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The susceptibility of bioprosthetic heart valve leaflets to oxidation

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ABSTRACT

The clinical use of bioprosthetic heart valves (BHV) is limited due to device failure caused by structural degeneration of BHV leaflets. In this study we investigated the hypothesis that oxidative stress contributes to this process. Fifteen clinical BHV that had been removed for device failure were analyzed for oxidized amino acids using mass spectrometry. Significantly increased levels of ortho-tyrosine, meta-tyrosine and dityrosine were present in clinical BHV explants as compared to the non-implanted BHV material glutaraldehyde treated bovine pericardium (BP). BP was exposed in vitro to oxidizing conditions ($FeSO_4/H_2O_2$) to assess the effects of oxidation on structural degeneration. Exposure to oxidizing conditions resulted in significant collagen deterioration, loss of glutaraldehyde cross-links, and increased susceptibility to collagenase degradation. BP modified through covalent attachment of the oxidant scavenger 3-(4-hydroxy-3,5-di-*tert*-butylphenyl) propyl amine (DBP) was resistant to all of the monitored parameters of structural damage induced by oxidation. These results indicate that oxidative stress, particularly via hydroxyl radical and tyrosyl radical mediated pathways, may be involved in the structural degeneration of BHV, and that this mechanism may be attenuated through local delivery of antioxidants such as DBP.

1. Introduction

Heart valve replacement surgery is the primary treatment option for progressive, symptomatic heart valve diseases, including aortic valve stenosis and mitral valve prolapse. Each year more than 300,000 valve replacement surgeries are performed in the U.S. with both mechanical valves and bioprosthetic heart valves (BHV) [1]. BHV, which are fabricated from glutaraldehyde treated bovine pericardium (BP), bovine jugular vein, or porcine aortic valve leaflets, have significant advantages over the mechanical valves including a lower risk of thrombosis [2]. In addition, BHV are currently the only type of prosthetic valves that can be catheter deployed as an interventional device [3]. Unfortunately, the use of BHV is limited by poor durability leading to a relatively short device lifespan. BHV begin to fail clinically an average of 10 years following the original valve replacement due to leaflet malfunction caused by structural degeneration associated with either calcification or primary leaflet degeneration [4].

Clinical pathology studies have identified calcification as a major contributor to BHV failure. Therefore the primary focus of the BHV field has been both investigations of calcification mechanisms and the development of anti-calcification strategies involving either alternative fixatives to glutaraldehyde, material pre-treatment, or local drug delivery [5]. Despite the advances in anti-calcification treatments, BHV structural degeneration remains a significant problem. There are no clinical results at this time demonstrating clear efficacy of any of the anti-calcification strategies. Clinical-pathology studies of BHV have suggested alternative mechanisms of BHV degeneration including mechanical stress [6], inflammation [7–9], immune responses [10], and collagen damage not associated with calcification [11,12]. Therefore, the development of strategies targeting alternative mechanisms of BHV structural degeneration may be effective in mitigating clinical BHV failure.

Oxidative stress has been identified as an important cause of material failure for synthetic biomaterials such as polyurethane pacemaker leads and metal alloy joint prostheses [13], but has not

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been studied as a mechanism of BHV structural degeneration. Implantable biomaterials such as BHV and synthetic materials in general elicit a foreign body reaction associated with acute and chronic inflammation [14]. Inflammatory cells that are recruited to the site of biomaterial implants produce reactive oxygen and nitrogen species (ROS/RNS) that can cause post translational oxidative modifications to proteins. For synthetic materials, oxidative stress can lead to material cracking, pitting, and impaired function [13,15]. The effects of ROS/RNS on BHV have not been investigated, but the mechanisms likely involved have been studied in systems using purified collagen, a major component of BHV. Reactions of oxidants with collagen result in the formation of structurally specific oxidative modifications including o,o′-dityrosine, a tyrosyl radical mediated cross-link, the non-physiological isomers o-tyrosine and m-tyrosine formed by hydroxyl radical modification phenylalanine, as well as an increase in susceptibility to degradation by proteolytic enzymes [16–19]. Based on these previous studies, we hypothesize that oxidative or nitrative stress by specific pathways may cause structural modification to BHV.

