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Ascorbate enhances elastin synthesis in 3D tissue-engineered pulmonary fibroblasts constructs

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ABSTRACT

Extracellular matrix remodeling is a continuous process that is critical to maintaining tissue homeostasis, and alterations in this process have been implicated in chronic diseases such as atherosclerosis, lung fibrosis, and emphysema. Collagen and elastin are subject to ascorbate-dependent hydroxylation. While this post-translational modification in collagen is critical for function, the role of hydroxylation of elastin is not well understood. A number of studies have indicated that ascorbate leads to reduced elastin synthesis. However, these studies were limited to analysis of cells grown under traditional 2D tissue culture conditions. To investigate this process we evaluated elastin and collagen synthesis in primary rat neonatal pulmonary fibroblasts in response to ascorbate treatment in traditional 2D culture and within 3D cross-linked gelatin matrices (Gelfoam). We observed little change in elastin or collagen biosynthesis in standard 2D cultures treated with ascorbate, yet observed a dramatic increase in elastin protein and mRNA levels in response to ascorbate in 3D cell-Gelfoam constructs. These data suggest that the cell-ECM architecture dictates pulmonary cell response to ascorbate, and that approaches aimed toward stimulating ECM repair or engineering functional cell-derived matrices should consider all aspects of the cellular environment.

1. Introduction

The extracellular matrix (ECM) is the hydrated webbing comprised of proteins and polysaccharides that surrounds most cells in multicellular organisms. The chemical and mechanical properties of the ECM define tissue architecture and instruct cell function. Thus, tissue disruption during injury and disease is communicated to resident cells, in large part, through alterations in ECM (Hynes, 2009). Consequently, ECM remodeling is a continuous process that is critical to maintaining tissue homeostasis, and alterations in this process have been implicated in chronic diseases such as atherosclerosis, lung fibrosis, and emphysema (Arroyo and Iruela-Arispe, 2010). In chronic disease a self-perpetuating feed forward loop has been proposed where ECM dysfunction leads to improper remodeling and progressively compromised tissue function. For example, during the progression of emphysema uncontrolled proteolysis degrades ECM leading to mechanical failure and collapse of alveolar septal walls and airspace enlargement. As this process progresses, the dysfunctional ECM may signal the resident cells in an effort to stimulate tissue repair, yet the ECM produced is not properly organized or able to meet the demands of the tissue leading to continued failure and disease progression (Suki and Bates, 2008).

Collagen, elastin, and proteoglycans are three major components of the ECM. Collagen bears the majority of the tensile load, while the viscoelastic nature of elastin imparts compliance with its ability to stretch 2–3 times in length with nearly no loss of energy (Suki et al., 2005). There are at least 28 genetically distinct collagen types, while a single gene encodes elastin. Both collagen and elastin are subject to post-translational modifications that are required for function (Suki and Bates, 2008). Of particular importance to collagen stability is the enzymatic hydroxylation of proline and lysine residues that requires ascorbate (vitamin C) as a cofactor (Eyre et al., 1984). Elastin is also subjected to ascorbate-dependent hydroxylation yet the function of this modification is less well understood. In particular, a number of studies have indicated that ascorbate leads to enhanced collagen production and reduced elastin synthesis (Bergethon et al., 1989; Dunn and Franzblau, 1982; Leesa et al., 1985). However, these studies were limited to analysis of cells grown under traditional 2-dimensional tissue

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culture conditions. Additional studies in animals have produced somewhat inconclusive results. Vitamin C deficiency in guinea pigs results in reduced elastin mRNA expression in blood vessels (Mahmoodian and Peterkofsky, 1999) and no change in elastin levels in skin (Barnes et al., 1969), while supplemental vitamin C reduced elastin deposition in neonatal rat aorta (Quaglino et al., 1991). Thus, it remains unclear how ascorbate-mediated modification ultimately influences elastin biosynthesis. It is likely that cell response to ascorbate will be dependent on cell type, tissue architecture and ECM organization.

Loss of elastin is a major contributing factor to the progression of emphysema and yet little is known about how ascorbate influences elastin biosynthesis in pulmonary cells. To investigate this process we evaluated elastin and collagen synthesis in primary rat neonatal pulmonary fibroblasts. We observed little change in elastin or collagen biosynthesis in standard 2D cultures treated with ascorbate. However, when we cultured these cells within 3D, cross-linked gelatin constructs (Gelfoam), we observed a dramatic increase in elastin deposition and expression of tropoelastin mRNA. These data suggest that the cell-ECM architecture dictates pulmonary cell response to ascorbate, and that approaches aimed toward stimulating ECM repair or engineering functional cell-derived matrices should consider all aspects of the cellular environment. Analysis of ECM remodeling within a 3D environment will provide insight into how cells in normal and diseased tissues respond to external stimuli and may be critical to the future development of treatments for ECM based diseases.

