Full Length Research Paper

Cellular response of Murine Osteoblasts to Cryopreservation: the influence of attachment to Hydroxyapatite (HA) scaffolds

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This paper presents data relevant to the rational design of cryopreservation processes for tissue-engineered bone. The effects of cell-scaffold interactions and cell-cell interactions on osteoblast viability and attachment to hydroxyapatite (HA) scaffolds following cryopreservation processing are defined experimentally. It is found that cryopreservation processing detaches osteoblasts from the HA scaffold and that cells suffer significantly more damage than when they are cryopreserved in liquid suspension. Thus, some aspect of cell-surface interaction is detrimental. The detrimental influence involves thermal modulations because when osteoblasts attached to HA are exposed to cryoprotective agents for two-step freezing without temperature change, morphological changes occur but little detachment or damage is observed. Enhanced post-thaw attachment and viability are realized for higher number densities of osteoblasts on the HA scaffold surface, indicating that some aspect of cell-cell interaction is beneficial. Higher porosity HA scaffolds are better for cell attachment but porosity is not a statistically significant factor for post thaw viability.

Keywords*: osteoblast, cryopreservation, attachment, hydroxyapatite.

INTRODUCTION

Each year ~300,000 bone grafts are performed in the U.S. Although auto-grafts and allo-grafts seem ideal to repair bone defects, they are limited in supply, carry additional surgical/patient risks and diminished proper-ties. Osteoblast (OB)-seeded hydroxyapatite (HA) impl-ants are a new type of engineered cortical bone substi-tute because such implants have the potential to create a similar mineral content and crystal structure when compared to bone. The evolving field of tissue enginee-ring has created the need for cryopreservation processes

to store tissue-engineered products. The unpredictable demand for specific tissues will make it necessary for manufacturers and distributors to create tissue banks in order to maintain large stocks as a means of ensuring a steady supply of product (Karlsson and Toner, 1996). Current techniques for the preservation of tissues are primarily empirical extensions of protocols developed for cellular systems. It is important to realize that these protocols cannot necessarily be applied with universal efficacy to tissues (Liu et al., 2005). However, valuable insight into the issues important for developing cryopre-servation methods for bio-artificial tissue products can be gained from studying the freezing response of cells in suspension. Rational design of such preservation techno-logy should include consideration of the responses to

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cryopreservation protocols of the osteoblasts, the HA scaffold itself as well as cell-scaffold and cell-cell interactions. Previous research has demonstrated that high survival (80-90%) of osteoblasts can be achieved after preservation processing by slow freezing when cells are processed in liquid suspension (Liu, 2002). However, tissue-engineered bone constructs involve osteoblasts attached to one or more types of scaffold materials that can be configured in a multitude of geometrical forms.

In tissue engineering, it is crucial for cells to remain attached to the surface of biomaterials. On the one hand most tissue-derived cells are anchorage-dependent and require attachment to a solid surface for viability and growth (Porter, 2006), while on the other hand, cell adhesion precedes cell spreading, migration, differenttiation and mineralization (Porter, 2006; Shahab 2006), all of which are very important to the final formation of engineered tissues.

Although mechanical strain is used to modify the cell morphology by many researchers (Lacouture et al., 2002; Derderian, 2005; Kakisis et al., 2004), excessive forces may cause detachment of cells from the surfaces or damage the cells. Cryopreservation of NIH-3T3 cells and Mouse fibroblast cell line L929 fixed to glass and silicon were studied by Hornung et al. (1996). Their results showed that both cell types detached from the surface because of the mechanical and osmotic stress. However no detach-ment was reported for the immortalized human endothe-lial cell line (EVC304) fixed on microcarrier beads (Pegg, 2002). So attachment is cell type, surface character and cryopreservation procedure specific.

It was assumed that physical contact with ice crystals might cause damage to endothelial cells when cryopre-serving the mammalian liver using high subzero freezing method (Ishine et al., 1999). No such damage was obser-ved for the cells not attached to a surface (Randy, 2004).

