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Ultrastructural identification of cells involved in the healing of allogeneic bone grafts augmented by basic fibroblast growth factor

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Abstract
This study was designed to identify the phenotype of cells involved in the healing process of allogeneic bone grafts augmented by basic fibroblast growth factor (bFGF). Eighteen critical size (15×10mm) defects were created bilaterally on rabbit mandibles. Three groups of six defects each were grafted with allogeneic bone alone, allogeneic bone and demineralized bone matrix prepared from intramembranous bone (DBM_{IM}), or allogeneic bone and bFGF reconstituted in DBM_{IM}. Cellular identification was carried out at three weeks postgrafting by light and electron microscopy. The results showed that there were many bone appositional cells and capillaries across the whole defect where the abundant newly formed bone were evident in allogeneic IM bone and bFGF in DBM_{IM} grafts. In conclusion, composite allogeneic IM bone-bFGF-DBM_{IM} graft healed through an intramembranous ossification route, which was greatly accelerated by the presence of bFGF and DBM_{IM} through recruitment of osteoprogenitor cells, differentiation and proliferation of osteoblastic cells, and capillary invasion.
Key words: allogeneic bone graft; bFGF; cell identification; TEM

Introduction

The use of bank bone allografts in endoprosthetic surgery is gaining importance, particularly in mandibular reconstruction, where host bone cannot be obtained[1].Fresh–frozen allogeneic bone offers an alternative to autogenous bone with the advantage of little immunologic response and unlimited quantities from bone banks [2]. However, compared with autogenousbone, fresh-frozen allogeneic bone shows disadvantages such as less revascularization[3], slower new bone formation[4] and lack of integration with the host bone. Recent work in our laboratory explored a new graft material that eliminates the need to harvest autogenous bone from patients[5]. In that research, composite graft material, consisted of allogeneic intramembranous (IM) bone, similar to that obtained from bone banks and mixed with demineralized bone matrix prepared from IM bone (DBM_{IM}), and enriched with human recombinant basic fibroblast growth factor (hrbFGF), produced 550% more new bone than allogeneic intramembranous (IM) bone alone in a rabbit mandibular defect model[5]. This graft material showed faster integration with host bone. We hypothesize that the success of this graft material over allogeneic bone grafts might result from an active cellular response in the healing process. Advances in bone cell

biology have recognized that active growth and morphogenetic factors can modulate bone cell's function[6],[7]. Systemic administration of bFGF has been shown to stimulate endosteal lamellar bone apposition in rat long bones through an increase in the number of osteoblastic cells[8]. It has been reported that DBM implanted in cranial defects directly induces the proliferation and differentiation of primitive mesenchymal stem cells to osteoblasts and the formation of bone [9],[10]. Furthermore, the healing of autogenous IM bone grafts in both the presence and absence of DBM_{IM} in the rabbit calvarial defects has been carefully examined[9], and it was found that both autogenous IM bone grafts and IM bone grafts in the presence of DBM_{IM} healed directly through an intramembranous ossification route, where preosteoblasts, osteoblasts and osteocytes were observed in bone apposition that did not pass through a cartilaginous intermediate stage[9]. Moreover, DBM_{IM} was found to accelerate the appearance of those osteogenic cells as well as blood vessels[11]. To understand the mechanism of healing of allogeneic bone mixed with bFGF we need to identify the cells involved in its healing process and compare that to the healing of allogeneic bone graft alone. Therefore, the purpose of the current study was to identify the phenotype of cells involved in the early healing process of allogeneic bone grafts augmented by bFGF using morphological evidence at light microscopic and ultrastructural levels.

Material and methods

Animals and materials

Twenty-eight adult New Zealand White rabbits, 5-12 months old that weighed 3.5-4.0 kg, were used in this study. The project was approved by the Committee for the Use of Living Animals in Teaching and Research at the University of Hong Kong (CULATR No.368-99). Nine rabbits were used as we previously described for preparation of fresh-frozen allogeneic IM bone graft from bilateral mandibles[5]. Ten rabbits were used for preparation of DBM_{IM} powder with the same procedures as previously reported[5]. The remaining nine rabbits were divided into three groups. Two defects per rabbit were created in the bilateral mandibles making a total of eighteen defects. These three groups of six defects each were grafted with fresh-frozen allogeneic IM bone alone, fresh-frozen allogeneic IM bone and DBM_{IM} powder only, and fresh-frozen allogeneic IM bone and bFGF (Sigma) reconstituted in DBM_{IM} powder.

Surgical procedures

The surgical procedures were the same as previously described[5]. In brief, after being anesthetized, each rabbit underwent a central incision in hemicervical regions. A critical size 15×10 mm ostectomy, which did not interrupt the mandibular continuity, was created bilaterally in mandibular body. Two bone grafts were implanted in the mandibular defects bilaterally in per rabbit. Holes were drilled in the graft and host bone to allow for fixation of the bone grafts with stainless steel wires. In allogeneic bone graft group, the fresh-frozen allogeneic IM bone was placed into the defect and fixed. In the composite allogeneic IM bone + DBM_{IM} graft group, 500 mg DBM_{IM} powder was placed in each defect with allogeneic IM bone. In the composite allogeneic IM bone + bFGF + DBM_{IM} graft group, 1250 ng hrbFGF reconstituted with 500 mg DBM_{IM} powder was placed in each defect with allogeneic

IM bone. All wounds were closed with 3-0 nylon sutures. No attempts were made to suture the periosteum. Three weeks later, animals were sacrificed and tissues were harvested.

