolytic activity in the course of tooth movement. Numerous factors are probably correlated and co-reactive biologically *in vivo*. Furthere studies are required. Although the functional significance of collagenolytic enzyme is not clear at present, the onset of collagenolytic activity in the granulation-like connective tissue which appears during tooth movement suggests its involvement in collagen resorption of either degenerated periodontal membrane or alveolar bone, or both.

There is much discussion regarding the mechanism of tooth movement *in vivo*. Gottlieb[15] believed that the tooth could move within the limits of the periodontal membrane. Schwarz[14] postulated the optimal force for tooth movement to be not more than the blood pressure of peripheral capillaries (20–26 g/cm^2). Reitan[17] has demonstrated histologically that bone resorption does not occur all along the pressure side until the so-called "cell-free area" has been eliminated by undermining bone resorption and has estimated that if the hyalinized zone is small, the underlying bone is readily eliminated by resorption. Thus, he recommended that if a fairly rapid tooth movement is to be obtained, the hyalinized zone must be avoided or kept as small as possible. This would involve the use of light forces only.

Since the hyalinized tissue is to be replaced, sooner or later, by the proliferation of granulation tissue capable of tissue collagen resorption, it might be said that the theoretical ideal of tooth movement involves the compression of the periodontal membrane to such a degree as to cause degradation of its original cellular content initially within the physiologically acceptable level and to induce its replacement by granulation tissues (mesenchymal connective tissue); thereafter to maintain such tissues uncrushed for the continuation and preservation of increased enzymatic environment. To some degree, therefore, the present results could suggest that the utilization of the tissue potentiality in the healing of wounds caused by extrinsic force might be involved in the mechanism of tooth movement.

Conclusions

Specimens of rabbit periodontal membrane taken following experimental tooth movement were assayed for collagenolytic activity by sterile incubation on reconstituted collagen gel plates. The results are summarized as follows;

1. The periodontal membrane of rabbits first molars subjected to compressive force for varying intervals developed collagenolytic activity *in vitro*.

2. The appearance and degree of collagenolytic activity of the tissues obtained from the compressed area was dependent upon the duration of force application.

The tissue fragments derived from the pressure side after 7 days of pressure application exhibited the highest frequency of visible lysis of the collagen substrate, consisting of granulation-like connective tissue rich in vascularity and osteoclasts, while the tissue fragments after 3 days showed collagenolysis to a lesser extent, consisting of compressed and partially hyalinized connective tissues.

3. The results could suggest, therefore, that the appearance of collagenolytic activity may be closely related to the deposition of granulation tissues. This fact also confirmed the assumption, based on the histological findings heretofore, that a series of histological alteration during tooth movement might be a kind of regenerating or wound healing process in the supporting tissues.

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Explanation of Figures

Plate 1

Fig. 4. Photomicrograph showing the normal periodontal membrane around the medial crest area of lower first molar; AL: alveolar bone, C: cementum, P: periodontal membrane. Haematoxylin and eosin, ×300

Fig. 5. At the end of 1st day. Medial arest crea. The periodontal membrane is compressd to half of its original width. Haematoxylin and eosin, ×300

Fig. 6. At the end of 3rd day. Medial crest area. Crushed and hyalinized periodontal membrane is observed and frontal bone resorption with osteoclasts can be seen beneath the hyalinized area. OC: osteoclast, HY: hyalinized tissue. Haematoxylin and eosin, ×300

Fig. 7. At the end of 7th day. Medial crest area. Undermining bone resorption and granulation tissue, accompanied by vascularity and osteoclasts, are prominent. Haematoxylin and eosin, ×300

Plate 2

Fig. 8. a) Photomicrograph showing the tissue fragment of periodontal membrane excised from the control side. Before incubation. Haematoxylin and eosin, ×70
b) High magnification, ×200

