Transforming Growth Factor Beta 1 and Hyaluronan Oligomers Synergistically Enhance Elastin Matrix Regeneration by Vascular Smooth Muscle Cells

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Elastin is a vital structural and regulatory matrix protein that plays an important role in conferring elasticity to blood vessel wall. Previous tissue engineering approaches to regenerate elastin *in situ* or within tissue engineering constructs are curtailed by innate poor elastin synthesis potential by adult vascular smooth muscle cells (SMCs). Currently, we seek to develop cellular cues to enhance tropoelastin synthesis and improve elastin matrix yield, stability, and ultrastructure. Our earlier studies attest to the elastogenic utility of hyaluronan (HA)-based cellular cues, though their effects are fragment size dependent and dose dependent, with HA oligomers deemed most elastogenic. We presently show transforming growth factor beta 1 (TGF- β 1) and HA oligomers, when provided concurrently, to synergistically and dramatically improve elastin matrix regeneration by adult vascular SMCs. Together, these cues suppress SMC proliferation, enhance synthesis of tropoelastin (8-fold) and matrix elastin protein (5.5-fold), and also improve matrix elastin yield (45% of total elastin vs. 10% for nonadditive controls), possibly by more efficient recruitment of tropoelastin for crosslinking. The density of desmosine crosslinks within the elastin matrix was itself attenuated, although the cues together modestly increased production and activity of the elastin crosslinking enzyme, lysyl oxidase. TGF- β 1 and HA oligomers together induced much greater assembly of mature elastin fibers than they did separately, and did not induce matrix calcification. The present outcomes might be great utility to therapeutic regeneration of elastin matrix networks *in situ* within elastin-compromised vessels, and within tissue-engineered vascular graft replacements.

Introduction

ELASTIN IS AN IMPORTANT structural component of the extracellular matrix (ECM) of connective tissues (e.g., blood vessels, lung, and skin), which facilitates their elastic recoil and provides the resilience necessary for cyclic distension and contraction.^{1,2} It is a chemically inert, highly hydrophobic, and alkali-insoluble fibrous protein stabilized in the presence of lysyl oxidase enzyme (LOX), by desmosine crosslinking of its soluble precursor—tropoelastin, produced by smooth muscle cells (SMCs).³ In the extracellular space, the newly secreted tropoelastin molecules aggregate as “amorphous component” on preexisting elastic fibers or on bundles of electron-dense long microfibrils (composed of fibrillin and ~76 nm long), which appear to function as scaffolds for elastic fiber assembly.³ A typical growing elastic

fiber thus consists of an amorphous elastin core surrounded by dark microfibrils at the periphery. Elastin forms around 30–50% of dry tissue mass in healthy vascular ECM and serves to maintain SMCs quiescent,⁴ and regulates their migration,⁵ particularly during vascular morphogenesis.⁶ Under inflammatory conditions, elastin gene expression can be down-regulated by factors, such as basic fibroblast growth factor⁷ and tumor necrosis factor- α ,⁸ and also degraded by matrix metalloproteinases⁹ and macrophage-derived elastases to subsequently interrupt elastin-SMC signaling pathways. Such elastin disruption due to inflammatory diseases^{10,11} and direct mechanical injury,¹² or its malformation in congenital and inherited conditions can encourage SMC hyperproliferation and medial thickening, leading to reduced arterial compliance and hypertension.¹³ Compromised elasticity contributes significantly to ageing of connective tissues and in the

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development of aortic aneurysms and emphysema, wherein vessels can weaken and ultimately rupture with fatal consequences. In such cases, vascular elastin must be restored or regenerated as a priority.

Efforts to regenerate lost elastin structures *in vivo* and within tissue-engineered constructs are limited by the progressive destabilization of tropoelastin mRNA expression in adult vascular cells,^{14,15} the current unavailability of appropriate cellular cues for upregulating elastin precursor production and its assembly into structural matrices, that are faithful mimics of native elastin networks.¹⁶ Due to the frequently exaggerated and unnatural cell responses to cues such as those provided by synthetic biomaterial scaffolds, we seek to employ an alternate regenerative strategy based on ECM-derived biomolecular cues, which we expect would be more useful to faithfully regenerate matrices that replicate the functional architecture and mechanics of native elastin. A logical elastin regeneration approach is thus to select elastogenic cues from amongst a subset of ECM molecules that participate in or regulate elastic fiber deposition during development.¹⁷ Glycosaminoglycans (GAGs) represent such classes of molecules, which closely associate with proteoglycans and elastin in pulmonary, vascular, and dermal tissues.^{18,19} Among GAGs, hyaluronan (HA) suggestably plays a key role in elastogenesis through its intimate binding of versican, which in turn interacts with other elastin protein-associated microfibrillar proteins (fibulin-1 and -2) to form macromolecular structures responsible for elastic fiber assembly.^{20,21}

