Osteoporotic bone microstructure by collagenase etching

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SUMMARY Collagenase etching has been used to show the microstructure of bone from patients suffering from primary osteoporosis. Both polished and unpolished surfaces of trabecular bone from femoral heads were treated with collagenase solution before study in the scanning electron microscope. The polished surfaces show the mineral component of this bone as small rounded units ~10–20 nm across, which aggregate to form a continuous phase of contiguous spheroidal particles ~100 nm across. Lamellations are clearly seen to be due to the removal of collagen fibres up to ~200 nm across, fibres in adjacent lamellae being arranged approximately perpendicular to each other. The unpolished surfaces also show small rounded units, which aggregate into rods of mineral ~100 nm across. Although these rods form a connected system, they are loosely packed, compatible with their being interspersed with the collagen fibres in vivo. This model for the detailed microstructure of bone is consistent with specimens from a number of other sources and shows no features unique to osteoporosis.

There are many published studies demonstrating the reduction in trabecular bone volume and bone mineral content in osteoporosis.^{1–5} The ultrastructure of the mineral and its relation with the collagen, however, have received little attention.

The structure of the mineral component of normal bone material has long been the subject of investigation, giving rise to a diversity of opinion. Early investigators, relying on line broadening and low angle diffraction experiments⁶ and transmission electron microscopy of thinly sectioned material,^{7–9} have suggested the presence of a variety of needle and plate shaped hydroxyapatite crystals with dimensions varying between ~2 nm and 150 nm. Recent experiments on the disaggregation of bone from a variety of sources have been interpreted as resulting from hydroxyapatite crystals in a tabular or book-like form ~45 nm long by 30 nm wide.¹⁰

On the other hand, a number of contemporary investigators have proposed models in which spheroidal shaped particles ~100 nm in diameter aggregate to form the mineral component of macroscopic bone. Boyde observed such 'spheroidal particles' in

fetal bone¹¹ and isolated similar particles from solutions of NaOCl used to wash bone and dentine.¹² Sela reported observing 'calcospherites' 100–300 nm in diameter in osteogenic tumours.¹³ Pautard isolated similar particles from 6 day old mouse calvarial bone.¹⁴ Contiguous spheroidal particles have also been noted in bovine and human compact lamellar bone following ion bombardment.^{15 16} Criticism that the techniques used for the investigation of the structure of bone mineral have damaged the mineral and hence produced artefacts led us to employ a more subtle, biochemical etching technique involving the incubation of both polished and unprocessed bone surfaces in a solution of collagenase. Collagenase etching has been successfully used in this laboratory to demonstrate the structure of bovine bone,^{17 18} fish and chicken bone,^{19 20} human bone of archaeological interest,²¹ and human bone free from disorders.²² Here we report its application to osteoporotic human bone.

Materials and methods

Samples of trabecular bone were obtained from 16 female patients aged 58–92. They were all admitted to the casualty unit following fracture of the neck of the femur. The patients were diagnosed as

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osteoporotic on the basis of radiographic and histological examination.⁵ They subsequently underwent total hip replacement.

The intact femoral heads were sectioned with a hand hacksaw in the mediolateral plane. Specimens were then removed from the centre of the head and prepared for examination in the scanning electron microscope.

Specimens were first polished using carbide papers and polishing alumina. Subsequently they were thoroughly washed in distilled water to remove any adherent alumina, then boiled in distilled water for five minutes to denature the collagen. Aliquots (5 ml) of collagenase solution were made up in a calcium containing trometamol (TRIS) buffer (pH 7.4) containing 2100 units of collagenase. Each

Fig. 1 Low magnification scanning electron micrograph of collagenase etched osteoporotic trabecular bone. The central area has been polished and shows some lamellation and in the background are unpolished surfaces.

Fig. 2 Higher magnification of the polished surface of Fig. 1 showing the lamellar structure more clearly.

Fig. 3 More detailed micrograph showing the origins of the lamellar structure. Light lamellae include large numbers of holes from which collagen fibres up to ~200 nm across running approximately perpendicular to the surface have been removed, whereas the dark lamellae have few such holes.

Fig. 4 The polished collagenase etched surface of osteoporotic trabecular bone at much higher resolution shows spheroidal shaped units ~100 nm across.

Fig. 5 The polished surface at highest resolution shows that the basic building blocks for the mineral component are small rounded units ~10–20 nm across.

Fig. 6 The unpolished surface of collagenase treated osteoporotic trabecular bone. Several osteocytic lacunae are seen and the mineral forms a pattern suggesting rods of varying preferred orientation.

