

Structural aspects of the reversal phase of alveolar bone remodelling

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ABSTRACT

The ultrastructure of the reversal line during alveolar bone remodelling was investigated in the rat. Surface bone remodelling along the periosteum of the mandible was induced by the extraction of opposing maxillary molars. With transmission EM the reversal line was seen to be composed of a superficial electron-dense amorphous layer and a deep filamentous layer at 7 d after extraction. The reversal line exhibited strong alkaline phosphatase activity and contained acid mucopolysaccharide. Scanning EM of the surface of the line, exposed by sonication in distilled water, showed papillary structures, the surface of which appeared granular and exhibited a crystalline appearance. The tips of collagen fibrils of new bone were attached to the top of the papillae in the front area of bone formation. It is suggested that the reversal line is involved in the coupling of bone resorption and formation.

Key words: Rat; periosteum; reversal line; collagen.

INTRODUCTION

Bone undergoes continuous turnover or remodelling. Newly formed bone is distinguished from the rest by a reversal line with an irregularly scalloped outline (Parfitt, 1983). Since this line surrounds the outer border of each osteon as a result of cortical remodelling, it is also referred to as the cement line in compact bone (Jee, 1988). It has been suggested that the reversal line, formed during the intermediate stage between bone resorption and the formation of remodelled bone, may be involved in the coupling of bone resorption and formation (Baron et al. 1983; Parfitt, 1984; Chen et al. 1993) in which a variety of systemic and local factors released into the local milieu either from cells or the bone matrix are implicated (Mundy & Roodman, 1987; Huffer, 1988). However, definite evidence as to a coupling function of the reversal line has not been provided.

From transmission EM observations, Tran Van et al. (1982*b*) reported that the reversal line is deficient in collagen and highly calcified, but few reports have described the micromorphology of the line in detail. Moreover, scanning EM has not so far been used for the examination of the surface structure of the line. As

to its composition, there appears to be agreement that it is deficient in collagen. Thompson et al. (1975) reported the presence of acid phosphatase. Schaffler et al. (1987) were unable to demonstrate selective staining of the line using periodic acid-Schiff, Sudan black B, or Alcian blue. According to Chen et al. (1993), bone sialoprotein appears to be concentrated in the reversal line.

The purpose of this investigation was to examine the ultrastructure, histochemical nature and the surface morphology of the reversal line. The largest possible areas of the reversal line were obtained by using a synchronised model of bone remodelling in rat alveolar bone, and the surfaces of the reversal line were exposed for scanning electron microscopic and histochemical studies.

MATERIALS AND METHODS

Induction of bone remodelling and histomorphometry of the reversal line

Female Sprague–Dawley rats weighing 100 ± 10 g were used. Their right maxillary molars were extracted with the tip of a no. 17 dental explorer under ether anaesthesia. According to Tran Van (1979), the

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extraction of maxillary molars provokes synchronised bone remodelling in the periosteal surface of the buccal plate of the mandible. For histomorphometry of the reversal line, 30 animals were used. 3 rats were killed by cardiac perfusion with 2.5% glutaraldehyde–2% paraformaldehyde under ether anaesthesia 1, 3, 5, 6, 7, 8, 10, 14, 22 d after the extraction. Three animals forming a control group were killed in the same way. The buccal plates of the molar region of the right mandible were isolated and fixed in 2.5% glutaraldehyde (0.1 mol/l cacodylate buffer, pH 7.2). After decalcification with formic acid–sodium citrate for 3–4 d, the buccal plates were postfixed in OsO₄, dehydrated in graded concentrations of alcohol, and embedded in Epon 812. Semithin sections perpendicular to the long axis of the root were obtained at a level 1 mm below the alveolar crest and stained with toluidine blue. Photographs were taken at a magnification of $\times 400$ along the entire periosteal surface of 3 sections obtained at intervals of 50 μm from each animal and used for morphometric assessment of the reversal line. In this way, the lengths both of the entire bone surface and of the basophilic line lining the lacunae without osteoid or osteoclasts were measured in each section, with an image analyser (Analytical Measuring System) and the VIDS VI program. The centile length of the reversal line as a proportion of the total length of the periosteal surface was obtained. The average percentage for each animal was derived from the values for the 3 sections and a mean value obtained for each day from the 3 animals examined. The statistical significance between the mean values was analysed by a multiple range test.

