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Engineered Composite Fascia for Stem Cell Therapy in Tissue Repair Applications

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Abstract

A critical challenge in tissue regeneration is to develop constructs that effectively integrate with the host tissue. Here, we describe a composite, laser micromachined, collagen-alginate construct containing human mesenchymal stem cells (hMSCs) for tissue repair applications. Collagen type I was fashioned into laminated collagen sheets to form a mechanically robust fascia that was subsequently laser micropatterned with pores of defined dimension and spatial distribution as a means to modulate mechanical behavior and promote tissue integration. Significantly, laser micromachined patterned constructs displayed both substantially greater compliance and suture retention strength than non-patterned constructs. hMSCs were loaded in an RGD-functionalized alginate gel modified to degrade *in vivo*. Over a 7 day observation period *in vitro*, high cell viability was observed with constant levels of VEGF, PDGF- β and MCP-1 protein expression. In a full thickness abdominal wall defect model, the composite construct prevented hernia recurrence in Wistar rats over an 8-week period with *de novo* tissue and vascular network formation and the absence of adhesions to underlying abdominal viscera. As compared to acellular constructs, constructs containing hMSCs displayed greater integration strength (cell seeded: 0.92 ± 0.19 N/mm vs. acellular: 0.59 ± 0.25 N/mm, $p = 0.01$), increased vascularization (cell seeded: $2.7\text{--}2.1/\text{hpf}$ vs. acellular: $1.7\text{--}2.1/\text{hpf}$, $p < 0.03$), and increased infiltration of macrophages (cell seeded: $2021\text{--}3630 \mu\text{m}^2/\text{hpf}$ vs. acellular: $1570\text{--}2530 \mu\text{m}^2/\text{hpf}$, $p < 0.05$). A decrease in the ratio of M1 macrophages to total macrophages was also observed in hMSC-populated samples. Laser micromachined collagen-alginate composites containing hMSCs can be used to bridge soft tissue defects with the capacity for enhanced tissue repair and integration.

Graphical abstract

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Keywords

Collagen; alginate; human mesenchymal stem cells; full thickness abdominal defect; tissue repair; integration; vascularization; immune response

1. Introduction

Abdominal wall hernia repair is the most common operation performed by general surgeons and approximately 31 to 55% of primary repairs may result in recurrence [1–3]. In order to reduce recurrence rates, repair most often mandates the use of a prosthetic mesh to achieve a tension-free repair [4]. The most common meshes in current use include synthetic textiles of polypropylene, poly(tetrafluoroethylene) or poly(ethyleneterephthalate) [5]. However, synthetic mesh remains limited by infection, seroma, adhesion to underlying viscera, as well as shrinkage and stiffness resulting in pain and discomfort [6–8]. Moreover, a synthetic mesh is contraindicated in contaminated or infected fields due to the risk of bacteria biofilm formation and infection with attendant local or systemic septic complications [9]. Finally, while a synthetic mesh provides mechanical support, tissue repair is not actively induced.

Use of a biologically derived material is often considered if reconstruction is required in the presence an infected wound. Current biologic materials originate from human cadaver, porcine, or bovine donors and are sourced from tissues such as the dermis, pericardium or intestinal submucosa. Neovascularization of the biologic material is often greater than that observed for a synthetic mesh, which facilitates access of the host innate immune response to residual contaminating microorganisms. Moreover, permanent biofilm formation is avoided due to the inherent biodegradability of the biologic [10, 11]. Although these

materials have been proposed for use in infected wounds, their high cost and loss of mechanical strength over time has limited their widespread adoption [12].

Recently, control over collagen processing has provided new opportunities to design and fabricate synthetic ECM constructs for tissue reconstruction [13]. Significantly, high-density collagen constructs can be designed that display slower degradation, tunable strength and flexibility, and can be modified for the delivery of therapeutics or cells. Mesenchymal stem cells (MSCs) secrete a well-defined repertoire of bioactive chemokines and growth factors and, as a consequence, display the capacity to induce a controlled response of the innate immune system [14, 15]. Therefore, we postulated that incorporation of human mesenchymal stem cells (hMSCs) within a collagen-based construct would lead to improved tissue repair. Alginate gels can be engineered to tailor rates of degradation, viscosity, stiffness, as well as cellular responses through the conjugation of biomacromolecules [16, 17]. Moreover, alginate gels can be used as a vehicle to deliver cells and facilitate the local release of soluble factors [18, 19]. In this report, we postulated that a multilamellar collagen-alginate composite containing human MSCs would display the capacity for locally inductive tissue repair. Laser micromachining was used to generate patterned, microporous constructs designed with pores of defined size and distribution as a means to tune mechanical responses, accommodate and protect incorporated cells, and enhance tissue integration. Acellular or hMSC-seeded laser patterned constructs were implanted in a rat full thickness abdominal wall defect model. Significantly, we observed an enhanced reparative response in hMSC-seeded constructs with increased neovascularization and associated matrix production, and increased strength of tissue integration.

2. Materials and Methods

2.1 Isolation and purification of monomeric collagen

Rat tail tendon monomeric Type I collagen was isolated by acid extraction from Sprague-Dawley rats (Pel-Freez Biologicals, Rogers AR) following an adapted procedure from Silver and Treslad [20]. Frozen rat tails were allowed to thaw at room temperature. Tendons were extracted with sterile pliers and placed in 10 mM HCl for 4 h at room temperature (pH 2.0, 6 tendons in 1 L). Soluble collagen was isolated by centrifugation at 30,000 g at 4 °C for 30 min followed with sequential vacuum filtration through 20 µm, 0.45 µm, and 0.2 µm filter membranes. Precipitation of sterile collagen was achieved by addition of concentrated NaCl to a final concentration of 0.7 M with stirring for 1 h. The precipitated collagen was centrifuged at 30,000 g for 1 h and the pellet re-dissolved in 10 mM HCl (~150 mL) overnight. The solution was subsequently dialyzed (Spectra/Por Dialysis membrane, MW cut off 50,000) against 20 mM phosphate buffer at room temperature for 8 h and then at 4°C for at least 8 h. This was followed by 10 mM HCl dialysis and by deionized water dialysis at 4 °C overnight. The solution was frozen and collagen was lyophilized.

