Morphologic Characteristics and Proliferation of Rabbit Corneal Stromal Cells Onto Complexes of Collagen–Chitosan–Sodium Hyaluronate Under Simulated Microgravity

Xiaoxia Li,1,2 Yan Yang,1,3 Qinhua Li,4 Ying Dai,5 Chan Wang,5 and Jiansu Chen1,3,5

Correspondence: Jiansu Chen, Institute of Ophthalmology, Medical College, Jinan University, 601 West Huangpu Avenue, Guangzhou, 510632, China; chenjiansu2000@163.com.

XL and YY contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Submitted: June 30, 2013
Accepted: September 16, 2013

PURPOSE. We investigated the morphologic characteristics and proliferation of rabbit corneal stromal cells (CSCs) onto scaffolds under simulated microgravity.

METHODS. Rabbit CSCs were cultured under simulated microgravity (SMG) and static condition. Complexes of collagen–chitosan–sodium hyaluronate with pores were used as scaffolds. Rotational speed was set at 15, 20, and 30 rpm in the first, second, and third weeks of culture, respectively. Histology, immunofluorescence staining, atomic force microscope (AFM), and scanning electron microscope (SEM) examinations were performed. The cell proliferation was analyzed by cell counting kit-8 (CCK-8) assay.

RESULTS. In the SMG group, more CSCs adhered to the carriers in 24 hours. Confocal microscopic evaluation showed aggregated cells positively immunostained with vimentin. The SEM displayed the complex network of triangular or polygonal dendritic morphology of the cell bodies with many fine and long processes, which adhered to the scaffolds tightly. After 18 days of SMG culture, keratocyte-like CSCs with rich cell interconnections not only grew on the surface, but also into the interior of scaffolds. There were degradation phenomena in scaffolds in the SMG condition. Under static condition, cells just grew on scaffolds forming a monolayer. Cells showed elongating spindle shape and developed less processes. The absorbance values of the CCK-8 assay in the SMG group were significantly higher (P < 0.01) than in the conventional group.

CONCLUSIONS. The condition of SMG and porous collagen–chitosan–sodium hyaluronate scaffolds facilitate the proliferation of CSCs. Cells showed robust growing characteristics and morphologic properties of keratocytes. The techniques for microgravity culture of keratocyte-like CSCs on scaffolds can yield cell aggregates or cell sheets that are favorable to the reconstruction of tissue engineering of the corneal stromal layer.

Keywords: tissue engineering, simulated microgravity culture, collagen–chitosan–sodium hyaluronate, rabbit corneal stromal cells

Keratoplasty is an effective way to treat corneal diseases, such as corneal ulcer, trauma, and scar formation, which may result in blindness. However, the shortage of corneal donors worldwide, and some uncertainties and insecurities associated with keratoplasty have limited its application.1 Therefore, trying to recruit new sources of corneal donors and new corneal replacements has been a continuously strived after effort.2 Tissue engineering of cornea is one of the primary efforts. Corneal tissue engineering refers to the fabrication of biologic living cornea equivalents by means of three-dimensional (3D) culture of three corneal cell types (epithelium, keratocyte, and endothelium) and biomaterials, and it can be considered as a substitute for donors in keratoplasty.3–4 Among the three components of the cornea, limbal stem cell sheets or oral epithelial cell sheets of tissue engineering have been constructed by culture on thermal responsive materials, human amniotic membrane (AM), or fibrin, and used clinically to create the corneal epithelium in patients with total limbal stem cell deficiencies.5–9 Tissue-engineered corneal endothelial cell sheets or cell injection therapy has restored corneal transparency successfully in animal models.10–13 However, constructing corneal stroma or a whole cornea close to the natural cornea in vitro and transplanting in vivo still is difficult.14,15

