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Impact of Pre-Existing Elastic Matrix on TGF- β 1 and HA Oligomer-Induced Regenerative Repair by Rat Aortic Smooth Muscle Cells

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Abstract

Regenerating elastic matrices lost to disease (e.g. in aneurysms) is vital to re-establishing vascular homeostasis but is challenged by poor elastogenicity of post-neonatal cells. We previously showed exogenous hyaluronan oligomers (HA-o) and TGF- β 1 to synergistically enhance tropo and matrix elastin deposition by healthy adult rat aortic SMCs (RASMCs). Towards treating aortic aneurysms (AAs), which exhibit cause- and site-specific heterogeneity in matrix content/structure and contain proteolytically-injured SMCs, we investigated impact of pre-existing elastic matrix degeneration on elastogenic induction of injured RASMCs. Elastin-rich RASMC layers at 21 days of culture, were treated with 0.15 U/ml (PPE15) and 0.75 U/ml (PPE75) of porcine pancreatic elastase to degrade the elastic matrix variably, or left uninjured (control). One set of cultures was harvested at 21d, before and after injury, to quantify viable cell count, matrix elastin loss. Other injured cell layers were cultured to 42d with or without factors (0.2 μ g/ml HA oligomers, 1 ng/ml TGF- β 1). We showed that (a) ability of cultures to self-repair and regenerate elastic matrices following proteolysis is limited when elastolysis is severe, (b) HA oligomers and TGF- β 1 elastogenically stimulate RASMCs in mildly-injured (i.e., PPE15) cultures to restore both elastic matrix amounts and elastic-fiber deposition to levels in healthy cultures, and (c) in severely injured (i.e., PPE75) cultures, the factors stimulate matrix elastin synthesis and crosslinking, though not to control levels. The outcomes underscore need to enhance elastogenic factor doses based on severity of elastin loss. This study will help customize therapies for elastin regeneration within AAs based on cause and location.

1. Introduction

Elastin is a major component of elastic fibers of the extracellular matrix (ECM) of vascular and other connective tissues, which provides the tissues elasticity and resilience. In addition, intact elastic fibers modulate cell behavior in maintaining vascular smooth muscle cells (SMCs) in a healthy, quiescent phenotype. Thus, accelerated elastic fiber breakdown and loss due to inflammation following disease, trauma, and congenital or genetic abnormalities, can severely impact vascular homeostasis, necessitating elastic matrix regeneration or repair as a priority. Despite the advent of tissue engineering technologies with their immense potential to regenerate tissues/organs, little progress has been made towards regenerating

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such elastic matrix structures (e.g. elastic fibers, sheets) due to the significant challenges imposed by the poor elastin regenerative capacity of post-neonatal cell types^{1, 2}.

In light of literature suggesting possible roles for glycosaminoglycans (GAGs), specifically hyaluronan (HA), in facilitating elastin synthesis, assembly, and maturation in vivo, during development and beyond^{3–10}, our lab has sought to understand their influence on vascular elastic matrix homeostasis under healthy and diseased conditions, and their potential utility as elastogenic factors for adult cells. Working with HA biomaterials incorporating chemically crosslinked native high molecular weight (>1 MDa) HA, and smaller, variably-sized HA fragments^{11–13}, our lab showed that these hydrogels encouraged cellular deposition of a fibrous elastin matrix by cells seeded thereupon. Accordingly, in follow-up studies, our lab explored size- and dose-specific effects of uncrosslinked HA on elastin synthesis. These studies specifically found HA 4mers to enhance synthesis of both tropo- (precursor) and matrix-elastin, to improve tropoelastin recruitment and crosslinking into a matrix, in part by enhancing production and activity of lysyl oxidase (LOX), an elastin crosslinking enzyme, to encourage elastic fiber assembly, and to stabilize the elastin matrix by inhibiting the elastin-laminin receptor (ELR) activity, while not stimulating cell proliferation^{14, 15}. In light of the modest elastogenic benefits of transforming growth factor- β 1 (TGF- β 1)^{14, 15}, our studies further investigated co-delivery of HA oligomers and TGF- β 1 to elastin matrix regeneration, and showed them to synergistically improve upon the effects of the individual factors and to further enhance matrix elastin yields. Beneficially, these factors also suppressed expression of active elastolytic MMPs 2-, and 9 down to levels exhibited by healthy cultured cells, and served to attenuate matrix mineralization^{16–19}. Though these results demonstrate the utility of HA oligomers and TGF- β 1 for tissue engineering elastic tissue constructs using healthy, patient-derived vascular cells, it is unknown if these factors will be similarly elastogenic in the context of regenerating elastin matrices in situ within elastin-compromised tissues (e.g., in vascular aneurysms). It is also not known as to how the severity of proteolytic elastic matrix degradation and hence quality/content of the pre-existing elastic matrix, would impact subsequent basal- and induced-cellular elastin regenerative outcomes. This is relevant since cell phenotype and remodeling of the ECM are influenced by the biochemical and biomechanical stimuli cells perceive from their microenvironments. In fact, numerous parameters such as the etiology of an aneurysm, size and location, proximity to site of injury, stage in development, and inflammatory cell and thrombus involvement can impact proteolytic activity and generate temporal- or location- specific variability in cell phenotype and matrix degradation properties within aneurysms^{20–22}. These individual parameters can be studied step-wise in a cell culture model of porcine pancreatic elastase (PPE)- injured aortal SMCs. This model has been previously shown to degrade intact elastic structures, and reduce elastin content in cell cultures to mimic the diseased state²³. Though simplistic, this cell culture model is advantageous because it isolates the effects of individual parameters (e.g., inflammatory cells, thrombus-derived cellular factors, etc.) on elastin degradation and cell phenotype, including their basal- and induced- elastic matrix synthesis responses.

