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Molecular assessment of collagen denaturation in decellularized tissues using a collagen hybridizing peptide

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ABSTRACT

Decellularized extracellular matrix (ECM) derived from tissues and organs are emerging as important scaffold materials for regenerative medicine. Many believe that preservation of the native ECM structure during decellularization is highly desirable. However, because effective techniques to assess the structural damage in ECM are lacking, the disruptive effects of a decellularization method and the impact of the associated structural damage upon the scaffold's regenerative capacity are often debated. Using a novel collagen hybridizing peptide (CHP) that specifically binds to unfolded collagen chains, we investigated the molecular denaturation of collagen in the ECM decellularized by four commonly used cell-removing detergents: sodium dodecyl sulfate (SDS), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), sodium deoxycholate (SD), and Triton X-100. Staining of the detergent-treated porcine ligament and urinary bladder matrix with carboxyfluorescein-labeled CHP demonstrated that SDS and Triton X-100 denature the triple helical collagen molecule while CHAPS and SD do not, although second harmonic generation imaging and transmission electron microscopy (TEM) revealed that all four detergents disrupt collagen fibrils. Our findings from the CHP staining were further confirmed by the circular dichroism spectra of intact triple helical collagen molecules in CHAPS and SD solutions, and the TEM images of CHP-conjugated gold nanoparticles binding only to the SDS and Triton X-100 treated collagen fibrils. CHP is a powerful new tool for direct and reliable measurement of denatured collagen molecules in decellularized tissues. It is expected to have wide applications in the development and standardization of the tissue/organ decellularization technology.

Statement of Significance

Preservation of the native ECM structure in decellularized tissues is highly desirable, since denaturation of ECM molecules (e.g., collagen) during decellularization can strongly influence the cellular response. Unfortunately, conventional techniques (SEM, SHG) are not conducive to identifying denatured collagen molecules in tissues. We demonstrate the first investigation into the *molecular* denaturation of collagen in decellularized ECM enabled by a novel Collagen Hybridizing Peptide (CHP) that specifically binds to unfolded collagen chains. We show that SDS and Triton X-100 denature collagen molecules while CHAPS and SD cannot. Such detection has been nearly impossible with other existing techniques. The CHP technique will advance our understanding about the effect of the cell-removing process on ECM, and lead to development of the decellularization technology.

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1. Introduction

Extracellular matrix (ECM) obtained by the decellularization of tissues has become an important biomaterial for tissue engineering and regenerative medicine [1]. Dozens of products derived from decellularized tissues, commonly known as biologic scaffolds, are currently used in clinical practice [1] for applications in wound care [2,3], pericardial reconstruction [4], and heart valve replacement [5], among others. By presenting a combination of structural and biological factors, such as the three-dimensional (3D) ultrastructure, mechanical integrity and a specific ECM composition, these acellular biologic scaffolds can provide a near-native and complex microenvironment for cell growth and tissue development, which is difficult to recapitulate with synthetic materials or a single ECM component. The ECM can also be partially digested with pepsin, and reconstituted *in situ* to form a hydrogel [6,7], which retains numerous biochemical constituents found in native tissues, such as growth factors and glycosaminoglycan. When the hydrogel forms in the damaged tissue, these bioactive cues can promote a constructive host remodeling response while curbing inflammation and scarring [6]. One example is the use of decellularized myocardial matrix hydrogels as a post-myocardial infarction biomaterial therapy [8]. This new therapy has produced encouraging preclinical results [9,10] and is currently under a clinical trial [6]. In recent years, improvement in whole organ decellularization techniques has enabled 3D organ scaffolds, which preserve the native tissue architecture including vascular networks [11,12]. These decellularized scaffolds can be repopulated with selected cell types *in vitro* to regenerate functional organs. So far, a variety of organs, including heart [13], kidney [14], liver [15], lung [16,17], limb [18], and pancreas [19], have been created from decellularized whole organs, and their short-term functions have been demonstrated after transplantation *in vivo*. In light of critical shortage of organ donors, this technological breakthrough provides hopes of transplanting engineered animal organs to patients with end-stage organ failure.