Tissue preparation for light and electron microscopy

The defect areas, approximately 15×10mm, including the surrounding tissues, were harvested for histological preparation. Tissues were fixed in 4% paraformaldehyde, decalcified in EDTA, double-embedded in celloidin-paraffin, cut into 5 μm thick sections, and stained with hematoxylin and eosin. Histological sections were used to locate the area of the interface between the graft and the host bone, and the area of the middle on the tissue blocks selected for transmission electron microscopy. The areas of interest were dewaxed, post-fixed in 1% osmium tetraoxide, trimmed into 1 mm blocks, and then embedded in resin. Semi-thin sections (1 μm thick) from the resin block were sectioned and stained with toluidine blue for further orientation and reduction of the block surface. Ultra-thin sections (90 nm thick) were cut with a diamond knife in an ultramicrotome (Reichert Ultracut S, Leica AG Reichart Division, Austria) and mounted on 200 metal mesh grids. Sections were then stained with uranyl acetate and lead citrate for observation in a transmission electron microscopy (TEM; EM208S, Phillips Electron Optics B.V., Achtseweg Noord 5, 5651 GG Eindhoven, the Netherlands).

Results

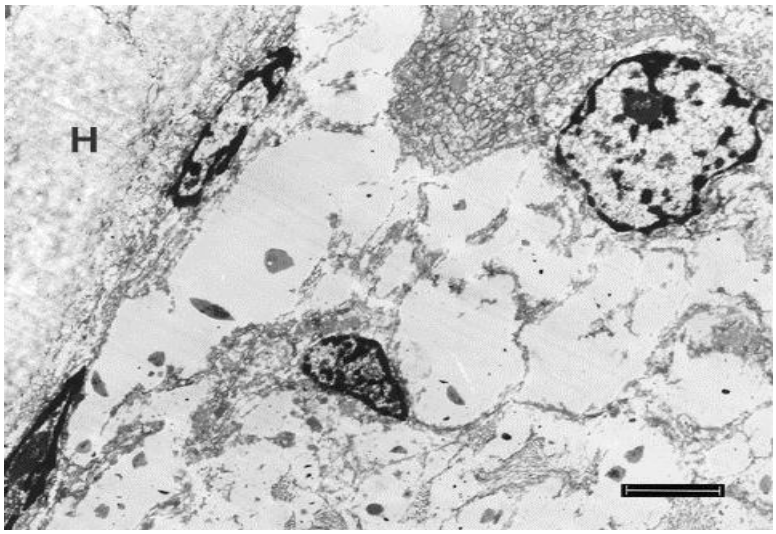
Allogeneic IM bone graft group. A few osteoprogenitor cells were only seen near host bone (Figure 1a), where only a small amount of new bone was evident (Fig I). The undifferentiated mesenchymal cell appeared as flattened cell with elongated nuclear and very thin cytoplasmic process was present near the allogeneic bone graft (Figure 1b).

Allogeneic IM bone graft+ DBM_{IM} group. More new bone was interposed between DBM_{IM} particles on the side of bone graft and host bone (Fig II). Bone-making cells including undifferentiated mesenchymal cells, pre-osteoblasts and osteoblasts were evident near the new bone matrix based on DBM_{IM} particle, showing the involvement of blood vessel (Figure 2a). Higher magnification showed active osteoblast with prominent rough endoplasmic reticulum (rER) interposed between newly formed matrixes, which was present on resorbing DBM_{IM} particle (Figure 2b).

Allogeneic IM bone graft+ bFGF in DBM_{IM} group. Abundant new bone was localized to both host bone-graft interfaces and across the defect (Fig III). Abundant osteogenic cells including undifferentiated mesenchymal cells, pre-osteoblasts and osteoblasts were present in the bone marrow and new bone matrix in presence of capillaries (Figure 3a). Higher magnification showed full osteoblasts with profiles of prominent rough endoplasmic reticulum (rER), Golgi complex, mitochondria and other subcellular organelles, and the matrix newly synthesized together with presence of capillaries (Figure 3b). Capillary was presented by the migration of prevascular and vascular endothelial cells into bone apposed sites (Figure 3c). Higher magnification showed the vascular endothelial cells are thin and flattened, with flattened, crescented nuclei with much condensed chromatin, and a thin layer of cytoplasm around the

lumen with few mitochondria or rER, and a large number of pinocytotic vesicles (Figure 3d). A lineage of osteogenitor cells can be seen in bone marrow, including undifferentiated mesenchymal cells, pre-osteoblasts, osteoblasts and osteocyte, without a cartilage intermediate stage. The capillaries and blood vessels were also involved in the bone-making process (Figure 3e). Higher magnification showed the newly embedded osteocyte in the lineage (Figure 3f) and the front of mineralisation evident to two full osteoblasts on the surface of bone cells (Figure 3g). Another active osteocytic osteoblast newly entrapped by the matrix that itself has synthesized became a young osteocyte with large area of rough endoplasmic reticulum and Golgi complex (Figure 3h).

a)



b)