Prior studies have reported on how HA influences cellular functions, such as proliferation, migration, and their inflammatory response, in a manner influenced by HA fragment size.^{22–24} Accordingly, we confirmed that elastin protein production by vascular SMCs is also influenced by HA fragment size²⁵; particularly, HA oligomers in the 4- to 6-mer (756–1220 Da) range increased production of soluble elastin protein precursors (tropoelastin) and crosslinked elastin matrix, and enhanced formation of fibrillin-rich elastic fibers and of elastin matrix stability by increasing desmosine crosslink densities.²² Other studies have shown that elastin matrix synthesis can be modestly enhanced by stimulating vascular SMCs with growth factors, such as transforming growth factor beta 1 (TGF- β 1),^{26,27} at physiologically relevant concentrations, typically in the order of 1 ng/mL [10^{-11} M].²⁸ Also at low doses [~ 1 pg/cell; 1 pg = 10^{-12} g], TGF- β 1 does not stimulate SMC proliferation^{29,30} in a manner it does at higher doses. These guidelines are useful to consider prevention of undesired hyperplastic outcomes if and when such cues are applied in tissue engineering applications that may involve partial or complete elastin regeneration *in situ* within inflamed, elastin-compromised vessels. TGF- β 1 has also been shown to regulate synthesis of other ECM proteins (e.g., collagen and proteoglycans).³¹ Despite their standalone benefits, it is not known if TGF- β 1 and HA fragment cues together will synergistically improve elastin matrix synthesis and assembly, while retaining the benefits (e.g., suppressed cell proliferation and elastin-laminin receptor activity²²) of their standalone delivery.

To assess these aspects, we presently investigate the proelastogenic effects of HA fragments supplemented exogenously to the cultured adult rat aortic SMCs (RASMCs) concurrent with an elastogenically effective dose of TGF- β 1.

Our findings are expected to greatly benefit efforts to regenerate elastin matrix *in situ* within diseased or injured vessels, to stabilize them, and also to augment synthesis and assembly of close mimics of vascular elastin matrices using tissue engineering principles.

Materials and Methods

Cell culture

HA with molecular weights of 2000 kDa, 200 kDa (Genzyme Biosurgery, Cambridge, MA), 20 kDa (Lifecore Technologies, Chaska, MN), and 0.756 kDa, designated as HMW, LMW, VLMW, and oligomers, respectively, were dissolved in sterile serum-free culture medium. HA oligomer mixtures were prepared by testicular hyaluronidase digestion (37°C, 16 h, 160 U/mg) of HMW HA as we previously reported,²² and contained $75 \pm 15\%$ w/w of HA 4-mers, with 6-mers and 8-mers forming the balance.

Adult RASMCs (Cell Applications, San Diego, CA) from passages 3 to 5 were seeded onto six-well tissue culture plates (Becton Dickinson, San Jose, CA; $A = 10 \text{ cm}^2$) at 2×10^4 cells/well. The total volume of medium added per well was 5 mL. A 1 mL bolus of HA/fragments/oligomers prepared in serum-rich Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA; 10% v/v fetal bovine serum) was added to cell cultures at an ultimate dose of 0.2 $\mu\text{g/mL}$. In addition, TGF- β 1 (Peprotech, Rocky Hill, NJ) was supplemented at a final dose of 1 ng/mL. Thus, cells theoretically received an equivalent of 0.25 fg of TGF- β 1/cell, which closely agrees with the dose supplied in prior published studies.³⁰ Other cultures received either HA/fragments/oligomers or TGF- β 1 alone, or no supplements (controls). Spent medium from each well was replaced twice weekly during the duration of culture, pooled, and frozen. At 21 days, these aliquots and their corresponding cell layers were biochemically analyzed.

