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Pentagalloyl glucose increases elastin deposition, decreases reactive oxygen species and matrix metalloproteinase activity in pulmonary fibroblasts under inflammatory conditions

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ABSTRACT

Emphysema is characterized by degradation of lung alveoli that leads to poor airflow in lungs. Irreversible elastic fiber degradation by matrix metalloproteinases (MMPs) and reactive oxygen species (ROS) activity leads to loss of elasticity and drives the progression of this disease. We investigated if a polyphenol, pentagalloyl glucose (PGG) can increase elastin production in pulmonary fibroblasts. We also studied the effect of PGG treatment in reducing MMP activity and ROS levels in cells. We exposed rat pulmonary fibroblasts to two different types of inflammatory environments i.e., tumor necrosis factor-α (TNF-α) and cigarette smoke extract (CSE) to mimic the disease. Parameters like lysyl oxidase (LOX) and elastin gene expression, MMP-9 activity in the medium, lysyl oxidase (LOX) activity and ROS levels were studied to assess the effect of PGG on pulmonary fibroblasts. CSE inhibited lysyl oxidase (LOX) enzyme activity that resulted in a decreased elastin formation. Similarly, TNF-α treated cells showed less elastin in the cell layers. Both these agents caused increase in MMP activity and ROS levels in cells. However, when supplemented with PGG treatment along with these two inflammatory agents, we saw a significant increase in elastin deposition, reduction in both MMP activity and ROS levels. Thus PGG, which has anti-inflammatory, anti-oxidant properties coupled with its ability to aid in elastic fiber formation, can be a multifunctional drug to potentially arrest the progression of emphysema.

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Emphysema, a subset of chronic obstructive pulmonary disease (COPD) is characterized by chronic inflammation, oxidative stress, elastin damage, and progressive alveolar destruction [1]. Improvement in lung function is not possible even after smoking cessation [2]. Cigarette smoke insult triggers the inflammatory response in lungs and over a period of time recruitment of inflammatory cells causes excessive release of enzyme mediators leading to a disruption of ECM balance in the lungs. Cigarette smoke also activates cellular apoptosis and inhibits alveolar elastin repair, which makes the emphysema condition irreversible [3–5].

Elastic fibers provide elastic recoil to organs like the lungs. Loss of lung elasticity has been correlated to a loss of lung function in emphysema patients [6]. The inability of mature cells to regenerate elastin in large quantities has been attributed to the lack of reparative process [7]. Moreover, elastin degradation products (EDPs) act as chemo-attractants for monocytes and therefore EDPs further increase the inflammatory burden on lungs [8]. Inflammatory cells release matrix metalloproteinases (MMPs) that has been shown to cause protease-antiprotease imbalance in emphysematous lungs. Many varieties of MMPs have now been shown to overexpress in emphysema; however, MMP 12 and MMP 9 are more pronounced [9–12]. Stopping elastin degradation has received little attention as a potential therapy for treatment of emphysema.

Pentagalloyl glucose (PGG) is a derivative of tannic acid found in green tea, red wine, and nuts. We have previously shown from *in vitro* and *in vivo* experiments that PGG can preserve elastin, inhibit MMP activity, and help restore lost elastin in aneurysmal aorta of rats [13–15]. We hypothesize that PGG treatment, due to its anti-oxidant and anti-inflammatory properties combined with its affinity to bind to elastin, would stop the emphysema progression by inhibiting MMP activity and restoring elastin deposition [16,17]. As a preliminary step, we investigated the effects of PGG on rat

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pulmonary fibroblasts cell cultures for inhibition of matrix metalloproteinases (MMPs) and reactive oxygen species (ROS) activity, elastin gene and protein expression, and elastin deposition under inflammatory conditions mimicking emphysema.

1. Methods (expanded methods are provided as a supplement)

Preparation of cigarette smoke extract (CSE) Cigarette smoke extract was prepared using a custom designed instrument with slight modifications to the procedure described in literature [18].

1.1. Pulmonary fibroblast cell culture

Primary rat pulmonary fibroblasts (Cell Biologics Inc©, IL, USA) were grown in Dulbecco's Modified Eagle Medium (DMEM), (ScienCell™, CA, USA) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich®, St. Louis, MO), 1% fibroblast growth substrate (FGS) (ScienCell™, CA, USA), and 1% Penicillin-Streptomycin. Cells from passages 2–6 only were used for all experiments. The cells were allowed to grow for one day in normal growth medium before the start of the treatments.

