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NEW BONE FORMATION IN AN OSTEOPOROTIC PATIENT TREATED BY INTRAOSSEOUS INJECTION OF BIOACTIVE MATERIALS; A CASE REPORT

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Abstract

We have been investigating the hypothesis that injection of a material which is both osteoconductive and osteoinductive, into the head, neck and trochanteric region of the hip in elderly osteoporotic patients, may lead to new bone growth and thereby strengthen the bone and reduce the risk of subsequent fracture.

A mixture of hydroxyapatite (HA), calcium sulphate (CS) and growth hormone (GH) was injected through a small drill hole into the unfractured proximal femur of women having surgery for contralateral hip fracture.

One patient died four months after such an injection due to an unrelated cause. It was possible to obtain her hip for further examination by scanning electron microscopy (SEM) and X-ray microanalysis.

Abundant new bone formation across the 4.5 mm drill hole was seen by SEM. There appeared to be a gradation of change from the centre, where there was a fine network of new bone between the HA particles, to the periphery of the hole, where dense new woven bone engulfed or bridged across injected HA particles and also linked across to old trabeculae. X-ray microanalysis was used to confirm the composition of the newly-formed material.

Key Words: Osteoporosis, hydroxyapatite, calcium sulphate, growth hormone, bone formation, scanning electron microscopy, X-ray microanalysis, intraosseous injection.

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Introduction

The incidence of osteoporotic fractures in the elderly is rising. They are estimated to cost the U.K. National Health Service over £600 million per annum (Dixon, 1992). These fractures are associated with significant morbidity and mortality.

Osteoporosis leading to reduced bone strength is one of the factors responsible for hip fractures in the elderly. It is generally believed that once an elderly person has become osteoporotic there is no opportunity for that bone to recover, and therefore in the elderly prevention of fracture is directed towards prevention of falls. Treatment for osteoporosis focuses on the perimenopausal population, in the hope that hormone replacement will prevent further reduction in bone mass.

It is possible however that in the elderly osteoporotic, local injection of osteoinductive and osteoconductive factors might induce new bone growth in locally weak areas to improve their strength, reducing the risk of subsequent fracture.

Bone graft substitutes are now being increasingly used to reconstitute bony defects. Hydroxyapatite (HA) along with calcium sulphate (CS) has been shown to be an osteoconductive material, acting as a mineral scaffolding into which new bone can grow. This has been used to reconstruct various skull, zygomatic and mandibular defects (Georgiade *et al.*, 1993) and also to fill simple bone cysts (Inoue *et al.*, 1993). When HA is used to coat implants such as the femoral component of a hip replacement, there is clear evidence to show that bone growth occurs into the HA layer (Furlong and Osborn, 1991).

Animal studies have shown that HA is replaced by bone even in non-stress-bearing skeletal tissue. When implanted directly onto the surface of the skull in cats, foci of bone have formed at the interface between the bone and the implant without any foreign body reaction, extrusion, infection or toxic reaction (Costantino *et al.*, 1991). HA was implanted into the frontal sinus of nine cats, and the whole sinus became progressively replaced

with woven bone over 18 months without loss of its volume (Friedman *et al.*, 1991).

The ability of bone to grow into a block of HA and to bond with it depends on the surface area of the HA, and therefore its porosity (Schliephake *et al.*, 1991). There is therefore a possible advantage of combining HA and CS. A mixture of HA and CS and distilled water forms a paste which can be injected into the bone. It sets rapidly like plaster of Paris, but the CS is resorbed *in vivo* leaving a porous HA with a large surface area for osteoconduction. Najjar *et al.* (1991) demonstrated that the addition of CS to HA improved its working properties without adversely affecting its osteointegration. The composite showed a higher rate of bone ingrowth than HA alone.

Certain proteins have been shown to possess the capability of inducing bone formation. These osteoinductive factors include the family of bone morphogenetic proteins, growth hormone (GH), insulin-like growth factors 1 and 2 (IGF-1 and 2) and transforming growth factor-ß.

GH is a potent anabolic hormone for calcium and bone metabolism. Decreased osteoblastic activity is of significant importance in the pathogenesis of post-menopausal and senile osteoporosis. GH stimulates osteoblastic proliferation and differentiation *in vitro* and increases the production of IGF-1 and 2. This has a profound stimulatory effect on osteoblasts and is important for regulation of bone remodelling. It also affects other osteotropic hormones (Brixen *et al.*, 1993).

Recombinant GH is now being used therapeutically to correct GH deficiencies. GH has also been used in the treatment of osteoporosis by subcutaneous injections and results suggest that it can increase bone density (O'Halloran *et al.*, 1993; Thoren *et al.*, 1993). We are not aware of any study in the treatment of osteoporosis that advocates the intraosseous use of GH.

Use of osteoconductive and osteoinductive materials together have been shown to be promising in the reconstruction of bony defects (Damien *et al.*, 1990).

We have therefore been investigating the hypothesis that injection of these materials, into the head, neck and trochanteric regions of the unaffected proximal femora in osteoporotic women who have had a contralateral hip fracture, may lead to new bone growth and thereby strengthen the bone and reduce the risk of subsequent fracture. A clinical trial is now under way; this paper presents a case report of the findings from the first treated hip we have been able to retrieve at post-mortem.

