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A targeted mass spectrometry method to screen collagen types I-V in the decellularized 3D extracellular matrix of the adult male rat thyroid

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ABSTRACT

Here we have developed and validated an original LC-MS/MS SRM procedure flexible enough to quantitatively screen collagen types I-V in copies of the same type of stromal matrix prepared with different protocols of cell removal to retain the native 3D architecture of the ECM. In a first step, identification of tryptic sequences exclusive to specific chains (either α1 or α2) of mammalian collagen standards types I-V was pursued using a combination of LC-LIT-Orbitrap XL and LC-MS/MS SRM analyses. In a second step, the adult male rat thyroid was decellularized using three different protocols specifically set for engineering of bioartificial 3D thyroid organoids. In a third step, DNA analysis of the decellularized 3D thyroid stroma was pursued to exclude contamination by cell nuclear debris. In a final step, collagen standards and 3D thyroid matrices were digested using the same mechanical / enzymatic protocol, and quantitative profiles of collagen types I-V ensued using comparisons of ionic intensities between tryptic peptides of collagen standards and matrices, as derived from targeted LC-MS/MS SRM analysis. Collectively, the procedure allowed for detection and quantitation of collagen types I-V at a femtomolar level in thyroid gland stromal matrices initially maintaining their original 3D architecture, tryptically digested through a method common to collagen standards and thyroid ECM, with satisfactory reproducibility of results, moderate procedural cost, and limited analytical time.

1. Introduction

Relative or absolute quantification of extracellular matrix (ECM) proteins including collagens in normal and neoplastic, rodent and human viscera (e.g. skin, tail tendon, aorta, heart, mammary gland, liver, thyroid) has been achieved associating liquid chromatography to mass spectrometry (LC-MS/MS), to yield variably sensitive, specific, and accurate results [1–4]. A mainstay of all these methods is an immediate and/or substantial disruption of the original three-dimensional (3D) structure of the organ-specific stroma that is rapidly extracted, and/or solubilised, and/or fractionated. In addition, although most recent LC-MS/MS techniques based on isotope labelling provide high analytical specificity and accuracy, they imply a quite high preparative cost, and a long procedural time [5].

Integrity of the 3D architecture of the ECM, collectively known as “stromal matrix” when including both interstitial and basement membrane ECM [6], is considered a key factor in tissue engineering for organ reconstruction based on re-cellularization with stem cells / progenitors [7–9]. Indeed, a de-cellularized organ-specific 3D stromal matrix may act as an ideal biocompatible scaffold for 3D organ reconstruction [10] including innovative engineered biostructures like 3D organoids and organs-on-chip, whose potential as laboratory tools for pharmacological / toxicological screening and diagnostic purposes is under active investigation [11,12]. Finally, 3D bio-artificial organs engineered on the laboratory bench (i.e. ex situ) promise to become a feasible therapeutic option in the clinical setting, where they may substitute malfunctioning native organs through transplantation, and thus provide a definitive solution to the paucity of organ donations [13].

Therefore, to have an LC-MS/MS procedure aimed at quantitatively screen the protein composition of the de-cellularized stromal matrix

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starting directly from the same type of 3D matrix preparations eventually used for re-cellularization protocols might be very useful. It would allow for comparison of the role of different amounts of specific stromal proteins with the capacity of the matrix to act as a 3D guide to induce *in vitro / ex situ* 3D self-assembly of cells. Indeed, changes in amounts of a specific collagen type within the same 3D environment has been shown to be critical for *in vitro* self-assembly of ovarian cells [14], and we have predicted through theoretical modelling that this phenomenon might play a critical role also for *ex situ* formation of thyroid follicles [9]. In addition, a similar LC-MS/MS approach could eventually be applied to a real-time quantitation of stromal collagen breakdown from 3D organs-on-chip during their medium and long-term maintenance in micronized circuitries [12]. This, particularly when using endocrine cells like those of the thyroid gland whose follicular stability is ensured by a complex 3D network of different collagens [8,15,16].

Finally, tissue engineering increasingly search for standardization of production chains, and for technical procedures with moderate cost and a limited analytical time [17]. An LC-MS/MS procedure simple to standardize, of contained analytical cost, and limited procedural time but retaining sensitivity, specificity, and accuracy enough to provide a reliable quantitation of the relevant ECM proteins would be of great help, primarily to set strategies for 3D bio-printing of copies of the organ-specific, stromal matrix using biomaterials functionalized with organ-specific collagens, as in the case of a bio-artificial thyroid gland [18,19].

To these purposes, we have applied a combination of mass spectrometry approaches (LC-LIT-Orbitrap XL and LC-MS/MS Selective Reaction Monitoring, SRM) to identify and quantitate the five most relevant types of collagen (I to V) in the stromal matrix of the adult, male rat thyroid gland [16]. The glands were de-cellularized using protocols specifically developed by our group for preparation of 3D bio-artificial thyroid organoids [8,20,21] and, thus initially retaining a thyroid-specific 3D architecture ideal for re-cellularization [9,15,17]. Only in final stages, the 3D stromal matrix was disrupted using standard solubilisation methods, and accurate quantitation of the five collagen types studied was achieved retaining an analytical sensitivity up to the femtomolar level.

2. Materials and methods

2.1. Animals

Seven adult Sprague Dawley male rats weighting 50–75 g (3 animals, 3–4 weeks old) and 225–250 g (4 animals, 8 weeks old) were purchased from Harlan Labs (Udine) and Charles River Labs (Lecco), respectively and housed in temperature-controlled rooms (22 °C) with a humidity of 50% and a 12:12 h light–dark cycle, according to the stabulary rules. All animal procedures were conducted in accordance with the current European Community Council Directives, and approved by the Ethical Committee for Animal Care of the University of Parma, Parma, Italy (approval code n. 18/2016-PR released on 12/01/2016).

