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Original Article

Diversity in intracortical remodeling in the human femoral bone: A novel view point with the morphological analysis of secondary osteons

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ABSTRACT

Introduction: In humans, intracortical bone remodeling is performed by a basic multicellular unit (BMU) composed of osteoclasts and osteoblasts penetrating through cortical bones. As a result, secondary osteons and their boundaries, cement lines, can be observed on the transverse section. There have been few reports mention whether there is diversity within a single individual and on the relevance to bone remodeling. The purpose of this study is to investigate the morphological diversity of secondary osteons in human femoral bone and to examine the relationship with bone remodeling.

Material and methods: First of all, we developed an original method to get the cross-sectional images of the cortical bones around the whole circumference for the purpose of evaluating the morphology of the secondary osteon exhaustively. Then, a total of ten cross-sectional slices from one right human femoral bone of male were prepared and stained with this method. The osteon population density and complexity of cement lines in osteons were evaluated in detail.

Results: Within this femoral bone, the osteon population density was significantly higher in the periosteal side and in the posterior area. Conversely, the cement line density and the osteon complexity were higher in the endosteal side; the proportion of complexed osteon significantly increased from the periosteal side toward the endosteal side.

Discussion: The results suggested that there were diversities in osteon population densities and osteon morphological pattern within one human femoral bone. It seemed that the BMUs ran to avoid the existing regions of osteon in the periosteal sides and to overlap the existing osteon in the endosteal sides. This seemed to be one of the novel viewpoints in the morphological analysis of secondary osteons. It might be better for the orthopedic surgeons to be aware that the osteon distribution in the cortical bone is not uniform.

1. Introduction

Bone resorption by osteoclasts and bone formation by osteoblasts are repeated throughout life, even after the growth of human bones. This series of turnover is called bone remodeling [1]. Since bone remodeling was previously considered to mainly occur in cancellous bone, research was actively performed on spinal vertebrae and other cancellous bones [2,3]. However, evidence to show that secular bone remodeling in cortical bone largely contributes to decreases in bone strength is increasing [4]. Furthermore, 70–80% of insufficiency fractures occur in cortical bone [5]. Based on these findings, the focus of bone research is shifting to analyses of cortical bones.

In humans, intracortical bone remodeling is performed by a basic multicellular unit (BMU) penetrating through cortical bones.

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Osteoclasts at the apex of BMU resorb bone parallel to their longitudinal axis. Lamellar bone is then centripetally formed from the absorption surface by a number of osteoblasts, and a Haversian canal is formed at the center. As a result, osteons, the basic structure of cortical bone, and their boundaries, cement lines, can be observed on the transverse section [1]. Although the composition of the cement line has not yet been clarified, Skedros et al. reported that it is more highly mineralized than the surrounding bone and is produced by differentiating osteogenic cells in the outermost layer of osteons [6]. Thus, osteons and cement lines are trajectories through which BMU passes, providing evidence of cortical bone remodeling in that region. There have been various reports on the morphology of secondary osteon so far [7,8], but few reports mention whether there is diversity within a single individual and on the relevance to bone remodeling. The purpose of this study is to investigate the morphological diversity of secondary osteons in human cortical bone and to examine the relationship between its diversity and bone remodeling.

2. Materials and methods

2.1. Specimen preparation-1, undecalcified polished specimens

A right human femur in the Department of Macroscopic Anatomy, Nagasaki University Graduate School of Biomedical Sciences was selected as the sample source for this study. It was obtained from the cadaver that was provided to Nagasaki University School of Medicine for anatomical dissection by medical students between the 1950s and 1970s, and it were voluntarily donated, and nowadays it is an anonymous subject. Although it was impossible to consolidate information on this bone, it was estimated anthropologically to be that of a young adult male.

The femoral bone shaft was divided into 10 strips from the center of the lesser trochanter to the adductor tubercle (blocks 1–10 from the cranial side). Six blocks from blocks 2 to 7 with sufficient thicknesses for cortical bone were evaluated.

Undecalcified polished specimens were prepared using the following procedure. The center of each block was cut out with a band saw (TBS-50, RYOBI) at a width of 5–10 mm perpendicular to the bone axis and embedded in a photopolymer resin (Technovit 7200 VLC®, Heraeus Kulzer). Embedding was performed under negative pressure to remove air in the bone, thereby allowing resin to penetrate the bone as much as possible. A fluorescent light was used for polymerization, and specimens were polymerized and cured in 2 days.

Cured specimens were adhered to an acrylic plate with an instantaneous adhesive (Aron Alpha®, TOAGOSEI) and then polished manually to a thickness of approximately 100 μm using grindstones and sandpaper (from #100 to 800, Japanese Industrial Standards).

Completed undecalcified polished specimens were designated slices 2–7, respectively.

