Published in final edited form as:

Acta Biomater. 2014 January ; 10(1): 163–172. doi:10.1016/j.actbio.2013.08.042.

Composition of Intraperitoneally-Implanted Electrospun Conduits Modulates Cellular Elastic Matrix Generation

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Abstract

Improving elastic matrix generation is critical to developing functional tissue engineered vascular grafts. Therefore, this study pursued a strategy to grow autologous tissue *in vivo* by recruiting potentially more elastogenic cells to conduits implanted within the peritoneal cavity. The goal was to determine the impacts of electrospun conduit composition and hyaluronan oligomer (HA-o) modification on the recruitment of peritoneal cells, and their phenotype and ability to synthesize elastic matrix. These responses were assessed as a function of conduit intraperitoneal implantation time. This study showed that the blending of collagen with poly(ϵ -caprolactone)(PCL) promotes a faster wound healing response, as assessed by trends in expression of macrophage and smooth muscle cell (SMC) contractile markers and in matrix deposition, compared to the more chronic response for PCL alone. This result, along with the increase in elastic matrix production, demonstrates the benefits of incorporating a little as 25% w/w collagen into the conduit. In addition, PCR analysis demonstrated the challenges in differentiating between a myofibroblast and a SMC using traditional phenotypic markers. Finally, the impact of the tethered HA-o is limited within the inflammatory environment, unlike the significant response found previously *in vitro*. In conclusion, these results demonstrate the importance of both careful control of implanted scaffold composition and the development of appropriate delivery methods for HA-o.

Keywords

Elastin; Peritoneal Cavity; Electrospinning; Collagen; Hyaluronan; Vascular Grafts

1. Introduction

Surgical options for coronary heart disease are currently limited in more than 30% of patients due to unavailability of suitable autologous vessels for bypass grafting [1]. While tissue engineered grafts have been investigated as potential alternatives, several challenges have yet to be overcome. One challenge is the poor generation of mature elastic matrix by

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most adult cells [2, 3]. Elastic matrix in healthy arteries enables both vessel recoil after stretch and regulation of smooth muscle cell (SMC) phenotype [4]. Mature elastic matrix within engineered vascular grafts can serve a similar role, and critically determine graft mechanics after degradation of the initial scaffold material. Strategies have been developed to improve the inherently limited cellular production of elastic matrix (i.e. elastogenesis) [5], including stimulation by tetramers of hyaluronan (HA-o), transforming growth factor β [6, 7], and insulin-like growth factor 1 [8]. While these factors improved elastogenesis, and can be incorporated within grafts, long-term small-diameter graft viability can still be compromised unless more mature elastic matrix is assembled within the three-dimensional constructs. A second challenge is the inflammatory response that engineered constructs elicit post-grafting, which must be controlled. While inflammation is necessary for wound healing and tissue remodeling, a chronic response, or one that is too severe, can compromise these processes [9]. For vascular tissues, the consequences of a negative healing response can include graft occlusion via thrombosis [10] and tissue disruption from chronic cellular overexpression of proteolytic enzymes [11].

We have sought to address the above limitations of tissue engineered grafts by recruiting a potentially more elastogenic, autologous cell population within the peritoneal cavity. Most elastin in the body is generated during development, within microenvironments rich in stem and progenitor cell populations [12, 13]. In addition, it has been recently shown that cell therapy with stem cells can improve elastic matrix production [14]. Thus, we hypothesize that stem or progenitor cell populations recruited from the peritoneal fluid to implanted conduits would represent a more elastogenic cell source than terminally-differentiated cells isolated from adult vascular tissues [15]. Growing tissue within the peritoneal cavity can also provide additional benefits, including avoiding the high cost and extended culture times required for *in vitro* manipulation of seeded cells. Finally, since the initial wound healing response to the construct may subside while still in the peritoneal cavity, this strategy also has the potential to minimize inflammation, and potential thrombosis, after subsequent arterial grafting of the constructs.

Intraperitoneal implantation of silicone tubes has been previously performed to generate constructs that contain a mixture of cell types (e.g. macrophages, mesothelial cells, and myofibroblasts) [15–19], and exhibit short-term viability after grafting into arteries. However, limited elastic matrix production was observed within these constructs, both in rat and dog models. Despite these findings, the recruited peritoneal cells may still exhibit enhanced elastogenicity if the microenvironmental cues (e.g. conduit composition and architecture) are carefully controlled. In fact, elastic matrix production *in vitro* has been shown to be significantly modulated by scaffold composition (e.g. fibrin for SMCs [20]). Most of these *in vitro* studies utilized terminally differentiated cells, and single-cell types, unlike the mixed cell population recruited within the peritoneal cavity. Certain sub-populations of peritoneal cells have a greater potential for differentiation and inter-cell signaling [18], but it is not known how scaffold / conduit composition would modulate tissue generation in this complex microenvironment.