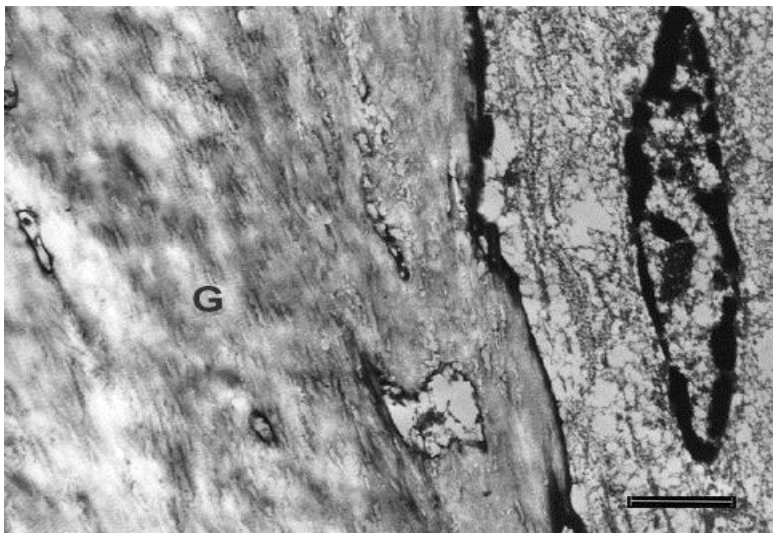
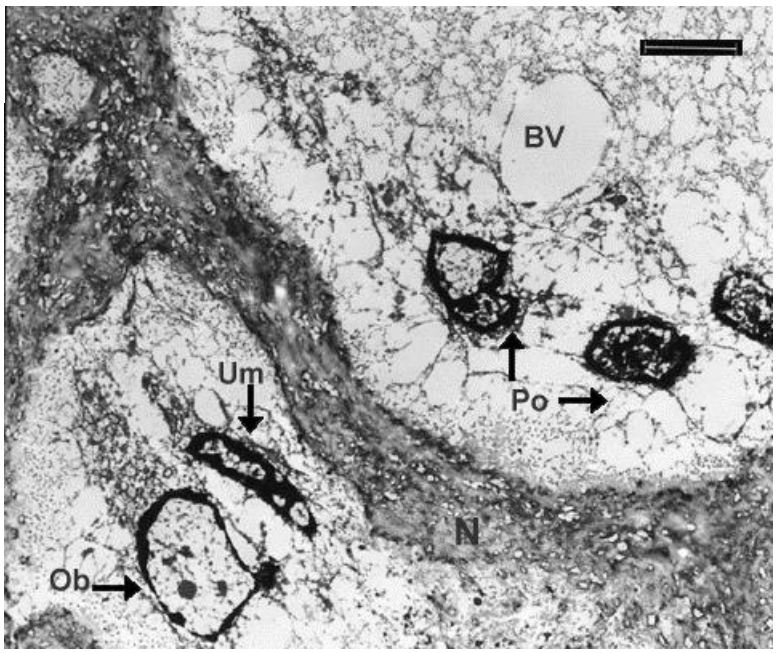


Figure 1. Electron micrograph of cells involved in the healing of allogeneic bone. **a**) A few osteoprogenitor cells were only seen near host bone (H) (scale bar: 2µm, ×5,200). **b**) An undifferentiated mesenchymal cell appeared as flatten cell with elongated nuclear and very thin cytoplasmatic process was present near the allogeneic bone graft (G) (scale bar: 1µm, ×11,500).

a)



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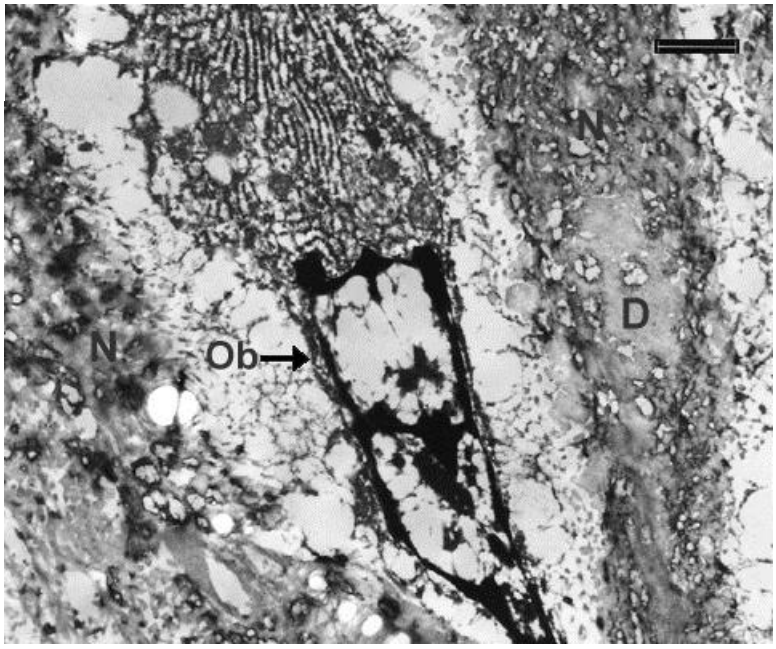
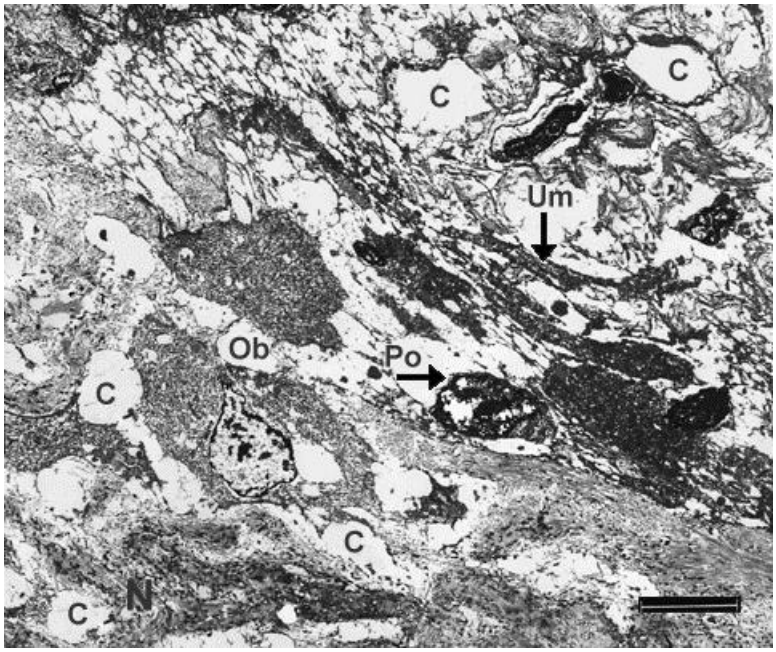


