Fine Structure of Remodeling Sites on Iliac Cancellous Bone in Senile Osteoporosis: A Study by Scanning Electron Microscopy Using an Improved Organic Specimen Preparation Method

Toshihiko Takita
Tottori University, Yonago

Tomonori Naguro
Tottori University, Yonago

Kichizo Yamamoto
Tottori University, Yonago

Follow this and additional works at: https://digitalcommons.usu.edu/cellsandmaterials

Part of the Biological Engineering Commons

Recommended Citation

FINE STRUCTURE OF REMODELING SITES ON ILIAC CANCELLOUS BONE IN SENILE OSTEOPOROSIS: A STUDY BY SCANNING ELECTRON MICROSCOPY USING AN IMPROVED ORGANIC SPECIMEN PREPARATION METHOD

Toshihiko Takita[1,2,*] Tomonori Naguro[1] and Kichizo Yamamoto[2]

[1]Department of Anatomy, [2]Department of Orthopedic Surgery,
Faculty of Medicine, Tottori University, Yonago 683 Japan

Abstract

We improved on the organic specimen preparation method in order to investigate the details of the functional state of bone in osteoporosis at high magnification level. The usefulness and possibilities of this method are discussed showing fine structural images of remodeling sites. Iliac bones of senile osteoporotic patients obtained by transilial biopsies were studied by scanning electron microscopy (SEM) using the improved preparation method. Excepting the region near the cortico-endosteal surface, most of the iliac cancellous bone was composed of thin rod-shaped trabeculae. Remodeling sites on trabeculae appeared as concavities of various sizes and were easily distinguished from inert surfaces. The region near the cortico-endosteal surface showed a higher remodeling activity as compared with other regions.

The morphological analysis of remodeling site by SEM made it possible to know its functional state. Five areas of different stages were distinguishable in remodeling sites, each corresponding to a different stage in the remodeling cycle: area in resorbing phase, in reversal phase, in early forming phase, in late forming phase, and in resting phase. The distribution patterns of the five areas in remodeling sites were investigated. More than half of the remodeling sites showed the heterogeneous composition: they were occupied by plural areas in different phases. Some remodeling sites were entirely occupied by a single area in reversal phase or in forming phase or resting phase. The remodeling site occupied by an area in resorbing phase alone was not found.

Key Words: Scanning electron microscopy (SEM); osteoporosis; iliac bone biopsy; trabecular bone; remodeling; reversal phase.

*Address for correspondence:
Toshihiko Takita
Department of Anatomy and Orthopedic Surgery,
Faculty of Medicine,
Tottori University,
Yonago 683, Japan

Phone No.:0859-34-8002

Introduction

As the number of elderly people grows, it is clear that osteoporosis is a major health problem. Until today, bone histomorphometry of undecalcified sections obtained from human iliac bone biopsies has been used by many researchers as a suitable method for investigating morphological bone changes of osteoporosis (Wakamatsu and Sissons, 1966; Courpron et al., 1981; Parfitt, 1984). Although the method has provided much important information about the functional state of bones, it was difficult to get the three dimensional images of bone dynamics.

The surface topography of bones by scanning electron microscopy (SEM), which provides three dimensional information, is one of the most useful means to study morphological changes of osteoporosis. Up to now, many methods of bone specimen preparation for SEM have been reported (Boyde, 1972, 1984; Boyde et al., 1986; Dempster et al., 1986; Mosekilde, 1990; Reid, 1987). They are roughly classified into two types of preparation: the anorganic and organic specimen preparations. The former is suitable for observing mineralization of bones, but has the deficiency that the real surface of the bone cannot be observed and, moreover, the samples prepared by this method are extremely fragile especially in bones from osteoporotic subjects. On the other hand, the latter has the merit that the real surface of the bone can be observed, though it is difficult to know the details of functional state of the bone.

In the present study, we improved on the organic specimen preparation method in order to investigate the details of functional state of bone from osteoporotic subjects at high magnification levels. The aim of the present report is to demonstrate the merit and possibilities of this method.

Materials and Methods

The specimens were obtained from 14 female patients (ages: 72-94 years old) with clinically diagnosed senile osteoporosis by iliac bone biopsy using a trephine of 8 mm inner diameter during surgeries for femoral neck fracture. The patients gave their consents to iliac

bone biopsy beforehand.

The cylindrical specimens with bone marrow were horizontally cut into two with a bone chisel, and then, bone marrow cells were immediately removed from the freshly cut pieces by washing in a phosphate buffer solution, followed by immersion in 2.5% glutaraldehyde in M/15 phosphate buffer for 2 hours and 1% osmium tetroxide in M/15 phosphate buffer for 1 hour.

