RESEARCH PAPER

Cell Viability in a Cryopreserved Human Cancellous Allograft

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Purpose. To determine the persistence of living cells in cryopreserved bone grafts as well as their biological potential.
Materials and methods. Several cancellous bone fragments were processed that had been extracted from a bone-bank cryopreserved femoral condyle used in a revision total hip arthroplasty. After fragment lavage and digestion, a group of cells were isolated that were deposited in an α-MEM culture medium supplemented with 10% bovine fetal serum (BFS) (GIBCO) and 1% penicillin-streptomycin (GIBCO) and cultured under standard conditions at serum-free 37° C and 5% CO_2 enriched air (BCO).
Results. Some spindle-like cells bound to the plastic surface of the culture plate at 48 hs from processing. After 10 days' culture, cells quickly started proliferating and generating colonies. Once the culture was expanded, the isolated population of cells was phenotyped. The expression of the surface markers was analyzed by means of flow cytometry and showed an expression pattern similar to that obtained for the population of mesenchymal stem cells derived from human bone marrow.
Conclusions. Bone cryopreserved allografts contain viable cells that could correspond, on the basis of their mesenchymal lineage and their low degree of morphological differentiation, to osteogenerating cell precursors.

Key words: *allograft, cryopreservation, cell viability.*

Viabilidad celular en un aloinjerto de hueso esponjoso humano criopreservado

Objetivo. Determinar la persistencia de células vivas en injertos óseos criopreservados y su potencialidad biológica.
Material y métodos. Se procesaron diversos fragmentos óseos esponjosos provenientes de un cóndilo femoral crioconservado, de nuestro banco de huesos, utilizado en la cirugía de un recambio de prótesis de cadera. Previo lavado y digestión de los fragmentos, se aislaron células que fueron depositadas en un medio de cultivo α-MEM suplementado con un 10% de suero fetal bovino (FBS) (GIBCO) y un 1% de penicilina-estreptomicina (GIBCO) y cultivadas en condiciones estándar a 37° C y 5% CO_2 en aire enriquecido (BCO) sin suero.
Resultados. Algunas células con aspecto fusiforme se adhirieron al plástico de la placa de cultivo a las 48 horas del procesamiento. Después de 10 días en cultivo, las células empezaron a proliferar rápidamente y a generar colonias. Una vez expandido el cultivo, se procedió al fenotipaje de la población celular aislada. La expresión de los marcadores de superficie analizada mediante citometría de flujo mostró un patrón de expresión similar al obtenido para la población de células madre mesenquimales derivadas de médula ósea humana.
Conclusiones. Los aloinjertos óseos criopreservados contienen células viables que pueden corresponder, atendiendo a su estirpe mesenquimal y a su escaso grado de diferenciación morfológica, a precursores de células osteoformadoras.

Palabras clave: *aloinjerto, criopreservación, viabilidad celular.*

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Use of cryopreserved cancellous bone grafts to reconstruct acetabular bone defects with osteolysis secondary to loose total hip arthroplasties has shown a high degree of success in restoring the bone defects involved.

Bone graft incorporation is multifactorial and complex. The degree and pattern of incorporation that is achieved de-

pends on the histological behavior of the graft; the status of the bed, in particular its blood supply; the biomechanical requirements and the stability achieved between the graft and the host, apart from any potential immunological processes.

Allograft processing (cryopreservation) reduces their immunogenicity, but also their biological potential and their osteogenic capacity, given that all living cells get destroyed[1-3]. Nonetheless, there are some facts that raise some doubts about these considerations.

Several studies have confirmed, both clinically and histologically, the incorporation of fragmented cryopreserved impacted allografts used in acetabular revision surgery. The technique by Sloof[4,5] has been shown to deliver good results when impacting a cryopreserved cancellous allograft onto the acetabulum and placing a cemented implant on the surface thus created[6,7]. We[8] have published excellent results with a similar technique, placing a hemispherical threaded hydroxyapatite-coated acetabular component onto the impacted cryopreserved bone grafts. The same Nijmegen group[9] showed in a study of 24 biopsy samples of patients treated with Sloof's technique the rapid revascularization of the graft, followed by resorption and bone formation from the third to the fifteenth month post-op.