Figure 2. Electron micrograph of cells involved in the healing of allogeneic bone+DBM_{IM} group. **a**) Bone making cells including undifferentiated mesenchymal cells (Um), pre-osteoblasts (Po) and osteoblasts (Ob) were evident near to new bone matrix (N) based on DBM_{IM} particle, showing the involvement of blood vessel (BV) (scale bar: 2µm, ×5,200). **b**) Higher magnification showed the active osteoblast (Ob) with prominent rough endoplasmic reticulum was interposed between newly formed matrix (N) which was based on resorbing DBM_{IM} (D) particle (scale bar: 1µm, ×8,900).

a)



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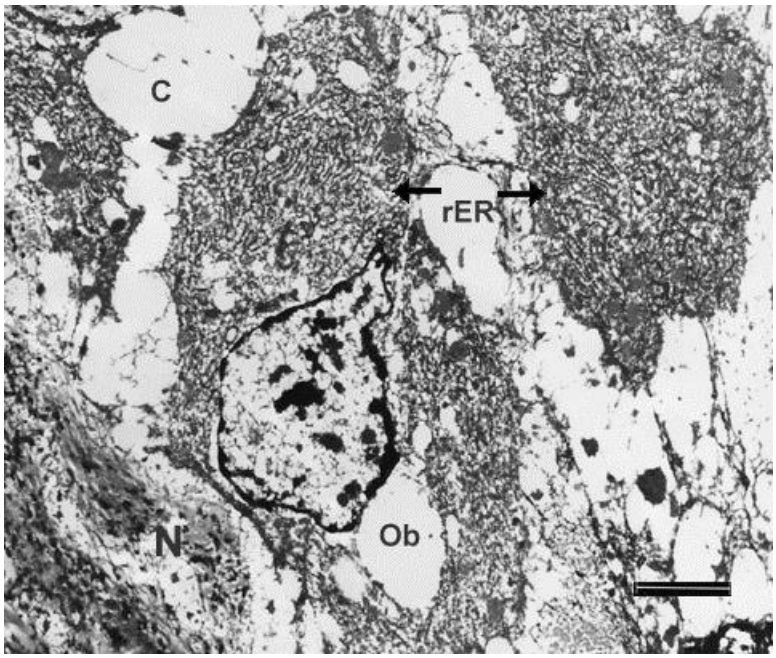
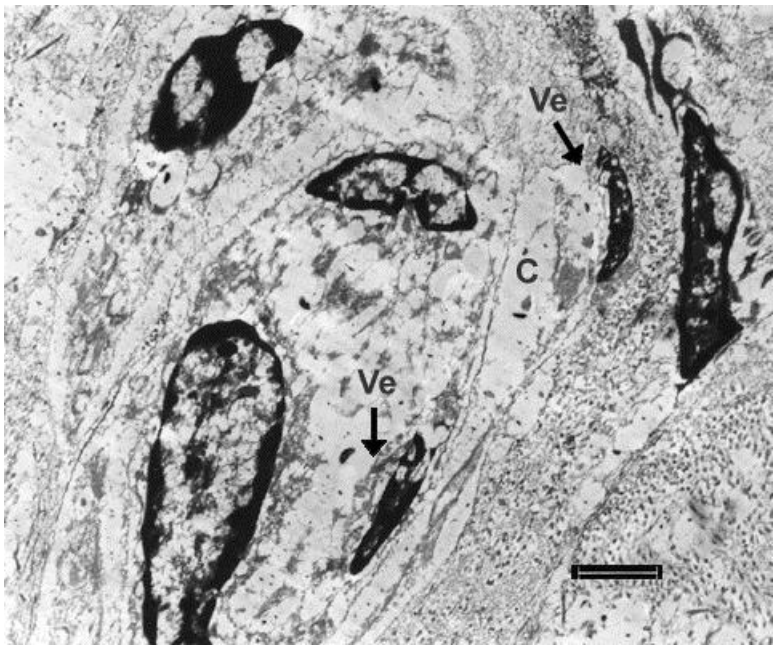


Figure 3. Electron micrograph of cells involved in the healing of allogeneic bone+bFGF in DBM_{IM} group. **a**) Abundant osteogenic cells including undifferentiated mesenchymal cells (Um), pre-osteoblasts (Po) and osteoblasts (Ob) were present in the bone marrow and new bone matrix (N) in presence of capillaries (C) (scale bar: 5µm, ×2,200). **b**) Higher magnification of the full secretory osteoblast (Ob) with profiles of prominent rough endoplasmic reticulum (rER), Golgi complex, mitochondria and other subcellular organelles, and the matrix (N) newly synthesized together with capillary (C) invasion (scale bar: 2µm, ×5,200).

c)