Each slice was observed with a circular polarizing microscope (OLYMPUS BX 43, ZEISS Axio Cam Erc 5S), images were acquired, and images of the whole cross section were created using synthesis software (Image Composite Editor 2.0®, Microsoft). These images were observed using image editing software (GNU Image Manipulation Program, free software) and qualitative evaluations of osteons were performed omnidirectionally in the femoral section (Fig. 1a–c).

In the present study, osteons had Haversian canals and surrounding lamellar bone and cement lines, which are classically defined as secondary osteons. The structure considered to be a primary osteon without a cement line was excluded from the evaluation because it was not considered to be an area undergoing remodeling.

2.2. Specimen preparation-2, hematoxylin-eosin-stained specimens

In order to visualize the cement line, which is the boundary line of osteons, more clearly, undecalcified polished specimens were further demineralized with 0.5 M EDTA for 4 days and stained with hematoxylin-eosin (HE) (Fig. 1d–e). Images were acquired and synthesized in the same manner as that described above, except that a light microscope was used, and osteons and cement lines were quantitatively evaluated throughout the femoral cross section.

2.3. Qualitative evaluation of osteons in undecalcified polished specimens

According to the method of Kerley and Maat et al. [9,10], age was estimated from histological images and evaluated for contradiction as a specimen of a young adult male. We also investigated whether the sizes of osteons and Haversian canals showed regional differences.

2.4. Quantitative evaluation of osteons in HE-stained specimens

In each of the anterior, posterior, medial, and lateral compartments of the HE specimens of slices 2–7, three regions of interest were set as the periosteal, central, and endosteal sides. Twelve regions of interest were set in one slice and the area of each region was 6.5 mm^2 (Fig. 2a). We defined the osteon existing in each area as “osteon complexity” based on the number of cement lines. Grade 1 indicates osteons that have a single cement line around them regardless of their morphologies. Grade 2 indicates osteons that have two cement lines, suggesting that these osteons piled up on another osteon; in other words, intracortical remodeling occurred at an area where an osteon had preexisted. Osteons that had more than three cement lines were considered to be formed by repeated intracortical bone remodeling, and, thus, were classified as osteon complexity grade 3 (Fig. 2b). Osteon population density (number of osteons per 1 mm^2) [11], osteon complexity, and cement line density (number of cement lines per 1 mm^2) were calculated by measuring the number of identified osteons within the region of interest in each slice. Evaluations were performed on each slice in each of the three regions in the periosteal, central, and endosteal sides (combined anterior, posterior, medial, and lateral each compartment). Therefore, regional diversity in remodeling characteristics was examined in the entire femur.

2.5. Statistical analysis

The region in which osteon population and cement line densities were high was identified using SAS® (Statistical Analysis System) and by performing an ANOVA (analysis of variance) and Tukey's test, a multiple comparison test. Osteon complexity in the periosteal, medial, and endosteal sides was investigated using the Jonckheere test. The significance level was set at less than 0.05. Data were shown as the mean ± standard deviation.

2.6. Ethical approval

This study was approved by the Ethics Committee of Nagasaki University Graduate School of Biomedical Sciences (approval number: 13102353). The right and welfare of the cadaver in this study was protected by the ethical guidelines outlined in the Declaration of Helsinki.