In order to minimize metal coating influence for observing fine structures at high magnification, the tannin-osmium conductive staining method, which was first introduced by Murakami (1973), was employed with slight modifications as follows. Fixed samples were treated with 2% tannic acid aqueous solution for 1 hour and 1% osmium tetroxide in M/15 phosphate buffer for 1 hour. Prolonged treatment with tannic acid had been done before the fixation with osmium in the original method (Murakami, 1973). The present method was modified so that the treatment with tannic acid could be carried out in a short time.

After the conductive staining, the samples were dehydrated in graded ethanol and dried by the critical point drying method. The dried samples were coated with about 3 nm of platinum in an ion coater (VX10R, EIKO Engineering Co. Ltd., Japan) and examined by a Hitachi S-430 scanning electron microscope at an accelerating voltage of 25 kV.

To confirm the mineralization, some samples were reinvestigated after having been made anorganic by immersion in 5% sodium hypochlorite for 30 minutes (Boyde, 1984).

Results

Observations at low magnification (below X 500)

Iliac cancellous bone in senile osteoporosis was composed of a three dimensional lattice of plate- and rod-shaped trabeculae (Fig. 1). The relative proportion of the two types of trabeculae seemed to vary individually. However, there was a tendency for the cancellous bone to divide into two parts. The region near the cortico-endosteal surface (less than 500 μm from the cortico-endosteal surface) was mostly occupied by plate-shaped trabeculae, while the remaining part (midway region about 5 mm between the cortices) was dominated by thin rod-shaped trabeculae. Irrespective of age, this distribution pattern of the two types of trabeculae was fairly constant in all individuals.

Because the remodeling sites appeared as concavities of various sizes and could be clearly distinguished from the smooth inert surfaces, the location, size and shape of remodeling sites were visible even at low magnification (Fig. 2). In many cases, remodeling sites were found in the region near the cortico-endosteal surface and a few in the midway region. Plate-shaped trabeculae near the cortico-endosteal surface had large remodeling sites around the vascular channel (Fig. 2). In the midway region, the remodeling site was mainly found at the portions of transition between plate- and

Figure 1. The cancellous bone lattice of a 89-year-old woman. R, rod-shaped trabecula; P, plate-shaped trabecula and disconnected trabecula (arrow).

Figure 2. A plate-shaped trabecula near the cortico-endosteum (72-year-old woman). A large remodeling site around a vascular channel (V).

Figure 3. One of the disconnected trabecula near the cortico-endosteum (82-year-old woman). The end of the disconnected trabecula (arrow) has no remodeling site.

Figure 4. A large remodeling site situated at the transition between plate- and rod-shaped trabeculae near the cortico-endosteum. R, rough surfaced area; S, smooth surfaced area.

Figure 5. Higher magnification of a border between rough surfaced area (R) and smooth surfaced area (S) in a remodeling site. Howship's lacunae (RB) are visible in the rough surfaced area. Newly formed collagen fibers are visible in the smooth surfaced area.

Figure 6. Area in R-1 (resorbing phase). It consists of sharply edged RB whose collagen fibers are cut off.

rod-shaped trabeculae (Fig. 3). Few remodeling sites were observed at the ends of disconnected rod-shaped trabeculae (Fig. 3).

Observations at high magnification (above X 2500)

Two different areas could be distinguished in remodeling sites: rough surfaced area (R) consisting of numerous characteristic excavated pits, so called Howship's lacunae or resorption bays (RB), and smooth surfaced area (S) free of RB (Figs. 4, 5). The two areas (R and S) were further subdivided by their fine morphological appearances as follows.

R-1: rough surfaced area consisting of sharply edged RB whose collagen fibers are cut off (Fig. 6).
R-2: rough surfaced area consisting of dully edged RB whose collagen fibers run closely parallel to each other showing wavy appearances (Fig. 7).
R-3: rough surfaced area having collagen fibers of various thickness which extend over adjacent RB forming a network (Fig. 8).
S-1: smooth surfaced area having collagen fibers of various thickness (Fig. 9).
S-2: smooth surfaced area having uniformly thick bundles of collagen fibers which has a similar configuration to inert surfaces (Fig. 10).

Observations by using anorganic preparation

As shown in Figure 11, there was no difference between area S-2 and inert surface in their appearances after anorganic treatment with sodium hypochlorite. Uniform thick bundles of mineralized collagen fibers were arranged in a lattice-like pattern. Area S-2 seemed to be fully mineralized because it showed almost the same appearance as that of the organic preparation.