Materials and Method

The material injected consists of HA, CS and GH. We have obtained injectable grade HA (Fig. 1) and CS (Fig. 2) from Plasma Biotal U.K. Ltd. The HA was sintered and the size of the particles was 180 to 300 μm. In order to obtain an average space of about 250 μm between HA particles to facilitate bony ingrowth, various combinations of these materials mixed with water have been studied by scanning electron microscopy. It has been possible to obtain a satisfactory mixture of biomaterials using 22 g HA, 14 g CS and 15 ml distilled water; this produced about 21 ml of reasonably runny paste which set (*i.e.*, became a mass that did not flow) after about 7 minutes.

4 units of GH was added to the mixture of HA and CS; this was stored at 4°C in theatre and mixed with water just before the injection.

Method

With the help of an X-ray image intensifier, a 4.5 mm diameter hole was drilled into the trochanteric, cervical and head regions of the femur, the entry point being in the soft cancellous part of the greater trochanter (Fig. 3). The biomaterials were injected into this hole using a specially made instrument.

When HA and CS are mixed with water, it initially forms a paste and after a few minutes it sets into a hard mass. The material was injected into the bone while it was still a paste and, under pressure, it flowed into the hole and surrounding trabecular spaces. It was possible to inject about 6 to 8 ml of the material into the hip.

One of the treated women died 4 months after the injection was carried out, due to an unrelated cause, at the age of 95. It was possible to obtain the injected hip for further examination. A CT scan of the hip showed that the material had spread well beyond the drilled hole. The extreme end of the hole in the head had not filled with any of the injected material and this acted conveniently as a control.

After removal from the patient, the hip was stored at -20°C until processing was commenced. Approximately 8 mm thick sections were sawn across the neck perpendicular to its long axis. These were frozen in liquid nitrogen and fractured across the injected material to reveal a surface undamaged by sawing (Fig. 4). The pieces were then placed in a mixture of sodium dodecyl sulphate (1% w/v) and proteinase K (1 mg/ml) at 37°C for 24 hours to digest organic material. After 3 washes of distilled water, remaining lipid was removed by 4 changes of acetone followed by 4 changes of diethyl ether over several days. Residual vapours were removed by drying at 60°C for 30 minutes and pumping in a vacuum down to 10^{-5} mbar. The pieces were trimmed prior to mounting on Al stubs with colloidal silver adhesive, sputter-coated with 20 nm platinum and examined in a JEOL JSM-35CF SEM operated at an accelerating voltage of 10 kV. Subsequent to imaging it was

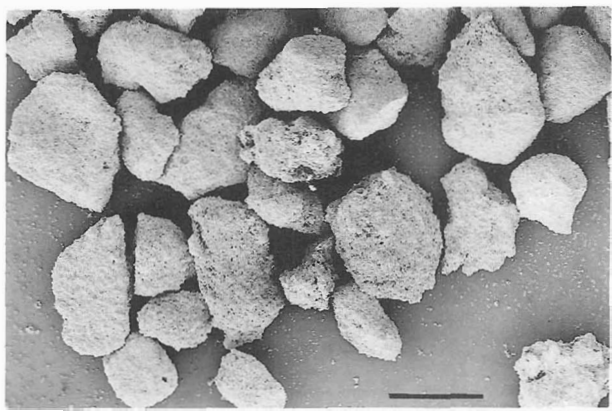


Figure 1. The HA particles injected. Bar = 300 μm

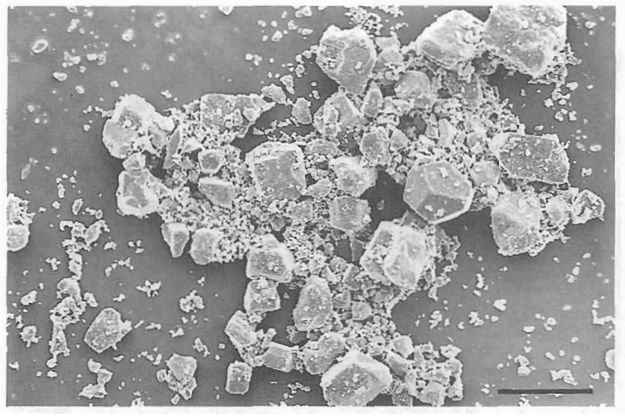


Figure 2. The CS crystals injected. Bar = 50 μm.

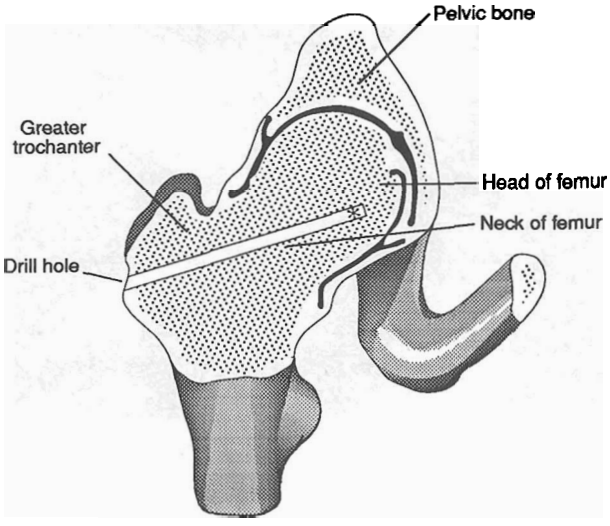


Figure 3. Diagram illustrating site of the hole drilled in the femur to inject the materials. Asterisk indicates where the injected material did not penetrate the extreme end of the hole in this patient.

realised that X-ray microanalysis might be useful although these unrepeatable specimens had not been ideally mounted or coated for that technique. Qualitative X-ray microanalysis was carried out using a Philips XL20 SEM equipped with Oxford Instruments Link eXLII X-ray system fitted with a Pentafet thin window detector capable of detecting elements down to Boron (Z = 5).