The corneal stroma should be the current focus of researchers attempting to produce a corneal tissue analog.16 More than 90% of the cornea is stroma, a highly organized, transparent connective tissue maintained by specific corneal stromal cells (CSCs), keratocyte, which provides the principal functions of the corneal tissue.17 Keratocytes are specialized neural crest–derived mesenchymal cells. They exit the cell
cycle and become quiescent G0 cells after eyelid opening (at 24 weeks in the human embryo).\(^\text{18}\) Keratocytes can be stimulated to undergo proliferation, migration, and myofibroblast transformation in response to corneal wounding in the corneas of many species examined, including mouse, rat, rabbit, pig, and human.\(^\text{19,20}\) Keratocytes bodies had a flattened pyramidal or stellate shape, and the fine cell processes formed extensive distal ramifications in the central stroma.\(^\text{21}\) Keratocytes usually rapidly lose their dendritic morphology and acquire fibroblastic morphology when they are cultured on plastic substrate in serum-containing medium, especially when seeded at a low density.\(^\text{22,23}\) Therefore, for corneal tissue engineers, it is preferable that stromal constructs provide an environment that promotes and maintains the keratocyte phenotypes.\(^\text{18}\) The techniques for the cell expansion and in vitro culture environment may influence keratocyte biological characteristics greatly. For instance, human keratocytes maintained dendritic morphology and keratocan expression when subcultured on AM stromal matrix, even in the presence of 10% fetal bovine serum (FBS).\(^\text{24}\)

In our previous study, we successfully cultured rabbit CSCs on the surface of culture plates, AM, collagen–chitosan–sodium hyaluronate complexes, acellular corneal matrix, and so forth.\(^\text{25,26}\) In the presence of 10% FBS, almost all CSCs on plastic in static culture showed spindle shape, and rare were interconnected or unconnected with each other. However, also with 10% FBS in existence, CSCs cultured on the acellular bovine corneas interconnected to form reticular structures and cells grew into a dendritic shape.\(^\text{27}\) At the same time, we found that the proliferation of rabbit CSCs could be promoted by using dehydrated bovine acellular corneal matrix as a scaffold to culture rabbit CSCs under simulate microgravity (SMG) of a rotary cell culture system (RCCS; Synthecon, Houston, TX). Rabbit CSCs on acellular corneas were dendritic- or spindle-shaped and grew into the porous acellular corneal matrix in SMG culture.\(^\text{25}\) We also reported that keratocyte-like CSCs formed a rich interconnection and 3D aggregates on acellular corneas on day 4 of SMG culture. They expressed keratocan and lumican in immunofluorescence stains, and RT-PCR analysis even in the presence of 10% FBS.\(^\text{27}\)

In this study, we further researched microgravity bioreactor culture of CSCs in the porous collagen compound scaffolds. We examined cell proliferation and the morphologic changes of CSCs on collagen compounds. In our previous study, we have shown that the complexes of 20% collagen, 10% chitosan, and 0.5% sodium hyaluronate had good cytocompatibility with three corneal cell types and good biocompatibility with corneal tissue.\(^\text{26}\)

### Materials and Methods

#### Ethics Statement

Primary cultures were established from the corneas of New Zealand White rabbits (10 eyes) aged 3 to 4 months with a weight range of 2 to 2.5 kg. Rabbits were handled in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. The protocol was approved by the Institute Animal Care and Use Committee of Jinan University. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

#### Materials

Culture reagents, except those stated otherwise, were from Gibco (Grand Island, NY). Corneal CSCs were cultured in a complete growth medium that consisted of Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 3.7 g/L NaHCO\(_3\), 100 U/mL penicillin G sodium, 100 mg/mL streptomycin sulfate, and 10% vol/vol fetal calf serum (Sijiqing, Hangzhou, China). Unless otherwise stated, all other reagents were from Sigma-Aldrich (St. Louis, MO). Cell counting kit-8 (CCK8) was from Dojindo Laboratories (Kumamoto, Japan). Monoclonal anti-vimentin (NeoMarkers) was from Lab Vision Corp. (Fremont, MO).

#### Isolation and Primary Culture of CSCs

Eyes from New Zealand White rabbits were obtained and the cornea was excised for CSCs. Connective tissue and external muscles then were removed. The corneas were rinsed with saline containing antibiotic solution (prepared with 100 U/mL penicillin G sodium and 100 mg/mL streptomycin sulfate). The CSCs were isolated according to the methods of Jester et al.\(^\text{28}\)

Briefly, corneas stripped of endothelial and epithelial tissues were put into a solution of 0.2% type I collagenase (CCK8) was from Dojindo Laboratories (Kumamoto, Japan). The complex scaffolds were placed into a culture dish containing cultivation media and exposed to ultraviolet light for 24 hours before cell culture.

