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ORIGINAL PAPER

Allografts supercharged with bone-marrow-derived mesenchymal stem cells possess equivalent osteogenic capacity to that of autograft: a study with long-term follow-ups of human biopsies

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Abstract
Purpose Bone-marrow-derived mesenchymal stem cells (BM-MSCs) have been proposed to enhance bone formation in allografts. However, it is not known whether a combination of MSCs, contained in bone marrow concentrate (BMC) and structural allograft could be better than an allograft without MSCs and equivalent to a femoral head autograft in terms of histologic bone formation and long-term cellularity in the graft. After ten years of follow-up, three types of grafts: those initially loaded with BM-MSCs; dead, irradiated allografts; autografts.
Materials and methods Twenty patients received acetabular grafting during hip surgery and subsequently underwent femoral hip revision eight to 13 years later (average 10 years). Revision surgery was for reasons other than graft failure. These 20 patients had received eight allografts initially loaded with BM-MSCs: six dead irradiated allografts and six autografts. The number of MSCs present in the three types of graft were evaluated at the time of initial surgery and at revision. New bone formation associated in the acetabular graft was assessed by histology and calculated as a percentage of total available bony area.
Results At the most recent follow-ups (average 10 years), concentration of MSCs in allografts previously loaded with BM-MSCs was higher than that found in autografts. There were low or no MSCs found in uncharged allografts. New-bone-formation analysis showed that allografts loaded with BM-MSCs produced more new bone (35 %; range 20–50 %) compared with either uncharged allografts (9 %; range 2–15 %) or autografts (24 %; range 12–32 %).
Conclusions Our observations with allografts charged with BM-MSCs provides evidence in support of a long-term benefit of supercharging bone allografts with autologous BM-MSCs

Keywords Mesenchymal stem cells · Allografts · Autografts · Hip revision

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Introduction

Bone allograft in association with a reinforcement device [1–8] has been used widely for reconstruction of major osteolytic lesions of the acetabulum. Biopsy sampling of allografts has revealed evidence of remodeling, with viable tissue growth into the graft [9–12]. Scintigraphic examination [13, 14] demonstrated that an allograft showed increased activity compared with the surrounding bone, suggesting new bone formation within the grafts. During hip revision, bone-marrow-derived mesenchymal stem cells (BM-MSCs) loaded into allografts [3, 15–18] have been proposed to improve ingrowth of new host bone in the graft and clinical improvement observed. However, the benefit of supercharging structural allograft with BM-MSCs concentrated from a patient's bone marrow aspirate has not been compared with the performance of a femoral head autograft over the long term. Of particular

interest is the persistence of MSCs in the supercharged allograft and the presence of new bone formation.

This study examined the impact of supercharging allograft with autologous MSCs prior to implantation into the acetabular cavity during revision surgery to test the hypothesis that this method could generate more new bone compared with allograft only and as much bone as autograft. We examined 20 patients who received one of three different types of grafts implanted during hip surgery after an average of ten years (range 8–13 years) and who later underwent hip revisions for reasons other than acetabular failure (femoral revision). The first group received allografts loaded with BM-MSCs; the second irradiated allografts; the third bulk femoral head autografts used for acetabular revision in total hip arthroplasty (THA) with acetabular loosening. At the time of the initial surgery, we evaluated the number of MSCs present in each group (allograft and autograft of patients who had acetabular reconstruction during hip revision). During hip revision, we analysed bone formation in the different grafts and the number of MSCs present in the three graft types. The main study goal was to determine whether supercharging structural allografts with autologous BM-MSCs could be equivalent to a femoral head autograft used in hip surgery revision. We therefore evaluated three types of grafts after a ten year follow up: allografts initially loaded with BM-MSCs; dead, irradiated allografts; autografts. We evaluated what histological new bone formation occurred and how many MSCs were present after ten years in each type of graft.