In this study, we specifically determined the impact of incorporating collagen with poly(ϵ -caprolactone)(PCL) into electrospun conduits, and the effects of tethering elastogenic HA-o

to the conduit surface, on (a) the wound healing response within the peritoneal cavity and (b) the extracellular matrix (ECM) production. Collagen was specifically selected for incorporation since it is a dominant component of vascular ECM, and since it is an enzymatically degradable structural protein with specific cell binding sequences. While collagen-rich matrices have been previously shown to influence the general wound healing process [9, 21], its impact may differ when blended with a synthetic polymer and implanted within the peritoneal cavity. In this study, PCL conduits with and without incorporated collagen, and with and without immobilized HA-o, were implanted in rat peritoneal cavities for 2, 4, or 6 weeks. We determined the impact of the conduit composition on matrix accumulation and distribution, particularly the elastic matrix, and on recruited peritoneal cell phenotype and differentiation.

2. Materials and Methods

2.1 Materials

All disposables, chemicals, and biological supplies were obtained from VWR International (West Chester, PA) unless specified otherwise. PCL (inherent viscosity 1.0 – 1.3 dL/g in chloroform) was from Lactel Absorbable Polymers (Pelham, AL), and acid-solubilized collagen (derived from calf skin) was from Elastin Products Co., Inc. (Owensville, MO). All antibodies were from Abcam (Cambridge, MA), unless specified otherwise. Hyaluronan oligomers (HA-o) were prepared by digestion of high molecular weight hyaluronan (1,500 kDa, Sigma-Aldrich, St. Louis, MO) with testicular hyaluronidase (Sigma-Aldrich), using a method we have published previously [22]. The digestate contained primarily HA-4 mers (83.9%, MW 657 Da) with the remainder as HA-6 mers, as determined with fluorophore-assisted carbohydrate electrophoresis.

2.2 Electrospinning and Surface Modification of Conduits

2.2.1 Electrospinning—Electrospinning was performed from a 22% w/v solution of a collagen/PCL blend (25/75% w/w) in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) to generate conduits that present cell binding sequences, and exposed primary amines that can be used for biomolecule functionalization. Electrospinning was performed using a 15 kV voltage gradient, 0.8 mL/h flow rate, 22 gauge needle, and 15 cm throw distance. Control conduits containing PCL alone were electrospun from a solution of 22% w/v poly(ϵ -caprolactone) (PCL) in 90% v/v chloroform and 10% v/v dimethylformamide. HFIP was avoided as a solvent for the conduits containing only PCL because of its toxicity, but it was required for the blend conduits since it is a good solvent for both components. For the PCL conduits, electrospinning was performed as described above, except for the use of an 11 kV voltage gradient, which was necessary to prevent charging, and a higher 3 mL/h flow rate. Electrospinning was performed for 7–30 min for the PCL solution, and 15 min – 1 h for the collagen/PCL solution, onto an aluminum rod with a 1.6 mm diameter. A longer electrospinning time was necessary for the collagen/PCL blend to compensate for the lower flow rate and generate conduits with thick walls. However, the blend conduits still had thinner walls than the PCL ones (i.e. 157 ± 72.1 and 428 ± 139 μ m, respectively). As described previously [7], the rod was both rotated slowly (i.e. < 300 rpm) and moved laterally to maintain a consistent thickness throughout the circumference of the conduit and

along the length of the drum. The conduits were removed from the rod after electrospinning, with ethanol if necessary, cut into 1 cm long sections, and stored in a desiccator until further use.

2.2.2 Modification with HA-o—The electrospun conduits described in Section 2.2.1 were crosslinked using a procedure adapted from Croll et al. [23]. Briefly, the conduits were initially wetted with isopropanol, and then washed with a zero length crosslinking solution – 200 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Thermo Scientific, Rockford, IL), 100 mM N-hydroxysuccinimide (NHS; Thermo Scientific), and 10 mM trisodium citrate (pH 5.0, Sigma). The conduits were then reacted for 2 h with crosslinking solutions containing either 2.5 mg/mL of HA-o, or no HA-o (negative control). Afterward, the conduits were washed in phosphate buffered saline (PBS) then deionized water (2 times, 5 min each) prior to lyophilization. The mass and length of the dry conduits were measured prior to implantation.

2.3 Characterization of Electrospun Conduits

2.3.1 Size and Alignment of Electrospun Fibers—Conduit wall thickness was determined from brightfield images of cross-sections of the conduits obtained using an Olympus IX51 microscope (Olympus Corp., Center Valley, PA). The average diameters and degree of orientation of the electrospun fibers were determined from scanning electron images (SEM). For SEM, the electrospun conduits were mounted onto aluminum stubs with their outer surface exposed, sputter-coated, and then imaged in a JEOL JSM 5310 (Peabody, MA) with a working distance of 10 mm and operating at 15kV. Analysis of the SEM and light microscopy images was performed with ImagePro Plus[®] software (Media Cybernetics, Bethesda, MD). The degree of orientation was characterized by angular standard deviation (ASD), where a reduced angular standard deviation is indicative of a conduit containing more aligned fibers [24].

2.3.2 Confirmation of Collagen Incorporation and HA-o Immobilization—The presence of collagen within the blend conduits was confirmed with energy-dispersive x-ray spectroscopy (EDS). Samples were sputter-coated, mounted in a FEI Quanta 200 3D SEM (Hillsboro, OR), and elemental analysis was performed with an EDS detector. The accelerating voltage was maintained at 15 kV, and the spot size and time constant were kept constant. The presence of collagen was determined by an N1s peak in EDS spectra and in elemental maps produced in NSS 3[®] software (Thermo Scientific). For the detection of HA (i.e., HA-6 mers or crosslinked HA-4 mers), the electrospun meshes were labeled first with biotinylated HA binding protein (HABP; bovine nasal cartilage, EMD Chemicals Inc, San Diego, CA), then reacted with a streptavidin conjugated Alexa 633 probe (Invitrogen, Grand Island, NY), and finally visualized with an Olympus IX51 microscope.