2. Materials and Methods

2.1 Cell Isolation and Culture

Aortae were harvested from adult Sprague-Dawley rats. All animal protocols were approved by the *Institutional Animal Care and Use Committee* at Clemson University. Aortae were opened lengthwise and the intima scraped gently with a scalpel blade. The medial layer was dissected out from the underlying adventitia, and was then chopped into ~ 0.5 mm-long sections, rinsed with sterile phosphate buffered saline (PBS), pooled, then enzymatically degraded in DMEM-F12 medium (Thermo Scientific, Logan, UT) containing 357 U/mg collagenase (Worthington Biochemicals, Lakewood, NJ) and 4.5 U/mg elastase

(Worthington Biochemicals, Lakewood, NJ) for 45 min at 37 °C. The digestate was centrifuged at 400 g for 5 min, the tissue segments then reconstituted and cultured in 6-well plastic plates with minimal volumes of DMEM-F12 medium containing 10% v/v fetal bovine serum (PAA, Etobicoke, Ontario) over 4–6 weeks. Primary rat aortic SMCs (RASMCs), derived by outgrowth from these tissue explants, were cultured until confluence and passaged. Low passage (2–8) RASMCs were then seeded onto 6-well tissue culture plates (area = 10 cm²) at a density of 3×10^4 cells/well and cultured in DMEM-F12 medium containing 10% v/v FBS and 1% v/v Penstrep (Thermo Scientific, Logan, UT) for time periods designated in Table 1.

2.2 Experimental Design and Time Points

Table 1 summarizes the experimental cases, and corresponding conditions of culture and treatments. Briefly, RASMCs (Passage 2–8) were cultured within 6-well plates (3×10^4 cells/10 cm²) for 21 days, over which time the cells deposited a robust matrix. Select wells (n = 3/case) were then incubated for 45 min with 2 mL of porcine pancreatic elastase (PPE) (Worthington Biochemicals, Lakewood, NJ), prepared at doses of 0.15 U/ml (PPE15) and 0.75 U/ml (PPE75) respectively, to proteolytically degrade the elastin matrix to different degrees of severity. Cultures designated as injury controls (No PPE) instead received PPE-free medium. One set of cultures (n = 3/treatment) was harvested before, and after proteolytic injury to assess the effects of proteolytic injury on cell viability and elastin matrix amounts. Other cell layers (n = 3/case) were cultured for a further 21 days with or without factors (0.2 µg/ml of HA oligomers, 1 ng/ml of TGF-β1; see below) with medium changes performed twice weekly. In this study, the effects of these factors alone were not assessed due to our previous findings with healthy cells that the factors, delivered individually at the present doses, had limited effect, and were much more effective when delivered together¹⁶. Spent medium aliquots removed from each well, at the time of each medium change, were pooled and frozen, and biochemically assayed together with their corresponding cell layers when harvested 42 days post-seeding.

HA oligomer mixtures used in this study contained $\sim 75 \pm 15\%$ w/w of HA 4-mers (tetramers), with 6-mers and 8-mers forming the balance, and were prepared using our previously-described protocols based on digestion of HMW HA (>1 MDa; Genzyme Biosurgery, Cambridge, MA), with testicular hyaluronidase (20 mg hyaluronidase, 40 U enzyme/mg; Worthington Biochemicals, Lakewood, NJ) for 20 min at 37 °C¹⁴. HA oligomers and TGF-β (Peprotech, Rock Hill, NJ) dissolved in serum-free DMEM-F12 medium, were syringe-filtered and supplemented to DMEM-F12 culture medium containing 10% v/v FBS and 1% v/v Penstrep. Final serum levels were normalized to 10% for all culture groups, so the TGF-β content in the serum would not be a confounding factor. The presence of FBS was deemed necessary since these poorly elastogenic cell types, produce even less elastic matrix, at the threshold of detection, when cultured in the absence of FBS. A 5 mL-volume of medium was added to each well. As with the above cultures, spent medium from each of these wells was also changed twice weekly. The spent medium collected from each well was pooled with prior collected aliquots from the respective wells and stored frozen at –20°C until biochemical analysis was performed.

2.3 Immunofluorescence Detection of SMC Markers

Immunofluorescence techniques were used to confirm the isolated cells as SMCs. RASMCs were cultured in sterile, 2-well glass chamber slides (Nalge Nunc International, Naperville, IL) at a low cell density overnight. They were fixed with 4 % w/v paraformaldehyde for 10 minutes (4°C), and detected with polyclonal rabbit antibodies for SM22α and α smooth muscle actin (Abcam, Cambridge, UK), and visualized with FITC-conjugated goat anti-

rabbit IgG secondary antibodies (Chemicon, Temecula, CA). Cell nuclei were visualized with Vectashield hard-set mounting medium with DAPI (Vector Laboratories, Switzerland).

2.4 DNA Assay for Cell Proliferation

The DNA content of the RASMC cultures listed in Table 1 were compared to determine the impact of PPE and the elastogenic factors on cell viability and proliferation. Select cell layers were harvested at 21 days (before and after PPE injury) and 42 days of culture, sonicated, and their respective DNA contents quantified using a fluorometric assay described by Labarca and Paigen²⁴. Actual cell counts were then calculated assuming 6 pg of DNA/cell²⁴.

