

Will Mesenchymal Stem Cells Differentiate into Osteoblasts on Allograft?

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Tissue engineering approaches for bone blocks previously have used synthetic scaffolds. Bone graft (allograft) is used to fill bone defects, but standard processing can lessen this scaffold's osteoinductive potential. We wanted to test if allografts could be used to produce a viable bone block using mesenchymal stem cells. We hypothesized that mesenchymal stem cells differentiate into osteoblasts producing extracellular matrix when cultured on allografts. We also hypothesized that the addition of osteogenic supplements would increase the rate of differentiation. To test these hypotheses, mesenchymal stem cells were isolated from bone marrow aspirated from 10 patients and cultured on allografts from five donors (Group 2), producing 50 samples. This was repeated on allografts heat-treated to denature bioactive proteins (Group 1), and repeated again on allografts to which osteogenic supplements (Group 3) were added. Group 2 mesenchymal stem cells differentiated into osteoblasts producing higher levels of alkaline phosphatase, osteopontin, and Type I collagen matrix protein than Group 1. The rate of differentiation of Group 3 mesenchymal stem cells increased with the supplements. Overall, it was established that the bioactive proteins in the allograft stimulated mesenchymal stem cell differentiation into osteoblasts, with production of extracellular matrix, and that this differentiation increased with the addition of osteogenic supplements.

Bone grafts are used commonly in orthopaedic procedures, but their limitations have led to research into tissue engineering of bone.^{9,18,19} The use of cadaveric allograft for bone tissue engineering is ideal because, unlike other scaffolds, it has the closest structural and mechanical properties to living bone, is not associated with donor site morbidity, and currently is used in clinical practice to fill bone defects.¹²

There has been some success using fresh marrow in bone regeneration, but this is limited because the osteoprogenitors required to produce bone represent only 0.001% of nucleated cells in bone marrow.⁹ However, techniques have been developed to isolate and culture-expand mesenchymal stem cells (MSCs) from bone marrow more than one billion-fold.^{16,28} This dramatic expansion has allowed MSCs to be used in synthetic¹ and natural⁶ scaffolds or in an injectable form³⁷ to improve healing of segmental bone defects in animals. However, when MSCs were used in this environment, differentiation occurred after implantation of the graft; therefore, the use of pregrown bone has an obvious advantage in the potential rate of bone healing. Investigators also have caused MSCs to differentiate into osteoblasts on ceramic scaffolds in vitro,^{21,36} but as ceramics do not have the same mechanical properties or structure as bone or allograft, their usefulness is limited in orthopaedic procedures.

An alternative scaffold is fresh allograft, which is osteoinductive but also immunogenic, the adverse effect of which can be reduced substantially by processing methods.¹² The risk of transmission of infection by allograft also can be reduced by donor screening and by secondary sterilization.¹⁹ In the United Kingdom, allograft is collected, stored, and supplied regionally, overseen nationally by the British Association for Tissue Banking (BATA), as part of the blood transfusion service. The BATA processes cadaveric donor allograft bone blocks harvested from long bones, pelvis, and sternum, washing them to remove blood and marrow. They then freeze-dry the allograft to increase its shelf life and sterilize it using 2.5-Mrad gamma irra-

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diation, which sterilizes by damaging large molecules such as DNA in bacteria, but aims not to denature collagen normally present in the extracellular matrix of bone.¹⁵ However, gamma irradiation destroys cellular DNA and osteoprogenitor cells, reducing osteoinductivity. Nonetheless, some osteoinductive potential may be retained by the allograft,^{18,36} attributable predominantly to bone morphogenetic proteins (BMPs) and also fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF) in the collagen matrix.²² These proteins may play an important role in the differentiation of MSCs down the osteogenic lineage, when cultured on allograft bone *in vitro*.¹⁹

We wanted to clarify whether bone could be grown efficiently, such that it potentially could be used in orthopaedic procedures. To test this, we developed the hypothesis that MSCs are stimulated to differentiate down an osteoblastic lineage when cultured on washed, freeze-dried, gamma-irradiated cancellous allograft bone. These hypotheses raised some key questions: First, were the isolated human marrow cells used MSCs, already established as being capable of differentiation?²⁸ Second, when cultured on allograft, did the cells differentiate into osteoblastic cells? Third, would cells grow on allograft? Fourth, does allograft have any properties that may affect the differentiation of MSCs into osteoblasts? Fifth, recognizing the benefit of faster healing rates in orthopaedic operations, could the rate of differentiation be influenced?

