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Probing the role of scaffold dimensionality and media composition on matrix production and phenotype of fibroblasts

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ABSTRACT

Porous sponges, hydrogels, and micro/nanofibrous matrix are most commonly used three dimensional (3D) biomaterials in tissue engineering; however, reciprocal interaction between internal dimensionality of biomaterials and fibroblasts remains largely unexplored. Such studies would have potential to generate valuable insights about wound healing, tissue morphogenesis and homeostasis. To the best of our knowledge this is the first study to evaluate functionality of porous collagen matrix and collagen gels for *in vitro* culture of fibroblasts while investigating the role of culture media composition in modulating morphology, phenotype, extracellular matrix (ECM)-related gene expression and protein synthesis by fibroblasts. Encapsulation of fibroblasts in collagen gel was found to be more effective for ECM production compared to scaffold-based culture, as evidenced by enhanced collagen type I, elastin, lysyl oxidase, aggrecan gene expression. High glucose media induced spindle like morphology of typical *in vivo* fibroblasts and enhanced collagen production compared to other media. This variation in biosynthesis in different glucose concentrations was possibly due to endogenous activation of TGF-β or by an increase in ATP consuming anabolic pathways in high glucose concentration.

1. Introduction

Three dimensional (3D) culture conditions are becoming crucial tools for cell biology, drug discovery, and tissue engineering research to simulate physiologically relevant conditions with respect to chemical, geometrical, and signaling responses [1]. Extensive work has been reported on culturing fibroblast in 3D context, to develop dermal substitute matrix [2]. Some studies used seeding of fibroblasts on 3D porous scaffolds [3,4], whereas in other studies cells were encapsulated within hydrogels [5,6]. Various collagen-based 'skin tissue equivalents' are commercially available, for example, Orcel™ (Ortec International, New York, USA) and Apligraf™ (Organogenesis Inc., Massachusetts, USA) are made of bovine collagen sponge [7–9], whereas Permaderm™ (Regenicin, Little Falls, New York, USA) is based on collagen gel synthesized by autologous fibroblasts [7]. To the best of our knowledge no comparative study has been reported to evaluate functionality of porous matrix and collagen gels.

The porous 3D scaffolds, in spite of being used to a large extent for tissue engineering, cannot be considered fully as 3D, since the cell proliferation pattern in 3D porous scaffolds may still follow monolayer conditions, covering the curved contours of the scaffold interstices, especially in the initial time period. However, cell encapsulation within hydrogel may simulate *in vivo* tissue niches more closely [10]. But mass transport and maintenance of cell viability issues are pertinent questions. The differences in simulating *in vivo* architecture and environment by the 3D porous and gel based matrices could be explained at molecular level, like the difference at the level of cell adhesion to the matrix or at the cellular signaling level. Thus, it will be important to understand how the geometry of artificial matrix affects the availability of the adhesion sites and cytoskeleton, cell morphology, which in turn affects cell signaling and regulation.

Other than the mode of interaction between cell-biomaterials, composition of culture medium may further modulate the cellular phenotype and ECM synthesis. Many efforts have been made in the past to standardize the best culture medium that promotes proliferation of cells, as well as provide necessary cues to replicate the complex 3D microenvironment of the tissue [11,12]. Historically the basal medium formulated by Harry Eagle failed to support culture of fibroblasts. Minimum essential medium (MEM), the modified version of basal medium, was among the first widely used media [13]. Then a modified MEM with a higher concentration of amino acids than that of the basal medium was introduced [14]. Dulbecco's Modified Eagle's Medium (DMEM) has double the concentration of amino acids and four times the amount of vitamins as MEM, as well as ferric nitrate, sodium pyruvate, and some supplementary amino acids [15]. Alpha MEM is the Alpha modification

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of MEM, whose composition includes Earle's balanced salts, ascorbic acid, lipoic acid, non-essential amino acids, vitamin B12 and biotin. In spite of all these commercially available media being used for the purpose of fibroblast culture [16,17] there is no justification for the use of specific media in most of the reports. There is certainly a need to understand the effect of media constituents on fibroblast morphology and ECM composition.

The main objective of this study was to identify the best suitable matrix and media composition for 3D culture of dermal fibroblasts. The response of dermal fibroblast over collagen gel and porous scaffold under the influence of three different culture media, namely Alpha minimum essential medium (MEM), DMEM low glucose (DMEM LG) and DMEM high glucose (DMEM HG), was observed. Cell proliferation using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) and DNA quantitation, gene expression pattern of dermal fibroblasts, ECM synthesis using biochemical assays for total collagen, immunofluorescence based confocal imaging, and ECM remodeling were studied to find efficacious culture condition (gel based and 3D porous collagen scaffold, ideal media) that provided distinct growth requirements, allowed the development of dermal tissue microenvironment, as well as to generate insights about the signaling mechanisms involved.

2. Experimental section

2.1. Materials

Rat tail collagen type I solution (A1048301, 3 mg/ml) was purchased from Gibco, Life technologies, India. Ultrafoam (UF) collagen matrix (collagen type I), FDA approved hemostatic porous sponges, was procured from Avitene, Davol Inc., USA. DMEM high glucose (CC 3004.05 L, 4500 mg/L of glucose) was purchased from Cell Clone, India. DMEM low glucose (AL006A, 1000 mg/L glucose) and Alpha MEM (AL080, 1000 mg/L glucose) were purchased from HiMedia, India.

