The soft tissue seal around a dental implant along with osseointegration have been shown to be crucial for long-term implant success. The understanding of the biology and mechanisms of soft tissue sealing around implants is vital for ensuring high implant stability and esthetics under oral conditions. Cell adhesion between the soft tissue/bone interface and the implant surface is considered the most biologically important stage in the process. This structural and functional integration is influenced by the activity of adjacent cells and by the biophysical and biochemical properties of the implant surface itself. Implant surface texture and chemistry play a role in bone and soft tissue integration. Surface topography of the implant improves wettability and increases cell growth and proliferation, which eventually accelerates the process of osseointegration. The connective tissue around a dental implant is characterized by collagen fibers aligned parallel to the implant. Collagen deposition and orientation are fundamental for a good sealing and to prevent biofilm formation that could lead to peri-implantitis. Cell-matrix adhesion mediated by integrin receptors allows cells to bind and respond to both biochemical and biophysical cues in the tissue and on the implant. Integrin-mediated adhesion controls cellular mechanotransduction, allowing the cells to respond to surface stiffness and topography features, such as grooves, converting them to biochemical signals and influencing cellular response to the implant surface. Prior in vitro research has focused on achieving optimal osseointegration and connective tissue attachment to implant materials. Simple in vitro 2D-culture methods have been used extensively to test this. However, these 2D models do not resemble the physiologic 3D microenvironment and, hence, may not be ideal for testing final products with different geometries and surfaces. Previous studies assessing fibroblast interactions with implants have mainly relied on measurements such as cell migration, gene expression, and cell adhesion. For these studies, testing cellular behavior at the implant surface was done by imaging the cell-implant interface using standard microscopy techniques in 2D tissue culture dishes. The true behavior of cells relative to the implant can best be assessed in a more physiologic 3D microenvironment.

**Purpose:** Previous studies assessing fibroblast interactions with implants have mainly relied on measurements such as cell migration, gene expression, and cell adhesion. For these studies, testing cellular behavior at the implant surface was done by imaging the cell-implant interface using standard microscopy techniques in 2D tissue culture dishes. The true behavior of cells relative to the implant can best be assessed in a more physiologic 3D microenvironment.

**Materials and Methods:** The embedding of the implant disks in 3D collagen gels was standardized with labeled fibroblasts to allow the imaging of fibroblast morphology and behavior when proximal to or binding to the implant disks. This allowed comparison of the behavior of laser-microgrooved and machined implant disk surfaces quantitatively in an in vitro 3D microenvironment.

**Results:** This in vitro imaging assay revealed for the first time in a 3D microenvironment setting the statistically significant impact laser-microgrooved disk surfaces have on both cell adherence and recruitment of cells in proximity to the disk. It also allowed visualization of membrane protrusivity and cytoskeletal organization in cells adherent to the implant disk.

**Conclusion:** This assay provides a simple and effective way of observing cell behavior on and around the implant disk surface in a more physiologic 3D setting. Within the limits of this study, it revealed that the laser-microgrooved implant surface demonstrates significant superiority in fibroblast recruitment and binding in a 3D microenvironment.
organizational behavior of fibroblasts and epithelial cells around implant abutments. However, some aspects of cell behavior are best studied at the single cell level. Fibroblasts have a distinctly different morphology and adhere and migrate differently in a 3D microenvironment. The fibrillar structure of the 3D collagen matrix provides a mechanically compliant environment, which induces a reciprocal effect on cells. These cells not only adhere through integrin-dependent 3D cell-matrix interactions, which differ from classical focal adhesions in 2D, but also change in mechanical stability due to cell-matrix entanglement. This further induces local and global matrix remodeling to support cell migration and eventual binding to the implant surface.

