THREE DIMENSIONAL OPTIC TISSUE CULTURE AND PROCESS


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A process for artificially producing three-dimensional optic tissue has been developed. The optic cells are cultured in a bioreactor at low shear conditions. The tissue forms as normal, functional tissue grows with tissue organization and extracellular matrix formation.

22 Claims, 3 Drawing Sheets
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RELATED PATENTS AND APPLICATIONS


ORIGIN OF THE INVENTION

The jointly made invention described herein was made by employees of the United States Government and may be manufactured and used by or for the Government of the United States of America for governmental purposes without the payment of any royalties thereon or therefor.

The invention described herein was also made by inventors in the performance of work under a NASA contract with Krug Life Sciences and a memorandum of understanding in 1958, Public Law 85-568 (72 Stat. 435; 42 U.S.C. 2457).

Within corneal tissue, the extracellular matrix serves as scaffolding to provide mechanical strength and structural organization. After being synthesized and secreted from corneal cells, the matrix forms distinct three-dimensional, lattice-like arrangements in the extracellular space of corneal tissue [Komai, Y. and Ushiki, T., Invest. Ophthalmol. Vis. Sci., 32, 2244 (1991)]. Considering the stroma, fibrils of extracellular matrix are interwoven to form dense felt-like sheets in Bowman’s layer. At the interface between the stroma and Descemé’s membrane, the matrix becomes a loose fibrillar network oriented in various directions and interfaced. In between these two regions, the stroma is composed of successively stacked layers of flat lamella bundles of matrix fibrils.

There are primarily two classes of macromolecules present in the extracellular matrix: glycosaminoglycans (GAG’S) including chondroitin sulfate and dermatan sulfate, and fibrous proteins such as fibronecin and collagen. The role of the latter group is mainly structural and adhesive. For example, fibronecin binds to cells and to other matrix macromolecules, promoting cell attachment to and subsequent migration along the matrix [Alberts B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. D., Molecular Biology of the Cell (Garland Publishing, New York, 1989)]. In the cornea, GAG’s regulate spacing between fibrils and the three-dimensional organization of stroma lamellae [Hahn, R. A. and Birk, D. E., Development, 115, 383 (1992)]. In addition, they regulate the kinetics of fibril formation [Birk, D. E. and Lande, M. A., Biochim. Biophys. Acta, 670, 362 (1981)].


Corneal transplants are the most frequently performed human transplant procedure. Since 1961, there have been more than 421,300 corneal transplantations performed in the U.S. In 1991 alone, there were 41,300 such transplantation, more than all other organ transplantations performed in that year [Eye Bank Association of America, Annual Report...
Intra-Ocular Implant with donor tissue. The use of donor tissue results in several complications, including donor shortage. There are in excess of 5,000 patients on waiting lists for donor tissue throughout the U.S. [Eye Bank Association of America, Annual Report (Washington, D.C., 1992)]. These people wait between two weeks and two years to obtain suitable tissue. And when this tissue becomes available, there is still the possibility of transplant rejection and disease transfer of HIV [Saha, S. Z., Palese, A. G., Heck, E., Ablashie, D., Luckenbach, M., McCulley, J. P., and Nussenblatt, R. B., Am. J. Ophthalmol., 104, 149 (1986)], hepatitis B [Raber, I. M. and Friedman, H. M., Am. J. Ophthalmol., 104, 255 (1987)], herpes [Leibowitz, H. M., Corneal Disorders: Clinical Diagnosis and Management (W. B. Saunders, Philadelphia, 1993)], and other ailments from donor to patient. In addition to these complications, many patients with corneal disease or injury are not amenable to transplantation. This can occur, for example, when there is a chemical burn resulting in severe scarring and vascularization [Brightbill, F. S., Corneal Surgery: Theory, Technique, and Tissue (Mosby-Year Book, St. Louis, 1993)].

To overcome these difficulties, alternatives to donor tissue have been developed. One such alternative is a prosthetic implant made of an optical cylinder and supporting flange [Polack, F. M. and Heilmke, G., Ophthalmology, 87, 693 (1980); Trinkaus-Randall, V., Banwatt, R., Capecci, J., Leibowitz, H. M., and Franzblau, C., Invest. Ophthalmol. Vis. Sci., 32, 3245 (1991)]. For post-operative stability, implant materials must be biocompatible and promote cell adhesion. When these two specifications are met, good vision can be retained for 7 to 8 years after implantation. Permanent stability, however, has yet to be obtained. In refractive keratoplasty procedures, a synthetic intracorneal lens can be implanted to change the refractive power of the cornea [McCary, B. E., Refract. Corneal Surg., 6, 40 (1990); Inset, M. S., Busbors, G., and Caldwell, D. R., Am. Intra-Ocular Implant Soc. J., 11, 159 (1985)]. As before, biocompatibility and cell adhesion are required for implantation to be successful, but another requirement is permeability so that nutrients can flow across the lens to the anterior portion of the cornea. Synthetic lenses have been stable in animal models for almost a decade. Clinical trials are in an early stage.