One might infer that incorporation is due to osteoconduction-related phenomena, ruling out any role for osteoinduction and, above all, for osteogenesis. However, in the light of the clinical results, it could be assumed that there are other phenomena involved apart from a simple invasion of the graft by the surrounding tissues. Recent findings related to the persistence of living cells in cryopreserved allografts provide further arguments for this debate[10,11].

This study is an attempt to further the study into the biological capacity inherent in cryopreserved allografts like those used regularly in our clinical practice.

MATERIAL AND METHODS

The samples were obtained from a fragment of cancellous bone from a femoral condyle taken from our hospital's bone bank, in the course of a total hip replacement that required a cryopreserved bone graft. They came from a 61-year old male, blood group B and Rh +. The extraction was made 3 months after implantation.

The criteria of the *Musculoskeletal Council of the America Association of Tissue Banks* (AATB)[12] and the *European Association of Musculoskeletal Transplantation* (EAMST)[13] were followed for the storage of bone tissue. It was frozen at –80°C, immediately after being harvested, in a double sterile container. Serum antibody tests were negative for IgG class antibody to the human immunodeficiency virus 1 and 2 (HIV-1/HIV-2); Australia antigen; IgG class antibody to hepatitis C; lues (RPR *[rapid plasma reagin]* and *Treponema Pallidum* Hemagglutination (TPHA); human T-cell leukemia virus (HTLV I/II); HIV p-24 Ag; IgG class antibody to the hepatitis B core antigen; and positive for the IgG class antibody to cytomegalovirus (CMV).

Cultures for the biological contamination test turned out negative for anaerobes and aerobes. The histological exam of the bone harvested revealed that the sample was a piece of cancellous bone of normal structure.

The cancellous bone fragment was cut up into small pieces (3 x 3 x 2 mm). These were washed with calcium and magnesium free saline phosphate buffer and digested for 24 hours at 37 °C with 1 mg/ml collagenase (type II, Worthington) in a serum-free ·-MEM culture medium (GIBCO).

The isolated cells were recovered by centrifugation, resuspended in a complete ·-MEM culture medium supplemented with 10% de fetal bovine serum (FBS) (GIBCO) and 1% penicillin-streptomycin (GIBCO) and cultured in standard conditions at 37 °C and 5% CO_2 in air. The culture medium was exchanged 48 hours later in order to select the adhering cells and, subsequently, every 3-4 days. When the cells reached 80% confluence, they were tripsinized, designated as a primary culture and reseeded for their ulterior expansion.

The expression of surface markers was studied with flow cytometry. 2 x 105 cells were washed with phosphate buffered saline supplemented with 1% BSA and 0.1% sodium azide [Sigma, St. Louis, MO, USA.]) in polypropylene tubes and marked with 10 Ìl FITC or PE conjugated antibodies specific for CD105 (Serotec, Oxford, UK), CD166, CD29, CD44, CD90, CD117, CD14, CD34 and CD45 (BD Pharmingen, San Jose, CA, USA) for 30 minutes at room temperature and in the dark. The expression levels of each marker were compared with those of the IgG isotopic controls (Caltag Laboratories, Burlingame, CA, USA). Cells were analyzed using a Coulter EPICS XL cytometer (Beckman Coulter, Miami, FL, USA). Data was collected by means of the Expo32 software (Beckman Coulter, Miami, FL, USA). The level of expression of each marker (fluorescence intensity) is defined as the quotient between the signal of the antibody and that of the isotype control (1 = no difference).

RESULTS

Some spindle-looking cells bound to the plastic of the culture plate at 48 hours from processing. After 10 days in culture, the cells rapidly started proliferating and generating fibroblastic type colonies (fig. 1).