Transmission electron microscopy (TEM)

Ultrathin sections were cut from selected areas of the reversal zone of the buccal plates in 10 rats that had survived for 7 d after the extraction, when the ratio of the reversal lacunae appeared highest in the morphometric assessment. After staining with uranyl acetate and lead citrate, the grids were examined with a JEOL 1200 transmission electron microscope.

Ultrahistochemistry

To remove the soft tissues covering the buccal plate and expose the surface of bone matrix, the buccal plates from 10 animals at d 7 postextraction were immersed in distilled water for 3 h and sonicated for 30 s. They were fixed in 2.5% glutaraldehyde or 4% formaldehyde and decalcified. Following this they were immersed in a dialysed iron solution for staining for acid mucopolysaccharide (Rinehart & Abul-Haj,

1951) and incubated in a medium containing β -glycerophosphate for alkaline phosphatase ultra-histochemistry (Kurahashi & Yoshiki, 1972). Negative controls, omitting each substrate, were also prepared. The specimens were subsequently dehydrated and embedded in Epon 812. Ultrathin sections from the reversal zone were examined by TEM.

Scanning electron microscopy (SEM)

Ten buccal plates in which the matrices had been exposed were processed for observation of surface morphology. They were fixed in 2.5% glutaraldehyde, dehydrated in a graded alcohol series, transferred to isoamyl acetate, and dried in a CO₂ critical point dryer. After coating with gold-palladium, they were examined with a JEOL 840A scanning electron microscope.

RESULTS

In control animals the periosteal side of the buccal plate was seen to be covered with a continuous layer of osteoid tissue and osteoblasts, implying that active bone formation was in progress (Fig. 1*a*). Following the induction of synchronous bone remodelling by the extraction of molars on the opposite side, bone resorption, reversal, and new bone formation occurred sequentially along the periosteal surface of the buccal plates. The centile length of the reversal line as a proportion of the total periosteal surface reached a peak 7 d after extraction ($42.5 \pm 4.5\%$, mean \pm S.E.M.; see Table). This maximum value showed significant differences ($P < 0.05$) from the other groups, except for the group examined at 8 d after extraction.

In the semithin sections of the buccal plates at 7 d after extraction, the reversal lacunae were seen to be lined by a basophilic layer occupied either by mononuclear cells alone (Fig. 1*b*), or by mononuclear cells and osteoclasts in contact with the bone surface. The distinct border of the reversal line became obscured by new bone formation, especially towards the newly forming bone rather than towards the old bone (Fig. 1*c*).

The mononuclear cells in the reversal lacunae had euchromatic nuclei and were rich in rough endoplasmic reticulum, free ribosomes, and mitochondria (Figs 2, 3). In most instances their plasma membranes facing the bone surface were slightly separated from the bone surface and showed short and irregular cell processes. The surface of the reversal line beneath the mononuclear cells was very irregular and appeared to consist of 2 layers. The superficial layer was thin, electron dense, and amorphous, while the deep layer

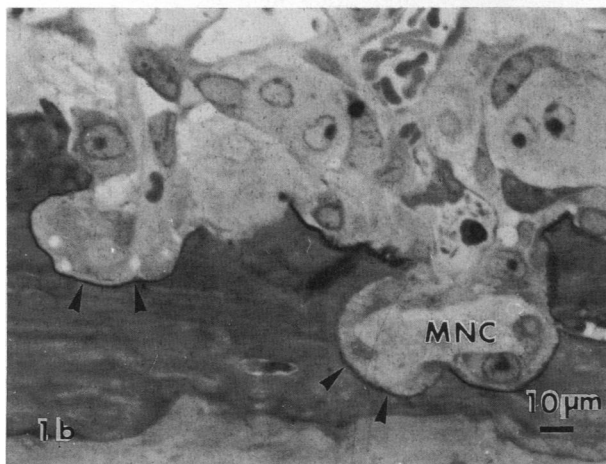
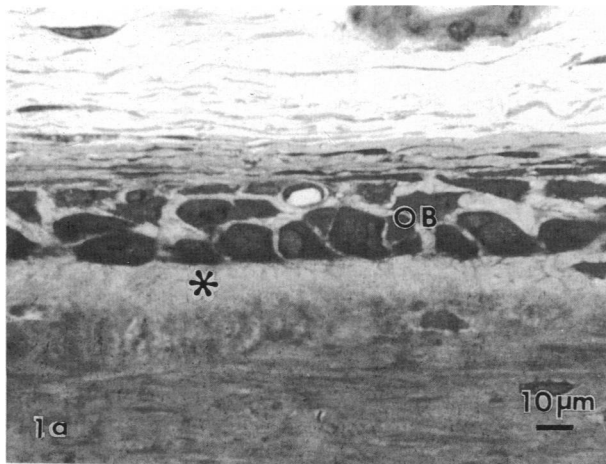