2.2 Fabrication of collagen sheets and laminated collagen constructs

Monomeric Type I collagen was dissolved in 10 mM HCl (2.5 mg/mL) and a gel casted with a neutralizing buffer (4.14 mg/mL monobasic sodium phosphate, 12.1 mg/mL dibasic sodium phosphate, 6.86 mg/mL TES (n-Tris(hydroxymethyl)methyl-2-aminoethane sulfonic

acid sodium salt, 7.89 mg/mL sodium chloride, pH 8.0) at 4 °C in a rectangular mold (10 × 7 × 0.8 cm) for 24 h. Gels were subsequently removed and allowed to warm to room temperature for 2 h and then incubated at 37 °C for 24 h. Gels were then rinsed in deionized water for 4 h with three water changes and then allowed to dry on a glass substrate overnight. Once the collagen gel was dry, the collagen film was cut into rectangles (25 mm × 15 mm) with a CO₂ laser (Universal Laser Systems, Scottsdale, AZ). The films were placed in glass container filled with ddH₂O for 30 min. Nine films were carefully layered on an acrylic plate and were allowed to dry completely. After the layered construct had dried, fiber incubation buffer (FIB, 7.89 mg/mL sodium chloride, 4.26 mg/mL dibasic sodium phosphate, 10 mM Tris, pH 7.4) was added to re-hydrate the construct. Subsequently another acrylic plate was placed on top of the partially hydrated patch and then secured with screws (Fig. 1). The compression device was then incubated in FIB for 48 hrs at 37°C to promote fibrillogenesis, with slight tightening after the first 24 h. Laminated constructs were removed from the compression device and rinsed in deionized water for 4 h with three water changes and were allowed to dry on a glass slide. The constructs were then micropatterned with a CO₂ laser (Versa laser).

2.3 Imaging of laminated collagen patches

Optical and scanning electron microscopy (SEM) was used to image the laminated collagen constructs. For optical imaging, samples were hydrated in PBS for 24 h and the imaged with a stereoscope (Zeiss Axio Zoom V16). For SEM studies, constructs were hydrated in DI water for 24 h and dehydrated by serial incubation in ethanol-water mixtures from 30% to 100%. Samples were then critical point dried (Auto Samdri 815 series A, tousimis, Rockville, MD), sputter coated with 8 nm of gold (208HR Cressington, Watford, England), and imaged at an accelerating voltage of 10,000 eV using a field emission scanning electron microscope (Ziess Supra 55 FE-SEM, Peabody, MA).

2.4 Mechanical testing of laminated collagen constructs

Collagen constructs were cut using a dog-bone press to yield samples with a gauge length of 13 mm and 4.5 mm width. Tensile testing of collagen patches was done using an Instron 5566 (Instron, Norwood, MA). Samples were preconditioned 15 times to 66% of the average maximum failure strain determined from pilot samples and then tested to failure at 5 mm/min. Hydrated thickness was measured using optical microscopy for calculation of cross-sectional area. Young's modulus was determined from the slope of the last 4% of the stress-strain curve. Suture retention strength testing was done using sutures (Prolene 4-0) that were passed through 5 mm square nine layer collagen samples, 2.5 mm from the patch edge and the suture fastened to the actuating arm of the Instron and pulled at a rate of 1mm/s. The maximum force measured before the suture tore out of the patch was recorded as the suture retention strength, reported in grams-force (g-f).

2.5 Generation of RGD-derivatized alginate

Ultrapure alginates (ProNova Biomedical) were chemically modified as previously described [21]. Briefly, MVG alginate (M/G: 40/60) was used as the high molecular weight (HMW, 2.65×10^5 g/mol) component to form gels. Low molecular weight (LMW, 8.5×10^4 g/mol) alginate was obtained by γ -irradiating HMW alginate with a cobalt-60 source for 4 h

at a dose of 5.0 Mrad [22]. Alginate treatment with sodium periodate (Sigma) for 17 h in the dark at room temperature oxidized 1% of the sugar residues in the polymer. An equimolar amount of ethylene glycol (Fisher) was added to stop the reaction and the solution was then dialyzed (MWCO 1000, Spectra/Por) over three days. Following oxidation, the adhesion peptide sequence GGGGRGDSP (Peptides international) was coupled to both the HMW and LMW alginate by carbodiimide chemistry. Following peptide modification, alginate was dialyzed, treated with activated charcoal, filter sterilized (0.22 μ m), freeze-dried, and stored at -20°C .

2.6 Human mesenchymal stem cells

Frozen vials of passage one human mesenchymal stem cells (hMSCs) from bone marrow aspirates from the iliac crest were obtained from the Center for the Preparation and Distribution of Adult Stem Cells (Texas A&M, 5701 Airport Rd, Temple, TX, <http://medicine.tamhsc.edu/irm/msc-distribution.html>), which supplies standardized preparations of MSCs under the auspices of an NIH/NCRR grant (P40 RR 17447-06). MSCs were obtained from two different donors (nos. 8001R, 8004L). After a 24 h recovery period, hMSCs were seeded a low density (100 cells/cm), incubated in complete culture medium (CCM), and allowed to proliferate to 50 to 70% confluency over 6 to 7 days. hMSCs were cultured in α -MEM medium (Gibco, CA) containing deoxy- and ribonucleosides, supplemented with 16% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 100 units/mL penicillin and 100 mg/mL streptomycin (Gibco). Cell cultures were incubated in a humidified 37°C and 5% CO_2 environment. Cells were harvested using 0.25% trypsin in 1 mM EDTA at 37°C for 2 minutes. The trypsin was inactivated by adding CCM and the cells were washed with phosphate buffer saline (PBS) by centrifugation at 1,200 rpm for 5 minutes. The cells were frozen in α -MEM with 30% FBS and 5% dimethyl sulfoxide at a concentration of 1×10^6 cells/mL. Only passages two or three were used to initiate experiments.

