Comparison Of Osteogenic Cell Differentiation Within 2D And 3D Culture Systems

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Abstract

The physiology of different cell types has been shown to vary remarkably within three-dimensional (3D) environments compared with two-dimensional (2D) monolayer cultures. Chemically synthesized 3D scaffolds, which offer a variety of features to match tissue specific applications, are recently drawing tremendous attention in the bone tissue engineering field. Currently, there are only a few reports comparing osteoblastic differentiation of stem cell progenitors within 2D and 3D culture systems. The objective of this study was to compare the growth pattern of osteoblastic cells within 2D and 3D culture systems. Human mesenchymal stem cells (hMSCs) and 7F2 osteoblasts were seeded on 2D polystyrene (PS) culture plates and on 3D poly- ε -caprolactone (PCL) scaffolds (3D Insert™-PCL) with non random pores engineered using a layer by layer fabrication technology. Osteoblastic differentiation was induced through culture media supplemented with L-ascorbic acid and β -glycerophosphate. Quantitative and qualitative assays of osteogenic differentiation were conducted at multiple time points during 4 weeks of *in vitro* culture. Osteocalcin secretion and alkaline phosphatase (ALP) activity were detected at significantly higher levels within 3D cultures compared with 2D cultures ($P < 0.05$, *t*-test). Von Kossa staining indicated more extensive mineralization of 7F2 cells on 3D PCL scaffolds than in 2D PS monolayer cultures. In conclusion, our results demonstrate that the osteogenic differentiation of hMSC and osteoblastic cells is enhanced in a 3D culture system compared with a 2D culture environment. 3D PCL scaffolds are biocompatible and osteo-conducive for bone formation *in vitro*. Furthermore, these findings suggest that 3D cell culture is a superior system for performing stem cell differentiation and assays.

Introduction

A cell's environment in a living organism has a 3D architecture where cells interact with each other and with extracellular matrix (ECM). The majority of cell culture studies, however, have been performed on 2D surfaces such as tissue culture plates/flasks because of the ease, convenience, and high cell viability of 2D culture. These conventional 2D culture systems have notably improved the understanding of basic cell biology. Nevertheless, 2D plastic substrates are considerably limited in reproducing the complex 3D environments existing in vivo. In addition, cells grown on 2D plastic substrates are forced to adapt to an artificial flat, rigid surface, resulting to some degree, a misrepresentation of findings, including altered metabolism and declined functionality [1-3]. Cells cultured in 2D systems have been shown to differ remarkably in their morphology, proliferation, and differentiation from those growing in 3D environments [4, 5].

3D cell culture matrices, also known as 3D scaffolds, were recently introduced as a way to overcome the limitations of traditional 2D cell culture. Extensive studies have shown that growing cells within 3D scaffolds diminishes the gap between cell cultures and physiological tissues. Therefore, a 3D cell culture system may prove to be of tremendous advantage over conventional 2D cell culture system.

In the development of stem cell based therapies and tissue regeneration products, the differentiation potential of the stem cells has to be assessed to ensure the quality of the isolated or expanded stem cells. These differentiation assays normally include osteogenic, chondrogenic and adipogenic assays. In an effort to develop a standard 3D osteogenic differentiation assay, we have used 3D PCL scaffolds (**Figure 1**) manufactured using 3D precision micro-fabrication technology. Human bone marrow derived mesenchymal stem cells (hMSCs) are one of the adult stem cells that has been extensively studied for tissue regeneration in bone and cartilage [7, 8]. In this study, we evaluated the differentiation behavior of both human MSCs and 7F2 osteoblastic cells in both 2D polystyrene tissue culture plates (PS-TCPs) and on 3D PCL scaffolds.