The goal of tissue decellularization is to remove cells and cellular remnants while retaining the 3D ultrastructure and composition of the native ECM as much as possible. Complete removal of all cellular materials is not possible, and decellularization processes inevitably cause disruption of the matrix architecture and surface ligand landscape [1,11]. Consequently, preferred methods of decellularization vary among tissues and organs. A variety of decellularization methods have been reported so far [1], including physical disruption (e.g., freeze-thaw) [20], enzymatic treatment [21], and treatment with chemical agents, such as acids and bases [22], and various detergents [15,17,23,24]. When these methods are used appropriately, the resulting ECM scaffold can be used as a cell-guiding template that contains the bioactive cues and 3D configuration beneficial for cell infiltration and proper tissue remodeling. However, for many tissues and organs, achieving adequate decellularization can disrupt the native ECM structures dramatically. Since it is difficult to make accurate assessment of structural changes to ECM and their effects on cell-matrix interactions, selecting ideal decellularization method heavily relies on anecdotal experience. Despite an increasing number of studies in the literature that directly compare different decellularization methods [22,25–28], there are many conflicting reports about the structure-disrupting effects of a given agent even when it is used to decellularize fairly similar tissues [29,30]. These disparate results are, in large part due to the lack of convenient and accurate methods for determining the structural changes in the ECM that may occur at multiple scales during the cell-removing process.

Because the fibrillar collagen is the most predominant component of ECM [31], it has been the primary target for assessing the structural damage in decellularized tissues. Histological collagen stains such as picrosirius red, and immunohistochemistry based on anti-collagen antibodies are widely used, but they can only report the changes in collagen content after decellularization, and do not provide any information about collagen structure. Scanning electron microscopy (SEM) can reveal the higher order architecture of the collagen fibers in the decellularized ECM. Unfortunately, different decellularization methods often result in drastic differences in ECM morphology, and it is nearly impossible to compare the levels of structural disruption solely by observation of the ECM morphology under SEM [32]. Microscopic methods such as second harmonic generation (SHG) and transmission electron microscopy (TEM) have been used to detect structural damage to collagen fibers after decellularization [33,34]. The reduction of SHG signal [25], as well as the disappearance of the d-banding pattern in TEM, are indications of the loss of collagen structure. However, both methods measure collagen damage by loss of signals or features, a mechanism that is not sensitive enough to detect low levels of disruption which could be the outcome of certain decellularization agents. Most importantly, all of the existing methods can only detect the structural change of collagen at the fiber level, and there has been hardly any investigation of whether a decellularization procedure can compromise the structure of a collagen molecule, the most fundamental building block of the collagen fibers.

Here we present a new study to assess the effects of decellularization agents on the molecular structure of collagen. The study was made possible by the collagen hybridizing peptide [CHP, sequence: $(GPO)_9$, O: hydroxyproline] that our group developed. The CHP is designed to have a strong propensity to fold into a triple helix, a unique super-secondary protein structure that is nearly exclusively found in collagen. A CHP strand can readily bind to unfolded collagen chains by forming a hybridized triple helix through inter-strand hydrogen bonds, in a fashion analogous to primers binding to DNA strands during PCR [35,36]. The CHP has essentially no affinity to intact collagen and other biomolecules, due to lack of any binding sites and the peptide's neutral and hydrophilic amino acids composition [37]. Our previous studies demonstrated that the CHP can bind to collagen molecules disrupted by heat [36,37], enzymatic degradation [36], or mechanical overloading (unpublished results). In this study, we show that the carboxyfluorescein labeled CHP (designated as CF-CHP) can effectively report the level and location of molecular denaturation of collagen in tissues decellularized by commonly used detergents: sodium dodecyl sulfate (SDS) [13,16,29], 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) [17], sodium deoxycholate (SD) [24,38], and Triton X-100 [39,40]. Our findings demonstrate that CHP can provide molecular level information about the structural damage in decellularized ECM. It could be a useful tool for advancing our understanding of the effects of structure alternation on the performance of the acellular biologic scaffolds, and for developing new and improved tissue decellularization technologies.

2. Materials and methods

2.1. Synthesis of fluorescently labeled CHP

The CHP [sequence: GGG-$(GPO)_9$, where GGG is a linker, O is hydroxyproline] and the sequence-scrambled control peptide (SSCP, GGG-PGOGPGPOPOGOGOPPGOOPGGOOPPG) were synthesized using standard solid-phase Fmoc and HBTU chemistry as described previously [36,41]. The fluorophore, 5,6-carboxyfluorescein (CF) was coupled to peptides on resin in a 24 h reaction with 6 molar equivalence of CF, 6 molar equivalence of PyAOP, and 12 molar equivalence of DIPEA. The peptides were cleaved from the resin by incubation with trifluroacetic acid