MATERIALS AND METHODS

The study design compared the differentiation of MSCs taken from 10 patients, cultured on allograft in three treatment groups. Each patient's cells were cultured on five different allograft scaffolds resulting in 50 scaffolds per group.

In Group 1, the allograft was heated at 60°C for 1 hour to denature bioactive proteins in the graft without affecting osteoconductivity,^{26,36} and the MSCs were cultured in standard medium. Standard medium consisted of Dulbecco's modified Eagles medium (Sigma, Poole, UK), 10% fetal calf serum (Sigma), penicillin 50 U/mL (Sigma), and streptomycin 50 µg/mL (Sigma). In Group 2, the allograft was cultured with MSCs in a standard medium, without any heating of the allograft. In Group 3, the allograft was cultured with MSCs and additional osteogenic supplements (OS), which consisted of 100 nmol/L dexamethasone, 10 mmol/L β-glycerophosphate, and 0.05 mmol/L L-ascorbic acid, which were added to the standard medium.

The potential for osteoblastic growth was assessed in the three Groups as follows. Cell differentiation was measured through the presence of osteoblastic proteins (alkaline phosphatase and osteopontin). Existence of extracellular matrix was observed under transmission electron microscopy (TEM) and through comparison of Type I collagen matrix formation. Cell growth was assessed by testing DNA levels and alamar blue

assay absorbance, and cell adhesion between the MSCs and the allograft was assessed through observation using scanning electron microscopy (SEM).

Power analysis on 50 samples in each Group was greater than 0.8 for each comparison test and therefore an alpha value of 0.05 and beta value of 0.2 were used for significance throughout the study.

After local committee on ethics approval and informed patient consent, we harvested bone marrow from the posterior iliac crest of 10 consecutively selected patients, excluding those with medical or drug history that may have interfered with the properties of MSCs while under general anesthesia for planned orthopaedic operations. Two-milliliter aspirates were taken from each patient, as this is the most efficient volume to draw, harvesting 85% of the stem cells from that area.²³ The marrow aspirates then were loaded onto Ficoll-Hypaque® (Pharmacia Biotech, Amersham, UK) and spun in a centrifuge, after which the buffy coats were isolated and cultured.¹⁴

Characterization of the MSCs was done using immunoglobulin M (IgM) monoclonal antibody STRO-1 (University of Iowa, Iowa City, IA).^{32,33} Thirty thousand MSCs from each patient were plated on sterile cover slips in well plates and incubated in standard conditions (at 37°C, 95% humidity, and 5% CO₂) for 36 hours with either standard culture medium or with standard culture medium with OS. Afterward, STRO-1 primary antibody was added and labeled with a fluorescent antibody to facilitate viewing of MSCs under fluorescent microscopy.

The allograft scaffolds were cancellous bone cubes each measuring 1 cm³. The upper surface of each graft was seeded with 1 × 10⁶ MSCs in 0.25 mL medium, which was observed to filter through the porous structure, distributing the cells through the graft. All samples were incubated in the same standard conditions for 1 hour, allowing the cells time to adhere. Following this, the wells in which the scaffolds stood were covered with either standard or osteogenic culture medium, depending on group, and cultured on the well plates for 14 days.

To assess whether there had been osteoblastic differentiation, we measured alkaline phosphatase (ALP) protein levels in duplicate at Days 1, 7, and 14 in Groups 1, 2, and 3, using p-nitrophenol cleaved from p-nitrophenol phosphate.²⁶ We also measured osteopontin (OPN) enzyme immunometric ELISA,⁵ and PICP radioimmunoassay²⁴ in all groups at Day 14, as these are markers of maturing osteoblasts.²

To standardize measurement of protein levels across samples, we used the equation of an accepted level of DNA to number of cells to standardize a per-cell measure of the protein present, in addition to using it as a measure of cell proliferation. This standardization was used as, although the same number of cells was seeded initially on each allograft, the growth rate may have varied between samples and an increased level of protein may have been reflected by an increased volume of cells, rather than by strong demonstration of osteoblastic potential.