Particulate samples (for Figs. 1 and 2) were suspended in isopropyl alcohol and a drop of each suspension allowed to dry on 9 mm diameter glass coverslips. These were mounted, sputter-coated and examined as above.

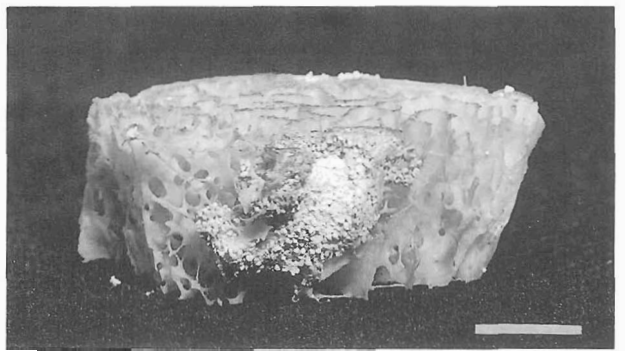


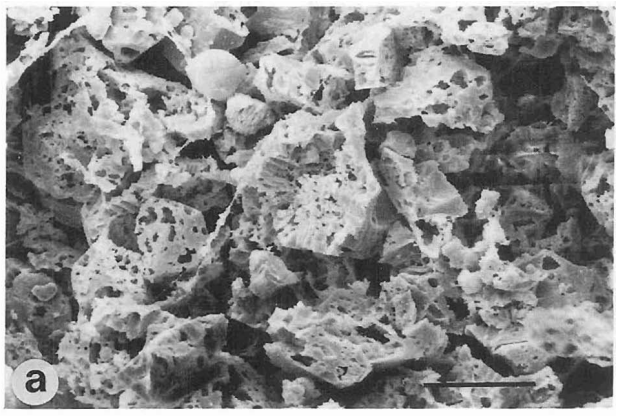
Figure 4. Macro-photograph of a freeze-fractured bone section showing the injected material within osteoporotic trabecular bone. Bar = 5 mm.

Results

Several changes had occurred in and around the injected material when examined by SEM and X-ray microanalysis.

Most of the injected particles of CS had disappeared. It was possible to identify some of the remaining crystals (Fig. 5a) by X-ray microanalysis (Fig. 5b). The surface showed an eroded appearance (Fig. 5a *cf* Fig. 2). In some places there were aggregates of crystals having a flake-like appearance (Fig. 6). X-ray microanalysis confirmed these to contain calcium, sulphur and oxygen. It is known that CS can occur in several crystalline forms, and this new crystalline form had developed *in vivo*.

The particles of HA did not show any changes in their appearance. Several particles appeared to be nearly engulfed by new bone formation (Fig. 7) towards the periphery of the injected mass.



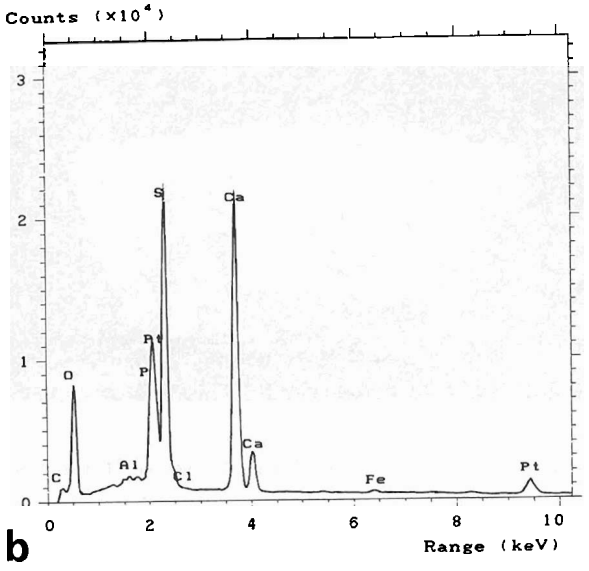


Figure 5. (**a**) CS crystals with an eroded surface. Bar = 20 μm. (**b**) X-ray spectrum confirming that these eroded crystals are composed of calcium sulphate.

New bone formation was evident in and around the injected material. In the centre of the injected material, some areas showed a fine net-like structure surrounding HA particles (Fig. 8). In places this net coalesced to form bridges between HA particles (Fig 9a.). X-ray microanalysis (e.g., at point 1 on Fig. 9a) showed these bridges were mineralised, having a similar chemical composition to bone (Fig. 9b *cf* Fig. 12); point 2 on Fig. 9a gave no significant X-ray signal. Towards the edge of the injected mass the nets coalesced into sheets of calcified material (Fig. 10). Near the edges of the drill hole the newly-formed bone was even more dense (Figs. 7 and 11a); its calcium content was confirmed by X-ray mapping (Fig. 11b). An X-ray spectrum of new bone (Fig. 12a) was found to be indistinguishable from that of old trabecular bone (Fig. 12b). The appearance