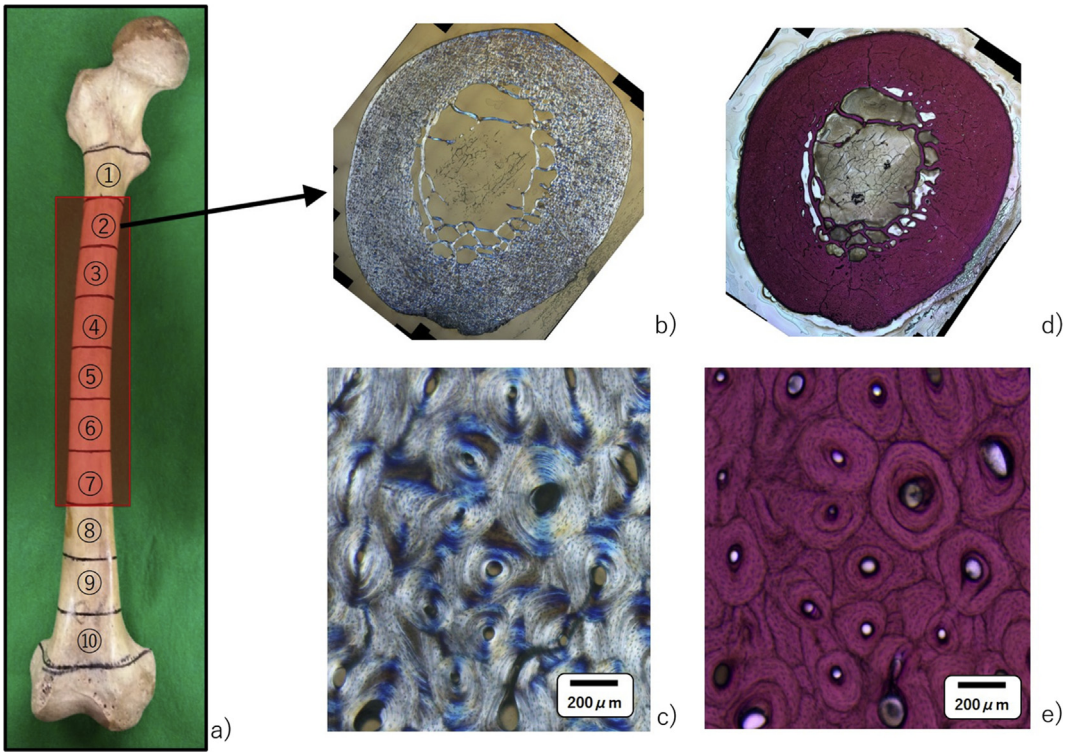

Fig. 1. a) The right femoral shaft of the adult male was divided into 10 equal parts, and cross sections at the center of each block were evaluated from slices 2 to 7. b) Circular polarized light microscopic image of an undecalcified polished specimen. Images captured under the conditions of an eyepiece lens ×10 and an objective lens ×4 are combined to obtain an overall image of the cross section. c) Highly magnified image of b), in which various forms of osteons are observed. d) Light microscopic images of specimens demineralized and stained with hematoxylin-eosin. e) Highly magnified image of d), in which cement lines are visualized and a detailed evaluation is possible.

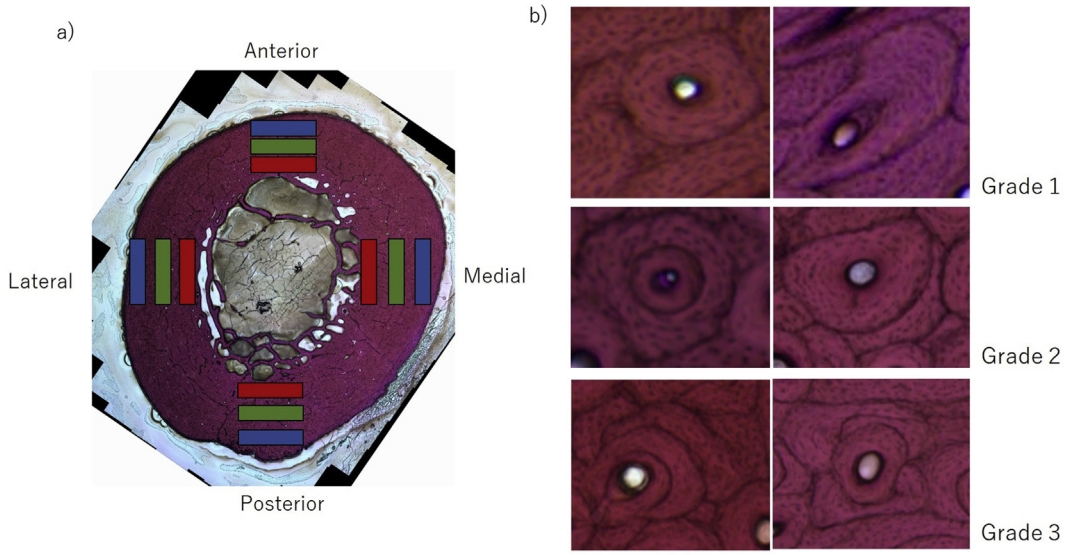

Fig. 2. a) In each slice, regions of interest in the periosteal, central, and endosteal sides were set at equal intervals of 6.5 mm at the anterior, posterior, medial, and lateral compartments. Each region of interest did not deviate outside the periosteum or medullary cavity and was set as close as possible to the periosteum and medullary cavity. b) Osteon complexity was defined according to the number of cement lines afferent to one Haversian canal. Grade 1 indicates osteons that have a single cement line around them regardless of their morphologies (upper). Grade 2 indicates osteons that have two cement lines, suggesting that these osteons are piled up on another osteon (middle). Several osteon fragments were in the background, and those with three or more cement lines were grade 3 (lower).