2.4 Intra-Peritoneal Implantation of Electrospun Conduits

Prior to intra-peritoneal implantation, 3 conduits of the same condition (i.e. polymer composition and with and without HA-o immobilization) were enclosed within poly(tetrafluoroethylene) (PTFE) pouches. These pouches were used to prevent the formation of adhesions to the surrounding peritoneal tissue, as we have described previously

[19]. The pouches and enclosed conduits were ethylene oxide-sterilized and intra-peritoneally implanted into male Sprague-Dawley rats (200–250 g; Charles River Laboratories, Wilmington, MA), as described previously [19]. All animal procedures were in compliance with the NIH Guide for Care and Use of Laboratory Animals and approved by the Cleveland Clinic IACUC. Briefly, a ventral laparotomy was performed and two pouches were inserted into each rat. The muscle and skin layers were closed and the animals were allowed to recover. The pouches were removed after 2, 4, or 6 weeks and the tissue constructs within were processed for analysis. The constructs were stored in RNeasy[®] buffer (Qiagen, Valencia, CA) for PCR, flash-frozen in liquid nitrogen for biochemical assays, and mounted in OCT (Tissue-Tek, Torrance, CA) for sectioning.

2.5 Harvest of Abdominal Aortae for Control Cells

At the time of intra-peritoneal implant removal, aortae were also harvested from the same rats. A segment of the abdominal aorta was exposed, clamped, and excised. The aortic segments were quickly stored for PCR in RNeasy[®] buffer to avoid RNA degradation, and processed the same as the peritoneal implants (see Section 2.6). Due to the rapid processing of the aortae, some intimal and adventitial cells, which presumably included fibroblasts and endothelial cells, were included in addition to SMCs (i.e., the cell type of interest). However, since a significant percentage of cells in the collected aortic tissue would be SMCs, the tissue was deemed to be a good positive control for SMC markers. Moreover, within the tissue, SMCs remain in their native microenvironment.

2.6. Quantitative Real-time PCR (RT-PCR) Analysis

Gene expression of ECM components and cell phenotypic markers by recruited peritoneal cells were determined using RT-PCR. RNA was isolated from constructs 4 and 6 weeks post-peritoneal implantation of conduits. Briefly, the constructs were removed from the RNeasy[®] buffer, cut into small pieces with a surgical blade, transferred to 350 μ L of RLT buffer (Qiagen), and centrifuged (8,000 $\times$ g) for 5 min. The supernatant was transferred to different tubes and the rest of the isolation was performed using the RNeasy[®] Mini Kit (Qiagen) according to the manufacturer's instructions. Quantification of RNA was performed using a RiboGreen assay (Invitrogen), as described previously [25]. Equal amounts of total RNA were reverse transcribed using iScript[®] cDNA kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. RT-PCR was performed with an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster, CA) and Power SYBER[®]-Green Master Mix (Applied Biosystems) as described previously [7, 25, 26]. Specific primers were included for SMCs (i.e. smooth muscle myosin heavy chain (*Myh11*) and smoothelin (*Smtn*)), myofibroblasts / SMCs (i.e. α -smooth muscle actin (*Acta2*)), general macrophages (i.e. CD68 (*Cd68*)), pro-inflammatory M1 macrophages (i.e. CD80 (*Cd80*)), and pro-healing M2 macrophages (i.e. macrophage mannose receptor C type 1 (*Mrc1*)). Also included were primers for collagenous matrix (i.e. collagen 1 α 1 (*Col1a1*)), elastic matrix (i.e. tropoelastin (*El*)), Fibrillin-1 (*Fbn1*), and Fibulin-5 (*Fbln5*)), and the matrix crosslinking enzyme lysyl oxidase (*Lox*). The gene expression profiles were analyzed with a linear regression of efficiency (LRE) method [27] using a Matlab code developed by Leigh et al. [28]. The LRE method allows comparisons between the expression levels of different genes, unlike a traditional fold change analysis. The resulting copy number was

normalized by the 18s ribosomal RNA (*Rn18s*) copy number to report a relative fluorescent value. The *Acta2*, *Myh11*, *Col1a1*, and *Eln* primers were designed previously [7]. The rat specific *Smtn*, *Cd68*, *Fbln5*, and *Lox* primers were ordered from Real Time Primers, LLC (Elkins Park, PA). The other sequences were designed in PerlPrimer [29] (Table 1) and ordered from Applied Biosystems. Amplification without cDNA was performed as a control for the amplification process.

2.7 Imaging

For imaging, explanted constructs were embedded in OCT (Sakura Finetek USA, Inc., Torrance, CA), and 8 μ m cross- and longitudinal-cryosections were prepared. The sections were fixed in 4% w/v EM-grade formaldehyde (Polysciences, Inc, Warrington, PA) for 5 min and then washed two times in PBS prior to either immunofluorescence or histology.

