Short Stimulation of Electro-Responsive PAA/Fibrin Hydrogel Induces Collagen Production

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Acrylic acid/fibrin hydrogel can mechanically stimulate cells when an external electrical field is applied, enabling them to migrate and align throughout the depth of the gel. The ability of electro-responsive polyacrylic acid (PAA)/fibrin hydrogel to promote collagen production and remodeling has been investigated by three-dimensional (3D) culturing and conditioning of smooth muscle cells (SMCs). SMCs-seeded hydrogels were subjected to an alternating electrical field (0.06 V/mm) for 2 h for one, two, or three times per week during 4 weeks of culturing. Fluorescent images of collagen structure and accumulation, assessed by CNA-35 probe, showed increased collagen content (>100-fold at 1× stimulation/week) in the center of the hydrogels after 4 weeks of culture. The increase in collagen production correlated with increasing extracellular matrix gene expression and resulted in significantly improved mechanical properties of the stimulated hydrogels. Matrix metalloproteinase (MMP)-2 activity was also significantly enhanced by stimulation, which probably has a role in the reorganization of the collagen. Short stimulation (2 h) induced a favorable response in the cells and enhanced tissue formation and integrity of the scaffold by inducing collagen production. The presented set up could be used for conditioning and improving the functionality of current tissue-engineered vascular grafts.

Introduction

DEPOSITION OF EXTRACELLULAR MATRIX (ECM) by cells in tissue-engineered (TE) constructs plays an important role in providing a long-term functional substrate to regulate cell-tissue interaction.^{1,2} For replacement of mechanically active tissues such as blood vessel grafts, it is compulsory that TE constructs are sufficiently strong to withstand physiological forces such as blood pressure.³ The medial layer of blood vessels consists primarily of smooth muscle cells (SMCs), collagen (mainly type I and III), elastin, and proteoglycans that influence vessel function by providing appropriate stiffness, elasticity, and compressibility.³ Collagen is the most abundant ECM protein in vascular tissue, and its structural arrangement is responsible for the tensile strength.⁴

Environmental conditioning of seeded TE constructs can effectively influence ECM production and tissue formation. For example, mechanical stimuli such as cyclic strain have been shown to enhance collagen production in SMC-seeded scaffolds.^{4–7}

Previously, we have reported on the preparation, optimization, and characterization of an electro-sensitive polyacrylic acid (PAA)/fibrin hydrogel, showing that short electrical stimulation resulted in high cell alignment and distribution.⁸ PAA is a known biocompatible anionic poly-

mer. Acrylic hydrogels and their derivative polymers have been already used as an FDA approved basic component of dental and bone cement implants and intraocular lenses.^{9,10} In addition, based on the high charge density of PAA, they have been used as good candidate for mucoadhesives.¹¹

By applying an electrical field to the hydrogel, carboxylic groups of PAA network dissociate into mobile positive H⁺ and negatively charged –COO[–] groups. The attraction of ions toward their opposite electrodes induces swelling of the hydrogel at the cathode side and shrinkage at the anode side. Thus, the PAA hydrogel is pulled toward the positive electrode anode (Fig. 1). By changing the direction of the electrical field, rhythmic deformation (bending) of the hydrogel is achieved, which regulates cell behavior by inducing alignment of fibrin fibers, cells, and smooth muscle α -actin filaments.⁸

In this study, we investigate the long-term effect of different regimes of electrical stimulation on cellular density and collagen production of SMC-seeded electro-sensitive hydrogels. Synthesis, accumulation, and organization of collagen play an important role in regulating tissue growth and providing a mechanically functional graft with appropriate strength. Collagen production was visualized by staining the samples with a CNA-35 probe, a high-affinity collagen-binding protein that binds to the most abundant fibrillar collagens I, III, and IV present in the native vessel

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FIG. 1. Graphic overview of the hydrogel mounting fixing and cellular seeding in the culture plate equipped with carbon electrodes (a). Electrical current is applied to the cells, 4 h after seeding. Hydrogel swells at the side of the positive electrode side and shrinks at the negative one due to the attraction and migration of negative/positive ions toward opposite electrodes (b, c). Changes in the direction of the current (0.0167 Hz) cause reversible bending of the hydrogel. Color images available online at www.liebertpub.com/tec

wall.^{12,13} The effect of electrical stimulation on collagen remodeling was assessed by measuring the activity of two major matrix metalloproteinases (MMPs) produced by SMCs (i.e., MMP-2 and -9).^{14,15}

