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THE FIBER DIAMETER OF SYNTHETIC BIORESORBABLE EXTRACELLULAR MATRIX INFLUENCES HUMAN FIBROBLAST MORPHOLOGY AND FIBRONECTIN MATRIX ASSEMBLY

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Abstract

Background—The ideal scaffold material should provide immediate capacity to bear mechanical loads, while also permitting eventual resorption and replacement with native tissue of similar mechanical integrity. Scaffold characteristics such as fiber diameter provide environmental cues that can influence cell function and differentiation. In this study, the impact of fiber diameter of scaffolds constructed from a tyrosine-based bioresorbable polymer on cellular response was investigated.

Methods—Electrospun bioresorbable poly(DTE carbonate) scaffolds comprised of microfibers or nanofibers were constructed and seeded with human dermal fibroblasts. The impact of fiber diameter on actin cytoskeletal morphology, focal adhesion size, fibronectin matrix assembly, and cell proliferation were evaluated using immunofluorescent microscopy and computer-assisted image analysis.

Results—Actin stress fibers were more easily observed in cells on microfiber scaffolds compared to those on nanofiber scaffolds. Cells on nanofiber scaffolds developed smaller focal adhesion complexes compared to those on microfiber scaffolds ($p < 0.0001$). The temporal patterns of fibronectin matrix assembly were affected by scaffold fiber diameter with cells on microfiber scaffolds showing a delayed response in dense fibril formation compared to nanofiber scaffolds. Cells on nanofiber scaffolds showed higher proliferation compared to microfiber scaffolds at time points under 1 week ($p < 0.01$), but by 2 weeks significantly higher cell proliferation was observed on microfiber scaffolds ($p < 0.01$).

Conclusions—The fiber diameter of bioresorbable scaffolds can significantly influence cell response and suggest that the ability of scaffolds to elicit consistent biological responses depend on factors beyond scaffold composition. Such findings have important implications for the design of clinically useful engineered constructs.

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Keywords

extracellular matrix; bioresorbable scaffolds; fiber diameter; cell response; tissue engineering; fibronectin; fibroblast; scar

INTRODUCTION

The development of an ideal scaffold material that can be implanted to provide or augment load-bearing capacity while also allowing eventual resorption and replacement over the long term with native tissue of similar mechanical integrity would benefit patients undergoing plastic and reconstructive procedures for a broad range of problems, and recent years have seen the publication of several studies examining the use of bioresorbable matrices for hernia repair (1,2), breast reconstruction (3,4), and the replacement of musculoskeletal structures such as ligaments and tendons (5–7). Nonetheless, an ideal synthetic extracellular matrix (ECM) that provides biomechanical and structural support allowing cell adhesion and migration as well as environmental cues that regulate cell differentiation and metabolic function remains elusive, and the development of such a matrix would benefit from a clearer understanding of how cells interact with these matrices.

Cells adhere to the ECM by forming focal adhesions composed of clustered transmembrane integrins linked to juxtamembrane cytoplasmic proteins such as vinculin, talin, paxillin, and tensin, which are attached in turn to the actin cytoskeleton (8). In addition to providing anchorage, focal adhesions provide signaling pathways in which cells are informed about the state of their external environment and play an important role in the fibril and matrix assembly of fibronectin, a natural ECM protein whose secretion is upregulated by wound cells during tissue repair (9,10). The formation of a fibronectin matrix precedes the formation of, and may act as a template for, the mature scar collagen matrix (11,12). The ability of cells to assemble a fibronectin matrix is therefore believed to play an important role in wound healing, and information regarding the impact of synthetic ECM scaffolds on fibronectin matrix assembly would be valuable in optimizing their design.

In an effort to examine the influence of ECM and bioresorbable scaffold dimensions on cell response, three-dimensional (3D) electrospun scaffolds were investigated that were constructed using the bioresorbable synthetic polymer material known as polydesaminotyrosyl-tyrosine ethyl ester carbonate (M_w 218,000 Daltons), or poly(DTE carbonate) which was first described as a degradable biomaterial by Ertel and Kohn (13). Published studies have suggested potential clinical application for scaffolds constructed from this tyrosine-based polymer including, for example, use in reconstruction of the anterior cruciate ligament of the knee (6). Poly(DTE carbonate) scaffolds have been previously characterized with demonstrated biocompatibility *in vivo* and an extended degradation rate that can be adjusted by manipulating the monomer acid content (14,15). However, specific data regarding how cellular interactions with this material may contribute to eventual resorption and replacement with native tissue by human cells remain relatively incomplete. In this study, we report data that characterize the response of normal human fibroblasts, a common wound cell type, to scaffolds comprised of bioresorbable synthetic fibers of different diameters. This is the first known study to characterize normal human cell response to poly(DTE carbonate) fiber diameter, and to our knowledge, the only known study to report data on the ability of cells to assemble a fibronectin matrix in a manner that is dependent on the fiber diameter of a scaffold.