Area R-2 of the anorganic preparation also had the same appearance as that of the organic preparation (Fig. 12); mineralized collagen fibers in this area run closely parallel to each other showing wavy appearances.
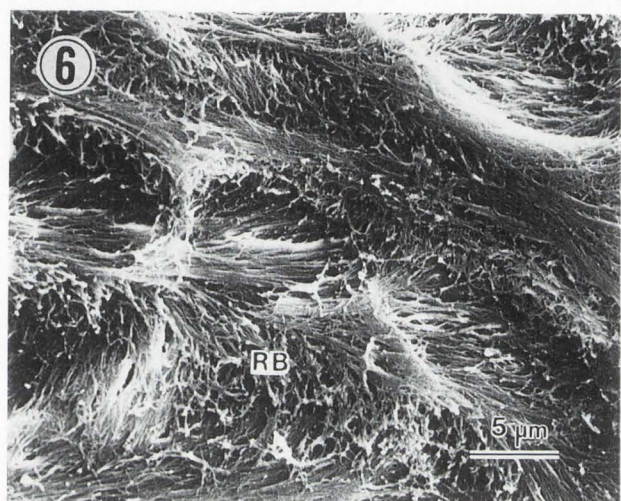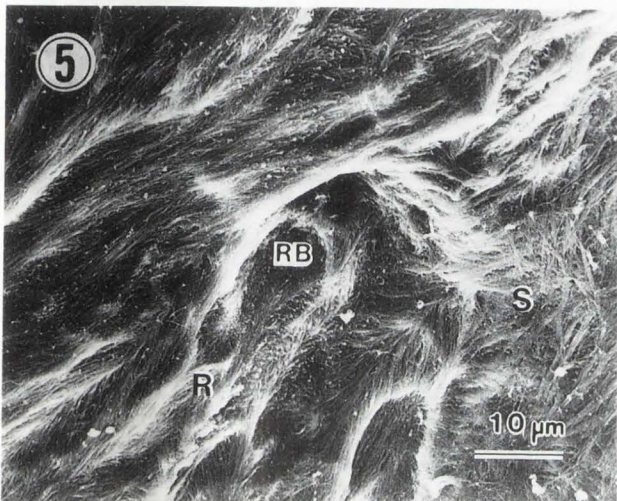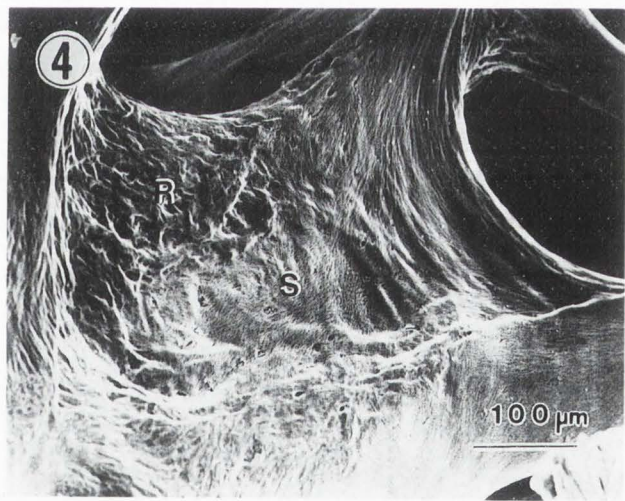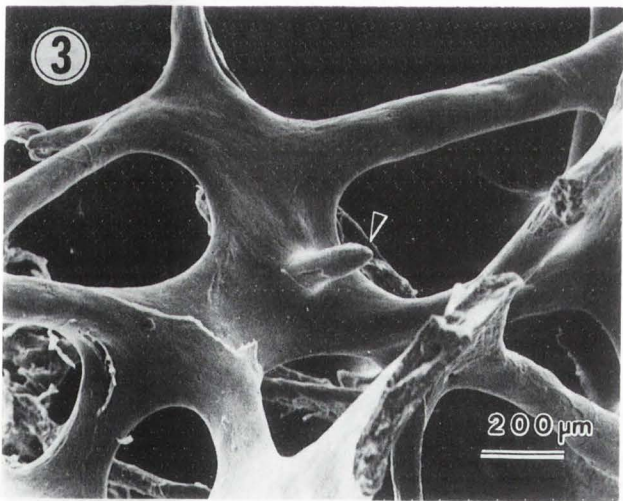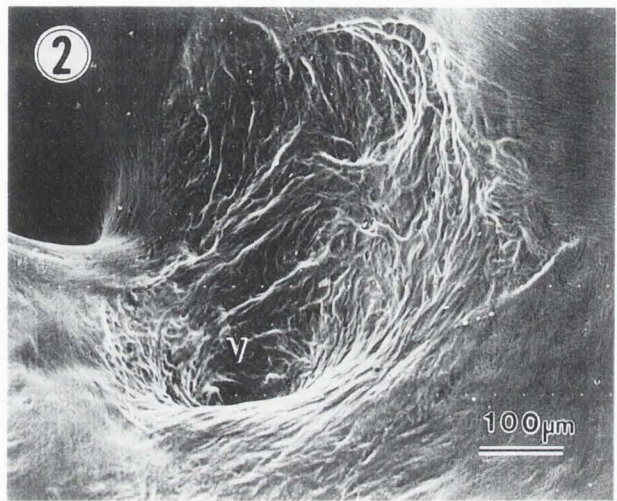