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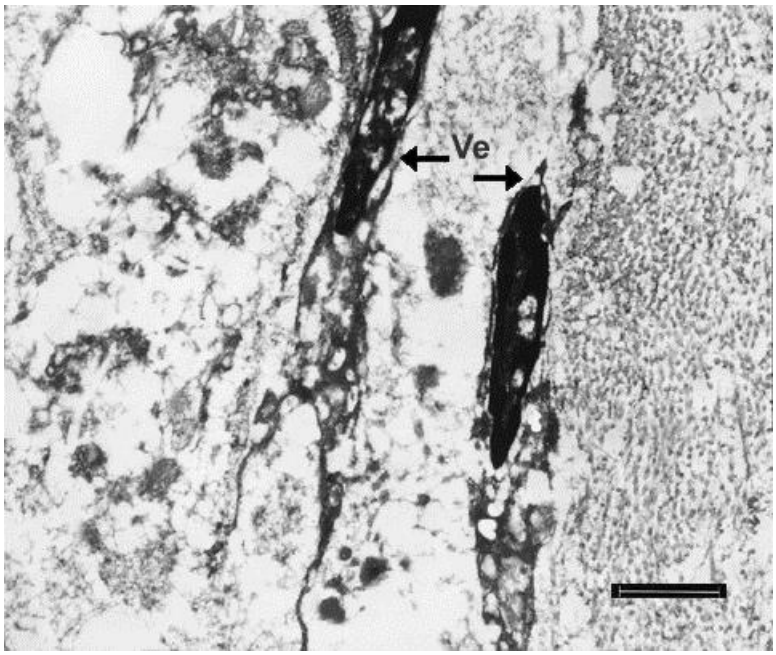
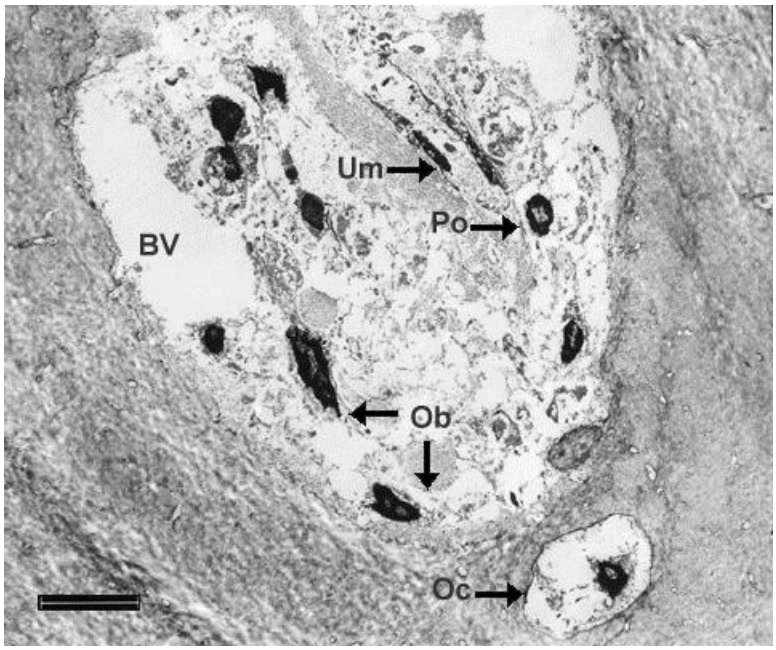


Figure 3. c) Capillary (C) invasion accomplished by the migration of prevascular and vascular endothelial cells (Ve) into bone making site (scale bar: 1µm, ×8,900). **d**) Higher magnification showed the vascular endothelial cells (Ve) are thin and flattened, with flattened, crescented nuclei with much condensed chromatin, and a thin layer of cytoplasma around the lumen with few mitochondria or rER, and large number of pinocytotic vesicles (scale bar: 1µm, ×11,500).

e)



f)

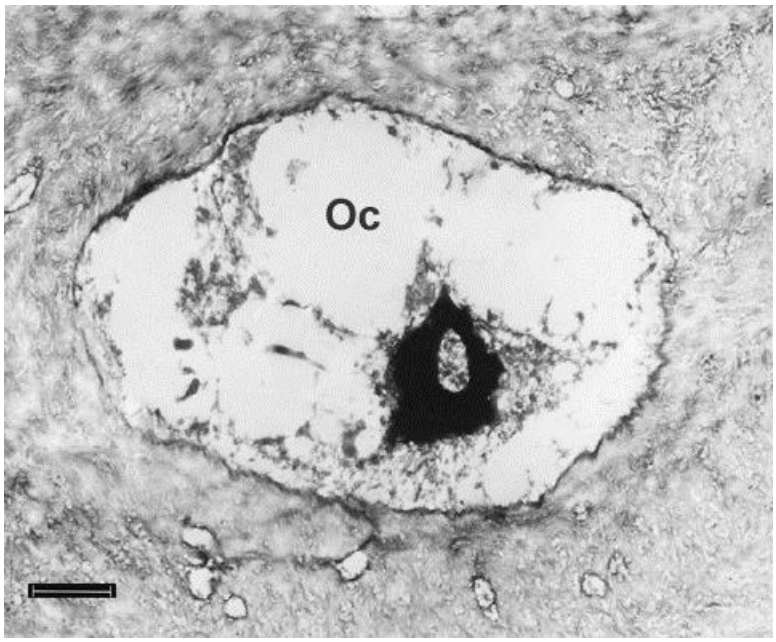
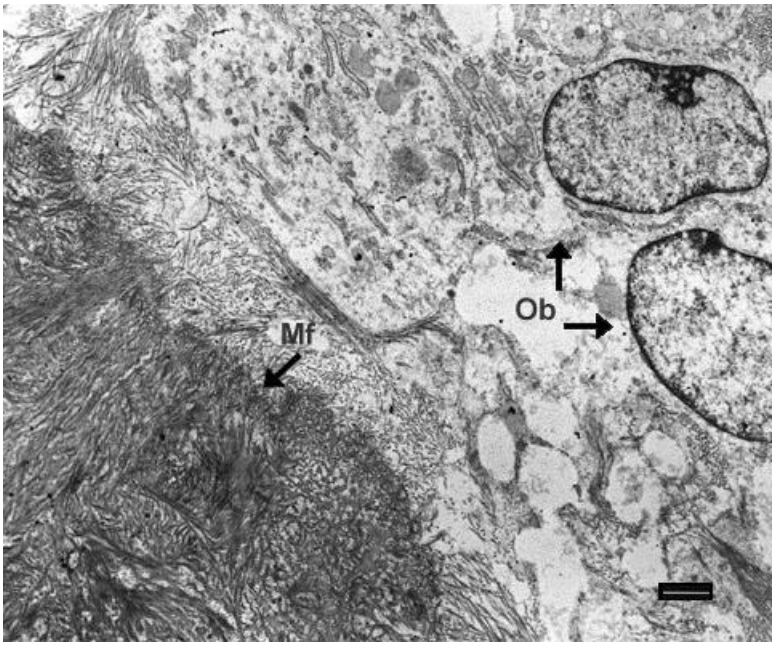