Here we investigate the effects of oxidative stress on BHV using clinical-pathologic BHV explants as well as experimental models with BP and BP covalently modified with the oxidant scavenger DBP. Clinical BHV explanted for device failure were analyzed for the presence of oxidized amino acids with stable isotope dilution mass spectrometry. Experimental systems involving BP exposure to oxidizing conditions were used to assess the effects of oxidation on BHV and the capacity of DBP to provide resistance to oxidative damage.

2. Materials and methods

2.1. Materials

Biosol and Bioscint were purchased from National Diagnostics (Atlanta, GA). Amplex Red was purchased from Life Technologies (Philadelphia, PA). Glutaraldehyde was purchased from Polysciences, Inc (Warrington, PA). ^{3}H-glutaraldehyde and [2,3-^{14}C]-methyl acrylate were purchased from American Radiolabeled Chemicals (St. Louis, MO). All chemicals unless otherwise specified were purchased from Sigma Aldrich (St. Louis, MO).

2.2. Synthesis of DBP

DBP was synthesized through the reaction of 2,6-di-tert-butylphenol with methyl acrylate under catalysis with a mixture of NaOH and KOH. The resulting methyl 3-(4-hydroxy-3,5-di-tert-butylphenyl)propionate was reduced with lithium aluminum hydride in ethyl ether to 3-(4-hydroxy-3,5-di-tert-butylphenyl)propanol, which was transformed into the mesylate by treatment with methanesulfonyl chloride and triethylamine in dichloromethane. The mesylate was reacted with phthalimide potassium salt in 1-methylpyrrolidinone and the phthalimide derivative was cleaved with hydrazine in ethanol to DBP-amine base, which was finally transformed into its water-soluble hydrochloride.

^{14}C-DBP hydrochloride (7 μCi/mmol) was prepared similarly, using [2,3-^{14}C]-methyl acrylate (10–20 mCi/mmol) diluted with the non-labeled compound. The only difference between this radiolabeling synthesis and the non-radioactive procedure was at the first step, where an excess of 2,6-di-tert-butylphenol was applied to react the labeled acrylate as completely as possible.

2.3. Clinical BHV explants

Between 2010 and 2012, 15 explanted bovine pericardial BHV were collected from patients according to the University of Pennsylvania IRB approved protocol #809349. Informed consent was obtained from patients requiring repeat aortic valve replacement due to a failing BHV at the Hospital of the University of Pennsylvania. Patients with bioprosthetic aortic valve failure due to pannus, thrombus, and endocarditis were excluded from the study. Explanted bioprosthetic aortic valves were fixed in 10% buffered formalin overnight, followed by dehydration in 70% ethanol solution, and stored at 4 °C. BHV leaflets were embedded in paraffin according to standard procedures.

2.4. Quantification of oxidized amino acids in clinical explants

Oxidized amino acids were quantified by established stable isotope dilution liquid chromatography tandem mass spectrometry (LC MS/MS) methods [20] on an AB SCIEX API 5000 triple quadrupole mass spectrometer interfaced with an Aria LX Series HPLC multiplexing system (Cohesive Technologies Inc., Franklin, MA). Briefly, paraffin-embedded BHV leaflets were deparaffinized by xylene. [$^{13}C_6$]-labeled oxidized amino acid standards and universal labeled precursor amino acids ([$^{13}C_9$,$^{15}N_1$]tyrosine and [$^{13}C_9$,$^{15}N_1$]phenylalanine) were added to samples after protein delipidation and desalting with a single phase mixture of H_2O/methanol/H_2O-saturated diethyl ether (1:3:8 v/v/v). Proteins were hydrolyzed under argon gas in methane sulfonic acid, and then samples were passed through C18 solid-phase extraction column (Discovery – DSC18 minicolumn, 3 ml, Supelco, Bellefone, PA) prior to MS analysis. Individual oxidized amino acids and their precursors were monitored by characteristic parent to product ion transitions unique for each isotopologue monitored. Results are expressed relative to the content of the precursor amino acids, tyrosine and phenylalanine.