In testing for extracellular matrix, because of the length of time required for the matrix to start forming, we used TEM at Day 14 on MSCs cultured on allograft from all groups to identify the presence of any matrix. Samples were stained using 1% osmium, dehydrated with serial alcohols as much as 100%, dried

in propylene oxide, and embedded in resin. One-millimeter sections were cut and these were reembedded in resin, from which thin sections were cut, using an ultramicrotome with a diamond blade. These sections then were placed on copper grids, random samples of which were viewed in a Phillips CM12 electron microscope (Phillips Electronics NV, Eindhoven, The Netherlands) to check for the presence of extracellular matrix. The calcium and phosphate content of matrix was determined qualitatively using energy dispersive xray analysis (EDAX).

As noted, SEM was used to assess any adherence to the graft and morphologic features of the cell. Using a random number generator, three allograft samples were selected from each group at Days 1, 7, and 14. The samples were prepared using standard techniques to enable them to be viewed under SEM in a Joel Winsem JSM-35C 6300 series SEM (Joel, Welwyn, Herts, UK).

Cell density can be observed using SEM, but proliferation also was assessed using an alamar blue assay, which measures cellular metabolic activity and has been used to measure the cell proliferation rate.²⁵ It is nontoxic to cells and therefore can be used to measure cell proliferation with time. Alamar blue absorbance was measured during 14 days by replacing each grafts' medium with phenol red-free medium with 10% alamar blue. After 3 hours incubation, the medium was extracted and tested for absorbance at 570 nm and compared with the control assay, which had been incubated identically, but with cell-free allograft. This was repeated at Days 1, 7, and 14 for each of the three groups.

As an additional measure of cell proliferation, total cellular DNA was measured in duplicate in all groups, on Days 1, 7, and 14, using Hoechst 33258,²⁹ and the samples were analyzed using a fluorometer.

The impact of heat-treating on osteoblastic differentiation was assessed through statistical comparison of the ALP, OPN, and PICP results for Groups 1 and 2, which were first analyzed for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests. As the results did not follow a normal distribution, a non-parametric Mann-Whitney U test was used to compare the two groups.²⁷ Nonparametric tests similarly had to be used when comparing the effect of OS on differentiation in Group 3. When analyzing the proliferation as measured by production of DNA and alamar blue, normality tests were negative and therefore the nonparametric data again were tested using the Mann-Whitney U test. Taking into account our power analysis, a probability value less than 5% was considered significant throughout the study.²⁷

RESULTS

All bone marrow-isolated cells were immunofluorescent with STRO-1, confirming a population of MSCs. The MSCs cultured with standard medium showed an expected spindle-shaped appearance (Fig 1) but, when osteogenic medium was added for 36 hours (Fig 2), this changed universally to more rounded morphologic features, indicative of early-stage osteoblastic growth.

At Days 7 and 14, there was evidence of more osteoblastic differentiation on standard allograft (Group 2) com-

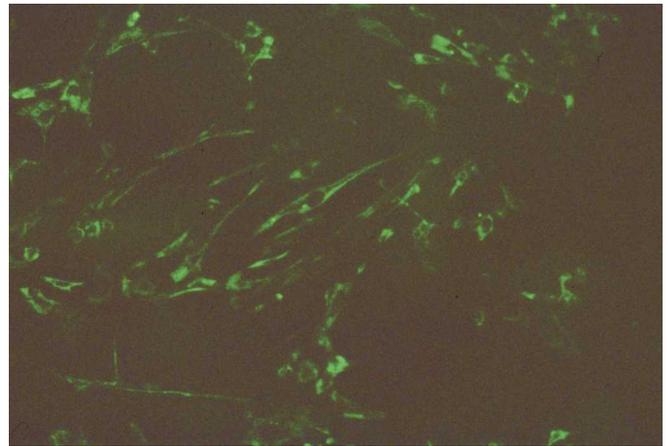


Fig 1. Immunofluorescence shows the IgM monoclonal antibody STRO-1 marker on our isolated marrow cells cultured with standard medium for 36 hours characterizing the population as MSCs. Also the spindle shape of our cells can be seen typical for MSCs (Magnification, $\times 20$).

pared with heat-treated allograft (Group 1), as seen by cells on heat-treated allograft producing significantly less ALP ($p < 0.005$) and OPN ($p < 0.005$) compared with on standard allograft (Group 2) (Figs 3 and 4).