2.5 Immunofluorescence Detection of Elastic Matrix

Immunofluorescence techniques were used to visualize and confirm PPE-induced elastic matrix loss within treated cell layers. RASMCs were cultured in sterile, 2-well glass chamber slides (Nalge Nunc International, Naperville, IL) under identical experimental conditions as described for cultures meant for biochemical analysis, though the seeding density and treatment doses were adjusted proportionally to the substrate surface area and the cell number respectively. At 21 days post-seeding, when the cell layers were either treated with PPE75 or were untreated with PPE, they were fixed with 4 % w/v paraformaldehyde for 10 minutes (4°C), and elastin and fibrillin in the matrix then detected with polyclonal mouse antibodies (courtesy of Dr. Robert Mecham, St. Louis, Missouri), and visualized with FITC-conjugated donkey anti-mouse IgG secondary antibodies (Abcam, Cambridge, UK). Cell nuclei were visualized with the nuclear stain DRAQ5 (Biostatus Limited, Leicestershire, UK).

2.6 Fastin Assay for Elastin

A Fastin assay (Accurate Scientific and Chemical Corporation, Westbury, NY) was used to quantify the total amount of elastin deposited within cell layers (matrix elastin), and that released into the culture medium as a soluble precursor (tropoelastin). For each treatment group, tropoelastin in the spent medium was collected and pooled over the culture period and frozen at -20°C. The spent medium fractions collected from individual wells were pooled separately for the pre- and post-injury phases of cell culture. To isolate matrix elastin following either 21 days, or 42 days of culture, the cell layers were trypsinized, scraped, re-suspended in NaCl/Pi buffer, and centrifuged (2500 rpm, 10 min). The cell pellet was digested with 0.1 N NaOH (98°C, 1 h), and centrifuged to yield a less crosslinked, alkali-soluble supernatant fraction (soluble elastin), and a mature, highly crosslinked, insoluble pellet (insoluble elastin). Since the Fastin assay quantifies only soluble α -elastin, the insoluble elastin was first converted to a soluble form prior to quantification. To do this, the insoluble elastin pellet was dried, solubilized with 0.25 M oxalic acid (95°C, 1 h), and the pooled digests then centrifuge-filtered (3000 RPM, 10 min) in microcentrifuge tubes fitted with low molecular weight (10 kDa) cut-off membranes (Millipore, Bedford, MA). All three elastin fractions (tropoelastin and soluble and insoluble matrix elastin) were quantified using the Fastin assay. Between each of the cases, the ratios of matrix elastin (soluble + insoluble elastin) to total elastin (tropoelastin + matrix elastin), and the ratios of the soluble and insoluble fractions of matrix elastin were compared to gauge efficiency of crosslinking into a structural elastin matrix, and the extent of maturation of the matrix elastin respectively.

2.7 SEM Analysis of Elastic Matrix

Scanning electron microscopy (SEM) was used to visualize proteolytic degradation of elastin matrix by PPE and its subsequent regeneration, under basal and induced conditions. Spent medium was removed from atop cell layers after 21 or 42 days of culture. The cell

layers were rinsed with PBS, and fixed with 4% w/v paraformaldehyde in 0.1 M phosphate buffer (4°C, 15 min). After fixation, the cell layer was rinsed several times with 0.1M phosphate buffer, then submersed in 25% w/v KOH (60°C, 5 min) to remove cellular and ECM debris and yield relatively pure, matrix elastin structures. Following a phosphate buffer rinse, the isolated elastic structures were then treated with 1% w/v tannic acid (1 h) and stained with 1% w/v osmium tetroxide (1 h). The osmium tetroxide was used as an alternative to sputter coating in order to create a high electron emission without the need for a metal coating. The samples were dehydrated successively in graded ethanol (70–100% v/v 1 min each) and finally hexamethyldisilazane (Sigma Aldrich, St. Louis, MO) (3 min), under a fume hood. The samples were finally mounted on aluminum stubs and imaged using a Hitachi SEM TM1000 (Hitachi Technologies, Pleasanton, CA).

2.8 LOX Enzyme Activity Assay

Frozen aliquots of spent culture medium, pooled over the respective periods of culture (21 and 42 days) were assayed for the elastin crosslinking enzyme, lysyl oxidase (LOX), using a fluorometric assay (Amplex® Red Assay, Molecular Probes, Eugene, OR) based on measurement of H₂O₂ released when LOX oxidatively deaminates alkyl monoamines and diamines²⁵. The fluorescence intensities were measured at $\lambda_{\text{excitation}} = 560 \text{ nm}$ and $\lambda_{\text{emission}} = 590 \text{ nm}$.

2.9 Assay for Elastase Activity

Elastase activity following proteolytic injury of the cell cultures was assayed using an EnzChek® Elastase Assay kit (Molecular Probes, Eugene, OR). Aliquots (50 μL) of spent medium (collected and pooled over the 21 or 42 days, after injury or recovery) were mixed with 50 μL of diluted bovine neck ligament elastin, incubated for 30 min at 37°C, and the fluorescence intensity measured at $\lambda_{\text{excitation}} = 485 \text{ nm}$ and $\lambda_{\text{emission}} = 510 \text{ nm}$. One unit of elastase was defined as the amount of porcine pancreatic elastase required to solubilize 1 mg of elastin at pH 8.8 and 37 °C.

