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Elasto-regenerative properties of polyphenols

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ABSTRACT

Abdominal aortic aneurysms (AAA) are progressive dilatations of infra-renal aorta causing structural weakening rendering the aorta prone to rupture. AAA can be potentially stabilized by inhibiting inflammatory enzymes such as matrix metalloproteinases (MMP); however, active regression of AAA is not possible without new elastic fiber regeneration. Here we report the elastogenic benefit of direct delivery of polyphenols such as pentagalloyl glucose (PGG), epigallocatechin gallate (EGCG), and catechin, to smooth muscle cells obtained either from healthy or from aneurysmal rat aorta. Addition of 10 μg/ml PGG and ECGC induce elastin synthesis, organization, and crosslinking while catechin does not. Our results indicate that polyphenols bind to monomeric tropoelastin and enhance coacervation, aid in crosslinking of elastin by increasing lysyl oxidase (LOX) synthesis, and by blocking MMP-2 activity. Thus, polyphenol treatments leads to increased mature elastin fibers synthesis without increasing the production of intracellular tropoelastin.

1. Introduction

Abdominal aortic aneurysms (AAA) is a fatal disease of the artery characterized by accelerated inflammation mediated loss of matrix proteins such as elastin and collagen leading to structural weakening and eventual rupture of the artery [1]. There are approximately 18,000 deaths each year due to aneurysms in the United States making it the 13th largest cause of death [2]. Screening and early detection with elective surgical intervention is an effective way to decrease mortality in abdominal aortic and iliac arteries (AAA), where rupture is a great threat to the patient's life. Whether open or endovascular treatment is chosen, the interventional procedure is not without complications, including mortality, thus the benefit has to outweigh the risk of surgical repair. Most importantly, none of these clinical interventions provide therapeutic relief in preventing or reversing the pathology.

As high as 90% of detected AAAs are small without indications for surgery, and the obvious and clinically relevant question is whether expansion of those small aneurysms can be prevented. AAA onset is associated with elastic lamina degradation by metalloproteinases (MMPs), which are derived from activated vascular cells and infiltrating inflammatory cells [3]. Once degraded, elastic lamina cannot be restored as adult cells have no ability to remodel elastic fibers [4]. To revert aneurysmal aorta to a healthy state, we must not only stop degradation; we also must reduce inflammatory enzyme activity and facilitate regeneration of elastic lamina.

In prior studies, we have demonstrated the ability of plant derived polyphenols such as pentagalloyl glucose (PGG) to bind to elastin and prevent it from elastolytic degradation [5]. We have also shown, in a rat model, that a single time application of pentagalloyl glucose (PGG) prevents AAA expansion and reduces aortic diameter [6]. That study showed a significant restoration of elastic lamina after PGG treatment. Thus, we wanted to explore the ability of polyphenols to not only prevent elastin degradation but increase elastin synthesis by vascular smooth muscle cells at the disease site. Here we tested ability of different polyphenols to bind to tropoelastin and to increase insoluble elastin production in healthy and aneurysmal vascular smooth muscle cells in vitro.

2. Materials and methods

2.1. In vitro coacervation and maturation of tropoelastin

The kinetics of tropoelastin coacervation and maturation were performed using UV–Vis plate reader (BioTek, Winooski, VT) equipped with temperature, stir controllers and kinetic measurement features. 1 mg of human recombinant tropoelastin (Advanced BioMatrix, Poway, CA) was dissolved in 100% glacial acetic acid (Fisher Scientific, MA). Polypeptides were diluted in

Abbreviations: AAA, abdominal aortic aaneurysm; MMP, matrix metallo-proteinase; PGG, penta-galloyl glucose; EGCG, epigallocatechin gallate; LOX, lysyl oxidase; RASMC, rat aortic smooth muscle cell; Anu-RASMC, aneurysmal rat aortic smooth muscle cell.

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coacervation buffer (50 mM Tris, pH 7.5) to either 25 μM for one set of experiments and 10 μM for another set of experiments. The temperature of coacervation was 37 °C. Samples were stirred at the rate of 1000 rpm and absorbance was measured at 440 nm every minute throughout the reaction time. 100 μl polyphenols at 10 μg/ml were added to 100 μl polypeptides (in ice) immediately before the absorbance measurement.

2.2. Cell culture

Primary rat aortic smooth muscle cells (RASMC) were freshly isolated. Briefly, freshly harvested abdominal aorta from healthy adult male Sprague Dawley rats were isolated and cleaned. Endothelium was scraped off and the adventitia was removed with scalpel blade. The medial layer was minced and digested in 125 U/ml Collagenase (Worthington, Biochemicals Lake-wood, NJ) and 3 U/mg elastase (Elastin products company, Owensville, MO) in Dulbecco's modified Eagle's Medium-F12 (Hyclone, Thermo Scientific, Rockford, IL) with 10% fetal bovine serum. The RASMCs leave aorta and zattach and grow in petri dishes. Routine characterization of RASMCs was performed by staining cells for α-smooth muscle actin (SMA), myosin heavy chain II (MHC), and smooth muscle-22α (SM22) expression.

