CULTURED CORNEAL EPITHELIA FOR OCULAR SURFACE DISEASE*

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ABSTRACT

Purpose: To evaluate the potential efficacy for autologous and allogeneic expanded corneal epithelial cell transplants derived from harvested limbal corneal epithelial stem cells cultured in vitro for the management of ocular surface disease.

Methods: Human Subjects. Of the 19 human subjects included, 18 (20 procedures) underwent in vitro cultured corneal epithelial cell transplants using various carriers for the epithelial cells to determine the most efficacious approach. Sixteen patients (18 procedures on 17 eyes) received autologous transplants, and 2 patients (1 procedure each) received allogeneic sibling grafts. The presumed corneal epithelial stem cells from 1 patient did not grow in vitro.

The carriers for the expanded corneal epithelial cells included corneal stroma, type 1 collagen (Vitrogen), soft contact lenses, collagen shields, and amniotic membrane for the autologous grafts and only amniotic membrane for the allogeneic sibling grafts. Histologic confirmation was reviewed on selected donor grafts.

Amniotic membrane as carrier. Further studies were made to determine whether amniotic membrane might be the best carrier for the expanding corneal epithelial cells. Seventeen different combinations of tryspinization, sonication, scraping, and washing were studied to find the simplest, most effective method for removing the amniotic epithelium while still preserving the histologic appearance of the basement membrane of the amnion. Presumed corneal epithelial stem cells were harvested and expanded in vitro and applied to the amniotic membrane to create a composite graft. Thus, the composite graft consisted of the amniotic membrane from which the original epithelium had been removed without significant histologic damage to the basement membrane, and the

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Supported by an unrestricted grant from Research to Prevent Blindness, New York.

expanded corneal epithelial stem cells, which had been applied to and had successfully adhered to the denuded amniotic membrane.

Animal model. Twelve rabbits had the ocular surface of 1 eye damaged in a standard manner with direct removal of the presumed limbal stem cells, corneal epithelium, and related epithelium, followed by the application of n-heptanol for 60 seconds. After 6 weeks, all damaged eyes were epithelialized and vascularized. Two such treated eyes were harvested without further treatment, to be used for histologic study as damaged controls. The remaining 10 rabbits received composite grafts (consisting of amniotic membrane with expanded allogeneic rabbit corneal epithelial cell transplants) applied to the ocular surface in a standard manner followed by the application of a contact lens. At 16 days following transplantation, 5 of the rabbits were sacrificed and the corneal rims were removed for histologic study. At 28 days, the remaining rabbits were sacrificed and the previously damaged eyes were harvested for histologic and immunohistochemical study.

Results: Human subjects. Of the 19 total patients admitted to the study, the presumed corneal epithelial stem cells of 1 patient did not grow in vitro. Of the remaining 18 patients (20 procedures, 19 eyes), 3 patients had unsuccessful results (3 autologous procedures), 1 patient had a partially successful procedure (allogeneic procedure), and 1 patient had a procedure with an undetermined result at present (allogeneic procedure).

One unsuccessful patient had entropion/trichiasis and mechanically removed the graft and eventually went into phthisis. The other 2 unsuccessful patients suffered presumed loss of autologous donor epithelium and recurrence of the ocular surface disease (pterygium). The partially successful patient receiving an allogeneic transplant had infectious keratitis delay of his re-epithelialization; he has only minimal visual improvement but has re-epithelialized. The patient receiving the second allogeneic graft lost his donor epithelium at day 4. Additional donor epithelium was reapplied, but the result is undetermined at present.

Amniotic membrane as carrier. The in vitro preparation of the amniotic membrane with corneal epithelial stem cell graft overlay was successful. Histology documented removal of the amniotic epithelium and reapplication of corneal epithelial cells.

