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Utility of Hyaluronan Oligomers and Transforming Growth Factor-Beta1 Factors for Elastic Matrix Regeneration by Aneurysmal Rat Aortic Smooth Muscle Cells

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The progression of aortic aneurysms (AAs) is typically associated with an activated smooth muscle cell (SMC) phenotype, diminished density of mature medial elastic fibers, and an elevated presence of matrix-degrading enzymes, which ultimately leads to vessel rupture. Currently, no surgical or nonsurgical methods are available to regress aneurysms via regeneration of new elastic matrices, particularly because of inherently poor elastin synthesis by adult vascular cells and absence of tools to stimulate the same. We seek to address this void in this study. We recently showed 0.2 $\mu\text{g}/\text{mL}$ of hyaluronan oligomers and 1 ng/mL of transforming growth factor- $\beta 1$ (termed elastogenic factors) to dramatically enhance elastin synthesis and matrix formation by healthy aortic SMCs. In this study, the effect of these factors, alone or together, on suppressing procalcific and elastolytic activities of aneurysmal vascular cells, and improving their elastin matrix synthesis and assembly is examined. Periadventitial injury with calcium chloride was used to induce AAs in rats, and $\sim 45\%$ increase in aortic diameter was observed after 4 weeks. Aneurysmal SMCs isolated from these AA segments produced higher levels of inflammatory markers matrix metalloproteinases-2 and 9 elastase activity and calcific deposits, while synthesizing significantly less collagen, tropoelastin, and matrix elastin proteins over a 3-week culture period, relative to healthy SMCs. While hyaluronan oligomers alone significantly suppressed aneurysmal cell proliferation and promoted 20–50% increases in collagen and elastin synthesis ($p < 0.01$), transforming growth factor- $\beta 1$ alone had no effect on cellular proliferation and elastin synthesis. However, provision of factors together resulted in significantly higher amounts of collagen/elastin protein synthesis and crosslinking, by upregulating lysyl oxidase and desmosine. Compared to their individual contributions, the factors together were highly effective in minimizing the release of inflammatory enzymes, and encouraging elastic fiber formation. Since elastic matrix amounts were one order of magnitude lower than that observed with healthy cells, even upon elastogenic stimulation at doses optimized previously for healthy cells, increased doses are likely required and must be reoptimized for diseased cells. Despite this, the results point to the potential utility of these elastogenic factors in regenerating elastic matrices within AAs.

Introduction

AORTIC ANEURYSMS (AAs) are pathological conditions wherein segments of elastic arteries dilate and structurally weaken to fatally rupture.¹ AAs are also frequent outcomes in inherited conditions such as Marfan syndrome, characterized by defects in assembly and stabilization of arterial elastin,² and conditions wherein inflammatory cells (e.g., macrophages) infiltrate in response to calcified lipid deposits within the abdominal aortic wall.³ In the United States alone, more than 70,000 surgeries are performed annually to treat AAs,³ and despite this, nearly 16,000 people

die every year due to this condition.⁴ Elastin, being a major component of the extracellular matrix (ECM) in vascular connective tissues, is severely degraded to form soluble peptides,⁵ which further promote macrophage-mediated matrix destruction via secretion of cytokines, chemokines, interleukins, and proteinases.^{6,7} The pathogenesis of abdominal aortic aneurysms (AAAs) could also arise from enzymatic degradation of healthy elastic fibers and excessive accumulation of proteoglycans,⁸ leading to loss of elasticity and strength of the aortic wall, and progressive dilation to form a rupture-prone sac of weakened tissue.¹ Thus, absence or breakdown of elastin critically regulates aneurysm formation,

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progression, and fatal rupture, while its restoration in these segments will likely stabilize and restore homeostasis.

Current AA treatment methods are primarily surgical, and include open aneurysm repair, wherein the weakened aortic segment is replaced with a sutured synthetic mesh graft, and endovascular abdominal AAs repair, wherein a woven polyester graft mounted on a self-expanding stent is deployed within the aneurysm site.⁹ Despite their relative merits and demerits, long-term cardiac/pulmonary complications and other surgical and graft-associated complications cause survival rates to drop drastically within 10 years postsurgery.¹⁰ Other prominent nonsurgical approaches to inhibit inflammation and elastin matrix degradation within aneurysms include pharmacologically inhibiting release and activity of proteolytic matrix metalloproteinases (MMPs)¹¹ by tissue inhibitors of matrix metalloproteinases (TIMP), or other drugs,¹² chemically stabilizing existing matrix structures,^{13,14} and repairing already-developed aneurysms by endovascularly seeded healthy cells.¹⁵ Unfortunately, none of these strategies present an integrated approach to modulate aneurysmal cell phenotype, reinstate healthy matrix architecture, and provide conditions for stabilizing the local vascular environment. We believe that active, cell-mediated regeneration of lost elastin within aneurysmal sites could potentially provide a nonsurgical means of AA treatment. However, this is challenging since adult vascular cells do not produce much elastin on their own and mature elastic fibers rarely undergo active remodeling.

In past work, we identified and optimized ECM-derived biomolecular factors based on hyaluronan (HA) fragments^{16,17} and growth factors (transforming growth factor- β 1 [TGF- β 1] and insulin-like growth factor),^{18,19} for stimulating elastin regeneration by healthy vascular cells. Literature suggests that HA might play a key role in elastogenesis through intimate binding with elastin-associated proteins to form macromolecular structures responsible for elastic fiber assembly,²⁰ while TGF- β 1 has been shown to upregulate elastin matrix synthesis by smooth muscle cells (SMCs).^{21,22} Specifically, we showed that a combination of HA oligomers (4–6 mer; MW ~ 756–1221 Da; 0.2 μ g/mL) and TGF- β 1 (1 ng/mL), hitherto referred as elastogenic factors, synergistically attenuated vascular SMC proliferation and dramatically increased elastin synthesis, matrix assembly, maturation, and stability.¹⁸ Despite their benefits to elastin synthesis by healthy adult SMCs, it is yet unknown if these factors would likewise suppress procalcific and elastolytic activities of diseased vascular cells, such as those isolated from aneurysmal segments, and enable synthesis and assembly of new elastin matrix. In this context, it would be important to first demonstrate that SMCs isolated from aneurysmal aortae are continue to remain activated in culture, and to assess their extent and manner of elastic matrix synthesis relative to healthy SMCs.

To ascertain this, in this study, we adopted an established abdominal aortic injury protocol involving periadventitial application of calcium chloride (CaCl_2), to induce an aneurysm within in rats, over 28–38 days.²³ We then investigated the stand-alone and combined elastogenic benefits of TGF- β 1 and HA oligomer factors on SMCs isolated and cultured from these aneurysmal aortae, in an *in vitro* culture model. Based on the outcomes, we believe that this approach may be employed stand-alone or in consort with existing surgical or

pharmacological approaches to regenerate elastin matrices within aneurysmal aortic vessels.

Materials and Methods

Aortic injury by periadventitial application of CaCl_2

All animal studies were approved by the IACUC at Clemson University. Adult Sprague-Dawley rats (300–350 g) were procured and acclimatized for 1 week before surgery. The rats were placed under general anesthesia (2–4% iso-flurane), and the infrarenal abdominal aortae exposed surgically. The aortae were treated using a protocol adopted by various groups,^{23,24} wherein sterile cotton gauze presoaked with 0.5 mol/L CaCl_2 is rubbed on the aorta for 15 min. Sufficient care was taken not to expose other organs to this caustic agent. After application, the abdominal cavity was thoroughly washed with sterile saline to remove the residual CaCl_2 . The cavity was then closed, subcutaneously sutured, and stapled, and the rats were allowed to recover. After 28 days of rehabilitation, the animals were humanely euthanized by CO_2 asphyxiation. The abdominal aorta was excised from the arch to the celiac axis and processed for SMC isolation. The abdominal aortae were photographed before surgery and before harvesting at 28 days to compare their diameters.

Histological characterization of injured aortae

Harvested aortae were compared histologically with NaCl-treated (control) aortae to confirm CaCl_2 -induced elastin damage and calcific deposition, indicative of a pathologic microenvironment. Such characterization replicated methods used by Vyavahare *et al.*^{13,23} Harvested aortae were frozen in Optimal Cutting Temperature embedding solution (Electron Microscopy Sciences, Hatfield, PA) at -20°C , and then at -80°C , and cryosectioned transversely. The 10- μ m-thick sections were transferred onto positively charged histological slides (VWR Scientific, West Chester, PA), stained with hematoxylin and eosin for general tissue morphology, Verhoeff–Van Gieson's stain to gauge loss of integrity of elastic matrix structures, and Von Kossa stain for calcific deposits, and then cover-slipped with mounting medium (Richard-Allen Scientific, Kalamazoo, MI). All histological stains were procured from Poly Scientific (Bayshore, NY).

Immunofluorescence (IF) methods were also used to confirm elastic matrix degradation after CaCl_2 injury. To do this, cryosections of the tissue were fixed in 4% w/v paraformaldehyde (10 min, RT: room temperature), treated with a rabbit anti-mouse elastin antibody that cross reacts with rat tissues (courtesy of Dr. Robert Mecham, Washington University, St. Louis, MO), then labeled with an FITC-tethered goat anti-rabbit IgG secondary antibody (Chemicon, Temecula, CA), and mounted with Vectashield mounting medium (Vector Labs, Burlingame, CA) containing the nuclear stain 4',6-diamidino-2-phenylindole.