DNA assay for cell proliferation

The DNA content of cell layers was quantified at 1 day and 21 days of culture using a fluorometric assay described earlier by Labarca and Paigen.³² Briefly, cell layers at 1 day and 21 days of culture were detached with 0.25% v/v trypsin-EDTA, pelleted by centrifugation, resuspended in 1 mL of NaCl/Pi buffer (4 M NaCl, 50 mM Na_2HPO_4 , 2 mM EDTA, and 0.02% Na-Azide, pH 7.4), sonicated for 3 min over ice, and a 100 μL aliquot assayed. Cell numbers were calculated on the basis of an estimated 6 pg DNA/cell.³²

Hydroxyproline assay for collagen

A hydroxyproline (OH-Pro) assay was used to estimate the collagen content within test and control cell layers, and in the supernatant medium fraction, as described in previous publications.²⁵ The measured amounts of OH-Pro were corrected to account for the 4% w/w of OH-Pro contained in solubilized elastin. 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 protein

A Fastin assay (Accurate Scientific and Chemical, Westbury, NY) was used to quantify the total amount of matrix-elastin

protein (present as alkali-soluble and -insoluble fractions), and soluble tropoelastin in lyophilized pooled medium fractions from each well, as we previously described.²⁵ The measured elastin protein amounts were normalized to corresponding DNA amounts to provide a reliable basis of comparison between samples.

Desmosine assay for elastin crosslinks

In select cases, desmosine crosslink densities within elastin matrices were quantified to determine if any of the provided cues enhanced efficiency of crosslinking of elastin molecules. As described previously,²⁵ 21-day-old cell layers cultured with no additives (control), TGF- β 1 alone, or TGF- β 1 together with HMW HA or HA oligomers were digested with collagenase and elastase, the digestates were acid hydrolyzed, the DNA-normalized desmosine content in the reconstituted dried residue was quantified by ELISA, and compared to corresponding trends in amounts of insoluble matrix elastin protein.

LOX enzyme activity

Spent culture medium pooled over 21 days of culture was assayed for LOX activity using a fluorometric assay based on measurement of H₂O₂ released when LOX oxidatively deaminates alkyl monoamines and diamines.³³ H₂O₂ was detected using an Amplex Red[®] kit (Molecular Probes, Eugene, OR).³⁴ The fluorescence intensities were recorded with excitation and emission wavelengths at 560 and 590 nm, respectively.

Western blot analysis for tropoelastin and LOX

Western blot analysis was performed to semiquantitatively confirm observed biochemical trends in tropoelastin synthesis and to assess LOX protein synthesis. As previously detailed,²⁵ spent medium from cultures, pooled at biweekly over 21 days of culture, was lyophilized; assayed for protein content; protein bands detected with primary rabbit anti-rat polyclonal antibodies to elastin protein (Elastin Products, Owensville, MO) and the 31-kDa active LOX protein (Santa Cruz Biotechnology, Santa Cruz, CA); and visualized in a Chemi-Imager IS 4400 system (Alpha Innotech, San Leandro, CA).

RT-PCR for elastin mRNA expression

RNA was extracted from RASMC cultures using an RNeasy kit (Qiagen, Valencia, CA), and samples were loaded onto RNeasy mini columns (Qiagen). After DNase treatment, total RNA was eluted with 30 μ L of RNase-free water, and its concentration determined by absorbance measurement at 260 nm, and stored at -80°C . cDNA was transcribed with Omniscript Reverse Transcriptase (Qiagen) using 1 μ g of DNase-treated total RNA and random hexamer primers (Roche, Nutley, NJ). Samples (20 μ L) were incubated (42°C ; 50 min) and then reverse transcriptase (RT) inactivated (70°C ; 15 min). RT semiquantitative PCR (RT-PCR) was performed in the ABI Prism 7700 sequence detection system (PE Applied Biosystems, Foster City, CA) using GoTaq DNA polymerase and PCR nucleotide mix (Promega, Madison, WI). Reaction mixtures (20 μ L) contained 1 μ L cDNA, 18 μ L of master mix (Promega), and 1 μ L of appropriate primers.

Optimal primer sets were designed for each target gene using Primer Express (PE Applied Biosystems) and concentration optimized. DNA sequences were obtained from GenBank (www.ncbi.nlm.nih.gov), and products <150 bp with melting temperatures of $55\text{--}65^{\circ}\text{C}$ were selected. For gene expression, we used the primer sequences for elastin rattus: 5'-CCTGGTGGTGTTACTGGTATTGG-3' (forward) and 5'-CCGCCTTAGCAGCAGATTTGG-3' (reverse). For each sample, gene expression was normalized to control samples (cells in medium alone), and the reaction success was compared to expression of the GAPDH housekeeping gene.

Amino acid analysis

OH-Pro and amino acid (AA) contents were evaluated in protein hydrolysates (18 h in 6 M HCl at 110°C) of respective cell layers using liquid chromatography/mass spectrometry performed via methods described previously,³⁵ but modified to detect desmosine in tandem mass spectrometry (MS) mode.