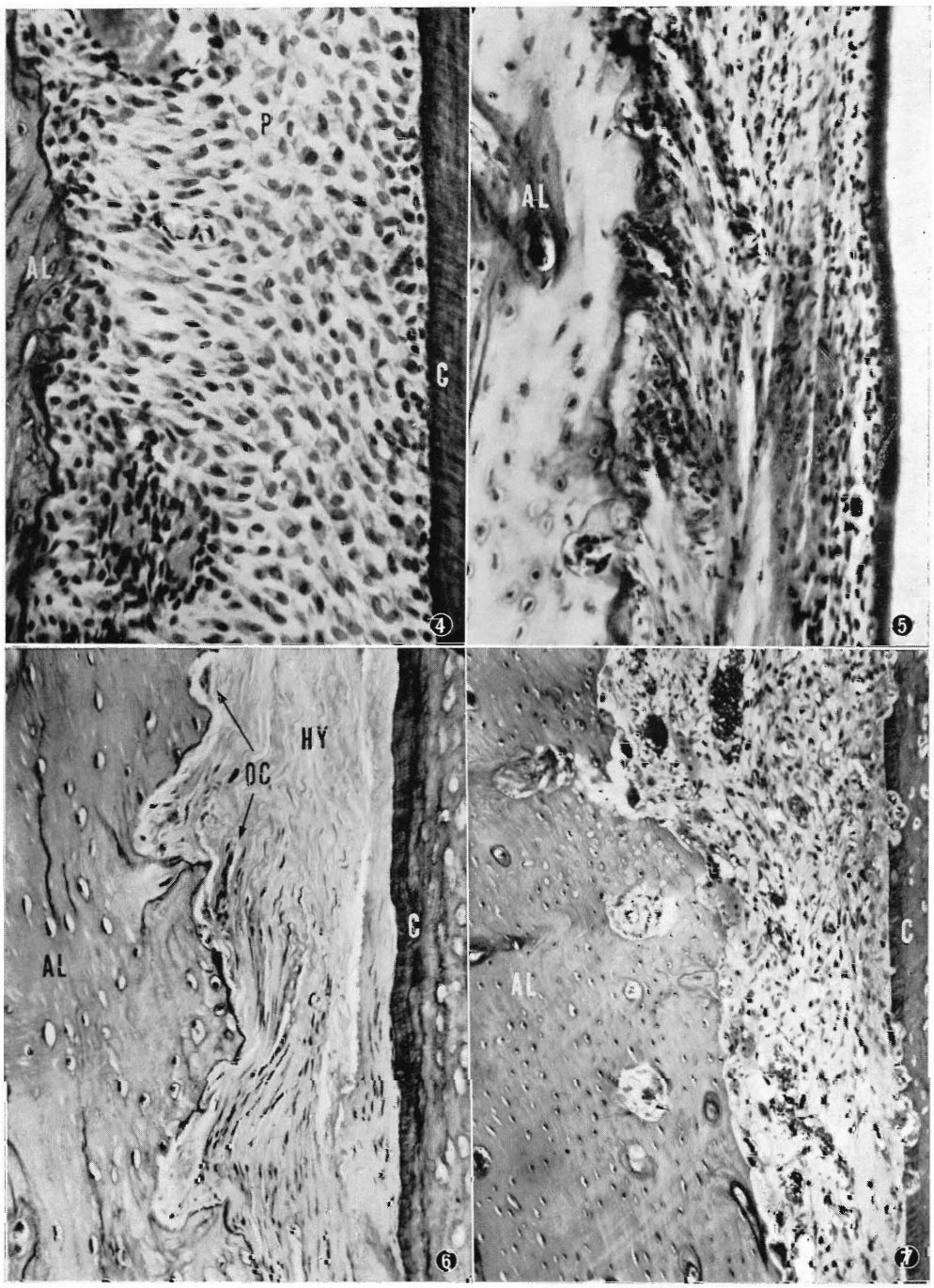
Plate 3

Fig. 9. Photomicrograph showing the tissue fragment excised at the end of 7th day.

Before incubation. Note the numerous osteoclasts lining the surface of the tissue, which consists of newly proliferated connective tissue. Haematoxylin and eosin, ×450

Fig. 10. Photomicrograph showing the tissue fragment after incubation with visible lysis of collagen gel. Connective tissue fibers are almost degraded and the connective tissue cells and osteoclasts are degenerated. Haematoxylin and eosin, ×140