Once the culture was expanded, the isolated cell population was phenotyped. The expression of surface markers, analyzed through flow cytometry, was positive for CD105, CD44, CD166, CD29 and CD90, and negative for CD117, CD34, CD45 and CD14 (fig. 2). This expression pattern is

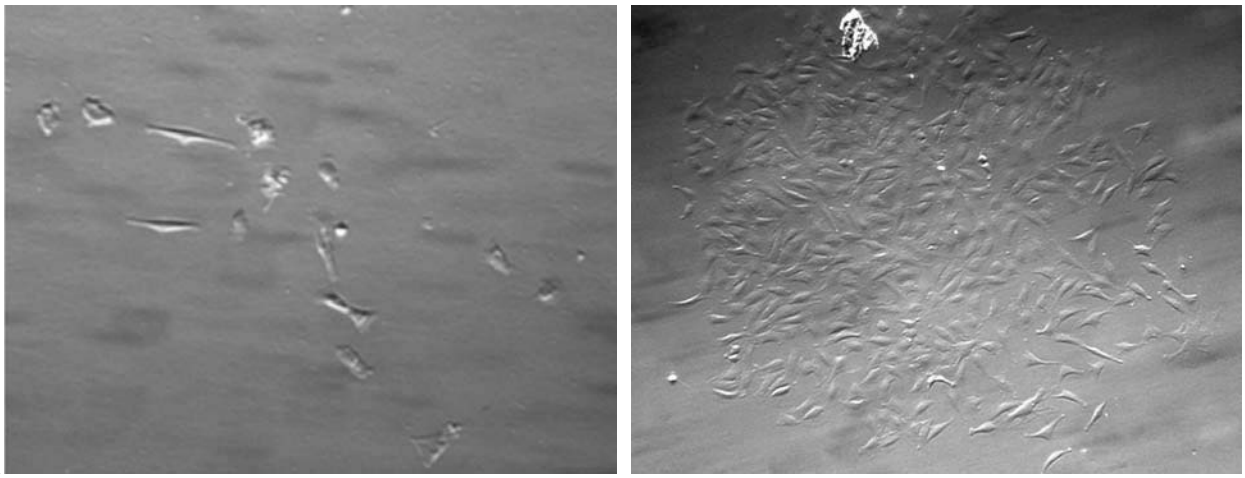

Figure 1. *Primary culture of the cells derived from the cryopreserved .bone graft The images show the spindle-like morphology of the isolated5 days into the culture (A),and the appearance of the colonies 2 weeks into the culture on the basis of the cells that did get bound (B).*

similar to that obtained for the population of bone marrow stromal cells[14] (table 1).

DISCUSSION

The excellent incorporation of fragmented cryopreserved cancellous bone grafts used in the reconstruction of acetabular defects witnessed in our clinical practice made us think whether potential cell viability could play a role in the graft's incorporation and remodeling. To find out, we processed fragments of cryopreserved cancellous bone and cultured them. The cultures showed that some mesenchymal cells survived the standard freezing protocols in bone banks.

Table 1. Comparative fluorescence intensity levels of surface markers as expressed by the cells of cryopreserved bone and human bone marrow stromal cells

Surface antigen	FBC	BMSC
	(level of expression/ratio)	
CD29	3.8	9.4
CD44	24.7	55.6
CD90	23.5	ND
CD105	2.3	4.2
CD166	10.4	27.8
CD14	1.03	1
CD34	1.7	0.58
CD45	1.0	1.08
CD117	1.1	ND

BMSC: bone marrow stromal cells; FBC: frozen bone cells; UD: undetermined. The expression level of each marker corresponds to the quotient between the signal of each antibody and that of its isotopic control (1 = no difference with the isotopic control).