Immunofluorescence detection of elastin protein, fibrillin, and LOX

As we described previously,²⁵ immunofluorescence techniques were used to confirm the presence of elastin protein within cell layers, and LOX expression for those treatments that appeared to upregulate elastin protein synthesis. Fibrillin presence within the cell layers was also verified to confirm whether the process of elastin matrix formation was mediated by predeposition of a fibrillin scaffold on which amorphous elastin protein was deposited to form a composite fiber. At 21 days, the cell layers were fixed with 4% w/v paraformaldehyde for 10 min, and labeled with Alexa 488 Phalloidin (Molecular Probes; 1:20 dilution; 20 min, 25°C), a marker for SMC actin, and then incubated with blocking serum (5% donkey serum, 0.3% Triton-100 in phosphate-buffered saline; 20 min; 25°C). Elastin and fibrillin proteins detected with respective polyclonal antibodies (Elastin Products) as well as LOX (Santa Cruz Biotechnology) were visualized with a rhodamine-conjugated donkey anti-rabbit IgG secondary antibody (Chemicon, Temecula, CA).²⁵ Cell nuclei were visualized with the nuclear stain 4',6'-diamino-2-phenylindole dihydrochloride (DAPI) contained in the mounting medium (Vectashield; Vector Labs, Burlingame, CA).

Matrix ultrastructure

Transmission electron microscopy (TEM) was used to characterize the ultrastructure of the elastin matrix. Control and test cell layers were fixed with 2.5% v/v glutaraldehyde, postfixed in 1% v/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 visualized on a Hitachi H7600T TEM.²⁵

Statistical analysis

All biochemical data were obtained from nine independent repeat data per case, and were analyzed using Student's *t*-test and one-way ANOVA, assuming unequal variance and differences deemed significant for $p < 0.05$. The data were observed to follow a near-Gaussian distribution in all the cases, and the mean and standard deviation were calculated

FIG. 1. Effect of HA fragments and TGF- β 1 on RASMCs. Proliferation ratios represent DNA content at 21 days relative to day 1 of culture. Values shown represent mean $\pm$ SD of nine repeat samples/case. Differences versus controls were significant (*) for $p < 0.05$.

accordingly. For immunolabeling and TEM, test and control samples were analyzed at least in triplicate.

Results

Cell proliferation

Figure 1 shows proliferation of RASMCs in the presence of TGF- β 1 or HA/fragments, alone or together. Over 21 days of culture, nonadditive control cells proliferated 3.4 ± 0.5 times the original seeded cell number, while addition of TGF- β 1 suppressed this ratio to 0.5 ± 0.1 times ($p = 0.005$ vs. controls). When HA oligomers alone were present, the proliferation ratio was modestly lower than controls ($p = 0.02$ vs. control). Except in the presence of HMW HA, concurrent delivery of TGF- β 1 and HA fragments inhibited cell proliferation to extents that inversely correlated to HA size.

Matrix synthesis

Relative to collagen matrix synthesis in control cultures (647 ± 21 ng/ng of DNA), TGF- β 1 alone inhibited the same by 0.5 ± 0.2 -fold ($p = 0.009$ vs. control; Fig. 2A), while HA oligomers alone induced a marginal 1.2 ± 0.2 -fold increase ($p = 0.12$ vs. controls). However, when TGF- β 1 was provided together with HA oligomers, collagen matrix synthesis was upregulated drastically (14 ± 3 -fold). Concurrent delivery of TGF- β 1 and nonoligomeric HA inhibited collagen synthesis in inverse correlation to HA fragment size (Fig. 2A). The treatment-specific trends in synthesis of collagen matrix were identical to their differences in total collagen output (pooled medium and matrix fractions). Figure 2B shows fold changes in total collagen production relative to nonadditive control cultures (22059 ± 668 ng/ng of DNA).

TGF- β 1 or HA oligomers alone stimulated tropoelastin production in controls (3.9 ± 0.9 μ g/ng of DNA) by 1.2 ± 0.3 - and 1.5 ± 0.2 -fold, respectively ($p = 0.12$ and 0.01 vs. controls; Fig. 3A). When provided together with TGF- β 1, HA oligomers stimulated an 8.0 ± 0.1 -fold increase in tropo-

FIG. 2. Impact of HA fragments and TGF- β 1 on (A) collagen matrix protein synthesis and (B) total collagen output (pooled medium and matrix fractions). Cells were cultured for 21 days. Data shown represent mean $\pm$ SD of nine repeats/case and are shown normalized to controls. Differences versus controls were significant (*) for $p < 0.05$.

elastin synthesis relative to controls ($p < 0.001$), while HMW HA inhibited the same to a 0.4 ± 0.05 fraction of control values ($p = 0.001$ vs. controls); nonoligomeric HA had no significant effect on tropoelastin output. Western blot analysis of tropoelastin in pooled spent medium confirmed these trends observed from biochemical assay (Fig. 3B).