2.10 Gel Zymography for MMP-2, 9 Activity

Proteins from spent medium fractions collected from control and test cultures (at 21 days post-seeding, and between 21 and 42 days post-seeding with and without PPE-treatment at 21 days) and from cell layers themselves (before and after PPE-treatment at 21 days of culture) were extracted in a RIPA buffer, dialyzed, and analyzed by gelatin zymography using 4 μg of protein per lane (as per BCA assay) as described previously²⁶. Intensity of the MMP bands were quantified and expressed as relative density units (RDUs) normalized to protein content, prior to comparison between cases.

2.11 Statistical Analysis

Although non-parametric tests (e.g., Mann-Whitney Test) are less powerful than parametric tests based on Gaussian distribution, especially for smaller sample sizes, **they are** still recommended for analyzing biologic data since these data typically do not follow Gaussian (bell-shaped) distribution. Accordingly, we used non-parametric methods for statistical analysis in the current study. The Kruskal-Wallis test was initially used to broadly assess differences between cell layers with or without proteolytic degradation and cell layers with or without the exogenous supplementation of TGF- β and HA oligomers. Subsequently, more detailed, pair-wise analysis between specific groups was conducted using the Mann-Whitney (Rank-Sum) test. Assumptions were that the data was representative, observations were independent, and the matrix elastin distributions were identical except for a shift in location. Statistical significance was deemed for $p \leq 0.1$. This level of significance is different than the typical $\alpha = 0.05$, representing a 5% type I error rate, because the current sample size of n

= 3/case, selected in consideration of the large number of test cases, long-culture times, and high cost of analysis, is inadequate to demonstrate significance with greater than 90% confidence level. Further analysis on the most promising results from this study will be subsequently conducted at a higher confidence level (i.e., 95%).

3. Results

3.1 Experimental Design and Time Points

The cells harvested from rat aortae expressed SM22 α and α smooth muscle actin confirming their identity as SMCs (Figure 1A). At 21 days of culture prior to PPE-treatment, the cells were confluent and exhibited a spindle-like phenotype. The cell layers were elastolyzed with two different strengths of PPE (0.15 U/ml: PPE15 and 0.75 U/ml: PPE75; 45 min) to degrade the elastin matrix to different levels of severity. Following incubation with PPE, the majority of the cells in both sets of cultures remained viable and spindle-shaped, with a small number of rounded cells (Figures 1B and C).

3.2 DNA Assay for Cell Proliferation

A fluorometric DNA assay was used to quantify cell proliferation of each of the three replicate cell layers from each treatment group listed in Table 1. In the PPE-untreated group, cell layers cultured for 42 days (with and without factors) contained a slightly lower number of cells relative to 21 day cultures ($p = 0.001$ and 0.00004 for 42 day and 42 day + factors vs. 21 day cultures; Figure 2). There were no significant differences in cell proliferation between cell layers cultured for 42 days with HA oligomers and TGF- β factors and those that were not (p -value = 0.8). Additionally, there were no differences in cell proliferation between No PPE, PPE15, and PPE75-treated groups (Kruskal- Wallis, p -value = 0.4).

3.3 Immunofluorescence Detection of Elastic Matrix

Figure 1C illustrates the decreased elastin content found in cell layers treated with PPE75 as compared to controls. With cell numbers both before and after PPE treatment at 21 days of culture appeared constant (Figures 1B, C), the elastic matrix was significantly decreased after PPE75 treatment.

3.4 Elastin Production

Prior to PPE-treatment at 21 days of culture, all cell layers contained similar cell numbers (Figure 1B) and produced similar amounts of tropoelastin (Figure 3). Similarly, regardless of whether they were treated with PPE or not, cell layers also synthesized similar amounts of tropoelastin in the time period between 21 and 42 days of culture, leading to very similar totals when measured at 42 days (Figure 3). Culture of cell layers (with or without PPE injury) for a further 21 days in presence of HA-o and TGF- β , did not enhance tropoelastin synthesis relative to the corresponding cell layers cultured without any factors (Figure 3). Matrix elastin content was also identical in all cultures prior to PPE-treatment. Incubation with PPE (PPE15 and PPE75) at 21 days of culture was found to cause a significant reduction in matrix elastin content relative to untreated control cultures (Kruskal-Wallis, p -value = 0.002); matrix elastin content in cell layers treated with 0.15 U/ml of PPE (PPE15) was $74 \pm 9\%$ of that in non-injured cell layers, while that treated with 0.75 U/ml of PPE was only $31 \pm 13\%$ (Mann-Whitney, $p = 0.1$ vs. non-injured cell layers for both cases) (Figure 4).

Broad statistical comparison of matrix elastin amounts between 21 day cultures and 42 day cultures (culture with or without added factors, and either following PPE-treatment at 21 days or not), indicated significant differences (Kruskal-Wallis, $p = 0.04$). More specifically, in PPE-untreated cell layers, cumulative accumulation of elastin matrix over 42 days of

culture in the absence of added factors, was no different than that at 21 days, though in the presence of TGF- β and HA oligomer factors, matrix elastin content increased by a further 23% ($p = 0.1$ for 42 day + factors vs. 21 day, no PPE group). Culture of PPE-treated cell layers for a further 21 days without any supplemented factors failed to enhance elastin matrix amounts (Figure 4). However, when instead cultured with TGF- β and HA factors, elastic matrix amounts in PPE15 and PPE75-injured cell layers was increased by $58 \pm 20\%$ and $100 \pm 0\%$, respectively. In the PPE15 group, induction of injured cells with the supplemented factors enhanced cumulative matrix elastin amounts to levels beyond that measured in 21 day-old cell layers prior to PPE-treatment, just as was observed for the no PPE-treatment group (p -value = 0.1 vs. no PPE treatment, 21 day cultures in both cases) (Figure 4).