Isolation and culture of aneurysmal SMCs

The isolated aortae ($n = 3$) were opened lengthwise, and the intima scraped gently with a scalpel blade. The medial layer, dissected from the underlying adventia, was chopped into ~0.5-mm-long sections, and washed twice with warm phosphate buffered saline (PBS). The resulting tissue slices

were pooled and enzymatically degraded (30 min at 37°C) in a mixture of 125 U/mg collagenase, and 3 U/mg elastase was prepared in serum-free Dulbecco's modified Eagle's medium (DMEM)-F12 culture medium (Invitrogen, Carlsbad, CA) and then pelleted by centrifugation at 400g for 5 min. The tissue pieces were then cultured in T-75 flasks with DMEM-F12 containing 10% fetal bovine serum over 15 days. Rat aortic SMCs (RASMCs) derived by outgrowth from these tissue explants were cultured over 2 weeks, and the cells passaged when confluence was attained. Passage 2 SMCs were then seeded onto six-well tissue culture plates (area = 10 cm²) at a seeding density of 2 × 10⁵ cells/well and cultured in DMEM-F12 medium containing 10% FBS and 1% Penstrep. The total volume of medium added per well was 5 mL.

The goal of this study was to evaluate the effects of HA oligomers and TGF-β1 on SMCs derived from induced AAs. Thus, we have restricted our current study to cultures of this cell type alone; since we have already investigated and reported¹⁸ on elastogenic upregulation of SMCs isolated from the healthy, in the present study, we only selectively study healthy SMC cultures as a control cell type to establish that SMCs derived from aortae containing induced "aneurysms" indeed exhibit an activated phenotype. However, in the Discussion section, we do compare elastogenic induction of cultured aneurysmal SMCs with healthy SMC cultures.¹⁸

HA oligomer mixtures supplemented to aneurysmal RASMC cultures were prepared in-house by digestion of long-chain HA (MW: 2 × 10⁶ Da; Genzyme Biosurgery, Cambridge, MA) with testicular hyaluronidase (Sigma-Aldrich, St. Louis, MO), as previously reported.¹⁷ The mixtures contained 75 ± 15% w/w of HA 4-mers (henceforth referred to oligomers), with 6-mers and 8-mers forming the balance. The experimental groups were aneurysmal SMCs cultured with no additives (treatment controls), and aneurysmal cells cultured with supplements of HA oligomers alone, TGF-β1 alone, or TGF-β1 and HA oligomers together. The nonadditive aneurysmal SMC cultures served as negative treatment controls for the effects of HA oligomers, TGF-β1, or both. TGF-β1 (PeproTech, Rocky Hill, NJ) was added at a final concentration of 1 ng/mL, while oligomers were added at a final dose of 0.2 μg/mL. Medium aliquots were replaced twice weekly, and spent medium from each well was pooled and stored at -20°C. At 21 days, these pooled aliquots from each well and their corresponding cell layers were biochemically assayed.

DNA assay for cell proliferation

The DNA content of cell layers was quantified at 1 and 21 days of culture to assess the proliferation of SMCs over the culture period. Briefly, cell layers were detached with 0.25% v/v trypsin-ethylenediaminetetraacetic acid (Invitrogen), pelleted by centrifugation, resuspended in NaCl/Pi buffer, and assayed using a fluorometric assay,²⁵ with the cell density calculated on the basis of an estimated 6 pg DNA/cell.²⁵

Hydroxy-proline assay for collagen

A hydroxy-proline (OH-Pro) assay was used to estimate the collagen content within test and treatment control cell layers, and in the supernatant medium fraction. As described

earlier,¹⁶ the cell layers were homogenized in distilled water after 21 days of culture, pelleted by centrifugation (10000 g, 10 min), and digested with 1 mL of 0.1 N NaOH (1 h, 98°C). The digestate was then centrifuged to isolate a mass of insoluble, crosslinked elastin. The supernatant containing solubilized collagen and uncrosslinked matrix elastin was neutralized with an equal volume of 12 N HCl, and divided into two equal volumetric halves. One half-volume was hydrolyzed at 110°C for 16 h and dried in a constant stream of N₂ gas overnight, and 20 μL aliquots of the reconstituted residue were assayed for OH-Pro content. The total and matrix collagen amounts were then calculated on the basis of the 13.2% OH-Pro content of collagen, and normalized to DNA content of corresponding cell layers.

Fastin assay for elastin

The amounts of matrix elastin (alkali-soluble and insoluble fractions) and soluble tropoelastin (in pooled spent medium) were quantified using a Fastin assay (Accurate Scientific Corp, Westbury, NY), according to manufacturer's recommendations. Since the Fastin assay quantifies only soluble α-elastin, the insoluble elastin was first reduced to a soluble form by digesting with 0.25 N oxalic acid (1 h, 95°C), and filtering the pooled digestate in microcentrifuge tubes fitted with low-molecular-weight cut-off membranes (10 kDa). The insufficiently crosslinked, soluble elastin fraction retained in the oxalic acid-free fraction and in the water-reconstituted hydrolysate (from the collagen assay above) was also quantified using the Fastin assay. Spent fractions of media pooled at bi-weekly intervals over the 3-week culture period were lyophilized and processed for tropoelastin using the Fastin assay. The measured elastin amounts were normalized to corresponding DNA amounts to provide a reliable basis of comparison between samples.

Von Kossa staining for matrix mineralization

After 21 days of culture, RASMCs were incubated with 1% w/v silver nitrate solution and placed under UV light for 20 min. After several changes of distilled water, the unreacted silver was removed with 5% w/v sodium thiosulfate for 5 min, and the cells were rinsed. The slides were counterstained with hematoxylin. The presence of black stain confirmed the presence of calcium phosphate deposits.

Desmosine assay for elastin crosslinks

The desmosine crosslink densities within elastic matrices were quantified using an ELISA-based protocol, as described previously,¹⁶ to determine if any of the provided factors enhanced elastin crosslinking efficiency. Briefly, the 3-week-old cell layers were digested with collagenase (12 h, 37°C) and elastase (12 h, 37°C), and the final digestates were acid-hydrolyzed (6 N HCl, 110°C, 16 h). The desmosine content in the reconstituted dried residue was determined using primary antibody for desmosine-hemocyanin conjugate (Elastin Products Company, Owensville, MO) tagged with calorimetric compound 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (Sigma-Aldrich), and the absorbances were read in a UV spectrophotometer (λ = 405 nm) and compared to corresponding trends in insoluble matrix elastin.

Lysyl oxidase enzyme activity

Lysyl oxidase (LOX) and LOX-like proteins are endogenous enzymes that crosslink and insolubilize soluble elastin precursor molecules, tropoelastin. Estimation of activities of endogenous LOX released into the extracellular space and thence into the culture medium is one indirect measure of cell-mediated crosslinking of elastin within cell layers to form mature matrix structures. Spent culture medium aliquots pooled for each cell layer over 21 days of culture were assayed for LOX activity using a fluorometric assay (Amplex[®] Red Assay; Molecular Probes, Eugene, OR) based on measurement of H₂O₂ released when LOX oxidatively deaminates alkyl monoamines and diamines.²⁶ The fluorescence intensities were recorded with excitation and emission wavelengths of 560 and 590 nm, respectively.

Assay for elastase activity

Elastase activity in the cell cultures was assayed using an *EnzChek*[®] Elastase Assay kit (Molecular Probes). Briefly, 50 μ L of supernatant was mixed with 50 μ L of diluted bovine neck ligament elastin and incubated for 30 min at 37°C, and the fluorescence intensity was measured using a fluorometer excitation and emission wavelength settings of 485 and 510 nm, respectively. 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.

Western blot analysis for LOX synthesis

Since our prior studies showed HA oligomers and TGF- β to enhance LOX protein synthesis and subsequently greater elastic matrix crosslinking by healthy rat SMCs, here we performed Western blot analysis on spent medium aliquots collected of control and test cell layers to semi-quantitatively assess impact of similar delivery of provided factors on basal LOX protein synthesis by aneurysmal SMCs.¹⁹ While such analysis is not quantitative, in the sense that it does not measure absolute amounts of LOX, a fraction that is retained within cells or associates with the ECM within the cell layer, the method allows semi-quantitative comparison of LOX protein between cultures receiving different treatments. Briefly, the spent medium from cultures, pooled over the 21-day period of culture, was lyophilized and assayed for protein content using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA), to optimize loading sample amounts (15 μ g) for sodium dodecyl sulfate polyacrylamide gel electrophoresis/Western blot. Protein bands were detected with primary rabbit anti-rat polyclonal antibodies to the 31 kDa active LOX protein (Santa Cruz Biotechnology, Santa Cruz, CA) and viewed using a Chemi-Imager IS 4400 system (Alpha Innotech, San Leandro, CA), and the images were analyzed and quantified using Image J (NIH).

Gel zymography for MMP activity

MMP-2 and -9 were detected in the culture medium by gelatin zymography methods described elsewhere.²⁷ Briefly, aliquots of culture medium were assayed for protein content using the biochronic acid assay, and all lanes were loaded in triplicate with 15 μ g of protein from each extract alongside with prestained molecular weight standards (Bio-Rad Laboratories). After development and staining, densities of the MMP-2 and -9

bands, seen on a dark background of stained gelatin, were quantified using Gel Pro Analysis software (Media Cybernetics, Bethesda, MD), and reported as relative density units.