Materials and Methods

Preparation of PAA/fibrin hydrogels

PAA/fibrin hydrogels were synthesized as previously described.⁸ Briefly, acrylic acid (AA, purity 99.9%; Merck) was cross-linked and polymerized in the presence of fibrin (20 mg/mL fibrinogen mixed by 20 IU/mL thrombin, bovine plasma; Sigma-Aldrich) by adding *N*-*N*'-methylenebisacrylamide (MBAA; 2 wt% monomer; Sigma-Aldrich), ammonium persulfate (APS, 0.2 mol% of the AA; Sigma-Aldrich), and tetramethylenediamide (TMEDA, 0.4 mol% of AA; Sigma-Aldrich) as cross-linker, initiator, and accelerator, respectively. All components were dissolved in deionized water (1:1 weight ratio of AA to fibrin), molded between two plates with a 1 mm spacer, and heated at 80°C for 3 h. All chemicals used in hydrogel preparation were of molecular cell biology grade without further purification.

Hydrogel cell seeding

Lyophilized hydrogels were prepared by freeze drying and UV sterilization as previously described.⁸ Hydrogel strips (15×4×1 mm) were fixed at one end by tissue glue (Vetbond Tissue Adhesive) in a four-well plate. Primary porcine smooth muscle cells (pSMCs) were isolated from pig aorta, cultured, and expanded until passage 8–10, on fibronectin-coated flasks containing smooth muscle basal

medium (Lonza) that was supplemented with 5% fetal bovine serum, human epidermal growth factor, human fibroblast growth factor-B, insulin, and gentamicin. Cells were suspended in 100 μ L with a final cell density of 1.5×10^6 cells/sample and seeded on the surface of the hydrogels. Samples were incubated at 37°C with 5% CO₂ (Fig. 1a).

Stimulation of cell-seeded hydrogels

To determine the optimal stimulation regime, different patterns of electrical field were applied to the pSMC-seeded hydrogels. We studied the effect of four different stimulation conditionings, including a single stimulation at the first day of seeding, followed by one, two, or three stimulations per week. For each stimulation, the seeded hydrogel which had been fixed at one end in the four-well plate that was equipped with carbon electrodes was exposed for 2 h to an alternating electrical field of 0.06 V/mm. An electrical power supply (Bio-Rad Laboratories) connected to an in-house developed programmable switch provided the electrical field with a changing current direction every 60 s to induce reversible swelling/deswelling at both sides of the gel.⁸ To enable initial cell attachment and infiltration, samples were exposed to their first electrical stimulation 4 h after cell seeding. To block SMC contraction by electrical stimulation and to investigate only the effect of the produced mechanical force, Verapamil (10 μ M; Sigma-Aldrich) was added to the culture medium. SMCs in fibrin gels without the electroresponsive PAA component were used to evaluate the effect of the electrical field only, as fibrin itself does not undergo any conformational change in an electrical field. Nonstimulated cultured samples served as controls for all the experiments.

Microscopy

The stained samples were scanned and imaged with a two-photon laser scanning microscope (2PLSM; Bio-Rad) that was coupled to a Nikon E600FN microscope (Nikon) with a Ti-Sapphire laser excitation source of 140-fs-pulsed (Spectra Physics Tsunami) tuned and mode-locked at 800 nm (for visualization of fluorescent probes).^{16,17} To determine the collagen production, pSMC-seeded hydrogels were washed with phosphate-buffer solution (PBS) 1× (Lonza) and fixed in 2% paraformaldehyde for 20 min at 4°C. Samples were then washed thrice in PBS 1× and incubated with the CNA35-conjugated Alexa Fluor® 647 collagen probe (CNA35 in house synthesis, diluted 1:10 in HBSS; Alexa Fluor Lifetechnologies) for 60 min, followed by DAPI for cell nucleus staining (diluted 1:1000 in PBS) for 20 min at room temperature.¹⁷

To scan the samples by 2PLSM, hydrogels were put in a plate, filled by liquid 2% agarose gel, and cooled down at room temperature to solidify and fix the sample. A small amount of deionized water (MiliQ) was added on top of the gels to enable scanning with a 40× water-dipping objective (2.0 mm working distance, numerical aperture 0.8; Nikon). Hydrogel surface images were collected in the *x-y* plane. To scan the hydrogel center section, *z* stack images were recorded from the depth center (~400 μm from top-seeded surface) in three different parts of the sample and continued through the thickness of the structure (*z* direction) with interval *z*-steps of 2–4 mm for approximately 300 μm.