In the PPE-untreated cultures, at 21 days, approximately 88% of the total elastin in the cell layer was crosslinked into an alkali-insoluble form (Table 2). This proportion was maintained over longer-term culture, both in presence and absence of TGF- β and HA factors. In cell layers that were only moderately proteolyzed (PPE15), the fraction of matrix elastin in the alkali-insoluble form was initially no different than in PPE-untreated cell layers, though the value gradually decreased to approximately 71% following 21 days of post-injury culture in the absence of any stimulating factors (Table 2). Differently, however, in those PPE-treated cell layers that were cultured with factors, the fraction of matrix elastin in the alkali-insoluble form, as analyzed at 42 days of total culture, was again similar to that measured in 21-day-old, PPE-untreated cultures. In cell layers subject to more severe proteolysis (PPE 75), such injury to elastic matrix caused an immediate reduction in to the absolute content of fractional alkali-insoluble elastin. Regardless of further culture with HA and TGF- β 1 factors, the maturation levels of the elastic matrix as exhibited within cell layers prior to PPE treatment, was not reinstated.

3.5 SEM Analysis of Elastic Matrix

Figure 1C shows representative scanning electron micrographs of elastic matrices isolated from 42 day cultures of RASMCs prior to and following proteolytic degradation with PPE75. These images show that elastic matrix structures are severely degraded upon treatment with PPE. Figure 5A shows elastic fibers deposited by RASMCs over 21 days of post PPE-injury culture in the absence of HA oligomer and TGF- β 1 factors; elastic matrices in cell layers that were not PPE-injured at 21 days (A, B) contained more crosslinked, elastic matrix structures than cell layers treated with PPE15 (C, D) or PPE75 (E, F). Figure 5B shows scanning electron micrographs of elastic fibers deposited by RASMCs over 21 days of post PPE-injury culture (C, D for PPE15 and E, F for PPE75), in the presence of HA oligomer and TGF- β 1 factors. A comparison of panels in Figures 5A and 5B indicates that culture of RASMCs with HA oligomer and TGF- β 1 factors, following injury with PPE15 (panels C, D) and PPE75 (panels (E, F) enhanced cellular deposition of crosslinked elastic fibers relative to culture without the factors.

3.6 LOX Enzyme Activity

There was no difference in LOX activities between the different treatment groups. On average, the LOX activity was $(1 \pm 0.24) \times 10^{-4}$ U/ml.

3.7 Elastolytic Activity

As shown in Figure 6A, there was no difference in endogenous elastase activity in cultures representative of each of the tested groups. Gel zymography (Figure 6B) also indicated that the activities of MMPs 2, 9 measured in spent medium fractions collected from 21 day-old cell layers, before and after PPE-treatment were similar. Further, activities of MMPs 2, and 9 measured in the pooled spent medium aliquots collected from PPE-treated cell layers over

21 days of culture in the presence and absence of elastogenic factors, were no different from that measured immediately following PPE-treatment. No error bars are present on Figure 6B because the data was collected from a single gel with $n = 1/\text{condition}$. This gel served as a secondary confirmation of the data collected with the elastase assay, therefore was not completed in triplicate.

4. Discussion

A major challenge in treating aortic aneurysms (AAs) is the inherently poor elastin regenerative potential of adult cells^{1, 2} that deters their ability to repair or replace degraded elastic matrix structures and thereby regress the pathological condition. Our prior work has accordingly focused on identifying growth factors that can be therapeutically delivered to the diseased cells within AAs to stimulate cellular elastin production in situ. In a major step towards being able to stimulate elastin regeneration, we showed that hyaluronan (HA) oligomers and transforming growth factor- β 1 (TGF- β) synergistically induce healthy rat aortic smooth muscle cells (RASMCs) to enhance elastin precursor and matrix synthesis, enhance elastic fiber formation, and increase cellular synthesis of the elastic crosslinking enzyme, lysyl oxidase (LOX), to enhance elastic matrix stability. Despite these positive outcomes, therapeutic utility of these factors is contingent upon demonstrating that injured/diseased SMCs can be elastogenically up-regulated. This is especially relevant because cell phenotype and behavior are critically determined by their matrix microenvironments which differ significantly between diseased and healthy vessels. A gradient of proteolytic enzymes and matrix proteins has been shown to exist within AAs as a function of distance from the rupture site^{20–22}. In such cases, vascular SMC behavior can be modulated by their binding to the ECM via specific integrin receptors; the impacted behavioral patterns can include their own synthesis and organization of matrix proteins, responsiveness to growth factors and cytokines, altered Ca^{2+} ion uptake to possibly promote matrix calcification²⁷, and importantly the type and extent of matrix (e.g., collagen, elastin)-degrading enzymatic activity they exhibit. Thus, establishing the utility of elastogenic protocols, and the need for their customization is contingent on understanding of inter-relationships between extracellular matrix content/quality, and cell phenotype, and hence the nature of basal and induced elastogenic responses. This study seeks to explore these aspects.

