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Collagenase treatment enhances proteomic coverage of low-abundance proteins in decellularized matrix bioscaffolds

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ABSTRACT

There is great interest in the application of advanced proteomic techniques to characterize decellularized tissues in order to develop a deeper understanding of the effects of the complex extracellular matrix (ECM) composition on the cellular response to these pro-regenerative bioscaffolds. However, the identification of proteins in ECM-derived bioscaffolds is hindered by the high abundance of collagen in the samples, which can interfere with the detection of lower-abundance constituents that may be important regulators of cell function. To address this limitation, we developed a novel multi-enzyme digestion approach using treatment with a highly-purified collagenase derived from *Clostridium Histolyticum* to selectively deplete collagen from ECM-derived protein extracts, reducing its relative abundance from up to 90% to below 10%. Moreover, we applied this new method to characterize the proteome of human decellularized adipose tissue (DAT), human decellularized cancellous bone (DCB), and commercially-available bovine tendon collagen (BTC). We successfully demonstrated with all three sources that collagenase treatment increased the depth of detection and enabled the identification of a variety of signaling proteins that were masked by collagen in standard digestion protocols with trypsin/LysC, increasing the number of proteins identified in the DAT by ~2.2 fold, the DCB by ~1.3 fold, and the BTC by ~1.6 fold. In addition, quantitative proteomics using label-free quantification demonstrated that the DAT and DCB extracts were compositionally distinct, and identified a number of adipogenic and osteogenic proteins that were consistently more highly expressed in the DAT and DCB respectively. Overall, we have developed a new processing method that may be applied in advanced mass spectrometry studies to improve the high-throughput proteomic characterization of bioscaffolds derived from mammalian tissues. Further, our study provides new insight into the complex ECM composition of two human decellularized tissues of interest as cell-instructive platforms for regenerative medicine.

1. Introduction

The extracellular matrix (ECM) is a complex 3-D network of proteins and polysaccharides that functions as a supporting structure for cells, providing tissue-specific biochemical and biophysical cues that modulate cell behaviour [1]. Harnessing the innate regenerative potential of the ECM, decellularized tissue bioscaffolds have shown promise as cell-instructive platforms for wound healing and tissue-engineering applications [2–5]. These pro-regenerative biomaterials are typically fabricated by subjecting tissues to a series of processing steps involving physical cell disruption, chemical extraction of cellular components, and/or enzymatic digestion to release cells or degrade residual nucleic acid or lipid content [6]. To date, virtually every tissue in the body has been decellularized using customized protocols tailored to the unique compositional and structural properties of each tissue [7,8].

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To develop a deeper mechanistic understanding of ECM-derived scaffold bioactivity, there has been growing interest in the compositional analysis of decellularized tissues [9]. Traditionally, techniques such as immunohistochemistry (IHC), western blotting (WB), or enzyme-linked immunosorbent assay (ELISA) have been applied to probe for specific proteins, such as growth factors, postulated to mediate the cell or tissue response [10,11]. However, these approaches do not allow for the high-throughput global characterization of these complex biomaterials. Recognizing the need for a more complete analysis of the ECM proteome within decellularized tissues, several groups have begun to apply mass spectrometry-based screening techniques to assess the composition of specific decellularized matrices [12,13]. To aid in these efforts, Naba et al. developed a bioinformatics approach to predict the *in silico* "matrisome", defined as the ensemble of ECM proteins and associated factors, with a total of 1021 proteins sorted into 6 main categories: collagens, proteoglycans, ECM glycoproteins, ECM-affiliated proteins, ECM regulators, and secreted factors [14].

Although progress has been made on the characterization of the matrisome within a number of decellularized tissues, limitations still exist. In most approaches to date, a combination of detergents, solvents, and/or chaotropic agents are used to extract proteins for proteomic analysis [15]. However, many of these reagents are not compatible with mass spectrometry investigations and must be removed prior to analysis using techniques such as gel electrophoresis and in-gel digestion of proteins [16,17]. Unfortunately, the poor recovery of peptides from in-gel digestion limits the detection of low-abundance proteins present in the ECM [18]. Other groups have used a variety of buffers and detergents to compartmentalize proteins from whole tissues based on their relative solubility to obtain an ECM-enriched fraction [19,20]. A major limitation of this technique is the high abundance of collagen within these samples. More specifically, collagen type I and other family members are readily detected in multiple fractions and can interfere with the identification of low-abundance ECM proteins that may be key regulators of cell behaviour involved in the observed bioactivity of decellularized biomaterials.