2.2. Decellularization of the rat thyroid gland

All rats were deeply anesthetized with intraperitoneal tiletamine-zolazepam (40 mg kg^{-1}), and sacrificed by cervical dislocation. The thyroids were surgically removed and cleaned from the capsule and adjacent parathyroid glands, the two lobes of each gland separated at the level of the isthmus, each lobe put into plastic vials containing phosphate-buffered saline 0.1 M pH 7.4 (PBS), rapidly frozen in liquid nitrogen, and stored at −80 °C until use. Four lobes coming from the thyroids of two 225–250 g rats, and 6 lobes coming from the thyroids of three 50–75 g rats were de-cellularized using three different de-cellularization protocols as follows:

- protocol 1 (two lobes from one 225–250 g animal, 2 lobes from one 50–75 g animal): thawing of the thyroid lobes at 4 °C for 24 h, followed by 0.02% Trypsin EDTA 0.25% (Sigma Aldrich p.n. T4049) in PBS for 1 h at 37 °C, 3% Triton X-100 (Sigma Aldrich p.n. T8532) in PBS for 48 h at 4 °C, and 0.03% sodium deoxycholate (Sigma Aldrich p.n. 30970) in PBS for 24 h at 4 °C;
- protocol 2 (one lobe for one 225–250 g animal, 2 lobes from one 50–75 g animal): thawing of the thyroid lobes at 4 °C for 24 h, followed by 0.01% Trypsin EDTA 0.25% (Sigma Aldrich p.n. T4049) in PBS for 1 h at 37 °C, 3% Triton X-100 (Sigma Aldrich p.n. T8532) in PBS overnight (ON) at 4 °C, and 0.3% sodium deoxycholate (Sigma Aldrich p.n. 30970) in PBS ON for 24 h at 4 °C;
- protocol 3 (one lobe for one 225–250 g animal, 2 lobes from one 50–75 g animal): thawing of the thyroid lobes at 4 °C for 5 h, followed by 0.01% Trypsin EDTA 0.25% (Sigma Aldrich p.n. T4049) in PBS for 1 h at 37 °C, 0.1% Triton X-100 (Sigma Aldrich p.n. T8532) in PBS for 24 h at 4 °C, 2% sodium deoxycholate (Sigma Aldrich p.n. 30970) in PBS for 24 h at 4 °C, sodium chloride (NaCl) 1 M in PBS for 1 h at room temperature (RT), and 30 μg mL^{-1} DNAase (Sigma Aldrich p.n. D4263) + 1.3 mM $MgSO_4$ + 2 mM $CaCl_2$ in distilled H_2O (dH_2O) for 1 h at RT.

SEM has been used to investigate differences in the morphological architecture between the three decellularization protocols. After decellularization, 3 thyroid lobes coming from two 225–250 g rats have been fixed in glutaraldehyde 4% (in cacodylate sodium buffer 0,1 M) O/N at 4 °C, then dehydrated with several rinses of a growing percentage of ethanol, starting from 30% (v/v in dH_2O) to 100% v/v and subjected to Critical Point Drying (Balzers) with 100% ethanol / liquid CO_2 exchange system. Dried samples have been mounted on aluminium stubs and metallized (Balzers) to cover scaffolds with a 60 nm gold film. Samples have been observed with a Philips SEM501.

After each treatment, samples were washed at least 5–10 min two times with PBS. De-cellularized matrices were sterilized under an UV laminar flow hood using 0.1% peracetic acid in sterile PBS for 3 h at RT. Finally, samples were rinsed with sterile dH_2O two times for 5–10 min, and stored in 0.01 mg mL^{-1} gentamicin (Sigma Aldrich p.n. G1297) in PBS at 4 °C until analysis.

2.3. Analysis of the residual DNA content by CyQUANT® cell proliferation assay kit

In a first step, the wet weight of 3 intact thyroid lobes coming from two 225–250 rats and that of 4 de-cellularized thyroid lobes coming from the other two 225–250 g rats was determined using an analytical balance. Then, each lobe was digested in 1 mL of Lysis Solution (LS) at 56 °C ON. The LS was composed by 1 mg mL^{-1} proteinase K (Sigma Aldrich p. n. P2308) in 50 mM Tris (Sigma Aldrich p.n. A5456-3) pH 7.6 and a protease inhibitor cocktail made with 1 mM EDTA + 1 mM iodoacetamide + 10 μg/mL Pepstatin A (Sigma Aldrich p. n. P5318) diluted in DMSO (FLUKA p. n. 41640).

For DNA measurements, a calibration curve was prepared in a 96 multiwell plate using 200 μL serial dilutions (0, 50, 200, 600, 1000 ng mL^{-1}) of the bacteriophage λ DNA in CyQUANT dye solution. The latter was prepared by mixing the Working Buffer with the CyQUANT® GR dye 400X supplied by the kit. For analysis, 5 μL of each digested sample (control thyroid and de-cellularized matrices) were dissolved in 195 μL of CyQUANT dye solution, and three replicates were prepared for each sample. Analysis of the calibration curve and samples were performed using a Victor 3 V Multilabel Plate Readers (Perkin Elmer) set at the Fluorescein protocol (485/535 nm of wavelength – λ). The calibration curve gave an interpolation (y = 1923x + 77,356) with an R^2 = 0,9986. For each sample, the amount of DNA was normalized to the dilution factor and wet weight of the native tissue (intact thyroid and de-cellularized matrix), and expressed as ng DNA/mg wet tissue.