Fig. 1. Photomicrographs of periosteal surface of rat mandible. (a) In the control group, the periosteum is covered with a continuous layer of osteoid (asterisk) and osteoblasts (OB). (b) Reversal lacunae 7 d after extraction of maxillary molars to induce bone remodelling. The lacunae are lined by a basophilic reversal line (arrowheads) and mononuclear cells (MNC). (c) In the early stage of bone formation, the reversal line is widened and extends into new bone tissue (small arrows). The osteocytes partially embedded are shown (arrowheads).

was composed of filamentous material, distinct from the coarse bone collagen fibrils below (Fig. 3). The thickness of the deep layer was not uniform. Where a

Table. Centile length of reversal line relative to total periosteal surface undergoing bone remodelling after tooth extraction*

Days after extraction	Centile length of reversal line
0	0.0±0.0
1	0.0±0.0
3	12.9±3.6
5	27.4±7.7
6	27.4±5.1
7	42.5±4.3
8	39.9±3.6
10	26.8±1.7
14	26.5±1.2
22	7.3±4.3

* All values are means ± S.E.M. for the 3 experiments.

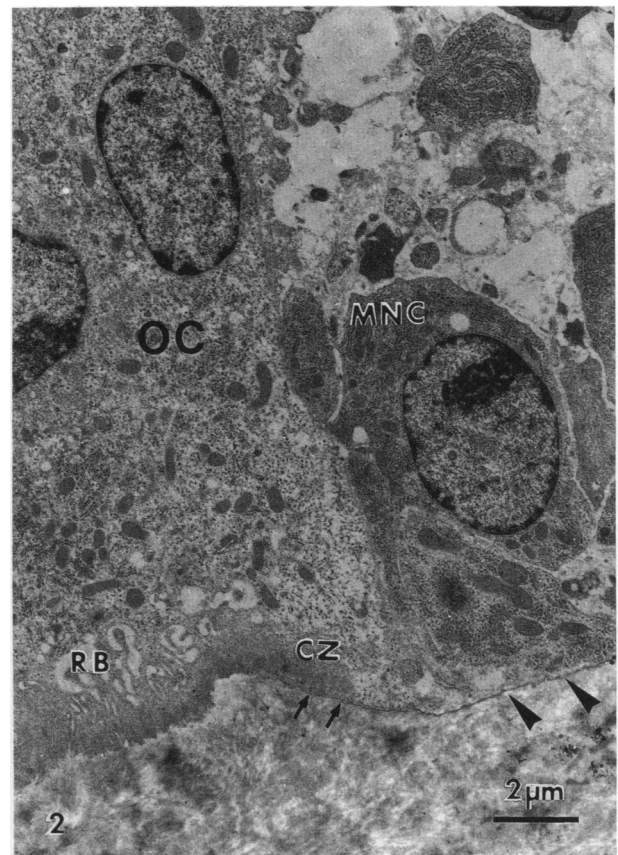


Fig. 2. Transmission electron micrograph of an osteoclast (OC) and an immediately adjacent mononuclear cell (MNC) in close apposition to the reversal line (arrowheads). The reversal line appears to extend to some extent into the area under the osteoclast with a gradual decrease of thickness (small arrows). RB, ruffled border; CZ, clear zone.

mononuclear cell was adjacent to an osteoclast, the reversal line seemed to extend from the area under the mononuclear cell to the area under the clear zone of the osteoclast and the thickness decreased towards the osteoclast (Fig. 2). After the onset of bone formation, a layer of osteoid and newly mineralised bone of lesser

