Biomaterials 32 (2011) 2885–2893

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Differences in valvular and vascular cell responses to strain in osteogenic media

Zannatul Ferdous [a,*], Hanjoong Jo [b], Robert M. Nerem [a,b]

[a] Institute of Bioengineering and Biosciences, Georgia Institute of Technology, 315 Ferst Drive, Mail code 0363, Atlanta, GA 30332, USA
[b] Emory University School of Medicine, Atlanta, GA 30322, USA

ARTICLE INFO

Article history:
Received 3 November 2010
Accepted 12 January 2011
Available online 1 February 2011

Keywords:
Calcification
Tissue engineering
Cyclic strain
Aortic valve
Collagen gels

ABSTRACT

Calcification is the primary cause of failure of bioprosthetic and tissue-engineered vascular and valvular grafts. We used tissue-engineered collagen gels containing human aortic smooth muscle cells (HASMC) and human aortic valvular interstitial cells (HAVIC) as a model to investigate cell-mediated differences in early markers of calcification. The HASMCs and HAVICs were isolated from non-sclerotic human tissues. After 21 days of culture in either regular or osteogenic media with or without 10% cyclic strain at 1 Hz, the collagen gels were assessed for DNA content, collagen I, matrix metalloproteinase (MMP)-2 and glycosaminoglycan (GAG) content. The collagen gels containing HASMCs contained significantly greater amounts of collagen I and GAG compared to HAVICs. Although strain increased MMP-2 activity for both cell types, this trend was significant ($p \leq 0.05$) only for HAVICs. Cultured gels were also assessed for osteogenic markers calcium content, alkaline phosphatase (ALP), and Runx2 and were present at greater amounts in gels containing HASMCs than HAVICs. Calcium content, Runx2 expression, and ALP activity were also modulated by mechanical strain. The results indicate that cell-mediated differences exist between the vascular and valvular calcification processes. Further investigation is necessary for improved understanding and to detect biomarkers for early detection or prevention of these diseases.

1. Introduction

Cardiovascular disease is a major cause of mortality in the elderly population in the developed world. Vascular and valvular diseases affect approximately 25% of the over 65 year population [1–3], where calcification is manifested in both of these diseases. Calcification is also a major challenge and main cause of failure for tissue-engineered and bioprosthetic vascular grafts and heart valves [4]. However, unlike vascular calcification, relatively little is known about the cellular and molecular mechanisms of valvular calcification. Vascular and valvular calcification shows similarities in risk factors suggesting a continuum of the same disease [5]. Both of these disease processes are now accepted to be an inflammatory condition [4,6] and are mediated by similar morphogens and growth factors. Vascular and valvular cells also demonstrate osteogenic differentiation during calcification [5,7]. Despite these similarities, how these diseases initiate and whether vascular or valvular cells are more susceptible to calcification is still not clearly understood.

Valvular cell behavior has commonly been related to that of vascular cells due to the proximity of these two physiological systems; however, recent studies suggest a more complex and unique behavior by valvular cells as compared to vascular cells [8,9]. Valvular endothelial cells show unique cell alignment and gene expression in response to shear stress as compared to vascular cells [10,11]. Similarly, vascular and valvular progenitor mesenchymal stem cells elicit phenotypic differences supporting unique behavior by these two cell types [12]. Valvular interstitial cells (VICs) are responsible for maintaining valve structure and function [13]. They also demonstrate a heterogeneous population in culture, whereas a subpopulation of these cells (activated VICs) show greater osteogenic behavior compared to others [14]. Therefore, VICs would be expected to elicit a more complex response to osteogenic environment than a homogeneous population of vascular smooth muscle cells. Likewise, differences have been observed between VICs and smooth muscle cells (SMCs) in 2D culture in spontaneous calcification, cell viability, and calcific nodule formation [12,15].

Mechanical forces are an important physiological component of the environment experienced by cells and likely contribute toward the calcification process. In the aortic valve, calcification predominantly occurs on the fibrosa side of the valve [16–18]. Since the two sides of the aortic valve experience differences in their mechanical environment, where the ventricularis sees predominantly uniform and the fibrosa encounters disturbed blood flow, it suggests that mechanical factors might contribute toward the side-dependent

* Corresponding author. Tel.: +404 894 5271; fax: +404 778 5674.
E-mail address: zferdous3@mail.gatech.edu (Z. Ferdous).