Established aneurysmal tissues represent disease outcomes and thus do not allow us to distinguish between disease causes and secondary degenerative changes, and to isolate the standalone impact of individual factors that influence inherent or induced matrix repair and/or regeneration. Small animal models of AAs also rely on artificial hemodynamic manipulations that influence cellular behavior²⁸. Such in vivo models also render it difficult to distinguish the effects of matrix content and quality/extent of degradation on one hand, and matrix architecture, and the incidence and extent of matrix debridement by inflammatory cells recruited to the site of tissue/matrix injury on subsequent elastic matrix regeneration or repair. In vitro cell culture models, such as one previously described by Thompson et al.²⁹, may be usefully adopted to circumvent these problems. Such models have been previously studied to understand progression of elastic fiber injury by elastases and inherent cellular mechanisms for repair of degraded elastic fibers. They have never been adapted to the study of elastin regenerative processes, either inherent or induced, which we propose here. In mimicking the progressive changes in existing ECM and assessing the subsequent matrix repair and regeneration, such models, though simplistic, are superior to ones based on culture of aneurysmal cells, which represent a terminal outcome of a disease.

In the present study, we have sought to specifically investigate the impact of elastic matrix degeneration, independent of leukocyte debridement, on its subsequent repair possible via regeneration of new elastin, and if/how this phenomenon may be stimulated by exposure to

growth factors we have deemed 'elastogenic' based on prior study of effects on healthy SMCs. While it may exhibit minimal, non-specific proteolytic activity, PPE was selected for this study, since it specifically targets and rapidly degrades elastic matrix structures, unlike post-inflammatory cell-mediated matrix degradation that gradually, and simultaneously degrades elastin, collagen, and other ECM structures due to the collective action of multiple matrix metalloproteases (MMPs) exhibiting diverse substrate specificities.

Mature RASMC layers were incubated with two different concentrations of PPE. The lack of change in cell counts before and after enzyme treatment attested to the lack of any immediate deleterious effects on the SMCs; yet, DNA analysis at 21 days post-PPE-treatment (42 days post-seeding) showed the cell numbers to have nearly halved. One possibility is that the PPE-treatment had no short-term (i.e., 45 minutes) effect on the cells, yet triggered degenerative effects that compromised their viability in long-term culture. However, the occurrence of a similar decrease in cell number in control cultures that were not treated with PPE, eliminated this hypothesis. Rather, a likely explanation for the decrease in cell number is competition of cells for life-sustaining resources, as the already-confluent cell layers at 21 days, further proliferated to form multi-layers with the cells in the interior possibly succumbing to limitations to diffusional supply of essential nutrients/gases.

Identical tropoelastin amounts synthesized in all (test and control) cell layers prior to PPE-treatment suggested deposition of identical elastin matrix amounts as well, although this was only measured in one set of cell layers ($n = 3$) that was harvested at 21 days of culture, and prior to PPE-treatment. With this assumption as a basis, we deemed PPE-treatment to induce an enzyme dose-dependent decrease in elastic matrix amounts, particularly of insoluble matrix elastin relative to non-treated cultures, as confirmed via biochemical assays and electron microscopy (Figures 1, 4, and 5). Elastase activity and gel zymography analysis for MMP activities performed on medium aliquots drawn from the PPE-treated cultures (both doses) following 1 hr of recovery after PPE treatment and after 21 days (i.e., 42 days post-seeding) did not indicate any increase in values beyond that measured in non-treated cultures (Figure 6). This suggests that PPE-treatment likely does not result in sustained injury to cells, beyond the actual 45 minute period of incubation of the PPE with the cell layers. These results lend credence to our above explanation that decreased cell number in long-term culture post-PPE treatment is not due to any sustained effects of the treatment, but rather due to nutritional limitations. However, the lack of any measured increase in elastase activities in the culture medium removed from cell layers as early as 1 hr after PPE-treatment, relative to control cultures, was unexpected. To assess this further, an independent set of experiments was performed, wherein confluent RASMC cultures were treated with the higher dose of PPE (PPE75), and following 1 hour of recovery, the cellular, rather than the medium fraction was analyzed using gel zymography. These results (not shown) again indicated no differences in activities of the MMPs-2, and 9 between PPE-treated and – untreated cell layers. Collectively, these results suggest that PPE-treatment at the present doses/duration is effective from the standpoint of inflicting elastic matrix damage, but does not significantly activate cells, to be able to quantify temporal increases in activities of elastolytic MMPs.

Although tropoelastin production by PPE-untreated (control) cell layers between 21 and 42 days of culture was less than that in the first 21 days of culture, the lack of elastic matrix accumulation during the same period could be possibly attributed to either (a) a decrease in LOX-mediated crosslinking activity or of LOX protein itself, (b) sustained elastolysis due to PPE, MMP release by cells, or (c) spatial limitations for matrix deposition, imposed by high cellularity and already dense matrix in cell layers at 42 days of culture. Since elastase and LOX activity assays showed no change in cell layers at 42 days of culture relative to cell layers at 21 days of culture, the lack of any significant accumulations of elastic matrix

between 21 and 42 days of culture may be purely due to a contact inhibition of the SMCs within the super-confluent and dense cell layers. Although, we did not specifically quantify other mature elastic fiber proteins such as fibrillin and microfibril-associated glycoprotein (MAGP) it is quite possible that cellular synthesis of these and other proteins critical to elastic fiber formation could have also been inhibited when cell layers attained super-confluence. This will be elucidated in a follow-up study.