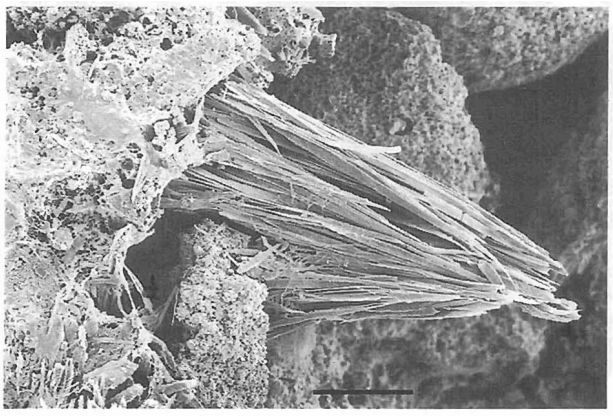


Figure 6. Aggregation of crystals having a flake-like appearance. Bar = 100 μm.

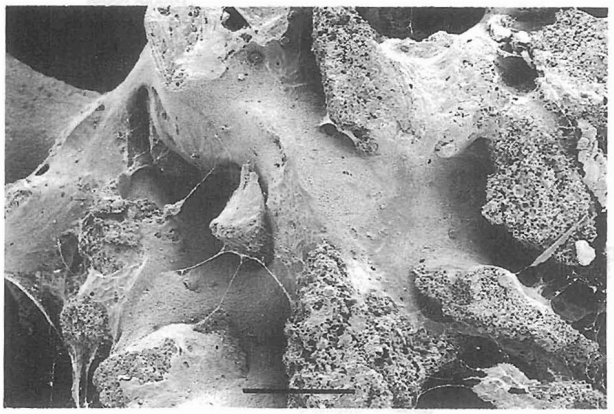


Figure 7. HA particles becoming engulfed by new bone. Bar = 200 μm.

Figure 8. Fine network of calcified material on the surface of a HA particle. Bar = 30 μm.

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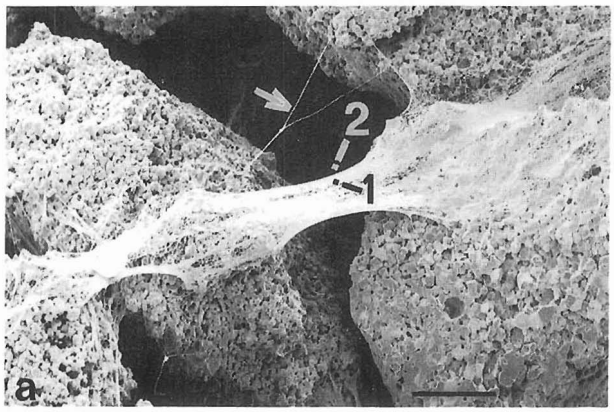
of the surface of new bone at high magnification (Fig. 13a) showed a similar woven texture to that of old bone (Fig. 13b). It was also possible to identify bunches of



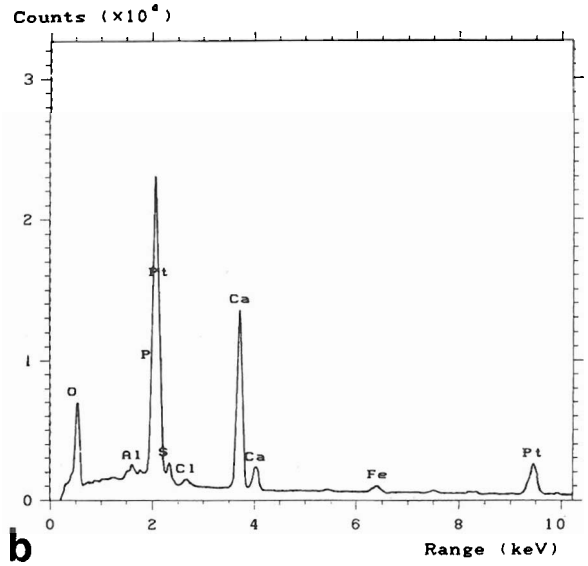


Figure 9. (**a**) A coalesced net of calcified material forming a bridge between HA particles. The X-ray spectrum shown in (**b**) was obtained from point 1; no significant X-ray signal could be obtained from point 2. Arrow indicates the single thread which was stable in the focussed beam (see **Discussion**). Bar = 50 μm.

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small crystals in localised areas within the network of new bone (Fig. 14). X-ray microanalysis confirmed that this area contained only calcium, phosphorus and oxygen with no evidence of sulphur or carbon. Although the platinum and phosphorus peaks are not resolvable, in bony areas containing phosphorus the size of this combined peak relative to the size of the calcium peak was much higher than in other areas where it was due to platinum alone, for example, compare Figure 5b with Figures 9b, 12a and 12b. This may represent an early stage of bone formation. It was possible to visualise clearly the close association between new bone and the HA particles (Fig. 15).