Adding OS to the culture medium in Group 3 resulted in greater production of ALP ($p < 0.05$) and OPN ($p < 0.05$) on allograft (Figs 3 and 4), indicative of a higher rate of differentiation of osteoblastic cells.

A layer of extracellular collagen matrix was visible adjacent to osteoblasts when cultured on allograft with OS (Group 3), and there was evidence under TEM that

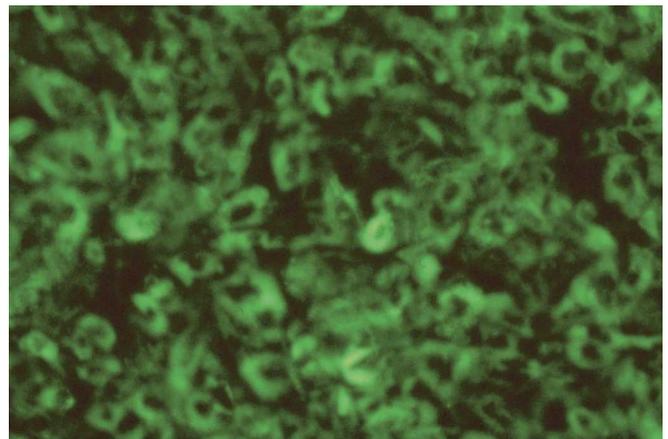


Fig 2. Immunofluorescence shows the IgM monoclonal antibody STRO-1 marker on human MSCs cultured with OS for 36 hours showing a rounded morphology characteristic of osteoblasts (Magnification, $\times 20$).

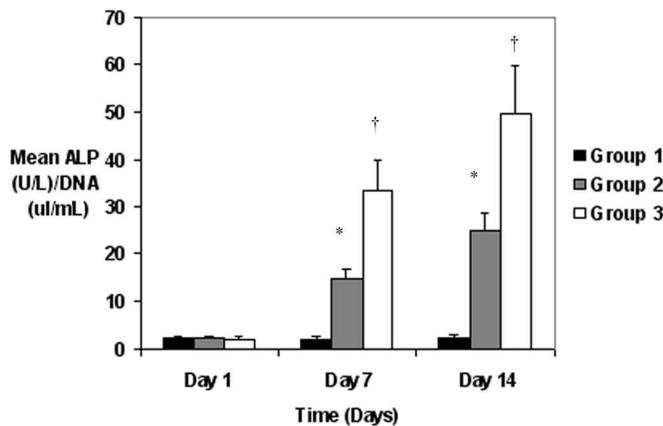


Fig 3. A box and whisker plot of the mean ALP for the three treatment groups (bar = mean \pm SD; * $p < 0.005$ between Groups 2 and 1 at Day 7 and Day 14; † $p < 0.005$ between Groups 2 and 3 at Day 7 and Day 14). Confirming reduced protein production when allograft was heated compared with standard allograft tested and increased production in OS culture. The ALP levels also increased in each group during the culture period ($p < 0.05$).

the newly formed matrix had started to calcify, which was confirmed with EDAX analysis calcium and phosphate ratio of 1.8. These results did not occur in cells grown in the heat-treated allograft or with cells grown in nonsupplemented allograft (Groups 1 and 2). Similarly, at Day 14, the mean PICP was greater ($p < 0.05$) in standard allograft (Group 2) compared with heat-treated allograft (Group 1), and further enhanced ($p < 0.05$) by the addition of OS (Group 3) (Fig 5). At this point, the TEM showed osteoblastic cells in Group 3 attaching via their processes to the graft (Fig 6).

Cells in all three groups were seen, under SEM, adhering to each allograft after 14 days. Cell density was ob-

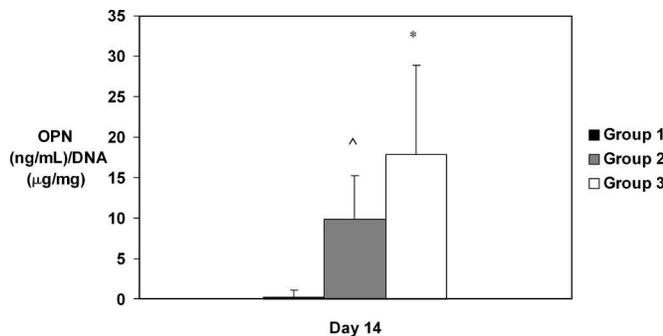


Fig 4. A box and whisker plot shows the variation in OPN at Day 14 for the three treatment groups. There was minimal OPN synthesis when allograft was heated and increased in OS culture as compared with standard allograft (* $p < 0.005$ between Groups 1 and 2; † $p < 0.05$ between Groups 3 and 2).