Immunofluorescence was used to visualize phenotypic markers and ECM proteins expressed by recruited peritoneal cells, similar to previous studies [26]. Briefly, the sections were permeabilized with 0.1% v/v Triton X-100 (VWR) for 7 min, and then blocked with 5% v/v goat serum (PAA Laboratories Inc., Dartmouth, MA). Cell phenotype was assessed with primary antibodies that detected markers of contractile cells [30] – i.e. α -smooth muscle actin (α -SMA) – and macrophages – i.e. CD68. The presence of matrix proteins was detected with primary antibodies against elastin (Millipore, Billerica, MA). For these primary antibodies, α -SMA is mouse monoclonal (IgG2a, Abcam, 1:100 dilution), CD68 is mouse monoclonal (IgG1, Abcam, 1:100 dilution), and Elastin is rabbit polyclonal (IgG, Millipore, 1:100 dilution). The markers were visualized with anti-rabbit or anti-mouse secondary antibodies conjugated to Alexa 633 probes, and affinity purified against rat IgG (Invitrogen). Cell nuclei were visualized with the nuclear stain 4',6-diamino-2-phenylindole dihydrochloride (DAPI) contained in the mounting medium (Vectashield, Vector Labs, CA). Imaging was performed on an Olympus IX51 fluorescent microscope. The brightness and contrast were adjusted equally for all cases and for the negative immunofluorescent labeling control (i.e. no primary antibody).

Histology was used to visualize the distribution of the cells and the cell-generated ECM. Sections were stained with hematoxylin and eosin (H&E) (Sigma, St Louis, MO), avoiding xylene since it dissolves the electrospun PCL fibers. Tissue sections were cover-slipped using a mounting medium with limonene as the solvent (Electron Microscopy Sciences, Hatfield, PA).

2.8 Biochemical Analysis

The flash-frozen conduits / constructs described in Section 2.4 were lyophilized after harvest, measured for mass and length, and then digested for biochemical assays (see Section 2.8.1). The construct mass and length were used to normalize the amount of tissue generated within the conduits over 2, 4, and 6 weeks in the peritoneal cavity. The mass of the construct was also used for normalization of the number of cells and collagen and elastin content.

2.8.1 Cell Concentration—Cellularity of explanted peritoneal constructs was quantified using a DNA assay, as described previously [19]. Briefly, the constructs were digested in 200 μ L of 5 mg/mL proteinase-K (Invitrogen, Grand Island, NY) in PBS for 12 h at 37 °C. After sonification for 1 min, an aliquot was removed to quantify elastin and collagen content. The remaining volume was retained for DNA quantification with Hoechst 33258 dye and a procedure previously described by Labarca and Paigen [31]. Cell densities were calculated based on the estimate of 6 pg of DNA/cell.

2.8.2 Collagenous Matrix Content—The amount of collagenous matrix deposited by recruited peritoneal cells was estimated with a hydroxyproline (OH-Pro) assay, as described previously [19]. Briefly, sample volumes remaining from section 2.7.1 were digested in 0.1 M sodium hydroxide (98 °C, 1 h). After centrifugation, 600 μ L of this digestate was further hydrolyzed in 500 μ L of 6 M HCL (110 °C, 16 h) and then dried under a nitrogen stream at 37 °C. The residues were reconstituted in 200 μ L of deionized (DI) water and assayed with the OH-Pro assay, as described previously [32, 33]. Collagen content was calculated on the basis of the 13.2% w/w OH-Pro content of collagen.

2.8.3 Matrix Elastin Content—Matrix elastin was quantified using a Fastin[®] assay (Accurate Chemical and Scientific Corporation, Westbury, NY) as described previously [19]. Briefly, an aliquot from the digestate after sodium hydroxide treatment (see Section 2.8.2) was solubilized with 0.25 M oxalic acid (98 °C, 1 h) prior to assaying with the Fastin[®] assay. The Fastin[®] assay was performed as described in the manufacturer's protocol.

2.9. Statistics

Results are presented as the mean $\pm$ standard deviation, except the PCR results that are presented as the mean $\pm$ standard error. Reproducibility was verified by replicating all studies once using different rats and batches of electrospun conduits (i.e., two experiments for each assay and $n = 3$ samples/condition for each experiment). A total of $n = 6$ samples/condition were used for SEM analysis of electrospun conduits (> 70 fibers/sample), light microscope analysis of conduit wall thickness, PCR analysis, and for biochemical assays. Observations from histological and immunofluorescence images were made by analysis of 4 and 8 images/sample, respectively. Statistical analysis was performed with SPSS software, and statistical significance was determined for a significance criterion of $p = 0.05$. Results with p -values close to significance (i.e., p -value between 0.05 to 0.1) are briefly mentioned as “trends” and not discussed in detail. In the study results (see Section 3), the p -values primarily indicate the comparison of specific conditions through one-way ANOVA and the Tukey post-hoc comparisons method (e.g., 4 week, HA, PCL vs. 4 week, HA, blend). However, when individual conditions are not different, our factorial study design allowed us to analyze the overall difference for specific variables (e.g., PCL vs. blend), which cannot be noted in the figures. In the manuscript, p -values corresponding to a comparison of specific conditions are identified by listing the conditions compared within the parentheses.