Elastin protein incorporated into the deposited matrix was measured as a sum of a highly crosslinked alkali-insoluble fraction, and an alkali-soluble fraction. The total DNA-normalized output of soluble matrix elastin protein (Fig. 3C) broadly mirrored HA fragment size-specific trends reported for tropoelastin. While TGF- β 1 alone or with HA/fragments had no significant effect relative to controls (3403 ± 121 ng/ng of DNA), HA oligomers induced 8 ± 0.2 -fold increase in soluble matrix elastin protein synthesis ($p < 0.001$ vs. controls). Relative to controls, synthesis of alkali-insoluble, cross-linked elastin protein in the matrix was enhanced 4.4 ± 0.1 -fold by TGF- β 1 alone (Fig. 3D), and was further increased to 5.5 ± 0.4 - and 4.5 ± 0.4 -fold on addition of HA oligomers and VLMW HA, respectively ($p < 0.001$ vs. controls for all these cases); HMW HA, however, significantly inhibited deposition of crosslinked elastin protein relative to controls ($p < 0.01$).

As shown in Figure 3E, relative to nonadditive control cell layers (28 ± 4 pg desmosine/ng of DNA), cells cultured with TGF- β 1 alone showed modest, but significant, increases in desmosine synthesis (1.07 ± 0.03 -fold; $p < 0.02$ vs. controls), while TGF- β 1 together with HA oligomers (or HMW HA) significantly inhibited desmosine synthesis (0.71 ± 0.02 -fold; $p < 0.01$ vs. controls).

Elastin mRNA expression

RT-PCR studies showed TGF- β 1 and HA oligomer cues to synergistically upregulate elastin mRNA expression by 1.52 ± 0.1 -fold versus control cultures ($p = 0.001$), more than that induced by TGF- β 1 alone (1.14 ± 0.07 -fold; $p = 0.03$ vs. controls) or HA oligomers alone (1.05 ± 0.06 -fold; $p = 0.07$ vs. controls).

LOX functional activity and protein expression

Relative to control cultures, addition of TGF- β 1 significantly inhibited cellular LOX activity ($p < 0.001$; Fig. 4A), while HA oligomers with or without TGF- β 1 had no significant effect ($p = 0.7$ and 0.42 vs. controls). Again, HMW HA had no effect on LOX activity ($p = 0.12$ vs. controls), though marginal inhibition was noted when TGF- β 1 was also provided ($p = 0.025$ vs. controls). Western blot analysis of LOX protein exhibited trends similar to that for synthesis of matrix elastin protein (Fig. 4B). TGF- β 1 alone or together with HA oligomers stimulated 1.7 ± 0.1 - and 2.2 ± 0.1 -fold increases, respectively, in LOX protein amounts compared to controls ($p < 0.001$ vs. controls in both the cases); larger HA fragments had no effect. HMW HA and TGF- β 1 together inhibited control levels of LOX synthesis by $40 \pm 7\%$ ($p < 0.001$ vs. controls).

FIG. 3. Effect of exogenous HA fragments and TGF- β 1 alone and together on synthesis of tropoelastin (A: Fastin assay; B: SDS-PAGE), alkali-soluble (C), and -insoluble elastin matrix proteins (D). Case-wise trends for DNA-normalized amounts of desmosine mirrored that in insoluble matrix elastin protein (E). DNA-normalized data represent mean $\pm$ SD of nine repeats/case, and are shown as fold change compared to controls. Differences versus controls were significant (*) for $p < 0.05$.

FIG. 4. (A) LOX enzyme activities in test cultures at 21-day culture periods. (B) SDS-PAGE/Western blots showed that TGF- β 1 alone or together with HA oligomers/fragments enhanced LOX protein synthesis. All the data are shown normalized to LOX activities in controls. Data shown represent mean $\pm$ SD of nine repeats/case and are shown normalized to controls. Differences versus controls were significant (*) for $p < 0.05$.