Hence, measurements done so far with laser-microgrooved implant surfaces in 2D may not provide a true representation of cellular behavior in the context of their microenvironment. This could also mean that implant surfaces that favor fibroblast/osteoblast interplay in 2D could behave differentially in 3D. This suggests the need to evaluate cellular behavior with implant surfaces in a 3D microenvironment. The soft tissue seal around the implant is regulated by fibroblast binding to the implant surface. This is a result of the migratory and matrix remodeling capability of fibroblasts around the implant collar. Fibroblast-matrix crosstalk at the implant surface is regulated among others by caveolin-1–mediated regulation of membrane endocytosis and metalloproteinase function. The ability to visualize cell protrusivity, membrane trafficking, secretion, and matrix remodeling in 3D microenvironments could further enhance the understanding of cellular behavior with implant surfaces.

The hypothesis was as follows: An in vitro assay in 3D collagen gels with embedded fibroblasts would provide a physiological system to compare cell behavior between microgrooved and machined implant surfaces. Confocal imaging of cells would allow quantitative evaluation of single cell behavior in 3D gels relative to the implant disks.

To the best of the authors' knowledge, assessment of cellular behavior such as migration and binding at the single cell level on the implant surface has never been done before in a 3D microenvironment, although 3D culture techniques have been previously utilized for studying cell adhesion and motility. Thus, the objectives of this study were to:

- Evaluate and compare fibroblast behavior in a 3D collagen microenvironment on laser-microgrooved (test) and machined implant (control) surfaces
- Visualize cells in 3D collagen gels in relation to the implant and obtain quantifiable comparative data in relation to the laser-microgrooved surface

**MATERIALS AND METHODS**

Assessment of the following listed parameters for fibroblast behavior (wild type mouse embryonic fibroblast [WTMEF]) was done with two groups: group 1—laser-microgrooved surface (test), and group 2—machined surface (control). The laser-microgrooved surface has standardized 8- to 12-micron grooves designed to provide a functional surface for cellular attachment and has been tested in previous studies:

- Standardization of the embedding of the implant and fibroblasts in 3D collagen with the implant
- Labeling of the cell membrane and imaging of fibroblasts in 3D collagen gel to evaluate their morphology in proximity to the implant surface
- Evaluating the differential association of cells in proximity to and on the surface of the implant in 3D collagen gels

**Cell Culture**

Mouse embryonic fibroblasts (MEFs) obtained from Dr Richard Anderson (University of Texas Health Sciences Centre) were cultured in complete Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 5% fetal bovine serum (FBS; Invitrogen) and penicillin-streptomycin (Invitrogen) at 37°C in a 5% CO₂ incubator.

**Embedding of Implant Disk in 3D Collagen Gel with Fibroblasts**

MEFs growing for 48 hours in DMEM (5% FBS) were detached using 1 mL Accutase (Sigma Aldrich), washed, and reconstituted in DMEM (5% FBS). Meanwhile, collagen (8.56 mg/mL stock; 71 µL) was mixed with 10× PBS (30 µL), Milli Q Water (MQW; 197 µL), and 100 µL of cell suspension containing 25 × 10⁵ cells. Finally, 1 M NaOH (1.75 µL; Sigma-Aldrich) was added to the mixture to allow collagen to polymerize at a final concentration of 1.5 mg/mL (400 µL total volume). This gel solution was mixed gently and added to a well in an 8-well Lab-Tek glass bottom chamber, and an implant disk was embedded into collagen solution before it polymerized. The implant was carefully placed into the 1.5 mg/mL collagen mixture such that it stood along its broader edge in the middle of the chamber. Sterile forceps that were wiped down with 100% ethanol (Thermo Fisher Scientific) were used for this. The Lab-Tek chamber (Thermo Fisher Scientific) was gently moved to a CO₂ incubator at 37°C and incubated for 30 minutes to allow the collagen to completely polymerize. Treated (test) and machined (control) disks were embedded in neighboring chambers of the Lab-Tek chambers. After 30 minutes, DMEM (5% FBS) was added to the gel and incubated for 2 hours at 37°C and then fixed using
4% paraformaldehyde + 5% sucrose solution (Sigma-Aldrich) for 20 minutes at room temperature. Cells embedded in the gel were washed with 1× PBS for 5 minutes at RT.