ImageJ (National Institutes of Health) software was used to quantify and analyze the collected images. Cell density was calculated by counting DAPI-stained cell nuclei of each image (*x-y* plane) using Nucleus Counter Plug-in. Collagen was quantified by converting the images presenting CNA-35-stained areas to 8-bit images and measured the adjusted threshold area in each section by using the Area Calculator plug-in. Total cell density and collagen area in the (top-seeded) surface were calculated on more than six spots (*x-y*) for each sample (*n*=3). For the center section of the hydrogels, an average value of at least 18 images for each condition, including six images in the *z*-direction from three spots (*x-y*), was used. Three-dimensional images of stained hydrogels were built up and rendered using the VolumeJ builder plug-in (v. 1.7a; Michael Abramoff).

Reverse transcriptase–polymerase chain reaction

Protein gene expression in the TE constructs was measured by performing Real-time reverse transcription–polymerase chain reaction (RT-PCR) by using a BIORAD MiQ5 (Bio-Rad) with Quanta reagents (VWR international

BV). Data analysis was performed by using Biogazelle Qbase (Biogazelle). pSMC-seeded hydrogels were cultured, and RNA was collected after the last stimulation at 4th week. To extract total RNA, samples were freeze dried, dissolved by RNeasy lysis buffer (RLT; Qiagen), and mechanically destroyed by Ultraturrax homogenizer to disrupt tissue and cell membranes. A total of 100 ng RNA per sample was subjected to RT. Quantitative PCR was performed by two steps: Syber Green kit protocol from Quanta with a 5-min 45°C incubation prerun step and 40 PCR cycles with 59°C annealing temperature using the primers as listed in Table 1. Quantitative PCR reactions were run on a BioRad MiQ Real-Time PCR detection System (Bio-Rad Laboratories). Expression levels of *COLLAGEN1α1* (*COL1α1*), *COLLAGEN1α2* (*COL1α2*), *COLLAGEN3α1* (*COL3α1*), and *ELASTIN* were normalized to the expression of *β-ACTIN* and *HPRT*, which stayed constant during stimulation and control culture conditions. The samples collected before stimulation were considered controls and used to normalize the data (set at 1).

Hydrogel bending and compaction

pSMC-seeded hydrogels were either not stimulated (controls) or exposed to stimulation at the first day of seeding and, subsequently, one or three stimulations per week, each for 2 h. All samples were cultured for 4 weeks in total. Digital images of hydrogels were taken every other day by a microscope with a built-in camera (EVOS, AMG). Hydrogel width was measured from all images using ImageJ (*n*=3 for each condition). The compaction ratio was calculated as follows: $(W_t - W_i)/W_i \times 100\%$, where W_i and W_t represent the width of the hydrogel at initial condition and time *t*, respectively. Reversible deformation of the hydrogels was recorded during their exposure to electrical field and measured as a deviation of the hydrogel strip from a fixed middle line along the hydrogel as a reference and defined as bending angle (Fig. 1).

Uniaxial tensile test

Mechanical properties of pSMC-seeded PAA/fibrin hydrogels were determined by a uniaxial testing machine (Tainstruments Q800) with the use of an 18-N load cell at a cross-head speed of 1 mm/min. All the samples were ~3 × 10 × 1 mm (width × length × thickness) and had been cultured for 4 weeks, in either stimulated or static condition. Samples were mechanically measured in their swollen hydrated state. At least four samples were tested for each condition.

TABLE 1. FORWARD AND REVERSE PRIMERS USED FOR RT-PCR OF SMOOTH MUSCLE CELL-SEED PAA/FIBRIN HYDROGELS

Gene	FWD	REV
<i>COL1α1</i>	AAGAAGAAGGCCAACAAGG	CATCGCACACACATTGC
<i>COL1α2</i>	GAGATGGTGATGATGGTATCC	ACTGAGCAGCAAAGTTCC
<i>COL3α1</i>	GAGCTTCCCAGAACATCACA	CACCTTCATTTGATCCCATC
<i>ELASTIN</i>	GTGGAAGCTTTTGCTGGAAT	GCAGTTTCCCAGTGCTGTAG

PAA, polyacrylic acid; RT-PCR, reverse transcription–polymerase chain reaction.