Materials and methods

Grafts were provided to patients undergoing acetabular component revision performed for aseptic failure of cemented implants associated with massive periacetabular osteolysis and Paprosky type 3A or 3B classification [19] without pelvic discontinuity. The three types of graft were prepared, and the first group of eight patients received allograft supercharged with BM-MSCs during hip revisions performed between 2000 and 2005. The second group of six patients had received standard allograft without BM-MSCs between 1998 and 2000. The third group of six patients had received autograft for hip revision between 1994 and 1998 in which the fresh contralateral bulk femoral head autograft was harvested during the primary hip arthroplasty for osteoarthritis and performed at the same time as the revision on the other side. Patient basic demographics were similar between groups: age 71 years (range 64–81), 69 years (range 60–78), and 70 years (range 62–75); reason for revision (cup loosening); and previous bearing surface [ceramic/polyethylene (PE)]. The rationale for the investigation and the accompanying risks factors were discussed with each patient, who signed an informed consent form approved by the University Hospital. These patients underwent hip revisions after 10 years for femoral revision without graft failure or cup loosening [20].

Allograft preparation [21] was performed as previously described using a bone bank approved by the National Health Agency of France. These allografts complied with the European Union Directive 2004/23/EC.32 at the time of revision surgery. All grafts were obtained from the National Blood and Tissue Services. The grafts had undergone γ-irradiation using 25 KGy and were ground, packed under sterile conditions, fresh-frozen and stored at −80° C.

At the time of implantation, the number of progenitor cells present in the grafts was evaluated. For allografts loaded with BM-MSCs, patients gave their informed consent to have aspiration of bone marrow at the time of hip arthroplasty and revision. Aspiration was performed from the iliac crest during surgery under general anaesthesia. Bone marrow was harvested from the posterior iliac crest before hip incision but after installation of the patient for hip arthroplasty. Aspirate was concentrated as previously described [22, 23]. Allografts were supercharged with BM-MSCs by injecting 10 ml of concentrated bone marrow (BMC) several times into a femoral head allograft through the cartilage at different points, as previously described [17]. BMC used for the first group of patients was assessed for the number of BM-MSCs present in the concentrate and injected into the graft [16, 17, 23, 24]. Unloaded allografts used for the second group had zero cells present, since sterilisation (at the dose used for sterilisation) killed all nucleated cells. To determine the number of native MSCs present in the autograft implants used for the third group, aspiration of a small aliquot of bone marrow from the femoral head was performed at the beginning of surgery under general anaesthesia. The analytical techniques to determine MSC content have been reported previously [23, 25]. In brief, two parameters were measured directly or calculated from the results of cell culture: (1) bone-marrow-nucleated cell count (the number of bone marrow nucleated cells per 1 cc of marrow aspirate) by counting marrow smears on a haemocytometer and (2) frequency of MSCs per one million nucleated cells estimated by counting the number of colony-forming units/fibroblasts (CFU-Fs) present in culture. The concentration of MSCs was calculated for each sample as the product of the nucleated cell count and the frequency of MSCs.

Subsequently, at the time of re-revision, bone marrow aspiration was performed on the grafts from all three groups of patients and MSCs content in the grafts was determined. In the context of being certain that these cells were stem cells, we checked that they remained plastic-adherent under standard culture conditions; that these expanded BMSCs were strongly positive for CD90, CD105 and CD73 (hallmarks of BMSCs) and that the cultures did not contain haematopoietic lineage cells, as indicated by the absence of CD34-expressing cells. Osteoblastic gene expression was analysed after culture in the presence of AA, βGly and Dex for BMSCs, and these cells exhibited markers consistent with

osteoblastic differentiation, such as the following: alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx2), fibronectin and osteonectin (SPARC). Bone formation was evaluated by histology. After casting the biopsy specimens in resin, sections were cut through the transverse plane. Radiographs were used to measure the position of each cut. Prior to histomorphometric analysis, the sections were stained with toluidine blue for ten minutes (which stains fibrous tissue blue) and Paragon for 20 minutes (which stains new bone bright pink).

Statistical analysis

For nucleated cell and MSC numbers, group mean values and standard deviations (SD) were calculated. The results are reported as means ± SD and range. Percentages were compared using the Mann–Whitney test. Corresponding p values were considered significant at <0.05.

Results

Histological assessment of new bone formation

New bone formation in the implanted grafts was assessed: Allografts supercharged with BM-MSCs (Fig. 1) produced statistically significantly more new bone (mean 35 %; range 20–50 %) compared with bone formation measured in the uncharged allografts (mean 9 %; range 2–15 %; $p < 0.01$) (Fig. 2) and autografts (24 %; range 12–32 %; $p = 0.03$). Histology (Fig. 3) also demonstrated that haematopoietic cells and osteogenenesis were present in the centre of BM-MSC-charged allografts and in autografts but absent in the centre of uncharged allografts. For unloaded stem-cell allografts in the second patient group, new bone was observed only adjacent to the interface with the acetabular recipient bone. In contrast, the first patient group, with BM-MSC-charged allografts, and the third group, with autografts, showed no significant spatial difference in bone formation across grafts. Moreover, histological sections of the allograft loaded with BM-MSCs showed new bone formation integrated onto the surface of dead bone. These results indicated that the allogenic dead bone graft supercharged with autologous BM-MSCs demonstrated an osteogenic potential similar to autograft.