Immunofluorescence detection of elastin, fibrillin, and LOX

IF techniques were used to confirm presence of elastin within the cultured cell layers. RASMCs were seeded within four-well-chamber slides at an initial seeding density of 5×10^3 cells/well and cultured for 21 days under the respective sets of conditions as cells cultured for biochemical analysis ($n = 3$ /case/set). The cell layers were then fixed with 4% v/v paraformaldehyde for 10 min, and labeled with a monoclonal primary antibody against rat SMC α -actin (1:200 dilution; 30 min, 25°C; Abcam, Cambridge, MA). Elastin and fibrillin were detected with polyclonal primary antibodies (1: 100 v/v; Elastin Products Company) as well as LOX (1: 100 v/v; Santa Cruz Biotechnology) viewed with a Rhodamine-conjugated donkey anti-rabbit IgG secondary antibody (1: 1500 v/v; Abcam). Cell layers deemed as negative IF controls were not treated with primary antibodies. The LOX protein identified within the cell layers using IF labeling likely includes a fraction of LOX released by cells into the extracellular space and that sequesters with the ECM (the rest being lost into the culture medium), and the LOX within the cells themselves. Cell nuclei were viewed with the nuclear stain 4', 6-diamino-2-phenylindole dihydrochloride contained in the mounting medium (Vectashield; Vector Labs). For each labeled protein, images of each of the treatment control (no additives) and test cell layers were collected under identical image capture settings on a Leica confocal microscope and were identically brightness/contrast enhanced for the purpose publication. An average of 15 random locations were imaged on each of the three replicate cell layers/case/set.

Matrix ultrastructure

Transmission electron microscopy was used to characterize the ultrastructure of matrix elastin, since the goal of this study was to achieve deposition of elastin fibers and not random deposits of amorphous elastin. Aneurysmal RASMCs cultured for 21 days in the absence of any supplements (controls) and in the presence of TGF- β and HA oligomer factors were fixed with 2.5% w/v glutaraldehyde, postfixed in 1% w/v osmium tetroxide (1 h), dehydrated in graded ethanol, embedded in Epon 812 resin, sectioned, placed on copper grids, stained with uranyl acetate and lead citrate, and viewed on a Hitachi H7600T transmission electron microscope (Hitachi High Technologies America, Schaumburg, IL).

For immunogold labeling of fibrillin within cell layers, the microtomed sections of fixed cell layers (80–100 nm) were blocked in PBS (with 3% skim milk and 0.01% Tween-20) for 1 h, and washed with PBS multiple times. The sections were then incubated overnight at 4°C with rabbit anti-VHb antibody (1:500 in PBS containing 0.3% skim milk and 0.01% Tween-20), followed by washing. The bound antibodies were viewed by incubating the sections for 1 h with goat anti-rabbit gold conjugate (10 nm; Sigma, St. Louis, MO), diluted 1:20 in PBS (containing 0.3% skim milk and 0.001% Tween-20) at room temperature. The grids were finally washed on water drops (five changes, 5 min each) before being stained with 2% aqueous uranyl acetate at room temperature.

Statistical analysis

Since the experimental data ($n = 3/\text{case}$) were determined to exhibit a near-Gaussian distribution, they were analyzed using Student's *t*-test, assuming unequal variance. Statistical significance was deemed for $p < 0.05$. Asterisks in figures denote statistical significance ($p < 0.05$) for each group compared with nonadditive aneurysmal SMC cultures.

Results

Aneurysm development and SMC phenotype

Figures 1A and B show representative rat abdominal aortae before and 28 days after CaCl_2 injury. It was observed that an $\sim 45\%$ local increase in aortic diameter was attained over this period, agreeing with prior observations.²³ Histological analysis (Fig. 2) confirmed increased size of CaCl_2 injured aortae relative to those treated with NaCl (treatment controls). Hematoxylin and eosin and Verhoeff–Van Gieson's staining indicated that CaCl_2 injury caused extensive breakdown of medial elastin, visible as gaping holes in the elastic lamellae (see arrows); these outcomes were confirmed via IF labeling of medial elastin (green), which appeared intact in NaCl-treated aortae, but again exhibited holes within CaCl_2 -treated aortae. As shown in Figures 3A and B, SMCs outgrowing from aneurysmal aortic explants initially appeared rounded, then more spindle-shaped, and thereafter somewhat more spread attaining 50% confluence by 15 days. In contrast, SMCs isolated from healthy rat aortae exhibited more spread morphology on day 7 after seeding (Fig. 3C). The SMC phenotype was confirmed by staining for SMC α -actin (Fig. 3D).

Aneurysmal SMC proliferation and matrix synthesis

Figure 4A shows the proliferation ratios of passage 2 aneurysmal SMCs cultured in the presence of TGF- β 1 alone, oligomers alone, or both. Nonadditive control aneurysmal SMCs proliferated 2.5 ± 0.32 -fold over 21 days. At this time, all cell layers, regardless of treatment received, were confluent and exhibited hill and valley distribution typical of SMCs, as evident in Figures 7D–G. No morphological differences were noted between cell layers supplemented with elastogenic factors and control cell layers, even early in culture. Addition of TGF- β 1 to SMC cultures had no effect on

these proliferation ratios (0.97 ± 0.08 -fold at 21 days vs. controls; $p = 0.56$). However, HA oligomers supplemented alone, or together with TGF- β 1, suppressed cell proliferation ratios significantly, relative to nonadditive controls (0.81 ± 0.1 -fold and 0.66 ± 0.15 -fold, respectively; $p = 0.002$ and 0.0001).

When HA oligomers or TGF- β 1 alone were provided to SMCs, a significant increase in collagen synthesis (1.4 ± 0.07 -fold and 1.33 ± 0.05 -fold, respectively; Fig. 4B) was observed, relative to control cultures ($p = 0.008$ and 0.005 , respectively). Addition of both the factors furthered collagen synthesis to 1.78 ± 0.18 -fold relative to controls ($p = 0.008$).

As shown in Figure 5A, addition of HA oligomers alone or together with TGF- β 1 promoted tropoelastin synthesis by 1.17 ± 0.02 -fold and 1.47 ± 0.05 -fold, respectively, relative to nonadditive controls ($p = 0.007$ and 0.0001 vs. controls, respectively). However, addition of TGF- β 1 alone had no effect on tropoelastin synthesis (0.94 ± 0.02 -fold; $p = 0.13$). Interestingly, collagen and tropoelastin synthesis (779 ± 62 ng/ng DNA and 3516 ± 149 ng/ng DNA, respectively) observed within additive-free aneurysmal SMC cultures was much lower than that we previously measured within healthy SMC cultures of similar passage (22509 ± 668 ng/ng of DNA and 39070 ± 8707 ng/ng DNA, respectively).¹⁸

As explained earlier, elastin protein incorporated into the ECM was measured as a sum of two individual fractions, that is, a highly cross-linked, alkali-insoluble elastin (which represents structural elastin), and an alkali-soluble fraction. As shown in Figure 5B and C, the trends in matrix elastin protein production mirrored those observed for tropoelastin synthesis under identical conditions. Addition of $0.2 \mu\text{g}/\text{mL}$ of HA oligomers to aneurysmal SMC cultures increased soluble and insoluble matrix elastin synthesis by 1.5 ± 0.11 -fold and 1.23 ± 0.09 -fold, respectively, relative to that measured in nonadditive control cultures (157 ± 26 ng/ng DNA and 216 ± 54 ng/ng DNA; $p = 0.017$ and 0.031 , respectively). Also, addition of TGF- β 1 ($1 \text{ ng}/\text{mL}$) alone had no effect on basal matrix elastin synthesis levels (soluble elastin: $p = 0.56$; insoluble elastin: $p = 0.54$). On the other hand, HA oligomers and TGF- β 1 factors together increased production of soluble and insoluble matrix elastin fractions by 1.78 ± 0.29 -fold and 1.56 ± 0.34 -fold, relative to controls ($p = 0.014$ and 0.026 , respectively, vs. control). Overall, relative to controls, the total elastin output (sum of tropoelastin and matrix elastin fractions) increased by 1.19 ± 0.03 -fold, 0.96 ± 0.03 -fold,

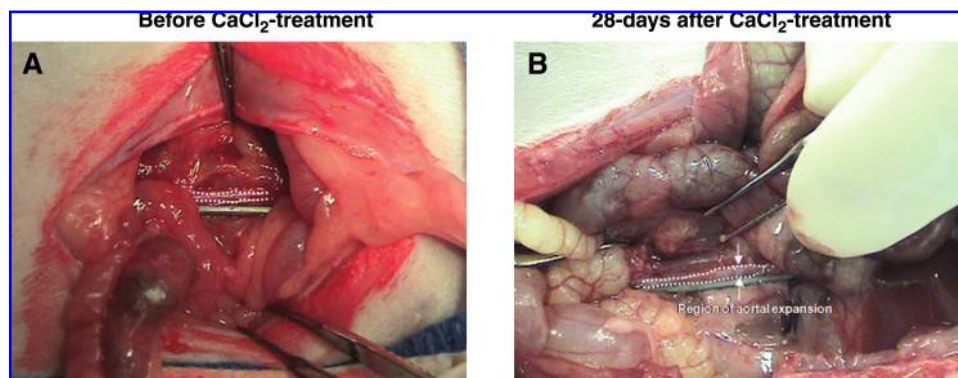


FIG. 1. Representative images of abdominal rat aortae before calcium chloride (CaCl_2) treatment (A) and 28 days postinjury (B), when significant elastin degradation was observed. The aortae showed a local diameter increase of 45%. Color images available online at www.liebertonline.com/ten.