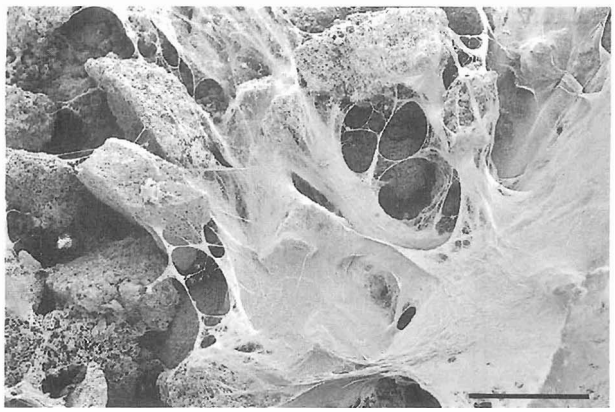
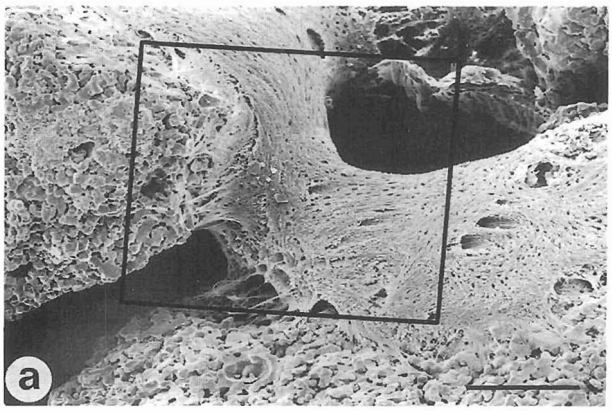


Figure 10. Network of calcified material coalesced into extensive sheets. Bar = 300 μm.



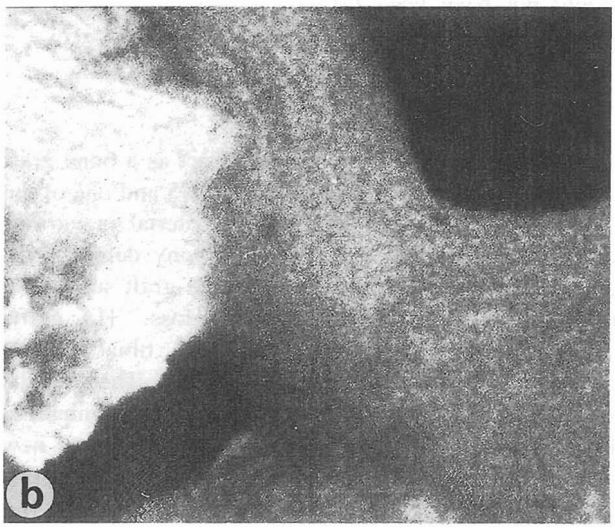


Figure 11. (**a**) Bridge of dense new bone between HA particles. Bar = 50 μm. Box indicates the area of the calcium X-ray map shown in (**b**).

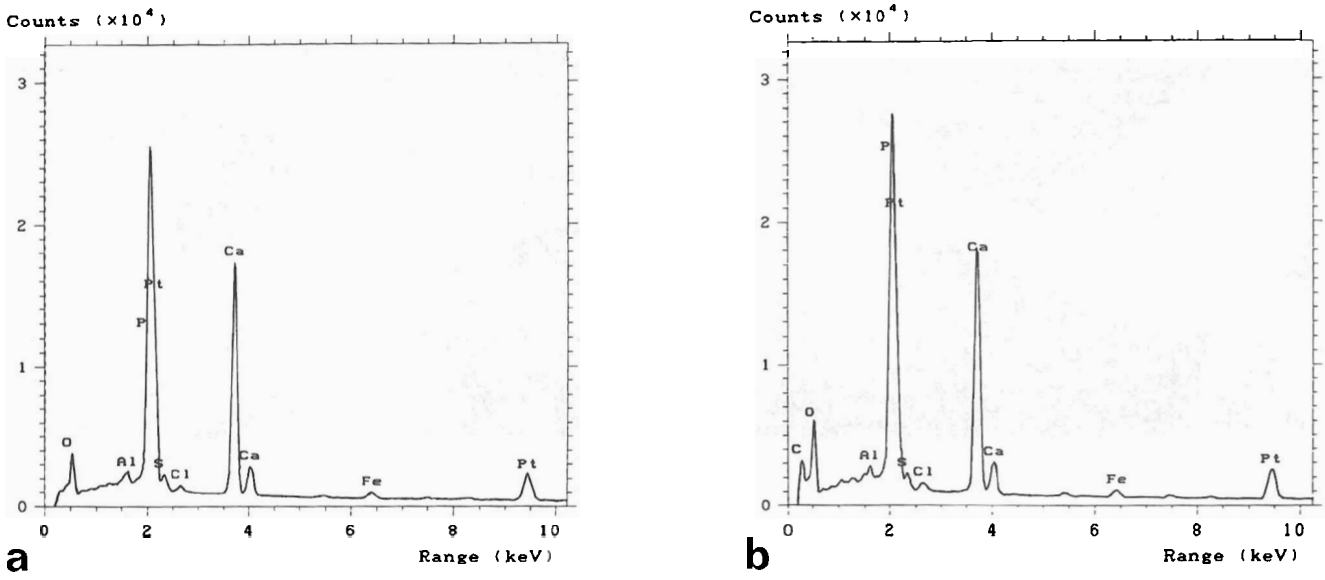


Figure 12. X-ray spectra of (a) dense new bone and (b) old trabecular bone.

There was evidence of new bone formation in some places across the entire width of the 4.5 mm drill hole where injected material was present, suggesting that it is possible to bridge a gap of that size with this treatment in 16 weeks. The extreme end of the drill hole in the head had not filled with the injected material in this patient (see Fig. 3). Although there was a thin layer of new bone formation around the periphery of the hole (Fig. 16), the gap remained unbridged by new bone. Being immediately adjacent to the filled region, this provided a control suggesting that the 4.5 mm hole would not have been bridged with bone at this time without the injection of bioactive materials.