0142-9612/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2011.01.030

valve pathology. Similarly in blood vessels, calcification is observed in areas of major branches that experience lower hemodynamic forces [19–21].

In this study, we used tissue-engineered collagen gels as a model to investigate events associated with vascular versus valvular calcification. Even though similarities exist in the calcification process between these two cell types, identifying differences might result in early detection and possible treatment options for either system. Since both cell types experience cyclic strain in their native environment, we also investigated the contributions of a representative physiological strain of 10% cyclic strain on calcification events for these two cell types. The collagen gels prepared for each cell type were divided in three groups: a) static gels cultured in base media (static ctrl), b) static gels cultured in osteogenic media (static ost), and c) gels cultured under 10% cyclic strain in osteogenic media (strain ost). Our research was based on the hypothesis that calcification is a process dependent on both the cell type and the mechanical environment experienced by the cells.

2. Materials and methods

2.1. Primary cell isolation

Primary cell culture of HASMC and HAVIC were established from freshly obtained non-sclerotic tissues from heart transplant patients (one female, age 44 years, and three male, ages 22, 62, and 66 years) using a similar protocol as previously reported [8]. The study was approved by the institutional review board at Georgia Institute of Technology and Emory University and patient consent were obtained prior to surgery. The ascending aorta and the aortic valve tissues were obtained during transplant and were immediately processed for cell culture. Both the aorta (near the annulus) and the aortic valve leaflets were first minced under sterile conditions and digested in type II collagenase solution in an incubated shaker for 4 h. The resulting cell suspension was then filtered, centrifuged, and plated in T-75 flasks. Both types of cells were cultured in medium containing MCDB 131 (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (Mediatech, Inc.), 1% penicillin-streptomycin (Mediatech, Inc.), 1% L-glutamine (Mediatech, Inc.), 0.0005 μg/ml FGF (PeproTech Inc., Rocky Hill, NJ), 0.01 μg/ml EGF (Invitrogen, Carlsbad, CA), 5 μg/ml Insulin (Invitrogen). Cells from four patients at passage 6 were used to prepare collagen gels.

2.2. Tubular collagen gel preparation and pneumatic bioreactor culture

A tubular mold for the collagen-cell mixture was created using a hollow glass rod, rubber stoppers, and a glass tube, as previously reported [22]. The mold also contained silicone sleeves inserted on the glass rods, where the surface of the sleeves was modified in 10N H_2SO_4 to promote adherence of collagen to the surface. The collagen gels were prepared using acid-soluble bovine dermal collagen type I (2 mg/ml, MP Biomedicals, Solon, OH) and either HASMC or HAVIC cells (1 million cells/ml) using an established protocol [23]. The collagen gels were cultured under static condition for 5 days before applying osteogenic conditions.

Cyclic distension was applied to the compacted collagen gels using a pneumatic bioreactor developed in our lab [22]. The base culture medium used for experimental conditions was same as previously mentioned culture medium without insulin. The osteogenic media consisted of dexamethasone (10 nm), β-glycerol phosphate (5 mM) and ascorbate-2-phosphate (50 μg/ml), all purchased from Sigma, St. Louis, MO [24]. As previously mentioned, the collagen gels prepared for each cell type were divided in three groups: a) static gels cultured in base media (static ctrl), b) static gels cultured in osteogenic media (static ost), and c) gels cultured under 10% cyclic strain at 1 Hz in osteogenic media (strain ost). Some preliminary studies were also performed where collagen gels were cultured under 10% strain in base media (strain ctrl). However, no significant difference was observed in the osteogenic markers in strain ctrl group as compared to static ctrl group and therefore was removed from the experimental groups. To determine the length of culture, we did preliminary studies, where the collagen gels were cultured for 9 or 21 days in the strain bioreactor. Since the trends for gene expression were similar for 9 versus 21 days but the osteogenic markers demonstrated stronger expression at 21 days, we selected 21 days as the culture duration for this study.

2.3. Hematoxylin & Eosin (H&E) and Von Kossa staining of tissue sections

The collagen gels were fixed in 10% formalin and processed using standard histology protocols. Paraffin-embedded tissue sections (8 μm) were used for H&E and Von Kossa staining. For H&E, the sections were stained with Hematoxylin for 5 min and Eosin for 10 min using a Leica Autostainer. For Von Kossa staining, silver nitrate (5%) was added to the deparaffinized tissue sections and exposed to UV until the calcium salts turn black. The undissolved salts were then removed using 5% sodium thiosulfate, and the sections were counter-stained using nuclear-fast red.