The invention described in this patent application is another alternative to the use of donor tissue for transplantation. It can be used to prepare corneal tissue from in vitro cultures of the patient’s own corneal cells or from a well-defined primary culture derived from another human source. With in vitro produced tissue, shortage of tissue and disease transfer to the patient would be minimized. Also, post-operative stability should be greatly enhanced with artificially generated tissue over that currently obtained with a prosthesis or synthetic intracorneal lens.

Reported corneal tissue regeneration has been limited to date to two-dimensional models. In particular, regenerated corneal endothelial cells have been successfully transplanted into animal models [Insler, M. S., and Lopez, J. G., Curr. Eye Res., 5, 967 (1986)]. Since the endothelium consists of a single layer of cells in the cornea, a two-dimensional culture can be used for transplantation. Since both the corneal epithelium and stroma are composed of multiple layers of cells, three-dimensional tissue is required to replace these cell structures when they become damaged.

To study cell structure and function in normal and abnormal cornea, three types of tissue models are currently employed: intact tissue in living animals [Trinkaus-Randall, V., Banwatt, R., Capecci, J., and Franzblau, C., Invest. Ophthalmol. Vis. Sci., 32, 3245 (1991)], donor tissue [Komai, Y. and Ushiki, T., Invest. Ophthalmol. Vis. Sci., 32, 2244 (1991)], and tissue from in vitro culture [Goralski, D. H. and Hadley, A., Curr. Eye Res., 11, 61 (1992)]. The demand for these models is increasing yearly. The number of donor eyes that were used for research and education grew from 34,147 in 1989 to 40,239 in 1991 [Eye Bank Association of America, Annual Report (Washington, D.C., 1992)]. In vitro cell culture has certain advantages over the other two models: it uses avoids unnecessary loss of sight in lab animals and avoids variation in tissue characteristic of corneas from different donors. To date, the majority of cell-culture models have been two-dimensional. These models are limited in their applications, however, in that they preclude an accurate representation of three-dimensional phenomena within the cornea such as wound healing and mass transport of nutrients from the endothelium to the stroma and to the epithelium. For these phenomena, a three-dimensional model is required. Furthermore, the synergistic interaction between different cell types in a three-dimensional model could more accurately reflect the cell function in vivo than a two-dimensional model.

The use of conventional stirred or sparged bioreactors have not been generally successful for culture of three dimensional, functional tissue. Some tissues such as the Chinese hamster ovary cells grow robustly in conventional stirred bioreactors [O’Connor, K. C. and Papoutsakis, E. T., Biotechnol. Tech., 6, 323 (1992)]. In contrast, SP9 fall armyworm ovary cells will not grow at all in these reactors under the same operating conditions; in fact, they die unless supplemented with a liquid surfactant [Murhammer, D. W. and Guochie, C. F., Biotechnol. Prog., 6, 391 (1990)]. Only limited work has been done to date to develop three-dimensional corneal models. Bioreactors have not been used for optic tissue growth such as cornea tissue. For example, a model of multiple layers of rabbit corneal epithelial cells grown on a support of contracted collagen lattices was employed to investigate wound healing [Ouyang, P. and Sugrue, S. P., “Identification of an Epithelial Protein Related to the Desmosome and Intermediate Filament Network,” J. Cell Biol., Vol. 118, pages 1477–1488, 1992] rather than bioreactor produced cells.
Three-dimensional corneal tissue has been produced from an in vitro culture of primary rabbit corneal cells to illustrate the production of optic cells from aggregates and tissue. This tissue is a three-dimensional spheroid that more closely approximates intact corneal tissue in size and in morphology than has yet been achieved in vitro. To generate the tissue, corneal cells were cultured in a bioreactor called the High Aspect Ratio Vessel (HARV) described in the published patent application by the National Aeronautics and Space Administration in the Scientific and Technical Aerospace Reports Volume 29/Number 9, May 8, 1991, ACC NOS. N91-17531, U.S. Ser. No. 625,345, now U.S. Pat. No. 5,153,131, entitled “A Culture Vessel With Large Perfusion Area to Volume Ratio,” invented by David A. Wolf, Clarence F. Sams and Ray P. Schwarz and filed on Dec. 11, 1990 and issued October 6, 1992, previously incorporated by reference.

