Published in final edited form as:

Biotechnol Bioeng. 2012 October ; 109(10): 2683–2698. doi:10.1002/bit.24533.

Disorganized collagen scaffold interferes with fibroblast mediated deposition of organized extracellular matrix *in vitro*

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Abstract

Many tissue engineering applications require the remodeling of a degradable scaffold either *in vitro* or *in situ*. Although inefficient remodeling or failure to fully remodel the temporary matrix can result in a poor clinical outcome, very few investigations have examined in detail, the interaction of regenerative cells with temporary scaffoldings. In a recent series of investigations, randomly oriented collagen gels were directly implanted into human corneal pockets and followed for 24 months. The resulting remodeling response exhibited a high degree of variability which likely reflects differing regenerative/synthetic capacity across patients. Given this variability, we hypothesize that a disorganized, degradable provisional scaffold could be disruptive to a uniform, organized reconstruction of stromal matrix. In this investigation, two established corneal stroma tissue engineering culture systems (collagen scaffold-based and scaffold-free) were compared to determine if the presence of the disorganized collagen gel influenced matrix production and organizational control exerted by primary human corneal fibroblast cells (PHCFCs). PHCFCs were cultured on thin disorganized reconstituted collagen substrate (RCS - 5 donors: average age 34.4) or on a bare polycarbonate membrane (5 donors: average age 32.4-controls). The organization and morphology of the two culture systems were compared over the long-term at 4, 8 and 11/12 weeks. Construct thickness and extracellular matrix organization/alignment was tracked optically with bright field and differential interference contrast (DIC) microscopy. The details of cell/matrix morphology and cell/matrix interaction were examined with standard transmission, cuproline blue and quick-freeze/deep-etch electron microscopy. Both the scaffold-free and the collagen-based scaffold cultures produced organized arrays of collagen fibrils. However, at all time points, the amount of organized cell-derived matrix in the scaffold-based constructs was significantly lower than that produced by scaffold-free constructs (controls). We also observed significant variability in the remodeling of RCS scaffold by PHCFCs. PHCFCs which penetrated the RCS scaffold did exert robust local control over secreted collagen but did not appear to globally reorganize the scaffold effectively in the time period of the study. Consistent with our hypothesis, the results demonstrate that the presence of the scaffold appears to interfere with the

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global organization of the cell-derived matrix. The production of highly-organized local matrix by fibroblasts which penetrated the scaffold suggests that there is a mechanism which operates close to the cell membrane capable of control fibril organization. Nonetheless, the local control of the collagen alignment produced by cells within the scaffold was not continuous and did not result in overall global organization of the construct. Using a disorganized scaffold as a guide to produce highly-organized tissue has the potential to delay the production of useful matrix or prevent uniform remodeling. The results of this study may shed light on the recent attempts to use disorganized collagenous matrix as a temporary corneal replacement *in vivo* which led to a variable remodeling response.

INTRODUCTION

A critical basic science objective associated with connective tissue research (development, growth, disease, repair and tissue engineering) is gaining a full-understanding of how organized, anisotropic collagenous matrix can arise from a population of synthetically active fibroblastic cells (Cowin 2000; Cowin 2004). The answer to this question is paramount if we are truly to gain control over the production and organization of the structural molecule of choice in vertebrate animals: collagen. In the process, we may also learn how collagen can be effectively controlled *de novo*. In load-bearing tissues, fibrillar collagen (comprising types I, II, III, V and XI or heterotypic combinations thereof) is often arranged in aligned, lamellar sheets which maintain their direction over long length scales to effectively resist mechanical force. In some tissues, these sheets alternate in direction to accommodate loads which are parallel to the plane of the structure and multiaxial (i.e. primary lamellae and secondary osteons in compact bone, annulus fibrosus in intervertebral disk, lamellae in cornea). In the cornea, the ultrastructure of the collagenous matrix in the stroma is under the further constraint that the fibrils must be spaced reasonably uniformly and possess a nearly monodisperse diameter distribution to effectively transmit light (Cox et al. 1970; Goldman et al. 1968; Hart and Farrell 1969). These simultaneous requirements are met by the nanoscale architecture of the corneal stroma making the prospect of engineering a cornea particularly difficult in comparison to other connective tissue (Ruberti and Zieske 2008).

The normal adult human cornea exhibits very little regenerative capacity and typically resolves injury via a complex response involving stromal keratocyte apoptosis, differentiation to repair fibroblasts and possibly further dedifferentiation to myofibroblasts (for extensive reviews of keratocyte behavior in wounded corneas see Fini (1999) and Jester et al. (1999) (Fini 1999; Jester et al. 1999; Zieske 2001)). This process often results in relatively permanent and sight threatening corneal scarring (Dupps and Wilson 2006). Although classical tissue engineering approaches (seeding fibroblastic cells into biocompatible scaffoldings) to corneal regeneration have been taken by several investigators, the successful reproduction of corneal stromal architecture has not been demonstrated in the laboratory (Crabb et al. 2006a; Crabb et al. 2006b; Griffith et al. 2002; Griffith et al. 1999; Li et al. 2003; Li et al. 2005; Orwin et al. 2003; Orwin and Hubel 2000; Ren et al. 2008). The Griffith laboratory has been experimenting with a different approach which entails the implantation of cell-free scaffolds (type III collagen crosslinked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide) directly into animal and more recently, human corneas (Fagerholm et al. 2010). This general change in philosophy from producing a viable, tissue-engineered cornea (by seeding a collagen scaffold *in vitro* with corneal cells) to direct implantation of a cell-free, disorganized scaffold indicates the high degree of difficulty associated with efforts to produce viable corneas in the lab. The recent approach of direct implantation of cell-free scaffolds into the cornea has been shown to induce a significant and variable remodeling of the implant in phase I human clinical trials. In one patient, at 24 months, the central corneal thickness was

measured at 211 μm while in another it was 560 μm . This suggests that there is significant variability in the remodeling of the implanted collagen gel. If corneal tissue engineering using a scaffold-based approach is going to be successful, it is critical to understand how fibroblasts interact with scaffolds both *in vitro*, and ultimately *in vivo*.