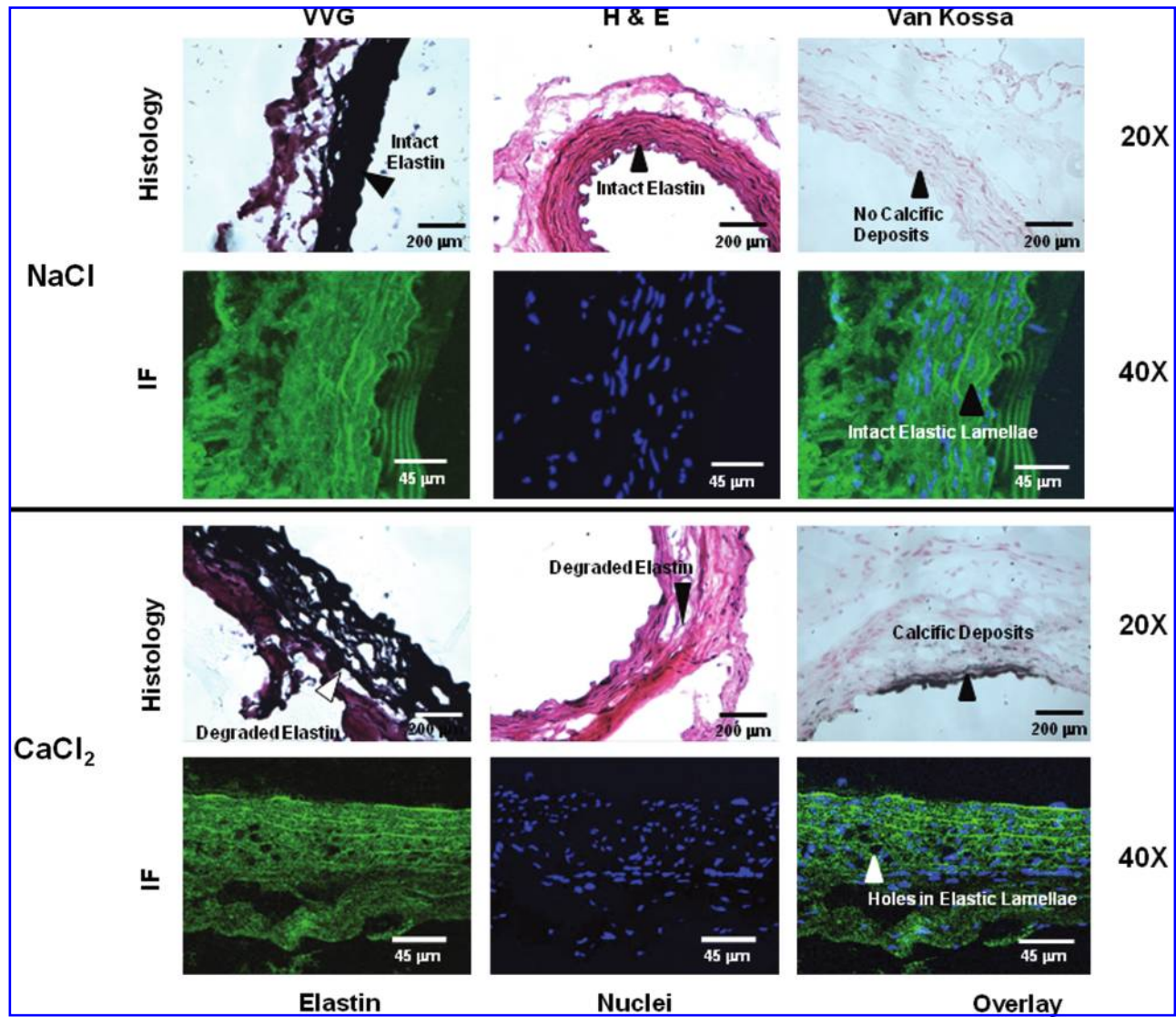


FIG. 2. Histological analysis of rat aortae shown 38 days posttreatment with 0.5 M CaCl_2 and 0.5 M NaCl (treatment control). The solutions were applied for 15 min, postadventitally. Hematoxylin and eosin–stained sections of I2-treated aortae showed extensive breakdown of elastic lamellar structures (pink; see arrows) in the tunica media layer, in contrast to those within NaCl-treated aortae, which remained intact. These results were confirmed by comparison of elastin (black structures) within Verhoeff–Van Gieson–stained sections, and using immunofluorescence (note holes in green-fluorescing elastic lamellae). Von Kossa staining revealed presence of calcific deposits within aortae treated with CaCl_2 , but not NaCl, confirming the induction of an abnormal, disease-like microenvironment in the former case. Color images available online at www.liebertonline.com/ten.

and 1.49 ± 0.06 -fold, respectively, upon addition of oligomers alone, TGF- β 1 alone, or both the factors together, respectively (Fig. 5D).

LOX protein expression and functional activity

Figure 6A compares outcomes of Western blot analysis of LOX protein expression with trends in biochemical quantification of matrix elastin. LOX protein expression increased significantly in cultures supplemented with TGF- β 1 alone or together with HA oligomers (1.59 ± 0.05 -fold and 1.72 ± 0.04 -fold, relative to controls; $p < 0.001$ in both the cases). However, addition of HA oligomers alone promoted only 1.03 ± 0.14 -fold increase in LOX protein expression relative to controls ($p = 0.73$). In all the cases, no significant differences in the cellular LOX enzyme activities were ob-

served though, relative to nonadditive controls (data not shown).

As shown in Figure 6B, aneurysmal cells, cultured both supplement-free and in the presence of oligomers, did not show any significant increase in desmosine synthesis (0.78 ± 0.01 -fold and 0.92 ± 0.08 -fold) relative to healthy SMC layers (11.85 ± 0.37 pg desmosine/ng DNA), while TGF- β 1 alone or together with HA oligomers enhanced desmosine synthesis by 1.07 ± 0.07 -fold and 1.21 ± 0.03 -fold, respectively ($p = 0.06$ and $p < 0.001$ vs. healthy controls).

Production of proteolytic enzymes

As shown in Figure 7A and B, gel zymography analysis revealed that the release of MMPs-2 and 9 was significantly higher within nonadditive aneurysmal cell cultures relative

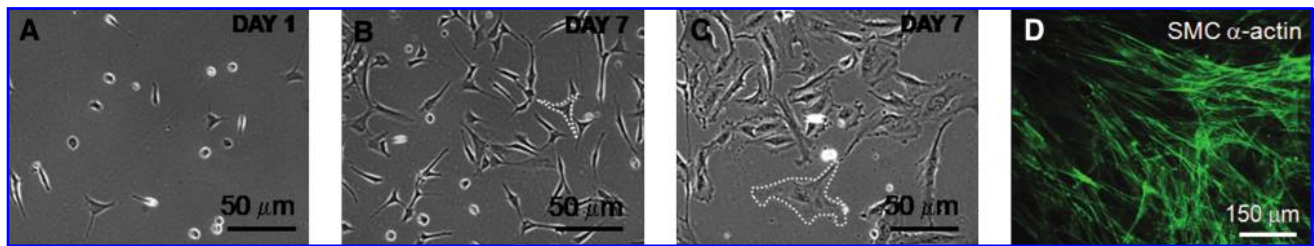


FIG. 3. Significant rounding of the smooth muscle cells (SMCs) isolated from the aneurysmal segment was observed on day 1 (A) and more spindle-shaped by day 7 (B). SMCs isolated from healthy aortae showed more spread morphology on day 7 of seeding (C). The cells were stained with SMC- α actin to confirm the SMC phenotype (D). Color images available online at www.liebertonline.com/ten.

to healthy cell cultures (1.67 ± 0.16 -fold and 2.07 ± 0.35 -fold, respectively; $p = 0.03$ and 0.01), confirming their activated/diseased phenotype. HA oligomers and TGF- β 1 individually modestly attenuated the basal MMP activity in aneurysmal SMC cultures. In the presence of both HA oligomers and TGF- β 1, production of MMPs-2 and 9 was attenuated relative to additive-free aneurysmal SMC cultures ($p = 0.02$ and 0.03), down to levels seen in healthy SMC cultures ($p = 0.67$ and 0.71 vs. healthy SMCs). Similarly, as shown in Figure 6C, elastase activity within additive-free aneurysmal SMC cultures was 1.19 ± 0.09 -fold higher than those in healthy SMC cultures (0.23 ± 0.02 U/mL; $p = 0.04$). HA oligomer- and TGF- β 1-supplements, provided separately, did not alter the basal elastase activity in aneurysmal SMC cultures, which remained higher than in healthy SMC cultures (1.29 ± 0.08 -fold and 1.39 ± 0.18 -fold, respectively, relative to healthy SMC cultures; $p = 0.015$ and 0.03 , respectively). Similarly, in the presence of both HA oligomer and TGF- β 1 factors, elastase activity was almost identical to additive-free aneurysmal cell cultures (1.01 ± 0.06 -fold vs. nonadditive aneurysmal SMCs; $p = 0.82$).

Figure 7 also shows the images of Von Kossa-stained aneurysmal SMC cultures. Untreated aneurysmal SMC cultures (panel D) exhibited multiple large calcific deposits (black); supplementing cultures with TGF- β 1 alone (panel F), did not alter patterns of matrix calcification, while addition

of HA oligomers (panel E) appeared to decrease the density of these deposits. However, the presence of both the factors significantly suppressed formation of calcific deposits (panel G), though complete inhibition was not observed.