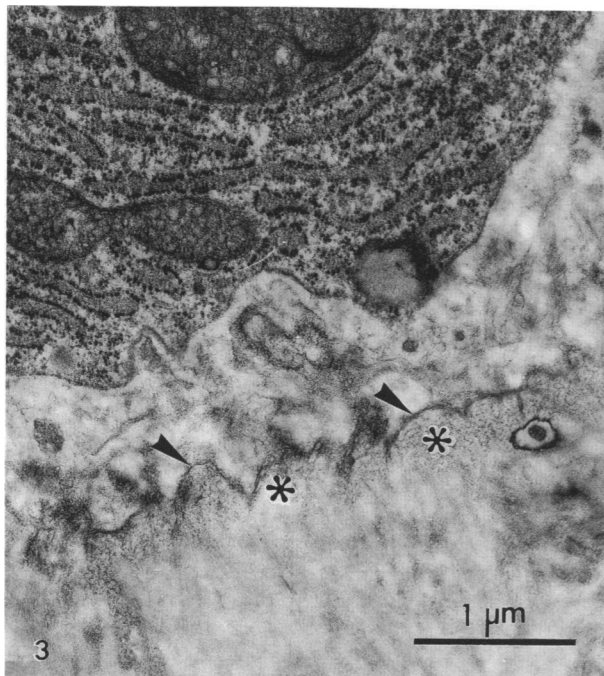


Fig. 3. Transmission electron micrograph of the reversal line with an associated mononuclear cell with osteoblastic characteristics. The reversal line is composed of a superficial electron dense layer (arrowheads) and a deep filamentous layer (asterisks).

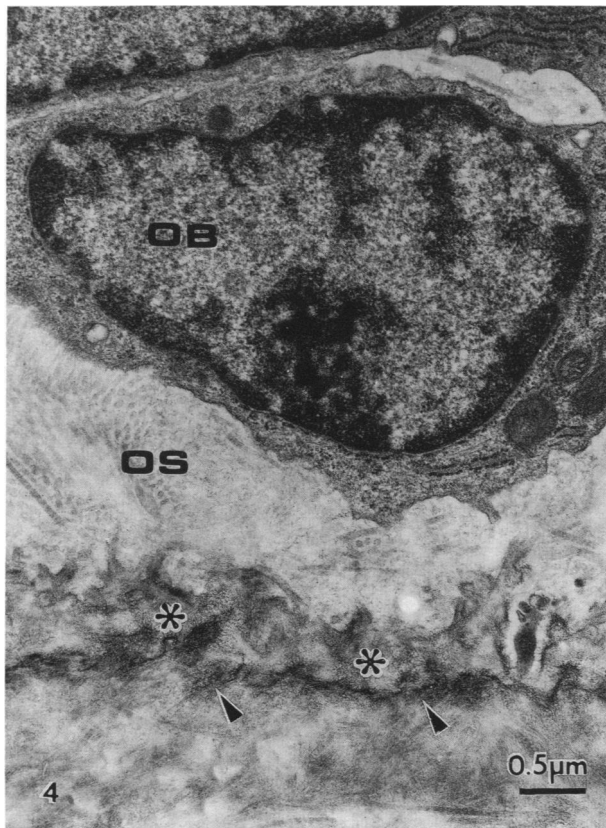


Fig. 4. Transmission electron micrograph of the early stage of bone formation. The osteoid (OS) and newly mineralised bone (asterisks) of lesser electron density than the reversal line (arrowheads) are formed on the line by the osteoblasts (OB).

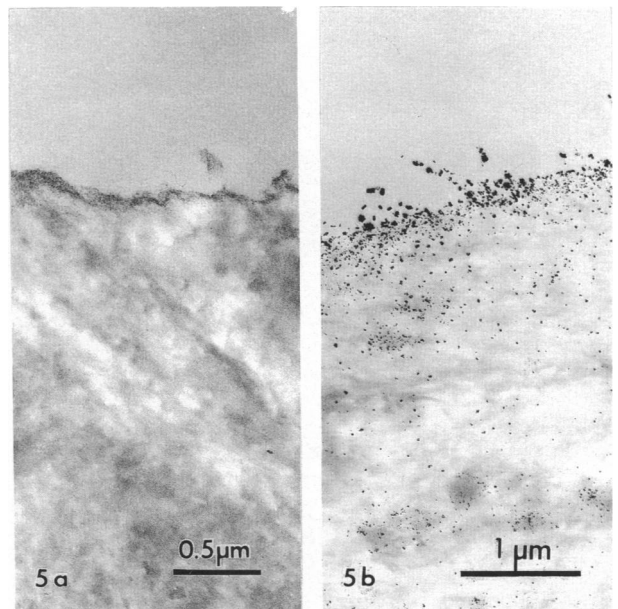


Fig. 5. Transmission electron micrograph for histochemical demonstration of acid mucopolysaccharide (a) and alkaline phosphatase (b). In (a), the granules are fine and attached only to the superficial layer, while in (b), they are attached to the entire matrix, but with higher density to the surface and with frequent agglutination.