**Labeling of Fibroblasts in 3D Collagen with Implant Disks**

Collagen gel with cells and an implant disk were incubated with phalloidin Alexa 488 (1:250) diluted with 2% bovine serum albumin (BSA) in PBS, overnight at 4°C. The gel and cells were washed with PBS and stored at 4°C, and confocal imaging was done as described below. Alternatively, fibroblasts growing for 48 hours in DMEM (5% FBS) were incubated with CTxB conjugated to Alexa594 (1:1,000; Invitrogen) for 30 minutes at 37°C. This labels the cell membrane, and endocytosis of CTxB-Alexa 594 marks the inside of the cells. These cells were washed with PBS, incubated with 1 mL Accutase for 2 minutes, detached, washed, and added to the collagen mixture. Cells were incubated in collagen gels, fixed, and imaged as described below.

**Confocal Imaging of Cells and Implant Disk in 3D Collagen Gel**

Zeiss Multiphoton LSM710 was used for fluorescence imaging of labeled cells at 3% laser power (pinhole 2AU). The implant was simultaneously imaged using a bright-field DIC filter. Images were captured at 60× magnification at a scan speed of 5 to get an optimal resolution and to get a clear image of the cells with implants. Z-stacks for fluorescence and DIC images were taken at 0.2-µm intervals and processed as described below.

**Deconvolution of Images Using Huygens Image Analysis Software**

All fluorescence z-stacks images were processed and analyzed using Huygens Professional version 16.10 (Scientific Volume Imaging, http://svi.nl). Deconvolution of confocal z-stacks was optimized using the following settings: average background value = 1, number of iterations = 30, signal-to-noise ratio (SNR) = 20, and quality change threshold = 0.0001. These settings were kept constant for all image convolutions. The deconvoluted file was opened using the maximum intensity projection (MIP) renderer tool in Huygens Software in the Visualization tab; these were set at a zoom of 1.38 and frame size of 1,600 × 1,200 and saved accordingly.

**Counting of Cells in Proximity to Implant Disk in 3D Collagen Gel**

Three-dimensional collagen gels with labeled mouse fibroblasts and dental implant disks were observed using a 60× oil immersion objective in the EVOS imaging system (Invitrogen), and images were recorded. The implant was imaged at five distinct points along its length and three points along its height. A 100-µm line was drawn vertically from the surface of the implant in the image, and cells in this region of proximity to the implant were counted in each image. The total number of cells in all images per implant were counted and compared between the treated disk (test) and untreated disk (control) and plotted. A total of five frames were imaged in each disk, and cells were counted per frame. Data from five independent experiments (and 25 frames) were compared. Statistical analysis was performed with the Mann-Whitney test using GraphPad Prism 5.0 (Graph Pad Software). The significance was set at P < .05.

**RESULTS**

To study fibroblast behavior with dental implants in a 3D microenvironment, the embedding of implant disks was optimized in 3D collagen gels. Earlier experiments in the lab had tested mouse fibroblast survival and migration in collagen gels of varying stiffness and identified 1.5 mg/mL collagen as the ideal for the time of incubation with embedded implants (data not shown). Mouse fibroblasts mixed with collagen gel at optimal cell numbers were incubated with an embedded laser-microgrooved implant disk (Fig 1a). Embedded cells with implant disks were fixed and stained overnight with phalloidin (Fig 1b). The implant disk had a small but distinct region of contact with the glass bottom that was accessible to and imaged with a confocal microscope (Fig 1b). This allowed the cells to be imaged in proximity and adherent to the coated and uncoated surface of the laser-microgrooved implant disk (Fig 1b). DIC images at 40× magnification showed clear separations in the treated surface of the disk relative to its untreated backside. The presence of cells labeled with phalloidin adherent to the treated surface of the disk was also detected. Phalloidin staining, while allowing the detection of cells adherent to the treated disk surface, did not give much clarity on the architecture of individual cells, such as possible membrane protrusions they might make when adherent.

