

Tissue Engineered Bone Replacements Systems

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Background and Introduction: Over one million operations for the repair of orthopedic irregularities such as trauma, fracture nonunions, and congenital defects are performed annually in the United States. Of these procedures, roughly 275,000 involve the use of bone grafts to assist the healing process [1]. Traditionally, bone grafts have been the material of choice for managing the repair of bone defects. Nearly \$300 million is spent annually in the United States on bone grafts and bone substitutes [2]. Grafts and other materials currently used for the treatment of orthopedic defects have select advantages, but autografts and allografts are deficient in several aspects. Autografts suffer from donor site shortage and surgery at the donor site often produces complications such as donor site damage, pain, infection, and hematoma [3-5]. Sterilization of allografts can lead to a lessening of the allograft's osteoinductivity or to a reduction in strength [6]. Avascular autografts and allografts remodel slowly due to lengthy vascularization times; as a result, healing of the defects is highly unpredictable [7]. Neither autografts nor allografts can be easily shaped to fit a bone defect, particularly under operating room conditions, and incomplete resorption of the graft may occur [8]. As a result of the drawbacks associated with the use of traditional grafts, efforts to tissue engineer a more desirable bone substitute are underway.

Rational: A composite of demineralized bone matrix (DBM) and polylactide may be useful as a tissue-engineered bone substitute. Polylactide and DBM are both biocompatible and osteoconductive. Polylactide beads are appealing because characteristics such as their degradation profile, mechanical properties, biological interaction, and their capacity to deliver growth factors or drugs can be customized to fit particular applications. DBM fragments are desirable because they contain osteoinductive BMPs that are delivered naturally. The osteoinductivity and mechanical properties of DBM can be tailored to suit specific applications by carefully monitoring the level of demineralization. A composite containing polylactide beads and DBM fragments may be advantageous in that polymer beads having different functions such as cell delivery, drug delivery, and growth factor delivery could be used

simultaneously with DBM fragments, thereby providing different mechanical properties and levels of osteoinductivity for the treatment of specific orthopedic defects or conditions. Thus, it is feasible to investigate the potential for a composite system consisting of DBM fragments and polylactide beads to support the attachment and proliferation of mesenchymal stem cells and to provide a structure for the cells' differentiation into the osteoblast lineage.

Methods:

Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed to monitor the expression of select osteoblastic genes by D1 and MC3T3 cells cultured on specific composite systems. The systems consisted of 100% DBM, 70% DBM:30% PL, 50% DBM:50% PL, and 100% PL. On days 4, 13, 24, and 36, the designated RT-PCR samples were rinsed twice gently with PBS. Ribonucleic acid (RNA) was then isolated from the samples at each timepoint using an RNeasy® Mini Kit (QIAGEN) and following the manufacturer's protocol. The purity and concentration of the isolated RNA was determined using an RNA 6000 Nano Assay Kit (Agilent Technologies) and following the manufacturer's protocol. Primer pairs were chosen to test for the expression of four mouse target genes in the RNA samples: osteoblast-specific genes including bone sialoprotein (BSP), osteocalcin (OCN), and runt-related transcription factor 2 (Runx2); and an endogenous control gene of β -actin (β -ACT).

Results: The level of BSP expression in D1 cells increased significantly ($p < 0.05$) between day 13 and day 24 for the 70/30 and 50/50 mixtures; BSP levels increased significantly ($p < 0.05$) between day 13 and day 36 for 100DBM and between day 4 and day 24 for 100PL. On days 4 and 13, BSP expression by D1 cells on 100PL was significantly higher ($p < 0.05$) than expression on 100DBM. By day 24, BSP expression by D1 cells on 100PL was significantly higher ($p < 0.05$) than expression on the other three mixtures.

Levels of Runx2 expression in D1 cells increased significantly ($p < 0.05$) between day 4 and day 24 for all mixtures. With the exception of the 50/50 mixture on day 13, Runx2 expression by

D1 cells on 100PL was significantly greater ($p < 0.05$) than expression on the other three mixtures on days 4, 13, and 24.

The level of OCN expression by D1 cells increased significantly ($p < 0.05$) (and exponentially) between day 13 and day 36 for the 70/30 and 50/50 mixtures; the OCN level increased significantly ($p < 0.05$) between day 13 and day 24 for 100PL. OCN expression by D1 cells on 100PL was significantly higher ($p < 0.05$) than expression on the other three mixtures on days 4, 13, and 24. By day 36, OCN expression in D1 cells on 100PL was significantly greater ($p < 0.05$) than expression on 100DBM only.

The level of BSP expression by MC3T3 cells decreased significantly ($p < 0.05$) between day 4 and day 13 for all DBM-containing mixtures. For the 100PL mixture, the BSP expression increased significantly ($p < 0.05$) between days 4 and 13. On day 4, BSP expression by MC3T3 cells on 100DBM was significantly higher ($p < 0.05$) than expression on the PL-containing mixtures. By days 24 and 36, the expression of BSP in MC3T3 cells on 100PL was significantly greater ($p < 0.05$) than expression on the DBM-containing mixtures.

Levels of Runx2 expression by MC3T3 cells increased significantly ($p < 0.05$) between day 4 and day 36 for the 100DBM and 70/30 mixtures. The Runx2 expression by MC3T3 cells on 100PL was significantly higher ($p < 0.05$) than expression on the DBM-containing mixtures on days 4, 13, and 24.

The level of OCN expression by MC3T3 cells increased significantly ($p < 0.05$) between days 24 and 36 for 100DBM and between days 4 and 36 for the 70/30 mixture. For the 50/50 and 100PL mixtures, OCN expression by MC3T3 cells increased significantly ($p < 0.05$) between days 13 and 24, but OCN expression decreased significantly ($p < 0.05$) between days 24 and 36; a similar pattern was observed for Runx2 expression. On days 13 and 24, OCN expression by MC3T3 cells on 100PL was significantly greater ($p < 0.05$) than expression on the DBM-containing mixtures. The large increase observed in OCN expression with time for D1 and MC3T3 cells signifies that extracellular collagen was becoming mineralized.

Discussion and Conclusion: A battery of qualitative and quantitative analytical methods indicates that D1 mouse marrow stromal cells attach, proliferate, and differentiate on four specific mixtures of DBM fragments and PL beads. Greater amounts of cell attachment occurred on DBM fragments than on PL beads due to factors

that may include: inconsistent hydrolyzing of the PL beads with EtOH, more favorable conditions for serum coverage of DBM, mechanical agitation of the medium, and contact of bead surfaces with other substrates. Larger cell populations led to increased amounts of lactic acid production and more acidic medium in the DBM-containing mixtures. The cellular lactic acid buildup exceeded the medium's buffering capacity, as evidenced by the distinct color change in the medium. Despite the inhibiting effects of acidic medium on osteoblast differentiation, D1 and MC3T3 differentiation occurred on all mixtures; however, ALP activity and gene expression levels were higher on 100PL. With medium having a more neutral pH level, the 100PL mixture may have provided the best culture environment for D1 differentiation to occur. Similar studies with lower cell densities and the addition of HEPES buffer to the culture medium should be performed in an effort to keep the extracellular pH at a physiological level. Finally, studies should be conducted without osteogenic medium supplements to determine if D1 differentiation can occur solely as a result of the osteoinductive factors in DBM.

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