The corneal tissue described herein is unique and the HARV produced tissue has distinctive features. Corneal tissue is one of the most dependent on the formation of three-dimensional extracellular matrices. As discussed herein, these matrices are responsible for the intricate order of cell layers in the cornea and, in turn, are responsible for...
its transparency and focusing power. This invention demonstrates that these matrices form and provide an internal structure to corneal tissue. This invention demonstrates that the HARV supports robust grown of corneal cultures.

The corneal tissue produced in the HARV was initiated from a mixed population of primary corneal cells (BioWhittaker, Walkersville, Md) containing endothelial cells, epithelial cells and keratocytes. These cells are attachment dependent and, as such, were grown bound to a support, Cytodex-3 microcarrier beads (Pharmacia, Piscataway, N.J.). To prepare the beads for culture, they were first hydrated in Ca²⁺, Mg²⁺-free phosphate buffer saline (PBS) for 3 hours with occasional gentle agitation at room temperature and a concentration of 50–100 ml/g bead. The beads were subsequently washed with fresh (30–50 ml/g bead) and sterilized at 115°C and 15 psi for 15 minutes in PBS at the same concentration. Before initiating a microcarrier culture, the PBS was washed from the sterile beads by rinsing in ward culture medium.

Alternative attachment means may be used such as dissolvable microcarriers. Also other cells introduced into the culture may be an attachment means so that the cells use each other as support.

Corneal cultures were grown in two types of vessels: the HARV and a Bellco spinner flask (Vineland, N.J.). The latter vessel served as a control with which to compare the HARV. The spinner flask is the conventional vessel of choice for the preparation of the microcarrier cell cultures. In both vessels, cultures were inoculated with 1x10⁵ viable cells/ml and a bead density of 5 mg/ml in 50 ml GTSF-2 medium (NASA, 30S). Prior to inoculation, spinner flasks were siliconized with Sigmacote (Sigma, St. Louis, Mo.) to prevent cell adhesion to the flask walls. Corneal cells were maintained in suspension by end-over-end rotation of the vessel at 11–25 rpm. The vessel was completely filled with medium, eliminating cell damage from hydrodynamic forces associated with air/liquid interfaces and boundary layer at the vessel wall. The HARV has three ports for medium replacement and sampling. Replacing medium in HARV required stopping vessel rotation, allowing the cells to settle, and removing 20% of the spent medium through the top port. Fresh medium was then injected under sterile conditions with a syringe. Within 24 hours of inoculation, cells attached to the microcarrier beads, but there was minimal aggregation of beads. As a result, no tissue formed in the shaker flask.

For HARV cultivation, cells were suspended in culture medium by end-over-end rotation of the vessel at 11–25 rpm. The vessel was completely filled with medium, eliminating cell damage from hydrodynamic forces associated with air/liquid interfaces and boundary layer at the vessel wall. The HARV has three ports for medium replacement and sampling. Replacing medium in HARV required stopping vessel rotation, allowing the cells to settle, and removing 20% of the spent medium through the top port. Fresh medium was then injected under sterile conditions with a syringe. Within 24 hours of inoculation, cells attached to the microcarrier beads. After an additional week, cell/bead aggregates developed as cells bridged between beads as shown in FIG. 3. These aggregates grew in size to form tissue in the HARV over 2.5 months.

The concentration of cells present in either vessel was measured by counting released nuclei. Specifically, aggregated corneal tissue was exposed to 25 mg/ml collagenase type IA (Sigma) in Ca²⁺, Mg²⁺-free PBS containing 0.02% (v/v) ethylenediaminetetraacetic acid at 37°C to release cell nuclei. Next, the nuclei were stained with 0.1% (v/v) crystal violet in 0.1 M citric acid (Sigma) and counted with a hemocytometer.