MATERIALS AND METHODS

Electrospinning & Scaffold Characteristics

Scaffolds were electrospun (under the guidance of Dr. Joachim Kohn at the New Jersey Center for Biomaterials using previously published methodology) from one solvent system in an effort to eliminate variability in fiber texture and residual solvents, and fiber diameters were controlled by adjusting polymer viscosity, flow rate, needle gauge and electric field (15). Briefly, nanofiber (diameter $< 1 \mu\text{m}$) and microfiber (diameter $> 1 \mu\text{m}$) scaffolds comprised of poly(DTE carbonate) (Isochem, Princeton, NJ) were electrospun from 70/30% methylene chloride/N,N-dimethyl formamide (Aldrich Chemical, Milwaukee, WI) onto clear cellophane film taped to a 3" circular aluminum target. Nanofibers were obtained by spinning 10 wt. % polymer solutions at 0.20 mL/h and 30 kV (27 G needle, 35 cm target distance). Microfibers were obtained by spinning 19 wt. % polymer solutions at 2 mL/h and 20 kV (23 G needle, 35 cm target distance). General scaffold characteristics including degradation rate and thickness were consistent with similar ones produced at the New Jersey Center for Biomaterials and previously described (13,15). Scaffold morphology and fiber diameter were evaluated at 3 locations ($n=10/\text{spot}$) via scanning electron microscopy (Amray 1830, acceleration voltage 20 kV, Amray, Inc., Bedford, MA). Nanofibers (diameter $0.66 \pm 0.14 \mu\text{m}$) and microfibers (diameter $2.62 \pm 0.39 \mu\text{m}$) appeared to be uniformly distributed, beadless, smooth and without defects or bifurcations (Figure 1).

Cell culture and immunofluorescent microscopy

Scaffold samples were dried in a 55°C vacuum oven overnight. Then, 8-mm scaffold squares were placed into Nunc Lab-Tek II Permanox Chamber Slides (Fisher Scientific, Pittsburgh, PA) and coated with $10 \mu\text{g/mL}$ rat plasma fibronectin in phosphate-buffered saline (PBS). After 5 minutes the cellophane film detached from the polymer scaffold and was removed. The scaffolds were then placed at 4°C overnight. After removing the fibronectin solution, the scaffolds were blocked with 1% bovine serum albumin in PBS. Scaffolds were sterilized under ultraviolet light for 3 minutes and then pre-wetted and immersed with Fibroblast Growth Medium-2 (Lonza, Walkersville, MD). As a reference substrate for the 3D fibronectin-coated nanofiber and microfiber scaffolds, two-dimensional (2D) Permanox glass slides (Fisher Scientific) were also coated with fibronectin and prepared in the same manner as described above for the 3D scaffolds. Fibronectin coating of all substrates allowed optimization of cell adhesive ability and to minimize possible differences in protein receptor interactions. Each substrate type (3D nanofiber scaffold, 3D microfiber scaffold, and 2D fibronectin-coated glass reference substrate) was seeded in triplicate with 1×10^5 human dermal fibroblasts (Lonza) using previously published techniques (15). After visual confirmation of uniform seeding and cellular morphology, cells were incubated in a 37°C , 5% CO_2 incubator (Thermo Scientific, Asheville, NC) for different time points. Cells were then fixed with 3.7% formaldehyde (Fisher Scientific) and incubated with rabbit anti-fibronectin antibody followed by goat anti-rabbit Alexa Fluor 488-conjugated secondary antibody (Molecular Probes, Eugene, OR) and the nucleic stain 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes) for purposes of cell counting. Fibronectin matrix was imaged and cell count recorded ($n=3$ slides for each substrate type, 10 random low power fields per slide) for each time point using inverted fluorescent microscopy (Nikon Instruments, Melville, NY). Alternatively, to study cytoskeletal and focal adhesion structures by immunofluorescent microscopy, cells were permeabilized with 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO). Actin cytoskeletal structure was stained using Alexa Fluor 488-labelled phalloidin (Molecular Probes). Focal adhesion plaques were detected with rabbit anti-vinculin primary antibody (Sigma-Aldrich) followed by goat anti-mouse Alexa Fluor 555-conjugated secondary antibody (Molecular Probes). Cell nuclei

were stained using Hoechst Stain solution (Sigma-Aldrich). Experiments were performed in triplicate.