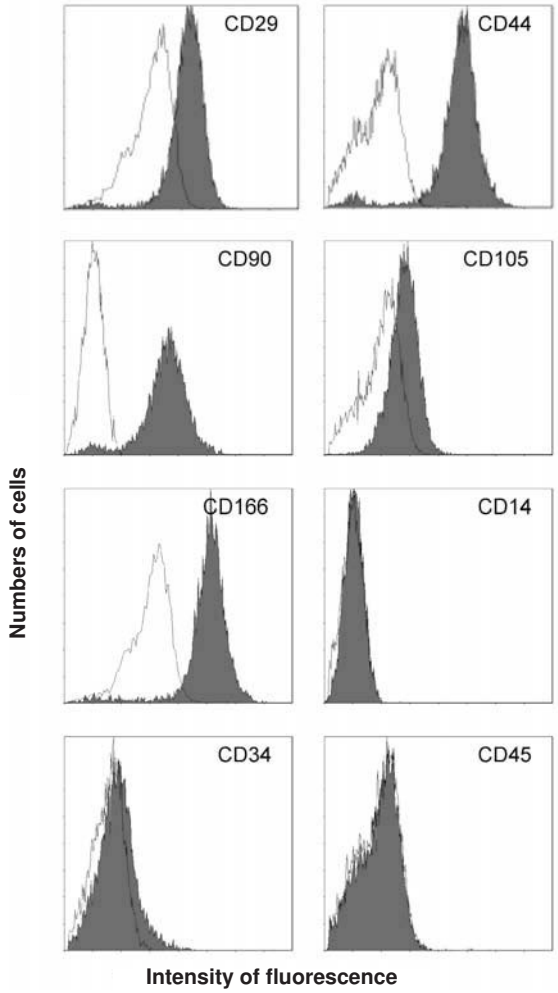

Figure 2. *Flow cytometry analysis of the expression of the surface markers of the cells derived from the bone graft. The image shows the purity of the cell population, isolated by means of a fluorescence intensity distribution analysis of the different surface antigens studied.*

Several techniques can be used to identify different cell types. We used flow cytometry with fluorescence detection. Flow cytometry allows us to individually analyze thousands of cells for the expression of proteins located on the cell surface, such as differentiation markers. Differentiation marker-based identification is dependent on the differential expression of the different to cell types[15].

Phenotypic analysis of the cells surviving in the processed graft indicates that the latter are characterized by a marker expression pattern similar to bone marrow stromal cells.

At this point, it is necessary to ascertain whether the isolated cells possess the pluripotentiality shown in the mesenchymal cells[16-18], including their osteogenic capacity, and whether they are able to actively participate in the remodeling process of the recipient bone instead of the graft.

We have found other studies in the literature that, like ours, attest to the survival of living cells in a frozen allograft. Weyts et al[10] detected cells whose allelotyping was identical to that of the donor in one of their two processed cryopreserved allograft samples. These cells were identified only on the basis of their morphotype as differentiated (evolved osteoblasts or immature osteocytes) and with little proliferative capacity. Heyliguers and Klein-Nulend[11] also detected living cells in cryopreserved grafts. Cell identification was obtained by means of an analysis with DNA markers, which showed identical alleles in both donor and graft and which, in cell cultures, demonstrated their osteoblastic capacity, expressed by high alkaline phosphatase levels in the culture medium.

The residual immunogenicity of frozen grafts, as shown by Deijkers et al[19], could be attributed to the persistence of living cells. In dogs it has been shown that hystocompatibility improves the incorporation of frozen allografts[20], which suggests that activation of the immune system negatively affects osteointegration. In human beings, the influence of immunohistocompatibility on the graft's success is not clear21, although Friedlaender et al reported that coincidence of immunocompatibility antigens improved clinical success[22].

In the light of these findings, radiological incorporation of cryopreserved bone grafts used in fragmentation and impaction techniques such as Slooff's[4-7,22,23] and our very own[8] could be construed as a biological incorporation. Van der Donk et al showed long before, in 24 biopsies of cases treated with their technique, how a graft could be replaced by new lamellar bone with a trabecular structure and with negligible remains of the graft[9], although they could not determine the origin of the regeneration (donor or host) by means of DNA.

These different kinas of behavior can be explained by the occasional survival of cells in different environments and pressure conditions, and surrounded by different nutrients, which may account for their viability and compatibility.

In any case, the survival of living cells in cryopreserved allografts calls into doubt the up-to-now accepted theory according to which cells died as a result of the breakage of the cell membrane caused by low temperature[1-3]. It also raises relevant issues as regards the graft's regenerating capacity, its immunogenicity and, above all, the transmission of infections.

Further research is obviously necessary to determine the significance and the implications of these preliminary findings.

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Conflict of interests

The authors have declared to have no conflict of interests.