Fig. 7 A higher magnification micrograph of the unpolished surface showing the mineral as loosely packed rods with numerous interconnections, forming a continuous mineral phase.

specimen was bathed in one aliquot at 37°C for one week to ensure adequate etching. After incubation the specimens were coated with gold and examined in the scanning electron microscope. The low magnification photographs of Figs 1, 2, 3, 6, and 7 were taken in a Jeol 35C operating at 30 kV. The higher resolution micrographs of Figs 4, 5, 8, and 9 were obtained on a Jeol 120C Temscan operating in the scanning mode, at an accelerating voltage of 100 kV.

Results

Figure 1 is a low magnification electron micrograph of a polished and collagenase etched section through trabecular bone. The central polished surface is evident, as are the unpolished areas beyond. Closer examination of the polished area shows lamellation, which appears as alternate light and dark areas ~10 μm wide. This lamellation can be more clearly observed in the higher magnification micrographs of Figs 2 and 3. It can be seen that the light layers show a large number of holes up to ~200 nm in diameter.

These holes indicate the in vivo position of the collagen, which exists in the form of fibres of up to this diameter. The mineral component is shown to be a continuous phase surrounding the holes vacated by the collagen. The mineral phase consists of contiguous spheroids ~100 nm across. At higher magnification (Fig. 4) these spheroids are seen to be composed of smaller units. In the high resolution picture of Fig. 5 these smaller units are observed as rounded particles ~10–20 nm in diameter. At this resolution micrographs of the light and dark areas look essentially the same.

Figure 6 is a low magnification micrograph of an unpolished surface of a collagenase treated trabecula. Osteocytic lacunae are evident, and the pattern of the mineral can be observed to vary over the bone surface. On closer examination (Fig. 7) the hydroxyapatite is seen to be in the form of rods, which are interconnected forming the continuous mineral phase. The structure is not tightly packed but shows clearly the areas from which collagen fibres have been removed. At yet higher magnification (Fig. 8) these rods can be seen to be ~100 nm in

Fig. 8 The unpolished surface of collagenase etched osteoporotic trabecular bone at high magnification shows a complex plexiform of mineral rods and suggests that the mineral rods are composed of still smaller units.

Fig. 9 The highest resolution micrograph of the unpolished surface showing that the mineral rods ~ 100 nm across are in turn composed of smaller rounded units ~ 10 – 20 nm across.

diameter, and in the very high resolution micrograph (Fig. 9) the ultimate units are visualised as the same type of rounded particles (~ 10 – 20 nm across) as observed in the polished sections (Fig. 5).

Discussion

The classical model for the structure of bone mineral and its relation with the collagen envisages a mineral phase consisting of discrete needle or plate-like crystallites intimately linked with the tropocollagen molecules. Recent investigations in this laboratory^{17–22} have proposed a model for bone in which the mineral exists in the form of a continuous phase composed of spheroidal particles of ~ 100 nm in diameter, which are themselves made up of smaller units ~ 10 – 20 nm across.

This latter model is more readily reconciled with the known mechanical properties of bone than the former classical model, in which the mineral is said merely to stiffen the collagen. The failure of this model becomes apparent if a bone which has been

heated to a temperature above which collagen denatures is subjected to mechanical testing. If the mineral merely stiffened the collagen one would expect the denaturation of the collagen to totally disrupt the mechanical integrity of the bone. This, however, clearly does not occur.

The holes observed in the mineral phase are produced by the removal of the collagen owing to the action of collagenase, and therefore indicate the *in vivo* position and direction of the collagen fibres. The lamellation observed in the polished sections is reminiscent of the lamellar structure of bovine secondary osteons, which have been studied in some detail using the same technique.^{17, 18} It has been shown that the lamellation in bovine secondary osteons is a result of the direction of the collagen fibre orientation changing through approximately 90 degrees from one lamella to the next.¹⁸ Although such detailed examination has not been carried out on this human material, it is reasonable to assume that the structural basis for the lamellation is similar.

It appears from examination of micrographs of

the unpolished surfaces (Figs 8 and 9) of this osteoporotic bone that the mineral phase is in the form of columns with the collagen interdispersed between them. This is compatible with the polished sections through trabeculae (Figs 4 and 5), in which the mineral is observed to form a continuous phase around the collagen fibres. All these pictures are directly equivalent to those previously obtained on normal human trabecular bone from patients with no known bone disorders.²²

The results of this investigation of the microstructure of trabecular bone derived from patients suffering from primary osteoporosis support the more recent model for the structure of bone. The detailed mineral structure is similar to that observed in bovine bone,^{17 18} fish bone,¹⁹ chicken bone,²⁰ and normal human bone.²²

Conclusions

Bone mineral obtained from patients suffering from primary osteoporosis was found to exist as a continuous phase consisting of columns of mineral interdispersed with collagen. The mineral columns are ~100 nm in diameter and appear to be of roughly circular section. They are composed of an ordered arrangement of smaller units ~10–20 nm in diameter, which appear to be the basic building blocks of bone mineral. There is no apparent difference between normal and porotic bone at this ultrastructural level.

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