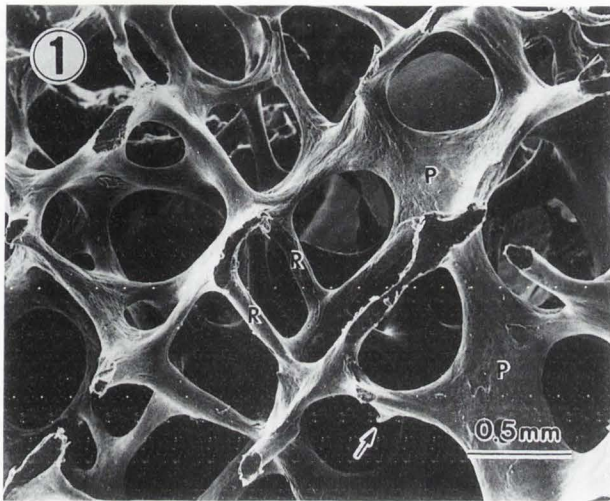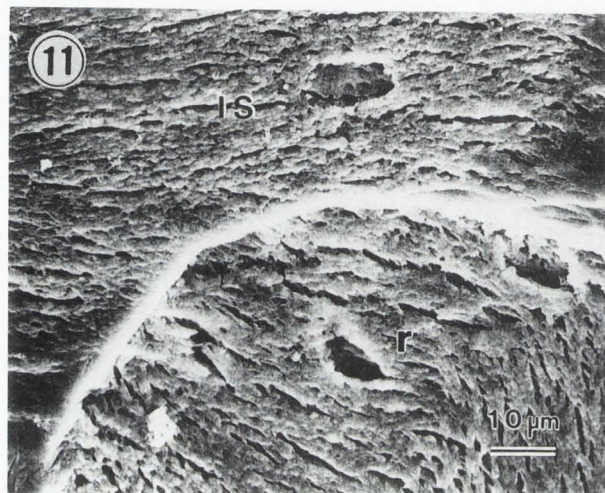
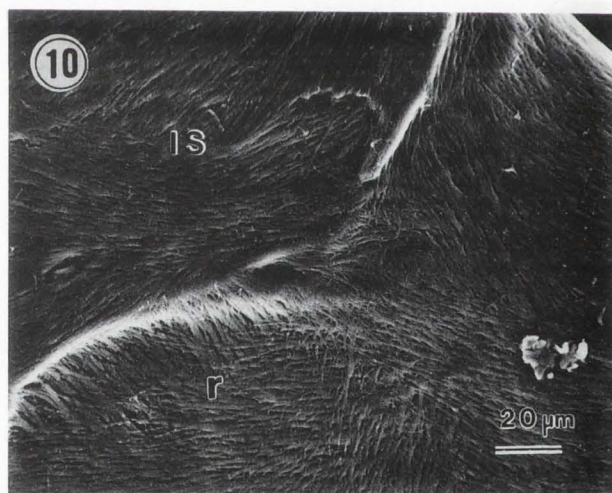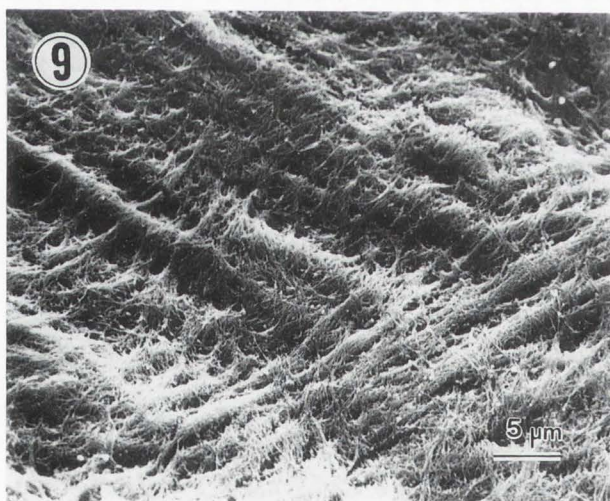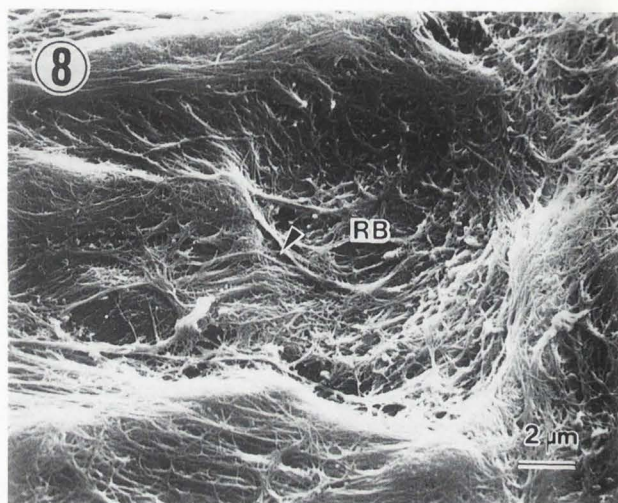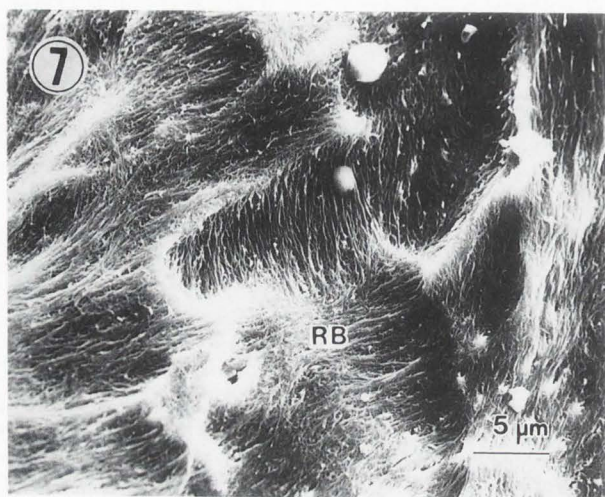**Figure 7.** Area in R-2 (reversal phase). It consists of dully edged RB whose collagen fibers run closely parallel to each other showing wavy appearances.