electron density than the reversal line was formed over the line (Fig. 4).

Acid mucopolysaccharide and alkaline phosphatase were included in the reversal line. Fine granules bound to acid mucopolysaccharide were attached to the line, forming a thin superficial layer, but not to the underlying bone matrix (Fig. 5a). For alkaline phosphatase, the granules were bound both to the reversal line and the bone matrix, but with higher density to the reversal line and with frequent agglutination (Fig. 5b).

After removal of the fibrous and cellular periosteum from the mandibles by immersion and sonication in distilled water, leading to exposure of the surface of the bone matrix, a band within the remodelling area near the alveolar crest was observed, at low magnification, where the surface was very irregular. Deep to the remodelling surface towards the underlying bone, there was an irregular network of collagen fibrils, implying that bone formation was in progress. In the remodelling surface, the resorption, reversal, and formation of lacunae could be distinguished and each kind of lacuna had a tendency to be aggregated together. Collagen fibrils covered with calcific globules were exposed with a parallel arrangement in the resorption lacunae (Fig. 6). The reversal lacunae were lined with papillary structures ~ 170 nm in diameter. These had either rounded or flattened blunt ends (Fig. 7a) and were covered with granular material 20–30 nm in diameter when viewed at high magnification.

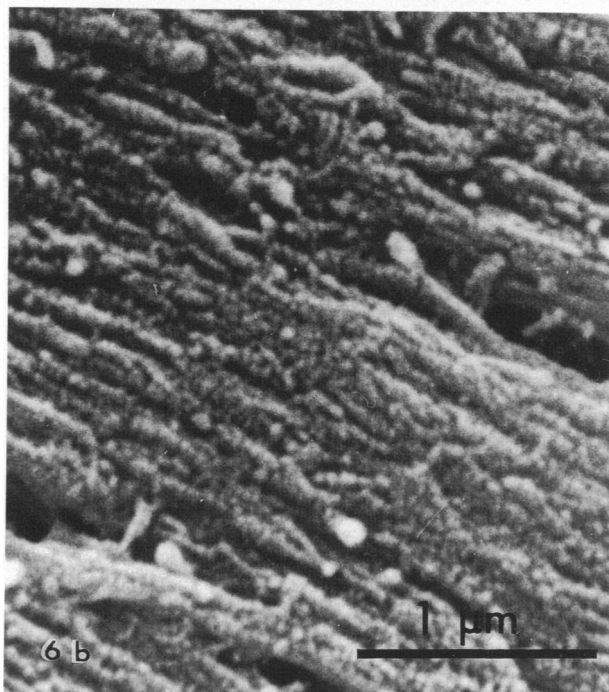
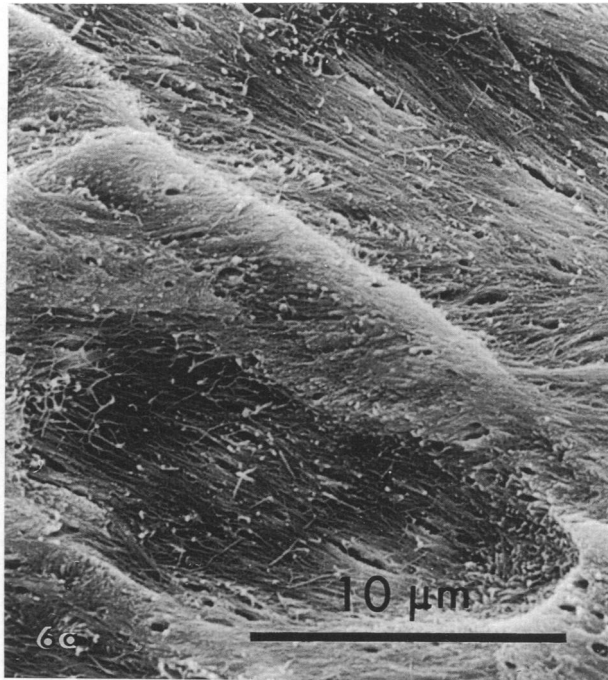


Fig. 6. Scanning electron micrograph of the surface of resorption lacunae, which is lined with collagen fibrils arranged in parallel (a). In (b), a high magnification view of (a), the fibrils are seen to be covered with calcific globules.