#### Preparation of Porous Collagen Compound Scaffolds

The methods of our previous preparation of compounds of collagen–chitosan–sodium hyaluronate were used.\(^\text{26}\) Briefly, chitosan (molecular weight [MW], 500,000–600,000) was prepared from chitin by deacetylation (85%–90% deacetylated). Type I collagen and chitosan were dissolved in 0.001 N hydrochloric acids separately. Then, the collagen and chitosan solutions were poured into a beaker and stirred by a homogenizer for 30 minutes. Sodium hyaluronate (purity, 95%) solution was dripped into the mixture and mixed for 30 minutes. The homogeneous solutions were cast on Perspex plates and heated for 24 hours at 35°C to obtain complete dry membranes. The dry membranes were immersed into distilled water and adjusted 7.5 < pH < 8.0. The components of membranes were prepared by 20% collagen, 10% chitosan, and 0.5% sodium hyaluronate weight percent. The membranes again were heated to obtain dry ones. Laser perforation on the surface of collagen–chitosan–sodium hyaluronate membranes was performed. The pore diameters and pore distances were designed using software CorelDraw 9.0 (diameter of 50 μm; Corel Corporation, Ottawa, Ontario, Canada). Samples were put on the platform. After regulating the focus and setting the parameters, collagen compound membranes were perforated by laser irradiation of CO\(_2\) Laser Marker CO\(_2\)-H10 (Han’s Laser, Shenzhen, China). The compound scaffolds then were placed in PBS buffer at 4°C. A circular scaffold piece of 5.5 mm in diameter and approximately 1 mm thick was prepared by using a trephine. The complex film scaffolds were rinsed in distilled water, contacted with 75% ethanol for 10 minutes, rinsed in a sterile solution of PBS buffer, and then equilibrated in the cultivation media. The complex scaffolds were placed into a culture dish containing cultivation media and exposed to ultraviolet light for 24 hours before cell culture.
CSCs Cultured Under Simulated Microgravity

Two groups were created in the experiment, the SMG culture group and the conventional culture group. The procedure of our previous SMG culture was used. Briefly, CSCs at passage 3 to 4 were trypsinized and suspended at $5 \times 10^5$ cells/mL. The prepared circular scaffolds (diameter = 5.5 mm, thickness = 1 mm per piece, $n = 18$) were put into the cellular suspension, mixed gently, and then cultured at 37°C in a 5% CO$_2$ incubator for 30 minutes. The CSCs and compound scaffolds were then cultured in the simulated microgravity culture system and conventional culture. The first step of the microgravity culture procedure was to inject slowly 10 mL serum-free DMEM into the 25 mL capacity high-aspect-ratio vessel (HARV) of RCCS (Synthecon, Houston, TX). Then, DMEM with CSCs and scaffolds were together put into this vessel. At last, DMEM was filled into the vessel at a concentration of $1 \times 10^5$ cells/mL in the culture medium supplemented with 10% FBS. Gas bubbles in the HARV vessel had to be removed. The vessel was put into the incubator for RCCS simulated microgravity dynamic seeding and culture. The rotational speed was set at 15, 20, and 30 rpm in the first, second, and third weeks of culture, respectively. Culture samples were obtained from the scaffolds every 3 days for examination of cell proliferation and morphology.

CSCs Cultured Under Simulated Microgravity

The CSCs cultured in a 96-well plate under conventional condition were referred to as the group of conventional culture. Each well of the 96-well culture plates received a piece of the prepared circular scaffolds (diameter = 5.5 mm, thickness = 1 mm per piece, $n = 18$). The same origin of CSCs were trypsinized, resuspended in medium, counted, and seeded onto the scaffolds at a density of $1 \times 10^3$ cells/mL. Culture medium was changed every second day.