Quantification of Focal Adhesion Size and Statistical Analysis

Fibroblast focal adhesions on the different substrate types were quantified by measuring vinculin-stained area on 100× immunofluorescent images with IPLab software (Scanalytics, Rockville, MD). Each image was normalized, inverted, segmented for area, and quantified to report average pixel area/focal plaque. Average areas for focal plaques were calculated and recorded as mean pixel area $\pm$ standard deviation. Statistical analyses for focal adhesion size and cell proliferation were performed with Prism 4.0 (GraphPad Software, San Diego, CA) using one-way analysis of variance (ANOVA) and Bonferroni adjustment for multiple comparisons.

RESULTS

Scaffold fiber diameter impacts cell morphology and cytoskeletal organization

Cells seeded onto 2D fibronectin-coated glass reference substrates for 24 hours exhibited typical bipolar morphology with well-defined actin stress fibers (Figure 2 A). When cells were seeded onto microfiber scaffolds for 24 hours, they extended multiple long processes along individual polymer fibers resulting in cell spreading and suspension between the polymer fibers (Figure 2 B). Similar to cells on the 2D surfaces, actin stress fibers were easily visible in these cells. In contrast, cells adherent to nanofiber scaffolds for the same period of time demonstrated few stress fibers. Rather, they were flat and displayed distinct “cork-screw” ruffles as membrane protrusions extended along the nanofiber scaffold surface (Figure 2 C).

Scaffold fiber diameter impacts focal adhesion size and morphology

Fibroblasts seeded onto 2D fibronectin-coated glass reference substrates for 24 hours exhibited long vinculin-rich focal adhesions associated and aligned with long actin stress fibers (Figure 3 A). Although some were found within the cell body capping shorter actin fibers, focal adhesions were predominantly found at the periphery of the cell. Cells seeded onto microfiber scaffolds for 24 hours developed focal adhesions (Figure 4) that were of similar size (pixel area = 108.5) to 2D glass surfaces (pixel area = 111.5), but in contrast to 2D glass surfaces, distribution of focal adhesions was limited to discrete points of cell attachment (Figure 3 B). In contrast, cells seeded onto nanofiber scaffolds for 24 hours demonstrated focal adhesions that were many in number and also limited to points of attachment but much smaller in size (pixel area = 5.8) compared to microfiber scaffolds ($p < 0.0001$) and associated with few stress fibers (Figures 3 C).

Scaffold fiber diameter impacts the temporal pattern of fibronectin matrix assembly

Fibroblasts assembled fibronectin matrices in distinctly different temporal patterns depending on whether they were seeded onto nanofiber or microfiber scaffolds. When seeded onto nanofiber scaffolds, cells assembled well-defined fibronectin fibrils as early as day 4 (Figure 5 A), and by day 7, a relatively dense fibronectin matrix could be observed (Figure 5 C) which continued to increase in fibril density over the following 7 days (Figure 5 E). In contrast, fibroblasts on microfiber scaffolds demonstrated a delayed response with regard to fibronectin matrix assembly. At day 4, only isolated and small fibronectin fibrils localized to polymer fibers throughout the scaffold were observed (Figure 5 B). By day 7, a fibronectin matrix was visible but its fibrils were relatively sparse compared to the fibronectin matrix seen at the same time point on nanofiber scaffolds (Figure 5 D). By day

14, however, matrix assembly appeared to have accelerated enough so that a dense and mature fibronectin matrix could be observed throughout the microfiber scaffold (Figure 5 F).