2. Materials and methods

2.1. Materials

The stable L-ascorbic acid analog (2-O-D-glucopyranosyl L-ascorbic acid) was purchased from Wako Chemicals (Richmond, VA). Compressed Gelfoam was obtained from Pharmacia and Upjohn Co., Division of Pfizer Inc. (New York, NY). Primary polyclonal rat lung alpha-elastin antibody was obtained from Elastin Products (Owensville, MO). Secondary peroxidase-conjugated AffiniPure Donkey Anti-Goat IgG antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). DAPI Vectashield and TO-PRO were from Life Technologies (Grand Island, NY). Compact Reaction Columns and 35 μm Compact Reaction Column Filters (upper) for three dimensional (3D) enzyme-linked immunosorbent assays (ELISAs) were obtained from USB Corporation (Cleveland, OH). Dulbecco's Modified Eagle's Medium (DMEM), phosphate buffered saline (PBS), penicillin/streptomycin, trypsin and nonessential amino acids, and ethylenediaminetetraacetic acid (EDTA) were obtained from Life Technologies (Grand Island, NY). Fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA). Tris buffered saline (TBS), bovine serum albumin (BSA), Tween 20, guanidine thyiocyanate, guanidine hydrocholoride, and beta-mercaptoethanol were obtained from American Bioanalytical (Natick, MA). Formaldehyde (37%) was obtained from Fisher Scientific (Fair Lawn, NJ). Tetramethylbenzidine (TMB) 2-component microwell peroxidase substrate kit was purchased from KPL (Gaithersburg, MD). Optimax microtiter plate reader (Molecular Devices, Sunnyvale, CA) was used for absorption measurements. RNase-free DNase 1 was purchased from New England Biolabs (M0303S, Ipswich, MA). Rat elastin (RN01499782_m1), collagen 1 (RN00801649), and 18S rRNA endogenous control (4308329) Taqman gene expression assays were purchased from Applied Biosystems (Foster City, CA) for use with Applied Biosystems 7300 Real-Time PCR.

2.2. Isolation of rat neonatal pulmonary fibroblasts

Neonatal rat pulmonary fibroblasts (NNRLFs) were isolated from 2–3-day-old Sprague-Dawley rats (Foster et al., 1990). All studies with animals were conducted as approved by the Boston University Medical Campus Institutional Animal Care and Use Committee, and our animal facility is approved by the American Association for the Accreditation of Laboratory Animal Care. Lungs were removed and washed three times with PBS. Scissors were used to mince the tissue into small pieces (1–2 mm^3) that were then suspended (1 lung/mL) in 0.05% trypsin/0.53 mM EDTA solution. The mixture was shaken at 350 rpm as it incubated at 37 °C for 20 min. A 100-μm Nucleopore filter was used to remove the digested tissue fragments. The resulting filtrate was centrifuged ($300 \times g$ for 10 min) and the cell pellet was washed with DMEM, 5% fetal bovine serum (FBS) infused with a 1% penicillin/streptomycin antibiotic cocktail and 1% nonessential amino acids (NNRLF medium).

Lung fibroblast medium was used to resuspend the pellet. The resulting suspension was seeded into flasks and allowed to sit for 2 h for adequate cell adhesion of fibroblasts before the media was changed. The media was replaced twice more that week with lung fibroblast medium. Trypsin was used to release the cells from the tissue-culture flasks. The cells were collected by centrifugation and resuspended in the lung fibroblast medium for 2–4 passages before being seeded onto plastic tissue culture dishes or Gelfoam matrix constructs.

2.3. Cell seeding and maintenance

NNRLF were seeded onto plastic tissue culture dishes at a density of 30,000 cells/cm^2. Gelfoam constructs were cut from a 12 cm × 8 cm × 0.3 cm sheet into 4 cm × 1 cm × 0.3 cm pieces the day prior to seeding. The constructs were fully submerged in NNRLF medium and were maintained in a tissue culture incubator at 37 °C overnight. Constructs were placed onto 100 mm Petri dishes and were seeded with 250,000 cells by spot pipetting over the entire 4 cm × 1 cm surface. Samples were placed in the incubator for 2–3 h to ensure adhesion had occurred. After the cell adhesion period, cell-Gelfoam constructs were placed in centrifuge tubes containing NNRLF medium, lids were left vented to allow gas exchange. Medium was changed 24 h after seeding and two times weekly thereafter. L-Ascorbic acid analog (10 mM) was added to half the samples at each feeding beginning on day 3 in culture. This concentration was selected based on previous studies that evaluated various concentrations of ascorbate (Welch et al., 1993) and compared the use of ascorbate with the analog used in the present study (Kumano et al., 1998).

2.4. ELISA analysis in 2D cultures

During days 7 through 28, NNRLF cells seeded onto 96 well tissue culture plates were washed with tris buffered saline (50 mM tris, pH 7.6, 150 mM sodium chloride) (TBS) followed by a fixation step of 3.7% formaldehyde for 20 min and a final TBS wash. Cells were blocked in a 3% bovine serum albumin (BSA) in TBS overnight at 4 °C. Samples were washed with TBS prior to adding 1 μg/mL RA-75 antibody in 3% BSA-TBS for 1.5 h at room temperature. Samples were then washed with TBS and incubated with 0.4 μg/mL peroxidase-conjugated donkey anti-goat IgG at room temperature followed by rinses with TBS containing 0.1% Tween-20 and with TBS. Finally, secondary antibody binding was detected with the TMB 2-component microwell peroxidase substrate kit and the reaction was stopped after 10 min by the addition of 1 N sulfuric acid. An