Scaffold-free corneal tissue engineering—There is an alternative to using scaffolds to engineer corneas *in vitro*. In the last few years, multiple investigations have been performed which suggest that corneal stromal cells in scaffold-free cultures can produce aligned, and somewhat orthogonal arrays of collagen fibrils (Du et al. 2007; Guo et al. 2007). In the latter (Du et al. 2007), human stromal stem cells were isolated then pellet-cultured to produce a high-density, mass of cells. In this system, collagenous arrays which alternated in direction were found at the perimeter of the pellet where cells were flattened and stratified. The collagen organization was co-localized with the presence of cell junctional proteins (connexin 43 and cadherin 11) suggesting that cell-cell contact/communication and local control of the space is important to produce organization in the matrix. In the former investigation, Guo *et al* (Guo et al. 2007) cultured primary human stromal cells from corneal buttons at high density onto bare polycarbonate membranes, subjected them to fetal bovine serum (FBS; to convert them to a fibroblastic phenotype) and enhanced collagen stability with ascorbic acid. The resulting constructs were stratified and comprised flattened fibroblastic cells in a matrix which contained arrays of aligned, stroma-like collagen. Both of these investigations, which began in a scaffold-free system and produced organized matrix, suggest that cells may need to work together in close proximity to control the local and global organization of collagen fibrils (as in development). Because of the relative success in the production of stroma-like collagenous matrix by scaffold-free systems and the fact that corneal development proceeds in the absence of disorganized matrix, it is of considerable interest to examine the effect of a collagenous scaffold on a culture system already known to produce organized collagen. In this comparative investigation, the details of collagen deposition and organization in a scaffold free system (using the method of (Guo et al. 2007)) were compared with those in a culture system using a type I collagen disorganized scaffold (using the method previously described by (Ren et al. 2008)).

MATERIAL AND METHODS

All procedures used in these studies adhered to the tenets of the Declaration of Helsinki. Human corneas were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA).

Isolation of Primary human corneal Fibroblast cells

The details of the isolation of the PHFCs from donor corneas have been described in (Guo et al. 2007; Ren et al. 2008) as this investigation compares data from those studies (although we do include a longer time period than was published in Guo et al). The scaffold-free system of (Guo et al. 2007) shall be considered the “control”, while the scaffold-based system of (Ren et al. 2008) shall be considered the “experimental”. Briefly, corneal epithelium and endothelium were removed from the stromas of 10 separate human donors by scraping with a razor blade. The stromal tissue was cut into small pieces and put into 6-well plates (4 or 5 pieces of 2×2 mm tissue per well). Explants were allowed to adhere to the bottom of the wells at room temperature for 5 minutes before EMEM medium (Sigma-Aldrich; St. Louis, MO) containing 10% fetal bovine serum (FBS:ATCC; Manassas, Virginia) was added. Each 500 ml bottle of medium was supplemented with 5 ml of 1% antibiotic/antimycotic (Sigma). Care was taken when adding the medium to make sure that

the explants remained attached to the wells. After 1–2 weeks cultivation (37°C, 5% CO₂), the fibroblasts were passaged into a T75 flask.

Preparation of reconstituted collagen substrate (RCS)

The preparation of the reconstituted collagen scaffold (RCS) has been described in the original publication of Ren et al 2008, but briefly: commercially available pepsin-extracted, bovine, monomeric type I collagen (3 mg/ml: Purecol; Fremont, CA) was polymerized in a thin, disorganized layer (10–30 microns) on porous polycarbonate membranes (pore size 0.4 µm: Costar; Charlotte, NC). To enhance adhesion of collagen fibrils to the polycarbonate, the surface was treated to covalently couple the collagen to the membrane (Murthy et al. 2004) using a Carbodiimide Kit (Polysciences; Warrington, PA). Briefly, membranes were rinsed in PBS twice then exposed to a freshly made solution of 2% 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride solution in 1x PBS for 3–4 hours on a rocking table. Membranes were then rinsed twice with PBS and covered dropwise with borate buffer. Collagen solution (750 µl) was tipped onto the prepared membrane surface followed by overnight placement in an incubator. Since the cross-linking is performed on the membrane, it would only affect the single layer of collagen that is adjacent to the membrane and therefore, the remainder of the scaffold thickness would be unaffected. Membranes were initially dried in a laminar flow hood for 4–6 hours, rinsed with DI and then air dried again.