Scaffold fiber diameter influences cell proliferation

The initial seeding efficiency after 1 hour for cells on nanofiber scaffolds was similar to what was observed on microfiber scaffolds (Figure 6). The mean cell number per low power field (mean #/LPF) was 250 on nanofiber scaffolds while the mean #/LPF on microfiber scaffolds was 245. After 4 days, the mean numbers of cells per LPF on nanofiber scaffolds exhibited a 28% increase compared to those on microfiber scaffolds (450 vs. 350; $p = 0.008$). After that time point, however, the rate of cell growth on microfiber scaffolds had increased while the cell growth rate on nanofiber scaffolds had begun to plateau so that by 7 days, cell numbers on the two types of scaffolds were approximately equal (590 vs. 610). By 14 days, the ratio of cell numbers between the microfiber and nanofiber scaffolds had inverted with the cell number on microfiber scaffolds surpassing the number of cells on nanofiber scaffolds by 29% (780 vs. 1010; $p = 0.007$).

DISCUSSION

In this study, we report data characterizing the response of normal human fibroblasts to bioresorbable poly(DTE carbonate) electrospun scaffolds of varying fiber diameters. We demonstrate that nanoscale diameters result in distinctly different responses compared to microscale diameters. This was seen in terms of cytoskeletal morphology, focal adhesion formation, fibronectin matrix formation, and cell growth with highly significant differences observed in focal adhesion formation ($p < 0.0001$) and cell proliferation ($p < 0.01$).

Microscale fiber diameters typically resulted in delayed responses in both fibronectin matrix assembly and proliferation compared to nanoscale fiber diameters. Since cell density affects the rate of fibronectin matrix assembly (16,17), it is possible that the delay in fibronectin matrix assembly is reflective of the delay in cell proliferation rate. Furthermore, although construction of the 3D nanofiber and microfiber scaffolds were identical in composition and technique and differed only in the specified fiber diameter, this variable, however, can affect other scaffold characteristics that influence cellular behavior such as scaffold porosity, stiffness, and surface topography, all of which are known to affect cell response to scaffolds (18). For electrospun scaffolds in particular, pore size is greatly influenced by fiber diameter and it can be difficult to attribute biological responses directly to one single parameter (19). However, the processes of matrix assembly and focal adhesion formation are closely linked (20,21), and the significant difference seen in the formation of focal adhesions directly upon fibers of different diameters in this study suggest that the observed difference in fibronectin matrix assembly is not merely a collateral effect of either the observed difference in proliferation or the impact of fiber diameter variability on other scaffold parameters. In addition, although focal adhesion formation on 3D microfiber scaffolds resulted in focal plaque areas that were similar in size to those on 2D surfaces (Figure 5), focal adhesion morphology between 3D microfiber surfaces and 2D surfaces were observed to be quite different (Figure 4) which corroborates previous reports demonstrating differences in adhesion formation between 2D and 3D surfaces with subsequent impact on biological responses including matrix assembly (18,22). Since the manner of fibronectin matrix assembly can have a significant impact on the nature of the subsequent scar collagen matrix (11,12), the current study may have significant relevance for the optimization of clinically useful scaffold constructs.

Studies over the past decade have demonstrated the importance of topographical cues in determining cell behavior, especially in the assembly and formation of ECM (21,22). Recently published data have demonstrated the influence of fiber diameter upon the

behavior of cells on non-bioresorbable scaffolds (23) and that fiber diameters below 1 μm can result in different rates of cell migration compared to fiber diameters greater than 1 μm (24). The current study reinforces these concepts in the context of a bioresorbable synthetic scaffold and adds the novel finding that fiber diameter can influence the assembly of native ECM in the form of fibronectin matrix, and thus may also influence the formation of the mature scar collagen matrix. How changes in fiber diameter and other scaffold parameters may affect *in vivo* cell responses including cell differentiation processes remains unknown, however, and requires further study.

Our current results demonstrate the importance of fiber diameter in fibroblast response on bioresorbable scaffolds and emphasize that the ability of scaffolds to elicit wound cell responses in a predictable fashion depends on the ability to maintain consistent parameters beyond scaffold composition. Such findings from this and other studies will have important implications for regenerative medicine and the development of clinically useful engineered constructs.

CONCLUSIONS

The fiber diameter of bioresorbable scaffolds can significantly influence cell response, underscoring the fact that the ability of scaffolds to elicit consistent biological responses depends on other factors besides scaffold material composition. In this study, normal human fibroblasts demonstrated altered cell responses to bioresorbable scaffolds that differed in the diameter of the fibers comprising the electrospun scaffolds. While overall biological response to a scaffold is a complex process, the impact on focal adhesion formation and fibronectin matrix assembly by scaffold fiber diameter seen in this study indicates that this factor can directly influence the assembly of native ECM matrix in the *in vitro* setting, suggesting this factor may have significant influence on the formation of an *in vivo* scar matrix. Findings such as this and others described in this study have important implications for the design of clinically useful engineered constructs.