3. Results and Discussion

3.1. Characterization of Electrospun Conduits

Electrospun conduits were generated from 25% w/w collagen/PCL blend and 100% PCL and exhibited average fiber diameters of 1.62 ± 0.87 and 2.19 ± 0.24 μm , respectively (Fig. 1A, B). The higher standard deviation in fiber size for the blend conduits is due to variations in average fiber diameter between replicate sample groups 1 and 2 (0.827 ± 0.0739 and 2.41 ± 0.0591 μm , respectively), although observed trends in the studied outcomes were comparable for conduits from both replicates. Therefore, the results from both replicates were combined in this manuscript. Both the blend and PCL conduits exhibited limited orientation, with average ASDs of 47.7 ± 8.94 and $51.9 \pm 11.9^\circ$, respectively. EDS analysis indicated the presence of nitrogen in the blend conduits (Fig. 1C), confirming the inclusion of collagen.

The presence of HA on electrospun meshes following surface immobilization of HA-o was confirmed using immunofluorescence (Figure 1D–G). Intense staining was observed for fibers in both PCL and blend conduits that were reacted with HA-o, but not for those subjected to the same reaction in the absence of HA-o. The intensity of staining also varied between neighboring fibers. Importantly, the HABPs do not bind to HA of molecular weights less than 2,000 Da. Since our study used HA oligomers of smaller size, the result suggests that (a) some of the HA-o was crosslinked during the tethering process, enabling detection [34], and (b) the actual amount of tethered HA may be significantly more than detectable by immunofluorescence.

3.2. Impact of Conduit Composition and HA-o Modification on Recruited Cell Phenotype

3.2.1 Expression of genes for cell phenotypic markers

3.2.1.1 General Trends: Implanted cell-free electrospun conduits recruited peritoneal cells that produced ECM within the conduits, which are subsequently described as “constructs.” In general, for all conduit types, recruited cells expressed markers for both macrophages (i.e. *Cd68*, *Mrc1*, and *Cd80*) (Fig. 2A, B) and contractile myofibroblasts or SMCs (i.e. *Acta2*, *Myh11*, and *Smtn*) (Fig. 2C, D) at 4 and 6 weeks after implantation. In particular, expression of the general macrophage marker *Cd68* (CD68) was high, indicating a significant macrophage presence. This is expected since previous studies have reported a significant increase in macrophages associated with peritoneal cavity wound healing responses, starting at 3–7 days post-implantation [18, 35]. The *Cd68*⁺ macrophages expressed *Mrc1*, a marker of an M2 pro-remodeling phenotype, at higher levels than the pro-inflammatory M1 marker *Cd80* in all conditions, although the differences were not statistically significant. This would be a positive finding since an established goal in biomaterials design is to promote a greater M2/M1 ratio, consistent with a pro-tissue remodeling response [9].

The cellular expression of contractile phenotypic markers was also observed. The highest expression was of *Acta2* (α -SMA; $p < 0.0001$), which is expressed by both myofibroblasts and SMCs. Interestingly, more specific SMCs markers were also expressed (i.e. *Myh11* and *Smtn*), although the expression of these markers were significantly lower within all peritoneal constructs compared to control aortic tissue ($p < 0.0001$ vs. abdominal aortae).

Previous articles have shown that the middle-stage SMC marker SM22 α is expressed by some recruited peritoneal cells [18, 19]. However, the expression of late-stage markers in our study confirms that SMC-like cells are present within the constructs. Our results also suggest that *Acta2*⁺ cells within the constructs exhibit a phenotype somewhere in a phenotypic spectrum ranging between myofibroblasts and SMCs. This concept has been described previously [36], and emphasizes the importance of determining the expression level of multiple SMC markers to establishing SMC phenotype. Finally, though our current study was not designed to determine the origin of these contractile cells, the results strongly suggest that partial differentiation or a transition in cell phenotype occurs within the peritoneal constructs.

3.2.1.2 Effect of collagen incorporation: Blending 25% w/w collagen with PCL in electrospun conduits significantly altered the wound healing response in the peritoneal cavity. Differences in wound healing and recruited cell phenotypes were observed between conduits of different compositions implanted for different lengths of time (Fig. 2A, D). *Myh11* (MYH11) expression was higher for cells recruited to blend compared to PCL constructs at 4 weeks ($p = 0.047$), but temporal increases for the PCL constructs provided an expression level that matched the blend constructs by 6 weeks. Importantly, the expression levels of contractile phenotypic markers did not decrease between 4 and 6 weeks for both conduit types (i.e., blend and PCL). In addition, a decreasing trend in the expression of macrophage markers (i.e. *Cd68* and *Cd80*) from 4 to 6 weeks was observed for the blend constructs ($p = 0.081$ and 0.09 , respectively), but not for PCL alone. At 6 weeks, a trend toward lower cellular *Cd68* expression within the blend versus PCL constructs was observed ($p = 0.09$).

