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14. ABSTRACT Trauma-induced injuries being sustained by our military men and women result in loss of multiple tissues, including associated vasculature and nerves. Current replacement technologies cannot address these kinds of injuries. Tissue engineering is still largely at the single cell stage and the tissues that are produced do not integrate well with surrounding tissues. Mesenchymal stem cells (MSCs) are an important tool but patients with large wounds may have reduced sources of stem cells and those stem cells that are present may be less robust as a consequence of trauma and medical treatment to suppress infection and inflammation. Our hypothesis is that effective repair of large defects requires the concerted processes of bone modeling and remodeling to create a functional marrow cavity, vascularization, and innervation and the best way to achieve this is by using autologous stem cells. Our objective is to develop technology to use adipose-derived MSCs to treat critical size segmental defects. Adipose-derived MSCs are attractive because of their relative abundance but there are still many issues that need to be resolved. We will: (1) develop methods for enriching the population of MSCs in adipose-derived cell preparations from rats; (2) determine if MSC-enriched adipose cells can be used to effectively treat large segmental defects using a rat segmental defect model developed in our group; and (3) optimize this technology for use in male and female animals. Adipose tissues from male and female normal Sprague Dawley rats and obese Zucker rats will be used as cell sources. MSCs will be enriched by selective removal of adipocytes and effectiveness will be determined using in vitro and in vivo assays. To test the ability of enriched MSCs to repair a critical size defect, cells will be loaded onto polymer composite scaffolds and implanted in a rat segmental defect (male in male; female in female; female in male; female in female) and healing assessed by microCT, histology, histomorphometry, immunohistochemistry, and biomechanical testing. This research will provide important new technology for treatment of bone injuries due to trauma. Most individuals have an adequate supply of fat tissue that can be used as a source of cells, but MSCs are in low abundance. Culture expansion dilutes MSCs with more committed cells. In contrast, our approach is to enrich the MSCs in the population by selective removal of more differentiated adipocytes.					
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PROGRESS REPORT

Year 2 Progress

Aim 1: Enrichment of adipose derived stem cells (ADSCs) from rat fat pads.

We have been able to demonstrate that osteogenic ADSCs can be isolated in an enriched population in a reproducible manner by treating the heterogeneous population of rat fat cells with resveratrol. The results of these experiments will be presented at the 2009 International Association for Dental Research meeting. We were also able to show that MSCs from different anatomic sites (bone marrow, umbilical cord, subcutaneous fat) differed in their osteogenic potential. These results will be presented at the 2009 Orthopaedic Research Society.

Aim 2: Use of ADSCs to treat segmental defects.

We have established a protocol for ADSC isolation and osteogenic enrichment, enabling us to reproducibly test scaffold loading efficiency. These studies have provided information on optimal delivery. We have also completed characterization of our segmental defect model, including analysis of vascular ingrowth during defect healing. We anticipate initiation of our in vivo study within the next four months.

Aim 3: Male v. female ADSCs and response to treatment.

We have completed studies showing a difference in the osteogenic ADSCs present in subcutaneous fat as a function of age. To do this, we developed an algorithm for quantifying the MSC population in each fat sample. These results are being submitted to Stem Cells for publication and to the 2009 Tissue Engineering and Regenerative Medicine Society meeting. We will now expand our analytical methods to compare male and female populations in each age group. These experiments are expected to be complete by 7/1/09.

Summary

All projects are on track as described in the Statement of Work. Tasks outlined for Year 2 are complete unless noted above. These are anticipated to be complete by the end of 12 months of Year 2 funding.

Year 3 Statement of Work

Year 3 objectives and associated tasks remain as described in the original statement of work, attached below.

STATEMENT OF WORK

Specific Aim 1. To develop methods for enriching the population of MSCs in adipose-derived cell preparations from rats.

Year 1

Task 1: Establish the ratio of MSCs/adipocytes in inguinal fats pads and bone marrow in normal male Sprague-Dawley rats and obese male rats Zucker rats (Cell sorting; Functional assays in vitro; Bone, cartilage and fat formation in vivo in male rats).

Task 2: Use assays above to test effectiveness of treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ (dose response/time course) for increasing the MSC/adipocyte ratio in cells preparations isolated from inguinal fat pads and bone marrow from male rats (Cell sorting; Functional assays in vitro; Bone, cartilage and fat formation in vivo in male rats).

Year 2

Task 1: Test effectiveness of treatment with two additional apoptotic agents (dose response/time course) for increasing MSC/adipocyte ratio in cell preparations isolated from inguinal fats pads and bone marrow from male rats.

Year 3

Task 1: Prepare MSC-enriched adipocytes for use in segmental defects below.

Task 2: Prepare summary report.

Specific Aim 2. To determine if MSC-enriched adipose cells can be used to effectively treat large segmental defects.

Year 1

Task 1. Develop methods for loading $1\alpha,25(\text{OH})_2\text{D}_3$ treated male MSC-enriched fat cells onto polymeric scaffolds.

Year 2

Task 1. Determine if treatment with alternate apoptotic agents modify loading efficiency.