2.5. BP treatments and DBP modification

Fresh BP obtained from an abattoir was treated with 0.625% glutaraldehyde or 0.625% ^{3}H-glutaraldehyde (specific activity 24 μCi/mmol) in HEPES buffer pH 7.4 for 7 days at room temperature with gentle shaking. BP was modified with DBP through a carbodiimide-driven reaction. The DBP modification reaction was prepared as two solutions: 42 mM DBP in 100% ethanol and 43 mM N-hydroxysuccinimide (SuOH) in deionized H_2O. Immediately before adding the BP, the two solutions were combined and 65 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was added. The reaction proceeded for 24 h at room temperature with gentle shaking. DBP modified BP was rinsed for 10 min in 100% ethanol to remove precipitate formed during the reaction. As a control for the effects of DBP, BP was treated with the carbodiimide reaction with the exclusion of DBP (Glut-EDC/SuOH). All BP samples were stored in 0.2% glutaraldehyde.

Glutaraldehyde fixed BP was reacted with [bis(trifluooacetoxy)iodo]benzene (BTI) to convert asparagine (Asn) and glutamine (Gln) residues to diaminopropionic acid and diaminobutanoic acid, respectively, and prevent the conversion of Asn to aspartic acid (Asp) and Gln to glutamic acid (Glu) during acid hydrolysis. BTI derivatization was performed in 5 M guanidine-HCl and 10 mM trifluoroacetic acid for 4 h at 60 °C. BP was then hydrolyzed in 6 N HCl, dried under air at 60 °C, and reconstituted for amino acid analysis by HPLC at the CHOP Metabolomics Core Facility.

BP was modified with ^{14}C-DBP through the carbodiimide-driven reaction to quantify the attachment of DBP to BP. Lyophilized ^{14}C-DBP modified BP was solubilized with Biosol at 50 °C with shaking for 72 h. Solubilized tissues were added to Bioscint and analyzed by liquid scintillation counting.

2.6. Oxidation capacity of DBP BP

The oxidation reporter molecule Amplex Red was used to determine the oxidant scavenging capacity of DBP modified BP in a system containing myeloperoxidase (MPO) (1 μg/ml), H_2O_2 (10 μM), and either Cl^- (120 mM NaCl) or NO_2^- (100 μM $NaNO_2$) ions. Amplex Red (50 μM) was added to the BP samples immediately after adding H_2O_2, MPO and NaCl or $NaNO_2$. Fluorescence of the solution at an excitation/emission of 560/590 nm was determined 30 min after adding Amplex Red in order to quantify the formation of resorufin, the product of Amplex Red oxidation.

2.7. Accelerated oxidative damage model

BP samples were placed in PBS or 1% H_2O_2/100 μM $FeSO_4$ for 7 days with solution changes every 2–3 days. Lyophilized samples were weighed at the start and end of treatments. Picrosirius red staining was done on formalin-fixed, paraffin-embedded samples using 0.1% Sirius red in saturated picric acid. BP treated with ^{3}H-glutaraldehyde was monitored for the release of ^{3}H throughout the assay, as well as in the solubilized tissues at the end of the treatments. Collagenase digestion was performed on lyophilized BP following the 7 day oxidation assay. Collagenase (600 U/ml) was added to BP samples and incubated for 24 h at 37 °C. Digestion by collagenase was measured as a loss of weight following collagenase treatment.

2.8. Statistical methods

Results are shown as the mean ± standard error for the mean. Single ANOVA with Tukey's test, Mann–Whitney rank sum test or a two tailed *t*-test were used to determine significance, which was defined as a p value less than 0.05.

3. Results

3.1. Analyses of clinical BHV explants

The patient population had roughly equal numbers of males and females (Table 1), and concomitant heart surgery was performed in 6 of the 15 subjects (Table 1). All of the explanted BHV were Carpentier-Edwards bioprostheses except for one Sorin bioprosthetic heart valve. Variable amounts of calcification were present in all explants per surgical pathology examination (data not shown), and quantitation of the calcium levels in the leaflets was