Animal model. The 2 rabbits that had no reparative surgery following standard ocular surface injury had histology and immunopathology consistent with incomplete corneal epithelial stem cell failure with vascularization and scarring of the ocular surface. Light microscopy and immunohistologic staining with AE5 confirmed the conjunctival phenotype of the ocular surface repair but also documented the incomplete model. The

allogeneic stem cell transplants had varying results. One rabbit had a suppurative infection and lost the graft. Reparative surgery failed in 2 of the rabbits, failed partially in 3 of the rabbits, was partially successful in 3 others, and was successful in 1 rabbit at 28 days. Histologic and immunopathologic study documented successful growth of corneal epithelium onto the recipient surface.

Conclusions:

1. Presumed corneal epithelial stem cells can be harvested safely from the limbus and expanded successfully in vitro.
2. Expanded corneal epithelial cell cultures can be grown onto various carriers, but currently denuded amniotic membrane seems to be the best carrier for ocular surface repair.
3. Expanded corneal epithelial cell transplants appear to resurface damaged ocular surfaces successfully, but cellular tracking and further confirmation are required.
4. Expanded allogeneic corneal epithelial cell transplants are technically possible and may represent alternative treatment modalities for selected ocular surface problems.
5. These techniques potentially offer a new method of restoring a normal ocular surface while minimizing the threat of damage or depletion to the contralateral or sibling limbal corneal epithelial stem cells.
6. The rabbit model was probably incomplete and should be interpreted with caution. The complete eradication of all corneal epithelial stem cells from any eye is difficult, making confirmation of such work challenging.
7. The results of the rabbit model suggest that allogeneic grafts may restore a nearly normal ocular epithelial surface to certain ocular surface injuries.

INTRODUCTION

The normal ocular surface is covered with highly specialized epithelia including a stratified squamous corneal epithelium and a stratified squamous, cubodial, or pseudocolumnar (depending on location) conjunctival epithelium containing goblet cells, lymphocytes, melanocytes, and Langerhans cells among others.[1] The corneal epithelium forms a tightly adherent, highly uniform refracting surface and functional barrier between the tear film and the corneal basement membrane as well as the corneal stroma. The conjunctival epithelium is less compact and has a specialized stratified squamous or cuboidal epithelium as a barrier to a substantia propria overlying the sclera. The limbus is a circumferential transition zone spanning 1.5 mm in width surrounding the cornea beginning

anteriorly at a point coinciding with the termination of Bowman's layer.[1] Both conjunctival and corneal epithelia have desmosomal attachments between adjacent cells and hemidesmosomes between the basal layer of epithelia and the basement membrane. These two phenotypically different epithelial cell types are believed to arise from stem cells in at least two different locations.[2-4] The corneal stem cells are thought to reside at the limbus, especially the superior limbus. The conjunctival stem cells are believed to be in the conjuctival cul-de-sac and perhaps elsewhere.[5]

The corneal epithelium is optically regular and is maintained by centripetal migration of stem-cell-derived epithelial cells onto Bowman's layer. The corneal epithelium is 5 to 6 layers thick and has well-defined basal columnar cells, wing cells, and superficial squamous cells.[6,7] The conjunctival epithelium varies in thickness from 3 to 10 cell layers and lacks the orderly progression from basal columnar epithelium to superficial wing cells. The conjunctiva is richly vascularized with loosely organized cell layers and is an important source of tear mucins, with goblet cells making up 5% to 10% of the total number of conjunctival cells. Throughout the conjunctiva, there are numerous resident specialized inflammatory and immunologic cells. The limbus, which represents specialized conjunctiva, consists of several layers of epithelial cells devoid of goblet cells and populated by Langerhans cells, melanocytes, and presumed corneal epithelial stem cells. Loss or damage of the corneal epithelial stem cell population at the limbus leads to re-epithelialization of the cornea by bulbar conjunctival cells.[7,8] The presumed stem cell population of the conjunctiva and the cornea are different and produce phenotypically different cells.[2,9] If the presumed corneal epithelial stem cells at the limbus are damaged, corneal repair by conjunctival epithelium results in neovascularization, chronic inflammation, recurrent epithelial defects, migration of goblet cells onto the corneal surface, and stromal scaring leading to poor vision and chronic irritation.[10]