Figure 3. e) Lineage of osteogenitor cells in bone marrow, including undifferentiated mesenchymal cells (Um), pre-osteoblasts (Po), osteoblasts (Ob) and osteocyte (Oc), without a cartilage intermediate stage. The capillaries and blood vessels (BV) were involved in the bone making process (scale bar: 5µm, ×2,200). **f**) Higher magnification of the newly embedded osteocyte (Oc) in the end of the lineage (scale bar: 1µm, ×8,900).

g)



h)

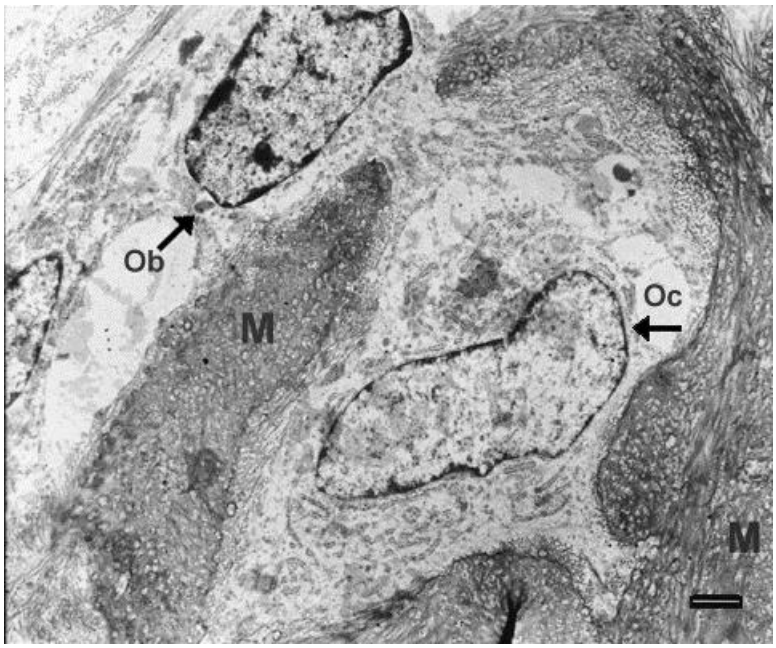
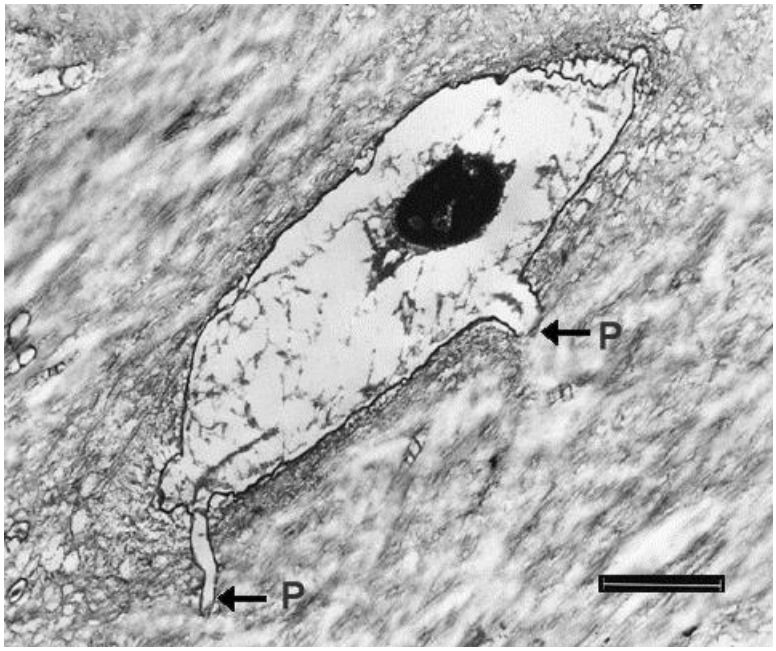