2.7 Fabrication of alginate-collagen composites

Partially oxidized and RGDderivatized, high and low molecular weight alginates were mixed at a 1:1 ratio in media without serum (2% w/v) to provide a low viscosity solution that facilitated impregnation of the collagen construct with uniform cell distribution throughout the pores. Collagen constructs were first sterilized in 70% ethanol for 30 min and then rinsed three times with $1 \times$ DPBS. The collagen construct was placed on a sterile gauze to remove excess $1 \times$ DPBS and then placed in a well on a Teflon mold ($2.5 \times 1.5 \times 0.5$ cm). A total of 425 μL of RGD-derivatized, 1% oxidized alginate (20 mg/mL) solution was added on top the collagen construct followed by 45 μL of CaCl_2 (100 mM) to crosslink the alginate. The Teflon mold was covered and the top of the mold secured with screws. The coated construct was molded for 20 min at room temperature. The coated construct was then turned over, placed in the well on the Teflon mold, and the process repeated. Cells were incorporated within the RGD-alginate coated collagen constructs by suspending hMSCs (passage 3) in the RGD-alginate solution at a concentration of 4×10^6 cells/mL prior to placing alginate solution on the collagen construct. Seeded constructs were removed from the mold and placed in a 12 well plate with 2 mL of complete culture media for in vitro studies or in HBSS buffer prior to surgical implantation.

2.8 In vitro assessment of hMSCs within alginate-collagen composites

Cell viability was initially analyzed by calcein AM and ethidium homodimer staining (Life Technologies, Carlsbad, CA) at 1, 3, and 7 days after impregnation of collagen constructs with cell containing alginate gels. Briefly, constructs were washed with HBSS buffer and then incubated for 30 min with HBSS containing calcein and ethidium homodimer. Cells were imaged using both a stereoscope (Zeiss Axio Zoom V16) and by confocal microscopy (Leica SP5 X MP Inverted Confocal Microscope). As a complementary approach, cell viability within the collagen-alginate constructs was also assessed by direct cell isolation at 1, 2, and 7 days. Briefly, hMSC-seeded constructs were placed in a solution of collagenase (1 mg/mL) and alginate lyase (250 µg/mL) prepared in serum free α -MEM media (5 mL/cm²) for 90 min at 37 °C with shaking, which was typically associated with patch dissolution. Cells were centrifuged for 5 min at 400 g, supernatant removed, and fresh media added to the pellet to re-suspend the cells. The number of live and dead cell and percent viable cells was obtained using Trypan blue exclusion by counting cells with a hemocytometer.

Analysis of cytokine secretion was performed on days 2 and 8 after cell incorporation within the construct. Complete culture media was added for collecting conditioned media. hMSC conditioned media was collected from cells cultured on tissue culture plastic and within alginate-collagen constructs. A total of 1.2×10^5 cells were either plated directly in a well or added to a well within a composite patch (5 mm $\times$ 5 mm) in a 6 well plate. Conditioned media was collected on days 2 and 7, after washing the construct twice in HBSS followed by a 24 hr incubation period in 3 mL of collection media at 37°C. On the day of collection, the media was collected, sterily filtered, and stored at -20°C. The media was concentrated 5 times and analyzed for three different growth factors and cytokines (Milliplex® multiplex assay, EMD Millipore, Billerica, MA). Data was normalized to cell number.

2.9 Rat abdominal wall repair model

Alginate-collagen composite constructs were evaluated in a full thickness abdominal wall defect (2 cm $\times$ 1 cm). Female Wistar rats (250 g) were repaired with either an acellular or hMSC-seeded alginate-collagen composite patch, as approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee. Anesthesia was induced and maintained with isoflurane inhalation (2.5% and 1.5%, respectively). A three to four cm vertical midline incision was used to expose muscular and fascial layers followed by creation of a full thickness, rectangular (2 $\times$ 1 cm), ventral abdominal wall defect. The defect was repaired with a planar construct using an onlay technique without relaxing fascial incisions. The skin was closed and animals closely monitored for 1 to 2 h and then daily. Samples were retrieved at 2, 4, and 8 weeks for histological analysis and measurement of integration strength at the host-implant interface. All studies were approved by the BIDMC Animal Care and Use Committee.

2.10 Immunohistochemistry

Specimens were fixed overnight in 10% neutral buffered formalin, processed for paraffin embedding, and 5 µm sections obtained and stained for extracellular matrix (Masson's trichrome), macrophages (CD68, iNOS), and endothelial cells (VWF) (Abcam, Cambridge,

MA). Blood vessels were analyzed by counting vessels that stained for VWF in 18 random fields of view at 20× magnification for all samples in each group using Image J (2 weeks samples: $n = 3 - 4$ animals/group; 4 weeks samples: $n = 34$ animals/group; 8 weeks samples: $n = 6 - 7$ animals/group). Macrophage (CD68) infiltration was measured in 12 random fields of view of three to four sections per sample using Image J (2 weeks samples: $n = 3 - 4$ animals/group; 4 weeks samples: $n = 3 - 4$ animals per group; 8 weeks samples: $n = 6 - 7$ animals per group). Data were presented as area covered per field of view at 20× magnification. The ratio of iNOS/CD68 staining was obtained in serially collected sections stained with CD68 and iNOS, respectively, by comparing CD68 rich areas to the same iNOS stained area in three fields of view for at least three sections per sample (2 weeks samples: $n = 3 - 4$ animals/group; 4 weeks samples: $n = 3 - 4$ animals/group; 8 weeks samples: $n = 6 - 7$ animals/group).

2.11 Strength of host tissue-construct integration

To measure the strength of integration, 4×20 mm strips of patch and adjacent tissue were excised and mounted on opposing platens of a uniaxial tensile tester (DMTA V, Rheometric Scientific, Piscataway, NJ) to determine tension at failure [23–25]. Since the cross-sectional thickness of the host abdominal wall and the implanted sample are often different, the strength of integration was presented in units of N/mm, which represents the force applied per width of the explanted host tissue-implant sample.