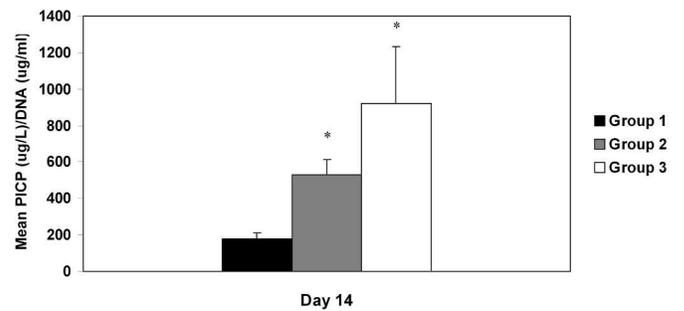


Fig 5. A box and whisker plot of the PICP values for the three treatment groups at Day 14 shows reduced matrix formation when allograft was heated and increased formation in OS culture (* $p < 0.05$ between Groups 1 and 2 and between Groups 2 and 3). These results complimented the TEM observations.

served under low magnification to be lowest on heat-treated allograft (Group 1) (Fig 7A), intermediate on the standard allograft (Group 2) (Fig 7B), and greatest after adding OS (Group 3) (Fig 7C).

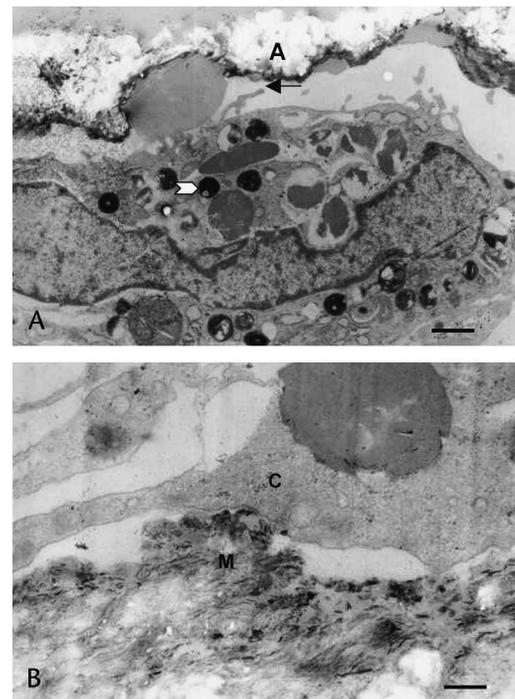


Fig 6A–B. Transmission electron microscopy of Group 3 at Day 14 shows (A) metabolically active cells attaching via their cell processes (black arrow) to allograft (A) (bar = 3 μ m). They contain many mitochondria (white arrowhead), rough endoplasmic reticulum, and storage vesicles. (B) Higher magnification (bar = 0.8 μ m) shows a layer of collagen matrix (M) with signs of calcification adjacent to the cell (C) (Magnification, $\times 120$).