The current study focused on the development of a novel processing method to selectively reduce the concentration of collagen within protein extracts from decellularized ECM scaffolds in order to enhance the identification of low-abundance proteins using mass spectrometry-based proteomic techniques. Several proteases have been identified that digest collagens, including the widely-studied collagenases derived from the bacterium *Clostridium Histolyticum* [21]. Depending on the processing conditions and enzyme purity, these enzymes can cleave collagen with high specificity into small oligopeptides [22], which can subsequently be removed through filtration. Since the aim of this study was to selectively deplete collagen while minimizing the digestion of other ECM proteins, we identified a commercially-available, ion-exchange chromatography-purified collagenase from *Clostridium Histolyticum* reported to recognize the sequence -R-Pro-8-X-Gly-Pro-R- (where X is most commonly a neutral amino acid, Pro-8 indicates hydroxyproline) [23]. While this is a very common amino acid sequence in collagen, this motif may also be found in other proteins [24,25]. However, we hypothesized that in our multi-enzyme digestion strategy, that collagenase would cleave collagen as the most abundant substrate, allowing for the increased detection of low abundance proteins using global mass spectrometry approaches. To demonstrate the flexibility of this sample preparation method with a range of ECM sources, we applied our new strategy to characterize the complex protein composition of human decellularized adipose tissue (DAT) [26] and human decellularized cancellous bone (DCB) [27], as promising bioscaffold sources of interest for regenerative applications, as well as commercially-available bovine tendon collagen (BTC) regarded as a more highly-purified ECM source that is commonly used *in vitro* culture studies [28,29].

2. Material and methods

2.1. Materials

Unless otherwise stated, all chemical reagents for this work were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) and were used as received.

2.2. Tissue procurement

Excised subcutaneous human adipose tissue was acquired with informed consent from female patients undergoing elective breast or abdominal reduction surgeries at the London Health Sciences Centre in London, ON, Canada (REB # 105426) ($N = 3$ tissue donors). Cadaveric human cancellous bone from the iliac crest or sternum was obtained from frozen, unfixed cadavers donated to the Body Bequeathal Programme in the Department of Anatomy and Cell Biology at Western University ($N = 3$). All samples were stored at −80 °C prior to decellularization processing. The donor information is summarized in Table 1.

2.3. Tissue decellularization and cryomilling

Human adipose tissue was decellularized as previously described [26]. Briefly, the tissue was subjected to a 5-day detergent-free decellularization protocol involving freeze-thaw cell lysis in hypotonic buffer, repeated polar solvent extraction of lipids, and enzymatic digestion with trypsin-EDTA, DNase, RNase, and lipase. Following decellularization, the DAT was frozen at −80 °C and lyophilized.

Human cancellous bone was surgically extracted from the iliac crest or sternum and submerged in freezing solution consisting of 10 mM Tris and 5 mM ethylenediaminetetraacetic acid (EDTA) supplemented with 1% antibiotic/antimycotic (Gibco®, ThermoFisher Scientific, Oakville, ON, Canada) and 0.014 mM phenylmethylsulfonyfloride (PMSF) at a concentration of 4 mL of solution per gram of tissue. The tissue was subjected to 3 freeze-thaw cycles from −80 °C to 37 °C, with replacement of the freezing solution between each cycle. Next, the tissue was incubated at room temperature in 0.5 M hydrochloric acid for 8 h under agitation at 300 RPM. The processed bone was then rinsed with absolute isopropanol supplemented with 1% antibiotic/antimycotic and 0.014 mM PMSF under agitation at 200 RPM and 37 °C for 24 h. Finally, the DCB was rinsed twice in phosphate buffered saline (PBS) for 15 min, followed by a single 15 min rinse in deionized water (DIH_2O), prior to freezing at −80 °C and lyophilization.

The lyophilized DAT, DCB, and commercially-sourced bovine Achilles tendon collagen (BTC) (Sigma-Aldrich) were cryomilled using previously described methods [30]. Briefly, the lyophilized tissue was finely minced with surgical scissors into 1–2 mm^3 fragments and transferred into a stainless steel milling chamber with two 10-mm stainless steel milling balls. The loaded chambers were submerged in liquid nitrogen for 3 min and then milled continuously using a Retsch® MM400 ball milling system for 3 min at 30 Hz. The process was repeated for a total of 3 cycles to yield a fine ECM powder.

2.4. Confirmation of cell extraction

The decellularization protocols for the DAT and DCB samples were validated through qualitative analysis by Masson's trichrome