Histology

Histology was performed as described previously. Samples were obtained for light microscopy with hematoxylin and eosin (H&E) staining. The samples were prepared as follows:

- Tissue samples were fixed in 4% paraformaldehyde at 4°C for 24 hours
- Dehydrated in a graded series of alcohol (50, 70, 80, 90, and 100%)
- Transparent with xylene
- Paraffin embedding
- Sectioning with microtome
- Staining with hematoxylin and eosin

Immunocytochemical Analysis

The samples of scaffolds with cultured CSCs, and cells or aggregates on coverglass-bottom Petri dishes were rinsed with PBS buffer and put in 95% ethanol for 20 minutes, washed twice for 5 minutes each time in distilled water, transferred to 0.45% hydrogen peroxide in methanol for 15 minutes, again washed twice for 5 minutes each time in distilled water, and incubated with normal goat serum at room temperature for 20 minutes to block nonspecific binding. The samples then were transferred to a moist chamber with monoclonal antivimentin (1:500) and incubated for 1 hour. After that, they were rinsed thrice with PBS buffer for 3 minutes. Then, the samples were incubated in the moist chamber for 30 minutes with the antimouse IgG Cy3 labeling corresponding secondary antibody.

Atomic Force Microscopy

The samples of compound scaffolds were washed with PBS twice and dispersed in distilled water. Then, samples were applied on silicon wafer and air dried in a vacuum desiccator. Atomic force microscopy (AFM) morphology of the samples was observed with AutoProbe CP in tapping mode.

Cell Proliferation Assay

The proliferation of CSCs was analyzed at various time points (at days 3, 6, 9, 12, 15, and 18 in cultures) by CCK-8 assay. The methods used were described previously. Briefly, scaffolds containing cells were picked out randomly from two groups of simulated microgravity and conventional cultures. The scaffolds were put onto 96-well culture plates. One well with scaffold without cells was used as control. The samples were stained with 10 μL CCK-8 solution and incubated on the plate in a CO$_2$ incubator at for 3 hours. The absorbance at 450 nm was determined using a multimode reader. Three parallel experiments in each sample were used to assess the cell proliferation. The data were processed with SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL). A $t$-test was used for comparing the mean of the groups.

RESULTS

Inverted Microscopic Evaluation

Rabbit CSCs were isolated successfully from cornea stromal layer after collagenase digestion. Cells grew well with DMEM–10% FBS on regular culture surface and reached almost confluence after 5 to 7 days of culture. The CSCs on plastic in static culture showed spindle shapes, and rarely were irregularly interconnected or unconnected with each other in the presence of 10% FBS (Fig. 1A). The CSCs on the scaffolds in the conventional culture distributed dispersedly and were rather difficult to spread on the surface of scaffolds (Fig. 1B). However, in the microgravity culture experimental group, CSCs on scaffolds were spherical, and singly and uniformly distributed at first, but shortly after (usually within 24 hours), many cells adhered to the scaffolds. Compared to the conventional culture group, a faster and higher yield of cell attachment on scaffolds was achieved when RCCS-simulated microgravity dynamic seeding was used. Cells showed the characteristics of aggregation on day 6 (Fig. 1C) or obvious attachment on compound scaffolds. In the SMG culture group, a few of aggregate cells fell off the scaffolds and grew up in...
suspensions. Some of these cell aggregates adhered to the long thread-like structure (Fig. 1D). After 18 days of culture under microgravity, some of the cell sheets could cling tightly on the scaffolds. There were partial thin degraded areas of the scaffolds (Fig. 1E). Other cell sheets could hold loosely onto the surface of the scaffolds (Fig. 1F). There were diverse growth states of CSCs in the dynamic SMG environment and they showed better cellular activity than that in the static one.

**Histologic Evaluation**

In the conventional culture group on day 6, there were almost no cells on the compact scaffolds of the H&E-stained sections (Fig. 2A). After 18 days of culture in the conventional culture group, a layer of CSCs attached and grew onto the surface of scaffolds, but no cells were present in the interior matrix or pores of the scaffolds. The scaffolds revealed mild degradation (Fig. 2B). Conversely, after 18 days of microgravity culture, CSCs were abundantly present onto or into obviously degraded scaffolds. Multilayers of CSC sheets adhered to the severely degraded scaffolds. Some of CSC layers evenly folded and formed thick cell sheets (Fig. 2C). A higher magnification of the same section showed many CSCs adhered to the surfaces of loose and fragile scaffolds, and some of them grew into the scaffolds (Fig. 2D).