3. Results

An evaluation using the age estimation method of Kerley and Maat et al. revealed that the bone was from a male in his early 30s [9,10]. Osteons with small circular Haversian canals were closely packed in many images obtained near the periosteal side. In contrast, osteons with large irregular shaped Haversian canals were detected in the endosteal side, and difficulties were associated with identifying the boundary between cortical bone and cancellous bone in some regions (Fig. 3a). Furthermore, osteons were detected

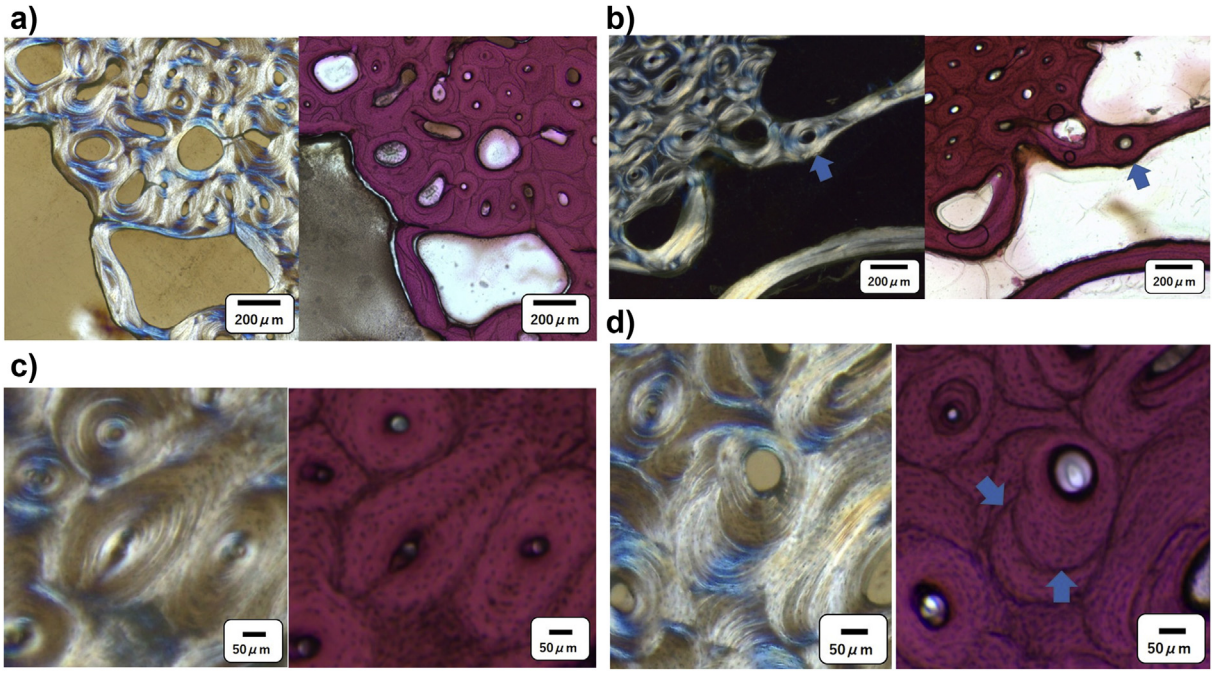

Fig. 3. a) Osteons with large Haversian canals were found in the endosteal side, and there were some regions in which it was difficult to identify the boundary between cortical bone and cancellous bone. b) An osteon is recognized in the cancellous bone-like structure (arrow). c) Drifting osteon. It has no cement line in lamellar bone. d) A circular polarized microscope image apparently showing a drifting osteon; however, a hematoxylin-eosin stain revealed a cement line in lamellar bone (arrow). This appears to be a newly appearing osteon overlapping with osteon fragments, not a drifting osteon.

within the area considered to be cancellous bone in some regions, suggesting that a site that was originally cortical bone was "trabecularized" [12] (Fig. 3b). "Drifting osteons," i.e., transverse osteons, were also found in some regions. However, a comparison of these sites with HE-stained specimens showed some osteons with overlapping cement lines. These "drifting osteons" were considered to differ from those defined by Robling (Fig. 3c,d) [13].

Osteon population densities in slices 2–7 are shown in Table 1. No significant differences were observed between slices.

An analysis of each region in the femur resulted in average osteon population densities were 10.3 ± 1.3/mm^2 in the periosteal side, 9.2 ± 0.9/mm^2 in the central part, and 8.2 ± 0.7/mm^2 in the endosteal side, and was significantly higher in the periosteal side than in the endosteal side ($p < 0.01$) (Fig. 4). The percentage occupied by osteon complexity grade 3 was compared in each region, and was 1.6% in the periosteal side, 9.6% in the central part, and 29.0% in the endosteal side. A Jonckheere test on all slices 2–7 demonstrated that grade 3 significantly increased from the periosteum to medullary cavity ($p < 0.0001$) (Fig. 5).

Cement line densities in slices 2–7 are shown in Table 2. Similar to the results obtained for osteon population density, no significant differences were observed among the slices. An analysis of each region in the femur resulted in average cement line densities were 11.5 ± 1.5 lines/mm^2 in the periosteal side, 13.7 ± 1.8 lines/mm^2 in the central part, and 16.0 ± 1.4 lines/mm^2 in the endosteal side. Cement line density was significantly higher in the endosteal side than in the periosteal side ($p < 0.01$) (Fig. 6).

Table 1
Osteon population density at each slice (n/mm^2).

