

Viable cells survive in fresh frozen human bone allografts

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Background Fresh frozen bone allograft is available for human recipients after at least 6 months of quarantine at -80°C . It is assumed that cryopreservation without cryoprotectant removes all viable donor cells.

Methods We studied the *in vitro* cell growth from samples of fresh frozen human femoral head allografts after they had been released for patient use, and compared it with cell growth from a control group of fresh cancellous bone specimens from excised femoral heads (8 samples in each group).

Results Cell outgrowths were seen in all of the fresh cancellous bone specimens (100% of replicates, 48 replicates per specimen) but only in a small minority of replicates from 4 of the allograft samples (mean 3.1%). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) investigations revealed that cell outgrowths from both groups contained mRNA for transcription factors Runx2 and Osterix, and also for matrix proteins collagen type I, osteocalcin and bone sialoprotein. This is consistent with the cells being osteoblast-related.

Interpretation This study confirms that fresh frozen human bone allograft cells have the potential to grow *in vitro*, but the significance of this in recipients is currently unknown.

Ullmark and Linder 1998, Linder 2000) and core biopsies (Mikhail et al. 1999, Ullmark and Obrant 2002) has shown that incorporation of the graft is never complete.

The biology of impacted fresh frozen bone allograft into the host is not well understood. The variability of graft incorporation could be due to mechanical factors, impaction technique, particle size, and well-being of the patient (Linder 2000). It has been shown that the main difference between autologous bone and all types of allograft is the lack of viable donor cells that can contribute to healing, and the potential for immunological reactions (Kerry et al. 1999). The nature of these immune reactions in the host are not well understood but are considered to be due to reaction to constituents of the donor bone. Studies have demonstrated an increased donor-directed antibody response to fresh frozen bone grafts in recipients, but the clinical significance of this is yet to be determined (Stevenson et al. 1996, Aho et al. 1998, Ward et al. 2005). However, both the incorporation of cancellous bone and the healing (union) of cortical grafts are generally slower and less complete than those of similar autogenous grafts. Immunological factors may play a part in this, and may to some extent explain the unpredictable outcome of all allografts in revision surgery.

Following release for human implantation after a quarantine period of at least 6 months at -80°C , frozen bone allograft without cryoprotectants is assumed to have no viable cells. We undertook the present study to investigate whether it is possible

Gie et al. (1993) provided the first report on satisfactory early- to medium-term results of impaction grafting with fresh frozen bone allograft in the proximal femur. However, histological examination of post-mortem femora (Ling et al. 1993,

to culture cells *in vitro* from fresh frozen bone allograft.

Material and methods

Ethical committee approval was obtained to harvest fresh cancellous bone specimens from patients undergoing primary total hip replacement. To be eligible, patients had to fulfill all our existing criteria to be a bone donor. Bone plug-sized cancellous bone cores were removed from femoral heads immediately after resection from 8 patients. The mean age of the patients was 64 (50–79) years. The cores were immediately transferred to a transport medium (Minimal Essential Medium (MEM) containing 50 µg/mL gentamicin and 2.5 µg/mL amphotericin) and stored at 4°C. The remaining femoral head was placed in the hospital bone bank according to routine protocols.

8 similarly sized samples of fresh frozen allograft were collected and details of the donors were recorded. The mean age of the patients was 66 (52–81) years. In all cases, the allograft had been stored in the hospital bone bank for at least 6 months and had been authorized for use following negative blood testing of donor patients for HIV and viral hepatitis. In all cases, the allograft was being used in patients undergoing revision hip surgery for aseptic loosening. A sample of allograft was taken at the time of impaction grafting, and was placed in the transport medium and stored at 4°C.

Culture

All specimens were plated out *in vitro* within 24 h. This involved each specimen being broken down into pieces approximately 2 mm in their largest dimension. 3 bone fragments were then placed in each individual well of a 24-well cell culture plate. 2 plates were used for each specimen, giving a total of 48 replicates. Each well was filled with 1 mL of growth medium (MEM supplemented with 10% fetal bovine serum), which was changed twice weekly for all specimens. The plates were incubated at 37°C in an atmosphere of 5% CO₂ and 95% air. Specimens from fresh and frozen allograft were incubated separately to prevent cross-contamination.

After 2 weeks specimens were assessed by microscopy for evidence of cell growth, either on the bone or on the surface of culture wells.