The tissue produced in the HARV from corneal cells is a three-dimensional spheroid greater than 5 mm in diameter containing multiple layers of cells as shown in FIG. 1. The maximum cell density that was achieved in the HARV was in excess of 1x10⁷ cells/ml. In contrast, the corneal cells grew for only a period of a week in the spinner flask, reaching a maximum cell density of only 4.5x10⁵ cells/ml. As mentioned above, aggregation in the spinner flask, reaching a maximum cell density of only 4.5x10⁵ cells/ml. As mentioned above, aggregation in the spinner flask was not substantial; on average, aggregates were 1 mm in diameter or less. Spinner flasks maintain cells in suspension through impeller mixing. This design generates turbulent eddies in the grown medium which cause cell-cell collisions that disrupt aggregates, inhibiting three-dimensional cell growth [Cherry, R. S. and Papoutsakis, E. T. Bioprocess Eng., 1, 29 (1986)].

Immunocytochemistry was used to characterize the composition and internal organization of artificially produced corneal tissue. For this analysis, tissue samples, each 1.0 ml in volume, were removed from the HARV after 50 days of cultivation. They were washed with PBS and then fixed in 1.0 ml of OmniFix II (An-Con Genetics, Melville, N.Y.), an alcohol-based fixative not containing aldehydes or mercury. Samples were paraffin embedded, blocked, sectioned and mounted on slides using standard methodology. After the mounted sections were deparaffinized and rehydrated, they were exposed to a primary mouse antibody directed against specific proteins expressed in the tissue as shown below in Table 1. This was followed by exposure to a secondary antibody, peroxidase-labeled, anti-mouse antibody (Boehringer Mannheim, Indianapolis, Ind.), which binds to the primary antibody. To visualize antibody binding, sections were incubated with diaminobenzidine (Sigma) solubilized in a solution of Tris buffer and H₂O₂. Diaminobenzidine reacts with peroxidase to form a brown color.

### Table 1

<table>
<thead>
<tr>
<th>Antibody Specificity</th>
<th>Dilution</th>
<th>Source, Location &amp; Order No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimentin</td>
<td>1:20</td>
<td>Boehringer Mannheim, Indianopolis, IN 1112457</td>
</tr>
<tr>
<td>Chondroitin-4-Sulfate</td>
<td>1:75</td>
<td>Chemicon, Temecula, CA MAB2030</td>
</tr>
<tr>
<td>Chondroitin-6-Sulfate</td>
<td>1:50</td>
<td>Chemicon, MAB2035</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>1:40</td>
<td>Boehringer Mannheim, 1087720</td>
</tr>
</tbody>
</table>

Analysis by immunocytochemistry revealed substantial extracellular matrix expression in the corneal tissue. Of the two classes of macromolecules that constitute the matrix, glycosaminoglycans' (GAG's) and fibrous proteins, both were present in the tissue.

The brown color in FIGS. 3 and 4 relative to the negative control in FIG. 1 shows expression of the GAG's chondroitin-4- and -6-sulfate. These two macromolecules are sulfated disaccharides that differ from each other in the position of their sulfate residue. Chondroitin-4-sulfate is sulfated at the C4 position of N-acetyl-D galactosamine, while chondroitin-6-sulfate is sulfated at the C6 position. In FIG. 3, stained for chondroitin-4-sulfate, the microcarrier beads are shown at 10, 12 and 14. The tissue between the beads, for example at 16 and 18, shows the strong staining for matrix component chondroitin-4-sulfate. In FIG. 4,
stained for chondroitin-6-sulfate, the tissue shows layers of cells with organized dimensional segregation. The layers of cells appear as walls of cells with staining.

As for fibrous proteins, fibronectin staining is depicted in Fig. 5. The darker stain as illustrated at reference numerals 20 and 22 is at the bead surface 24. This concentration of fibronectin at the cell bead interface is evidence of matrix organization and promotes cell adhesion to the bead surface.

These figures also reveal three-dimensional organization and segregation of these matrix macromolecules within the artificially produced tissue. In particular, Fig. 4 demonstrates that chondroitin-6-sulfate organized into parallel layers that were separated from each other by 10 μm on average. While Fig. 5 indicates that fibronectin was moderately expressed through the corneal tissue, it also shows that this protein concentrated at the cell/bead interface to promote cell adhesion to the bead surface.

Matrix formation permitted cell types within the regenerated tissue to organize and segregate. The cells migrated along the matrix to form distinct clusters of individual cell types. This is evident in Fig. 2. Recall that tissue was prepared from a mixed population of endothelial cells, epithelial cells and keratocytes. Fig. 2 shows strong positive staining for vimentin. This protein is an intermediate filament present in keratocytes and endothelial cells. The dense foci of brown color as shown at reference numerals 2, 4, 6, 8 and elsewhere in the figure depict distinct clusters that these cell types form in vivo. This shows that the cells have organized within the tissue.