Figure 8. Area in R-3 (early forming phase). It has newly formed collagen fibers (arrow head) of various thickness which extend over adjacent RB forming a network.

Figure 9. Area in S-1 (late forming phase). RB are completely covered with newly formed collagen fibers of various thicknesses.

Figure 10. Area in S-2 (resting phase) (r). It consists of uniform thick bundles of collagen fibers which have a similar configuration to inert surface (IS).

Figure 11. The same part of Fig. 10 after anorganic treatment with sodium hypochlorite. Uniform thick bundles of mineralized collagen fibers covering both the inert surface and the area in resting phase were arranged in the same manner as those of the organic specimen. Inert surface (IS), resting phase (r).
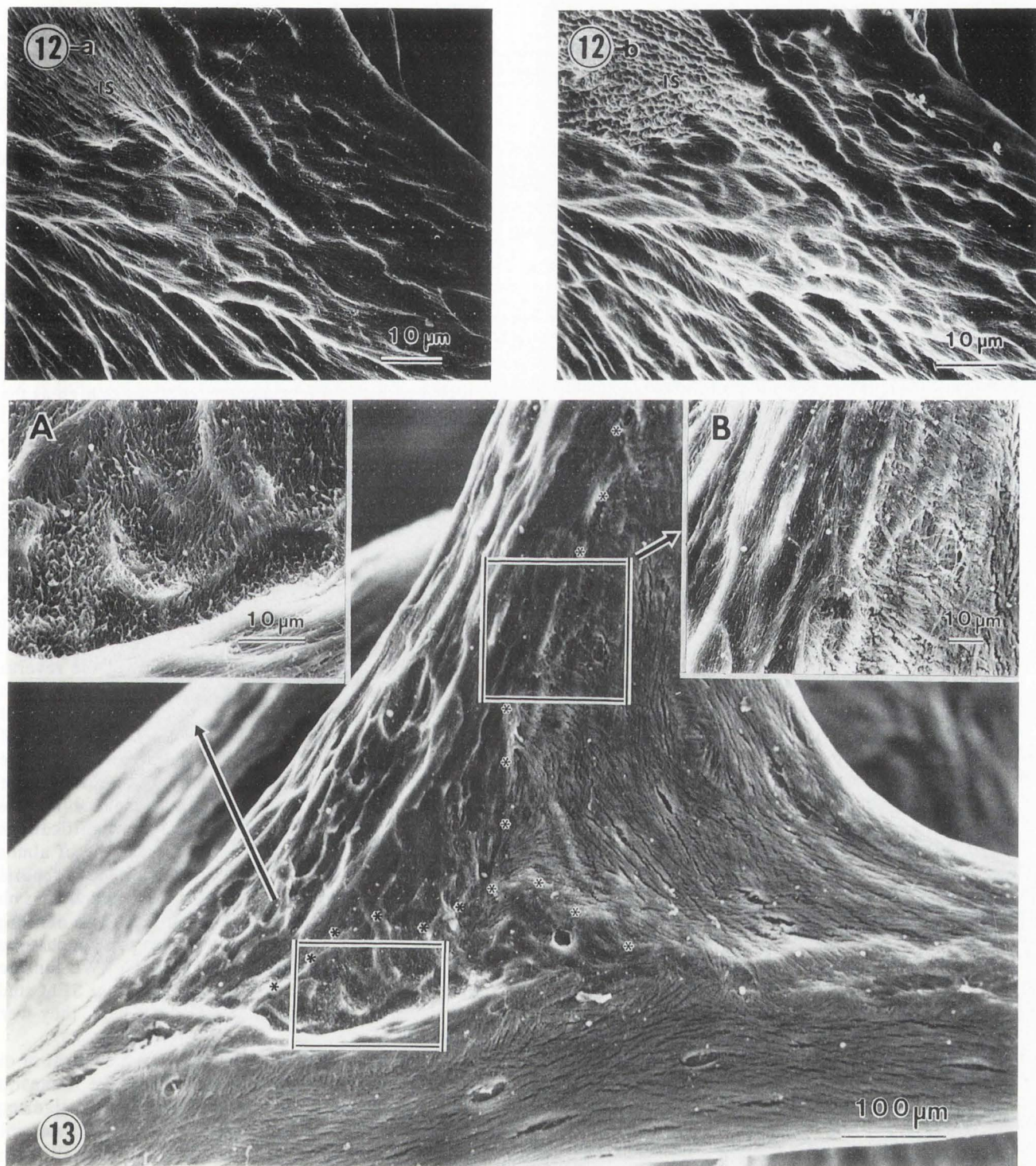
Figure 12a. A remodeling site occupied by an area in R-2 (reversal phase) alone. IS, inert surface.