(Fig. 7b). The granular surfaces exhibited a crystalline structure. Sometimes the papillae were interconnected with the adjacent papillae. The entire surface of typical reversal lacunae was coated with well-developed papillae. Often poorly developed papillae were found at the periphery of resorption lacunae. These lacunae were thought to be in the early stage of the reversal phase.

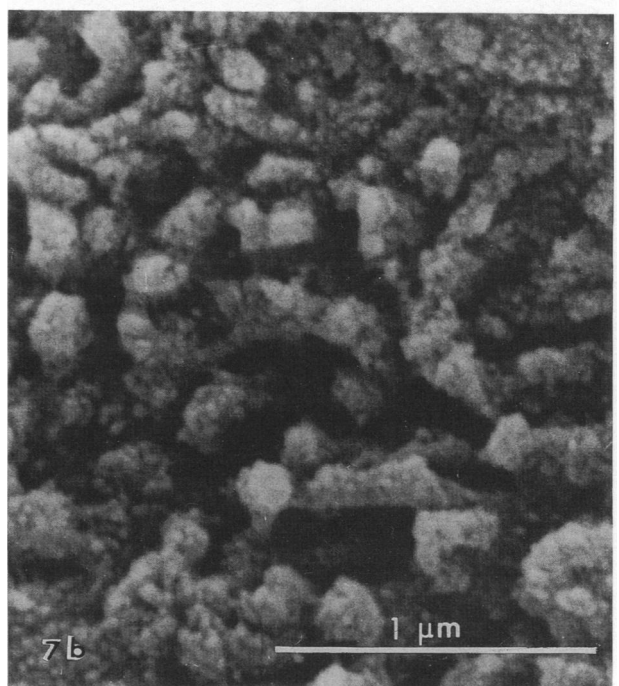
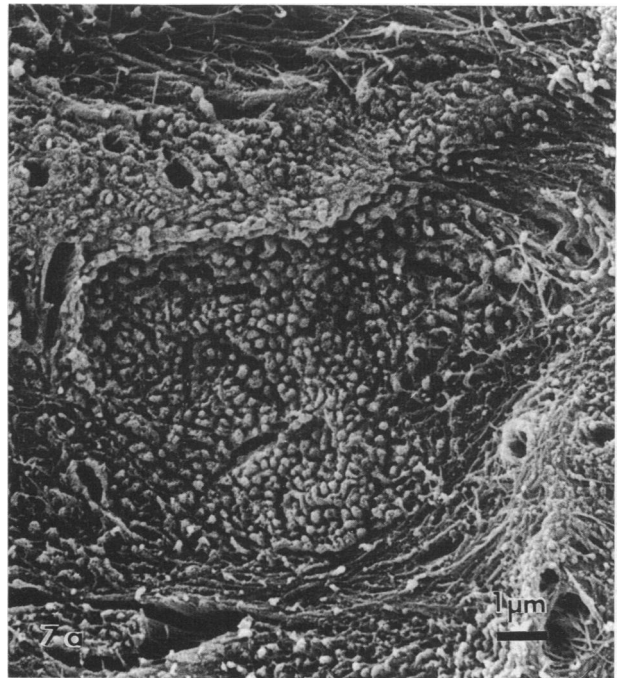


Fig. 7. Scanning electron micrograph of the surface of reversal lacunae. They are lined with papillary structures (a), which are seen to be covered with granules at higher magnification (b).