	Periosteal	Central	Endosteal	Average
Slice 2	11.8	9.7	9.5	10.3
Slice 3	11.8	10.1	8.2	10.1
Slice 4	10.9	9.2	7.8	9.3
Slice 5	8.8	7.8	7.8	8.1
Slice 6	9.0	10.0	8.3	9.1
Slice 7	9.7	8.4	7.5	8.5

4. Discussion

Bone remodeling is repeated for the purpose of restoring microdamage and maintaining mineral homeostasis such as calcium [14]. Reductions in bone mass due to remodeling in cancellous bone were previously considered to play an important role in bone strength [5,15]. However, since it is now possible to analyze the cortical bone microstructure due to advances in imaging technology, intracortical bone remodeling has been receiving increasing attention. Han et al. demonstrated that more remodeling was performed in cortical bone than in cancellous bone in postmenopausal women [16]. Turnbull and Cooper et al. showed that cortical porosity and thinning due to intracortical bone remodeling resulted in reductions in bone strength, and similar findings are being increasingly reported each year [17–19]. Therefore, cortical bone remodeling is an important factor affecting bone strength.

Skedros et al. elucidated the mechanisms by which osteons as a result of remodeling are distributed in cortical bone [20]. Their findings indicated regional differences in osteon population densities and Haversian canal diameters depending on the magnitude of stress applied even in the same bone. Previous studies have mostly evaluated the porosity and osteon density of bone by examining the distribution of Haversian canals in cortical bone. However, the overlapping of cement lines, which is the trajectory of BMU, has not yet been investigated. Specifically, the BMU passage process in which the Haversian canal, i.e., the "hole" in the cortical bone, was formed remains unclear [21]. The method we established in this study may be used to evaluate not only the osteon itself, but also the cement line within the same cross section, and, thus, the running performance of BMU. For example, the drifting osteon, as proposed by Robling et al., becomes bidirectional with absorption

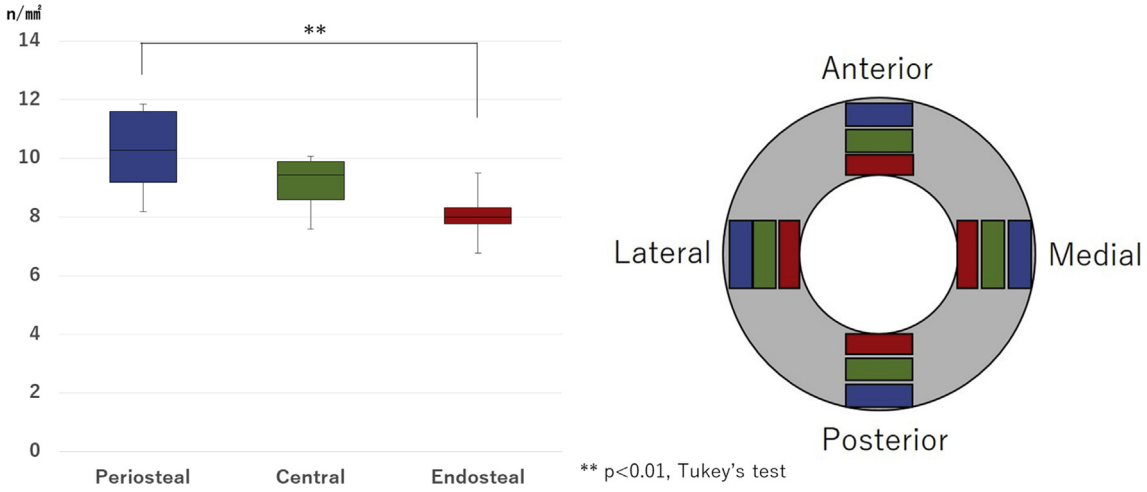

Fig. 4. Average osteon population densities in slices 2–7. Comparisons between the periosteal, central, and endosteal regions. Each region is a combination of four regions of interest: anterior, posterior, medial, and lateral, and each is 26 mm^2. Osteon population density was significantly higher in the periosteal than in the endosteal region ($p < 0.01$).