Immunodetection of elastin, fibrillin, and LOX in cell layers

Figure 8 shows IF micrographs of cell layers labeled for elastin, fibrillin, and LOX (red fluorescence) after 21 days of culture with oligomers alone, TGF- β 1 alone, or both the factors together. Nonadditive aneurysmal SMC cultures (treatment controls) and cultures untreated with primary antibodies (denoted as negative IF controls) are also shown in Figure 8. The negative IF controls exhibited no auto-fluorescence indicating the absence of false coloring from nonspecific binding of the secondary antibody-conjugated fluorophore to the SMC layers. While featureless amorphous elastin deposits were seen in nonadditive aneurysmal SMC cultures and TGF- β 1-treated cultures, more organized fibrous elastic structures were visible in cell layers cultured with HA oligomers alone, and together with TGF- β 1. The fluorescence intensity due to elastin within cell layers cultured with both TGF- β 1 and HA oligomer factors was also much greater than in the absence of the factors, or when either of the factors was provided alone. The fluorescence

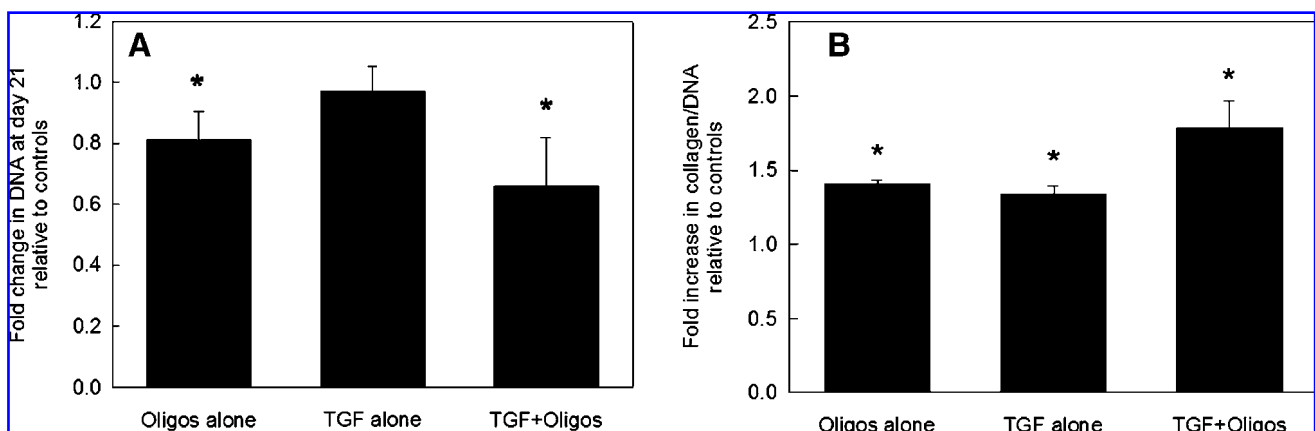


FIG. 4. (A) Proliferation ratios of aneurysmal SMC cultures supplemented with oligomers alone, transforming growth factor- β (TGF- β) alone, or cues together. Data shown represent mean \pm SD of DNA content of cell layers after 21 days of culture, normalized to initial seeding density, and further normalized to aneurysmal control cultures that received no additives ($n = 3$ /case). (B) Effects of oligomers alone, TGF- β alone, and cues together, on total collagen synthesis by aneurysmal SMCs. Data shown (mean \pm SD) are normalized to cellular DNA content at 21 days of culture and represented as fold change in protein production relative to aneurysmal controls ($n = 3$ /case). $p < 0.05$ represents significant differences from controls (*).

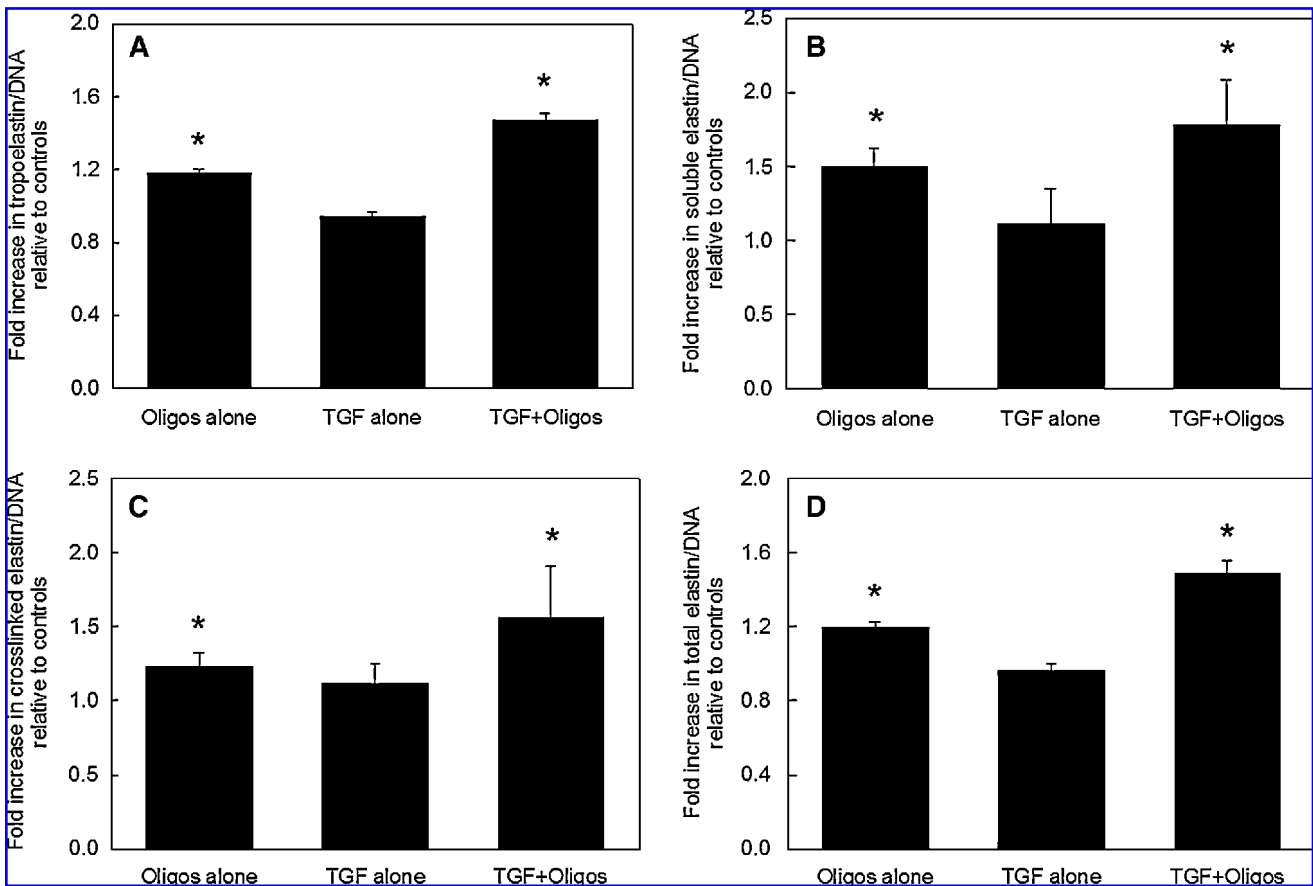


FIG. 5. Effects of oligomers alone, TGF- β alone, and both cues together, on tropoelastin (A), alkali-soluble (B), crosslinked matrix elastin (C), and total elastin (D) produced by aneurysmal SMCs. Data shown (mean \pm SD) are normalized to cellular DNA content at 21 days of culture and represented as fold change in protein production relative to aneurysmal controls ($n = 3/\text{case}$). $p < 0.05$ represents significant differences from controls (*).

intensity due to fibrillin microfibrils was similarly greater in cultures supplemented with HA oligomers alone, or together with TGF- β 1, compared to nonadditive aneurysmal SMC cultures or those cultured with TGF- β 1 alone. When either or

both the factors were provided to the SMC cultures, the fibrillin appeared organized into honey-comb-like structures unlike in nonadditive control cultures, where the fibrillin was not deposited in such an organized manner. However,

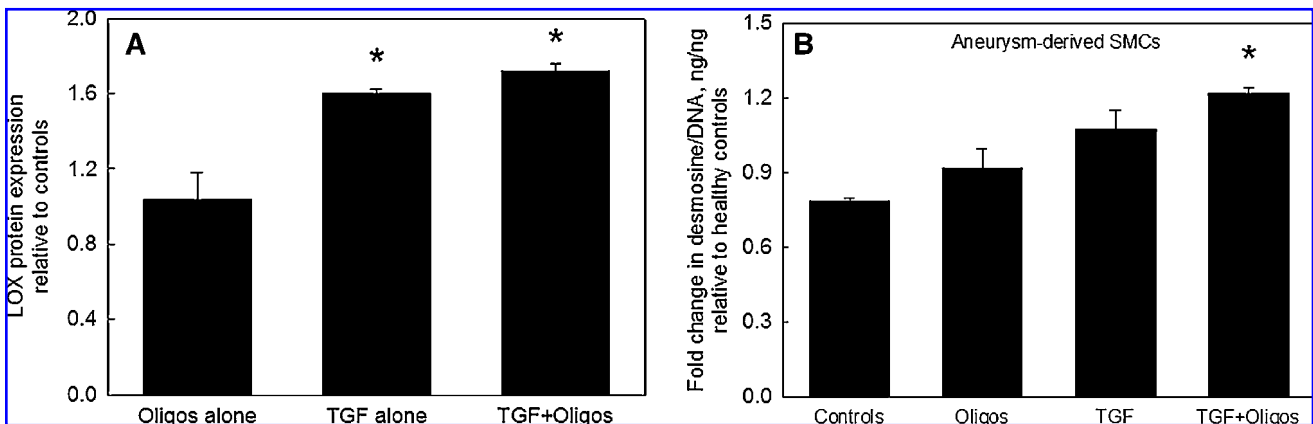


FIG. 6. (A) Sodium dodecyl sulfate polyacrylamide gel electrophoresis/Western blot analysis of tropoelastin and Lysyl oxidase (LOX) proteins within the pooled medium of aneurysmal cultures at the end of 21 days. Data shown represent mean \pm SD of three repeats/case and are shown normalized to controls. (B) Desmosine amounts measured in test cell layers were normalized to corresponding DNA amounts (ng/ng), and further a similar ratio obtained for the healthy nonadditive controls. Comparable trends were observed for the desmosine/DNA density and respective insoluble matrix elastin/DNA for selected cases. *Represents significance of differences in values relative to cultures of healthy additive-free cells, deemed for a p value < 0.05 .