Corneal and conjunctival epithelial cell injury, degenerations, and abnormalities are relatively common problems and may become a threat to vision. Persistent epithelial defects or abnormalities can be caused by infectious, chemical, iatrogenic, physical, and congenital insults, among others. Ocular surface diseases such as Stevens-Johnson's syndrome, chemical and thermal burns, recurrent pterygia, ocular tumors, immunologic conditions, radiation injury, inherited and congenital syndromes, aniridia, and ocular pemphigoid can severely compromise the ocular surface and cause catastrophic visual loss in otherwise potentially healthy eyes.[11,12] Treatment is expensive, frustrating, time-consuming, and often unsuccessful. Conjunctival scarring, foreshortening of the fornix, entropion, corneal epithelial keratinization, mucous depletion, and scarring of the

ocular surface all contribute to this problem.

The impaired epithelial cell regeneration and mucous deficiency caused by ocular surface disease prevents or discourages successful standard cadavaric donor corneal transplantation. In such cases, the successful corneal transplant often will have a clear corneal epithelium as long as the donor epithelium persists, but gradually the donor epithelium will be replaced by the recipient cells, which will resemble conjunctiva and include goblet cells and neovascularization, and result in corneal haze and vision failure. Conventional corneal transplantation simply is not successful in such situations because of the high risk of rejection and extremely poor prognosis.[13-16] Presumably, this occurs because the original corneal epithelial stem cells have been too severely damaged to produce the phenotypically correct corneal epithelial cell. This damage allows for, or may even stimulate, conjunctival cell growth and the accompanying vascularization to resurface the new donor cornea, resulting in corneal graft failure. There have been many different attempts to treat these ocular surface defects, including attempts to treat corneal surface failure, but most have met with discouragement and failure.

Recently much progress has been made in understanding corneal epithelial stem cell growth and the process of ocular resurfacing. This improved understanding has led to different approaches to the repair of the ocular surface, including conjunctival transplants, limbal autografts, and allogeneic limbal stem cell grafts.

Use of in vitro cultured corneal epithelial stem cell transplants has led to a novel approach to ocular surface repair. Evaluation of different carriers for the epithelial stem cells indicates that amniotic membrane may be the most successful. Although these techniques show great hope for ocular resurfacing, much about the biology of such work is not yet understood. We have documented both the successful maturation of these cells on the ocular surface in an animal model, and the problems inherent in this work. The longevity of these cells following autologous and allogeneic grafts can also be inferred from the successful human autologous and allogeneic transplants, but it is difficult to document with current technology.

Although much work remains, these techniques hold promise for patients with severe ocular surface injury who heretofore have had a hopeless prognosis.

BACKGROUND

Two aspects of corneal epithelial cell biology must be reviewed in order to understand this work. The integral concepts to be reviewed include (1) the regeneration of corneal epithelium, including the role of the putative

corneal epithelial stem cell, and (2) the previous surgical attempts to repair the damage from ocular surface disease.

REGENERATION OF THE CORNEAL EPITHELIUM

Fundamental concepts of corneal epithelial wound healing are essential to understanding the maintenance of corneal physiology.

The Cellular Aspects of Corneal Homeostasis

The corneal epithelium is constantly undergoing cellular division and renewal, maturation, and death usually through desquamation. Current thought holds that the limbal corneal epithelial stem cell is responsible for this process of renewal. This process must be maintained to assure a robust corneal surface. It is believed that uncomplicated corneal epithelial wounds heal quickly without residual scarring because of the activity of the epithelial cells at the wound margins. Cells at distant sites are, however, responsible for that transformation in ways that we are only beginning to understand at the intracellular level.