Discussion

HA is now increasingly used to act as a bone graft substitute usually in combination with CS and one of the many osteoinductive agents. The material in various combinations has been used to fill bony defects with good results. Following trauma, bone graft substitutes have been used to reconstitute bone loss. HA grafts have been used to restore depressed tibial plateau fractures (Itokazu and Matsunaga, 1993). Their results showed no difference in knee function when comparing HA graft substitute with bone graft. There was new bone formation on closed biopsy at six months. This suggested that it is possible for new bone formed in HA grafts to remodel and become a functional weight-bearing component. Furthermore, when implanted into canine long bones, HA increases the compressive strength of cancellous bone and the bending strength of cortical bone (Martin *et al.*, 1989).

In osteoporosis, the bone is weak at least partly because of decreased density. It would therefore seem appropriate to try to increase its bone content thereby making it stronger to avoid fractures; this is the underlying purpose of the present project.

In the specimen studied, obtained 16 weeks after injection, there was abundant new bone formation across the 4.5 mm drill hole, engulfing the particles of HA and linking with the peripheral trabeculae. The CS had been largely resorbed. There appeared to be a gradation of change from the centre of the injected mass, where there was a fine network of mineralised material around and between the HA particles, to the periphery of the hole, where there were thick bridges and sheets of new bone. The proximal uninjected part of the drill hole acted as a convenient control, and demonstrated that without the injected material, apart from a thin peripheral rim of new bone, the hole remained unbridged. It is possible that GH could have diffused into this region from the adjacent injected mass, but even if this had occurred, it is evident that by itself this was not enough to stimulate sufficient new bone growth to bridge the hole.

Clearly, once these unrepeatable specimens had been prepared for imaging, as described, they were not in an ideal condition for subsequent X-ray microanalysis. Nevertheless, we believe that the limited conclusions we have drawn from the major peaks of the spectra obtained regarding the qualitative composition of the structures observed provide useful additional information, particularly about the mineralisation of the network, sheets and bridges which precede recognisable bone. The fact that

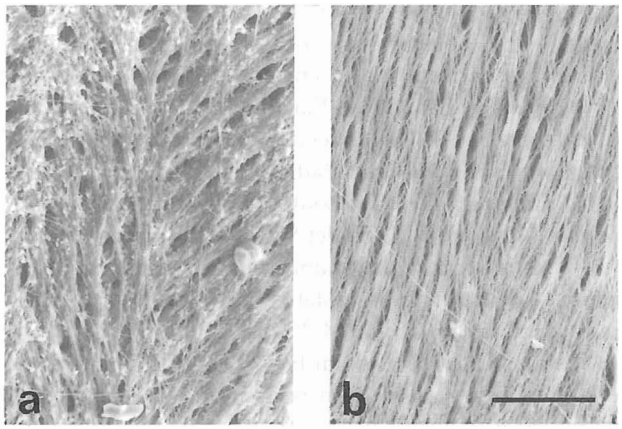


Figure 13. Woven texture of the surface of (**a**) dense new bone and (**b**) old trabecular bone showing their similarity. Bar = 10 μm.

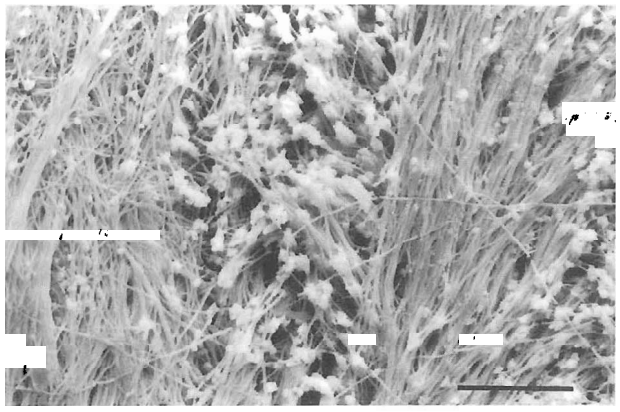


Figure 14. Localised area of small crystals within the network of new bone. Bar = 5 μm.

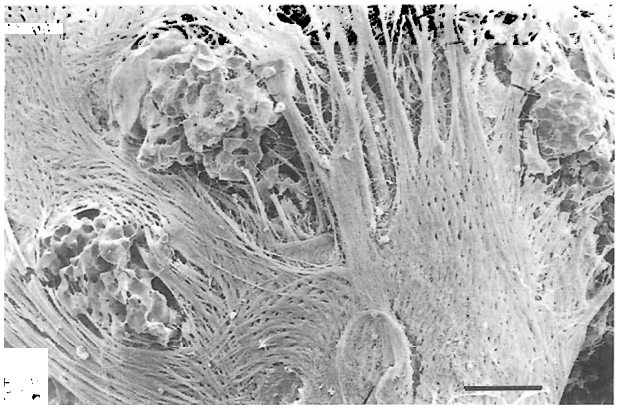


Figure 15. Dense new bone on the surface of a HA particle. Bar = 30 μm.