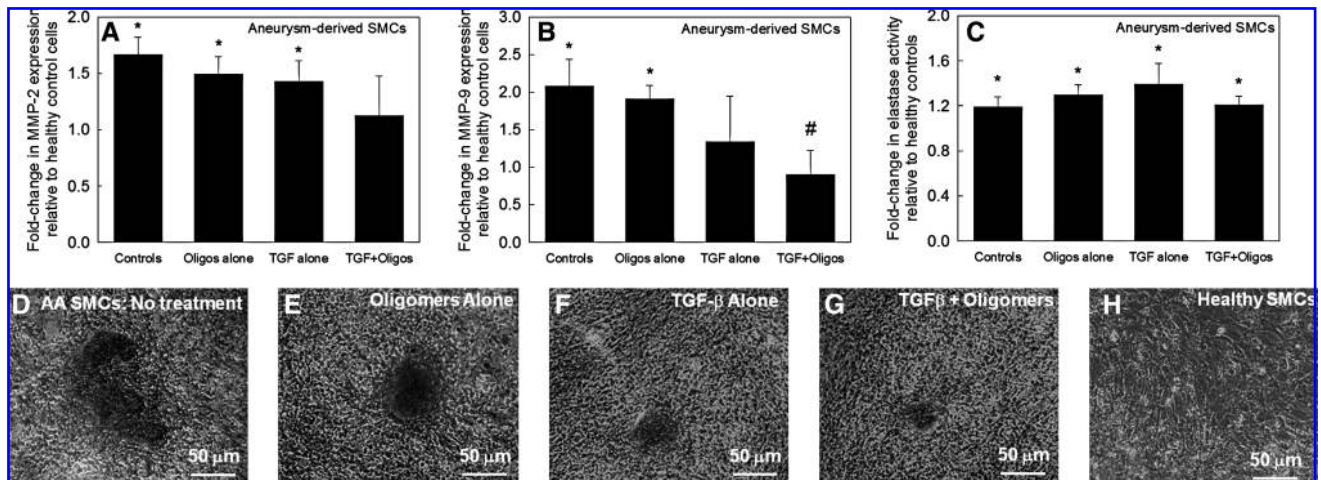


FIG. 7. Gel zymography analysis revealed the presence of matrix metalloproteinases-2 (A) and matrix metalloproteinases-9 (B) within aneurysmal cultures treated with or without cues. Data were shown normalized to the respective values observed in healthy nonadditive treatment controls ($n = 3/\text{case}$). (C) Elastase enzyme activity within aneurysmal SMC cultures treated with oligomers alone, TGF- β alone or together with hyaluronan (HA) oligomers. Data are shown normalized to the corresponding values in healthy nonadditive cultures ($n = 3/\text{case}$). Von Kossa staining images of untreated aneurysmal cell layers (D) or those treated with TGF- β alone (E), oligomers alone (F), or cues together (G), and healthy cell layers (H). Significant calcific deposits were evident in control cultures and those treated with TGF- β alone or oligomers alone, while cultures that received cues together showed a decrease in the calcific mineralization, almost to levels exhibited in healthy SMC layers. *Indicates significance of differences relative to healthy, additive-free control cell cultures, deemed for $p < 0.05$.

in all cases, LOX was rather faintly expressed and was sparsely distributed.

Ultrastructure of matrix elastin

Figure 9 shows representative transmission electron micrographs of aneurysmal SMCs cultured for 21 days in the absence and presence of the HA oligomer and TGF- β 1 factors. Like previously observed in healthy control cultures,¹⁸ non-additive aneurysmal SMCs also deposited aggregating clumps of amorphous elastin protein that were overall sparse and rather localized within the cell layers (panels A, B). When TGF- β 1 and HA oligomers together were provided to these cultures, mature elastic fiber formation was favored, with the matrix containing numerous fully-formed bundles (100–200 nm diameter) of aggregating fibrils (panels C, D). Fibrillin (immunogold particle-stained), which appeared as darkly stained nodules, was located at the periphery of aggregating elastin fiber bundles, signifying normal elastic fiber assembly.

Discussion

Our long-term goal is to enable elastin matrix regeneration on demand within elastin-degraded vessels such as within AAs, so as to stabilize and possibly even regress their growth and thus eliminate need for surgical intervention. Under healthy physiologic conditions, elastin turnover is very slow, and very little remodeling of elastin fibers occurs in adults.²⁸ This is because active elastin matrix synthesis is almost a one-time phenomenon that occurs pre- and postnatally, and which occurs to a very limited extent in adult tissues due to intrinsically poor tropoelastin mRNA expression by adult cells. Thus, regenerating elastin matrices after their enzymatic breakdown in certain inflammatory pathologies (e.g., atherosclerosis and aneurysms), or due to genetic abnormalities in matrix assembly is not currently possible. Recent studies on

aneurysm development and progression²⁹ reveal upregulated synthesis of elastolytic MMPs in inflamed vascular tissues, which rapidly degrade the elastin matrix to result in loss of tensile strength and elasticity of the vessel wall, and in progressive increases in vessel diameter.¹² Currently, surgical excision of aneurysmal vessel segments at near-rupture stages of development, and their replacement with vascular grafts is the major mode of AA treatment. Therapeutic approaches aimed at pharmacologically inhibiting MMPs¹² or at chemically stabilizing existing elastin matrix against enzymatic degradation have been attempted but are more useful to stabilize aneurysms rather than actively regressing them through new elastin regeneration. Thus, alternate tools are required to stimulate elastin regeneration and repair.

Rodents have in recent years been popular as animal models to study AAs.^{30,31} AAs can be induced by genetic manipulation (deficiencies in LOX, TIMP-1, low density lipoprotein (LDL)-receptor, etc.) or by chemical induction (intraluminal elastase infusion, periaortic aortic injury with CaCl_2). Induced AAs within rats have been shown to exhibit several key facets of human aneurysms, such as medial layer disruption, inflammation, thrombus formation, and rupture.³ Of these, elastase infusion into infrarenal segment of aorta³² and periaortic application of CaCl_2 ^{24,33} have been particularly found to induce significant and progressive aortic dilation, and localized inflammation at the site of application within 4 weeks.³⁴ In a prior publication, Vyavahare *et al.*^{13,23} showed elevated MMP production at CaCl_2 aortic injury sites in rats, and extensive destruction of the elastic lamellae within. In the current study, CaCl_2 -treated rat abdominal aortae developed aneurysms within 4 weeks post-injury, with an $\sim 45\%$ increase in aortic diameter, which is within the range reported by others as well.^{24,34} However, the impact of individual factors influencing cell behavior, for example, changes in matrix composition and architecture