Von Wyss in 1877[17] was probably the first to examine the reparative and regenerative proliferation of the epithelial cells but provided mainly histologic descriptions. Peters in 1885[18] was the first to record his observations, in frogs, of the sliding of the neighboring epithelium over a denuded area. In 1887, Neese, and eventually others, observed mitotic activity histologically in the healing of epithelial defects.[19-23] The first demonstration of the mechanical sliding of adjacent epithelial cells over a denuded area in human epithelial cells was performed by Ranvier in 1896.[24] In an extensive series of articles, Ranvier, between 1896 and 1898, also demonstrated that a clean wound affecting only the epithelium may be closed within 24 hours, but he thought that the process was entirely mechanical and was determined passively by the release of tissue-tension, which allowed neighboring cells to spill over the denuded area.[24-29] In 1903, Weinstein[20] studied the mitotic activity of epithelial healing, again at the local site of an uncomplicated wound. In a later work, in 1930, Lohlein[30] made clinical observations of epithelial sliding following an epithelial wound.

In the early part of this century, investigators understood the two basic tenets–sliding of epithelium and mitosis of epithelial cells that surround the defect–that supply the foundation for our understanding of local reparative and regenerative efforts of the epithelium. These early workers established what was believed to be a dual action of sliding and mitosis. These fundamental works were then amplified by other histologists, following World War I, when investigators were stimulated by the horrific ocular surface injuries caused by mustard gas and other gases used in combat.[22,31,32]

As a result of these investigations, corneal epithelial wound healing was believed to be a combination of two factors. Mitosis by the surrounding epithelial cells created new cells to fill the defect in the uncomplicated corneal wound, and an epithelial sliding process allowed the cells that surrounded the defect to migrate to cover it. These processes were also accompanied by a temporary pause in the natural process of exfoliation. These two steps (mitosis and sliding) accompanied by the delay in exfoliation rapidly restored the multilayered structure of the epithelium. Some of the initial investigators found that the process of renewal occurs all around the margins of the denuded area, both near the edge of the defect and adjacent to the limbus.[31,32] Later, in 1949, Buschke[33] witnessed two active cellular processes: the extension of active pseudopodial extensions onto the denuded area, and a spreading of the individual epithelial cells which is particularly notable in the basal cells. Once the area has been covered, the cells multiply and re-establish the normal multilayered arrangement.[23]

In the 1930s, Wigglesworth[34] was considering the stimulus for the epithelial sliding and discovered that, in insects at least, epithelial movement was due to chemotactic factors and, he thought, activated by some chemical product of the autolysis of the injured cells. In 1950, Buschke[33] demonstrated that the phenomenon was temperature-dependent in humans, and others[35-39] suggested that the healing process could be blocked by anesthetics and other agents.

Toward the end of the first half of this century, the understanding of corneal wound healing was evolving with some correct conceptual elements, but key portions of a unifying theory were missing.

The initial hypothesis regarding corneal wound healing centered on the centripetal movement of the cells immediately surrounding the wound or of the cells from the peripheral cornea, and it was believed that the cells moved by sliding. Numerous investigators provided tantalizing hints that the stimulus for corneal wound healing resided at the limbus.

In groundbreaking work, in 1944, Ida Mann[40] provided the first hint of current concepts when she demonstrated pigment movement from the limbus in a rabbit eye. She observed that pigment from the limbus slid toward a peripheral corneal abrasion as this area of corneal injury repaired itself. She was able to follow this pigment into the cornea from the limbus as the peripheral limbal epithelial cells proliferated and slid over the defect. She found that a macroscopic shift of the limbal pigment becomes evident within about 18 hours. She also thought that the migration was confined to an area within a 5 mm radius of the wound, but she did not speculate on the stimulus of mitosis and sliding.[40]