The roles played by cell-surface and cell-cell interact-tions in post-thaw cryopreservation response are poorly understood, but there are clear indications that such interactions may have both beneficial as well as detri-mental effects (Kotobuki et al., 2005; Kotobuki et al., 2004; Acker, 1998). The effect of cell-surface and cell-cell interactions on the attachment and viability of osteoblasts attached to HA surface was experimentally investigated. The results could be the guidance for the successful cryopreservation of tissue-engineered bone constructs.

MATERIALS AND METHODS

Culture of OB Cells

MC3T3-El cells, a mouse calvaria-derived osteoblasts cell line (Sudo, 1983), were cultured in α-MEM (α-Minimum Essential Medium, Gibeo BRL) supplemented by 5% fetal bovine serum (FBS, Gibeo BRL) with incubation at 37°C and 5% CO_2 atmosphere. Cells were harvested by rinsing with 1×PBS, exposing to 0.25% trypsin solution (Giblo BRL) for 3 min at room temperature. Cells were then suspended in α-MEM at a concentration of 4×10^4 cells/ml.

HA Scaffolds and Plastic Coverslips

Three types of HA discs with a diameter of 11 mm and thickness of 1 mm were fabricated in the Biomaterials and Tissue Engineering Laboratory at Michigan State University. Plastic coverslips (Therinanox, 13 mm diameter, NUNC Brand products) were used as a control group to compare with HA.

Seeding of OB cells on the Surface of HA Discs

All HA discs were sterilized before seeding with cells. OB cell suspensions containing 4×10^4 cells/ml were seeded on HA discs which covered the bottom of 24-well plates (Costar). The 24-well plates were then placed into a 37°C and 5% CO_2 atmosphere incubator. The media was changed every other day. All the OB:HA discs to be tested were incubated for 2 days unless otherwise noted. Plastic coverslips were placed on the bottom of 100 mm petri dishes to seed cells on their surfaces.

Addition and Removal of Cryoprotectant agents (CPAs)

5% Dimethyl sulfoxide (DMSO, J.T. Baker) in MEM was used for two-step freezing. Samples were placed directly into 5% DMSO in MEM for 15 min and then were transferred into sterile round-bottom tubes (FALCON, 15 ml, 1 disc/tube) with 1.5 ml, 5% DMSO in MEM before performing the freezing protocol.

Freezing and Thawing

For two-step slow freezing, the samples were cooled at a slow rate of 9°C /min to -80°C in the first step and then quenched into liquid nitrogen. All samples were thawed by shaking in 37°C water bath at the warming rate of ~60°C /min (Liu and McGrath, 2005).

Viability Assay

Live/Dead viability/cytotoxicity kit (L-3224; Molecular Probes) was used to stain cells as a viability assay. The assay was performed according to the manufacture's protocol. For control groups, at the end of the incubation times, the HA discs with OB cells were taken out of the wells, placed into a 35 mm diameter petri dish and washed twice with MEM, For frozen samples, after removal of CPAs, the discs were put into media and incubated for 5 min at room temperature. The Live/Dead solution was then dispensed on the surface of the OBs on the HA discs and cultured for 20-30 min at room temperature. The stained cells were transferred to a microscope with a UV light source. A digital camera (Spot, Diagnostic Instruments, Inc.) was attached to the microscope and used to produce digital images of live cells (green) and dead cells (red) by focusing on different locations on the discs with a 20 x phase contrast objective.

Statistics

For each experiment, 4-5 discs or coverslips (n= 4 or 5) were used to evaluate the cell attachment or viability. Five images were taken of each sample at random positions. The average cell number of the 5 images was used for data analysis. By calculating the area of each image, HA disc and coverslip, the number of cells attached to the surface can be estimated. Data shown in the Results section are representative of three separate experiments (mean ± SD).