Materials and Methods

Cells

hMSCs were purchased from Lonza Walkersville, Inc (Cat#: PT-2501), and re-suspended in MSCGM Basal Medium (Lonza Walkersville, MD) with 10% fetal bovine serum (FBS), 2mM of L-glutamine and 100IU/ml pen-strep. 7F2 cells were cultured in alpha-MEM with 10% FBS and 2mM L-glutamine. Both cultures were kept in a humidified 5% CO2 incubator at 37°C.

Schematic Scaffold Fabrication and Cell Seeding

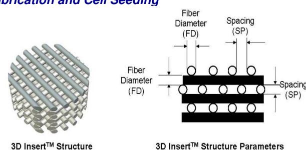


Figure 1. PCL scaffold design

PCL was purchased from Sigma-Aldrich and the 3D PCL scaffold was fabricated using 3D precision micro-fabrication technology. Within the scaffold, the struts within each layer were oriented 90° relative to the struts of the layer immediately below (**Figure 1**). Fiber diameter and spacing are approximately 300µm and 500µm, respectively. Scaffolds were sterilized by soaking in 70% ethanol for 1 h and air dried in a biosafety cell culture hood. Cells at a density of 4.75 x 10^2 (1.44x10^4/cm^2) in 200 µl were seeded into 2D 96-well PS tissue culture plate (TCP). Comparable cell densities of 0.1 million in 10 µl were seeded onto scaffolds measuring 5.1 mm in diameter and 2.1 mm in height. The seeded scaffolds were then incubated for 3 h to allow cell attachment. After 3 h, the scaffolds were flooded with 200 µl of maintenance medium and kept in culture until use.

Osteogenic Differentiation

24 hour after the cell seeding, the culture media were replaced with osteogenic differentiation induction media, which contains basal medium supplemented with 50µM ascorbic acid and 10mM β-glycerophosphate. 0.1µM of Dexamethasone was specifically used for hMSC osteogenic induction. Throughout the 4 weeks of in vitro culture, the induction media were changed every 2-3 days.

Cell Proliferation Assay

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyletrazolium bromide (MTT) assay was performed according to manufacturer's instructions (Cayman Chemical,MI).

Alkaline Phosphatase Activity Assay

Cell lysate was prepared using M-PER® Mammalian Protein Extraction Reagent (Pierce, IL) followed by centrifuge at 14,000 rpm for 5 minutes. The lysate in supernatant was collected and analyzed using p-Nitrophenyl Phosphate Liquid Substrate System (pNPP) and 4-nitrophenol solution (PNP) (Sigma,MO), according to manufacturer's instructions.

Osteocalcin Secretion Assay

Three days prior to culture medium supernatant collection, hMSC culture medium was replaced with osteogenic induction factor-supplemented Lonza BioWhittaker* General-Purpose Serum-Free Media (Lonza, MD). The osteocalcin concentration in culture medium supernatant was measured using Intact Human Osteocalcin EIA kit or mouse osteocalcin EIA kit (Biomedical Technologies Inc.,MA).

Von Kossa Staining

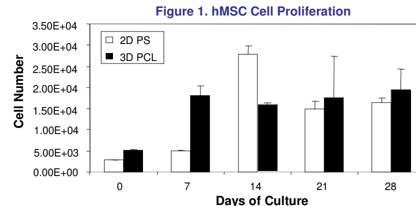
The PCL scaffold and 2D TCP were fixed with 10% phosphate-buffered formalin for 30 minutes. After washing with DI-H_2O, cultures were stained with 2% silver nitrate in DI-H_2O for 10 min in the dark. After DI-H_2O wash, the scaffolds and TCPs were exposed to bright light for 15 min. The cultures were washed and then dehydrated with 100% ethanol for 1 min followed by air drying.