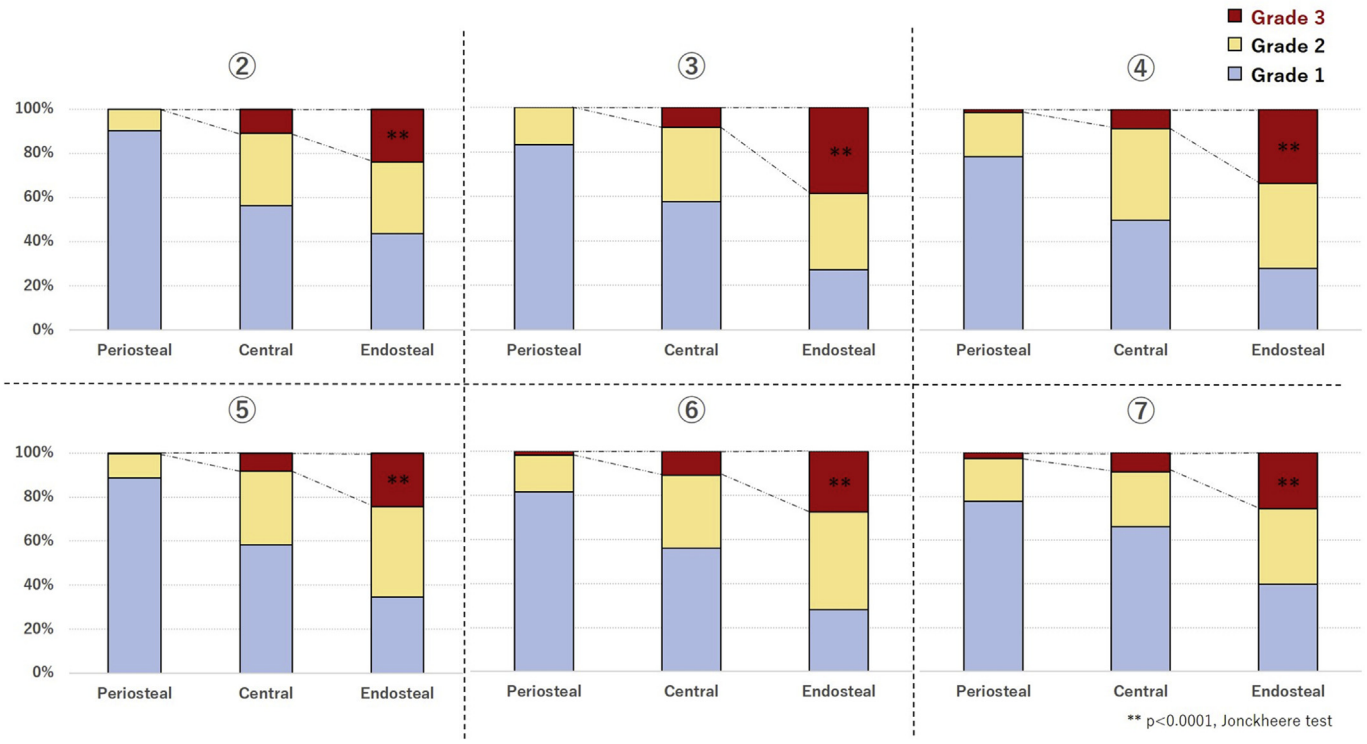

Fig. 5. The ratio of osteon complexity for each region in slices 2–7. In each slice, the proportion of grade 3 significantly increased from the periosteal side toward the endosteal side ($p < 0.0001$, Jonckheere test).

Table 2
Cement line density at each slice (lines/mm^2).

	Periosteal	Central	Endosteal	Average
Slice 2	10.9	15.0	17.1	14.3
Slice 3	11.9	14.7	17.5	14.7
Slice 4	13.4	14.6	16.0	14.7
Slice 5	9.8	11.7	14.7	12.1
Slice 6	10.8	15.4	16.7	14.3
Slice 7	12.1	12.0	14.0	12.7

surfaces at both tip and side of normally unidirectional BMUs, so that it transversely runs in the cross section [13]. It can be defined as a single bone unit because it has no cement line in the lamellar bone. As previously reported, some drifting osteons were observed by the circular polarization microscopic imaging of undecalcified polished specimen, and HE-staining of these specimens showed cement lines in the lamellae (Fig. 3c,d). These may have been formed on osteon fragments with multiple BMUs running in an overlapping manner. Such seemingly drifting osteons might include those formed with multiple BMUs running on the existing