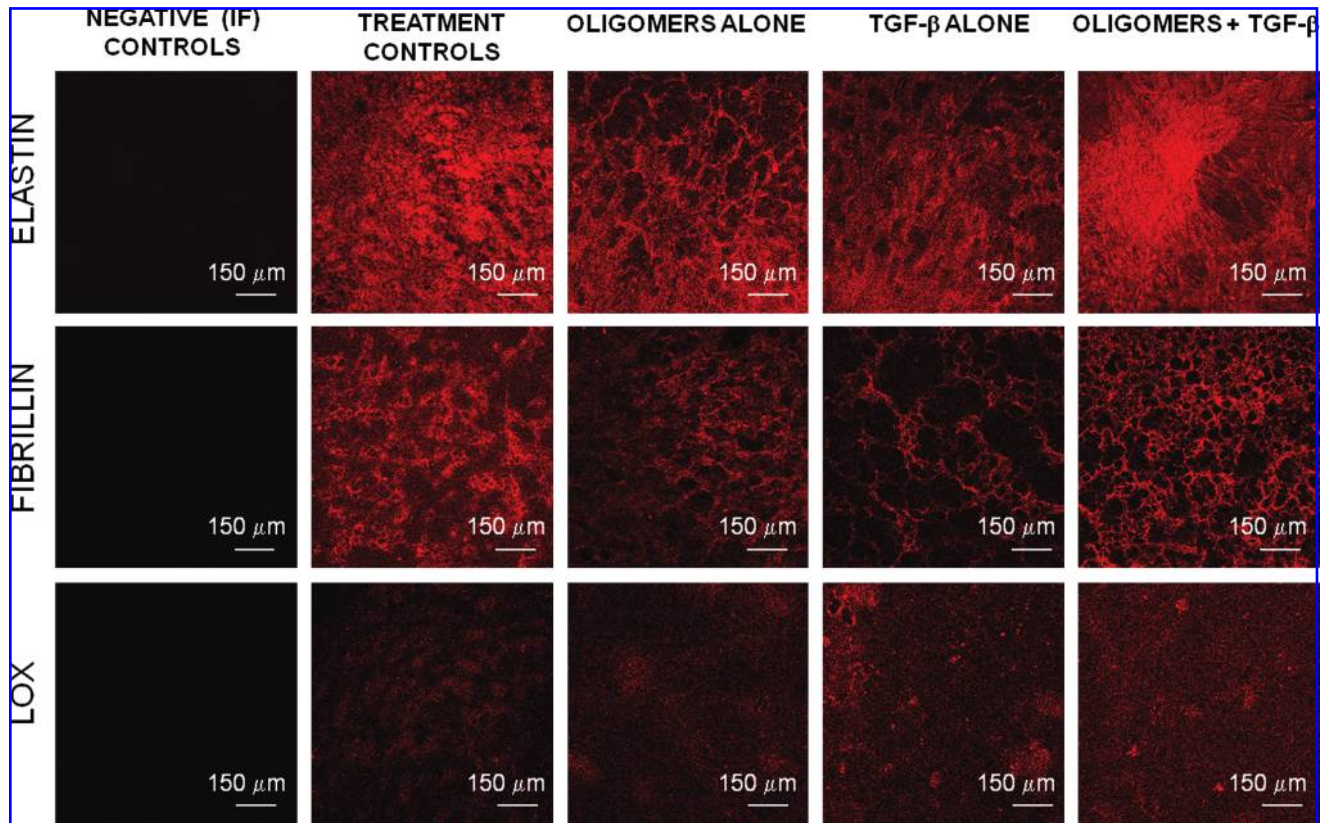


FIG. 8. Immunodetection of elastin, fibrillin, and LOX within control and test cell layers after 21 days of culture. For each labeled protein, images of each of the treatment control (no additives) and test cell layers were collected under identical image capture settings and were identically brightness/contrast enhanced for the purpose publication. An average of 15 random locations were imaged on each of the three replicate cell layers/case/set. An increase in matrix elastin, fibrillin, and LOX is evident in aneurysmal cultures that received both TGF- β and HA oligomers, relative to nonadditive aneurysmal SMC cultures (treatment controls). Cultures that received TGF- β alone or oligomers alone showed relatively less intense fluorescence relative to cultures receiving both cues. Lack of fluorescence in cultures untreated with primary antibodies (negative immunofluorescence controls) confirms lack of nonspecific binding of the fluorophore-conjugated secondary antibody. Scale bar: 150 μ m. Color images available online at www.liebertonline.com/ten.

and cellular activation by recruited inflammatory cells, on basal and induced elastin matrix regeneration can be difficult in such *in vivo* models. In this context, *in vitro* cell culture models of aneurysmal cells, as we study here, may help to circumvent these problems, though such models have never been studied to date in the context of matrix regenerative therapies. Since cells can be propagated in culture, limitations to tissue procurement do not restrict rigorous evaluation of parameters that influence cell phenotype and matrix regenerative potential. Although a two-dimensional cell culture model may not exactly replicate the physiologic three-dimensional matrix microenvironment, they still provide useful information since trends in scaffold-chemistry-dependent variations of cell phenotype and matrix synthesis are maintained, but not necessarily to the same levels, when translated into a three-dimensional culture system. Thus, two-dimensional cell culture models are invaluable for early study of the biochemical regulation of cells by supplemented biomolecules.

Although the cells isolated from our rat aneurysmal aortic explants expressed smooth muscle α -actin, confirming an SMC phenotype, a significant number among them exhibited decreased volume/spreading especially in the first 10 days after seeding, which were very different from the spread

morphology typically observed in healthy aortic SMC cultures. Such differences in phenotype between healthy and aneurysmal vascular SMCs have also been reported by others.³⁵ Studies have also shown that when SMCs are activated *in vivo*, or in culture, they release proteases, including MMPs and elastases that degrade their elastic matrix to generate elastin peptides (ELP).²⁷ The ELP-SMC interactions have been shown to in turn enhance expression of the elastin-laminin receptor (ELR), and release of elastolytic MMP-2, besides bone proteins such as osteocalcin and alkaline phosphatase. It has also been shown that TGF- β 1, at certain doses, can potentially enhance these osteogenic responses via ELR-mediated signaling. Thus, extent of calcific deposition within SMC cultures is indicative of the extent of their activation. Since our aneurysmal SMCs generated greater amounts of MMP-2 and -9 and exhibited greater elastase activity than did healthy control SMCs, and promoted deposition of a greater number of calcific deposits than in healthy SMC cultures, they were thus interpreted to exhibit an activated phenotype. Interestingly, the aneurysmal SMCs proliferated more slowly than healthy SMCs¹⁸ over the 21-day period ($33 \pm 4\%$ of healthy SMCs; $p < 0.001$), produced far less tropoelastin ($9 \pm 0.3\%$ of healthy SMCs; $p < 0.001$), collagen ($3.4 \pm 0.5\%$ of healthy SMCs; $p < 0.001$),

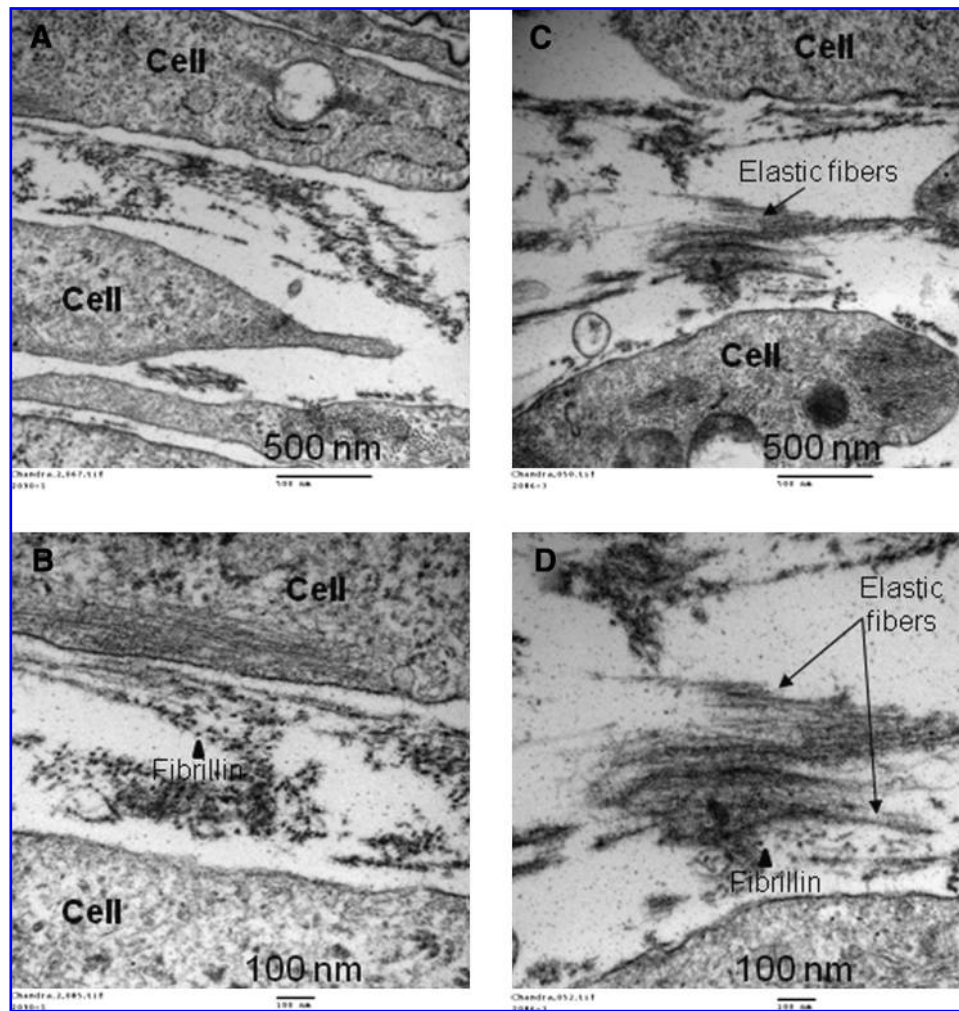


FIG. 9. Representative transmission electron microscopy images of 21-day-old aneurysmal SMC layers cultured additive-free aneurysmal SMC layers [treatment control; (A) 50000 \times ; (B) 100000 \times] and those cultured with both TGF- β and HA oligomers [(C) 50000 \times ; (D) 100000 \times]. Aggregating amorphous elastin clumps leading to the formation of elastin fibers can be clearly seen in aneurysmal cell cultures treated with the factors (C, D), while aneurysmal SMC cultures that received no additives (A, B) showed amorphous elastin deposits with sparse fiber formation. In all cultures, immunogold labeling showed presence of fibrillin microfibrils (black dots), indicative of normal mechanisms of elastic matrix assembly.

and matrix elastin ($11 \pm 2\%$ of healthy SMCs; $p < 0.001$) than did by healthy control SMCs.¹⁸ These results are supported by a significant decrease in the production of elastin cross-linking proteins (LOX and desmosine), as gauged from our biochemical analysis, and a visible decrease in elastin matrix density within these cultures relative to healthy cell controls.¹⁸ Overall, these results point to the activated state of these isolated aneurysmal SMCs when cultured *in vitro*.

In our recent studies exploring the elastogenic benefits of HA oligomers (0.2 $\mu\text{g}/\text{mL}$ or ~ 33 pg/cell) to elastin synthesis by healthy RASMCs,¹⁸ we found tropoelastin synthesis to be modestly enhanced (1.5 ± 0.2 -fold), while elastin yield was increased multifold (2.57 ± 0.3 -fold vs. nonadditive healthy SMC cultures), though on a per cell basis, matrix elastin synthesis remained unchanged. Unlike large HA fragments (>10 kD), which likely phosphorylate the primary CD44 cell surface HA receptor³⁶ to elicit downstream intracellular changes leading to enhanced cell proliferation, and decreased matrix, HA oligomers (2 $\mu\text{g}/\text{mL}$) appeared to elicit quite the opposite effects, possibly by interacting with and

clustering on the toll-like receptor 4 (a likely receptor for HA 4 mers) or CD44 (receptor for HA 6 mers and larger). HA oligomers also improved efficiency of tropoelastin recruitment and crosslinking into a matrix, likely by enhancing endogenous lysyl production of LOX (c) encouraged elastin fiber assembly, possibly by interacting with elastin-associated proteins to form structures important to elastic fiber assembly^{37,38} and inhibited activity¹⁷ of the ELR, to attenuate cellular release of elastolytic MMPs.