Subsequently, Maumenee and Scholz[41] provided direct human evidence of the current hypothesis when they noted the centripetal migration

of surface epithelial cells onto the cornea following damage of the central corneal epithelium. Other investigators also noted this centripetal migration following the injury of the central epithelium.[42,43] Increased DNA synthesis was demonstrated approximately 18 hours after an injury,[44] and it was thought that sliding was the first reparative step in wound repair. Much later, Buck[45] provided evidence for centripetal movement of the epithelium in the repair of corneal wounds in a murine model. He actually marked the epithelium and subjacent stroma with ink and noted the movement of the epithelial ink as compared to the stromal ink. Other investigators added more evidence that limbal and/or peripheral corneal cells migrate centripetally.[46-48] Hanna and associates,[46] for example, showed that the mitotic index of peripheral corneal epithelium is higher than that of central epithelium, suggesting that the force for the sliding originated at the limbus.

Cellular division was recognized as an important part of normal maintenance as well as wound healing. In 1944, Friedenwald and Buschke[36] discovered that the basal cells divide by mitosis at intervals of approximately 1 week. At about the same time, further evidence suggested that proliferation was limited to the basal cells.[37,38] By using autoradiography, Hanna and O'Brien[47] demonstrated in 1960 that the epithelial basal cells undergo mitosis and then migrate through the intermediate layers to the superficial layers with gradual modification. Once in the superficial layer, the normal epithelial cells desquamate within a few days in the normal corneal epithelium.

Eventually, Davanger and Evensen[48] raised the possibility that the limbus was the source of these migrating cells. They showed that in certain black patients, the heavily pigmented limbal cells form streaks toward the central cornea, and they observed that a centripetal movement of pigment from the region of the limbus onto the cornea of guinea pigs occurred following corneal epithelial wounding.[48] They also found an accompanying marked increase in mitotic figures within limbal basal epithelial cells with such a wound.[48] Bron[49] further extended this observation by suggesting that the vortex pattern of the corneal epithelium in toxic keratopathy, striate melanokeratosis, Fabry's disease, and the superficial iron lines of the cornea form similar streaking patterns, which could be attributed to a centripetal slide or migration of epithelium from the limbus towards the corneal apex. Other investigators have provided substantial but still circumstantial evidence that the peripheral cells do indeed migrate centripetally.[7,50-54]

The basement membrane was also recognized as playing an important role in the wound-healing process, representing perhaps the first recognition of the importance of the extracellular matrix. In 1961, Marena[55] observed that regeneration of the epithelial basement membrane begins 24

hours after injury and was thought to be complete in 62 hours. In later work, Lavker and others[56] showed that only cells that are in contact with the basement membrane have the ability for mitotic cell division, whereas cells that are displaced into the suprabasal layers become post-mitotic and lose their capability for cell division. This finding indicates at least some form of communication between the extracellular matrix and epithelial cell.

In 1977, Haik and Zimny[57] documented that epithelial healing begins with retraction of the corneal epithelium away from the wound during the first hour following injury. Retraction is followed by extension of the filopodia and lamellipodia to cover the bare wounded stroma.[57-59]

In 1977, Thoft and Friend[60] provided great impetus for the current concepts of corneal wound healing when they emphasized the potential interdependence of the corneal and conjunctival epithelium. They described the ocular surface as having a stratified nonkeratinizing epithelium with continuity of the conjunctival and corneal epithelium with the transition zone at the limbus. Most investigators of that era believed that conjunctival epithelium could transform into corneal epithelium by a process of transdifferentiation.[8,41,60-64] This had been an older concept begun by Mann and others.[41,65-67] The concept of corneal wound healing had evolved from those earlier works, and investigators believed that conjunctival cells adjacent to the cornea assumed corneal characteristics by transdifferentiation and migrated onto the cornea following a corneal wound, as suggested by Thoft and Friend and others.[8,61,64,68]