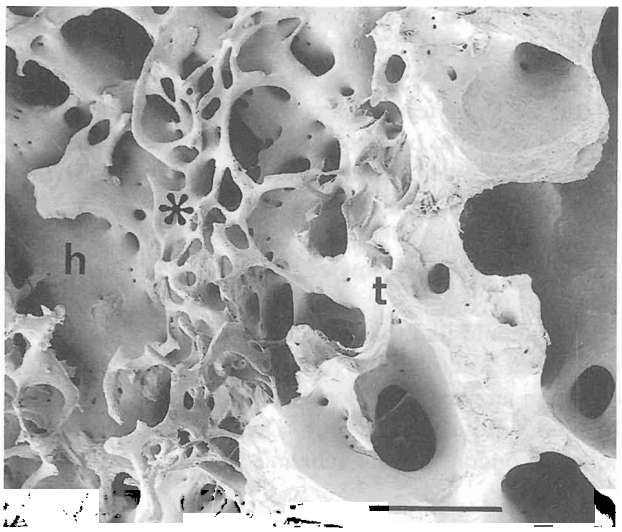


Figure 16. New bone formation (*) at the boundary between old trabecular bone (t) and the hole (h) where it did not contain injected material. Bar = 1 mm.

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we obtained the spectrum in Fig. 9b, from point 1 in Fig. 9a and no significant signal at all from point 2 in Fig. 9a shows that the beam was well collimated. The high levels of signal obtained could not be due to background fluorescence, and if a bridge, such as shown in Fig. 9a had been organic, it would not have stimulated a high calcium signal from the surroundings.

That these network structures were indeed mineralised is further supported by the fact that if the beam was focussed on a single thread (such as indicated by arrow in Fig. 9a) it was quite stable, whereas, if that is done to a single filament of organic material, such as collagen, it breaks and curls up or evaporates.

Our interpretation is further supported by the developmental progression observed between the centre and the periphery of the injected mass. After injection of the biomaterials, any cells interacting with it would have to infiltrate from the surrounding marrow, so one might expect more advanced development at the periphery. We did observe fine network structures (Figs. 8 and 9a) at the centre, more extensive coalesced sheets (Fig. 10) further out, and recognisable well-developed bone (Figs. 7 and 11a) at the periphery.

The results from this patient suggest that a mixture of HA, CS and GH is a potent stimulus to new bone formation in the elderly osteoporotic femur. We are not able to determine the relative importance of the three components. The HA, however, appears to be an important scaffolding on which new bone will quickly bond. Although CS had largely resorbed in 16 weeks, it may have provided a useful high concentration of

calcium ions and an appropriate pore size between the HA particles. GH may have been a significant osteoinductor.

In the short term this composite injected material has been shown to be a potent stimulus to new bone formation, may open new methods of treatment for this common clinical problem.

Acknowledgements

We should like to thank Dr. P. Carter, Department of Medical Microbiology, University of Aberdeen for advice on enzyme digestion of bone organic matter and Prof. A. Boyde, Anatomy Department, University College London, for his help and advice.

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Discussion with Reviewers

C. Scotchford: It is well known that bone mineral and related hydroxyapatites undergo dissolution and recrystallization when exposed to aqueous media. Considering the aqueous preparation techniques employed in this study how do the authors support the following observations as being *in-vivo* formations rather than preparative artifact? **a)** "It is known that CS (calcium sulphate) can occur in several crystalline forms and this new crystalline form had developed *in vivo*"; and **b)** "It was possible to identify bunches of small crystals in localised areas within the network of new bone".

Authors: Bone marrow is an aqueous environment. Bone mineral and hydroxyapatites have very limited solubility in aqueous media (estimates of the solubility product of HA range from 10^{-54} to 10^{-60}). In the unlikely event of dissolution and recrystallization occurring during preparation, one might expect such crystals to be randomly scattered over the specimen surface rather than be localised in very discrete patches as described here.

C. Scotchford: The use of platinum for sputter coating appeared to be an unfortunate choice in that it partly masked the P Kα peak. Although the X-ray microanalysis spectra for new and old bone did not appear different, do the authors feel that variations in the Ca/P ratios between new and old bone may have been masked by this choice of coating?

Authors: Platinum coating was chosen for imaging before there was any intention of performing X-ray microanalysis. It is possible that the choice of coating could have masked variations in the Ca/P ratio, but we have

made no attempt in this study to characterise new or old bone by their Ca/P ratio.

J.D. de Bruijn: In all X-ray microanalysis spectra, a distinct peak of Al is present. Since this element is known to potentially inhibit apatite formation [Blumernhat *et al.* (1984) Calcif Tiss Int **36**: 439-485; Sevenson *et al.* (1992) Arch Toxicol **68**: 706-712; Stea *et al.* (1992) Biomaterials **13**: 664-667], it might have adverse reactions on bone formation. Could this Al be derived from any of the biomaterials used; in other words, have the authors performed X-ray microanalysis on the starting materials or on the injected paste?
Authors: The authors have performed X-ray microanalysis on plaster composed of the injected materials and no aluminium signal was detectable. As described in **Method** above, with the original intention of imaging only, these specimens were mounted on aluminium stubs which may be the source of the Al peaks seen in our spectra.

J.D. de Bruijn: Results of a case report are presented and the conclusions are that (i) the CS may have provided a useful high concentration of calcium ions and an appropriate pore size between the HA crystals, and (ii) the GH may have been a significant osteoinductor. As these are all suppositions, have the authors performed any other (animal) experiments to determine the relative importance of the three separate components?
Authors: These suppositions are based on a considerable literature. There is no appropriate animal model for post-menopausal osteoporosis in humans.