Culture of human corneal Fibroblast cells

The culturing of the primary human corneal fibroblasts (PHFCs) has been described in Guo et al 2007 and Ren et al 2008. Briefly: PHFCs were plated into 6-well plates with either the RCS-coated polycarbonate membrane inserts (Costar; Charlotte, NC; n=5; ages 7, 18, 21, 57, 69; average: 34.4) or on scaffold-free (SF) polycarbonate membranes (control experiment; n=5; ages 1, 7, 22, 53, 79; average: 32.4) at a density of 0.5×10^6 cells/well. Fibroblasts were cultured in EMEM with 10% FBS and 0.5 mM 2-O- α -D-glucopyranosyl-L-ascorbic acid (G-Asc; Wako Chemicals USA, Inc.; Richmond, VA). Medium was changed 3 times a week. Control (SF) and experimental (RCS) cultures were allowed to assemble matrix and were harvested for assessment at 4, 8 and 11 or 12 weeks. Controls were run to 12 weeks but the experimental series in the SF system was terminated at 11 weeks due the detection of bacterial contamination in one sample well. Because of the relative stability of the constructs after 8 weeks, we felt comfortable directly comparing the two series as if the 11 and 12 week time points were equivalent. At each time point, the resulting constructs were processed using the following methods: (1) fixed in Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde in cacodylate buffer, pH 7.4) at 4°C for transmission electron microscopy (TEM) or (2) slam frozen for QFDE electron microscopy.

Light microscopy

Thick sections—To assess microscopic morphology in cross-section, thick sections were cut and photographed with a Nikon Eclipse E800 (Nikon, Melville, NY) equipped with a SPOT Camera (Micro Video Instruments, Avon, MA). *Differential interference contrast imaging (DIC)*. *En face* DIC imaging of cultures was performed to estimate the total construct thickness, to observe the long-range orientation of synthesized fibrils relative to the HCF organization and to examine the transition between the organized and disorganized matrix (Petroll and Ma 2003). To perform DIC constructs were gently removed from the underlying membrane and placed in a drop of 1x phosphate buffered saline on a glass coverslip. A second coverslip was placed over the drop and construct and the whole preparation was placed on the stage of a Nikon TE2000U inverted microscope (Microvideo

Instruments, Avon, MA) equipped with a Photometric 1394 video camera (Photometrics, Tuscon, AZ). Z-scans were used to find the bottom and top of each construct to determine thickness. Within the Z-scans, images were taken to demonstrate the general organization of the extracellular matrix and HCFs in the plane of the construct. The term globally-organized is defined as cell and matrix co-alignment which spans multiple cell widths. Globally-organized matrix thus indicates that two or more cells were operating in a manner consistent with the production of load-bearing collagenous tissue. To estimate the extent of globally-organized cell-derived matrix (CDM) in each construct, high-resolution DIC z-scans were examined by an objective observer. The constructs were divided into four categories: globally-organized matrix (entire visible field comprises aligned fibrillar arrays and cells which persist for multiple cell widths – See Figures 3C, D and E below), disorganized matrix (entire visible field fibrillar and locally-disordered – See Figure 3A), mixed (any combination of globally-organized and disorganized – See Figure 3B) and cellular (visible field occupied principally by cells). A minimum of 2 z-scans were evaluated per specimen, thus there were at least 10 thickness values at each time point for each series. The online supplementary movie shows representative z-scan from constructs grown on RCS.

Transmission electron microscopy (TEM)

The constructs were processed for TEM using a standard preparation as described in Gipson et al. (Gipson et al. 1983). This method of matrix examination will be referred to as *sTEM*. Briefly, constructs were fixed in ½ strength Karnovsky's fixative overnight at 4°C, rinsed in PBS then processed through postfixation in 2% osmium tetroxide, en bloc staining in 0.5% uranyl acetate, alcohol dehydration to propylene oxide and embedded in Epon. Sixty nm sections cut transverse to the plane of the construct with a diamond knife ultramicrotome (LKB ultramicrotome; Bromma, Sweden) were viewed and photographed with a Philips 410 Transmission Electron Microscope (Philips Electronics N.V.; Eindhoven, The Netherlands). To provide a global view of cell distribution within the constructs, thick sections (1µm) were also cut from the same blocks and viewed using bright field microscopy (Figures 1 and 2).

Quick-Freeze/Deep Etch (QFDE) transmission electron microscopy

Small specimens were cut from the tissue constructs. These specimens, while still attached to the membrane, were rinsed with PBS to remove any excess medium then mounted onto specimen carriers using a 10% Laponite solution (Rockwood Additives; Cheshire, UK) as an adhesive and cushioning material. After mounting the specimens, excess PBS was removed gently with filter paper, and the exposed surface of the tissue was rapidly slam frozen using a portable cryogun (Delaware Diamond Knives; Wilmington, Delaware). The frozen constructs were transferred to a custom, modified Cressington CFE-40 freeze fracture/freeze etch system (Cressington Scientific Instruments; Watford, England) for replication. During replication, the specimens were superficially fractured and etched at –100°C for 12 min. Rotary shadowed replicas of the etched surfaces of the constructs were created by evaporation of platinum/carbon (for contrast) at 20° angle onto the rotating construct, followed by evaporation of pure carbon (for replica strength) at a 90° angle. Tissue was digested overnight with household bleach. The cleaned replicas were picked up on copper 600-mesh grids. Grids were viewed and photographed with a JEOL JEM-1000 (JEOL, Tokyo, Japan) Transmission Electron Microscope.

