Vasculogenesis in PEGylated Fibrin

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The use of trade, product and/or other Army records in the preparation of this material is acknowledged, but is not to be construed or implying official Department of the Army approval of the conclusions presented.

Abstract

Adipose Derived Stem Cells (ASC) have received attention because of their ability to differentiate towards connective tissue cell types (1). In this present study we have developed a method using a Polyethylene glycol (PEG)–based fibrin extracellular matrix (ECM) mimic, to differentiate ASC towards vascular cell types. After 7 days, ASC seeded in PEGylated fibrin showed distinct morphologies. Cells began to form vascular tube-like networks in the PEGylated fibrin gel in the absence of additional soluble cytokines. Cell viability and proliferation of ASC that were embedded in the three dimensional matrix were measured utilizing MTT assay. The amount of vascularization was related to the initial cell number plating density. Accordingly, cell proliferation and viability increased with increasing cell seeding density. ASC inside the matrices were not only viable, but proliferated and formed microvessels. Expression levels of endothelial specific genes; von Willebrand factor (vWF) and CD31 significantly increased through course of differentiation. Further, immunohistochemical analysis identified ASC that had differentiated into vascular cells using endothelial cell specific markers, vWF and CD31, were also found to be positive for the perivascular specific NG2 and α-smooth muscle actin marker. This neovascularization to form capillaries is central to developing new therapies for wound healing and tissue engineering.

Introduction

There is mounting evidence that ASCs have an unexplored plasticity towards vascular cell types, and it may be that this plasticity can only be fully unlocked by a structural ECM mimetic. To date the majority of experiments have used soluble cytokines, hormones and growth factors to induce cell differentiation. We hypothesize that three dimensional matrices, instead of exogenous stimuli, can be used to direct ASC progenitor cells to differentiate towards vascular cell types. These mimetics may include tissue or other novel biodegradable biomaterials. These matrices must fulfill a number of functions including structural support and providing an appropriate 3-dimensional microenvironment (2). The approximation of PEGylated fibrin gel to the native tissue environment is expected to control and maintain the differentiated phenotype.

Methods

Briefly, SC-PEG-SC (3,400 Da, Sunbio, Anyang City, S. Korea) was added to fibrinogen (20 mg/ml in Tris-buffered saline, pH 7.8, Sigma, St Louis, MO) in the molar ratio of 10:1. The reaction product then underwent gelation by adding an equal volume solution of thrombin (25 U/ml in 40 mM $CaCl_2$, Sigma). The unbound free PEG was rinsed, and final concentration of fibrinogen was 10 mg/ml and that of thrombin was 12.5 U/ml (Figure 1).

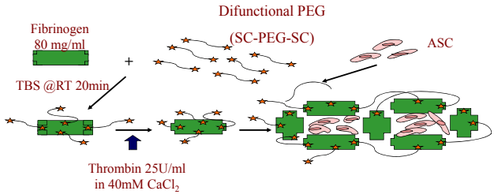


Figure 1: ECM mimetic formation.

Adipose derived stem cells (ASC) were isolated from Lewis rats using standard procedures as previously described (3). Gels were cast into 12- well plates at a cell density of 5,000-50,000 cells/ml. The ASC within the gels were analyzed for viability using MTT and tube formation by light microscopy after 1, 3, 5, 7 days. Expression levels of vascular cell specific markers (CD31, vWF) were analyzed using Real Time Polymerase Chain Reaction (RT-PCR). To determine if the vascular-like structures formed by ASC in the matrix expressed microvasculature cell phenotypes; samples were cryosectioned, processed for immunohistochemistry and analyzed for vascular (CD31, vWF) and perivascular (NG2, α-smooth muscle actin) specific markers.

Results

Scanning electron microscopic analysis showed that the PEGylated fibrin gel was found to have a dense interpenetrating network (Figure 2) and when ASCs were seeded in the gel showed distinct morphologies after 7 days.

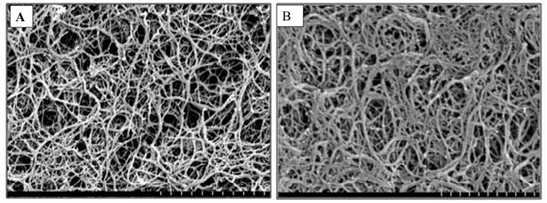


Figure 2: Scanning electron micrographs of Fibrin (A) and SC-PEG fibrin gel (B).