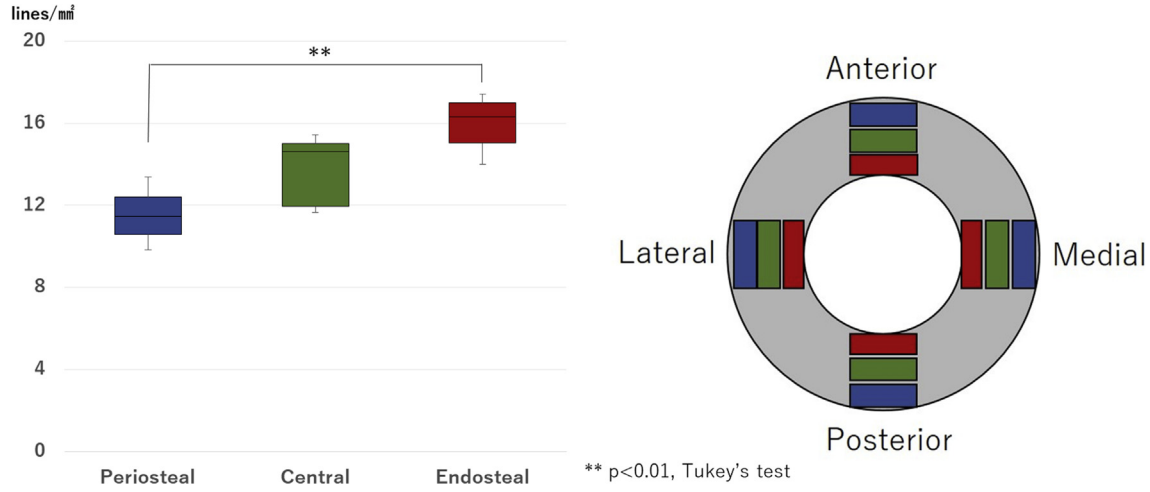

Fig. 6. Average cement line densities in slices 2–7. The method of dividing the region is the same as that in Fig. 4. Comparisons between the periosteal, central, and endosteal regions. Cement line density was significantly higher in the endosteal than in the periosteal region ($p < 0.01$).

osteons. Thus, overlapping osteon fragments in the background can be examined by visualizing the cement lines. The novelty of the study is in the analysis of regional diversity in the running performance of BMUs.

In order to investigate the running performance of BMU, we examined the regional characteristics of osteon population density and cement line density. An evaluation between the periosteal, central, and endosteal sides in the cross section revealed that osteon population density was significantly higher in the periosteal side than in the endosteal side. This result is consistent with that reported by Pazzaglia et al. in the femurs of rabbits, suggesting that humans adopt a similar remodeling format [22]. Qualitatively, osteons with small circular Haversian canals were more likely to gather in the periosteal side, while osteons with large irregular shaped Haversian canals were more frequently scattered in the endosteal side. It was consistent with the findings of Oers et al. [23].

Two mechanisms have been proposed for why osteon population densities differ between the periosteal and endosteal sides. One possibility is that repeated remodeling occurred more in the periosteal side than in the endosteal side. If BMU passes through the bone more frequently and repeats remodeling, the number of osteons inevitably formed within a single area is expected to be large.

Another hypothesis is a difference in the formation process of osteons between the periosteal and endosteal sides, namely, the running performance of BMU. If BMUs are running while "avoiding" the existing osteon regions on the periosteal side, the number of osteons will gradually increase. The osteons remain as structures depending on the number of remodeling. In contrast, if BMU cannot avoid the existing region of an osteon in the endosteal side and travels "in an overlapping manner", the existing osteon is absorbed and becomes osteon fragments, and a new osteon is formed thereon. Thus, the number of osteons remaining as structures decreases relative to the number of remodeling (Fig. 7).

Therefore, we defined "osteon complexity" based on the number of cement lines and identified each osteon, thereby indicating how many layers of osteon fragments are present in the background of the finally formed osteon. We also analyzed the distributions of osteon complexity and cement lines and examined regional diversity in the characteristics of remodeling in cortical bone. The results obtained revealed that almost no osteons of grade 3 with multiple osteon fragments stacked on the periosteal side were present; grades 1 and 2 accounted for the majority. The

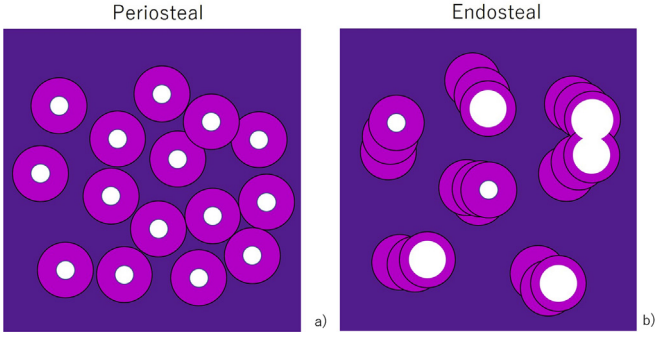

Fig. 7. Schema showing the hypothesis that regional differences in osteon population density arise due to differences in the running performance of BMU. a) If BMU runs to "avoid" the existing region of an osteon in the periosteal side, the osteon gradually increases the number gradually. b) If BMU cannot avoid the existing region of an osteon in the endosteal side and runs "in an overlapping manner", the existing osteon is absorbed and becomes osteon fragments, and a new osteon is formed on it. The number of osteons remaining as a structure is smaller than that of remodeling.

frequency of grade 3 significantly increased toward the medullary cavity. Similar results were observed in slices 2–7, over the entire length of the femur. Furthermore, a comparison of the number of cement lines in a single area in the periosteal, central, and endosteal sides showed that cement line density was higher in the endosteal side than in the periosteal side.

