Bone Marrow Stem Cells Differentiated Into Cartilage by Manipulation of Gas Phase

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INTRODUCTION

A continual need for bone in craniofacial repair exists; one solution to the problem of procuring enough bone for this purpose lies with tissue engineering (Doan et al., 2010). In most bone tissue engineering studies, osteoblasts are cultured on a scaffold with or without growth factors and then implanted (Doan et al., 2010). Because of concerns about rejection of implants, embryonic stem cells and stem cells from bone marrow have also been placed on scaffolds, differentiated into bone cells and implanted (Jukes et al., 2008). In a few instances, stem cells have been differentiated into bone-forming cartilage for bone repair (Oliveira et al., 2009; Jukes et al., 2008; Doan et al., 2010).

The rationale for using cartilage for bone repair is that during growth and development, the bony skeleton forms primarily through endochondral ossification, involving mineralization of a cartilaginous template, then its vascularization and replacement by bone (Duke et al., 1993). Fracture healing also includes a cartilaginous stage.

Previously, mouse embryonic limb bud cells were differentiated into cartilage in NASA’s rotating bioreactor and used to heal defects in the skulls of mice (Montufar-Solis et al., 2004; Doan et al., 2009). The purpose of the current study was to isolate mouse bone marrow stem cells (BMSCs), expand the cells in culture and differentiate them into cartilage, creating a flat cartilaginous implant. To grow cartilage from BMSCs, we used hardware developed for a 1992 space-flight, Flexcell (Flex I®) plates and 35 mm culture plates.

MATERIALS AND METHODS

Isolation of BMSCs was carried out according to Masoud and Samad (2009) for isolation and culture of MSC from mouse bone marrow. Isolated bone marrow cells from 7-9 day old C57BL mice were expanded in culture. After 2 passages, 0.5 ml differentiation medium (see below) with 88,000 cells was inoculated onto Silastic® membranes supported by hardware casings or in assembled hardware units that were placed in Petri dishes for culture. In other experiments, Flex I® plates (Flexcell International) or 35 mm culture dishes (Falcon BD) were used. In these experiments the cell number inoculated onto the plate ranged from $7.55 \times 10^6$ to $34.2 \times 10^6$. Cultures were incubated at 37°C with or without 5% CO2 for two hours; 5% CO2 was used thereafter. After medium addition, cells were cultured for 3-7 days with ½ the medium changed every other day, then fixed with 10% buffered neutral formalin and stained.

Medium for BMSC differentiation was BGJb-Fitton-Jackson modification (GIBCO) supplemented with 10% fetal bovine serum (Fischer), 150 μg/ml ascorbic acid, and 1% Penicillin-Streptomycin solution (Sigma). BGJb-FJM is a chondrogenic/osteogenic medium. In one experiment, DMEM medium replaced the BGJb-FJM.

Cells were stained in situ with methylene blue and cresyl violet, both of which stain neuronal Nissl bodies, and either Toluidine blue, a metachromatic dye specific for cartilage matrix, or Alcian blue at low pH, again a cartilage specific stain (Huang et al., 2010). Flex I® aggregates were sectioned and stained with Toluidine blue.

Hardware used in these experiments was the BEX unit designed for our CELLS experiment on the International Microgravity Laboratory-1 mission in 1992 (Duke et al., 1995). The hardware (Figure 1) consists of a two-welled polycarbonate chamber into which is inserted a gas exchanging membrane of a Silastic® compound (Dow-Corning 3150+360) in the shape of two domes or “bubbles” which allow for gas exchange, and inflate or collapse as medium is added or removed. Deflector rings inside the bubble control fluid forces related to medium removal or addition.
The complete assembly also includes a silicon rubber gasket, a polycarbonate bottom plate, and a stainless steel support plate. For flight, cells were cultured on a cover slip between the gasket and the bottom plate. For some of these experiments, complete hardware units were used, and for some, only Silastic membrane bubbles were used. Cells were cultured on the interior surface of the bubble and the units placed in a Petri dish.

Figure 1. BEX hardware. A. Position used to culture prechondrocytes on coverslips for spaceflight. B. Position used to culture BMSCs on membrane.

To form a flat piece of tissue, cells were cultured on Flex I® plates which look like regular culture dishes, but have a bottom surface of Silastic. For controls, 35 mm culture dishes were used.

RESULTS

Cells in units placed in CO₂ for the attachment period spread on the membrane assuming a neuronal-like appearance. Staining with methylene blue revealed numerous dark bodies (Nissl bodies) in the cytoplasm (Figure 2A). Cells in units not exposed to CO₂ during the attachment period did not spread, and the cell layer consisted of rounded cells with areas of aggregation typical of early cartilage formation (Figure 3). When cells in these layers were stained with Toluidine blue after 7 days, the entire layer was metachromatic, indicating the presence of sulphated glycosaminoglycans (data not shown). Alcian blue staining identified the aggregated cells as chondrocytes (Huang et al., 2010). When a non-differentiating medium (e.g. DMEM) was used, only neuronal-like cells formed (Figure 2B). With increased initial cell densities, the Flex I® group formed aggregates that, when sectioned and stained with Toluidine Blue, resembled hypertrophied cartilage with metachromatic matrix (Figure 4). Aggregates formed in the Petri dishes stained with Alcian blue at low pH, confirming the presence of cartilage matrix (Figure 5; Huang et al., 2010).

Figure 2. Neuronal like cells. A. Stained with methylene blue. B. Stained with cresyl violet. Bars = 50 µm.

Figure 3. Aggregates of rounded cells interspersed with neuronal-like cells. Bar = 100 µm.

Figure 4. Aggregate from Flex I® plate, sectioned and stained with Toluidine blue. Pink indicates cartilage matrix. Bar = 50 µm.
DISCUSSION

This study shows that BMSCs can be differentiated into cartilage or neuronal-like cells by manipulating the gas environment during the initial attachment phase. The exposure to CO₂ during this initial stage of culture changed the pH of the medium, making it more alkaline as indicated by the phenol red indicator in the medium. The exposure to CO₂ using a non-differentiating medium (e.g. DMEM) did not result in chondrocyte-like cells being present; instead all the cells were spread with numerous processes. The same result was seen in all culture vessels.

To advance these studies toward the ultimate goal of developing a cartilage implant for humans, the ability to grow more cartilage must be developed. One way this could be done is by the use of TGFβ. The medium used for human studies must be serumless, so that the implant is not exposed to animal products during culture. First, BMSCs must be grown in serumless medium, differentiated into cartilage which will be implanted into defects in the skulls of mice to determine its bone-forming capabilities.

Another major problem arising in this study was the tendency of the cartilage to come off the plate because of the cartilage’s loss of contact with the surface as matrix develops. Using Cell-Tac as in the spaceflight experiment could solve this problem.

The phenomenon of forming bone from a cartilaginous template continues to ignite hope for patients with skeletal and craniofacial disorders, offering a promising alternative approach in the realm of bone regeneration and reconstruction.

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REFERENCES