At the surface of the formation lacunae, collagen fibrils of ~ 70 nm in diameter were observed to be entangled with each other, unlike those found on the surface of resorption lacunae. The superficial fibrils that could easily be observed were not covered with calcific globules (Fig. 8b). In most of the lacunae at an early stage of bone formation, the new bone was formed on the reversal line (Fig. 8a). In these lacunae,

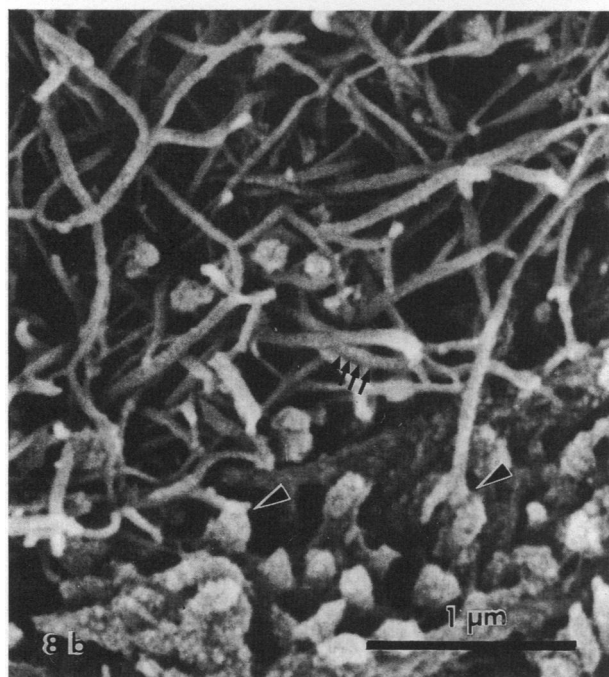
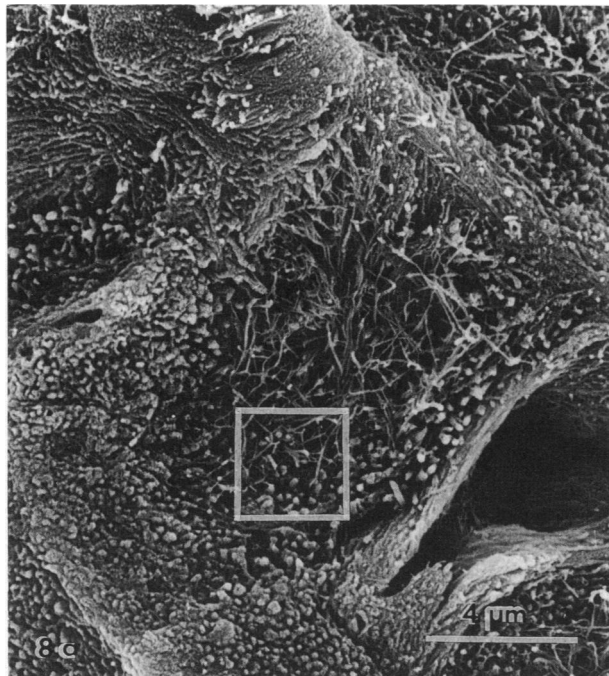


Fig. 8. Surface of lacunae where bone formation begins on the reversal line. Irregularly arranged collagen fibrils occupy the central portion of the lacuna of which the periphery is still covered by papillary structures (a). (b) High magnification view of the boxed area in (a). At the advancing region of bone formation, the tips of collagen fibrils are attached to the blunt ends of papillae (arrowheads). Small arrows indicate the cross bands of newly formed collagen fibres.

collagen fibrils always occupied a central position. It was anticipated that the advancing region of bone formation would reveal the relationship between papillary structures and collagen fibrils. The tips of the collagen fibrils approaching the papillae were seen



Fig. 9. Scanning electron micrograph of the surface at an advanced stage of bone formation, where the interconnecting network of collagen fibrils entirely covers the surface. Reversal surfaces are seen at the top of the figure.

to be attached to the blunt ends of papillae (Fig. 8b). As bone formation progressed, the outlines of lacunae became obliterated by a complex interconnecting network of collagen fibrils (Fig. 9).

DISCUSSION

The reversal line was observed to be composed of a superficial electron dense layer and a deep filamentous layer. This two-layered morphology closely resembled peritubular dentine (Thomas, 1983) and perilacunar bone (Mjör, 1962; Capen & Weisbrode, 1982). In these structures, the filamentous layer is known to be highly mineralised, while the superficial thin layer is unmineralised. In terms of morphological comparisons, the deep layer of the reversal line was considered to be hypermineralised. It may correspond to the calcified, dense granular collagen-free layer reported as the reversal line by Tran Van et al. (1982b); the superficial layer is composed of unmineralised material. The finding of a double-layered reversal line was attributed to the use of demineralised sections, as the fine morphology of the tissue tends to be masked by the mineral crystals in the undermineralised sections.