3.2.1.3 Effect of HA-o incorporation: HA-o immobilization on the conduits did not significantly impact the phenotype of the recruited peritoneal cells. This was unexpected since HA oligomers have been shown previously to uniquely impact SMCs phenotype and enhance their ECM synthesis when delivered exogenously in culture [7], and even when immobilized to glass culture substrates [37]. HA oligomers also reduce inflammation when injected in the spinal cord [38]. An article by Park et al. may explain our finding [34], since they showed that high molecular weight (MW) HA incorporated within a scaffold is masked *in vivo* by HA generated by dermal cells during wound healing. If the effects of high MW HA can be masked, it is also likely that the effects of lower MW HA oligomers can also be masked in the peritoneal cavity by a similar mechanism [39]. Potentially useful approaches to improve cell response to HA-o include augmenting the density of HA-o tethering, including a spacer/linker molecules to tether HA-o, or pursuing other methods of HA-o delivery (e.g., controlled release of HA-o from electrospun fibers) that have been adopted for delivery of other growth factors [40].

3.2.2 Visualization of phenotypic marker proteins—The immunofluorescence results confirmed the presence of myofibroblast or SMC (α -SMA) and macrophage (CD68) marker proteins within the constructs (Fig. 3). No noticeable differences were observed in intensity of α -SMA staining between different conditions – i.e. conduit compositions, implantation times of 4 and 6 weeks (Fig. 3A), and with/without HA-o modification (Supp.

Fig. 1). However, CD68 staining was more intense within the PCL constructs compared to the blend ones (Fig. 3B). These results are consistent with the trends from the gene expression results (Fig. 2). They suggest that synthetic PCL triggers a chronic inflammatory response, which is reduced by incorporating a natural macromolecular collagen component within electrospun conduits, even if collagen content is limited to (i.e., only 25% in this study). The specific cell attachment sequences presented by the collagen may promote the more acute inflammatory response and positive wound healing since the cell binding sequences found in collagen are known to modulate cell phenotype [41]. The collagen enzymatic degradation products may also impact wound healing. This hypothesis is supported by recent findings by the Badylak group, where degradation of collagen-rich, small-intestinal-submucosa scaffolds significantly enhanced and accelerated wound healing [9].

The immunofluorescent images also demonstrate that the cells in the outer capsule of all conduits exhibited more intense α -SMA staining than the cells within the construct wall, which generally exhibited more intense CD68 staining. Our results are consistent with a previous study [19] in strongly suggesting that a difference in phenotype develops between cells in the outer capsule and those directly in contact with the scaffold / conduit.

3.3 Impact of Conduit Composition and HA-o Modification on Cellular ECM Generation

3.3.1 Expression of ECM genes—Peritoneal cells recruited to the implanted conduits expressed genes encoding for ECM components (Fig. 4). While *Colla1* (collagen type 1) was the most highly expressed matrix component ($p < 0.0001$), *Eln* (tropoelastin) and other non-elastin components important for elastic fiber assembly – i.e. *Fbn1* (fibrillin 1) and *Fbln5* (fibulin 5) – were also expressed. The gene expression levels for matrix components by cells within the peritoneal constructs were comparable to control cells from abdominal aortae, except for *Eln*, which exhibited significantly lower expression in all peritoneal constructs ($p < 0.0001$ vs. abdominal aortae). It is important to note that a lower expression of tropoelastin mRNA does not necessarily indicate that less elastic matrix will be produced. In fact, previous articles have suggested that the crosslinking and organization of tropoelastin into elastic matrix, and not reduced gene expression, is a greater limitation to elastic matrix assembly by adult vascular cells [42]. Additionally, direct comparison between *Eln* expression by cells from our peritoneal constructs and native aortae likely does not reflect the inherent elastogenic potential of the two cell sources, since differences between the conduit and native aortic microenvironments can influence cell behavior. A controlled *in vitro* analysis of cells isolated from the two sources would provide a better comparison.

No significant differences in matrix gene expression levels were observed between peritoneal cells recruited to conduits both with different compositions and with/without tethered HA-o, although trends toward temporal changes were observed. *Fbln5* trended toward greater expression for the blend versus PCL constructs at 4 weeks ($p = 0.061$), but not at 6 weeks, after implantation. In addition, the expression of *Lox*, which encodes for the LOX enzyme that crosslinks collagen and elastin, exhibited a decreasing trend from 4 to 6 weeks in the blend constructs ($p = 0.069$) (Fig. 2C, D). No temporal changes in *Lox*

expression were observed within PCL constructs. Finally, no differences in gene expression of matrix components were observed with HA-o tethering, possibly due to masking (see Section 3.3.1).

3.3.2 Generation of ECM protein—Matrix generation by peritoneal cells over 2 to 6 weeks of implantation varied significantly between collagen-containing blend and PCL conduits. Unlike in blend conduits, increases in dry construct mass, normalized per construct length, occurred in the PCL conduits between 2 to 4 weeks of implantation (Fig. 5). The mass was also higher for PCL compared to the blend constructs ($p < 0.0001$). In particular, HA-o modified, PCL constructs exhibited significant increases in mass after 4 and 6 weeks of implantation ($p < 0.0001$ vs. no implantation). The PCL constructs without HA-o modification also exhibited increases in mass over time of implantation, although the differences between time points were not statistically significant. The blend constructs, regardless of HA-o modification, did not exhibit similar increases in mass after 2 weeks. This resulted in significantly lower normalized mass for blend vs. PCL constructs at 4 and 6 weeks ($p = 0.003$ and 0.001 , respectively vs. PCL conduits). H&E staining confirmed the increases in tissue mass described above (Fig. 6). Although H&E staining does not identify specific matrix protein components, these images highlighted differences in inflammatory responses and matrix production resulting from the incorporation of collagen with PCL within electrospun conduits. These results provide additional evidence that incorporating collagen into conduits prompts a more acute inflammatory response. This response may be desirable to avoid a chronic fibrotic response and to promote organization and maturation of the initially deposited matrix.