Fig. 11. Photomicrogaph showing the tissue fragment excised at the end of 3rd day. A partially hyalinized connective tissue can be seen in the fragment. Haematoxylin and eosin, ×450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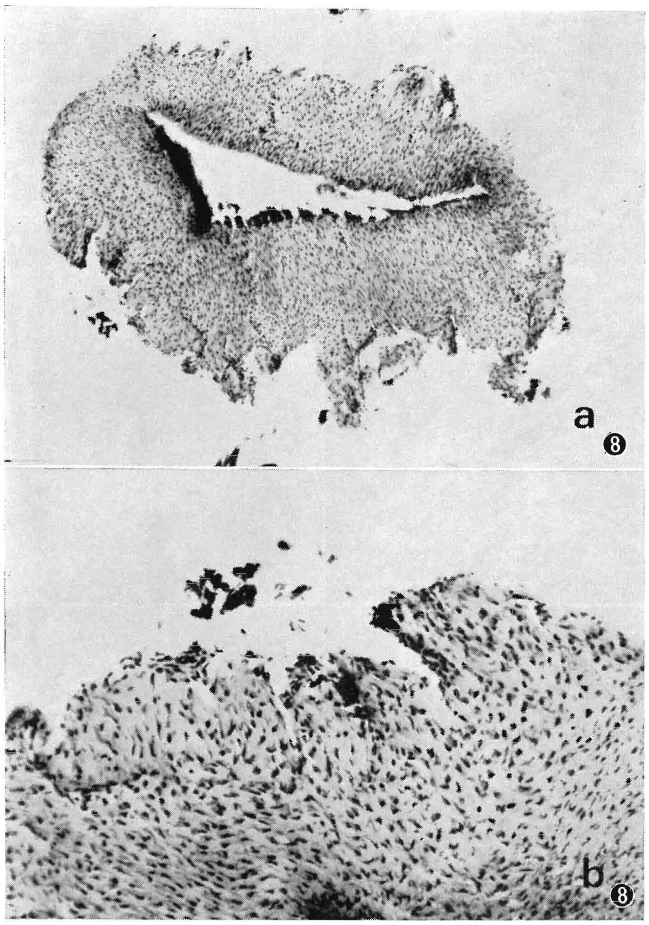
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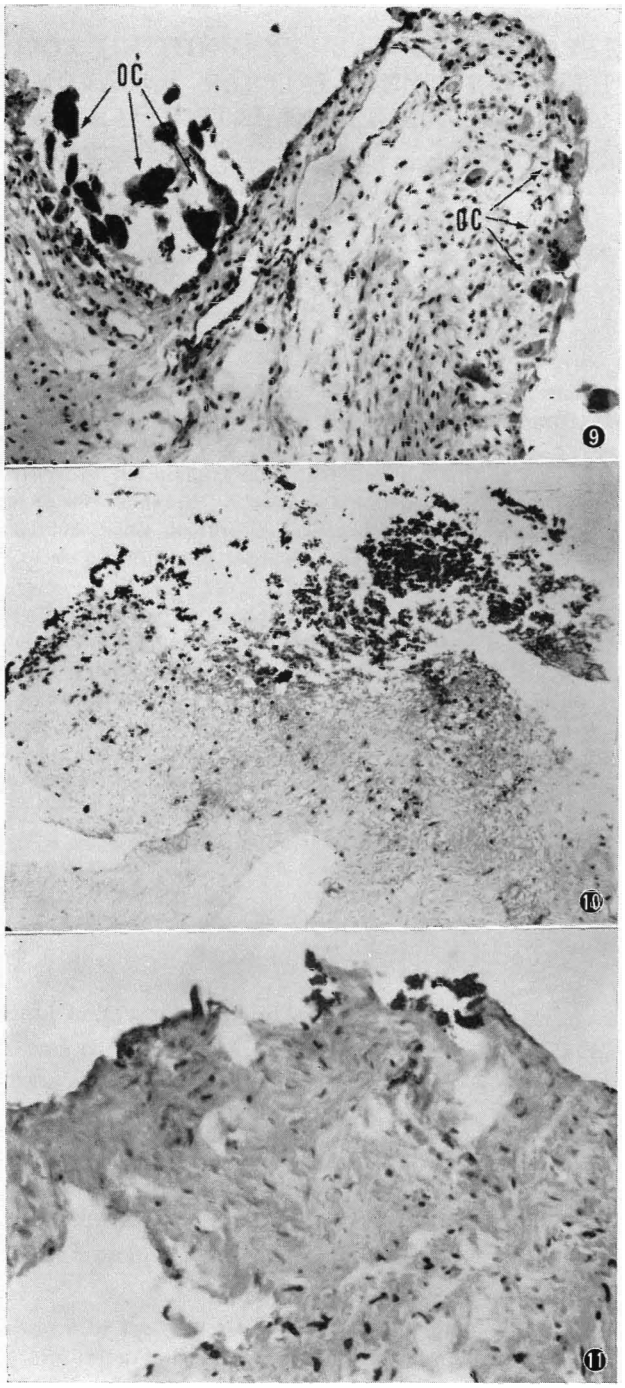
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