Results

Cell Proliferation by MTT

Cell proliferation assay showed that hMSCs proliferated in both 2D and 3D conditions (**Figure 2**). In 3D PCL scaffolds, cell number reached its peak at week 1, 7 days earlier than 2D PS-TCPs; however, cell number was highest in 2D than in 3D. After 2 weeks of osteogenic induction, cell number from 2D PS culture quickly decreased to a level that was comparable to 3D PCL scaffolds, indicating reduced cell viability due to over-confluency of cultured cells. In contrast, cell numbers in 3D PCL scaffolds remained at their highest level after reaching its maximum proliferation (Figure 2). These findings suggest that hMSCs proliferate faster in 3D than in 2D.

Figure 1. hMSC Cell Proliferation



Assay for Alkaline Phosphatase (ALP) and Osteocalcin Activity

ALP assays were performed from cell lysates to assess the osteogenic differentiation of both hMSCs and osteoblasts in both 2D and 3D cultures. Data showed that ALP activity, a early stage marker of osteogenic differentiation, was doubled in cells cultured in the 3D Insert™-PCL scaffolds as compared to 2D cultures at week 1 (**Figure 3**). In 3D culture, ALP activity reached its peak at week 2 while it took 3 weeks for ALP to reach peak in 2D culture. In addition, production of osteocalcin, a late stage marker of osteogenesis, was dramatically enhanced in the supernatant of cells grown on 3D Insert™-PCL at week 3 (**Figure 4**). Only a slight increase in osteocalcin secretion was observed at week 4 in 2D culture.

Figure 3. hMSC Alkaline Phosphatase

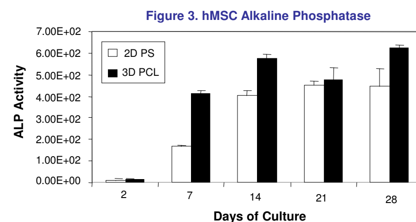
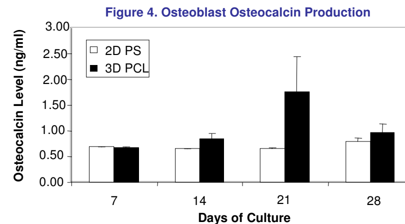


Figure 4. Osteoblast Osteocalcin Production



Von Kossa Staining

Von Kossa staining of hMSCs indicates that cells grown on 3D PCL scaffolds underwent more extensive mineralization during later stages of osteogenesis than cells on 2D polystyrene TCP (**Figure 5G-J**). Differentiated 7F2 mouse osteoblastic cells seeded on 3D Insert™-PS scaffolds were used as controls for comparison (**Figure 5A-F**).

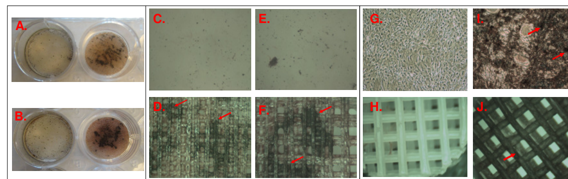


Figure 5. Von Kossa Staining of differentiated 7F2 mouse osteoblastic cells in 2D (**C, E**) and on 3D Insert™-PS (**D, F**) at Days 14 (**A, C-D**) and 21 (**B, E-F**) were used as controls for mineralized nodule formation. Images were taken with a stereomicroscope (**C-F**) (3X). hMSCs in 2D (**G, I**) and on 3D PCL scaffolds (**H, J**) were stained by Von Kossa on day 7 (**G, H**) and day 28 (**I, J**) of culture. The dark brown-black staining shows positive staining for mineralization on day 28 (**I, J**). No apparent positive staining was observed on day 7 (**G, H**).

Conclusions

Our results demonstrate that 3D PCL scaffolds provide a better 3D culture environment for the *in vitro* osteogenic differentiation of hMSCs and osteoblastic cells than conventional 2D polystyrene TCPs, as shown by enhanced osteogenic marker production in 3D cultured cells. This finding suggests that using 3D scaffolds is a better way for assessing the osteogenic potential of progenitor cells. Furthermore, this research study will contribute to the design of practical, applicable synthetic 3D scaffolds for bone engineering and related research.

References

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