2.12 hMSC tracking after implantation of cell containing constructs

hMSCs were incubated in a 12 μ M solution of carboxyfluorescein diacetate succinimidyl ester (Vybrant CFDA SE Cell Tracer kit; Life Technologies, Carlsbad, CA) in PBS for 15 min at 37 °C followed by incubation in media for 30 min at 37 °C. Labeled cells were incorporated in the composite constructs as detailed previously and cell-seeded constructs used to repair a full thickness abdominal wall defect in Wistar rats. Animals were sacrificed at 2 h, 3 days, 1 week, 2 weeks, and 6 weeks. Tissue was collected, fixed in 10% formalin, placed in OTC, frozen in liquid nitrogen and stored at -80°C . Samples were cryosectioned, placed on a glass slide, stained with DAPI (SlowFade® Gold Antifade Mountant, Life Technologies, Carlsbad CA) and imaged using a confocal microscope (Leica SP5 X MP Inverted Confocal Microscope). hMSCs were analyzed by counting stained cells in 12 random fields of view at 20× magnification for each time point group using Image J ($n = 1$ animal/group).

2.13 Statistical analysis

Mean values and standard deviation was obtained for all measurements, image analysis, and mechanical data. Comparisons were performed using the Student's t-test for unpaired data, ANOVA for multiple comparisons, and Holm's post hoc analysis for parametric data. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1 Fabrication of collagen sheets and multilayer constructs

We developed a mechanically robust collagen construct for tissue repair that can be easily tuned and modified for different applications. In fabricating the construct, 8 mm thick collagen gels were initially cast for 24 h (Figure 1A), rinsed, dried overnight on a glass substrate (Figure 1B), and 25 mm × 15 mm rectangular sheets cut with a CO₂ laser. Rehydrated collagen sheets were 65 μm thick and a total of nine hydrated sheets layered on an acrylic plate were allowed to dry to form a single multilamellar construct, which was then placed in a fibril incubation buffer within a compression set up that promoted physical bonding (Figure 1 C, D). After rehydration, the multilamellar collagen construct had a measured thickness of 500 μm.

3.2 Patterning of constructs and mechanical properties of collagen constructs

Through and through pores were patterned into the construct using a CO₂ laser to tune mechanical properties and facilitate integration of host tissue (Figure 1E). In one patterned format, 0.05 mm² hexagonal pores were generated with a side length of 140 μm (Figure 1F). Scanning electron microscopy demonstrated that the samples remained laminated after pore formation (Figure 1I–J). The mechanical responses of three different pore patterns were analyzed and compared to a non-patterned construct. Specifically, constructs with 0.05 mm² pores (240 pores/cm²) (Figure 2A) or 0.20 mm² pores (60 pores/cm²) (Figure 2B), each covering 10% of the total surface area, and constructs with 0.013 mm² pores (240 pores/cm²) (Figure 2C), covering 3% of the total surface area were analyzed. The distance between pores remained constant throughout the construct with pores linearly distributed with a staggered position from line to line. Each pore was surrounded by six other pores.

3.3 Mechanical testing of collagen constructs

Ultimate tensile strength decreased as the percentage of the surface area occupied by pores increased, but ranged between 1.39 ± 0.15 MPa and 2.19 ± 0.18 MPa for the porous constructs (Figure 2E). The Young's modulus showed little difference between the non-porous and the majority of patterned porous constructs (Figure 2F). The exception was a construct patterned with 0.05 mm² pores (240 pores/cm², 10% surface area), which had a significantly lower Young's modulus (7.72 ± 1.1 MPa vs. 13.02 ± 2.2 MPa for the non-patterned construct, $p < 0.05$), while maintaining a strain at failure that was comparable to the non-patterned sample ($p = 0.2$; Figure 2G). Suture retention strength was greater for all patterned samples when compared to the non-patterned construct (patterned constructs: 60.5–55.1 g-f vs. non-patterned constructs: 38.3 g-f, $p < 0.05$; Figure 2H). The enhanced flexibility along with acceptable tensile strength (1.39 ± 0.15 MPa), failure strain ($26.0 \pm 5\%$), and suture retention strength (60.5 ± 5.3 g-f) of the sample patterned with 0.05 mm² pores (240 pores/cm²) were seen as advantageous for hernia repair and, therefore, this construct was selected for subsequent studies.

3.4 Characterization of a cell-populated, alginate-collagen composite construct

The porous collagen construct was embedded within a partially oxidized and RGD-derivatized alginate gel containing hMSCs using an in-house fabricated Teflon™ mold (Figure 3A). Uniform cell distribution was observed over both sides and within the pores of the construct (Figure 3B, C). Cells were released following 1, 3, and 7 days in culture with dead cells identified by trypan blue staining. Overall loading efficiency was $72 \pm 11\%$. Over the seven day period, little proliferation was noted and a high level of cell viability (91%) maintained (Figure 3D, E). Consistent with these findings, an analysis of the secretome revealed that VEGF, PDGF- β and MCP-1 were produced at a relatively uniform rate from hMSC-populated constructs over the seven day period. hMSCs cultured on tissue culture plates displayed decreased levels over time or very low levels throughout, particularly in the case of MCP-1 and PDGF- β (MCP-1 day 8: construct 2867 pg vs. plate 509 pg, $p < 0.0002$; PDGF-B day 8: construct 163 pg vs. plate 0.0 pg, $p < 0.001$; Figures 4A–C).

3.5 Evaluation in an abdominal wall full thickness defect rat model

Alginate-collagen composite patches, with or without hMSCs, were implanted in a full thickness abdominal wall defect (1 cm $\times$ 2 cm) in the rat using an on-lay technique (Figure 5A). All composite constructs prevented hernia recurrence over an 8-week period (Figure 5B). Patches were explanted at 2, 4, and 8 weeks. The side of the construct interfacing with the subcutaneous tissue revealed *de novo* tissue formation on top of a preserved patch, particularly after 4 weeks and 8 weeks (Figures 5C). Vessels penetrating the construct and fatty tissue were observed on the side facing the peritoneal cavity (Figure 5D). Adhesions to the viscera were not observed.