Figure 12b. The same part of Fig. 12a after anorganic treatment. The surface of the area R-2 shows almost the same appearance as that of the organic specimen.

Figure 13. A remodeling site occupied by three different areas: area in resorbing phase (inset A); area in reversal phase (left side of inset B); area in late forming phase (right side of inset B) (72 year old woman). Asterisks (*) indicate the line of demarcation between the different areas.

Distribution maps of five areas

On the basis of the detailed SEM observations, the line of demarcation of the five different areas in each remodeling site could be drawn in SEM pictures (Fig. 13). By this method, it became possible to investigate the distribution patterns of five areas.

In the region near the cortico-endosteum, the frequency of remodeling sites was roughly as follows: mainly R-2 and partly R-3 (20%) > R-3 alone (18%) > mainly R-2 and partly R-3 and R-1 (13%) > mainly R-2 and partly R-1 (11%) > S-1 alone (9%) > mainly S-1 and partly R-3 (8%) > S-2 alone (7%) > R-2 alone (6%) > others (21%).

Some remodeling sites were occupied by plural areas and showed the heterogeneous composition and some were entirely occupied by a single area. The remodeling site occupied by R-1 alone was found nowhere. The partial R-1 was about 28%.

In the midway region, the order of frequency was: R-3 alone (22%) > mainly R-2 and partly R-3 (19%) > R-3 and S-1 or S-1 alone or S-2 alone or R-2 alone (10%) > others (19%)

Most of the remodeling sites were mainly occupied by R-3 alone or a combination of R-2 and R-3. The remodeling site occupied by R-1 alone was found nowhere. The partial R-1 was about 9%.

Discussion

Our results showed that the location of remodeling sites on iliac cancellous bone can be easily detected by SEM. Remodeling sites were mainly observed in the region near the cortico-endosteum. This means that the trabeculae in the region near the cortico-endosteal surface has a higher remodeling activity even in senile osteoporosis.

In a study on postmenopausal osteoporosis by bone histomorphometry, Keshawarz and Recker (1984) reported a progressive trabeculation of iliac cortical bone in the subendosteal area, and they called the trabeculated area the "transitional zone". The region near the cortico-endosteum in the present study probably corresponds to the transitional zone of Keshawarz and Recker (1984). Plate-shaped trabeculae near the cortico-endosteum may be structures which had been formed by trabeculation of iliac cortical bone.

Based on the results that remodeling sites were mainly observed around the vascular channels of plate-shaped trabeculae, we considered that an expansion of the vascular channels is one of the main factors responsible for trabeculation of cortical bone in senile osteoporosis.

The concept of ARF-remodeling sequence was first introduced by Frost (1964) and then it was modified hy Baron (1976). The sequence of Frost was: activation → resorption → formation, and that of Baron was: activation → resorption → reversal → formation.

The surface morphology of remodeling site by SEM is considered to reflect its functional state (Boyde,

1972), and therefore, in accordance with the concept of the remodeling sequence by Baron, the different areas in a remodeling site can be classified as follows, each corresponding to a different stage in the remodeling cycle.

R-1: resorbing phase (bone is being eroded by osteoclasts).

R-2: reversal phase (interval between resorption and formation).

R-3: early forming phase (initial stage of osteoid formation).

S-1: late forming phase (late stage of osteoid formation).

S-2: resting phase (resting stage after osteoid formation).

The frequency of occurrence of the remodeling site occupied by partial R-1 (resorbing phase) in the cortico-endosteum was about three times as high as that in the midway region. This result supports the conclusion that the trabeculae in the region near the cortico-endosteal surface have a higher remodeling activity.

In terms of the quantum concept (Parfitt, 1979), it has been believed that the slow reduction in mean trabecular thickness in osteoporosis occurs because osteoblasts deposit new layers of bone that are too shallow (Parfitt, 1984). Area S-2 in our study is considered to be the area where the new bone formation was incomplete and stopped. Enlargement of this area may be one cause of the reduction in mean trabecular thickness.