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FIGURE 1. Poly(DTE carbonate) nanofiber and microfiber bioresorbable scaffolds
 Nanoscale (A, B) and microscale (D, E) fiber scaffolds were imaged here by scanning electron microscopy at magnifications of 600× (A, D) and 6000× (B, E). Fibers were electrospun from one solvent system in an effort to eliminate variability in fiber texture and residual solvents, and fiber diameters were controlled by adjusting polymer viscosity, flow rate, needle gauge and electric field. Nanofibers ($0.66 \pm 0.14 \mu\text{m}$) and microfibers ($2.62 \pm 0.39 \mu\text{m}$) were uniformly distributed, beadless, smooth and without defects or bifurcations. Histograms (C, F) demonstrate frequency of nanoscale (C) and microscale (F) fiber diameters.

FIGURE 2. Bioresorbable scaffold fiber diameter impacts cell morphology and cytoskeletal organization

Human dermal fibroblasts were stained for actin with Alexa Fluor 488-labelled phalloidin and viewed at 60× magnification. Cells on fibronectin-coated 2D surfaces for 24 hours (A) exhibited typical bipolar morphology with well-defined actin stress fibers, while cells on microfiber scaffolds (B) over the same time period extended multiple long processes along individual polymer fibers resulting in cell spreading and suspension between the polymer fibers. In contrast, cells on nanofiber scaffolds (C) displayed few actin stress fibers and instead had membrane protrusions demonstrating distinct "cork-screw" ruffles (arrows).

FIGURE 3. Bioresorbable scaffold fiber diameter impacts focal adhesion morphology

Human dermal fibroblasts were seeded on fibronectin-coated 2D surfaces (A), microfiber scaffolds (B), or nanofiber scaffolds (C) for 24 hours and then fixed and stained for actin (green) and vinculin (red) to examine the relationship of vinculin-rich focal adhesions to the actin cytoskeleton. Cells adherent to fibronectin-coated 2D surfaces for 24 hours exhibited thick, long aligned actin stress fibers terminating in long vinculin-rich focal adhesions (A). Although some were found within the cell body capping shorter actin fibers, focal adhesions were predominantly found at the periphery of the cell. In contrast, distribution of focal adhesions for cells on microfiber scaffolds (B) was limited to discrete points of cell attachment (arrow). Cells seeded onto nanofiber scaffolds demonstrated focal adhesions that were many in number and also limited to points of attachment but much smaller in size and associated with few stress fibers (C).

FIGURE 4. Bioresorbable scaffold fiber diameter impacts focal adhesion size

Focal adhesion plaque formation on fibronectin-coated 2D glass, nanofiber, and microfiber scaffolds were quantified by measuring vinculin area on 100× immunofluorescent images. Image analysis software was used to normalize and invert the original black and white images. The inverted features were segmented for area and quantified. The average focal adhesion plaque areas in pixels were calculated for glass (111.5), nanofibers (5.8), and microfibers (108.5) with a highly significant difference seen between nanofiber and microfiber scaffolds ($p < 0.0001$).

FIGURE 5. Fibronectin matrix assembly on electrospun poly(DTE carbonate) bioresorbable scaffolds over time

Fibronectin matrix assembly by human dermal fibroblasts seeded on electrospun poly(DTE carbonate) scaffolds was assessed by immunofluorescent staining for extracellular fibronectin after various time intervals:: after 4 days on nanofiber (A) or microfiber (B) scaffolds at 10 $\times$ magnification (insets a & b = 100 $\times$); after 7 days on nanofiber (C) or microfiber (D) scaffolds at 10 $\times$ (insets c & d = 60 $\times$); after 14 days on nanofiber (E) or microfiber (F) scaffolds at 10 $\times$ (insets e & f = 100 $\times$).

FIGURE 6. Normal human dermal fibroblast proliferation on nanofiber and microfiber bioresorbable scaffolds

Fibroblasts were seeded on nanofiber or microfiber scaffolds in triplicate fashion and incubated for time points as indicated before being fixed, stained, and counted for cell nuclei at low power magnification. After 4 days, significantly greater cell numbers were found on nanofiber scaffolds compared to microfiber scaffolds ($p = 0.008$). However, by 14 days, cells on microfiber scaffolds exceeded those on nanofiber scaffolds ($p = 0.007$). LPF = low power field.