To overcome this limitation, the plasma membrane of MEFs was surface labeled with a fluorescently tagged cholera toxin B that binds GM1 (glycosphingolipid with one sialic acid, NANA, residue) that allowed the detection of the plasma membrane and observation of endocytosis of lipid membrane when cells were embedded in 3D collagen hydrogels. This labeling does not affect fibroblast function or mobility. Cells that were labeled when embedded in collagen gels to begin with were circular (lacking prominent protrusions), and their plasma membrane boundary was distinctly seen by confocal microscopy (Fig 2a). This further allowed morphologic
changes such as cellular membrane protrusions to be seen (Fig 2b). Labeled WTMEFs embedded in collagen gels for 4 hours with the implant disk migrated to bind the laser-microgrooved treated surface but not the untreated implant surface. GM1-CTxB labeled cells that were imaged using a confocal microscope and z-stack images deconvoluted, revealing the presence of distinct membrane protrusions when cells were adherent to the laser-microgrooved implant surface (Fig 2b). This was not observed in cells proximal to the untreated implant surface. GM1-CTxB labeling also allowed for the visualization of membrane endocytic vesicles localized in the intracellular pool including their localization along the membrane protrusions (Fig 2b—higher exposure).

To determine whether laser-microgrooved and machined disks have any significant difference in the way they recruited and bound fibroblasts, CTxB labeled cells in 3D collagen gels with disks were imaged as multiple frames along the entire optically accessible surface of contact between the disk and cells. It was observed that laser-microgrooved disks (with detectable grooves; test) had significantly more cells adherent to the implant surface compared with the machined (control) disks (Figs 3a and 3b). Cells adherent to the test disk were seen to make distinct protrusions (Fig 3a). Some cells were found near the machined control disks, but very few were adherent to the implant, which suggests that laser-microgrooved surfaces were better at allowing fibroblasts to adhere in the 3D microenvironment (Fig 3).
The present study further evaluated the number of cells in proximity, 100-µm distance from the implant disk surface (Fig 4a), and noticed significantly more cells were present near the test disk relative to the untreated control (control; Fig 4b graph). This further suggests that the laser-microgrooved surface was able to attract more fibroblasts toward it, allowing them to attach with this surface (test) of the implant, which could eventually help make a strong cellular seal around the implant. It also raises the possibility that the laser-microgrooved surface could influence the 3D matrix around the disk, allowing for cells to move better toward it and eventually bind to the implant surface.

**DISCUSSION**

Historically, studies looking at cell adhesion and function on implant surfaces used to focus primarily on washout tests, and subsequent measurement of cells remained attached to the substrate. Subsequently, assessment at the single cell level was done by the present group, which enabled quantifiable attachment measurement, while eliminating extraneous forces from other cells. Atomic force microscopy (AFM) and scanning electron microscopy (SEM) examination have also been used for assessing the interaction between fibroblasts and dental implant surfaces.

The transmucosal component of the implant does share some commonalities with the anatomical structure around teeth, such as the presence of a junctional epithelium and a connective tissue. However, there are some important differences. In teeth, the collagen fibers insert perpendicularly into cementum, while in the peri-implant zone, there is an adaptation of fibers into a more parallel arrangement. This, combined with reduced cellularity and vascularity in the peri-implant connective tissue, is said to make implants more susceptible to disease initiation and progression.