Gelatin zymography

Activity of MMP was analyzed in conditioned medium obtained from cultured pSMC-seeded PAA/fibrin hydrogels or pSMC-seeded fibrin gels. The media of samples 2 h before the stimulation, during the stimulation (2 h), and in the nonstimulated control condition were collected, stored at -20°C , and centrifuged before the measurement. MMP activity was assessed by Gelatin Zymography, as MMP-2 and MMP-9 are capable of degrading Gelatin. Zymography was performed by using 10% acrylamide/gelatin gel (Novex; Invitrogen). Equal volumes of media were loaded into the Zymogram gels and run at 125 V for 90 min. To reactivate MMP activity, gels were washed in re-naturing buffer and subsequently, incubated in developing buffer overnight. Gels were stained with SimplyBlue™ Safestain (Invitrogen) and rinsed in deionized water before detection with a BioRad camera and quantification by Quantity One® Analysis software (Bio-Rad Laboratories).

Statistical analysis

All quantitative experiments were performed in triplicate or more, and data were expressed as means $\pm$ standard deviation. To compare groups' multiple means with one or two parameters, statistical analysis of the results was performed by *t*-test, one-way analysis of variance (ANOVA), or two-way ANOVA (repeated measures) with Bonferroni *post hoc* analysis (Graphpad prism 4; Graphpad Prism Software, Inc.). The correlation of variables between two groups was examined by calculating the Pearson correlation coefficient (Graphpad Prism 4). *p*-values < 0.05 were considered significant in all the analysis.

Results

Cell density and collagen production after 4 weeks of culture

Hydrogels were stained with DAPI/CNA35 probes and scanned by 2PLSM at the end of 4 weeks of culture to check the cell density and collagen-matrix production in the surface and center section of the samples. Images were quantified by using ImageJ software.

Confirming our earlier results with a short culture period, the cells were aligned and homogeneously distributed in the stimulated samples compared with random orientation in the control samples after 4 weeks of culturing (Fig. 2a–d). Stimulated samples revealed profound fibrillar network formation of collagen compared with random spot deposition of collagen in nonstimulated hydrogels after 4 weeks of culture. We observed similar cell density at the surface of hydrogels in the stimulated and nonstimulated conditions (Fig. 2e); however, collagen production was significantly enhanced at the surface of the stimulated samples compared with controls (Fig. 2f; 3.7-fold; $p < 0.0001$). In the center section of the stimulated samples, cell density was more than six times increased compared with nonstimulated ones (Fig. 2h). The amount of collagen in the center of stimulated hydrogels was 85-fold increased compared with controls (Fig. 2i; $p < 0.001$) as a result of higher collagen production per cell (Fig. 2j; eightfold, $p < 0.01$) in the stimulated samples.

Applying mechanical load to the cells could augment collagen production through either increasing proliferation or increasing the procollagen synthesis per cell.¹⁸ Improved distribution of the cells across the depth of the stimulated hydrogels accompanied by enhanced capacity of cells to

FIG. 2. Two-photon laser scanning microscopy images of smooth muscle cells (SMCs) (DAPI, blue) and Collagen organization (CNA-35, red) from the surface and center cross-section (*z* direction) of the nonstimulated (control) (a, b) and stimulated constructs seeded with porcine smooth muscle cells (pSMCs) of one stimulation on the first day (c, d) after 4 weeks of culture. Total cellular density (e, h), collagen area (f, i), and collagen production per cell (g, j) quantified at the surface and in the center cross-section of hydrogels. Asterisks indicate *p*-value < 0.05 , stimulated versus control.

produce more collagen resulted in much higher collagen accumulation in the stimulated samples after 4 weeks.

Stimulation frequency affects collagen production after 4 weeks of culture

To evaluate the effect of stimulation frequency on pSMCs collagen and tissue production in the samples, different stimulation patterns were applied to seeded hydrogels during 4 weeks of culturing. Hydrogels were first of all exposed to electrical stimulation on the seeding day, and were followed by one, two, or three stimulations (each for 0.06 V/mm, 0.0167 Hz, 2 h) per week for the rest of the 4 weeks of culture. Nonstimulated samples served as controls. Hydrogels were stained with DAPI/CNA35 and scanned by 2PLSM at the end of the 4-week culture (Fig. 3a–l). Similar to samples with only one stimulation on the day of seeding (Fig. 2c, d), continued intermittent stimulation up to twice per week resulted in more condense collagen fiber formation

compared with nonstimulated controls after 4 weeks (Fig. 3a–i). These samples showed enhancement in collagen production both at the surface and in the center while at three stimulations per week collagen was disorganized, showing deposition mainly as cytoplasmic spots without fibrillar appearance (Fig. 3j–l).