2.2. Cell culture

2.2.1. Isolation of fibroblasts

4 cm^2 human full thickness skin patch was obtained from All India Institute of Medical Sciences, New Delhi, with ethical approval with informed patient consent. Skin piece was washed in wash buffer (PBS and antibiotics composition). Hypodermis was removed and the tissue containing epidermis and dermis was cut into small pieces (3 mm^2) and incubated in mixture of dispase 8.67 U/ml (15 mg in 1 ml PBS) and 1% collagenase for 90 min at 37 °C. The partially digested dermal tissue was plated separately for explant culture in Alpha MEM supplemented with 10% fetal bovine serum, HEPES buffer (1 M), 100 U/ml penicillin–streptomycin, 50 μg/ml gentamicin sulfate and 100 μg/ml amphotericin B, followed by the expansion of cells in the same media. Passage 2 cells were trypsinized using 0.25% trypsin for 5 min when they were subconfluent and used for further experiments.

2.2.2. Cell culture over collagen gel and collagen UF

Collagen gel was prepared as per the manufacturer's protocol. Briefly 3 mg/ml of collagen type I stock solution was diluted to a final concentration of 2 mg/ml using 10× PBS on ice, pH was adjusted to 7.0 using 1 N NaOH. Then 1×10^5 fibroblasts were added to this 100 μl collagen solution and the mixture was allowed to polymerize for 45 min. Collagen UF was cut into small pieces of 3 mm × 4 mm and sterilized by soaking in 70% ethanol for 30 min. Later the UF matrices were pre-wetted in respective media and 1×10^5 fibroblasts were seeded on the UF matrices. 1×10^5 fibroblasts seeded on 2D cell culture flask served as controls for all cell culture experiments. Cell density was kept same by taking the same volume into consideration for both gel and UF matrices. Cell culture was continued for 14 days for both experimental groups (gel and UF matrices) and controls using three different media, (n = 3, each experiment was repeated twice).

2.3. DNA content estimation

DNA content was estimated at time points of 7 and 14 days. DNA was isolated from constructs using DNA extraction kit (Agilent) according to manufacturer's protocol. Purity of DNA and DNA concentration was estimated using a Nanodrop 2000C (Thermo Scientific, Wilmington, DE, USA). Three samples from each condition were evaluated, and each experiment was repeated twice.

2.4. Metabolic activity

Cellular metabolic activity was determined at time points of 7 and 14 days using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay (Millipore). At each time point the metabolic activity of the cells seeded on UF matrices and encapsulated in collagen gel was measured by observing the production of formazan dye produced by reaction of metabolically active cells with the tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) in the MTT reagent. The constructs were harvested at 7th day and 14th day. The constructs were rinsed in phosphate-buffered saline (PBS) and incubated with MTT reagent and respective culture media in 1:10 ratio for 3 h at 37 °C that leads to the formation of crystals of formazan dye which was dissolved by the addition of equal volume of dimethyl sulfoxide. 100 μl of this suspension was aliquoted into fresh plate and the absorbance was measured using iMark microplate absorbance reader (BIORAD) at 560 nm (n = 3, each experiment was repeated twice).

2.5. Cell aspect ratio

Cell aspect ratio was determined by taking the ratio of the longer side of the cell (major axis) to the shorter side (minor axis). 20 cells from each condition were taken and aspect ratios were determined using Image J software (NIH, USA).

2.6. Scanning electron microscopy (SEM)

The cultured matrices were fixed with 2.5% glutaraldehyde at 7th and 14th days. The collagen gel samples were then snap-frozen in liquid nitrogen and vacuum dried. While collagen UF samples were dehydrated by using an increasing percentage gradient of alcohol followed by vacuum drying. This was followed by gold coating of the samples using a gold sputter coater (EMITECH K550X, UK) for 1 min with 25 mA electric current to form 15–20 nm thickness of coating. Cellular morphology was observed using SEM (model EVO 50, Zeiss, UK) under vacuum. Collagen UF was also analyzed for the presence of pores by SEM (Fig. 1c) and pore size was calculated using Image J software (NIH, USA) (n = 10 pores per image were measured (total of 3 images) to calculate the mean pore size). The fiber diameter of the collagen UF (Fig. 1c) and collagen gel (Fig. 1d) was calculated (n = 5) using Image J software (NIH, USA).

2.7. Immunofluorescence analysis

Constructs were processed for immunofluorescence after day 14 (n = 3, each experiment was repeated twice) by first rinsing in PBS thrice, then fixing in 4% formalin for 4 h, followed by washing with PBS. Constructs were permeabilized with Triton X-100 (0.1%), followed by PBS washing for three times. Constructs were then blocked with BSA (1%)/PBS followed by PBS washing for three times. Constructs were then incubated with primary antibodies: anti-collagen I (4 μg/ml, 0.2:100, Millipore), anti-elastin (4 μg/ml, 1:200, Millipore), anti-MMP-1 (2 μg/ml, 1:1000), anti-β Tubulin FITC conjugate (0.25 μg/5 μl, 1:10, BD Biosciences), goat anti-mouse IgG antibody-FITC conjugate (1:200, Millipore), Alexa Fluor 546 goat anti-mouse IgG (1:200, Invitrogen) by incubating for 1 h at room temperature. Nuclei were stained using DAPI (300 nm, 2:100, Invitrogen) for 5 min at room temperature.