3.6 Histological Analysis

Histologic analysis was performed at 2, 4, and 8 weeks after implantation (Figure 6A). At 2 weeks, constructs were present with alginate observed on both the subcutaneous and peritoneal sides of the implants, and host cell responses noted to acellular and cell-seeded samples. New blood vessels were also observed in the subcutaneous side, the peritoneal side, and in the pores of the construct. At 4 weeks, most of the alginate component has been replaced by new tissue and maintenance of a vascular network is observed. At 8 weeks, the collagen component of the construct remains, although there appears to be partial replacement with collagen produced by the host. Overall, acellular and cell-seeded constructs were vascularized by two weeks and remained vascularized over the 8 week study period. To further analyze blood vessels, immunohistochemical staining of von Willebrand factor (VWF) revealed no differences in the number of blood vessels observed in the subcutaneous side at all the time points analyzed. On the peritoneal side, no difference in the number of blood vessels was observed at 2 and 4 weeks, but an increase in the number of blood vessels in hMSC-seeded samples was noted at 8 weeks (3.5 ± 1.1 vs. 1.8 ± 0.5 VWF/hpf, $p = 0.01$). However, a significantly greater number of blood vessels were observed in the mid-section of cell-seeded as compared to acellular constructs at all time points (acellular: 1.7–2.1/hpf vs. cell seeded: 2.7–2.1/hpf, $p < 0.03$; Figure 6B, C).

Significant differences in macrophage infiltration were observed between acellular and hMSC-seeded patches at all time points. hMSC-seeded samples had increased macrophage

infiltration as compared to acellular constructs (acellular: 1570–2530 $\mu\text{m}^2/\text{hpf}$ vs. cell seeded: 2021–3630 $\mu\text{m}^2/\text{hpf}$, $p < 0.05$; Figure 7A, B). To investigate differences in macrophage phenotype, serially collected sections were immunostained for iNOS, as an M1 associated marker or for CD68, which is common to both M1 and M2 macrophages. A statistically significant decrease in the ratio of iNOS/CD68 was noted for hMSC-seeded samples at all the time points analyzed ($p < 0.05$, Figure 8A–D).

hMSCs were labeled with carboxyfluorescein diacetate succinimidyl ester and incorporated within composite constructs. hMSCs levels were greatest during the first 3 days after implantation and decreased substantially after 7 days (Figure 9).

3.7 Tensile strength of integration

We observed an increase in the strength of integration from 4 to 8 weeks as host tissue is incorporated into the construct and maintained by new blood vessel formation (Figure 10). Cell-seeded constructs had a significantly greater strength of integration at 4 and 8 weeks when compared to the acellular constructs at those time points. At 8 weeks, the tensile strengths of integration were 0.59 ± 0.25 N/mm and 0.96 ± 0.19 N/mm for acellular engineered composite constructs and hMSC-seeded constructs, respectively ($p = 0.01$). A decellularized hexamethylene diisocyanate (HMDI)-crosslinked porcine dermis (PermaCol™) with 1 mm thickness was also analyzed after an 8-week implant period ($n=7$). The tensile strength of integration was somewhat greater in strength than the acellular sample, but lower in strength than the cell-seeded construct (0.72 ± 0.29 vs. 0.96 ± 0.19 N/mm, $p = 0.1$). These differences were not statistically significant. During tensile testing, samples failed either within the patch itself or the abdominal muscle rather than at the tissue material interface.

4. Discussion

We describe in this report the fabrication of a composite construct for stem cell delivery and tissue repair. The engineered patch was comprised of a mechanically robust, multilamellar collagen sheet impregnated with an alginate gel containing human mesenchymal stem cells. Laser machining of pores within the collagen component provided a means to enhance construct flexibility and suture retention strength, while facilitating cell delivery and subsequent integration with host-derived tissue. The alginate provided a temporary interphase layer for localized release of hMSC secreted factors during the initial phase of wound healing.

Synthetic meshes were initially designed to reinforce an abdominal wall defect by induction of scar tissue. However, excessive fibrosis was associated with patient discomfort due to pain and restrictive motion of the abdominal wall [26]. To alleviate this problem, lighter meshes have been designed with large pores and reduced surface area [27]. Nonetheless, the use of commercially available synthetic meshes continues to be limited by infection, seroma formation, viscera adhesion, shrinkage, and stiffness resulting in pain and discomfort [6–9]. Current biologic materials used for soft tissue repair are derived from human cadaver, porcine, or bovine donors. Although the donor tissue is typically decellularized by a series of chemical extractions, undesirable host reactions to residual allo- or xenoantigens remain

limitations and the need to avoid viral or bacterial transmission creates the need for stringent requirements for appropriate tissue processing. Residual biologic factors are often incompletely characterized and include pro-inflammatory or antigenic factors, such as donor DNA [28] and the galactose epitope [29, 30]. As an alternative, multiple groups have sought to use purified monomeric collagen, as a building block for engineering more complex materials due to its capacity support cell adhesion, proliferation, and differentiation. Collagen based products, including fibers and single or multilamellar sheets or tubes can be fabricated with precise control over physical and mechanical properties [23, 31] [13].

In this report, the construct consisted of multiple dense collagen films that were physically laminated together to create a strong, but flexible, 500 μm thick sheet. We have previously shown that collagen concentration does not affect ultimate tensile strength, since increasing collagen mass resulted in an increase in sheet thickness. However, strength does increase after lamination, presumably due to interpenetration of adjacent layers with an observed decrease in layer thickness [13]. Significantly, load induced construct failure was not associated with delamination. Although three empirically selected pore patterns were analyzed in our study, multiple options exist that can be selected to a desired application. We determined that a particular pattern (0.05 mm² pores, 240 pores/cm⁻², 10% surface area) was sufficiently strong while providing suitable flexibility and suture retention strength. We speculate that increased suture retention strength for laser patterned as compared non-patterned constructs may be related to the presence of thermally induced crosslinks between collagen fibers and individual lamellae that constitute the multilamellar collagen sheet. As a consequence, this may be associated with a more controlled distribution of locally induced stress patterns that limit crack propagation.