Although total cell density in the surface was not affected by stimulation, a seven-fold increase in cell density in the center was observed (Fig. 3m, n). The collagen production at the surface was enhanced by approximately 3- and 2.5-fold in the one and two stimulations per week compared with nonstimulated controls, respectively (Fig. 3o). Notably, in the center of the gel, the increase in collagen production was strongly augmented to 120- and 100-fold, respectively, compared with the nonstimulated control condition (Fig. 3p). Although the three stimulations per week showed no significant difference in morphology and collagen amount at the surface of hydrogel compared with controls (Fig. 3a, j), in the hydrogel center section, the collagen amount was

FIG. 3. Two-photon laser scanning microscopy images of SMCs (DAPI, blue) and Collagen organization (CNA-35, red) from the surface, center, and center cross-section (z direction) of the nonstimulated (0 stimulations/week) (a–c) and stimulated constructs seeded with pSMCs of 1 (d–f), 2 (g–i), and 3 times stimulation (j–l) per week, over 4 weeks of culture. Total cellular density (m, n), collagen area (o, p), and collagen production per cell (q, r) quantified at the surface and in the center cross-section of hydrogels. Reverse transcription polymerase chain reaction (RT-PCR) analysis of gene expression of extracellular matrix genes, including *COLLAGEN1 α 1*, *COLLAGEN1 α 2*, *COLLAGEN3 α 1*, and *ELASTIN*, in polyacrylic acid (PAA)/fibrin hydrogel before and after the last stimulation at the 4th week of culture (s). Asterisks indicate $p < 0.05$ compared with no stimulation (m–r) or before stimulation (s).

significantly higher than in controls (Fig. 3p; 46-fold, $p < 0.01$).

Stimulated samples showed no significant difference in the collagen production per cell in the surface and center section, while in control hydrogels, there was a significant fivefold decrease in the hydrogel center compared with the surface (Fig. 3q, $r; p < 0.05$). Considering the fibrillar network of collagen, collagen content, and collagen production per cell, the optimal protocol appears to be one electrical stimulation per week.

Therefore, we also evaluated the effect of stimulation on the expression of ECM genes, including *COL1 α 1*, *COL1 α 2*, *COL3 α 1*, and *ELASTIN* in the samples stimulated once per week. RT-PCR analysis was performed on pSMC RNA isolated from the hydrogels to estimate their expression levels for the 4-week culture (Fig. 3s). RNA was collected from samples before and after stimulation. The samples collected before stimulation were considered controls and used to normalize the data.

Stimulation led to higher expression of the matrix genes *COL1 α 1*, *COL1 α 2*, *COL3 α 1*, and *ELASTIN* by 54%, 111%, 72%, and 185%, respectively, compared with the earlier stimulation condition. The obtained data corresponded with the higher production of collagen at 4 weeks on stimulation represented by 2PLSM images and confirmed the fibrillar collagen, as it mainly consisted of collagen type I and III.¹⁹

Hydrogel compaction and its electro-responsive bending after 4 weeks of culture

pSMC-seeded PAA/fibrin hydrogels subjected to alternating electrical field once at the day of seeding only or additionally one to three times per week during 4 weeks of culturing were evaluated for their degree of bending and compaction. During the stimulation, samples were evaluated by collecting light microscopy images for bending and every other subsequent day, for compaction (Fig. 4).

Just 2 days after seeding, a significant increase in the compaction ratio (>6 -fold) for stimulated samples compared with controls was noted (Fig. 4b). No significant difference was found between the compaction of samples stimulated with different regimes. Indeed, hydrogel stimulated once after cell seeding followed the same path as the samples stimulated thrice per week for 4 weeks. After the initial increase in stimulated samples, compaction of the hydrogel increased in the stimulated and control gels during 4 weeks of follow up, with a similar trend. The observation suggests that the reorganization of the PAA/fibrin network, which occurs during the first stimulation, plays an important role in the compaction. We previously showed high alignment of fibrin fibers in the direction of the electrical field after a one-time stimulation (0.06 V/mm, 0.0167 Hz, 2 h) in PAA/fibrin hydrogels.⁸