In testing the effect of HA oligomers on cultured aneurysmal SMCs, suitable negative controls could be (a) SMCs cultured with HA-free medium, (b) SMCs cultured with high-molecular-weight HA fragments, or (c) SMCs treated with an antibody targeted at the cell-surface HA 4 mer receptor. Since the HA 4 mer receptor has not been confirmed (although strongly suggested to be toll-like receptor 4), blocking these receptors is currently not an option. We have also shown high-molecular-weight HA to not induce elastogenic effects, but via opposite charge interactions, coacervate tropoelastin to enhance its recruitment and crosslinking into matrix structures.

Thus, we evaluated additive-free aneurysmal SMC cultures as negative treatment controls for HA oligomer effect.

Quite different from their effects on healthy RASMCs, addition of HA oligomers alone (0.2 $\mu\text{g}/\text{mL}$) to aneurysmal RASMCs suppressed aneurysmal cell proliferation, promoted tropoelastin and insoluble matrix elastin synthesis (~ 1.2 -fold), and increased collagen and soluble matrix elastin synthesis (~ 1.5 -fold). However, the elastin matrix yield remained similar to that in nonadditive aneurysmal SMC cultures (10.8 ± 0.8 vs. $9.6 \pm 2.1\%$), which might be due to the lack of any significant, parallel increase in desmosine and LOX protein content within these cultures. Interestingly, the production of the MMP-2 and -9 and the levels of elastase activity and matrix calcification in these cultures remained almost similar to that within additive-free (control) aneurysmal cell cultures, and significantly higher than that within additive-free healthy cell cultures. In contrast to their benefits to deposition of a fibrillar rather than amorphous elastin matrix deposition by healthy SMCs,¹⁷ HA oligomers alone provided no particular advantage to the quality of elastin matrix deposited by aneurysmal SMCs, since the elastin matrix was still largely nonfibrillar (not shown). These results lead us to speculate that when HA oligomers alone are provided, much higher doses may be necessary to stimulate aneurysmal RASMCs to enhance elastogenesis and elastin fiber deposition, in a manner that healthy RASMCs respond to when these oligomers are provided at a dose of 0.2 $\mu\text{g}/\text{mL}$.

In a previous study,¹⁸ we found TGF- β 1 (1 ng/mL or ~ 0.2 pg/cell) to significantly suppress healthy RASMC proliferation and increase synthesis of matrix elastin (~ 2.5 -fold) and collagen (~ 1.3 -fold). The predominant effect of TGF- β 1 appears to be the stabilization of elastin mRNA,^{39,40} although our own experiments and those of others suggest that TGF- β 1 modestly enhances tropoelastin mRNA,⁴¹ suppresses production of elastin-degrading metalloproteinases⁴² and elastases,⁴³ enhances mRNA expression for the TIMP-1,⁴⁴ and augments LOX protein synthesis and activity critical to elastin/collagen crosslinking and fiber assembly.⁴⁵

In the present study, we studied the same per cell dose of TGF- β 1 as that tested earlier on healthy RASMCs, on aneurysmal RASMCs. This TGF- β 1 dose was also relevant to study since others^{46,47} have also shown that this dose does not stimulate SMC proliferation in a manner it does at higher doses, preventing undesired, potential hyperplastic outcomes when the factors delivered for targeted *in situ* elastin regeneration within aneurysmal vessels. Our present results show that except for enhancing collagen synthesis (~ 1.3 -fold), TGF- β 1 had no impact on elastin yield within aneurysmal SMC cultures ($11.2 \pm 1.8\%$ vs. $9.6 \pm 2.1\%$ in additive-free aneurysmal cultures; $p = 0.72$), although the same dose of TGF- β 1 enhanced elastin matrix yield in healthy SMC cultures. The lack of elastogenic impact of TGF- β 1 on rat aneurysmal SMCs when provided at doses optimized for healthy rat SMCs suggests attenuated sensitivity of aneurysmal SMCs to these factors and thus the necessity to re-optimize and possibly increase doses of these factors to upregulate elastogenesis by these abnormal cell types. Nevertheless, the lack of elastogenic response of aneurysmal SMCs when induced with TGF- β 1 also appears to correlate with increases in MMPs-2 and 9-, and elastase activity relative to healthy SMC cultures, and greater distribution of nonfibrillar elastin (not shown), suggesting higher elastolytic

activity within these cultures. Although HA oligomers and TGF- β 1 factors, when provided separately, both attenuated MMP/elastase activity within aneurysmal cultures, the net elastolytic activity within these cultures remained greater than in healthy SMC cultures; similarly, soluble ELP thus are more rapidly generated, which would in turn discourage accumulation of a crosslinked elastin matrix.

TGF- β 1 and HA oligomer factors together suppressed proliferation of aneurysmal SMCs, though not as severely as they did to healthy SMCs.¹⁸ The suppression in proliferation of aneurysmal SMCs has vital implications to deterring hyperproliferation of these activated cell types when factors are delivered to an intact aneurysm site. These factors together also enhanced tropoelastin and matrix elastin synthesis and that of collagen matrix, although the level of increase in aneurysmal cell cultures was far less compared to the healthy cell cultures. Thus, our study shows that though HA oligomers and TGF- β 1, provided separately to aneurysmal rat SMC cultures at doses shown to elastogenically stimulate healthy rat SMCs, do not by themselves elicit likewise responses from aneurysmal SMCs, together they are able to increase elastin production both on an absolute and per cell basis.

The elastin crosslinking enzyme LOX is known to juxtapose with tropoelastin on fibulin-5 microfibrils, to activate it by deaminating its lysine residues, and to thus allow it to coacervate onto existing polymers and spontaneously crosslink.⁴⁸ Although LOX can regulate tropoelastin recruitment and crosslinking in this manner, to impact crosslinking efficiency, the extent of crosslink formation itself can be influenced by other factors such as presence of tropoelastin-binding glycosaminoglycans,^{49,50} which can locally coacervate tropoelastin molecules at the cell layer to facilitate their crosslinking by LOX. Prior studies have shown that exogenous TGF- β 1 augments LOX mRNA expression and activity by healthy cells and thus critically enhances elastin and collagen crosslinking and fiber formation.^{51,52} Strong positive correlations between TGF- β and LOX mRNA expression^{51,53} also suggest that enhancing endogenous LOX amounts/activity could in turn stimulate endogenous TGF- β release/receptor activity with subsequent benefits to elastic matrix synthesis and crosslinking. These factors motivated us to compare LOX protein synthesis and activity in control and test cultures. We previously showed HA oligomers and TGF- β to together improve elastin matrix yields in healthy SMC cultures, possibly by enhancing LOX protein synthesis and desmosine-mediated crosslinking.¹⁸ Based on these outcomes, we logically expected similar increases in elastin matrix yields within aneurysmal SMC cultures induced with the same factors, which however was not the case ($10.7 \pm 2.1\%$ yield with factors vs. $9.6 \pm 2.1\%$ for additive-free aneurysmal cultures); this was despite measured as increases in production of the elastin crosslinking enzyme, LOX. Perhaps due to increased LOX production, desmosine content per nanogram of insoluble elastin within these cultures was higher. However, the lack of improvement in matrix yield may not have been due to lack of benefits of the factors to recruitment and crosslinking of tropoelastin precursors, but rather due to innately enhanced elastolytic activity within aneurysmal SMC cultures. Regardless, on a positive note, these elastogenic factors reduced matrix calcification and MMP production by aneurysmal SMCs, results that were not observed when either of these factors was provided alone.

Imaging and ultrastructural analyses of synthesized elastin matrices qualitatively validated biochemical observations. Aneurysmal SMC layers cultured without any factors (and either of the factors alone) contained mostly amorphous elastin clumps, and very few mature fibers (Fig. 9A, B). This was despite the abundant presence of fibrillin microfibrils at the periphery of the amorphous clumps, which signified normal mechanisms of elastin matrix assembly, involving predeposition of a fibrillin scaffold for further deposition of amorphous elastin. When both TGF- β 1 and HA oligomers were provided concurrently, elastic fiber formation was favored (Fig. 9C, D). The matrix contained a visibly much greater number of fully formed fibers than in the treatment controls. Fibrillin microfibrils were localized at the periphery of aggregating elastin fiber bundles, signifying normal elastic fiber assembly.

Conclusions

The outcomes of this study support our hypothesis that HA oligomer and TGF- β 1 factors together can elastogenically upregulate aneurysmal SMCs. Our data suggest that SMCs isolated from induced rat AAs remain activated in culture, and synthesize elastin and elastic matrix, although these amounts are at least one order of magnitude less than that we have previously found to be produced by healthy RASMCs. In addition, the deposited elastic matrix is largely nonfibrillar. Our study also shows that these aneurysmal SMCs can be elastogenically stimulated *in vitro* to produce measurable amounts of elastin over 21 days of culture, although these amounts are still far less than that produced by healthy SMCs when identically stimulated, and deposit a fibrillar elastic matrix. In addition, these factors together also appear to restore these activated SMCs to a healthier phenotype (i.e., inhibited matrix calcification and MMPs production). Overall the data attest to the elastogenic utility of TGF- β 1 and HA oligomeric factors within sites of AAs, though the doses of these factors must be reoptimized and likely increased specifically for the aneurysmal SMCs.

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Disclosure Statement

No competing financial interests exist.

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