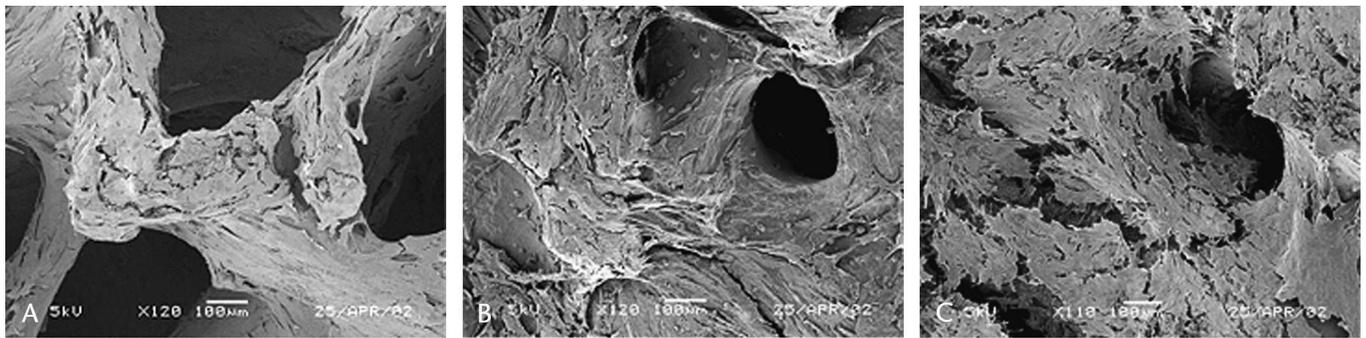


Fig 7A–C. Scanning electron microscopy at Day 14 shows the cell density is (A) lowest in Group 1, (B) intermediate in Group 2, and (C) highest in Group 3 (Magnification, $\times 120$). The morphologic feature of the cell changed to a more rounded shape, in keeping with osteoblasts.

During the 14-day culture period, cells were found to proliferate on allograft in all three groups, with total DNA increasing ($p < 0.05$). At Day 1, heat-treatment of allograft (Group 1) and addition of OS (Group 3) did not significantly affect cell proliferation, as measured by DNA or alamar blue. However, after Day 14, the amount of DNA and alamar blue measured in the heat-treated allograft was lower ($p < 0.05$) when compared with cells on standard allograft (Group 2) (Fig 8).

DISCUSSION

To assess whether improvement could be made to the osteogenic potential of allograft scaffolds, which could be

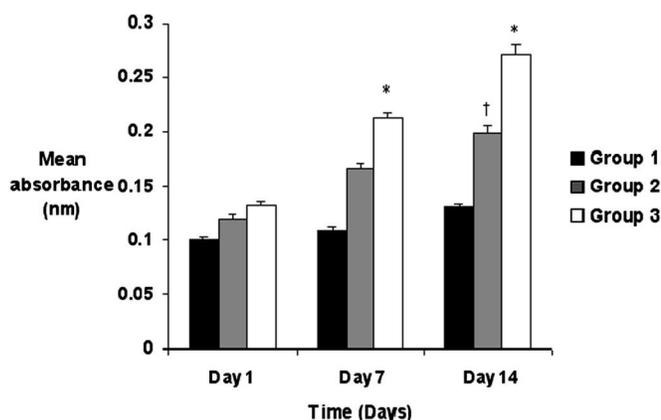


Fig 8. A box and whisker plot shows an increase in MSC proliferation measured by alamar blue absorbance levels for all three treatment groups during the culture period ($p < 0.05$). Further, proliferation was reduced by heat treatment after 14 days (* $p < 0.05$ between Groups 2 and 3 at Days 7 and 14; † $p < 0.05$ between Groups 1 and 2 at Day 14; in each group at Days 1 and 7, and at Days 7 and 14).

useful for orthopaedic procedures, we investigated whether human MSCs would grow and differentiate into osteoblasts when cultured on washed, freeze-dried, gamma-irradiated allograft, the success of which was measured by cell proliferation and levels of osteoblastic protein and matrix.

Also because bioactive proteins and osteogenic supplements are known to influence osteoblastic differentiation and growth,³⁵ the effects of these on the levels of proliferation and production of osteoblastic proteins were studied.

The main limitation of our study was that it only examined cell behavior on allograft in vitro and, as such, animal studies would need to be conducted to confirm the same results before clinical studies. The study did not determine the effect of individual bioactive factors in the allograft on MSC differentiation nor did the study investigate the potential for replacement of these factors by OS, proof of which could be useful where allograft does not display such high levels of these factors; additional studies are needed to investigate this. Our TEM results were also somewhat limited, as TEM only provides an observational assessment. However, the evidence of bone matrix was supported by EDAX analysis with a Ca/P molar ratio of 1.8, which is in the report range of normal bone 1.68–1.8³⁰ and correlates with PICP results.