Single cells   Colony   Confluent
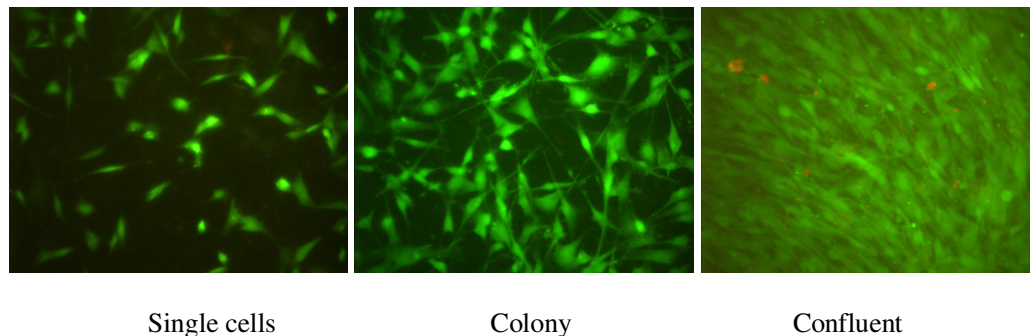
Figure 1. Three major categories of cell-cell configuration observed on HA scaffolds.

Elongated cells   Rounded Cells
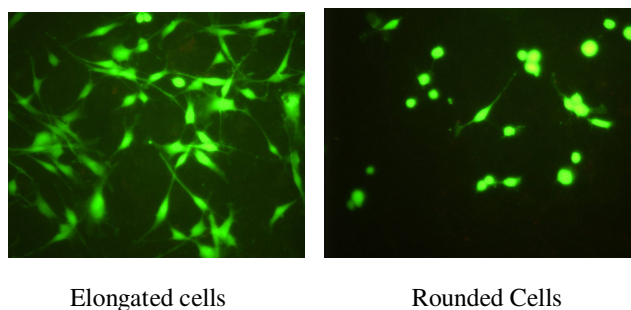
Figure 2. Cell Morphologies
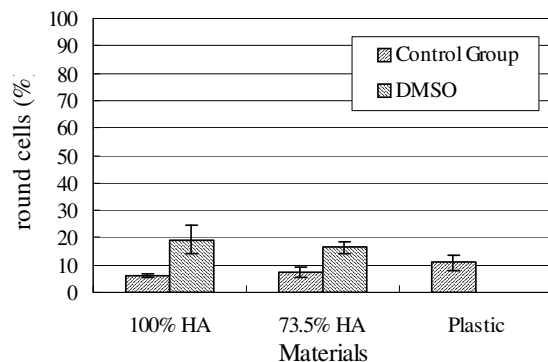
Figure 3. Morphology changes induced by CPAs.

RESULTS

Cell Density

Following incubation of osteoblasts on HA discs, the cell number density was found to vary over the surface of the discs. While the cell number density varies in a more or less continuous manner over the surface of the HA discs, three major classes of number density are defined here for convenience: single cells, cell colonies and confluent monolayers of cells. Images of these three configurations are shown below in Figure 1. The single cell configuration is defined as that where cells had little or no cell-cell interactions. The colony cells denote an intermediate

state when cell-cell interactions occur, but confluence is not established. Confluent cell monolayers represent cells with maximal two-dimensional cell-cell contact. Using these morphological delineations it was found after 2 days of incubation that the typical HA scaffold surface was covered with 15% single cells, 70% colony cells and 15% confluent monolayer of cells. The areas on the HA scaffold where these morphologies were found were distinct. Consequently it was possible to image represent-tative locations within these areas before (control) and after treatments to assess changes relative to untreated control conditions. In some cases described below, resu-lts are reported for the average of these 3 configurations (i.e. for the HA surface as a whole), while in other cases the influence of cell-cell contact is defined by distinguish-hing between these configurations.
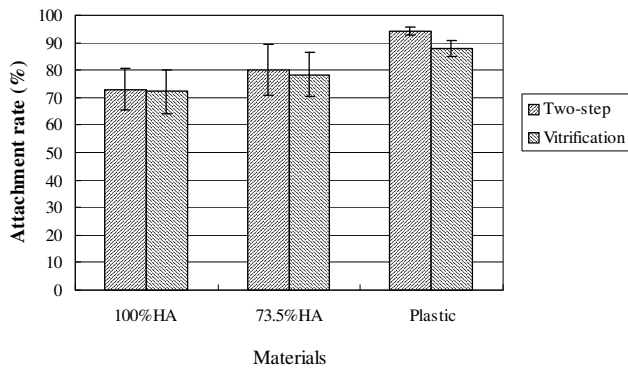
Cell Morphology