J.D. de Bruijn: The fine network (Fig. 8) and sheets of calcified material (Fig. 10) seems to represent residual connective tissue. Have the authors performed control experiments with the de-organizing solution to be sure that all organic material is digested? If the network and sheets were bone mineral, which contains significant amount of carbonate, carbon should be present in the X-ray microanalysis spectrum.
Authors: The efficacy of the proteinase-K/sodium dodecyl sulphate/solvent treatment used to remove organic matter was proved by the fact that no trace of organic residue from the marrow could be detected between the trabeculae of the uninjected bone by extensive searching in the SEM.

The small carbon signal which might be expected from bone would probably be largely absorbed by the platinum coating. The fine network was only found in close association with injected HA particles. Our evidence for believing this network to be mineralised matter rather than organic was based upon: (i) the strong calcium X-ray signal (Fig. 9b); (ii) its stability in a focussed beam (see **Discussion**); and (iii) the developmental sequence from fine net (in the centre of the injected mass) to recognisable bone (at the periphery of the injected mass; see **Discussion**).

However, since writing this paper we have examined samples from a second treated patient which confirm and amplify all findings reported in this paper, including the presence of a fine network. In these new samples, prepared for X-ray microanalysis with conventional carbon coating, the fine network gave a calcium peak ten times greater than from the immediately adjacent background (analogous to points 1 and 2 on Fig. 9a). This confirms unequivocally that the fine network is calcified; if it were composed of purely organic material (with carbon coating) it would not be possible for the resulting lower atomic number X-ray energies to fluoresce a calcium signal from the surroundings.

J.D. de Bruijn: Figure 6 shows crystals with a needle-shaped appearance, which are derived from calcium phosphate in the injectable substance. It is generally known that needle-shaped crystals give rise to a strong foreign body response, which would not be beneficial for bone formation. Do the authors have any information regarding the cellular response to this injectable substance, and what cellular response would they expect to be invoked by these crystals, if any?
Authors: The crystals shown in Figure 6 were flake-shaped and gave a X-ray spectrum of calcium sulphate (see above, not phosphate); presumably they are a form of gypsum. We do not have any information about the cellular response as all the organic material was digested out of these specimens prior to examination.

J.D. de Bruijn: The authors state that the small crystals present in Figure 14 only contain Ca, P and O, but not S (sulphur) or C (carbon), and are suggestive of an early stage of bone formation. Studies by Davies *et al.* [Cells and Mater **3**, 245-256, 1993] and de Bruijn *et al.* [Cells and Mater **3**: 115-127, 1993] have indeed shown that small mineralised globular accretions, with a diameter of 1 μm, are formed in the early stages of bone formation. Besides carbonate-apatite crystals, these globules are also composed of glycosaminoglycans (proteoglycans) and other sulphated proteins. All major proteoglycans that have been isolated from bone contain chondroitin sulphate, and several have a strong affinity for apatite [Sodek *et al.* In: The Bone-Biomaterial Interface, Davies JE (ed), pp 97-110, 1991]. Since demineralisation is needed to extract most of these proteins from bone tissue, one would expect sulphur to be present in the X-ray microanalysis spectrum, as a result of these proteins. Furthermore, carbon would be expected to be present as a result of the carbonate-apatite crystals.

Both elements, however, were not detected. Can the authors comment on this apparent discrepancy?
Authors: We do not know if the small crystals we observed in human bone are the same as the small globules reported by Davies *et al.* (1993) and de Bruijn *et al.* (1993) with cultured rat cells. The X-ray spectrum we obtained from a localised area with a cluster of crystals was indistinguishable from that obtained from an adjacent area without crystals; as stated above, the only significant peaks present in both were Ca, P and O. We would not expect to see a large carbon peak because of the absorption by the platinum coating.

J.D. de Bruijn: Our own experience with X-ray microanalysis on gold coated samples is that a considerable reduction of other X-ray signals occurs, especially in the case of lighter elements. For example, we are not able to detect C and O when we sputter coat with gold, although the latter element is detectable when we carbon-coat our samples. This reduction in intensity of X-ray signals is known to be the result of the relatively thick (approximately 6 nm!) gold layer and the high atomic number of gold. You have used a distinctly thicker sputter coated layer of platinum (approximately 20 nm thick), with an atomic number close to gold, and were able to detect very light elements, such as carbon and oxygen. Therefore, does a platinum sputter coated layer (of 20 nm) indeed allow light elements to be detected, or is your sputter coated layer significantly less thick than 20 nm. Please comment.

Authors: We only observed small peaks of oxygen and very small peaks of carbon. We believe X-ray signals from lighter elements would have been significantly reduced by the platinum coating. Its thickness was measured with a Balzers QSG 301 quartz crystal thickness monitor.

J.D. de Bruijn: Can the authors comment on the clinical applicability of this technique and its success thus far, as indicated by results from other injected patients with osteoporosis?
Authors: We are in the early stages of a clinical trial injecting this material into the contralateral femoral head of elderly women who have already suffered a fractured neck of femur, with the aim of delaying or preventing a second hip fracture. None of the 15 patients injected so far has suffered from any complications. To date, no patient has suffered a fracture of the treated hip. It is too early to assess fully the clinical applicability of this technique at this stage.