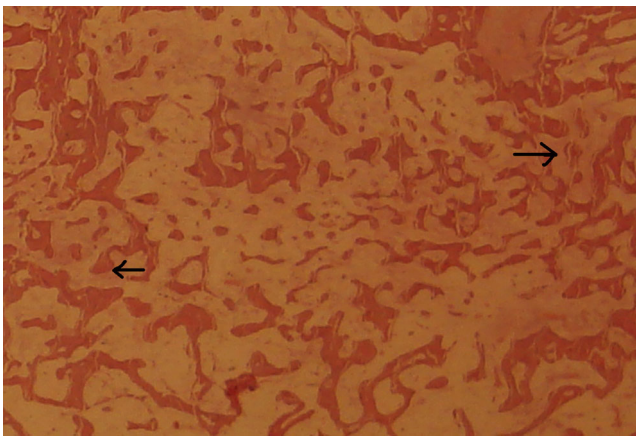

Fig. 1 Allograft loaded with bone-marrow-derived mesenchymal stem cells (BM-MSCs) at the time of implantation (10-year follow-up). There is viable bone with pink osteoid formation (*arrows*); the section was stained with Paragon for 20 minutes (which stains new bone bright pink). Magnification ×50

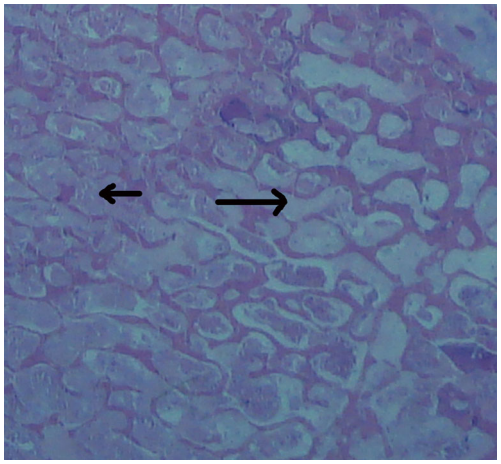

Fig. 2 Allograft implanted without bone-marrow-derived mesenchymal stem cells (BM-MSCs) (10-year follow-up). Fibrous tissue has grown (*arrows*) in the allograft, but there is no new bone formation; the section was stained with Toluidine Blue for ten minutes (which stains fibrous tissue blue). Magnification ×50

Cell analysis of bone grafts

At the time of grafting, MSC concentration in the unloaded allografts in the second patient group was zero, since irradiation of the graft during sterilisation killed any nucleated cells. MSC concentration in allografts loaded with BM-MSCs in the first

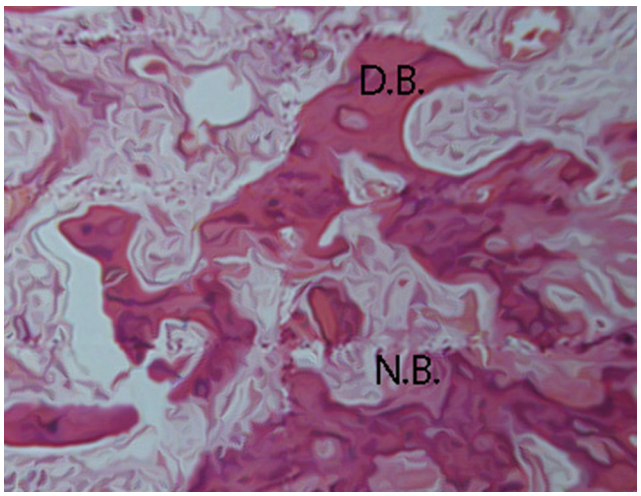

Fig. 3 Histology with dead bone (DB) and new bone (NB) on the same image

patient group was 3348 ± 112 progenitors per cubic centimetre (range 1200–6120). This value was much higher ($p = 0.001$) when compared with the MSC concentration obtained by aspiration of the bulk femoral head autografts in the third patient group (168 progenitors per cubic centimetre). When related to the volume of the femoral head, the total number of MSCs loaded in the allografts was 33,480, and the estimated number of MSCs in bulk autografts was an average of 8400.