Esfahanizadeh and coworkers assessed the morphology, proliferation, and gene expression of human gingival fibroblasts (HGFs) on Laser-Lok, titanium, and zirconia implant surfaces. HGFs on Laser-Lok surfaces were found to have a more mature morphology and greater proliferation and differentiation compared with those on other test surfaces. However, this study utilized standard 2D imaging methods to analyze fibroblast morphology.
A recent article\(^6\) presented an elegant model to study organizational behavior of fibroblasts and epithelial cells around an implant abutment. However, the focus of this article was to replicate the peri-implant zone and analyze how it might change with different abutment surfaces. Some aspects of cell behavior are best studied at the single cell level.\(^9\) Hence, the present study differs in the fact that it focused on analysis of single cell behavior in a 3D microenvironment.

In the present study, the Laser-Lok surface (laser-microgrooved), comprising a unique surface topography and thereby a unique biophysical property, demonstrated a significant difference in not just cell binding but also cell recruitment. This could reflect how the laser-microgrooved surface influences the organization of the matrix around the treated implant surface. Adhesion of cells to the implant surface could affect their secretion of regulatory factors (such as growth
factors) and matrix metalloproteases (MMPs)) that could further drive the recruitment of cells. The present authors’ ongoing studies aim to look at how this regulation works and its impact on matrix organization. Leong et al. compared two groups: laser-modified implant surfaces and machined implant surfaces using gene profiling analysis. Laser-mediated alterations in topography were shown to enhance collagen fibril-associated gene expression and alter epithelium/junctional gene expression. Gene expression of cells in 3D is known to be distinctly different from 2D microenvironments, which could further impact how cells behave in this assay and would be worth evaluating. The laser-microgrooved surface used in this experiment has been previously shown to demonstrate a physical histologic connective tissue attachment in a landmark article by Nevins and coworkers. The unique surface topography, along with the surface chemistry, may contribute to the difference seen in the cellular binding between the laser-microgrooved and machined surfaces.

The confocal imaging utilized in this study provided a means to assess this cellular behavior at the single cell level, which could allow better understanding of cell behavior (morphology and intracellular trafficking) in the context of the implant in a 3D microenvironment. The migration of cells in 3D is further influenced by how cells remodel the matrix and its organization, which such an assay could also help evaluate in context of the implant. Such an in vitro assay can also allow regulation of individual parameters such as cell density, matrix crosslinking, matrix composition, and stiffness to test their impact on cell-implant interaction.

This study has its share of limitations. Since this was an in vitro study, one needs to be careful not to extrapolate the results to an in vivo or clinical environment. Although good quantifiable data can be obtained at the single cell level, this also means that intercellular interactions need to be studied in more detail. The matrix composition in vivo is also more complex, and while these studies are done with 3D collagen, more complex 3D microenvironments could further help improve the context of these studies. This study, in establishing the imaging of fibroblasts in 3D gels with implants, highlights the possibilities that exist going forward with this assay system. What the study lacks in being a completely 3D in vivo assay system, it makes up for by allowing visualization of cells in a controlled 3D environment in a way that would not be possible in vivo. In doing so, this assay allows the study of cell-implant interactions in a way that has not been possible so far. The assay utilized here uses a single cell analysis. This assay could also serve as a tool to evaluate different implant coatings and get a better evaluation of how cells behave in 3D microenvironments relative to the implant surface, compared with a 2D analysis.

This could be a useful first step to help decide which of these coatings would be worth evaluating further in vivo. It could hence also allow for the repurposing of known implant coatings that have so far been tested only in 2D.

CONCLUSIONS

Within the limitations of this study, this assay provides an attractive tool to test the behavior of cells on implant disks. This 3D collagen gel with embedded cells and implant disk demonstrates that the laser-microgrooved surface provides a statistically significant impact on both cell adherence to the disk, as well as to the recruitment of cells in proximity to the disk. The implications of both would be that the laser-microgrooved surface could possibly influence the 3D matrix around the disk to improve recruitment and binding of cells to implant surfaces. The ability to visualize such changes in behavior and morphology in cells using confocal microscopy in 3D collagen gels also makes this a useful tool to characterize in detail the interaction of cells with implant disks.

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