In testing our hypotheses, we first wanted to verify we were using MSCs. We acknowledge that identification of MSCs through their characterization is difficult because there are no universally accepted specific markers of stem cells.⁷ However, our bone marrow-isolated cells were strongly positive for STRO-1, which has been suggested a reliable marker of MSCs.³¹ In addition, the morphologic feature of the marrow-isolated cells used in our study was spindle-shaped, consistent with accepted research on MSCs.^{8,11}

We next tested whether MSCs cultured on allograft differentiated into osteoblasts, a test of which was protein production as a marker of cell phenotype. We measured osteoblastic protein production with ALP as a marker of early osteoblasts, OPN as a marker of maturing osteoblasts, and PICP as a marker of new collagen matrix production. Findings show that the majority of cells in the midst of osteoblast lineage progression are highly positive for ALP,² an isoenzyme membrane bound onto osteoblasts.¹⁶ Furthermore, as OPN is a glycoprotein produced by osteoblasts and is a component of mineralizing extracellular matrix during bone formation, it is important in osteogenesis because it facilitates osteoblast attachment to the extracellular matrix.⁵

However, it is the qualitative measurement of PICP that is most compelling. PICP is produced in proportion to the amount of recently synthesized Type I collagen; when collagen forms a lattice, collagen Type I is derived from Type I procollagen and the carboxyterminal propeptide group (PICP) is released. Type I collagen is the most abundant extracellular matrix protein produced by mature osteoblasts. It is the major collagen in osteoid and is mineralized to form bone. Production of PICP therefore marks the synthesis of new matrix by osteoblasts,²⁴ and was used in our study to distinguish between collagen in allograft and collagen produced by differentiated MSCs.

To identify the drivers of the differentiation, we tested the MSCs on allograft. In preparing the MSCs for culture, it was observed they have a large proliferative capacity in vitro, following a long growth phase, which then plateaus.⁸ Mesenchymal stem cells have been shown to proliferate on various scaffolds, including on inorganic ceramics³⁴ and collagen constructs.¹³ Our use of allograft as the scaffold resulted in findings of increased cell proliferation on each group of allograft during the culture time.

The use of standard allograft was tested against the use of allograft that also had been subjected to heat treatment to establish whether the denaturing of bioactive proteins through heating^{17,38} had any effect on levels of differentiation. We found evidence of production of osteoblasts when using the standard processed allograft, which indicated that the inherent osteoinductive properties of fresh allograft are not completely destroyed by processing through washing, freeze-drying, and gamma irradiation,^{2,19,36} and that osteoinductive proteins, such as BMPs, may remain in the graft matrix.³⁰ Conversely, MSCs cultured on the heat-treated allograft displayed lower levels of differentiation, measured by minimal ALP, OPN, and PICP levels. These inferior results mirror the lesser differentiation observed when ceramic scaffolds are used.³⁶

Furthermore, the presence of bioactive proteins, including BMPs, have been shown to increase growth rate, measured by alamar blue among other tests.³⁵ Thus the lower

cell growth rate observed when the allograft was heat-treated is likely to be attributable to the loss of influence by BMPs in this group, compared with the standard allograft.

Having established the presence of osteoblasts differentiated from MSCs on allograft, we then investigated factors that might affect the rate of differentiation of the cells. We found that MSC differentiation was enhanced by the addition of OS, which augmented the osteoinductive properties of processed allograft and resulted in accelerated production of ALP, OPN, and PICP. This supports findings of previous work in which it was observed that OS facilitated MSC differentiation in vitro by increasing ALP production.¹⁶ The dexamethasone in the OS stimulates MSC differentiation down the osteogenic lineage, and L-ascorbic acid enhances production of Type I collagen and β -glycerophosphate, which is a requisite for formation of extracellular matrix and its subsequent mineralization.¹⁰ The effect of OS on the differentiation of MSCs was seen as early as at 36 hours by a change in the morphologic features of the cell as shown by immunofluorescence with STRO-1. Using OS resulted in the MSCs differentiating more rapidly on a favorable allograft substrate.

Finally, when the cell-seeded allograft samples were observed under SEM in all three groups of tests, adherence of cells to the allograft was observed. However, despite this positive result, more work needs to be done in this area, as cell attachment was not tested.

We successfully showed that, when cultured on washed, freeze-dried, gamma-irradiated cancellous allograft, bone marrow cells will differentiate into active osteoblasts. This was proven as MSCs obtained from the 10 patients' bone marrow and placed in this environment differentiated down the osteoblastic lineage, as defined by the production of different osteoblastic proteins and extracellular collagen matrix. The use of OS also increased the rate of cell differentiation and production of organized collagen matrix, and the cells were observed to adhere to the allograft. There is potential for the aspirated cells to be grown on allograft for a time supplemented with OS to ensure production of extracellular matrix before grafting in orthopaedic procedures.

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