AA analysis of elastin

AA analysis was performed on insoluble crosslinked elastin protein isolated from control cell layers, and those that received cues deemed to be most elastogenic (TGF- β 1 and HA oligomers). The AA profile in cultured elastin protein closely matched that previously reported for aortic elastin protein isolated from rats of different strains. The AA content of elastin protein cultured in the presence of elastogenic cues and in their absence was dominated by the non-polar AAs, glycine, alanine, proline, and valine (65% vs. 63.5%, respectively), similar to that described in literature (~71%).^{36,37} Desmosine (Des) and isodesmosine (Ide), vital but minor constituents of crosslinked elastin protein,³⁸ were barely resolved in the present analysis, while hydroxyproline (OH-Pro), which is present in elastin protein as a minor constituent (<3%), was sparingly detected (0.5%) as expected.

Immunofluorescence studies

All cultures (controls, TGF- β only, and TGF- β + oligomers) achieved confluence by day 21 of culture at which time

they were immunolabeled. However, cell densities in noncontrol cultures were visibly lower than in the control cultures (phase contrast images in Fig. 5A). Immunofluorescence micrographs of 21-day-old cell layers (Fig. 5; panel B) consistently showed significantly higher amounts of elastin protein and fibrillin (red fluorescence) in cultures that received either TGF- β 1 alone or TGF- β 1 and HA oligomers, than those that received HMW HA fragments and TGF- β 1 (not shown), or no additives (control). Although elastin distribution within test and control cell layers was mostly uniform, occasional clumps of higher cell density were seen, which corresponded to much greater elastin localization at such sites (Fig. 5D, E). However, LOX distribution within all test and control cell layers was fairly sparse. Cell layers untreated with primary antibodies (negative controls) exhibited no red fluorescence, confirming lack of nonspecific binding of the fluorophore-conjugated secondary antibody (Fig. 5C).

Ultrastructure of matrix elastin

Figure 6 shows representative transmission electron micrographs of elastin matrices from 21-day cultures. As did HA oligomers alone, TGF- β 1 stimulated deposition of multiple layers of elongated, aggregating elastin fibrils uniformly sandwiched between alternating cell layers, different from the discrete clumps of amorphous elastin protein that were distributed within control cell layers. When both TGF- β 1 and HA oligomers were provided together, elastin fiber formation was likewise favored, though the matrix contained much greater number of fully formed fibers (300–400 nm diameter) than in cultures provided with HA oligomers alone. Fibrillin (immunogold particle stained), which appeared in transverse sections as darkly stained nodules, was located at the periphery of aggregating elastin fiber bundles, signifying normal elastic fiber assembly.

Discussion

In a series of prior publications,^{22,25,39} we showed that HA upregulates elastin precursor (tropoelastin) and matrix synthesis by adult RASMCs. Specifically, HA oligomers enhanced elastin matrix assembly, maturation, and stability.²² Although larger HA fragments and long-chain (HMW) HA did not enhance tropoelastin synthesis, they enhanced elastin matrix deposition, possibly by coacervating soluble tropoelastin via opposite charge interactions,⁴⁰ which supports our prior hypotheses that HA indirectly facilitates elastogenesis.^{20,21} Other studies have shown that the cytokine TGF- β 1 modestly enhances tropoelastin mRNA,⁴¹ suppresses production of elastin-degrading metalloproteinases⁴² and elastases,⁴³ enhances mRNA expression for the tissue inhibitor of metalloproteinase (TIMP-1),⁴⁴ and augments LOX mRNA expression and activity critical to elastin/collagen cross-linking and fiber assembly.⁴⁵

Thus, we presently sought to investigate if concurrent delivery of HA fragments and TGF- β 1 can synergistically enhance tropoelastin synthesis and matrix assembly, fiber formation, and stability beyond that possible with either of the cues alone, while maintaining SMCs quiescent (non-proliferative) as in healthy vessels. Previously, we showed large HA fragments (20 and 200 kDa) to modestly increase cell proliferation in dose-independent manner,²⁵ possibly as

fluoresce blue. Immunofluorescence images are typical of ≥ 3 cultures/case. All cultures were imaged under identical conditions. Magnification: 20 $\times$ (panels A, D, and E) and 40 $\times$ (B, C). Color images available online at www.liebertonline.com/ten.

FIG. 6. TGF- β 1 and HA oligomers alone, or together, promote elastin fiber formation, as seen in transmission electron micrographs. Control cultures contained only amorphous clumps. Elastin was assembled as fibrils within a peripheral fibrillin scaffold (see black dots) and aggregated to form mature fibers ~ 400 nm in diameter, typical of fully formed fibers.

a downstream effect of phosphorylating the primary CD44 cell surface HA receptor,⁴⁶ while HA oligomers (2 μ g/mL) did not have any effect.²² Here we show 0.2 μ g/mL doses of HA oligomers to significantly inhibit SMC proliferation, indicating dose specificity of cell responses to these oligomer cues. We have also determined that 0.2 μ g/mL is the minimum dose of HA oligomers that induces a perceptible increase in tropo- and matrix elastin synthesis, though these effects are highly attenuated relative to a 2 μ g/mL dose.