Fibril diameter estimation

Fibril diameter was estimated directly from the standard TEM images using calibration grids and the measurement tool in Photoshop (Adobe Systems, San Jose, CA). For each experimental condition a minimum of fifty fibrils were selected randomly from multiple micrographs that were independent of position in the construct. For fibrils in cross-section,

the smallest possible diametric line was chosen. For longitudinal fibrils, the thickest part of the fibril was measured. It is well known that standard TEM processing causes shrinkage in collagen and generally alters the volume of collagen fibrils.

RESULTS

Optical Microscopy of Construct Organization

Thick sections—Figure 1 demonstrates the general construct organization across all five of the experimental series as a function of time in the scaffold-based culture system. Cross-sections of the constructs show varying degrees of integration of the fibroblasts with the RCS and varying amounts of secreted CDM. In some of the specimens, fibroblasts migrated into the RCS and colonized the membrane surface, while in other cases the fibroblasts remained on the surface of the RCS (series 4).

The synthetic response of the five sets of donor cells cultured on the RCS differed with specimens producing varying amounts of collagen and glycosaminoglycans (See Ren et al for data (Ren et al. 2008)). In general, fibroblasts in both the control (SF) and experimental (RCS) constructs had a flattened appearance and were aligned with and parallel to the membrane. In this way, both types of constructs showed similarity to developing corneal stroma (Sevel and Isaacs 1988). It is interesting to note that at the surface and the bottom of the construct for both experimental and control series that there were generally aligned and often confluent layers of cells (confirmed by DIC imaging). Figure 2 compares eight week specimens in series 4 and 5 (removed from the membrane) to an eight week control construct. It can be seen in series 4 that the fibroblasts did not penetrate the RCS whereas in series 5 they did. Note that the fibroblasts within the RCS were far apart while those above and below it in the CDM were much closer together. A reduction in cell density in the RCS was a common factor compared to cell density in CDM (by direct observation). It is also interesting to note that the orientation of the fibroblasts appeared to change with many cells in longitudinal section occupying the same strata and cells in cross-section occupying the same strata. The general organization of the CDM in control construct is similar to that of the experimental constructs as they both stratify on top of the RCS (Figure 2 – control series).

Differential Interference Contrast Imaging—DIC imaging allows detailed microscopic examination of the matrix texture and cell orientation in the plane of the construct. The excellent out of plane rejection of DIC combined with the thin nature of the constructs allow us to clearly distinguish disordered RCS from the organized CDM produced by the cells (see Figure 3 and supplemental movie). The overall construct thickness and the thickness of CDM vs RCS as a function of time were variable within and across specimens. Figure 3 shows a sequence of images from a z-scan which captures a sharp transition from RCS to CDM in experimental series 4. The disordered nature of RCS can be readily appreciated in Figure 3A while Figure 3B shows the transition from the RCS to CDM (depicted clearly in Figure 3C). Figure 3D shows the multilamellar nature of matrix produced in CDM. Figure 3E is a DIC image of globally-organized matrix in one of the SF control samples. One of the most striking aspects of the culture system is the fact that the CDM appears highly-aligned and can alternate direction en masse (i.e. above the RCS). In addition, the lateral (x,y) length scale over which the matrix appears to be aligned is often large (>100 microns). The measured z-thickness of globally-organized matrix versus disorganized matrix in both the SF and RCS constructs is plotted in Figure 4A. The large standard deviations indicate inconsistent matrix production in both the SF and RCS. Nonetheless, it was found that on average the cells in SF produced more globally-organized matrix than did the cells in RCS at all time points. The difference in globally-organized

matrix production reached statistically significant levels at 4 ($p<0.001$) and 11 ($p<0.05$) weeks (week 8 just missed significance ($p=0.089$)). It should be stated that when the experimental series did produce globally-organized CDM, it was typically found on top of the RCS scaffold (this was confirmed by TEM).

Transmission Electron Microscopy of Construct Organization

Interaction of cells and the cell-derived matrix with the reconstituted collagen substrate—The details of the interaction of the cells and the CDM with the RCS were revealed by examination of the constructs in cross-section using sTEM. Figure 5 depicts two four-week old experimental constructs at low resolution. The structure found in TEM is generally consistent with the optical thick sections (though there was some intraspecimen and interspecimen heterogeneity). In Figure 5A, a 4 week construct from series 1 possessed a similar thickness (~10 microns), a similar number of cellular strata (~6) and degree of penetration of the RCS by the fibroblasts as did the corresponding optical section (Fig 2). In this construct, there were few discernible collagen fibrils in the CDM, and the RCS was easily identifiable by its population of irregular cross-section collagen fibrils, which were on the order of 100–200 nm in diameter. In another sample a marked difference in the thickness of the CDM and its composition and organization is apparent (Fig. 5B). The fibroblasts did not penetrate the RCS in this location on the specimen; instead, globally-organized, stratified CDM was produced on top of the RCS. Five strata of cells were discernible with at least one change in the direction of the cells and collagen fibril orientation.