To mimic inflammatory conditions *in vitro*, cells were treated with either tumor necrosis factor (TNF-α) (Peprotech Inc.®, Rocky Hill, NJ) or Cigarette smoke extract (CSE). Cells were divided into six groups depending on the combination of substances they were treated with. The groups were: DMEM only (DMEM group), PGG (10 μg/mL) (PGG group), tumor necrosis factor (TNF-α) (50 ng/mL) (TNF-α group), TNF-α (50 ng/mL) + PGG (10 μg/mL) (TNF-α +PGG group), CSE (5% final concentration) (CSE group), and CSE (5%) + PGG (10 μg/mL) (CSE + PGG group). Cells were grown under these conditions for up to 21 days, and the medium was replenished twice every week. The cell cultures were analyzed at days 7, 14 and 21 for cell viability, total protein, total elastin in matrix and medium, collagen in matrix and medium, MMP activity in the medium, and ROS activity in cells.

1.2. mRNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

Cells grown in the aforementioned conditions were lysed at weeks 1 and 2, and mRNA was extracted using RNeasy RNA extraction kit (Qiagen, Valencia, CA) as per manufacturer's protocol.

1.3. Quantitative PCR

The quantitative PCR (qPCR) was performed and analyzed using a Rotorgene qPCR machine (Qiagen, Valencia, CA) to measure the relative expression levels of Lysyl Oxidase (LOX), Lysyl Oxidase 1(LOXL1) and Elastin (ELN) genes with respect to Beta-2 Microglobulin (B2MG). Description and primer sequences are included in the supplementary material.

1.4. Lysyl oxidase activity

Lysyl oxidase activity in the medium was measured using Amplite fluorimetric lysyl oxidase activity assay kit (AAT Bioquest, Sunnyvale, CA) according to manufacturer's instructions.

1.5. Gelatin zymography and reverse zymography

Active MMP activity was analyzed in the medium collected from cell cultures using gelatin zymography as described previously [19].

1.6. FASTIN assay

Total insoluble elastin deposited in the cell layers and soluble tropoelastin in the media were quantified using the Fastin™ Elastin assay kit (Biocolor, UK) according to the manufacturer's protocol. Matrix elastin was normalized to total lysate protein released by the cells, which is assumed to be directly proportional to the total cell count, while the tropoelastin in medium was normalized to the total protein content in the medium.

1.7. Reactive oxygen species (ROS) analysis

ROS activity in pulmonary fibroblasts was analyzed under aforementioned growth conditions using CellRox® deep red reagent, as per the manufacturer's protocol.

2. Results

Initially we checked the cell numbers in different conditions using Picogreen dsDNA assay. Addition of TNF-α did not affect cell growth or viability (Supplementary Fig. 1A). However, cell numbers in CSE group remained significantly lower than DMEM group (Supplementary Fig. 1B). PGG at the used concentration (10 μg/mL) did not show any toxicity to the cells.

2.1. Gene expression and activity of lysyl oxidase (LOX)

Quantitative PCR analysis of mRNA showed that expression of LOX and LOXL1 genes was upregulated in all of the groups as compared to the DMEM control group at week 1. The TNF-α+PGG group showed 6-fold upregulation of LOX expression. By week 2, the LOX expression returned to control levels (Fig. 1A–B). Treating cells with TNF-α did not seem to affect LOX activity in the medium. PGG addition slightly increased LOX protein activity levels at week 1 and 2 under TNF-α inflammatory condition (Fig. 1C). CSE and CSE + PGG groups showed an increase in LOX and LOXL1 gene expression(~3–4 fold) during week 1. However, they returned to control levels by week 2 (Fig. 1D–E). Addition of CSE impeded LOX protein levels in the media despite increases seen in the mRNA level; however, addition of PGG to CSE restored LOX protein levels (Fig. 1F).

2.2. Gene expression and deposition of elastin protein

Addition of TNF-α did not affect ELN gene expression. Addition of PGG in presence of TNF-α enhanced ELN gene expression by ~6-fold at week 1. (Fig. 2A). The ELN gene levels were down by week 2. Elastin levels in the media was not altered by addition of TNF-α by week 2 but it dropped by week 3. (Fig. 2B). When matrix elastin was quantified, PGG addition showed increased deposition of insoluble matrix elastin at week 3; clearly suggesting that PGG binds to soluble elastin and coacervates it leading to the deposition of insoluble elastin. (Fig. 2C).

Expression of elastin gene in CSE and CSE + PGG groups showed a significant increase (~4–6 fold) after one week. These levels came back to normal (same as DMEM group) by week 2 (Fig. 2D). Elastin protein levels in the media; however, were significantly lower CSE and CSE + PGG groups as compared to DMEM group at both 2 and 3 weeks (Fig. 2E). When matrix elastin was quantified, we observed a significant increase in cross-linked elastin deposition in the CSE + PGG group, suggesting that PGG mitigates the effects of CSE and helps elastin deposition similar to what was seen in TNF-α groups (Fig. 2F).

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