The images in Fig. 5 and 6 also suggest that cell infiltration and cell number within the PCL, but not the blend, conduits increase with increasing implantation time. However, analysis of DNA content did not show any significant temporal changes in construct cellularity (Supp. Fig. 2). This might be due to the variable amounts of extra tissue that tended to be removed when extracting the individual constructs from the pouches [19]. The large error bars in Supp. Fig. 2 are consistent with the construct removal. Despite these uncertainties, our current histology observations, and our previous study with implanted peritoneal constructs [19], strongly indicate that the cellularity of the constructs does increase with implantation time.

Collagen and elastic matrix generation within the conduits varied with the conduit composition. Collagenous matrix was produced within the PCL conduits, although no significant changes in collagen quantity occurred with increasing implantation times (Fig. 7A). In the blend conduits, collagen content decreased with time ($p = 0.01$), with the most significant decrease observed between 0 and 2 weeks of implantation. While collagen was absent in the PCL conduits pre-implantation, the total collagen content in the PCL and blend constructs at all post-implantation times were generally not statistically different. An exception was increased collagen content for the HA-o modified blend constructs ($p = 0.044$ and 0.034 , vs. PCL constructs with and without HA-o, respectively). Since the *Coll1a1* gene expression (Fig. 4) was comparable for both blend and PCL conduits, the differences in accumulated matrix between these two types of conduits are likely due to early degradation of the collagen that was electrospun into the blend conduits. Supporting these findings, a

study by van Amerogen et al. has shown that the collagen type 1 scaffolds degrade by 14 days after grafting in the myocardial environment, and that the recruited inflammatory cells release proteases that contribute to this degradation [43]. However, they also found that after 14 days the amount of cell-generated collagen increased with time. Thus, we expect that the initial decrease in collagen within blend conduits would be unlikely to continue beyond 6 weeks, and should not compromise graft mechanical integrity.

Elastic matrix was produced in both blend and PCL conduits, with general temporal increases in elastic matrix production observed between 2 to 4 weeks for all conditions (Fig. 7B). However, the increases were only statistically significant for the blend conduits ($p = 0.029$ and <0.001 , for no HA-o/ 4 week and HA-o/6 week vs. equivalent PCL conduits). Overall, significantly greater elastic matrix production occurred within the blend versus PCL constructs ($p = 0.002$). No statistically significant impact of HA-o modification on elastic matrix generation was observed, consistent with other results from this study.

Immunofluorescent images showed that the majority of generated elastic matrix was localized in the outer edge of the construct, which included the fibrous capsule and possibly also part of the conduit wall (Fig. 7C and Supp. Fig. 3), although weaker autofluorescence due to elastin was also detected in the construct interior. These images also detected fibrillin 1, a critical component of elastic fibers, although it was at the threshold of visualization (not shown). While limited elastic matrix generation within a conduit wall is not desirable for vascular grafts, our finding stress that this is an unaddressed concern that will require further improvement in methods to modify the scaffold with proelastogenic factors such as HA-o.

While previous studies have shown that fibrin scaffolds promote cellular elastogenesis [20], our results indicate that incorporating collagen with PCL in conduits also promotes the production of elastic matrix, even though *Eln* gene expression by recruited peritoneal cells was not statistically different between blend and PCL conduits (Fig. 4). One possible explanation for this increase in elastic matrix generation within blend conduits, without a corresponding increase in *Eln* expression, could be more efficient recruitment and crosslinking of tropoelastin within the blend constructs. This hypothesis is supported by the earlier upregulation of fibulin 5 mRNA at 4 weeks after implantation for the blend versus PCL constructs. Fibulin 5 is a critical component of microfibrillar scaffolds that have been shown to bind elastin [2]. Our results suggest that the machinery exists for elastic fiber assembly within the collagen – PCL blend conduits.

4. Conclusions

This study demonstrates that the incorporation of as little as 25% w/w collagen with PCL within electrospun conduits promotes a quicker progression of the wound healing response following intraperitoneal-implantation, with decreased expression of pro-inflammatory macrophage phenotypic markers at extended periods of implantation. Further, the expression of contractile markers, including late-stage SMC markers, remained elevated. Another important finding was that blend conduits incorporating collagen increased production of elastic matrix production by the recruited peritoneal cells. Overall, the collagen-containing conduits provide significant advantages for tissue generation *in vivo*. These include promoting a non-chronic wound healing response and possessing the ability to generate