2.4. Alkaline phosphatase (ALP) enzyme activity assay

ALP enzyme activity was measured using an assay as described by Osman et al. [24]. Briefly, the collagen gels were rinsed in calcium-free PBS and homogenized in RIPA buffer (Sigma). The ALP activity in the samples was measured against standard dilutions prepared from 10 M p-nitrophenol (Sigma). 10 mM p-nitrophenyl phosphate (Fisher Scientific, Pittsburgh, PA) in substrate buffer (0.1 M glycine, 1 mM $ZnCl_2$, 1 mM $MgCl_2$, pH 10.4, all purchased from Sigma,) was then added to the samples and the resulting absorbance at 405 nm was measured at 0, 15, 30, 60, 90 and 120 min. The gradient (nmol/min) was then calculated for each sample and normalized to total protein content. The total protein content was determined using modified Lowry assay kit (Thermo Scientific, Rockford, IL).

2.5. Calcium content

The calcium content in the collagen gels was measured using a calcium specific Arsenazo dye reagent (Fisher Scientific). The collagen gels were first homogenized in 1N acetic acid and the calcium content was immediately determined using the dye. The absorbance of the reaction product was measured at 650 nm using a plate reader. The calcium content was then normalized to the wet weight of the gels.

2.6. Quantitative reverse transcriptase polymerase chain reaction (qPCR)

We isolated RNA from the collagen gels using RNeasy Lipid Tissue Mini kit (Qiagen, Valencia, CA). cDNA synthesis was performed using SuperScript III First Strand kit (Invitrogen). The qPCR reaction were performed using SYBR green PCR master mix (Applied Biosystems, Foster city, CA) and a Step One Plus system (Applied Biosystems). The level of expression for each gene was quantified using known standards and normalized to reference gene QuantumRNA Classic II 18S Internal Standard (Applied Biosystems). The primers used were as follows: RunX2: Forward: GCACAAGTAAATCATTGAACTACAGAAA; Reverse: AGCCTGGCGATTTA-GAGTTTTG; Collagen I, alpha II: Forward: GCTACCCAACTTGCCTTCATG; Reverse: TTCTTGCAGTGGTAGGTGATGTTC.

2.7. DNA content

The DNA content in the collagen gels was measured using a DNA assay [23]. Briefly, the collagen gels were lyophilized overnight and rehydrated in 1 ml of buffer (50 mM Na_2HPO_4, 2 mM EDTA, 0.02% NaN_3, pH 7.4), then digested by adding 100 μl of Proteinase-K solution (10 mg/ml in ddH_2O, Invitrogen) and incubated at 60 °C for 2 h. The solubilized samples were fluorotagged with Hoechst 33,258 dye (Sigma) and the resulting fluorescence was measured at 458 nm. Double stranded DNA from calf thymus (Sigma) was used as standard to calculate the mass of DNA present in each sample. The DNA content was normalized to the dry weight of each collagen gel.

2.8. Glycosaminoglycan (GAG) content

The GAG content was determined using dimethylmethylene blue (DMMB) dye (Polysciences Inc., Warrington, PA) on the homogenized samples used in the DNA assay. The resulting absorbance was measured at 525 nm. Chondroitin sulfate (Sigma) was used as a standard to determine total GAG content in each sample. The GAG content measured was normalized to the dry weight of the collagen gels.

2.9. Gelatin zymography

Gelatin zymography was used to determine matrix metalloproteinase (MMP) activity in the collagen gels. All of the products used for zymography were purchased from Invitrogen unless otherwise mentioned. Equal amounts (5 μg of protein) of non-reduced samples digested in RIPA buffer and MMP-2/9 ladder (Chemicon International) were loaded onto 10% zymogram gels. After electrophoresis, the gels were shaken in a renaturing buffer for 30 min and then cleared overnight in developing buffer at 37 °C. The gels were then stained with colloidal blue staining kit to detect clear bands against a blue background. The band densities were calculated using gel analysis tool in Image J software.

2.10. Live/dead assay

Live/Dead viability/cytotoxicity kit (Invitrogen) was used to detect cell viability in the collagen gels. Briefly, at the end of culture, the collagen gels were sliced in the radial direction to obtain thin rings. The sections were then incubated in Calcein AM and Ethidium homodimer-1 solution according to manufacturer's protocol. The collagen gel sections were imaged using a Zeiss LSM 510 confocal microscope.