Figure 3. **g**) Two full secretory osteoblasts (Ob) on the surface of bone matrix showing the front of mineralisation (Mf) (scale bar: 1µm, ×5,000). **h**) An active osteocytic osteoblast (Ob) newly entrapped by the matrix that itself has synthesized became a young osteocyte (Oc) with large area of rough endoplasmic reticulum and Golgi complex. Note the surface of allogenei matrix (M). (scale bar: 1µm, ×5,000

i)



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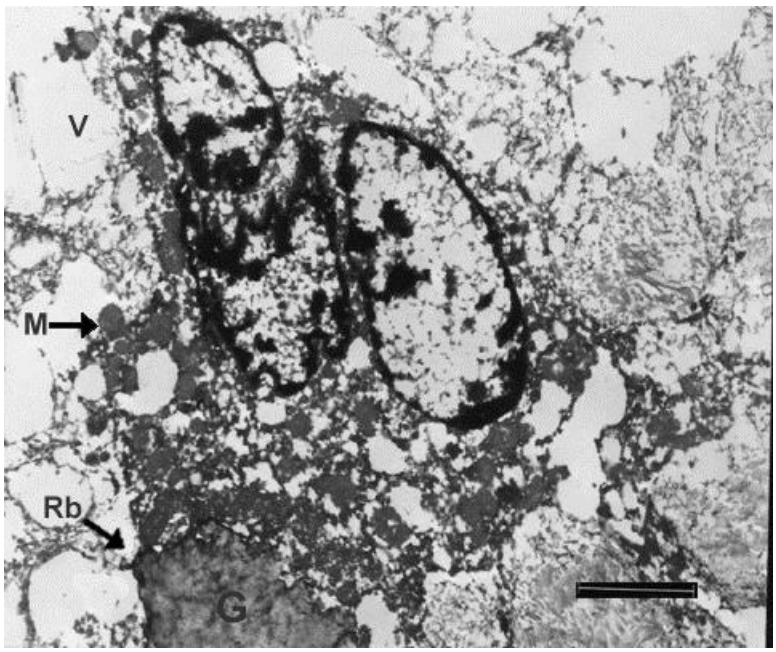

Figure 3. **i**) A mature osteocyte (Oc), in a lacuna with cell processes (P) forming canaliculus, was closely surrounded by demineralized matrix (scale bar: 2µm, ×6,600). **j**) A multinucleated osteoclast on the surface of allogeneic bone graft (G), showing a substantial amount of mitochondria (M), large cytoplasmic vacuoles (V) and the ruffled border (Rb) (scale bar: 2µm, ×6,600).

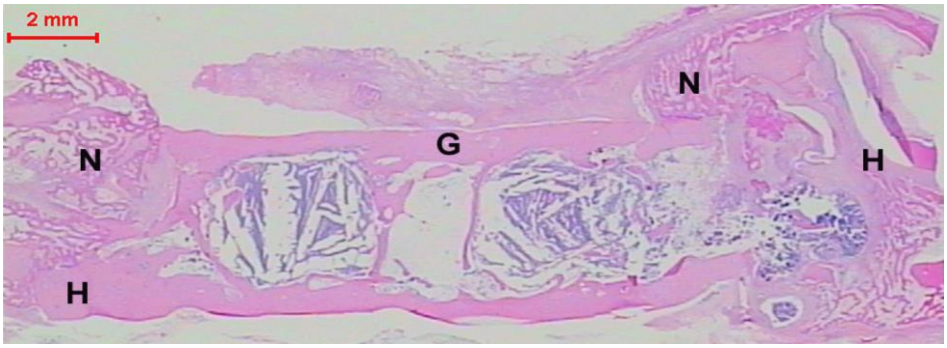

Figure I. Photomicrograph of defect grafted with allogeneic IM bone alone. A small amount of new bone (N) was localized near host bone(H).

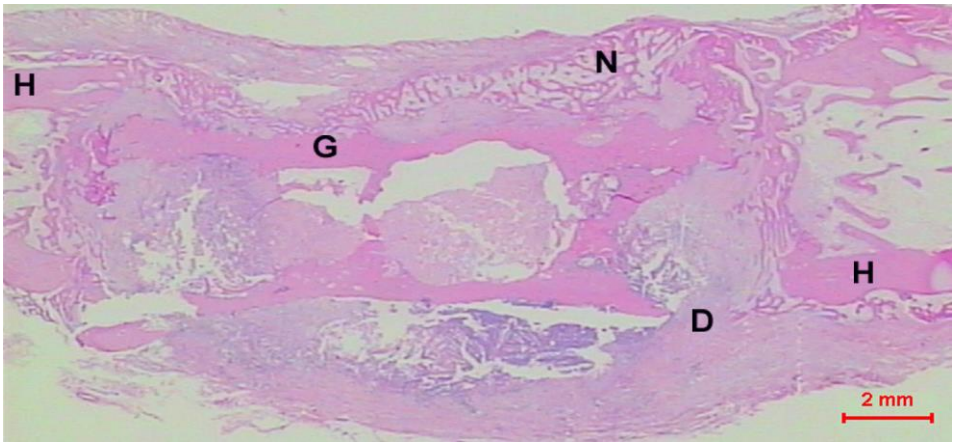

Figure II. Photomicrograph of defect grafted with allogeneic IM bone+ $DBM_{IM.}$ More new bone (N) was interposed between DBM_{IM} (D) particles on the side of bone graft (G) and host bone (H).