Task 2. Determine if MSC-enriched adipose cells support segmental defect repair (in vivo microCT at 4 weeks; microCT at euthanasia; vascularity at euthanasia; histology and histomorphometric assessment of bone healing; immunohistochemistry for re-innervation; biomechanics).

Year 3

Task 1: Prepare summary report.

Specific Aim 3. To optimize MSC technology for use in male and female animals.

Year 1

Task 1: Establish the ratio of MSCs/adipocytes in inguinal fats pads and bone marrow in normal female Sprague-Dawley rats and obese female rats Zucker rats (Cell sorting; Functional assays in vitro; Bone, cartilage and fat formation in vivo in female rats).

Task 2: Determine if the in vivo assay is sex-specific by testing enriched MSCs from male rats in female rats and enriched MSCs from female rats in male rats; compare bone, cartilage and fat formation by histomorphometry.

Year 2

Task 1: Use assays above to test effectiveness of apoptotic treatments (dose response/time course) for increasing the MSC/adipocyte ratio in cells preparations isolated from inguinal fat pads and bone marrow from female rats.

Task 2: Assess sex-specificity of apoptotic treatments by comparing female and male preparations in vivo for MSC/adipocyte ratio as described above.

Task 3: Assess effectiveness of female enriched MSCs in the segmental defect model using female rats.

Year 3

Task 1: Assess sex-specificity of enriched MSC effectiveness of by testing female enriched MSCs in the segmental defect model using male mice and male enriched MSCs in female rats.

Task 2: Prepare final report.

Osteoprogenitors are Enriched following Resveratrol-treatment of Adipose-derived Mesenchymal Cells

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Objective: Adipose tissue has been shown to contain a supply of mesenchymal stem cells (MSCs), however improved enrichment techniques are required before these cells can be used effectively. Resveratrol inhibits cyclooxygenase and activates Sirt1, which inhibit adipogenesis and induce apoptosis in adipocytes. This suggested that resveratrol could reduce the number of adipocyte progenitor cells and enrich the MSC population with osteogenic progenitors (OPCs). This study tested the hypothesis that resveratrol enriches MSCs and OPCs through an increase in cell number and percent of total population for both MSCs and OPCs. **Methods:** Adherent cells were isolated from the inguinal fat pads of Sprague-Dawley rats, plated at 5,000 cells/cm², and cultured in MSC growth media (GM) or osteogenic media (OM) (Lonza) containing 0, 12.5, or 25µM resveratrol for 7 or 14 days. Flow cytometry was used to assess expression of MSC (CD73+, CD271+, and CD45-) or osteoblast (osteocalcin) markers in the original population as well as following growth in GM or OM. **Results:** Resveratrol caused a dose and time-dependent increase in cell number and of the percentage of both MSCs and OPCs. In GM containing 25µM resveratrol, there was a 577-fold increase in MSCs at 7 days (5% of population), and a 106-fold increase in OPCs (21% of population) at 14 days. In OM, 25 µM resveratrol increased MSCs 27.8-fold (18% of population) at 7 days, and OPCs 29.9-fold (34% of population) at 14 days. **Conclusion:** These results show that MSCs and OPCs were present in the original adherent cell population and that resveratrol treatment enriched both cell populations. Resveratrol increased the cell number and the population percentage of MSCs and OPCs. Moreover, effectiveness of the treatment was reduced when the adherent cells were cultured in OM. Supported by Department of Defense; Children's Healthcare of Atlanta; NSF.

To be presented at the 2009 International Association for Dental Research

Reduced Surface Expression of CD44, CD49b, CD49e, and CD105 Associated with Growth of Human Bone Marrow Stem Cells Is Preserved During Growth in Osteogenic Differentiation Medium

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INTRODUCTION: Multipotent mesenchymal stem cells (MSCs) have been isolated from human bone marrow (BMSCs), and shown to have osteogenic and chondrogenic potential when cultured in defined differentiation media. Surface markers have been extensively characterized for the undifferentiated cells [1], but how expression of these markers changes during osteogenic and chondrogenic induction has not been described. It is clear that loss of the stem cell phenotype and acquisition of the bone or cartilage phenotype depend on the cell's ability to interact with its surrounding extracellular matrix [2,3]. For example, the alpha-2 subunit of the $\alpha 2\beta 1$ integrin pair (CD49b) and the $\alpha 5$ subunit of $\alpha 5\beta 1$ (CD49e) have been associated with osteoblast differentiation in a number of studies [4,5,11]. The objective of this study was to quantify changes in surface phenotypic expression during osteogenic and chondrogenic culture of human bone marrow-derived MSCs, focusing on those cell surface proteins that are specifically associated with cell/matrix interactions.