RNA analysis

Total RNA was collected from cultured cells after 4 weeks of culture from two fresh bone specimens and one allograft specimen that had given rise to cell growth, for the analysis of gene expression by reverse transcription quantitative polymerase chain reaction (RT-qPCR) using an ABI Prism 7000 (Applied Biosystems, Warrington, UK). The probes used for osteocalcin (OCN, Hs00179899_m1), bone sialoprotein (BSP, Hs001737201_m1), collagen type I (coll 1, Hs00164099_m1), and the transcription factors Runx 2 (Hs00231692_m1) and Osterix (Hs00541729_m1) were TaqMan Gene Expression Assays (Applied Biosystems, Warrington, UK). The abundance of transcripts was calculated relative to the control gene 18S RNA.

Statistics

Parametric tests (median, SD) were used when cell growth on wells and bone was the only variable involved in the analysis. When comparison with other variables was done, for which no assumption of normal distribution could be made—as in evaluating the difference in cell growth in 2 specimens—a non-parametric test (Mann-Whitney test) was used. Differences were considered statistically significant at $p < 0.001$.

Results

After 2 weeks of incubation, all plates were examined by microscopy. The fresh cancellous bone specimens all showed evidence of cell growth in the wells (Figure 1). All but a few (median 93%) showed cell growth on the trabecular bone itself (Figure 2). In the few specimens where cell growth was not noted, marrow fat obscuring the trabeculae was believed to be the cause. In the previously frozen allograft specimens, no cell growth was evident on any fragment of allograft bone. However, in 4 of the 8 frozen allograft specimens cell growth was noted in the occasional well (median 1.0%) (Figure 3). This is significantly lower than the median of the fresh bone cultures ($p < 0.001$,

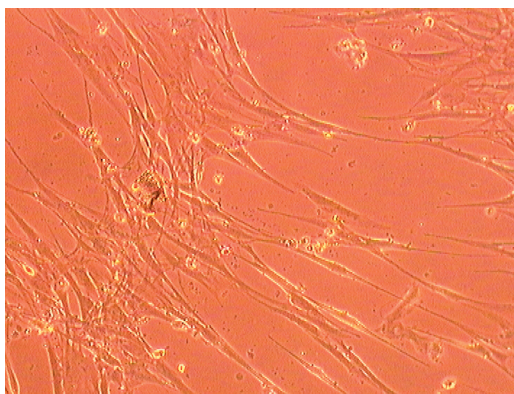


Figure 1. Cell growth on the surface of a cell culture well containing fresh bone.

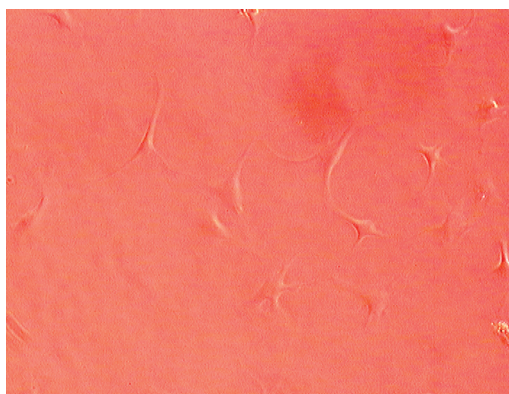


Figure 3. Cell growth on the surface of a cell culture well containing fresh frozen allograft.

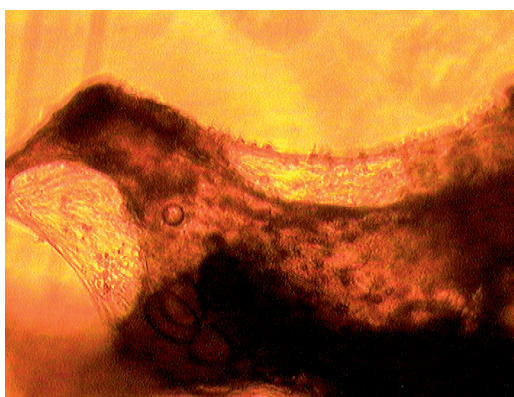


Figure 2. Cell growth on a fragment of fresh bone.

Mann-Whitney W-test) (Table 1).

There was no appreciable difference in the relative abundances of osteoblast-related mRNA tran-

scripts in cells from fresh or frozen bone (Table 2 and Figure 4). The transcription factor Osterix was detectable in cells from only one of the two fresh bone samples studied and in those from the frozen sample. This suggests that the cultured cells from both fresh and frozen bone are derived from similar progenitor cell populations.