While the morphology of osteoblasts attached to HA scaffolds is complex, two major types of morphology were observed. These morphologies were defined as "elongated" and "round" (Figure 2). After two days of culture on HA scaffolds, generally 90-95% of osteoblasts were elongated and less than 10% of cells were round.

Response of Osteoblasts to the Addition and Removal of CPAs

CPAs were added and removed in one step for two-step freezing as described in our recent paper (Liu and McGrath, 2002). Essentially all osteoblasts remained att-ached to HA scaffold and were viable after the addition and removal of CPAs using these procedures. Thus, the part of the overall cryopreservation process involving the addition and removal of CPAs is not detrimental to the attachment or viability of osteoblasts on HA scaffolds, at least for the protocols we developed previously.
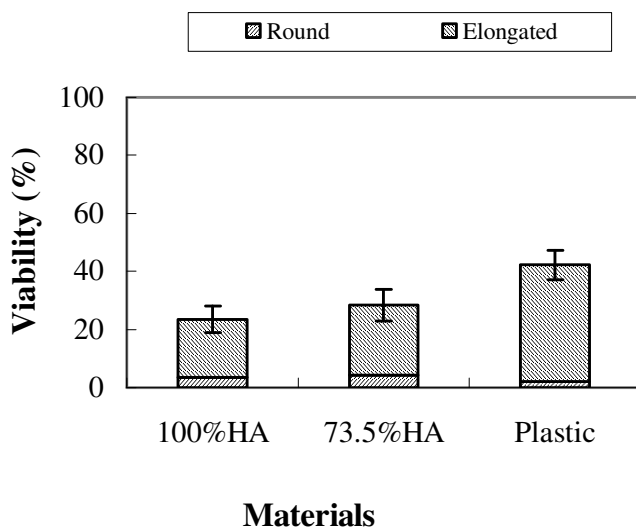
Figure 3 shows that the addition and removal of CPAs causes an alteration of morphology promoting the trans-formation of cells from elongated to round.

Figure 4. Post-thaw attachment of osteoblasts on HA scaffolds and plastic.
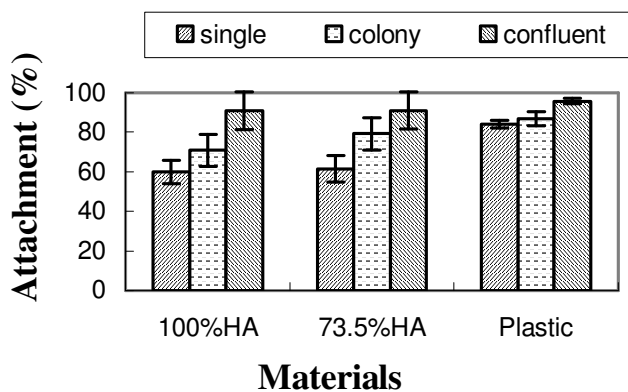
Figure 5. Two-step freezing: Post-thaw attachment of osteoblasts on HA scaffolds and plastic as a function of cell morphology. 3

Figure 6. Two-step freezing: Post-thaw viability of osteoblasts attached to HA scaffolds.

Post-thaw Attachment of Osteoblasts

Figure 4 reveals that most osteoblasts (~70-80%) remain attached to the HA scaffold immediately following cryopreservation processing. These results represent the average level of attachment for all 3 levels of cell-cell interaction (single cell, colony and confluent monolayer). These results probably represent the maximum amount of attachment to be expected because the samples were treated very gently during the whole processes.