The bending angles of pSMC-seeded hydrogels were also recorded during each stimulation. In the samples stimulated thrice per week, the bending angle significantly increased over time starting with 1.5° on the day of seeding and reaching a max of 2.7° ($\pm 4\%$) at 25 days (Fig. 4c; $p < 0.01$). In accordance with previous observation in rods made from PVA/PAA hydrogels, lower bending angles were also observed with increasing gel width.²⁰ However, the bending angle of hydrogels, which were exposed to one stimulation

FIG. 4. Light macroscopic images of pSMC-seeded PAA/fibrin hydrogel show compaction of the samples in non-stimulated (control) and one stimulation per week condition over 4 weeks of culture (a), the compaction rate of non-stimulated control and stimulated samples (single stimulation on the seeding day, 1 and 3 stimulations per week) (b), The bending angle during stimulation time of samples stimulated one and three times per week over 4 weeks of culturing (c). Color images available online at www.liebertpub.com/tec

per week, stayed constant over time, although they had the same compaction ratio as samples stimulated thrice per week. Since collagen amount was much higher in the once per week stimulation than in the thrice per week stimulation, the resulting mechanical strength likely resisted the bending (correlation coefficient between bending angle and collagen production at the end of 4th week: Pearson's $r = 0.93$, $p < 0.01$).

Mechanical properties of PAA/fibrin hydrogels

The mechanical properties of the pSMC-seeded PAA/fibrin hydrogels stimulated condition (one stimulation/week) and nonstimulated (static) condition were characterized by performing tensile testing along the longitudinal axis

of the hydrogels. In addition, nonseeded PAA/fibrin hydrogel incubated in SMCs medium for 4 weeks were tested as noncellularized controls and were compared with seeded and stimulated samples to evaluate the effect of cell seeding and stimulation on the mechanical properties of the tissues. The stress-strain curves of hydrogel behavior are illustrated in Figure 5a. The maximum tensile strength (Fig. 5b) and elongation at break (Fig. 5c) were significantly increased in seeded samples, which were stimulated once per week (26.6 kPa, 79.5%) compared with the statically cultured seeded (15.7 kPa, 40.8%) and nonseeded PAA/fibrin gels (12.5 kPa, 26.5%). No significant difference was found for ultimate strength and elongation between the seeded and nonseeded hydrogels cultured in the static condition.

The elastic modulus was not significantly different between the stimulated, nonstimulated, and nonseeded control gels (average of 45 kPa, data not shown), which likely reflects the lack of elastin deposition in the structures. The higher collagen density in the stimulated hydrogels as a result of the even distribution of cells within the stimulated samples,⁸ accompanied by a great increase in collagen production per cell, is likely the main reason for the increase (70%) in maximum strength and elongation at break (94%).

MMP activity in PAA/fibrin hydrogels during 4 weeks culturing

For the optimum stimulation protocol (one stimulation per week during 4 weeks), we measured the activity of MMP-2 and MMP-9 in conditioned media of PAA/fibrin hydrogel samples before and after every stimulation as well as in nonstimulated controls (Fig. 6). Compared with the control condition, MMP-2 activity was more than doubled after the first stimulation on the day of seeding followed by a 1.6- and 1.8-fold increase after the second and third stimulation, respectively (Fig. 6b). The fourth stimulation no longer resulted in a significant increase in MMP-2 activity. In the conditioned media collected from stimulated samples before stimulation and from nonstimulated controls, there was no significant difference between the levels of MMP-2. MMP-9 activity was substantially lower than MMP-2 and showed no effect of stimulation in the first and second stimulation, while expression significantly decreased after the third and fourth stimulation compared with before stimulation and nonstimulated control samples (Fig. 6c).

To check whether the effect of stimulation on MMP-2 activity is a result of the applied electrical field or the me-

chanical force, which is produced by swelling and deswelling of the PAA/fibrin hydrogel, we studied MMP activity of pSMCs in fibrin gel. Fibrin gel does not mechanically respond to the electrical field; therefore, the effect of the electrical field on MMP-2 activity by pSMCs can be studied. The pSMCs seeded fibrin gels were exposed to the same electrical stimulation regimes as PAA/fibrin hydrogels (0.06 V/mm, 0.0167 Hz, 2 h) on the day of seeding and MMP-2 activity was analyzed (Fig. 6d). No stimulatory effect on MMP-2 activity was observed. In addition, to establish the relationship between electrical field-induced contraction of pSMCs and MMP-2 activity, L-type voltage-gated calcium channels of pSMCs were blocked by adding verapamil (10 μ M) just before stimulation. Blocking pSMCs contraction had no effect on electrical field-induced MMP-2 activity in PAA/fibrin gel. These data suggest that the applied electrical field or the resulting contraction by pSMCs are not the driving forces for the observed enhancement of MMP activity.