Ideally, bioactive constructs are designed to improve tissue repair by presenting adhesive ligands, as well as soluble signals, such as cytokines and growth factors that guide the host cell inflammatory and reparative response. The incorporation of an RGD-derivatized alginate gel within and surrounding the collagen construct facilitates the delivery of cells in a manner that enhances cell survival and preserves cell functionality [32, 33]. Human MSCs can be generated with well defined and reproducible phenotypic properties [34–36] and low passage and low density cultures of these hMSCs are enriched for early progenitor cells [37]. As an isolated cell therapy, hMSCs have been shown to improve hind limb ischemia in a number of animal models due to their capacity to produce VEGF, basic fibroblast growth factor, and other angiogenic factors [38–40]. The secretome of hMSCs incorporated within the construct demonstrated constant levels of VEGF, PDGF- β and MCP-1 for at least seven days in vitro, while cells in 2-D culture were unable to sustain this response. Of note, the stability of the neovascular response depends on the coordinated interaction of VEGF and PDGF- β , which recruit endothelial cells and pericytes, respectively [41]. While MCP-1 promotes a monocyte and macrophage response [42], these cells, in turn, release multiple angiogenic factors [43].

Following repair of a full thickness abdominal wall defect, *de novo* tissue formation with associated neovascularization was observed for both acellular and cell-seeded constructs. Significantly, more blood vessels were noted within hMSCs-seeded samples as compared to acellular samples, which persisted through the 8 week study period. Likewise, macrophage

infiltration was greater among hMSC-seeded, but the ratio of M1 macrophages to total macrophages was lower in these constructs at all time points, which is suggestive of an increase in alternatively activated M2 macrophages. Although additional studies may be of value to further confirm this phenotypic switch, these results are consistent with prior studies that have shown that hMSCs modulate macrophages from M1 to M2 phenotype [44–46].

hMSCs were present in substantial numbers for at least one week after in vivo implantation and then at lower levels thereafter, despite the absence of immunosuppression in what was essentially a xenotransplant model. The results of these studies support the notion that hMSCs can produce a significant therapeutic benefit without cell engraftment or differentiation at sites of host tissue injury. The mechanism of the therapeutic effect is likely paracrine signaling through the hMSC secretome during the acute inflammatory phase. Overall, improved blood vessel formation and a lower ratio of M1 macrophage to total macrophages correlated with a significantly higher strength of integration at four weeks and eight weeks when compared to the acellular constructs. The collagen-alginate constructs provided outstanding mechanical support for the full thickness abdominal wall defect, even though they were 50% thinner than the commercially available patch. This new therapeutic platform can be customized for numerous applications and may be further modified to improve clinical outcomes.

5. Conclusion

Alginate-collagen composites containing human mesenchymal stem cells (hMSCs) effectively bridged a full thickness abdominal wall defect and prevented hernia recurrence in Wistar rats over an eight-week period. *De novo* tissue and vascular network formation was observed without peritoneal adhesions. Cell-laden constructs displayed improved strength of integration, which correlated with increased neovascularization, increased macrophage infiltration, and a reduced proportion of M1 macrophages. Laser micromachining facilitated the fabrication of porous collagen sheets and provided a convenient means to tailor the mechanical properties of the multilamellar construct, as well as its capacity to harbor cells and locally integrate with host tissue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Flum DR, Horvath K, Koepsell T. Have outcomes of incisional hernia repair improved with time? A population-based analysis. *Ann Surg.* 2003; 237:129–135. [PubMed: 12496540]
2. Kingsnorth A. The management of incisional hernia. *Ann R Coll Surg Engl.* 2006; 88:252–260. [PubMed: 16719992]

