Amplification of Osteogenic Cells from Human Embryonic Stem Cells

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INTRODUCTION

Embryonic stem cells (ESC) offer a potentially unlimited supply of cells that may differentiate to specific lineages to give rise to all cell types in the body. Recently there has been great interest in examining the osteogenic potential of ESCs derived from both mice [1-3] and humans [4-5] using two specific approaches. In one approach, osteogenic cells are derived from 3-dimenstional cell spheroids called embryoid bodies (EB) [4-5]. EB mimic the structure of the developing embryo and recapitulate many of the stages involved during its differentiation [6], and clonally derived EB can be used to locate and isolate tissue specific progenitors. An alternative system that has been tested but not well characterized avoids EB through the immediate separation of ESC colonies into single cells which are then plated directly into a cell adhesive culture dish [3,4]. Using this method, one can presumably exhibit better control over the cell microenvironment and thus obtain more pure populations of progenitor cells. Here we compared the differentiation process within osteogenic cultures of hESC with and without the formation of EB to determine if increased numbers of osteogenic cells could be obtained from hESC without the formation of EB.

METHODS

ES Cell Culture

hESC (line H9) were grown as cell aggregates on inactivated mouse embryonic feeders (MEF). Undifferentiated hESC aggregates were removed from MEF and a single cell suspension was obtained through incubation at 37^{o}C for 5min in a solution with 1:2 (vol:vol) trypsin to cell dissociation solution. Cells were plated at a concentration of 10^5 cells per cm^2 in α-MEM supplemented with 10% fetal bovine serum (FBS) & antibiotics consisting of 167U/ml penicillin G, 50µg/ml gentamycin, & 0.3 µg/ml amphotericin B. To examine the potential of the hES cells to produce mineralized extracellular matrix, two differentiation protocols were examined. ES cell aggregates were removed with collagenase IV & either (A) suspended as EB for 5 days & then plated as a single cell suspension or (B) directly placed onto tissue culture petri dishes for 1 day followed by plating as a single cell suspension. To stimulate differentiation into osteogenic cells, media was supplemented with 50µg/ml ascorbic acid (AA), 5mM β-glycerophosphate (βgP) & 10^{-8}M dexamethasone (DEX). Cultures were treated with or without osteogenic supplements to assess directed or spontaneous differentiation into osteogenic cells, respectively. Bone nodules were identified by the co-localization of alkaline phosphatase & von Kossa staining. Samples sputter-coated with carbon (≈250A) were examined with SEM and with energy dispersive x-ray detector for elemental analysis. Calcium to Phosphate ratios (Ca:P) were obtained by integrating the area under the Ca and P peaks. The FTIR spectra were obtained over the range of 900-1725 as previously reported [3]. All data gathered in triplicate from 3 independent expt. and ANOVA was performed with the Tukey's HSD test at significance levels of 95%.

RESULTS

Alkaline phosphatase, von Kossa, osteocalcin and saffinin-O stained nodules are shown in Figure 1. When cultured in the presence of supplements, as examined by SEM, differentiated hESC produce a mineralized extracellular matrix (not shown). Through removal of cells & matrix with a blast of compressed air, cultures displayed 1µm sized mineralized globular accretions which were reminiscent of the cement line formed by differentiating osteogenic cells. FTIR analysis demonstrated that the mineral peak from hydroxyapatite and the mineral and matrix peaks from human bone were comparable to the extracellular matrix produced by the hESC irrespective of whether the cells were derived from EB (not shown). However, hESC cultures derived from EB in the absence of supplements produced a substantially smaller mineral peak which corresponded to a low calcium to phosphate ratio. The frequency of bone nodules produced under various culture conditions is shown in Figure 2.

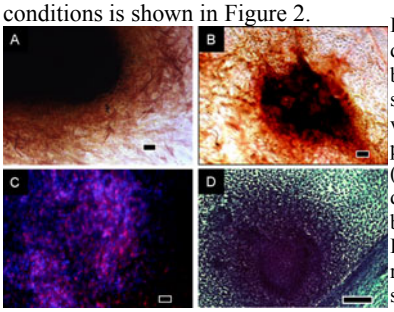

Figure 1: (A) After 28-30 days, the matrix produced by hESC cultured with EB stained for both ALP and von Kossa only in the presence of supplements. (B) In contrast, after 10-12 days, the matrix produced by hESC cultured without EB stained for both markers regardless of the supplementation regime. (C) Bone nodules also stained positively for OCN (red). (D) The mineralized matrix did not stain for saffrinin-O indicating that these regions did not contain glycoaminoglycans which are associated with cartilage. All scale bars represent 100µm.

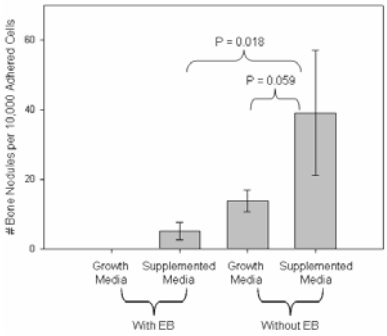

Figure 2: Cells grown from EB produced bone nodules only in the presence of osteogenic supplements (5.1 ± 2.4). In comparison, cells grown without EB cultured in growth media & media containing osteogenic supplements produced 13.8 ± 3.1 & 39.1 ± 17.8 bone nodules per 10,000 plated cells, respectively (n=3).

DISCUSSION AND CONCLUSIONS

Herein we found that culturing hESC without EB leads to over a 7 fold increase (P=0.018, n=3) in the number of osteogenic cells and to spontaneous bone nodule formation after 10-12 days. In contrast, when hESC were differentiated as EB for 5 days followed by plating of single cells, bone nodules formed after 4 weeks only in the presence of dexamethasone. Furthermore, regardless of the inclusion of EB, bone matrix formed including cement line matrix and mineralized collagen which displayed apatitc mineral (PO_4) with calcium to phosphorous ratios similar to hydroxyapatite and human bone. Together these results demonstrate that culturing hESC without an embryoid body step can be used to derive large quantities of functional osteogenic cells for bone tissue engineering.

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