Since these results were in contrast to those obtained for osteon population density, the first hypothesis, osteon population density in the periosteal side increasing as a result of repeated remodeling in the periosteum side, was not supported. Therefore, these results are considered to support the second hypothesis that osteon population density differs between the periosteal and endosteal sides because of "regional diversity in the running performance of BMU". The periosteum is occupied by a high density of less complex osteons, suggesting that BMUs are running while avoiding the existing osteon regions. On the other hand, BMU did not run without avoiding the existing osteon region in the endosteal side; therefore, osteon population density may be lower and osteon complexity grade 3 occupancy may increase (Fig. 8).

Kim et al. conducted a three-dimensional construction of the Haversian canal based on tissue specimens of the rat cortical bone and analyzed it locally [24]. They found that larger Haversian canals

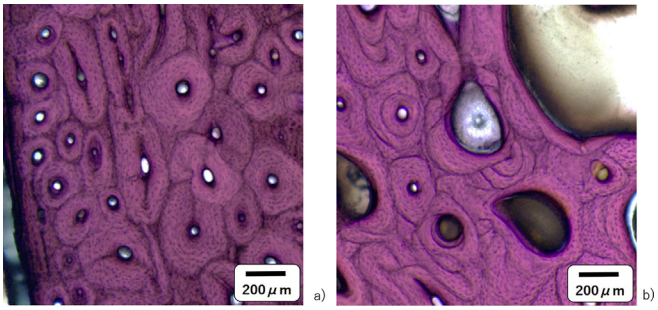

Fig. 8. a) Osteons in the periosteal side. Osteons with small Haversian canals were closely packed. The overlapping of cement lines was small, and BMU did not overlap and ran to avoid the existing osteon. b) Osteons in the endosteal side. Overlapped cement lines are scattered, which shows that BMU was running in order to overlap. Part of the Haversian canal fused and hollowed out. The Haversian canals were enlarged through partially fusion.

were more highly interconnected on the side of the medullary canal, and the periosteal side had many smaller Haversian canals and fewer interconnections than the endosteal side. Zebaze reported that Haversian canals gradually joined and cortical bone hollowing occurred when remodeling was repeated near the medullary cavity in the postmenopausal human radius [25]. As a result, the cortical bone exhibits an image of cortical remnants as if it was "trabecularized," suggesting the mechanisms of cortical porosity and thinning. Seeman described the temporal growth of osteoporotic and growing bones. Specifically, the lateral diameter of bone increases in the periosteum through continuous bone formation even after the completion of growth, while bone resorption gradually increases in the medullary cavity, resulting in the thinning of the cortical bone due to the expansion of the medullary cavity [26]. According to these findings, the lateral diameter of bone increases by continuing bone formation in the periosteal side, even after the end of growth, whereas bone resorption on the side of the medullary cavity gradually progresses, and the medullary cavity expands as cortical bone becomes thinner [27].

In the present study, osteons with large Haversian canal diameters were detected at some locations in the endosteal side, and, thus, it was difficult to identify the boundary between cancellous bone and cortical bone. In addition, considering the finding that osteons are observed in the seemingly cancellous bones, the cortical bone seems to be trabecuralized. These phenomena may be explained by the above regional differences in the running performance of BMUs. Since the histological evaluation in the present study was two-dimensionally conducted, it is superior to three-dimensional one in that the former can examine the whole circumference of a femoral cross section and the cement line over the entire length of the femur. We were able to discover the fact that there is diversity in intracortical remodeling in the femur within one individual. We think it is a novel view point with the morphological analysis of secondary osteons.

Unfortunately, it is difficult to analyze a large number of human femurs to derive new knowledge using the method of this research, as mentioned above, because of ethical restrictions. However, it is fully possible to apply the method established in this study to, for example, in vivo experiments targeting animals.

In recent years, research on osteoporosis targeting cancellous bone has made dramatic progress. On the other hand, regarding the new problem of "cortical porosity", there are many problems to be solved, such as elucidation of their pathology and construction of therapeutic strategies. This study presents a new direction in the evaluation of bone quality of cortical bone.

Once a fracture occurs in the cortex of the long bone, in many cases, surgical treatment is required and the treatment is left to the orthopedic surgeon's hands. However, with respect to the diversity of the osteon structure in the cortical bone and the cement line morphology that forms the boundary line between them, it is difficult to visualize with simple x-ray, CT, MRI, etc., which can be used in current daily practice is there. It might be better for the orthopedic surgeons to be aware that the osteon distribution in the cortical bone is not uniform. This may lead to the development of new future fracture fixation devices, new surgical methods, for example.

5. Limitations

There are several limitations in this study. First of all, more than 40 years has passed after the death of the individual. Although the material was an old femoral bone, osteons and cement lines around the whole circumference over the entire length could be evaluated clearly. Next, the material in this study was only one femur. However, under the ethical environment around the studies with human materials, it was meaningful for us to analyze the whole one bone comprehensively. We would like to emphasize that by using our established method, regional diversity in intracortical remodeling could be clarified in the femur even within one individual. We think that some elderly bones should be analyzed with the similar method in the future.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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