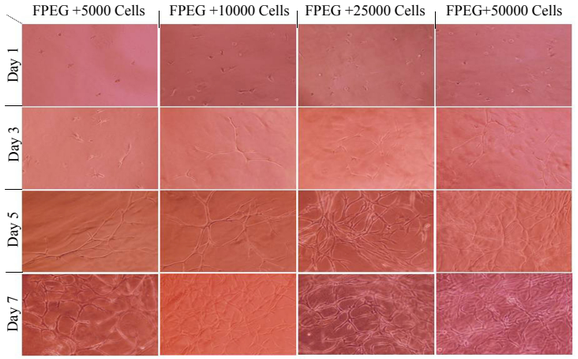
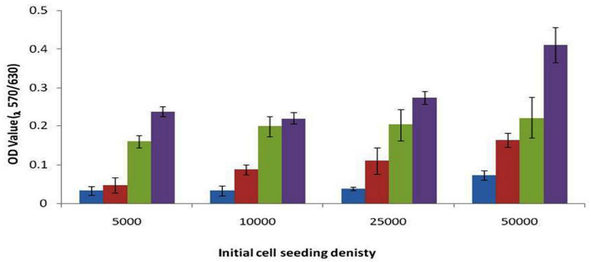


Figure 3: Differentiation time-course of ASCs into vascular like structures in SC-PEG fibrin gels.

Cells began to form vascular tube-like networks in the PEGylated fibrin gel in the absence of additional soluble cytokines (Figure 3). The amount of vascularization was related to the initial cell number plating density. Accordingly, cell proliferation and viability increased with increasing cell seeding density (Figure 4). There was significant increase in vascular cell specific markers; CD31 and vWF by day 7 (35 fold increase), after which a marginal increase (5 fold) was observed by day 11. There was a 8 fold and 10 fold increase in perivascular specific markers NG2 and PDGFR β respectively by day 11 (Figure 5), indicating the tube like structures formed were microvascular in nature and exhibit typical endothelial and perivascular like pehenotypes. Further immunohistochemical analysis showed positive expression of both vascular and perivascular specific markers to be expressed by the differentiated ASC in PEGylated fibrin gel after 7 days (Figure 6).



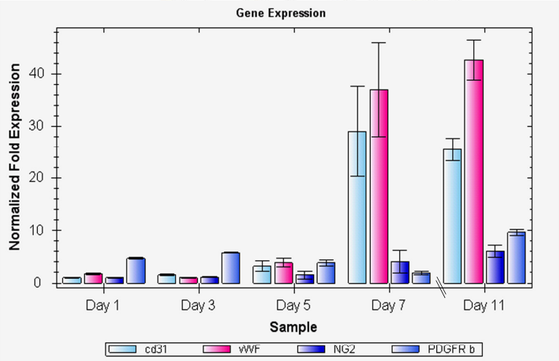


Figure 5: RT-PCR analysis indicating the significant increase in expression of vascular specific markers , CD31 and vWF.

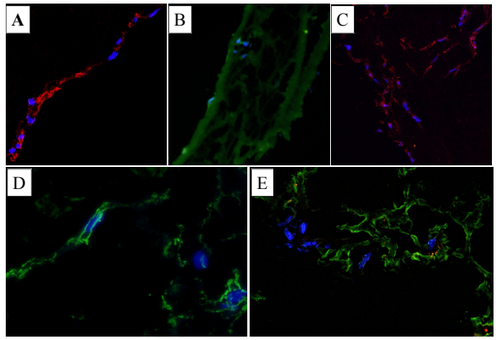


Figure 6: Immunochemistry against (**A**) NG-2, (**B**) CD31, (**C**) α-smooth muscle actin and (**D**) vWF of ASC population seeded in PEGylated fibrin. (20X) and (**E**) Overlay image of vWF (green), NG-2 (red) and Hoeschst (blue).

Conclusions

The data indicate that, PEG-fibrin embedded ASC exhibit positive staining for perivascular markers such as α-smooth muscle actin and NG-2. Immunohistochemical analysis confirms the existence of two phenotypically distinct cell populations organized around a lumen containing tube. Gene and protein analysis confirm the differentiation of two sub-populations of vascular cells from ASCs. Gene expression for the endothelial cell markers, VEGF and vWF demonstrated greater than 25-fold upregulation in both genes over 1 week. These results indicate that ASCs can form neovascular tubes without exogenous growth factors in a PEGylated fibrin matrix. Structural and histological analysis of the microvascular structures indicate that they are similar to capillaries found in vivo and occupy a pericyte fate during ASC differentiation. These results provide a novel approach to treat current combat injuries requiring tissue engineering based neovascularization of extensive skin wounds.

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