The previously reported features of the reversal phase (R-2) by SEM using the organic preparation method were that: the surface of the resorption cavity in reversal phase is smooth and even, without any visible collagen fibers (Mosekilde, 1990) and areas in which resorption has ceased (or is proceeding very slowly), present shallow, rather poorly defined lacunae (Boyde, 1972). Our results showed that at low magnifications, the area R-2 (reversal phase) appeared to be of almost the same configuration as those of the previous reports. However, at higher magnifications, we could always observe mineralized collagen fibers at the floors of RB in the area R-2 (Figs. 7; 12a, b). An area in which collagen fibers were not visible was not found. We consider that while thick metal coating in the previous SEM studies prevented observation of collagen fibers, they were in fact present.

Baron et al. (1981) reported that the extent of the reversal phase showed a tendency to increase in most of the osteopenic patients, thus suggesting a defective coupling between resorption and formation as a possible pathogenic mechanism in osteoporosis, and called this type of phenomenon "prolonged or aborted reversal phase". Parfitt (1983) described, in older persons (especially those with osteoporosis), a significant proportion of the total Howship's lacunar surface is covered by flat lining cells. These cells resemble those on the inert surface and so identify sites of arrested resorption (sites of permanent interruption of the normal remodeling cycle) which probably contribute significantly to the increase in total Howship's lacunar surface with age. The area

S-2 in our study is the same as the area of arrested resorption with respect to its resemblance to the inert surface, but in reality they differ much in the stage of remodeling sequence. The terms arrested resorption and aborted reversal phase have been used interchangeably to denote the same stage before bone formation. However, the area S-2 is a resting stage after new bone formation. The possibility cannot be excluded that some of S-2 areas were misinterpreted as "sites of arrested resorption".

Recently Mosekilde (1990) demonstrated the existence of a reversal phase of remodeling sites on disconnected horizontal trabeculae in a study of vertebral trabecular bone by SEM. Our results showed that remodeling site which is occupied by an area in reversal phase alone was about 10% in the midway region and 6% in the region near the cortico-endosteum. Whether the remodeling site occupied by an area in reversal phase alone indicates simply that the site is in prolonged reversal phase or is in aborted reversal phase, is still obscure and has a great importance. In order to clarify the relation of the reversal phase to bone volume loss in senile osteoporosis, further qualitative and quantitative investigations of remodeling sites based on ultrastructural observations by SEM are necessary.

As mentioned above, our modified organic preparation method has been proven to be useful for obtaining the information about the functional state of the bone. The advantages of our method are:

1) The removal of bone marrow from bone specimen can be done easily and effectively without using any special apparatus such as ultrasonicater or any chemicals such as trypsin.

2) By using the critical point drying method, the fine structure of collagen fibers is well preserved three-dimensionally without any deformation.

3) Phases of remodeling sites can be easily investigated at high magnification.

We are now going to apply this method to investigate the mechanism of bone volume loss in senile osteoporosis and other metabolic bone diseases.

Acknowledgments

References

Baron R (1976) Importance of the intermediate phases between resorption and formation in the measurement and understanding of the bone remodeling sequence. In: Bone Histomorphometry, 2nd Int. Workshop, Meunier PJ (ed.), Armour Montague, Paris, pp. 179-183.

Baron R, Vignery A, Lang R (1981) Reversal phase and osteopenia: Defective coupling of resorption to formation in the pathogenesis of osteoporosis. In: Osteoporosis: Recent Advances in Pathogenesis and Treatment. DeLuca HF, Frost HM, Jee WSS, Johnston Jr CC, Parfitt AM (eds.), University Park Press, Baltimore, pp. 311-320.

Boyde A (1972) SEM studies of bone. In: The Biochemistry and Physiology of Bone, 2nd edn., Vol. 1, Bourne GH (ed.), Academic Press, New York, pp. 259-310.

Boyde A (1984) Methodology of calcified tissue specimen preparation for SEM. In: Methods of Calcified Tissue Preparation. Dickson GR (ed.), Elsevier, Amsterdam, pp. 251-307.

Boyde A, Maconnachie E, Reid SA, Delling G, Mundy GR (1986) Scanning electron microscopy in bone pathology: Review of methods, potential and application. Scanning Electron Microsc. 1986;IV, 1537-1554.

Courpron C, Lepine P, Arlot M, Lips P, Meunier P (1981) Mechanisms underlying the reduction with age of the mean wall thickness of trabecular basic structure unit (BSU) in human iliac bone. In: Bone histomorphometry, 3rd Int. Workshop, Jee WSS, Parfitt AM (eds.), Armour Montague, Paris, pp 323-329.

Dempster DW, Shane E, Horbert W, Lindsay R (1986) A simple method for correlative light and scanning electron microscopy of human iliac crest bone biopsies: qualitative observations in normal and osteoporotic subjects. J. Bone Mineral Res. 1, 15-21.