Discussion

The art of tissue engineering lies in the design and complexities of the scaffold, where instructive signals could be provided to the cells. In our previous work, we showed that one single electrical stimulation (0.06 V/mm, 0.0167 Hz, 2 h) applied directly after seeding the electro-sensitive hydrogels improved the penetration of cells through the depth of the hydrogel compared with nonstimulated control samples after 1 week of culture.⁸ Uniform cell distribution of cells within the scaffolds is the potential key to produce uniform tissue. Apart from static cell seeding techniques, various methods such as perfusion and centrifugation of cell suspensions into the scaffolds have been used to improve the seeding efficiency.²¹ In our system, due to exothermic polymerization and cross-linking reactions of AA, as well as acidic pH in the prepolymerized solution, it was not possible to seed the cells during molding.²² However the responsive swelling/deswelling of the hydrogel facilitated cell infiltration into the structure, obviating the need for cell mixing during gel molding. In the current study, we analyzed the effect of prolonged culture time up to 4 weeks for hydrogels that were stimulated (0.06 V/mm, 0.0167 Hz, 2 h) once after cell seeding by checking for cell growth and tissue formation.

Cyclic strain plays a critical role in inducing collagen and elastin synthesis by vascular SMCs; however, the responses

FIG. 5. Stress-strain curve (a), elongation at break (b) and ultimate tensile strength (c) of seeded and nonseeded PAA/fibrin hydrogels cultured in stimulated (one stimulation per week, 2 h, alternative 0.06 V/mm) and control condition under uniaxial tensile test after 4 week of culturing. Asterisks indicate $p < 0.05$ compared with controls; ns, not significant.

FIG. 6. Zymography gels (a) of the conditioned media represent matrix metalloproteinase (MMP)-2 (b) and MMP-9 (c) activity in the pSMCs-seeded PAA/fibrin hydrogels in static condition (control), before stimulation, and after stimulation (one stimulation per week, 2 h, alternative 0.06 V/mm) during 4 weeks of culture. MMP-2 activity in the pSMC-seeded PAA/fibrin hydrogels and fibrin Gel on the first day of seeding, with or without verapamil treatment (10 μ M) during stimulation (d). Asterisks indicate $p < 0.05$; ns, not significant.

are highly dependent on the amplitude, frequency, and duration of the strain.⁴ Other different techniques such as micropatterning, perfusion systems, and ultrasound standing wave fields published recently have been shown to affect the ECM production.^{23–25} In our study, the mechanical forces on the cells were very briefly applied, but still resulted in increased tissue formation. Increasing the exposure time of mechanical loading to the cells (three stimulations per week) had a reduced effect on collagen production, as noted in previous studies.⁶

The elevation of transforming growth factor-beta (TGF- β) in the mechanically stimulated samples tends to be the key mediator of the stretch-dependent collagen production in SMCs.^{26,27} In addition, the strain-dependent release of TGF- β has been shown to stimulate L-proline and L-arginine transport during collagen formation and to maximize the capacity of cells to synthesize collagen.²⁸

Enhancement of ultimate tensile strength in the SMC-seeded collagen scaffold exposed to cyclic stretching associated with higher collagen content and integrity has been reported by others.^{5,6} We also observed higher ultimate tensile strength and elongation at break, which confirm higher collagen production and tissue formation within the stimulated samples. However, tensile strengths in the kPa range are still not sufficient to meet the mechanical requirements for TE blood vessels. Either prolonged culturing or combinations with other biomaterials are options for our technology to use in vascular grafts.

Our observations on increased MMP-2 with mechanical stimulation produced within the hydrogel are in accordance with earlier studies in which strain facilitated remodeling of the collagen substrate by the enhancement of MMP-2 activity.^{6,27,29} However, it has been shown that the response of SMCs in regulating the pro-MMPs protein and MMP activity is dependent on the regimen of the applied mechanical force with increased MMP-2 activity by stationary strain and decreased MMP-2 activity by cyclic strain.³⁰ The low frequency of the applied electrical field (0.0167 Hz) in our system likely produces a static strain in the hydrogel.