Figures 5 show further details related to Figure 4. In these two figures the distinction is made between the 3 levels of cell-cell interaction. Specifically, Figure 5 shows that only 60% of single cells remain attached to HA scaffolds following two-step freezing, whereas approximately 90% of osteoblasts in confluent monolayers remain attached to the scaffolds. This is true for HA densities of 73.5% and 100%. Interestingly, most osteoblasts remain attached to the plastic coverslips and the apparent dependence on cell-cell contact is much less pronounced.

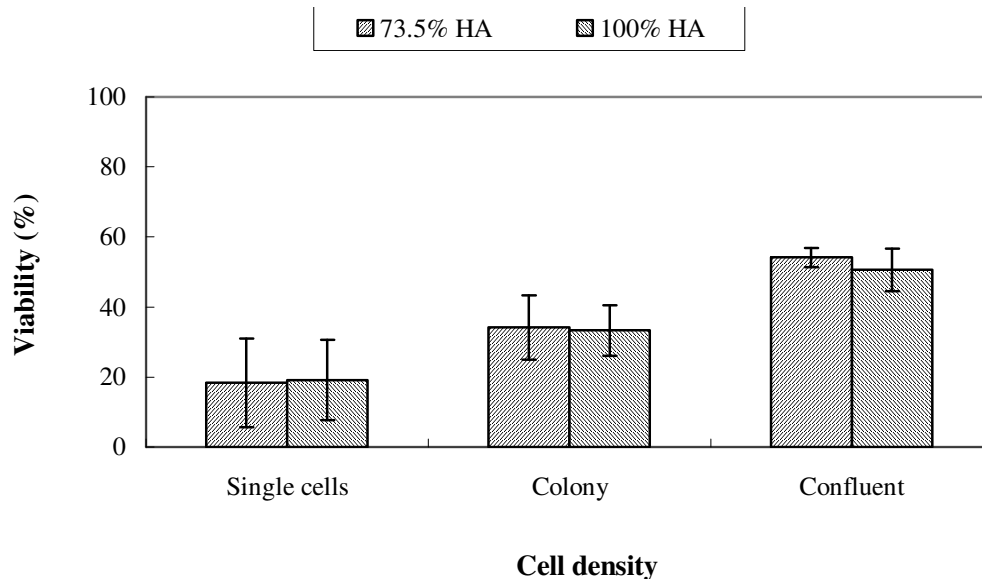## Post-thaw Viability: Attached and Detached Osteoblasts

The viability results are shown in Figure 6, revealing that 20% to 30% of osteoblasts attached to the HA scaffold remain viable after cryopreservation processing. Thus, while approximately three-quarters of the OBs remain attached to HA scaffolds, only about one-fourth of the attached cells are viable after cryopreservation. Most detached OBs were dead. Only 14% of the detached OBs that were frozen using the two-step process were viable. These results are averaged for all three configurations (single cell, colony and confluent monolayer). Viability on plastic is better than on HA. Figure 6 shows that most of the attached, viable cells are elongated rather than round cells.

Figure 7 illustrate in more detail the results shown in Figure 6. In particular, it quantifies the enhanced viability of attached OBs with increasing extent of cell-cell interaction. The viability increasing from some 20% for single cells to 50-60% for confluent monolayers.

DISCUSSION

Post-thaw Attachment of Osteoblasts

During cryopreservation, cell detachment may result from osmotic stress during the CPA addition and removal process and/or mechanical forces related to the cooling and warming processes. The CPA addition and removal processes alone did not detach osteoblast cells in this study. But it was found that addition and removal of CPAs did alter cell morphology from elongated cells to rounded cells (Figure 3). There are two possible reasons for morphology change of attached cells caused by CPAs.

Figure 7. Two-step freezing: Post-thaw viability of osteoblasts attached to HA scaffolds.

One is that cell-matrix attachment sites may be damaged as the cells swell and shrink in response to osmotic stress caused by addition or removal of CPAs. The other is that CPAs may depolymerize the proteins at the adherens junctions. Whatever the reason, the elongated cells became rounded cells after CPA treatment. The rounded cells were detached more readily than elongated cells during the cooling and warming processes.