Prior studies demonstrated that TGF- β 1 (~1 ng/mL) modestly upregulates elastin matrix synthesis by vascular SMCs.^{26,27} The stimulatory effect of TGF- β 1 on elastin protein synthesis, both at the mRNA and protein levels, has been demonstrated in various *in vitro* models.⁴⁷ The predominant effect of TGF- β 1 appears to be the stabilization of elastin mRNA,^{48,49} although a TGF- β 1-responsive element has been identified in the human elastin promoter.^{50,51} Though our current findings that TGF- β 1 (1 ng/mL, 0.25 fg/cell) inhibits cell proliferation confirm these prior observations,²⁹ it is interesting that HA fragments/oligomers, when concurrently provided, maintain these trends. It is likely that the respective cellular response-initiating mechanisms of these cues operate differently, with HA fragments/oligomers and TGF- β 1 binding to different cell surface receptors. In contrast, TGF- β 1 has no effect on cell proliferation in the presence of long-chain HMW HA. We believe this might be due to long-chain HA forming a pericellular coat to isolate cells from each other and from signaling biomolecules.⁵² Regardless, the utility of HA fragments/oligomers and TGF- β 1 together to attenuation of SMC hyperproliferation is clear.

Treatment-specific trends in cellular matrix protein synthesis are indicated in Table 1. Biochemical analyses showed that total cellular elastin protein output, as represented by the sum of tropoelastin and matrix elastin, was modestly enhanced by TGF- β 1 and was indicated to be at least partly due to upregulation of elastin mRNA. HA oligomers (0.2 μ g/mL) modestly enhanced tropoelastin synthesis (48 $\pm$ 23%), though these effects are likely elicited via posttranslational mechanisms (no increase in elastin mRNA). HA oligomers (0.2 μ g/mL) had no impact on synthesis of matrix elastin protein on a per cell basis, although we earlier showed doses of 2 μ g/mL to double elastin matrix production.²² Thus, elastin matrix synthesis by RASMCs positively correlates to HA oligomer dose, unlike HMW HA or larger HA fragments, as previously demonstrated.²⁵ We also showed that

HA oligomers enhance LOX production but not activity, and enhance desmosine crosslinking relative to controls. Collectively, our data indicate that at the tested dose, HA oligomers do not particularly increase recruitment of tropoelastin (i.e., yield of matrix from elastin precursors), as evidenced by lack of effect on elastin matrix amounts, but facilitate more robust crosslinking of generated matrices. In contrast, TGF- β 1 appears to specifically facilitate tropoelastin recruitment for crosslinking and matrix assembly, as borne out by increases in the ratios of matrix to total (i.e., matrix + tropo) elastin over controls (Fig. 3). Possibly, improved tropoelastin recruitment and crosslinking may be achieved by the observed TGF- β 1-induced increases in LOX protein synthesis and activity. TGF- β 1 does not appear to enhance the extent of crosslinking itself, since desmosine amounts on a per cell basis remained unchanged relative to controls. Possibly due to the exclusive nature of long-chain HA, concurrently provided TGF- β 1 did not have any impact on desmosine crosslinking.

TGF- β 1 and HA oligomers synergistically enhanced tropoelastin synthesis almost ninefold. Since these cues only upregulated elastin mRNA expression by ~1.6-fold, not commensurate with total tropoelastin synthesis, it is possible that these cues also impact cells posttranslationally, besides upregulating elastin mRNA expression. These cues together also greatly improved elastin precursor recruitment and crosslinking efficiency, as evidenced by multifold increases in both soluble and insoluble matrix elastin relative to controls, and of matrix to total elastin ratios (0.45 vs. 0.1 for controls). Further studies suggested that the improved crosslinking efficiency is possible, since a ~2.2-fold induction in LOX production was induced, though LOX activity itself remained unchanged. These upregulatory effects were not observed when TGF- β 1 and long-chain HA or non-oligomeric HA fragments were concurrently delivered. While the lack of effects observed with long-chain HA may be sourced to its ability to isolate cells from TGF- β 1, differences in results obtained with large HA fragments and oligomers are likely due to differences in their interactive mechanisms with cells. HMW HA and nonoligomeric HA fragments primarily interact with CD44, to intracellularly regulate cell proliferation, motility,⁵³ and other cell activities. Differently, HA oligomers likely interact via toll-like receptors 4 (TLR-4) and by CD44 clustering, to elicit responses quite different from those by larger HA fragments.²³ Since our oligomer mixtures here contain mostly 4-mers (75%