**Immunofluorescence Analysis and Confocal Microscopic Evaluation**

Immunofluorescence analysis showed that CSCs in conventional or microgravity culture were stained positively for vimentin. The CSC cytoplasm was stained red on day 6 in conventional culture (Fig. 3A). The CSCs grew on the surface of scaffolds in conventional culture and cells showed elongated spindle shapes after 6 days of culture (Fig. 3B). However, the growth characteristics of CSCs in microgravity dynamic culture obviously different were from that in conventional culture. A few cellular aggregations growing up in suspensions could be found every time when medium was changed. They could grow continuously, become bigger, and at last become spherical bodies of cells with diameters of 1 to 2 mm after 18 days of microgravity culture (Fig. 3C). Many CSCs grew into the interior of scaffolds on day 6 in RCCS. The CSCs showed a round shape and formed aggregates (Fig. 3D).

**The Observation of Surface Microstructure on the Scaffolds**

The surfaces of compound scaffolds of collagen-chitosan-sodium hyaluronate were imaged by AFM and SEM (Fig. 4).
The SMG condition allows the cells to extend their cellular bodies more extensively and form rich network structures of cells after 6 days of culture (Figs. 5A). The cells could grow over the pores of scaffolds (Fig. 6C). There were much more cells on the scaffolds and even extracellular matrix (ECM)-like membrane structures could be observed in the microgravity group after 18 days of culture (Fig. 6D). Keratocyte-like CSCs also showed the complex network of dendritic morphology of the cell body and processes (Fig. 6E). Many cells adhered to the surface of scaffolds tightly (Fig. 6F). Cell growth as multilayer or aggregations were seen on the rugged surface or in the pores of scaffolds (Fig. 6G). The multilayer of cells could gradually grow and enlarge (Fig. 6H).

The Observation of CSCs by SEM Evaluation

In the conventional culture group, some short CSC processes were adhering to the surfaces of compound scaffold after 6 days of culture. The surfaces of cells and scaffolds were smooth, and there were rare intercellular connections (Fig. 5A). There were almost no cells in the pores of the scaffolds (Fig. 5B). However, intriguingly, under microgravity culture conditions, the ultrastructures of CSCs were greatly different from the situation in conventional culture. The CSCs bodies were flattened and adhered to the scaffold surface tightly. Cells showed more intercellular connections after 6 days of culture in microgravity culture (Fig. 5C). The cells could elongate and began to shoot out many thin foot processes, gradually building intercellular adhesions. Cells showed rough cellular surfaces that were rich in globular prominences (Fig. 5D). There were cells in the pores of scaffolds (Fig. 5E), and the more highly magnified images showed many fine and long cell processes on one side of the cells (Figs. 5F–H).

The cultivated CSCs on scaffolds proliferated extensively, and rich network structures of cells could be observed in the microgravity group after 12 days of culture (Fig. 6A). Keratocyte-like CSCs showed triangular or polygonal shaped cell bodies. There were many intercellular connections between cell bodies, between cytoplasmic processes, and between cell bodies and the cytoplasmic processes (Fig. 6B).

The cells could grow over the pores of scaffolds (Fig. 6C). There were much more cells on the scaffolds and even extracellular matrix (ECM)-like membrane structures could be observed in the microgravity group after 18 days of culture (Fig. 6D). Keratocyte-like CSCs also showed the complex network of dendritic morphology of the cell body and processes (Fig. 6E). Many cells adhered to the surface of scaffolds tightly (Fig. 6F). Cell growth as multilayer or aggregations were seen on the rugged surface or in the pores of scaffolds (Fig. 6G). The multilayer of cells could gradually grow and enlarge (Fig. 6H).

Test of CSC Proliferation

The cell proliferations of the two groups were examined by the CCK-8 assay. The values of the CCK-8 assay were analyzed through a statistical approach. The results showed that the proliferation rate for the simulated microgravity group (0.6040 ± 0.0534, 0.7643 ± 0.8168, 0.7259 ± 0.3687, 1.0897 ± 0.1202, 1.5273 ± 0.2674, and 1.7026 ± 0.2684 on 3, 6, 9, 12, 15, and 18 days, respectively) was determined to be significantly higher ($P < 0.01$) than that of the conventional culture (0.4663 ± 0.0976, 0.5660 ± 0.0887, 0.6263 ± 0.3697, 0.8509 ± 0.1061, 1.0377 ± 0.6228, and 1.231 ± 0.0177 ($P < 0.01$, $n = 3$, Fig. 7). All data were presented as mean ± SD, $n = 3$.