Some fraction of osteoblasts detached from the surface of HA discs and plastics immediately following the cryopreservation process (Figure 4). Apparently the detachment occurred during the cooling and/or warming process. The mechanisms of detachment are not clear. Mechanical and osmotic stresses are possible reasons. We assume that detachment is caused primarily by differential thermal contraction between the cell structures involved in adhesion and the underlying matrix. The area contraction coefficient for human erythrocyte membranes with a cytoskeleton spectrin network is of order $10^{-3}/^{\circ}C$ (0.1% shrinkage per degree cooling) (Waugh and Evans, 1979). Plastic, glass and HA have much smaller thermal contra-ction coefficients (~10-1000-fold smaller). Additional ten-sion may develop within the cellular architecture upon cooling and the differential contraction between a cell and the substrate may break the attachment sites. Some of the cells may round up, while some cells may detach from the surface.

Improvement of Post-thaw Attachment of Osteoblasts

The data in Figure 4 show that attachment was significantly better when osteoblasts were cryopreserved on plastic coverslips rather than HA. This implies that post-thaw attachment could be enhanced by surface modification. The surface characteristics and material

properties can be improved by changing the components of HA. To enhance cardiomyocytes adhesion, polylactide-co-glycolide polymer surfaces might be coated with proteins (Brown, 2005).

It is shown in Figure 5 that confluent cells have signi-ficantly higher post-thaw attachment on HA discs com-pared to single and colony cells. Apparently more cell-cell interaction is beneficial for post-thaw attachment. Most cells attached to plastic coverslips are fully stretched with more attachment areas than those on HA, which assu-med an elongated, spindle morphology. So it seems that the single attached cells to plastic coverslips have enough focal adhesions to bear the mechanical stresses even without the help of cellular junctions. The goals defining what fraction of osteoblasts must remain attac-hed to the scaffold and what fraction of the attached cells must remain viable after thawing are not known at this time. However, it is assumed here that maximizing both cell attachment and cell viability are desirable goals.

Post-thaw Viability of Osteoblasts

The relatively low recovery of osteoblasts attached to HA scaffolds (Figures 6 and 7) compared to the recovery realized for OBs processed in liquid suspension (Liu and McGrath, 2002), clearly indicates that some aspect of cell attachment (cell-surface interaction) is detrimental, at least for the cryopreservation processes applied here.

The enhanced post-thaw attachment and viability (Figure 7) of confluent monolayers compared to single cells or small colonies may be the result of the increased strength of the monolayer due to load sharing by multiple cells. The cellular junctions may be involved in modula-ting the response to preservation, producing enhanced attachment and viability. Further experiments are needed

to explore the mechanism of attachment and viability to improve the cryopreservation quality of tissue-engineered bone and other tissue-engineered constructs.

Conclusion

Most osteoblasts (80-90%) in liquid suspension can be recovered from two-step freezing. The high survival is consistent with the lack of intracellular ice formation (IIF) and minimal toxicity associated with addition and removal of cryoprotectant agents (CPAs). The majority (~75%) of OBs remain attached to hydroxyapatite (HA) scaffolds immediately after thawing. Post-thaw attachment is enhanced by cell-cell interactions, with ~90% levels realized for confluent monolayers. Some aspect of cell-scaffold interaction is detrimental to post-thaw cell viability since viability immediately after thawing drops from ~80-90% for OB cells cryopreserved in liquid suspension to ~25% when OBs are attached to an HA scaffold. As is true for post-thaw attachment, post-thaw viability is enhanced for greater levels of cell-cell contact --- improving from ~20% for single cells to ~60% for confluent monolayers. Most of the viable OBs attached to the HA scaffold exist in an elongated rather than a round morphology. The detrimental nature of the cell-scaffold interaction relative to the cryopreservation viability response is not related to the addition and removal of CPAS, suggesting that some aspect associated with temperature change or phase change is involved in cell damage.

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