The invention of three-dimensional corneal tissue, produced in vitro cell culture and described herein, is closer in size and in morphology to intact corneal tissue than earlier attempts at in vitro production. This novel three-dimensional tissue can be used to model cell structure and function in normal and abnormal cornea. Also, it can eventually be used to develop tissue for patients requiring corneal transplantations. This process may also be used for non-mammalian optic and cornea tissue production for study and use with other animal forms.

The description of the process and embodiment is illustrative of the invention and is not intended to place any limitation on the claims of invention. Those skilled in the art will recognize other modes of practicing the invention described herein.

What we claim is:

1. A process for producing aggregates of optic cells comprising the steps of:
   (a) selecting the optic cells for culture;
   (b) introducing the cells and a cell attachment means and a culture media into a cylindrical culture vessel that rotates about its central horizontal axis; and
   (c) culturing the cells in the vessel during horizontal rotation modulated to create low shear conditions whereby extra-cellular, optic tissue matrices are formed and three dimensional cell growth is achieved.

2. A process for producing aggregates of optic cells of claim 1 wherein the culturing process provides for functional interrelationship by cell to cell contact producing the formation of three-dimensional extracellular matrices.

3. A process for producing aggregates of optic cells of claim 1 wherein the cell aggregates are organized with dimensional segregation.

4. A process for producing aggregates of optic cells of claim 1 wherein the vessel is rotated to create low shear at a low speed so that the circular motion of the culture media minimizes centrifugal forces sufficient to move cells outwardly from the rotational axis yet suspends the cells throughout the vessel during culturing.

5. A process for producing aggregates of optic cells of claim 1 wherein the cells are cornea cells.

6. A process for producing aggregates of optic cells of claim 1 wherein the cells are mammalian cells.

7. A process for producing aggregates of optic cells of claim 1 wherein the cells in step (a) are a mixed population of primary corneal cells containing endothelial cells, epithelial cells and keratocytes.

8. A process for producing aggregates of optic cells of claim 1 wherein the cell attachment means in step (d) is selected from the group consisting of cells, microcarriers, and dissolvable microcarriers.

9. A process for producing aggregates of optic cells of claim 1 wherein the three dimensional tissue growth is defined as positive by immunocytochemical staining for chondroitin sulfate.

10. A process for producing aggregates of optic cells of claim 1 wherein the three dimensional tissue growth is defined as positive by immunocytochemical staining for fibronectin.

11. A process for producing aggregates of optic cells of claim 1 wherein the three dimensional tissue growth is defined as positive by immunocytochemical staining for vimentin.

12. An artificially produced optic tissue made by the steps of:
   (a) selecting optic cells for culture;
   (b) introducing the cells and a cell attachment means and a culture media into a cylindrical culture vessel that rotates about its central horizontal axis; and
   (c) culturing the cells in the vessel during horizontal rotation modulated to create low shear conditions whereby extra cellular matrices are formed and three dimensional tissue growth is achieved.

13. The artificially produced optic tissue of claim 12 with three dimensional extracellular matrices.

14. The artificially produced optic tissue of claim 12 with cells organized with dimensional segregation.

15. The artificially produced optic tissue of claim 12 wherein step (c) the vessel is rotated to create low shear at a low speed so that the circular motion of the culture media minimizes centrifugal forces sufficient to move cells outwardly from the rotational axis yet suspends the cells throughout the vessel during culturing.

16. The artificially produced optic tissue of claim 12 wherein the cells are cornea cells.

17. The artificially produced optic tissue of claim 12 wherein the cells are mammalian cells.

18. The artificially produced optic tissue of claim 12 wherein the cells in step (a) are a mixed population of primary corneal cells containing endothelial cells, epithelial cells and keratocytes.

19. The artificially produced optic tissue of claim 12 wherein the cell attachment means in step (d) is selected from the group consisting of cells, microcarriers, and dissolvable microcarriers.

20. The artificially produced optic tissue of claim 12 wherein the tissue produced is defined as positive by immunocytochemical staining for chondroitin sulfate.

21. The artificially produced optic tissue of claim 12 wherein the tissue produced is defined as positive by immunocytochemical staining for vimentin.

22. The artificially produced optic tissue of claim 12 wherein the tissue produced is defined as positive by immunocytochemical staining for fibronectin.