RASMCs isolated from rat aorta with advanced abdominal aortic aneurysm were received from Dr. Ramamurthi at Cleveland Clinic, the experimental procedure for which has been described in detail earlier [7]. Briefly, the posterior lumbar aortic branches were ligated; infra-renal aorta was surgically exposed and injured via catheter mediated intra-luminal elastase perfusion. The aortic diameter was measured before aortotomy, after aortotomy and after harvest. The aneurysms were allowed to develop for 14 days before vascular smooth muscle cells were harvested with the same procedure as described above for normal aorta (denoted Anu-RASMCs).

Passage numbers 4–8 were used for all the experiments. Cells were cultured in 12 well plates (200,000/well) in Dulbecco's modified Eagle's-F12 medium containing 10% fetal bovine serum (HyClone Laboratories, Inc., Novato, CA), 100 units/ml penicillin and 100 units/ml streptomycin (Cellgro-Mediatech, Herndon, VA) in a humidifier incubator at 37 °C, with 5% CO_2. Media was replenished every 3 days.

2.3. Polyphenol treatment of cells

Healthy RASMCs / aneurysmal RASMCs (Anu-RASMC) were cultured in medium containing polyphenolic additives (10 μg/ml; $n = 3$/condition) for 14 days. Polyphenols were dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO) to prepare stock concentration of 10 mg/ml and filter sterilized using 0.2 μm membrane filters (Corning Incorporated, Corning, NY) prior to addition. Control groups received only vehicle (DMSO). Cell culture media was changed every 3 days and spent medium was collected at each media change, frozen at −20 °C and biochemically assayed for tropoelastin and lysyl oxidase. After 14 days, the cell layers and soluble proteins were collected and analyzed.

2.4. Protein isolation

Cell monolayers were washed twice in PBS and cells were isolated in a mammalian extraction buffer. To prepare the buffer, 1 tablet of protease inhibitor cocktail (Sigma, St. Louis, MO) was added to 10 ml of Solulyze-M mammalian extraction buffer (Genlantis, San Diego, CA). Cell layers were homogenized using PowerGen 125 homogenizer and centrifuged at 10,000 × g for 15 min. The supernatant was collected and assayed for different proteins of interest. Total soluble protein was quantified using Peirce BCA protein assay (Thermo Scientific, Rockford, IL).

2.5. Fastin assay for elastin

Total insoluble elastin deposited in the cell layers and soluble monomeric tropoelastin released in the media were quantified using Fastin assay (Accurate Scientific and Chemical Corporation, Westbury, NY). For each treatment group, tropoelastin was assayed individually after each media change to generate a trend curve, and also evaluated as cumulative tropoelastin released over 14 days. To quantify the mature elastin deposited within the cell layers, the cell pellet generated after the protein isolation procedure mentioned above, was lyophilized and digested in oxalic acid as per manufacturer instruction manual. Since fastin assay quantifies only soluble α-elastin, the dried insoluble pellet was subjected to 3 digestion cycles with 0.25 M oxalic acid (100 °C, 1 h in water bath) and the pooled digests were assayed in the exact same procedure as tropoelastin in media. The total α-elastin was normalized to the total soluble protein released by the cells which is assumed to be directly proportional to the total cell count.

2.6. Gelatin zymography

Active MMP-2 was analyzed in the cell lysates (intracellular soluble proteins) by gelatin zymography [8]. The total protein was quantified using BCA kit and 12 μg total protein was loaded per well alongside with pre-stained molecular weight standards (Precision Plus Protein Standard, Bio-Rad, Hercules, CA). All lanes were loaded in duplicates with equal amounts of protein in each well. After development, coommasie staining and de-staining, the gels were photographed and density of clear bands (MMP-2 at 68 kDa) was analyzed using ImageJ software and reported as relative density units.

In a separate set of experiments, proteins extracted from RASMC cell-cultures were loaded in gelatin gels, electrophoresed, and polyphenols were added to the development buffer. PGG, EGCG (250 μg) dissolved in DMSO, was added in 10 ml development buffer whereas control groups received volume matched DMSO. Following incubation, gels were stained, de-stained, and imaged.

2.7. Immunofluorescence for elastin, fibrillin-1

RASMCs/Anu-RASMCs were treated in similar conditions as mentioned earlier. After 14 days, the cell layers were washed twice with PBS and fixed in 4% formaldehyde for 15 min in room temperature followed by incubation with a 5% bovine serum albumin blocking serum. The primary antibody, rabbit anti-rat elastin antibody (United States Biological, Swampscott, MA) or a rabbit polyclonal anti-fibrillin I antibody (Abcam, Cambridge, MA) at 1:100 dilution was applied overnight at 4 °C. Alexafluor 488 chicken anti-rabbit IgG secondary antibody (Molecular Probes, Eugene, OR) was applied at a dilution of 8 μg/ml for 2 h at room temperature. Cell layers were mounted using aqueous mounting medium with anti-fading agents (Biomedia Corp., Foster city, CA). The samples were examined by fluorescent microscopy. Importantly, all samples were imaged under exactly similar conditions for impartial analyses.

2.8. Transmission electron microscopy

The ultrastructure of extracellular matrix was studied using transmission electron microscopy (TEM). After 14 days in culture, Anu-RASMCs were fixed with 2% Electron Microscopy grade glutaraldehyde for 1 h, rinsed in PBS, fixed in 1% aqueous osmium