In this construct, collagen fibrils appeared to be co-aligned with the fibroblast long axis (see inset). Fibroblast derived collagen appeared as small diameter fibrils and was densely packed at the bottom of the CDM. Of particular interest is the fact that there appears to be a reasonably sharp interface between the cell-derived collagen and the disorganized collagen in the RCS. The local and global organization of the CDM fibrils does not appear to be influenced by the close juxtaposition with the collagen of the RCS (as clearly demonstrated by the DIC images in Figure 3). In Figure 6, the interaction between fibroblasts and the RCS can be appreciated in a specimen where there are both bordering cells and cells that had migrated into the RCS while depositing cell-derived collagen. The micrographs are medium resolution and indicate that fibroblasts which had penetrated the RCS deposited aligned arrays of collagen in manner suggestive of leaving a trail. This behavior may provide a critical clue to the method by which collagen is deposited by individual migrating cells. However, the trails of locally-aligned, small-diameter collagen deposited in the RCS did not integrate to produce global organization. In contrast, at the border of the RCS, where the PHCSCs could operate in close proximity and without an intervening scaffold, the cell-derived collagen was globally-organized over long distances and appeared to be aligned with the cells. In Figure 7, the interaction between cell-derived collagen and RCS collagen is shown at high-resolution for two different conditions: at the border of the RCS and organized stratified CDM or around a single cell which has penetrated the RCS. In Figures 7A and 7B, the interaction of fibroblast-synthesized matrix at the border of a globally-organized CDM with collagen in RCS is apparent. In one case, the CDM fibrils were in longitudinal section and in the other they were in cross-section. It is clear from the images that the presence of the isotropic collagen fibrillar arrangement in the RCS exerted little influence over the local matrix organization in the vicinity of the border fibroblasts. It should be noted however, that numerous small-diameter fibrils, which were possibly cell-derived, could be found within the RCS. Since this RCS was not penetrated by the fibroblasts, these fibrils probably assembled some distance away from the cell border. Their alignment was thus not likely to be under the direct control of the fibroblast but rather reflective of the RCS organization. Figure 7C shows the interaction of a penetrating fibroblast with the surrounding RCS. It appears that fibroblasts can control the organization

of the matrix for a short distance before cell-derived fibrils intermingle with the RCS collagen (and possibly take on their orientation). Taken together, Figures 5, 6 and 7 demonstrate that the organization of CDM is under robust local control of the fibroblast producing the collagen. This boundary region around the cells appeared to contain a diffusely-stained fibrillar network which may have relevance to fibroblasts ability to control the local organization of fibrils. When cells are in close proximity and can self-organize (with no intervening disorganized RCS), the control over the collagen fibril orientation appears to be extended from local to global scales.

TEM examination of collagen fibrils diameter—*In vivo*, the diameter of collagen fibrils in the cornea are tightly controlled and exhibit a highly monodispersed diameter distribution (32 ± 0.7 nm) that is essential to corneal transparency. Therefore, it is of interest to investigate the diameter of collagen fibrils that are synthesized by corneal fibroblasts *in vitro*. Figure 4B, is a bar graph representing fibril diameter in the SF and in the RCS culture systems at 4, 8, and 11/12 weeks. At all time points the fibrils that are synthesized in the SF system have a significantly smaller diameter compared to those synthesized by the cells in the RCS system (38 ± 4.62 nm vs. 51.92 ± 4.10 nm at week 4, 38.70 ± 1.90 nm vs. 48.24 ± 4.28 nm at week 8, and 27.87 ± 2.15 nm vs. 48.54 ± 4.44 nm at week 11/12; $p < 0.05$ at all time points).

TEM examination of the cells and CDM—It has been noted that the cells can self-organize and produce globally-organized CDM above the RCS. In Figure 8, we contrast a standard transmission electron micrograph (sTEM) with a QFDE micrograph of the interface of what we believe is an actively synthetic cell and its local CDM. Some of the more interesting structures which can be observed in both the sTEM and QFDE include the numerous vesicle-like structures along the cell membrane which open out to the ECM, the secondary network of fine fibrils and small diameter poorly-staining fibrillar structures which appear to align with the collagen fibrils. Unlike the TEM image, the QFDE demonstrates just how packed the matrix around the cell might be (particularly if one considers the fact that QFDE is likely an underestimate of the space filling nature of the matrix as well). In Figure 9A, a clear, sustained change in the fibril alignment is apparent, while Figure 9B demonstrates the D-periodic banding in the CDM collagen fibrils. It is important to note that fibrils were far from monodisperse in their diameter distribution, which indicates that in this culture system, some loss of fibril morphology control (compared to native cornea and to the scaffold-free cultures (Guo et al. 2007)) has occurred.

One of the more interesting aspects of this investigation is the presence of what appear to be cell-derived fibrils well-within the RCS matrix. In Figure 10A it is possible to discern numerous small diameter fibrils within the RCS. As noted previously, cross-sections of small fibrils were often found adjacent to cross-sections of the larger RCS fibrils. In longitudinal section, one can easily find large RCS collagen fibrils and smaller collagen fibrils, which are possibly derived-from cell-synthesized collagen. Exactly how fibrils can assemble at such long distances from the fibroblasts, while intermingling with an existing collagen network, is not clear from models of collagen assembly in the literature (Birk and Trelstad 1984; Birk and Trelstad 1985; Canty and Kadler 2005; Canty et al. 2004; Giraud-Guille 1996). We suspect that the numerous vesicles that can be observed well into the RCS may provide a possible answer. In our culture system we observed such vesicles throughout both the CDM and the RCS. In Figure 10B, the QFDE shows clearly the penetration of vesicles into the RCS and the elaboration of a secondary anastomosing network of small fibrils. Figure 10C is a lower magnification which demonstrates the extensive investment of both vesicles and the secondary network into the RCS collagen. In Figure 10D QFDE reveals an open vesicle which appears to be the source of a group of polymerized small fibrils. Such fibrillar structures are not visible in the sTEMs (except perhaps faintly). In

addition, QFDE images have revealed extensive vesicular budding from the surface of some of the fibroblastic cells in our culture system (data not shown).