After an average of 10 years of follow-up, mean MSC concentrationsobserved in the first patient group with allografts supercharged with BM-MSCs at the time of implantation was 543 progenitors per cubic centimetre (range 110–1200; $p = 0.001$). Allografts used in the second patient group, those not charged with BM-MSCs, had very few MSCs at follow-up (<10 progenitors per cubic centimetre), while the mean concentration of MSCs in autografts from the third patient group, who received the contralateral femoral head graft at the time of implantation, was 135 progenitors per cubic centimetre (range 40–342).

Discussion

Allografts with or without metallic devices [26–28] have been used widely to stabilise implants adjacent to bone defects for revision total hip replacements. However, long-term biological properties and cellularity of the implanted allograft are not well known [29, 30]. Some histological studies on biopsy specimens [9–12] have shown remodelling of the allograft with the presence of new bone formation within the allografts, but the numbers of biopsy sites are limited and the location of the site unclear. BM-MSCs are multipotent cells [15, 31–33] and can be induced to differentiate into osteoblasts, chondrocytes etc. Many previous reports have indicated that combining cultured MSCs with porous ceramics [24, 34–38] showed consistent ectopic bone formation in the recipient sites after transplantation. Therefore, we hypothesised that osteogenic capacity could be enhanced through supercharging allogenic bone with autologous BM-MSCs by using the patient's BMC. A previous study [16] showed that intra-operative seeding of bone marrow stromal cells in an allograft could improve the healing process of that allograft.

In the study we present here, newly formed bone was not observed in the centroids of irradiated allogenic bone grafts that had not been charged with BM-MSCs at the time of implantation. They were observed, however, at the interface with the patient's natural bone. This indicates that osteogenic capacity is essentially lost when allogenic bone is sterilised by irradiation [2, 6, 21, 39], since the peripheral bone growth most likely is explained by migration of osteogenic cells from the adjacent natural tissue. We also show that when an allograft is charged with autologous BM-MSCs using the patient's own BMC, new bone formation is evident throughout the allograft, including new bony deposits in centroid and peripheral zones of the grafts. This is similar to the distribution pattern of new bony growth in autograft implants. Supercharging allografts with BM-MSCs from concentrated bone marrow [40] may be an important factor in enhancing implant fixation and stability.

This study has some limitations. Firstly, the number of patients is small and makes it difficult to draw general conclusions regarding efficiency of MSCs in different allografts. Secondly, it is difficult to compare at the time of implantation the number of MSCs aspirated from an autograft to the number of MSCs loaded into an allograft. However, comparison of aspiration in autograft and allograft is possible, and this study is, to our knowledge, the only one that gives information on the number of MSCs present in different grafts and the corresponding histology after ten years of implantation.

One concern of transplantation of BM-MSCs into an allograft is whether they can proliferate or induce proliferation of osteogenic cells within the target tissues. Although some studies demonstrated an approximate estimate [16, 25] for the number of MSCs required for bone regeneration, the number of viable cells remaining after implantation has not yet been reported. Stem-cell homing may function in two ways: Cell necrosis after trauma induces the release of a series of signaling molecules, and stem cells migrate into target tissue where specific receptors or ligands expressed in injured tissues play important roles in the healing process. We do not know whether MSCs observed after an average follow-up of ten years are directly descended from the BM-MSCs injected at the time of implantation or whether MSCs from adjacent healthy tissue migrated into the site. From a theoretical point of view, induction of cell migration from adjacent tissues to the treatment site should be better with an autograft compared with an allograft. However, this study demonstrated that the reverse is true when allografts are supercharged with autologous BM-MSCs at the time of implantation. Aspiration sampling performed at revision demonstrated that MSCs were present after 10 years in allografts charged with BM-MSCs and that the MSC concentration was higher compared with that in autografts. This phenomenon is probably due to the very high number of MSCs loaded in allografts compared with the native number of MSCs present in autografts. This means it is likely that some MSCs loaded in the allograft probably remained in situ and proliferated.

In conclusion, charging autologous BM-MSCs into a femoral head allograft during hip revision can enhance the

osteogenic capacity of the implant to a level that appears comparable with that of an autograft.

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