In light of the multi-fold increases in tropo- and matrix elastin production that we previously showed TGF- β and HA oligomers to induce in healthy cells¹⁶, here, 21 day culture of PPE-untreated (control) cultures with these very factors only modestly improved matrix elastin production (23%), and had no effect on tropoelastin synthesis. We believe that this muted effect is due to our high cell count, due to which factor doses on a per cell basis were rendered very low. Although the cell seeding densities in both our earlier and current studies was almost identical ($3 \times 10^4/10 \text{ cm}^2$), in this study, the factors were not provided to the cell layers immediately, but only after 21 days of culture, and by which time the cell counts had exponentially increased. Thus, effectively, the TGF- β and HA oligomer doses on a per cell basis were orders of magnitude lower than that provided in the prior study. It is thus likely that providing greater doses of HA oligomers and TGF- β on a per cell basis, to be equivalent to that provided in our prior study (0.2 $\mu\text{g/mL}$ per 40,000 cells and 1 ng/mL per 40,000 cells; 5 mL per well) would much more dramatically enhance tropo- and matrix elastin production in the control cell layers in this study also.

Our biochemical analysis clearly demonstrated that in absence of any provided factors, proteolytically-injured cell layers were incapable of regenerating the lost elastin matrix, though they continued to produce tropoelastin. As SEM analysis indicated, PPE-treatment resulted in etching of elastic fibers (see degenerated fibers vs. intact fibers in Figure 1C), their fragmentation and sometimes complete degeneration, particularly at the higher PPE dose. Prior studies have emphasized the importance of intact elastic fibers to the process of tropoelastin nucleation, crosslinking, and fiber formation³⁰, and the importance of glycosaminoglycans such as HA in facilitating the process of tropoelastin coacervation^{3–10}. Thus the reduced presence of intact fibers in PPE-treated cell layers, could likely have deterred further elastin matrix deposition. Perhaps for the same reason, we observed elastic matrix accumulation in PPE-treated cell layers cultured with HA oligomers and TGF- β factors. Likely, the provided HA oligomers interacted with cell surface receptors (Toll like Receptor 4 (TLR-4) for HA 4mers and CD44 for larger oligomers) to localize at the cell layer and further coacervate tropoelastin precursors through opposite charge interactions and facilitate their lateral aggregation and crosslinking with lysyl oxidase, to form mature fiber structures. Though likely muted in its effects, due to the low HA oligomer dose/cell ($\sim 0.2 \mu\text{g}/2 \times 10^6$ cells), it is clear that HA oligomers do benefit elastin matrix, specifically fiber formation (compare Figures 5A and 5B), in a manner that mimics our prior observations, though those outcomes were more significant. As we noted in a prior publication¹⁶, TGF- β likely interacts with cells independently via TGF- β receptors to elicit enhanced elastin synthesis, through changes to intracellular signaling pathways that, due to synergy between them and HA oligomers, likely show some degree of commonality. TGF- β may also enhance LOX production/activity to benefit tropoelastin crosslinking into a matrix. The lack of any perceivable increase in LOX activities in cell layers, post culture with TGF- β , and the absence of any significant increase in tropoelastin production over non-additive cultures may be again due to the low provided dose on a per cell basis.

Our results indicate that the provided elastogenic factors induce greater percent increase in elastic matrix synthesis within cell layers that are subject to more severe proteolytic injury. This could be due to a less dense matrix in the more severely proteolyzed cultures, which permits greater access of the elastogenic factors to the cells in the interior of the cell multi-

layer. Although cell counts immediately after PPE-treatment were similar in both PPE15 and PPE75-treated cell layers, cell numbers after an additional 21 days of culture ended up slightly lower in the latter case. It is thus possible that the somewhat lower cell numbers in PPE75-treated cell layers resulted in higher effective factor doses on a per cell basis, and accounted for the greater percent increase in matrix elastin relative to PPE15-treated cultures. Nevertheless, the provided dose of elastogenic factors was deemed insufficient to restore elastic matrix amounts to pre-injury levels within the more severely proteolyzed cell layers. These results suggest that the greater the severity of elastic matrix injury, the greater the dose of elastogenic factors that must be provided to induce complete recovery of lost elastic matrix. Fractional content of alkali-insoluble elastin in the matrix showed immediate and substantial (PPE75), or gradual and mild (PPE15) decreases from that in PPE-untreated (control) cell layers. In the absence of any added growth factors, there was no recovery after even 21 days of culture. As mentioned before, likely, more severe the PPE-injury, the greater the deterioration and removal of existing elastic matrix structure, and more afflicted the subsequent process of tropoelastin coacervation and crosslinking. The fact that the HA oligomers and TGF- β factors are able to restore fractional content of insoluble matrix elastin in PPE15 but not PPE75-treated cultures clearly demonstrates the vital involvement of the HA and TGF- β in the tropoelastin crosslinking process, and yet inadequacy of these factors (at the current doses) in overcoming the effects of severe elastic matrix injury. The study thus illustrates the importance of customizing factor doses to the severity of elastic matrix deterioration.

5. Conclusions

Our study suggests that ability of cell layers to self-repair and regenerate elastic matrices following their proteolytic damage is limited, particularly when elastic matrix injury is severe. Our results also provide evidence that HA oligomers and TGF- β factors together can elastogenically stimulate RASMCs in elastin-degraded cultures to restore both elastic matrix amounts and elastic fiber deposition to levels of accumulation observed in healthy cultures. However, doses of these elastogenic factors must be enhanced and optimized based on the severity of elastic matrix damage. These studies suggest that with early clinical diagnosis of aortic aneurysms and therefore moderate elastic matrix degeneration in the vessel wall, delivery of these elastogenic factors may be an appropriate therapeutic option.

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FIG 1.