The balance between collagen production by cells and degradation by proteases determines collagen accumulation in TE structures.³¹ Indeed, the degradation and reorganization of the collagen plays a key role in remodeling the ECM.^{32,33} The cellular response to mechanical forces induced in the PAA/fibrin hydrogel has resulted in higher expression levels of MMP-2, which could lead to higher reorganization of produced collagen later during culture.³⁴ The improvement in strength and elongation properties of the mechanically stimulated samples correspond well with the increasing MMP-2 activity level, which suggests that reorganization of the construct leads to an integrated collagen network.^{29,35}

In addition, it has been reported that increasing MMP-2 activity is linked to SMCs migration, where MMP-2 could mediate the migration of SMCs through barriers by degrading ECM and nonmatrix substrates.³⁶ MMP-2 and

MMP-9 can also degrade fibrin and fibrinogen.³⁷ Higher penetration of cells in stimulated PAA/fibrin hydrogel that was previously observed⁸ is consistent with the higher activity of MMP-2 induced by stimulation.

The proposed system has the potential to be translated into *in vivo* application by mounting electrode patches on the skin and stimulating the graft in a transcutaneous way to attract cells and stimulate tissue growth. Stickler *et al.* investigated the effect of external signals of mechanical forces to improve the development and growth of vascular grafts in Peritoneum *in vivo*.³⁸ Cells in the peritoneal cavity were attracted around a tubular scaffold by a designed cyclic stretching device connected to a pulsatile pump. A comparison between pulsed and nonpulsed scaffolds revealed significantly greater collagen organization, F-actin expression, and mechanical properties in pulsed scaffolds.³⁸ In addition, electro-responsive hydrogels have been tested as drug carriers to release drugs by swelling and deswelling under electrical stimulation,³⁹ and, therefore, could be used to facilitate attracting specific cells and stimulating tissue formation by releasing chemotaxis and growth factors.

On other hand, the body immune response to the implanted graft is an important criteria related to the biocompatibility of the biomaterial. UV exposure is being used as one classical technique to sterilize biomaterials and hydrogels before *in-vivo* applications^{40,41}; however, long UV irradiation could cause substantial changes in the polymer properties and its biological functionality as has been reported by Fischbach *et al.*⁴² Indeed, depending on the nature of the biomaterial and sterilization treatment (gamma, ethylene oxide, UV, autoclave, etc.), potential changes could be induced in molecular weight, mechanical strength, and surface topography of the polymer as has been recently reported.^{43–45}

In addition, the biodegradability of the implanted graft should also be taken into consideration for future clinical approaches. The current hydrogel is based on covalent cross-linking of AA monomers by MBAA as the cross-linker in the presence of fibrin fibers. MBAA cross-linked AA has been reported to show low inflammatory reactions with no systemic toxicity in a rat model, likely because cross-linking inhibits the solubility of the polymer and, therefore, reduces toxicity effects of the structure.⁴⁶ However, clinical application of these hydrogels is restricted due to their nonbiodegradability. Biodegradable cross-linkers and the use of natural electro-responsive biopolymers such as alginate are promising alternatives for future applications.

Conclusion

In this article, we evaluated the functionality of the electro-responsive PAA/fibrin hydrogel to enhance tissue formation as well as to improve collagen organization in the construct. Short application of the electrical field to seeded pSMCs in PAA/fibrin hydrogel produces a mechanical force, which efficiently induces higher collagen production, higher activity of matrix metalloprotease, and higher reorganization of the collagen content in the hydrogel. The improved collagen accumulation within the structures also enhances the mechanical properties of the hydrogel significantly compared with nonstimulated control samples. With

our designed hydrogel device and set up, we can facilitate and instruct 3D hydrogel tissues that could be used for tissue-engineering applications, such as creating TE blood vessels in which simultaneous alignment and improved collagen production are warranted.

Acknowledgments

This research forms a part of the Project P1.01 iValve of the research program of the BioMedical Materials institute, co-funded by the Dutch Ministry of Economic Affairs. The financial contribution of the Nederlandse Hartstichting is gratefully acknowledged. This work was also supported by the Research and Expertise Center for microscopic imaging of Maastricht University, Faculty of Health, Medicine, and Life Sciences (FHML). D.G.M. is supported by the Euregio Meuse-Rhine Interreg IV-A BioMIMedics project. The authors also thank Sheila Moli from Aachen University and Mohammad Ghomi Rostami from Sharif University of Technology for their support and assistance.

Disclosure Statement

No competing financial interests exist.

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Received: September 20, 2013

Accepted: December 12, 2013

Online Publication Date: February 6, 2014