DISCUSSION

In a recent publication we have demonstrated (Guo et al. 2007) that untransformed human corneal fibroblasts in a scaffold-free system can produce a substantial, globally-organized, three-dimensional matrix that bears qualitative resemblance to the architecture of a developing mammalian corneal stroma (Cintron et al. 1983). It has generally been our opinion that to produce such organized matrix, it is important that the culture system mimic development, where globally-organized matrix is readily and rapidly produced. Development of a cornea is decidedly different from corneal scar resolution which begins with a provisional matrix or scaffold that is remodeled over long periods of time (Cintron et al. 1981; Cintron et al. 1982). Several recent and laudable attempts to produce engineered corneas mimic stromal scar resolution and begin by seeding fibroblasts onto or into degradable scaffoldings (Germain et al. 1999; Griffith et al. 1999; Orwin et al. 2003). When the architecture of the resulting cell-derived matrix (which is intended to replace the disorganized degrading scaffolding) is examined, it does not comprise globally-organized arrays of collagen fibrils (Crabb et al. 2006a). To produce a functional cornea by classical tissue engineering methods, it is critical to reproduce the collagen organization in the stroma (Ruberti et al. 2007; Ruberti and Zieske 2008). Thus, it is important, both for our understanding of development and for translational approaches to producing an engineered cornea, to understand the interaction of fibroblastic cells with substrates (such as reconstituted collagen gels). In this investigation, we have examined the way ascorbic acid-stimulated primary human stromal fibroblasts produce and deposit matrix when in contact with a typical tissue engineering scaffold and compared to those in scaffold free cultures. Organizational control is found where the fibroblasts are able to stratify to multiple layers above the RCS scaffold. When the cells penetrate the RCS, they appear to produce organization only very close to the cell surface and appear to be surrounded by a faintly staining network. Surprisingly, we observed numerous small diameter collagen fibrils which appear to have assembled at substantial distances from the cells inside the RCS. The primary collagen source for these small fibrils is assumed to be the PHCFCs, however, it is possible (and would be quite intriguing) if they are derived from destabilized and remodeled RCS collagen (which was eventually decorated with PGs – unpublished data). These fibrils must interact with the scaffolding and at times appear to take on the direction of RCS collagen fibrils. Thus, it is likely that the scaffolding has a direct effect, and possibly influences the local organization of the collagen that is produced by the fibroblasts, but assembled at a distance from them. In this investigation we also observed the presence of vesicles in both the RCS and CDM. The purpose of the vesicles is not known, but it is likely they are involved in the export of matrix molecules. In addition to the presence of vesicles in the RCS, QFDE imaging revealed an extensive anastomosing secondary network of very small diameter fibrils the purpose of which is not yet known.

Unfortunately, very little is known regarding the mechanism of collagen deposition by fibroblasts, however, there are currently two established, but very different concepts: 1) *Fibripositor*: In this mode of collagen fibril deposition/organization, collagen monomers are formed into fibrils in cell invaginations which then vectorially discharge fibrils into the ECM (Birk and Trelstad 1984; Hay and Revel 1969). Matrix organization must be produced by cell-mediated deposition which is likely to involve organized migration. 2) *Liquid crystal collagen*: In this mode of fibril deposition/organization, collagen monomers are exported into the extracellular space where they are confined and concentrated to form liquid crystalline patterns before condensing into organized arrays of fibrils (Giraud-Guille et al. 2003; Trelstad 1982). In our culture system we could not directly identify fibripositors nor

did we not measure the concentration of collagen in the local space. Nonetheless, either method of collagen fibril deposition (fibripositors or liquid crystal condensation) might be affected by the presence of a scaffold. Disorganized scaffoldings have the potential to interfere with the deposition of highly-ordered collagen by 1) disrupting the ability of the cells to maintain contact, 2) preventing control of the local and global space, 3) interfering with migration and 4) by providing a template which might imprint disorganization onto liquid crystalline collagen monomer arrays prior to the condensation to fibrils.

A limitation of the current study is that globally-organized matrix was identified in a qualitative manner. In previous work (Saeidi et al. 2009) we have been able to quantify the degree of fiber alignment and principal direction of collagen orientation in our images by employing a 2D Fourier transform technique (Sander and Barocas 2009). Unfortunately that technique could not be applied to the images acquired here because a directional bias from the shearing gradient inherent in DIC obscured the fiber orientation distribution and quantification of the degree of fiber alignment. This bias could potentially be removed to allow quantification in future studies by acquiring images of the sample at multiple orientations (Shribak and Inoue 2006).

CONCLUSIONS

Classical tissue engineering methods, which require seeded or native fibroblasts to remodel an often disorganized scaffolding, are analogous to scar resolution which is a lengthy and inefficient process in highly-organized relatively avascular tissues such as the cornea (Cintron et al. 1982). In the present investigation, we have demonstrated that primary human corneal fibroblasts produce globally-organized matrix where they can stratify (in controls and above the RCS in experimental cultures) but that the fibroblasts produce only locally anisotropic matrix within an RCS scaffold. The ability of individual fibroblasts to control the alignment of fibrils immediately adjacent to their membranes even when surrounded by the RCS is remarkable and encouraging. However, our investigation provide an evidence that the disorganized collagen network interferes with the ability of cells to coordinate their behavior, to communicate (as suggested by (Du et al. 2007)), to migrate and to control the ECM space effectively. Though scaffolds are beneficial in that they allow the colonization of large 3-dimensional volumes by fibroblastic cells, we further hypothesize that they have the potential to imprint their own structural topology onto the resulting synthesized matrix and/or interfere with cellular control of matrix organization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This investigation was supported by NIH/NEI 1R01 EY015500-01. The authors would like to thank Pat Pearson for her excellent transmission electron microscopy work and Prof. Shashi Murthy for his aid in binding collagen gels to the polycarbonate membrane surface.