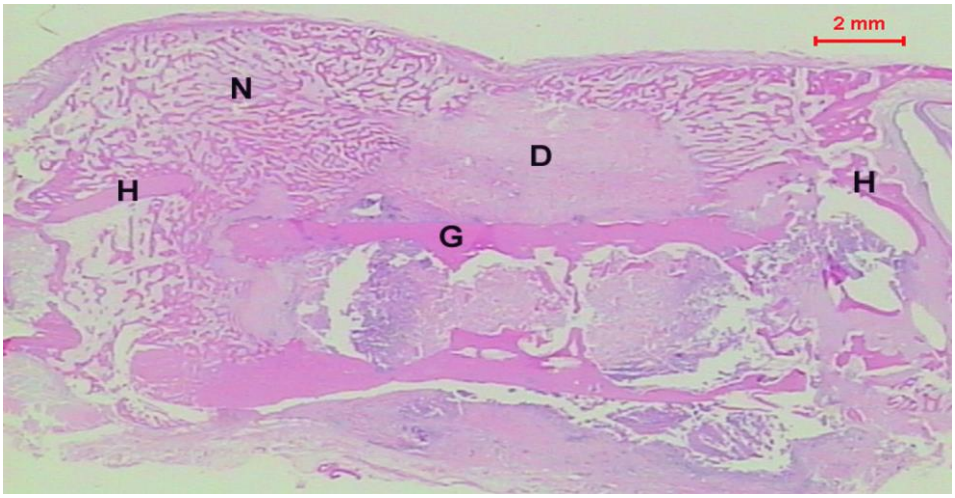

Figure III. Photomicrograph of defect grafted with allogeneic IM bone+ bFGF in DBM_{IM} powder. Abundant new bone (N) was localized to both host bone (H)-graft (G) interfaces and across the defect.

Discussion

We reported that we successfully produced a totally allogeneic bone-graft material that is osteoinductive [5]. This composite allogeneic graft material consisted of allogneic IM bone mixed with bFGF suspended in DBM_{IM} [5]. The current study identified the cells involved in the healing of the allogeneic bone in the presence and absence of bFGF. Basic-FGF has been known to have both osteogenic and angiogeneic properties in vitro and in vivo[12],[13],[14]. It has been shown to stimulate the proliferation of both preosteoblastic cells and more differentiated osteoblastic cells[15]. Earlier it was reported that integration of bone grafts was dependent on many interrelated processes, including the nature of the host bone and its osteoprogenitor cells, osteoconduction, osteoinduction and the nature of cextracellular matrix of the bone graft[16]. As such, in the current work, it was essential to identify the cells involved in the process of bone induction by the composite allogeneic IM bone and bFGF reconstituted in DBM_{IM} and to examine the effect of bFGF and DBM_{IM} on the integration of the allogeneic bone graft with the host bone.

Ultrastructural identification of cells involved in the healing of composite allogeneic bone graft showed multiple mesenchymal cells, preosteoblasts (Figure 3a, 3e) and full secretory osteoblasts (Figure 3b, 3g). These cells could be seen integrating with the allogeneic bone matrix (Figure 3h). Figure 3h shows osteoblasts and osteocytes on the surface of the allogeneic bone matrix and embedded within the matrix. These cells could only have made it into the allogeneic bone with the invading new blood vessels. As the new blood vessels penetrated into the allogeneic bone graft, the stem cells in the perivascular sites are a good source of osteoprogenitor cells which will differentiate into bone making cells[17] and start forming bone using the allogeneic bone as a scaffold (Figure 3a, 3e). However, in the allogeneic bone graft that itself lacked any cells within the grafted bone, the newly formed bone seen in the grafted area was due to bone conduction of the host bone rather than induction by the grafted allogeneic bone (Figure 1).

Another important role for the bFGF is its angiogenic ability[12]. Angiogenesis or vascularisation, is the sprouting and budding of endothelial cells from pre-existing vessels, usually the post-capillary and small terminal venules of the microvascular apparatus[18]. Basic-FGF has been known to be a potent mitogen for vascular and capillary endothelial cells in vitro[19] and can stimulate the formation of blood capillaries (angiogenesis) in vivo[20]. The role of DBM_{IM} in the early stages of angiogenesis associated with bone induction had already been examined[11]: when autogenous IM bone was mixed with DBM_{IM}, early vascularization was noted when compared with IM bone alone[11]. Furthermore, composite EC- DBM_{IM} was also found to vascularize earlier and almost 100% more than EC bone alone by quantitative analysis of immunolocalization of EN 7/44, an useful indication to the pattern of neovascularization[21]. In the current study, cells identified in the healing of composite allogeneic bone mixed with bFGF in DBM_{IM} graft clearly showed the presence of capillaries with prevascular and vascular endothelial cells evident in bone appositional sites (Figure 3c, 3d). Those capillary and vascular endothelial cells attracted by bFGF will form a vascular network of vessels, capillaries, and blood

sinusoids[19]. Apart from being a source of potential osteoprogenitor cells, these numerous capillaries and blood vessels in grafted sites incorporated between the newly apposed woven bone trabeculae (Figure 3a, 3b), not only provide the nutrient for newly formed bone, but also form the haematopoietic bone marrow, which is the origin of the osteoclasts responsible for bone resorption[22]. Later, this woven bone is remodeled and is progressively replaced by mature lamellar bone. The more capillary invasion, together with the more mesenchymal proliferation and differentiation, the more bone formation occurs at a later stage in the defect. Therefore, the positive effect of bFGF reconstituted in DBM_{IM} powder on the healing of allogeneic IM bone graft seen in our recent report[22] is due to the fact that bFGF augments both an angiogenic and an osteogenic response of this allogeneic graft material, thus making it a strong osteoinductive bone graft material that warrants further clinical examination.

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