METHODS: MSCs were isolated from bone marrow aspirates from the iliac crest of normal adult donors [3] and expanded for 4 passages in growth media consisting of low glucose DMEM with 10% FBS. A portion of undifferentiated MSCs was resuspended in PBS to assess expression of surface markers by flow cytometry at day 0. Remaining cells were re-plated at 5000 cells/cm² with either growth media (GM) or osteogenic media (OM) consisting of GM supplemented with 1nM dexamethasone, 3mM beta-glycerophosphate, and 50 μ g/ μ L ascorbic acid 2-phosphate. To assess effects of chondrogenic culture conditions, cells seeded in 1.2% Keltone alginate at a density of 12-15x10⁶cells/ml were loaded on 24-well transwell insert membranes [6]. Once hydrogel discs were cross-linked in 100mM CaCl₂, samples were cultured in either incomplete chondrogenic medium (ICM) consisting of high glucose DMEM with 40 μ g/ml L-proline, 50 μ g/ml ascorbate-2-phosphate, 0.1 μ M dexamethasone, and 1% ITS+ or complete chondrogenic medium (CCM) consistent of ICM with 10ng/ml TGF- β 3 and 50ng/ml BMP-6. After 7 days, cells in osteogenic culture were trypsinized and cells in chondrogenic culture were recovered by uncrosslinking the alginate with sodium citrate [7] and then incubated in 0.125% trypsin-EDTA solution with 2mg/ml collagenase at 37°C for 2 hours. Isolated cells were divided into aliquots of 1.25x10⁵cells, washed in PermWash buffer, and incubated with PE-labeled anti-human mouse monoclonal antibodies specific for CD34, CD44, CD73, CD49b, CD49e, CD105 and CD146. Percent change in surface expression was calculated by normalizing the difference between day 7 and day 0 expression percentage to the day 0 expression percentage. Data were analyzed by ANOVA; * p< 0.01.

RESULTS: At T=0, BMSCs were positive for CD105, CD73, and negative for CD34, CD49b and CD146 expression was low whereas expression for CD44 and CD49e was high (Fig 1). The pattern of surface marker expression changed with time. Both control and osteogenic cultures exhibited down-regulation of CD146 (data not shown). However, cells cultured in OM exhibited distinct differences in surface marker expression compared to GM control cultures (Fig 2). CD49e and CD44 were upregulated in OM cultures but down-regulated in GM cultures. CD49b was down-regulated in both media conditions; however this effect was reduced in OM cultures, although this was not a consistent observation. CD105 was regulated in a similar manner to CD49b but the change in surface expression, while statistically significant, was less pronounced. BMSCs undergoing chondrogenic differentiation exhibited comparable changes in surface markers to cells undergoing osteogenic differentiation.

DISCUSSION: Surface marker expression varied as a function of both time and the medium in which the cells were cultured. CD146, which is a cell adhesion molecule generally used as a marker of endothelial cell lineage [8], decreased with time in culture but was independent of the

medium used, suggesting that endothelial progenitor cells were present in low numbers and the GM media did not support endothelial lineage progression. BMSCs treated with OM in monolayer culture had significantly higher expression of the hyaluronan receptor (CD44), the $\alpha 5$ integrin subunit of the fibronectin receptor (CD49e), and the regulatory component of the TGF- β receptor (CD105) compared to GM cultures. The reduction in these surface markers observed in the cultures with time was mitigated by growth in OM or under chondrogenic culture. These results indicate that growth under appropriate conditions can help maintain or up-regulate cell adhesion molecules and surface proteins associated with tissue organization and cell differentiation [3,7,9,10]. Immunohistochemistry should be performed to confirm that the cell isolation process from tissue culture plates and hydrogels does not alter the surface phenotype. Gene expression of surface markers and proteins associated with osteoblasts and chondrocytes should also be investigated to determine if changes in the surface phenotype reflect post-translational regulation and to determine potential markers of differentiation. This study shows that exploring the temporal changes in surface phenotypic expression during osteogenesis and chondrogenesis can provide a better understanding of how cells interact with their surrounding environment throughout tissue development.

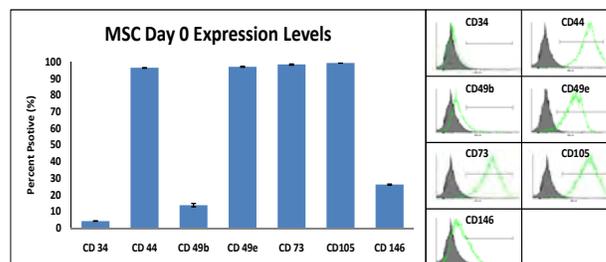


Figure 1

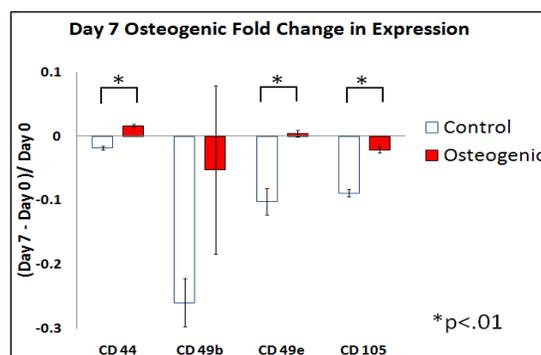


Figure 2

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