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Figure 1.

Thick sections of constructs. $1\mu\text{m}$ sections of the resin embedded constructs were cut and imaged using bright field microscopy to provide a global view of the cell distribution within and on the constructs. Series 1 through 5 are time sequences (time points at 4, 8 and 11 weeks) for the five different experimental specimens taken in cross-section. Areas where the RCS has limited interaction with the cells are identified by the arrows. There are several important things to note in these images. 1) There was a heterogeneous response across the specimens in general with series 4 and 5 producing the thickest constructs, 2) The fibroblasts sparsely populated the RCS (when they did enter it) but densely populated their own synthesized matrix, 3) Fibroblasts were generally flattened and aligned with the plane of the membrane and 4) At the limits of the constructs, cells tended to form more confluent, denser layers (confirmed by DIC imaging). In all but three specimens, the polycarbonate membrane is still attached to the construct. Membrane is approximately 9 microns thick.

Figure 2.

Higher magnification thick sections of eight week experimental (series 4 and 5) and eight week control constructs. Sections were prepared using a similar method as described in Fig. 1. In both the experimental and control systems, fibroblasts produced significant amounts of their own matrix. In the two experimental series shown, the fibroblasts appeared to prefer to stay on top of the RCS or to colonize membrane under the RCS. Confluent layers of cells on the top and bottom of the constructs are apparent (white arrows). Alternating fibroblast orientation suggests matrix alignment changes as well in individual strata (cells of similar orientation in an assumed stratum indicated by black arrows). In series 5 a fibroblast has migrated into and resides in the RCS (black arrowhead). In the control construct, the fibroblasts have stratified and produced their own matrix of which a significant portion is globally-organized (see Guo et al (Guo et al. 2007) for extensive ultrastructural assessment of control matrix at 4 weeks). Bars are 25 microns.

Figure 3.

Differential Interference Contrast Micrographs of RCS, CDM and CDM/RCS interface in 8 week construct. A) Location 0.5 microns above the membrane shows generally disorganized texture in the RCS. B) At about 4.5 microns above the membrane, the transition from RCS to more organized CDM (arrows) can be readily appreciated. C) By 5 microns, we have reached a globally-organized layer of CDM which extends over many cell widths (in the plane of the construct). D) Further excursion into the CDM in this construct (6 microns or so) the CDM appears to comprise arrays of aligned fibrils which cross one another. E) DIC image of the globally-organized matrix in a control construct at 4 weeks (modified from Guo et al, IOVS 2008). Bars are 10 microns. (See accompanying movie for full z-scan of experimental construct).

Figure 4.

Bar graph depicting the DIC assessed organization of the matrix (A) and diameter distribution of collagen fibrils (B) in both the control series and the experimental series. A) The term matrix refers to the optical texture detected by the DIC optics and includes both collagen arrays and embedded cellular components. There are 4 bars with reflect high-resolution DIC measurements of z-thickness for 1) the entire construct (labeled Total thickness: SF or Total Thickness: RCS scaffold) and 2) the thickness of just the organized matrix (labeled Globally-organized matrix SF or Globally-organized matrix RCS scaffold). The data indicate significant variation in the thicknesses across all experiments, however, it was clear that the amount of globally-organized matrix in the RCS corneas was lower at all time points relative to the scaffold free controls. The difference was significant at 4 and 11/12 week time points. The supplementary movie provided shows the clarity with which DIC can distinguish between disorganized and organized matrix. B) The diameter distribution of collagen fibrils as estimated directly from TEM images. Cells consistently produced smaller diameter fibrils in the scaffold-free system ($38 \pm 4.62 \text{ nm}$ vs. $51.92 \pm 4.10 \text{ nm}$ at week 4, $38.70 \pm 1.90 \text{ nm}$ vs. $48.24 \pm 4.28 \text{ nm}$ at week 8, and $27.87 \pm 2.15 \text{ nm}$ vs. $48.54 \pm 4.44 \text{ nm}$ at week 11/12; $P < 0.5$ at all time points). The collagen fibrils diameter is significantly reduced in the scaffold-free system at week 11 which is consistent with observation from the DIC images which showed that the ECM is significantly reduced at this time point.

Figure 5.

Low resolution sTEM of four week constructs. A) In series 1, fibroblasts penetrate the RCS where they have colonized the membrane surface. The reconstituted collagen fibrils in the RCS are indicated with the black arrowheads. Isolated CDM collagen fibrils are indicated by black arrows. The amount of CDM collagen in this image is minimal. This particular series produced small constructs with little CDM collagen. Bar is 2 microns. B) The construct from series 4 is much thicker and comprises strata which contain aligned collagen and fibroblasts. In this image, the fibroblasts did not penetrate the RCS, instead they colonized the collagen surface and produced significant amounts of collagen which appeared to be aligned with the cells. Cells in adjacent strata appeared to change direction, white arrowheads: cells in cross-section, black-arrowhead: cell in longitudinal section. Bar is 4 microns. (Above: Inset from B showing fairly sharp transition from CDM to RCS – white, translucent line. Collagen in CDM has small diameter and is packed along the cells).