TABLE 1. SUMMARY OF TREATMENT-SPECIFIC TRENDS IN PROLIFERATION OF ADULT RAT AORTIC SMOOTH MUSCLE CELLS AND IN MATRIX PROTEIN SYNTHESIS

Biochemical outcomes/ng DNA	TGF- β 1 addition				
	No HA	HA oligomers	VLMW HA	LMW HA	HMW HA
Proliferation ratio	=	↓↓	=	=	↑
Total collagen	↑	↑↑	↑	↑	↓
Tropoelastin	=	↑↑	=	=	↓
Matrix elastin	↑	↑↑	↑	↑	↓
Total elastin	↑	↑↑	↑	↑	↓

↑ and ↑↑ indicate moderate but significant and dramatic (>fivefold) increases, respectively; = indicates no significant changes, while ↓ and ↓↓ indicate likewise decreases in the biochemical outcomes; all measured on a per ng of DNA basis.

w/w) and 6-mers, likely, both CD44 and TLR-4 together elicit unique cell responses. Surprisingly, an approximately sixfold increase in desmosine crosslinks per nanogram of DNA induced by HA oligomers (twofold increase/ng of elastin matrix)²² was restored to baseline control levels upon concurrent delivery of TGF- β 1. Thus, TGF- β 1 may competitively inhibit HA oligomer-induced effects by preferentially interacting with cell surface proteins that modulate intracellular mechanisms that lead to desmosine crosslink formation. A future challenge is to dramatically enhance LOX activity and production to enhance desmosine crosslinking efficiency and thus matrix formation.

Sustained overexpression of TGF- β 1 can potentially induce matrix calcification.⁵⁴ However, Von-Kossa staining of 21-day-old cell layers (not shown), cultured with the elastogenic bolus of TGF- β 1 and HA oligomers, indicated no calcium accumulation. Thus, the 1 ng/mL dose (i.e., 10^{-11} M) of TGF- β 1 delivered in this study is elastogenically useful and yet safe so as to not induce matrix calcification.

Imaging and ultrastructural analyses of synthesized elastin matrices qualitatively validated biochemical observations. Bundles of elastin fibrils distributed between cell layers were seen in HA oligomer-supplemented cultures. In contrast, we previously showed cells cultured with large HA fragments and HMW HA to deposit amorphous elastin clumps, as did control cultures in the present study. The fibrillar elastin generated was continuous and associated laterally with surrounding fibrils to form larger and thickened elastin fibers. The abundant presence of fibrillin microfibrils at the periphery of the amorphous clumps confirmed that elastin matrix assembly, involving predeposition of a fibrillin scaffold for deposition of amorphous elastin, was maintained. Similar to HA oligomers, TGF- β 1 stimulated deposition of multiple layers of elongated, aggregating elastin fibrils and vastly different from the amorphous elastin clumps seen in controls. When both TGF- β 1 and HA oligomers were provided concurrently, elastin fiber formation was likewise favored, though the matrix contained a visibly much greater number of fully formed fibers than present in cell layers that received oligomers alone. As in oligomers- or TGF- β 1-only cultures, here too, fibrillin microfibrils were localized at the periphery of aggregating elastin fiber bundles, signifying normal elastic fiber assembly.

Conclusions

In summary, the outcomes demonstrate that TGF- β 1 and HA oligomers synergistically and dramatically improve elastin matrix protein regeneration by adult vascular SMCs, a cell type otherwise capable of only minimal elastin turnover. These synergistic effects are particularly striking since the HA oligomers themselves were of such low doses to impact elastin synthesis very modestly. Regardless, HA oligomers and TGF- β 1 cues together inhibit cell proliferation; upregulate synthesis of tropo- and matrix elastin proteins; and improve tropoelastin recruitment for matrix assembly and crosslinking efficiency, likely via increased LOX production.⁵⁵ In addition, these cues hasten maturation of elastin fibers relative to cultures receiving either of these cues alone. Importantly, sustained delivery of elastogenic doses of TGF- β 1 (1 ng/mL) and HA oligomers does not

appear to induce matrix calcification. These cues are of tremendous utility to restore elastin matrix homeostasis in de-elasticized vessels and tissue engineering constructs, and possibly even serve as an *in vitro* model to investigate elastogenesis during early morphogenesis and wound healing in adult tissues.

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