3. Rutkow IM. Demographic and socioeconomic aspects of hernia repair in the United States in 2003. *Surg Clin North Am.* 2003; 83:1045–1051. [PubMed: 14533902]
4. Luijendijk RW, Hop WC, van den Tol MP, de Lange DC, Braaksma MM, JN IJ, et al. A comparison of suture repair with mesh repair for incisional hernia. *N Engl J Med.* 2000; 343:392–398. [PubMed: 10933738]
5. Earle DB, Mark LA. Prosthetic material in inguinal hernia repair: How do I choose? *Surg Clin North Am.* 2008; 88:179–201. [PubMed: 18267169]
6. Halm JA, de Wall LL, Steyerberg EW, Jeekel J, Lange JF. Intraperitoneal polypropylene mesh hernia repair complicates subsequent abdominal surgery. *World J Surg.* 2007; 31:423–429. [PubMed: 17180562]
7. Hindmarsh AC, Cheong E, Lewis MP, Rhodes M. Attendance at a pain clinic with severe chronic pain after open and laparoscopic inguinal hernia repairs. *Br J Surg.* 2003; 90:1152–1154. [PubMed: 12945086]
8. Robinson TN, Clarke JH, Schoen J, Walsh MD. Major mesh-related complications following hernia repair: Events reported to the Food and Drug Administration. *Surg Endosc.* 2005; 19:1556–1560. [PubMed: 16211441]
9. Choi JJ, Palaniappa NC, Dallas KB, Rudich TB, Colon MJ, Divino CM. Use of mesh during ventral hernia repair in clean-contaminated and contaminated cases: Outcomes of 33,832 cases. *Ann Surg.* 2012; 255:176–180. [PubMed: 21677561]
10. Misra S, Raj PK, Tarr SM, Treat RC. Results of AlloDerm use in abdominal hernia repair. *Hernia.* 2008; 12:247–250. [PubMed: 18209948]
11. Parker DM, Armstrong PJ, Frizzi JD, North JH Jr. Porcine dermal collagen (Permacol) for abdominal wall reconstruction. *Curr Surg.* 2006; 63:255–258. [PubMed: 16843776]
12. Smart NJ, Marshall M, Daniels IR. Biological meshes: A review of their use in abdominal wall hernia repairs. *Surgeon.* 2012; 10:159–171. [PubMed: 22436406]
13. Kumar VA, Caves JM, Haller CA, Dai E, Li L, Grainger S, et al. Collagen-Based Substrates with Tunable Strength for Soft Tissue Engineering. *Biomater Sci.* 2013; 1:1193–1202.
14. Miyahara Y, Nagaya N, Kataoka M, Yanagawa B, Tanaka K, Hao H, et al. Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. *Nat Med.* 2006; 12:459–465. [PubMed: 16582917]
15. Chen L, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS One.* 2008; 3:e1886. [PubMed: 18382669]
16. Boonthekul T, Kong HJ, Mooney DJ. Controlling alginate gel degradation utilizing partial oxidation and bimodal molecular weight distribution. *Biomaterials.* 2005; 26:2455–2465. [PubMed: 15585248]
17. Vacharathit V, Silva EA, Mooney DJ. Viability and functionality of cells delivered from peptide conjugated scaffolds. *Biomaterials.* 2011; 32:3721–3728. [PubMed: 21334064]
18. Silva EA, Kim ES, Kong HJ, Mooney DJ. Material-based deployment enhances efficacy of endothelial progenitor cells. *Proc Natl Acad Sci U S A.* 2008; 105:14347–14352. [PubMed: 18794520]
19. Sun Q, Silva EA, Wang A, Fritton JC, Mooney DJ, Schaffler MB, et al. Sustained release of multiple growth factors from injectable polymeric system as a novel therapeutic approach towards angiogenesis. *Pharm Res.* 2010; 27:264–271. [PubMed: 19953308]
20. Silver FH, Trelstad RL. Type I collagen in solution. Structure and properties of fibril fragments. *J Biol Chem.* 1980; 255:9427–9433. [PubMed: 7410433]
21. Silva EA, Mooney DJ. Effects of VEGF temporal and spatial presentation on angiogenesis. *Biomaterials.* 2010; 31:1235–1241. [PubMed: 19906422]
22. Kong HJ, Kaigler D, Kim K, Mooney DJ. Controlling rigidity and degradation of alginate hydrogels via molecular weight distribution. *Biomacromolecules.* 2004; 5:1720–1727. [PubMed: 15360280]
23. Caves JM, Cui W, Wen J, Kumar VA, Haller CA, Chaikof EL. Elastin-like protein matrix reinforced with collagen microfibers for soft tissue repair. *Biomaterials.* 2011; 32:5371–5379. [PubMed: 21550111]

24. Liu Z, Tang R, Zhou Z, Song Z, Wang H, Gu Y. Comparison of two porcine-derived materials for repairing abdominal wall defects in rats. *PLoS One*. 2011; 6:e20520. [PubMed: 21637777]
25. Meyer T, Schwarz K, Ulrichs K, Hocht B. A new biocompatible material (Lyoplant) for the therapy of congenital abdominal wall defects: First experimental results in rats. *Pediatric surgery international*. 2006; 22:369–374. [PubMed: 16518595]
26. Brown CN, Finch JG. Which mesh for hernia repair? *Ann R Coll Surg Engl*. 2010; 92:272–278. [PubMed: 20501011]
27. Klosterhalfen B, Junge K, Klinge U. The lightweight and large porous mesh concept for hernia repair. *Expert Rev Med Devices*. 2005; 2:103–117. [PubMed: 16293033]
28. Zheng MH, Chen J, Kirilak Y, Willers C, Xu J, Wood D. Porcine small intestine submucosa (SIS) is not an acellular collagenous matrix and contains porcine DNA: Possible implications in human implantation. *J Biomed Mater Res B Appl Biomater*. 2005; 73:61–67. [PubMed: 15736287]
29. McPherson TB, Liang H, Record RD, Badylak SF. Galalpha(1,3)Gal epitope in porcine small intestinal submucosa. *Tissue Eng*. 2000; 6:233–239. [PubMed: 10941218]
30. Sandor M, Xu H, Connor J, Lombardi J, Harper JR, Silverman RP, et al. Host response to implanted porcine-derived biologic materials in a primate model of abdominal wall repair. *Tissue Eng Part A*. 2008; 14:2021–2031. [PubMed: 18657025]
31. Caves JM, Kumar VA, Wen J, Cui W, Martinez A, Apkarian R, et al. Fibrillogenesis in continuously spun synthetic collagen fiber. *J Biomed Mater Res B Appl Biomater*. 2010; 93:24–38. [PubMed: 20024969]
32. Hill E, Boontheekul T, Mooney DJ. Designing scaffolds to enhance transplanted myoblast survival and migration. *Tissue Eng*. 2006; 12:1295–1304. [PubMed: 16771642]
33. Hill E, Boontheekul T, Mooney DJ. Regulating activation of transplanted cells controls tissue regeneration. *Proc Natl Acad Sci U S A*. 2006; 103:2494–2499. [PubMed: 16477029]
34. Colter DC, Class R, DiGirolamo CM, Prockop DJ. Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. *Proc Natl Acad Sci U S A*. 2000; 97:3213–3218. [PubMed: 10725391]
35. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006; 8:315–317. [PubMed: 16923606]
36. Sekiya I, Larson BL, Smith JR, Pochampally R, Cui JG, Prockop DJ. Expansion of human adult stem cells from bone marrow stroma: Conditions that maximize the yields of early progenitors and evaluate their quality. *Stem Cells*. 2002; 20:530–541. [PubMed: 12456961]
37. Lee RH, Seo MJ, Pulin AA, Gregory CA, Ylostalo J, Prockop DJ. The CD34-like protein PODXL and alpha6-integrin (CD49f) identify early progenitor MSCs with increased clonogenicity and migration to infarcted heart in mice. *Blood*. 2009; 113:816–826. [PubMed: 18818395]
38. Lasala GP, Silva JA, Gardner PA, Minguell JJ. Combination stem cell therapy for the treatment of severe limb ischemia: Safety and efficacy analysis. *Angiology*. 2010; 61:551–556. [PubMed: 20498146]
39. Lu D, Chen B, Liang Z, Deng W, Jiang Y, Li S, et al. Comparison of bone marrow mesenchymal stem cells with bone marrow-derived mononuclear cells for treatment of diabetic critical limb ischemia and foot ulcer: A double-blind, randomized, controlled trial. *Diabetes Res Clin Pract*. 2011; 92:26–36. [PubMed: 21216483]
40. Williams AR, Hare JM. Mesenchymal stem cells: Biology, pathophysiology, translational findings, and therapeutic implications for cardiac disease. *Circ Res*. 2011; 109:923–940. [PubMed: 21960725]
41. Gaengel K, Genove G, Armulik A, Betsholtz C. Endothelial-mural cell signaling in vascular development and angiogenesis. *Arterioscler Thromb Vasc Biol*. 2009; 29:630–638. [PubMed: 19164813]
42. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): An overview. *J Interferon Cytokine Res*. 2009; 29:313–326. [PubMed: 19441883]
43. Kwon HM, Hur SM, Park KY, Kim CK, Kim YM, Kim HS, et al. Multiple paracrine factors secreted by mesenchymal stem cells contribute to angiogenesis. *Vascul Pharmacol*. 2014; 63:19–28. [PubMed: 24998908]