Immunofluorescence (IF) of SMC markers (SM22 α and α smooth muscle actin) was used to confirm isolated cells from rat aortae as SMCs (**A**). A count of these RASMC was conducted after 21 days of culture following variable PPE injury (**B**) and the elastic matrix degradation after treatment with PPE was validated with IF and scanning electron microscopy (SEM) (**C**). Representative 21day RASMC cultures with no PPE injury and severe PPE injury (PPE 75) are shown. Scale Bars: (**A**) 150 μ m (**B**) 150 μ m for IF; 30 μ m for SEM.

FIG 2.

Cell proliferation ratios of RASMC culture groups as described in Table 1. Data shown represent mean $\pm$ SD of cell count determined by DNA content of cell layers after 21 and 42 days of culture, normalized to 21 day control cultures that received no PPE treatment or elastogenic additives (n = 3/case). * indicates significance ($p \leq 0.05$).

FIG 3.

Tropoelastin measured from each individual sample outlined in Table 1 are shown. The mean and standard deviation are compared for each case (n = 3/case). There are no statistically significant differences.

FIG 4.

Matrix elastin data measured from each sample outlined in Table 1 are shown (A). The mean and standard deviation are compared for each case ($n = 3/\text{case}$). * indicates significance ($p \leq 0.1$). Representative scanning electron micrographs of RASM culture following PPE15 and PPE75 injury and supplementation with factors are shown (B). Comparison of SEM micrographs shown, with that corresponding to 21 day old PPE-digested cultures shown in Figure 1C confirms that addition of HA oligomers and TGF- β to PPE-treated cultures positively induces elastic matrix deposition.

FIG 5.

For SEM, the cell layers were incubated with KOH to digest away non-elastin ECM and cellular components. **(A)** Scanning electron micrographs of elastic fibers deposited by RASMCs over 21 days following proteolytic injury in the absence of elastogenic factors (42 days of culture, overall). Few highly-crosslinked elastic fibers remained after KOH digestion in PPE-injured cultures. **(B)** Micrographs of elastic fibers deposited by RASMCs over 21 days following proteolytic injury (42 days of culture, overall), in the presence of HA oligomer and TGF- β supplements. Relative to cell layers that did not receive the HA oligomers and TGF- β supplements during post-injury culture (see images in Figure 5A), these supplements enhanced cellular deposition of mature, crosslinked fibers that could survive KOH digestion. Black arrows and white arrows indicate elastic fibers and undigested cell debris, respectively. Magnification: 1000 X (A, C, E), 2500 X (B, D, F). Scale Bars: 100 μ m (A, C, E) and 30 μ m (B, D, F).

FIG 6. Endogenous elastolytic activity was measured using an elastase activity assay ($n = 3/\text{condition}$) (**A**) and gel zymography ($n = 1/\text{condition}$) (**B**) for all treatment groups.

Table 1

Experimental groups with corresponding treatments and conditions of culture. RASMCs were cultured within 6-well plates for 21 days, over which time the cells deposited a mature elastic matrix. Select wells (n = 3/case) were then incubated for 45 min with 2 mL of PPE, prepared at doses of 0.15 U/ml (PPE15) and 0.75 U/ml (PPE75) respectively, to proteolytically degrade the elastin matrix to different degrees of severity. Cultures designated as injury controls (No PPE) instead received PPE-free medium. One set of cultures (n = 3/treatment) was harvested before, and after proteolytic injury to assess the effects of proteolytic injury on cell viability and elastin matrix amounts. Other cell layers (n = 3/case) were cultured for a further 21 days with or without factors (0.2 µg/ml of HA oligomers, 1 ng/ml of TGF-β) with medium changes performed twice weekly.

Group Name	Culture Period	Enzymatic Treatment	Media Supplements
No PPE_21 day	21 day	-	-
No PPE_42 day	21 day	-	-
No PPE_42 day_+factors	21 day	-	HA oligomers & TGF-β1
PPE15_21day	21 day	0.15 U/ml PPE	-
PPE75_21day	21 day	0.75 U/ml PPE	-
PPE15_42 day	21 day + 21day	0.15 U/ml PPE	-
PPE75_42 day	21 day + 21day	0.75 U/ml PPE	-
PPE15_42 day_+factors	21 day + 21day	0.15 U/ml PPE	HA oligomers & TGF-β1
PPE75_42 day_+factors	21 day + 21day	0.75 U/ml PPE	HA oligomers & TGF-β1

Table 2

Basal and induced elastic matrix synthesis. Shown are cumulative, mean amounts of alkali-soluble and insoluble matrix elastin per well (n = 3/group), quantified in RASMC cultures either prior to or following PPE-treatment at 21 days post-seeding, and following culture of these cell layers for 21 more days (i.e., 42 days post-seeding), in the presence or absence of TGF- β (1 ng/ml) and HA oligomers (0.2 μ g/ml) factors. Yield of alkali-insoluble matrix elastin, i.e., insoluble elastin/total matrix elastin is indicative of the efficiency of crosslinking and hence, matrix maturation.

		Total Matrix Elastin (Ave μ g/sample)			
		Insoluble Elastin	Soluble Elastin	Total Matrix Elastin	Insol/Total Matrix
No PPE	21d	230.3	32.4	262.7	0.88
	42d	200.9	35.1	236.0	0.85
	42d + factors	285.4	38.4	323.8	0.88
PPE15	21d	167.9	26.3	194.2	0.86
	42d	79.9	33.2	113.1	0.71
	42d + factors	259.1	49.3	308.4	0.84
PPE 75	21d	60.6	21.6	82.2	0.74
	42d	69.1	27.5	96.6	0.72
	42d + factors	126.5	37.7	164.2	0.77