Figure 6.

Medium resolution sTEM of PHCSC interaction with RCS in eleven week construct. A) Construct comprising cells which have migrated into the RCS with bordering layer of CDM. The cells in the RCS appear to have entered the matrix and deposited a locally organized CDM. Bar is 5 microns B) Higher magnification of upper shaded box in which cell and collagen fibrils in the local CDM are in cross-section. C) Higher magnification of the middle shaded box in which the cell and collagen fibrils in the local CDM appear to be in longitudinal-section. D) Higher magnification of lower shaded box in which the CDM borders the RCS. Cells are in closer proximity and are all seen in cross-section. CDM fibrils are also in cross-section. B,C, and D bars are 1 micron.

Figure 7.

High resolution sTEM of RCS/CDM interaction in constructs. A) Border cell and associated CDM adjacent to the RCS. Cell-derived collagen fibrils, in longitudinal section, are indicated by the white arrowheads. These small diameter fibrils are embedded in a dense grainy material (*) that appears to share the distinct collagen fibril alignment (when imaged at higher magnification). The dense material does not penetrate the RCS matrix, but many smaller diameter fibrils (black arrows) which are possibly derived from fibroblast-secreted monomers are seen within the RCS. Large diameter collagen fibrils of the RCS are denoted by black arrowheads and are often jagged in appearance. Bar is 1 micron. B) Interaction of the RCS collagen (black arrowheads) with a fibroblast and the cell-derived matrix collagen. The CDM collagen fibrils, seen in cross-section, are densely packed and aligned in close proximity to the cell. The presence of the RCS collagen appears to invade the CDM collagen, altering its order. Further from the cell, the small diameter (presumably CDM) collagen (black arrows) appears to be losing its organization. Close to the cell, the CDM collagen appears to be enmeshed in a dense granular material. Bar is 500 nm. C) Fibroblast migrating into the RCS in an eleven week construct. Cell-derived small-diameter collagen fibrils appear to be clustered and organized in a layer less than 1 micron from the cell surface. Thicker collagen fibrils from the RCS (black arrowheads) approach the cell closely and are intermixed with small diameter fibrils (white arrows) which are presumably cell-derived. In some cases, the small diameter fibrils can be found well into the RCS matrix and away from the nearest fibroblast. There appear to be numerous vesicles or vacuoles in the RCS (V). Bar is 1 micron.

Figure 8.

Electron micrographs of cell-CDM interface in eleven week construct. A) sTEM image (in cross section) showing numerous small vesicle-like structures at the internal membrane surface (often opening to fuse with membrane – white-angled arrows). A couple of larger vesicles are also located at the membrane surface with one opening to the extracellular space (black asterisk). Adjacent to the membrane is a diffuse fibrillar material (white asterisks) which looks vaguely aligned in some locations. In the matrix there appear to be numerous collagen fibrils (vertical arrows) in various states of assembly (white arrows denote fibrils in cross-section, black arrows denote fibrils in longitudinal section). In many places there appear to be diffuse fibrillar structures which are co-aligned with the collagen fibrils (double white arrowheads). B) QFDE image of cell-CDM interface (en face fracture). Note numerous openings of various sizes in cell membrane (angled arrows). Webs of fine filaments can be observed adjacent to the cell and throughout the CDM (white asterisks). Thick (40–50 nm) fibrils are immersed in the fine filamentous network and show some alignment (white vertical arrows). An array of aligned and clustered thick fibrils can be seen in the right side of the image. Often thin filaments can be seen that are aligned and associated with thicker fibrils (white arrowheads). Bars 200 nm.

Figure 9.

sTEM of CDM. A) Clear demonstration of the change in fibril orientation which was seen routinely in the CDM matrix above the RCS in a 4 week construct. The image shows the presence of a cell process (black arrow) and vesicles (white arrows) which often contained dense material. The figure also demonstrates the poor diameter control over the collagen fibrils in the CDM (at some locations). Bar is 500 nm. B) High magnification of CDM in 8 week construct showing aligned collagen fibrils adjacent to a cell which display the characteristic D-periodic banding. Bar 200 nm.

Figure 10.

TEM micrographs of RCS. A) sTEM image of the RCS in eleven week construct which has not been penetrated by fibroblasts. The image shows numerous large diameter structures which are likely Purecol collagen (vertical arrows). Arrowheads indicate small diameter fibrils which possibly arise from cell-derived collagen but could be fibrils derived from fragmented RCS collagen, which often has a jagged appearance. Asterisks mark some of the numerous membrane bound vesicles found throughout the RCS. A black asterisk marks a vesicle with densely staining contents. Bar is 1 micron. B) QFDE micrograph of RCS. Three large diameter fibrils are visible (vertical arrows) as well as some small diameter fibrils (arrowheads). Asterisks mark vesicles. The image also shows an extensive secondary matrix of anastomosing fibrils of unknown composition. Bar is 1 micron. C) Lower magnification image of RCS showing secondary matrix with associated vesicles. Bar is 2 microns. D) A vesicle which appears to have released a network of polymerizing structures. Bar is 500 nm.