DISCUSSION

The stromal tissue of the cornea comprises 90% of the total thickness of the cornea, within which there is a 3D network of keratocytes. So, the reconstruction of corneal stroma in vitro should consider that the cultivating stromal cells can migrate through biomaterials of scaffolds and form 3D structures. However, in a static culture, the limited diffusion of nutrients and metabolic products constrain cells from growing into the constructs. Retardation of growth or even stagnation occurred among the remaining cell population of low cell density in the interior of the matrix.29–31 The SMG condition allows the cells to extend their cellular bodies more extensively and form rich network structures of cells after 6 days of culture (Figs. 5A). The cells could grow over the pores of scaffolds (Fig. 6C). There were much more cells on the scaffolds and even extracellular matrix (ECM)-like membrane structures could be observed in the microgravity group after 18 days of culture (Fig. 6D). Keratocyte-like CSCs also showed the complex network of dendritic morphology of the cell body and processes (Fig. 6E). Many cells adhered to the surface of scaffolds tightly (Fig. 6F). Cell growth as multilayer or aggregations were seen on the rugged surface or in the pores of scaffolds (Fig. 6G). The multilayer of cells could gradually grow and enlarge (Fig. 6H).
to proliferate with low shear stress and low turbulence environment. At the same time, its dynamic flow improves nutrient supply and increases metabolic waste removal for the cells in the biomaterials, and, thus, promotes cell growth in the interior of the scaffolds and becomes favorable for 3D engineering biomimetic constructs. In the middle of 1990s, microgravity tissue engineering was introduced in regenerative medicine. From then on, tissue engineering based on simulated microgravity has been developed, and more than 50 different types of cells had been cultured successfully by 2006.

Normal, quiescent corneal keratocytes are distributed within the cornea as a lattice network, interconnected by broad, cellular processes extending from a flattened cell body. Upon wounding of cornea, keratocytes can dedifferentiate into "repair" phenotypes, such as fibroblasts or myofibroblasts. Keratocytes cultured in serum-free medium usually appear as a dendritic morphology, which then become fibroblastic in appearance when exposed to medium containing serum. So, keratocytes maintain a more native phenotype and appearance when cultured in serum-free media. However, a culturing condition without serum is unfavorable for cell proliferation and subculturing. Therefore,
how to maintain keratocyte properties, but also enhance the growth of cells is one of the major concerns in tissue engineering of corneal stroma. Other investigators have reported that human keratocytes inoculated in the stromal matrix of human AM kept their native morphology of dendritic shape and secreted keratocan.

In our previous first study, we found that SMG facilitated the proliferation of rabbit CSCs and promoted cellular growth into the matrix of dehydrated bovine acellular corneal stroma. Later, in our next experiment, we confirmed that rabbit keratocyte-like CSCs revealed dendritic morphology and reticular cellular connections when cultured on bovine acellular corneal stroma supplemented with VPA and VC, even in the presence of 10% FBS. When cultured in SMG supplemented with VPA, VC, and 10% FBS, CSCs on the acellular scaffolds displayed aggregate growth. Keratocan and lumican were expressed in cells by RTPCR and immunostaining analysis. We demonstrated that the combination of SMG, VPA, VC, and acellular cornea provides a condition for CSCs to grow well, and CSCs can be manipulated to be aggregates or show physiological morphologic growth in vitro, which are important for the research of corneal stem cells and corneal tissue engineering. In this research, we studied further the growth of CSCs in porous collagen–chitosan–sodium hyaluronate complex scaffolds under SMG. This research displayed many significant changes when CSCs were placed in collagen compounds under simulated microgravity environment. First, SMG rotational seeding could produce a fast and efficient CSC attachment to scaffolds. Second, microgravity culture at dynamic environment, but low shear stress seemed to help keratocyte-like CSCs maintain morphologic characteristics, such as dendritic shape with rich cellular network structure and thin cell processes, which are similar to the adult human corneal keratocyte. Third, under simulated microgravity, CSCs formed spheroid aggregation easily. It is well known that the sphere-forming assay in static culture can be performed to produce precursors from corneal stroma. We also knew that microgravity culture promoted the growth of epidermal stem cells and periodontal ligament stem cells. Epidermal stem cells cultured in SMG inclined to aggregate on the microscaffolds and form multilayer 3D epidermis-like structures. Microgravity conditions were helpful for stem cells to keep proliferation at an undifferentiated state. Therefore, we may be able to expect that sphere-forming from SMG culture may be used to study the biology of corneal stromal stem cells.