Frost HM (1964) Dynamics of bone remodeling. In: Bone Biodynamics, Frost HM (ed.), Little Brown and Co., Boston, pp. 315-333.

Keshawarz HM, Recker RR (1984) Expansion of the medullary cavity at the expense of cortex in post-menopausal osteoporosis. Metab. Bone Dis. & Rel. Res. 5, 223-228.

Mosekilde L (1990) Consequence of the remodeling process for vertebral trabecular bone structure: a scanning electron microscopy study (uncoupling of unloaded structures). Bone and Mineral 10, 13-35.

Murakami T (1973) A metal impregnation method of biological specimens for scanning electron microscopy. Arch. Histol. Jpn. 35, 323-326

Parfitt AM (1979) Quantum concept of bone remodeling and turnover: Implications for the pathogenesis of osteoporosis. Calcif. Tissue Int. 28, 1-5.

Parfitt AM (1983) The physiologic and clinical significance of bone histomorphometric data. In: Bone Histomorphometry, Recker R (ed.), CRC Press, Boca Raton, Florida, pp. 143-223.

Parfitt AM (1984) Age-related structural changes in trabecular and cortical bone: Cellular mechanisms and biomechanical consequences. Calcif. Tissue Int. 36, S123-S128.

Reid SA (1987) Micromorphological characterization of normal human bone surfaces as a function of age. Scanning Microsc. 1, 579-597.

Wakamatsu E, Sissons HA (1966) The cancellous bone of the iliac crest. Calcif. Tissue Res. 4, 147-161.

Discussion with Reviewers

M. W. Lundy: The use of chisel to separate the biopsy would be expected to cause extensive damage to the fragile trabeculae since it breaks and crushes the bone rather than sawing through it.

C.M. Müller-Mai: Did this rather rough procedure cause artifacts (since the bone volume is reduced trabeculae break and displace)?

Authors: Bones with bone marrow are safely cut into two by using a chisel without any significant damage, though bones without bone marrow cause extensive damage. Some low speed saws are also available.

M. W. Lundy: "Few remodeling sites were observed at the ends of disconnected rod-shaped trabeculae". Resorption bays would be expected at these sites unless they are artifacts of preparation. Mosekilde (1990) described aggressive osteoclastic resorption at these sites. Are these disconnected trabeculae broken during preparation, or is formation occurring at these sites?

Authors: We think the reason why few remodeling sites were observed at the ends of disconnected rod-shaped trabeculae is that the disconnection of trabeculae occurred mainly at the beginning of postmenopause and so most of remodeling sites formed at the ends of disconnected rod-shaped trabeculae were usually repaired by the time of senile osteoporosis. Mosekilde (1990) described aggressive osteoclastic resorption at these sites. In the case of vertebrae, the disconnection of trabeculae increases gradually with advancing age. The differences between the results of Mosekilde and those of ours may be attributed to the difference in the materials used.

M. W. Lundy: An important question is whether bone remodeling occurs simultaneously or sequentially. A method to determine whether osteoclasts and osteoblasts are at the same remodeling site is needed.

Authors: We believe bone remodeling occurs sequentially (A → R → F) at the level of resorption bay, but not at the level of remodeling site. We would like to comment that both active resorption and formation occur simultaneously in one remodeling site.

C.M. Müller-Mai: Is there any confirmation of the clinical diagnosis senile osteoporosis by the pathologist?

Authors: We diagnosed our patients as having senile osteoporosis on the basis of the criteria of the Japanese Public Health Welfare Department.

C.M. Müller-Mai: The authors speculate, that the difference in the morphology of resorption cavities in reversal phase is related to the thicker sputter coating used in other experiments. Since collagen fiber bundles shown in Figs. 7 and 12a, b are easily recognized, it will require a tremendous amount of metal in the study of Mosekilde (1990) to coat the specimens and to cover the collagen totally. How do the authors relate other mechanisms, such as degree of mineralization, reprecipitation processes which were discussed in the study by Reid (1987) to cause smooth appearance of the floors of some resorption bays, and age related changes of the morphology to this observation?

Authors: As mentioned in the text, in the specimens prepared by the organic preparation method, we have always found collagen fibers on the surface of the resorption bays in the state of reversal phase. The smooth surfaced resorption bay without collagen fibers was found nowhere. On the basis of these observations, we are convinced that the smooth appearance of resorption bays in the previous SEM studies was due to the thick metal coating.

Reid (1987) reported the difference in the appearance of resorption bays between child and adult: the smooth appearance of resorption bays in the child and the rough appearance in the adult, as pointed out by the reviewer. But the findings of Reid were in the specimens prepared by the anorganic preparation method. We cannot directly relate our results to the findings of Reid, because of the different preparation methods used.

It is very important to consider the degree of mineralization or reprecipitation processes for investigating further details of functional state of bone surfaces. We are grateful to the reviewer for bringing these to our notice.