44. Kim J, Hematti P. Mesenchymal stem cell-educated macrophages: A novel type of alternatively activated macrophages. *Exp Hematol.* 2009; 37:1445–1453. [PubMed: 19772890]
45. Maggini J, Mirkin G, Bognanni I, Holmberg J, Piazzon IM, Nepomnaschy I, et al. Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile. *PLoS One.* 2010; 5:e9252. [PubMed: 20169081]
46. Nemeth K, Leelahavanichkul A, Yuen PS, Mayer B, Parmelee A, Doi K, et al. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med.* 2009; 15:42–49. [PubMed: 19098906]

Figure 1. Fabrication of multilamellar porous collagen constructs

(A) Collagen gel. (B) Collagen film following dehydration. (C) Formation of a multilamellar sheet followed by rehydration and lamination (D) in fibril assembly buffer in an acrylic compression set up. (E) Laser machining was used to create a porous patterned patch of (F) defined pore dimensions and spacing. Bright field images of the collagen construct were acquired in top view (G) and cross-section (H). SEM image of pores in the collagen construct at low (I) and high (J) magnification views.

Figure 2. Modulation of mechanical properties in collagen constructs as a function of laser machined pore size and distribution

(A) Area of a single pore is 0.05 mm^2 with 10% of surface area occupied by pores. (B) Area of a single pore is 0.20 mm^2 with 10% of surface area occupied by pores. (C) Area of a single pore is 0.013 mm^2 with 3% of surface area occupied by pores. (D) Non-patterned multilamellar collagen construct. (E) Ultimate tensile strength. (F) Young's modulus. (G) Strain at failure. (H) Suture retention strength. Significance indicated at the $p < 0.05$ level, error bars represent standard error of the mean ($n = 3 - 6$). Scale bars represent $250 \mu\text{m}$.

Figure 3. Distribution and viability of human mesenchymal stem cells in alginate-collagen composite constructs

(A) Schematic of collagen patch embedding within an alginate gel to form alginate-collagen composite constructs. (B) Fluorescent microscopy of hMSC distribution on both sides of the construct. hMSCs were stained with calcein AM. (C) Representative image of cells within a pore at 1, 3, and 7 days. (D) Number of cells per square centimeter and (E) cell viability in the composite construct as a function of in vitro culture period (n = 3). Scale bars represent 200 μ m. Error bars represent standard error of the mean.

Figure 4. In vitro cytokine release from cellular composite patches

(A) MCP-1, (B) VEGF, or (C) PDGF- β release were measured from hMSCs normalized to 10^5 cells when cultured with as a monolayer on a tissue culture plate or within a composite construct ($n=3$). Error bars represent standard error of the mean ($*p < 0.05$, ns = non-significant differences).

Figure 5. Gross tissue-material responses after implantation of composite patches
 (A) An abdominal wall (a) full thickness defect was (b) repaired with a composite construct.
 (B) Recurrent hernia formation was not observed at 8 weeks after repair using either
 acellular or cell-seeded patches. (C) Subcutaneous view of samples at 2, 4, and 8 weeks. (D)
 Peritoneal view of samples at 2, 4, and 8 weeks.

Figure 6. Extracellular matrix production and blood vessel formation within implanted composite patches

(A) Masson's trichrome staining of samples at 2, 4, and 8 weeks after implantation. (B) VWF staining of middle section of samples at 2, 4, and 8 weeks. (C) Number of blood vessels per 20× field at 2, 4 and 8 weeks (middle section, * $p < 0.05$, $n = 3 - 7$). Scale bar 200 μm . Error bars represent standard error of the mean.

Figure 7. Macrophage infiltration of acellular and hMSC-seeded patches after in vivo implantation

(A) CD68 staining of acellular and hMSC-seeded samples at 2, 4, and 8 weeks. (B) CD68 staining per 20× field at 2, 4 and 8 weeks (* $p < 0.05$, $n = 3 - 7$). Scale bar 200 μm. Bars represent standard error of the mean.

Figure 8. Proportion of M1 macrophages in acellular and hMSC-seeded patches after in vivo implantation
 Ratio of iNOS⁺ to CD68⁺ stained cells in serial sections normalized to acellular samples at (A) 2, (B) 4, and (C) 8 weeks (* $p < 0.05$, $n = 3 - 7$). Scale bar 200 μm . Bars represent standard error of the mean.

Figure 9. Persistence of hMSCs after implantation of hMSC-seeded patches
 (A) Confocal microscopy of cross-sectional images through hMSC-seeded patches at 2 hrs, 3 days, 1, 2, 4, and 6 weeks. Dashed lines represent a pore in the patch. (B) Number of labeled hMSCs over a 42 day period in vivo (n = 1). Scale bar 40 μ m.

Figure 10. Strength of tissue repair of acellular and hMSC-seeded patches after in vivo implantation

Strength of integration at 2, 4, and 8 weeks (* $p < 0.01$, $n = 3 - 7$). Error bars represent standard error of the mean.