We also discovered that there were some intriguing changes in CSCs after 18 days of SMG culture. The CSCs proliferated extensively and showed rich cell interconnections throughout the observation period. We observed that cell aggregates could adhere to the long thread-like structures after 2 to 3 weeks of microgravity culture. We do not know whether these thread-like structures are ECM or elongated CSCs. However, the phenomenon demonstrates that CSCs are able to survive actively for a rather long period in the SGM condition. Normally, CSCs have the appearance of highly active cells with abundant organelles, which show they are involved in turnover of the ECM. Moreover, keratocyte-like CSCs could form multilayer sheets. Some cell sheets could grow in suspension of medium under SMG and increase in size. They could be grasped with tweezers and showed some mechanical intensity. So, we also can expect that keratocyte-like CSC sheets from SMG culture may be used to construct corneal stromal tissue engineering. Furthermore, we observed that CSCs grew not only on the surface, but also into the interior of scaffolds. There were obvious degradation phenomena in scaffolds in the SMG condition. Our previous study had shown that the scaffolds of 20% collagen, 10% chitosan, and 0.5% sodium hyaluronate had good biocompatibility with corneal tissue after implantation. These scaffolds were biodegradable inside cornea. We speculated that the scaffold degradation in vivo or in vitro might be caused mainly by corneal stromal cells.

To sum up, bioreactors can provide the technologic means to reveal fundamental mechanisms of cell function in a 3D environment, and the potential to improve the quality of engineered tissues. The condition of SMG and scaffolds promote the proliferation of CSCs and establishment of 3D tissue engineering of the substitute for corneal stroma with cell aggregation or sheets with keratocyte-like CSC characteristics. There still are many works to explore on simulated microgravity rotating cell culture system-based corneal stroma tissue engineering in the future. How gravity vector influences cell morphology, phenotype, and function still is unclear. Future attempts to reconstruct stromal tissue should provide the equivalence with properties resembling those of native cornea, which include providing the structures of orthogonal lamellae of aligned collagen fibrils, maintaining the specific keratocyte markers (such as ALDH-1, keratocan, lumican, and so forth) and possessing the functions of mechanical protection, transmission of light, and refraction of light. Thus, such corneal stromal tissue engineering is a viable alternative for corneal transplantation and clinical applications. For the consideration of clinical applications, human corneal cells may be more conducive. So, in our next experiments, we will confirm the results in this study further by using human CSCs.

**CONCLUSIONS**

The condition of SMG and porous collagen–chitosan–sodium hyaluronate scaffolds facilitate the proliferation of CSCs. Cells showed robust growing characteristics and morphologic properties of keratocyte. The techniques for microgravity culture of keratocyte-like CSCs on collagen–chitosan–sodium hyaluronate scaffolds can yield cell aggregates or cell sheets, and establish 3D tissue engineering of corneal stroma.

---

**FIGURE 7.** The cell proliferations of the simulated microgravity group and conventional cultures group were examined by the CCK-8 assay following 1 to 18 days of culture. The absorbance values of the CCK-8 assay were analyzed through a statistical approach. The value for the simulated microgravity group was significantly higher than \((P < 0.01)\) that of the conventional cultures group. All data presented were mean \(\pm SD, n = 3\).
Proliferation of Rabbit Corneal Stromal Cells

Acknowledgments

The authors thank Jintang Xu for his helpful insights in this study of cornea; Zuwen Guo, Fuxing Tang, and Shuiliang Cao for their examination of scanning electron microscopy; Jing Wu and Yong Ding for their help in the experiment; and John Yeuk-Hon Chan for his help in the revision of the manuscripts.

Supported by the National Natural Science Fund of China (30973244) and a collaborated grant for HK-Macao-TW of Ministry of Science and Technology (2012DFH30060). The authors alone are responsible for the content and writing of the paper.

Disclosure: X. Li, None; Y. Yang, None; Q. Li, None; Y. Dai, None; C. Wang, None; J. Chen, None

References