elastic fibers, both of which are critical to the long-term integrity of a vascular graft. However, the mechanism requires further investigation. Tethered HA-o had significantly less impact on cell phenotype and matrix production in this *in vivo* study, in contrast to the significant elastogenic benefits demonstrated previously in culture studies. Better delivery methods for HA-o will be necessary to be able to induce recruited peritoneal cells to produce more mature elastic matrix. In conclusion, these results demonstrate that the peritoneal cavity can be used to produce constructs for use as small-diameter grafts, but also that careful control of implanted scaffold composition is necessary.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by NIH Grants through the National Heart, Lung, and Blood Institute (HL092051-01A1 and HL092051-01S) and the National Institute of Biomedical Imaging and Bioengineering (EB006078-01A1) awarded to Ramamurthi A. Additional funding includes an NIH postdoctoral fellowship (F32HL108548-01A1) awarded to Bashur CA. The authors would also like to acknowledge Dennis Wilk for preparing and characterizing the HA-o.

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Fig. 1.

Electrospun conduits were produced from a synthetic polymer and a natural/synthetic blend. Shown are SEM images of the outside surface of conduits electrospun from PCL (A) and a 25% w/w collagen/PCL blend (B). An EDS spectrum shows the presence of nitrogen in blend conduits after electrospinning (C). The fibrous surfaces of meshes with immobilized HA-o (F–G) exhibited significantly more intense HABP staining than those without (D–E). This occurred with both PCL (D–F) and blend (EG) conduits. Contrast and brightness were adjusted evenly for images with / without HA-o modification with each scaffold material. However, the PCL and blend meshes were adjusted separately because of their different thicknesses, which resulted in differing levels of background.

Fig. 2.

Quantitative gene expression profiles for constructs composed of PCL (A, C) and a 25% w/w collagen/PCL blend (B, D). Data shows differences in expression of macrophage (A, B) and contractile SMC (C, D) phenotypic markers. This data shows changes from 4 to 6 weeks of intra-peritoneal implantation of conduits with and without tethered HA-o (indicated by + or -, respectively). The error bars represent the standard error.

Fig. 3.

Temporal changes in phenotype of peritoneal cells recruited to conduits of different compositions. Shown are immunohistochemical images of myofibroblast, SMC, and macrophage markers – i.e. αSMA (A) and CD68 (B) – on HA-o modified surfaces. Cross-sections of PCL and blend conduits are shown at both 4 and 6 weeks implantation time. The outer surface of the fibrous capsule and implanted conduit is on the left edge in these images. Overlay channels also include DAPI stained nuclei (blue) and autofluorescent background (green).

Fig. 4. Quantitative expression profiles of matrix genes by cells within PCL (A) and 25% w/w collagen/PCL blend (B) constructs. This data shows temporal changes in gene expression between 4 and 6 weeks of intra-peritoneal implantation of constructs with/without tethered HA-o (indicated by + or –, respectively). The error bars represent the standard error. Individual conditions were not statistically different. However, there were statistical differences between experimental parameters, as described in the text.

Fig. 5.

Matrix synthesis within implanted conduits as a function of conduit composition, HA-o modification, and implantation time. Shown is normalized total tissue mass in the constructs.

* indicates statistical significance from non-implanted constructs and # indicates significance from HA-o-modified PCL conduits.

Fig. 6.

Hematoxylin and Eosin (H & E) staining of tissue generated within implanted conduits. Shown are representative images that demonstrate significant differences in cell and ECM synthesis responses to PCL (A–E) and blend (F–J) conduits that are HA-o modified. The images show constructs after 2 (A, F), 4 (B–C, G–H), and 6 week (D–E, I–J) implantation times. Cell nuclei stain blue and cytoplasm of cells and ECM stain pink. The outer surface of the conduits is on the right side of the images. Representative cell nuclei are circled in the high magnification images (C, H). Histology images of tissues generated within conduits without HA-o modification are not shown since there were no noticeable differences in tissue generation versus for the corresponding HA-o modified conduits.

Fig. 7. Matrix generation within intraperitoneal tissue constructs is influenced by conduit composition, the presence of HA-o, and duration of implantation. Shown within the constructs is collagen content via the hydroxy-Pro assay (A) and matrix elastin, via the Fastin[®] assay (B). Statistical significance is indicated by * from the HA-o modified blend conduits, # from PCL conduits for all time points, and @ from 2 weeks of conduit implantation. Immunohistochemical images of elastin on HA-o modified surfaces (C). Cross-sections of PCL and blend conduits are shown at both 4 and 6 weeks implantation

time. Inter-section variations in elastin content (red) were observed, and the included images represent areas that stained most intensely for elastin. The outer surface of the fibrous capsule and implanted conduit is on the left edge in these images. Overlay channels also include DAPI stained nuclei (blue) and autofluorescent background (green).

Table 1

RT-PCR primer sequences

Gene / Accession #	Forward Primer (5' → 3')	Reverse Primer (5' → 3')	Product Size (bp)
<i>Cd80</i> U88622	GTCTGTCAGAGCTGACTTCC	GGTGTACAGCTCAGATTCAGG	189
<i>Mrc1</i> NM001106123	AGCAACTTGACCAACAATGAG	TATGATGTCTTCCAGTAGCCA	133
<i>Fbn1</i> AF135059	ATAAATGAATGTGCCCAAGATCCC	ACTCATCCTCATCTTTACACATCC	132