The surface morphology of the reversal line could be deduced from the SEM findings in the present study. On the periosteal side of the rat mandible 7 d after dental extraction, with the fibrous and cellular

components removed, the lacunae undergoing resorption, reversal, and formation were clearly identified. The reversal line consisted of papillary structures with a granular surface. The outline of the surface was seen to be a little more irregular on SEM than in TEM and the granular surfaces appeared crystalline. The specimens for the latter were prepared by fixation and decalcification, and for the former, they were treated with distilled water. This difference in the method of preparation could influence the morphological analysis of reversal line, that is, a part of superficial layer containing organic material might be removed by the sonication process.

The histochemical investigations into the non-collagenous organic matrix in cement lines has yielded no consensus as to which possible organic components are present (Sokoloff, 1973; Schaffler et al. 1987). In our study, using en bloc staining of the exposed matrix, acid mucopolysaccharide and alkaline phosphatase were found in the reversal line. Acid mucopolysaccharide was seen to be contained specifically in the superficial layer, which was observed to be amorphous and electron dense on routine TEM. The finding by microprobe analysis that the cement line is a region of higher sulphur levels (Schaffler et al. 1987) supports the results for acid mucopolysaccharides in the present study. The perilacunar bone matrix, which is ultrastructurally similar to the reversal line, was also reported to contain higher amounts of proteoglycan (Sauren et al. 1992). The inclusion of acid mucopolysaccharide may explain the basophilia of the reversal line in light microscopic sections.

There can be 2 explanations for the extension of the reversal line up to the area under the clear zone of osteoclasts, although it is indistinct and thinner than under the mononuclear cells, opposite to the direction of migration. One is that the initiation, at least, of the formation of the reversal line may not be related to the activity of the mononuclear cells, that is, the initiation may occur on the resting bone surfaces spontaneously. This suggestion might be supported by the fact that the organic layer covering the surface or lumen of the peritubular dentine and perilacunar bone is formed by adsorption (Scherft, 1972; Parfitt, 1984; Chambers, 1985). The other possibility is that there may be activity of osteoclasts related to the formation of the reversal line. The appearance and movement of ^3H -glucosamine from within the osteoclast layer to the bone surface under the osteoclasts and then to the nonosteoclast surface, observed by Owen & Shetlar (1968) favours the latter. Regardless of the mechanism of the initiation, the maturation of the reversal line seems to require the activity of or microenvironment

formed by mononuclear cells, as can be conjectured from the finding that the thickness of the deep layer increases gradually as it moves away from the osteoclasts. In this view, the formation of the mineralised layer is also related to the activity of odontoblasts and osteocytes respectively in the peritubular dentine and perilacunar bone (Parfitt, 1976; Torneck, 1989).

The present finding that the tips of collagen fibrils in forming bone are attached to the tops of papillae may support a coupling function for the reversal line by providing the glue-like substrate to which the collagen fibrils can be attached. An intermediate substrate with a glueing action may be required for the connection of the newly oriented lamellar system to the cut surface of remnant lamellae. The network of collagen fibrils of newly formed osteoid was very irregular and random in comparison with that of resorbed lacunae. This difference means that the fibrils are rearranged after secretion, which may occur under the control of osteoblasts or osteocytes and may be related to mineralisation.

The extraction of maxillary molars unilaterally in the rat provides a well synchronised model of sequential bone remodelling in the mandibular periosteum through the egression of mandibular molars. In the analysis of kinetics of bone remodelling by Tran Van et al. (1982a), the resorption activity reached its peak 4–5 d after extraction and the reversal peaked at d 7 postextraction. We used this model and performed histomorphometry in order to obtain the largest area of reversal line. The peak time for reversal activity appeared to accord with their result.

In summary, this study has described the ultrastructural and histochemical features and surface morphology of the reversal line. On the basis of these findings, the reversal line is considered to begin to form on the resting bone surface beneath the clear zone of osteoclasts and to mature beneath the mononuclear cells. Our results also suggest that the reversal line is partly implicated in the initiation of bone formation.

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