Discussion

Cryopreservation is a process of controlled-rate freezing using DMSO (dimethyl sulfoxide) and glycerol to remove water during the freezing process in an attempt to preserve viable cells. The process works by altering water crystallization during the freezing process, which preserves up to 80% of cells (Shelton et al. 1998). The superiority of

Table 1. Growth of specimens on culture plates

Specimen	Fresh bone				Frozen bone			
	Growth on bone		Growth on well		Growth on bone		Growth on well	
	n	Ratio ^a	n	Ratio ^a	n	Ratio ^a	n	Ratio ^a
1	45	0.94	48	1	0	0	0	0
2	44	0.92	48	1	0	0	1	0.02
3	39	0.81	48	1	0	0	0	0
4	44	0.92	48	1	0	0	0	0
5	48	1	48	1	0	0	1	0.02
6	43	0.90	48	1	0	0	2	0.04
7	48	1	48	1	0	0	0	0
8	48	1	48	1	0	0	2	0.04
Median	44.5	0.93	48	1	0	0	0.5	0.01

^a Ratio = n/48

Table 2. Relative abundance of osteoblast-related mRNAs in comparison to that of structural 18S RNA transcripts; mean (SD)

Cells	18S	Osteocalcin	Runx2	Bone sialoprotein	Collagen type 1
Fresh bone A	1.00 (0.07)	1104 (75)	1.64 (0.28)	1070 (1713)	2.02 (0.13)
Fresh bone B	1.00 (0.05)	1493 (75)	5.89 (2.87)	419 (184)	2.35 (0.24)
Frozen bone	1.00 (0.04)	863 (41)	5.90 (0.71)	43 (67)	4.76 (0.39)

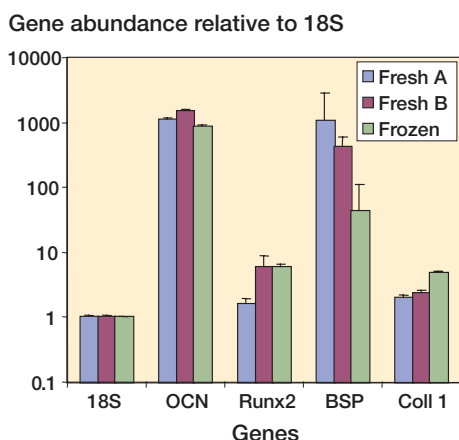


Figure 4. Relative abundance of gene products (mean and SD). OCN: osteocalcin; Runx2: transcription factor; BSP: bone sialoprotein; Coll 1: type-1 collagen

the autograft is not only due to viable cells and the absence of an immune response, but also to lack of freezing or preservation. However, cryopreservation without cryoprotectants should remove all viable donor cells.

Fresh frozen grafts are stronger, more immunogenic, and more completely incorporated than freeze-dried grafts. Ehrler and Vaccaro (2000) reported that allografts used alone or in combination with autografts for posterior lumbar spinal procedures have reduced fusion rates as compared to autografts. We hypothesized that fresh allograft would have more viable cells than frozen allograft, and decided to test the hypothesis by *in vitro* tests.

Following implantation, fresh frozen human allograft is thought to act as an osteoconductive conduit on which host bone is laid down as the graft is resorbed, although biopsies have shown that not all the graft is removed (Mikhail et al. 1999, Ullmark and Obrant 2002) Cryopreservation has been assumed to remove all viable cells from

the allograft, but our study has shown that osteoblast-related cells can be grown *in vitro* from fresh frozen allograft specimens after at least six months of quarantine in a bone bank. The cells derived from frozen grafts were morphologically indistinguishable from those grown out of freshly harvested trabecular bone, and had a similar mRNA profile with respect to osteoblast-related genes.

All our specimens were cultured in 10% fetal bovine serum, but autologous human serum is known to give better cell proliferation (Shigeno and Ashton 1995, McAlinden and Wilson 2000) and cell growth *in vivo* may be potentially more extensive. The fate of those cells may be determined by the rate of vascularisation of the graft, which will bring not only improved pO₂ and nutrition, but also cell-to-cell contacts and paracrine factors that may direct their proliferation and differentiation.

Disruption of cells is the first step in RNA isolation and one of the most critical steps, affecting both yield and quality of the isolated RNA. Finding the most appropriate method of cell or tissue disruption for the specific starting material is important for maximizing the yield and quality of the RNA preparation. The RNA samples thus obtained can be reverse-transcribed into cDNA and used successfully in the reverse transcription polymerase chain reaction amplification of transcripts (Barbaric et al. 2002).

A frozen autograft will resemble an allograft. The detrimental effects of preservation and the immune response to the allografts contribute to failure of these grafts *in vivo* (Stevenson et al. 1996). In allograft recipients, growth of donor cells may be one aspect of a whole spectrum of immunological reactions occurring following implantation, which may lead to a localized host/graft immune response and explain the inconsistent behavior of allografts in revision surgery. Further *in vivo* studies are

required to understand the extent and importance of this phenomenon.

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