Conceptually, transdifferentiation is the transformation of conjunctival epithelial cells into corneal epithelial cells, without the goblet cells, mucin cells, and vascular elements of the conjunctiva. When free conjunctival grafts were shown to be effective treatment for certain ocular surface diseases, it was assumed that the conjunctival cells transdifferentiated.[60] In the late 1970s and early 1980s, investigators believed that 4 to 5 weeks were required after conjunctival transplantation before the new corneal epithelium had the histologic appearance of corneal epithelium.[8] Metabolic and functional transformation lagged further behind the morphologic tranformation.[69] These same investigators also recognized that transformation was incomplete. When stressed, as by wounding, the transformed epithelium tended to revert to a conjunctival appearance.[70] Kinoshita and associates[72] and others[60,71-73] recognized that the process of transdifferentiation was temporary at best, and that the conjunctival cells never really became histologically or biochemically true corneal cells. Hence, skepticism continued, and eventually, most authorities agreed that conjunctival epithelium does not transdifferentiate into corneal epithelium.[29,74,75]

As a result of the work heretofore mentioned, in 1983, Thoft and Friend[68] presented the "XYZ hypothesis of corneal epithelial maintenance,"

suggesting that a stem cell that could be responsible for initiating the growth of corneal epithelial cells. This theory proposes that the desquamated cells (Z component) are continuously replaced not only by the basal cells (X component) that divide but also by cells that migrate from the periphery (Y). The source of the Y-component is believed to be the stem cells located in the basal epithelial layer of the limbus. They also predicted the cellular maturation of the epithelial cells as they approach the central cornea. This continued centripetal movement of peripheral corneal epithelium replenishes the central corneal epithelium as it gradually sloughs. Sharma and Coles[76] provided a mathematical model demonstrating that the rate of exfoliation of epithelial cells is consistent with their production from the limbal cells. Soon after the proposal by Thoft and Friend, investigators began discovering evidence to support this "xyz" theory.

The Intracellular Aspects of Corneal Epithelial Homeostasis

Eukaryotic cells depend on their cytoskeleton to create the properties of shape, internal organization, and movement.[77] The cytoskeleton consists of at least three classifications of filaments, including microtubules, microfilaments and intermediate filaments, so-called because they appear to be of "intermediate" size between the microtubules and microfilaments. Intermediate filaments, with diameters of 7 to 11 nm, are believed to play a role in differentiation or a functional specialization state, histogenesis, intracellular transduction of signals, and malignant transformation.[78-81] There are at least five subclasses of intermediate filaments, which include, for our interest, the cytokeratins(CK) found in epithelia, and vimentin, usually found in cells of mesenchymal origin. Cytokeratins can be further divided into acidic (type I) and basic (type II) subfamilies and are usually expressed in pairs.[82,83] The cytokeratins and vimentin are immunoreactive and can be identified by immunohistochemistry.[78-80] Investigators are able to use these antibodies to cytokeratins and vimentin to document the different classes and topographic distribution of the different classes of ocular surface epithelia.

In 1986, Schermer and associates,[84] and eventually others,[85] showed in cultured rabbit corneal epithelial cells that a basic 64 kD corneal epithelial cytokeratin (CK3) and an acidic 55-kD cytokeratin (CK12) are characteristic of suprabasal limbal cell layers and all corneal epithelial cells. They suggested that these two keratins may be regarded as molecular markers for an advanced stage of corneal epithelial differentiation and are specific markers for cells of corneal lineage. They found that CK3 is located suprabasally in limbal epithelium and throughout the entire thickness of the central corneal epithelium, including basal cells. This observation would suggest that the central cells had an advanced state of corneal

epithelial differentiation. Very weak staining for the CK3 keratin was found in conjunctival cells. These results suggest that limbal basal cells are less differentiated than corneal epithelial cells and might represent an early, stem cell compartment. Through this work and that of others, most investigators now accept that central corneal epithelial cells express CK5 along with CK pair 3/12, which appears to be specific for the corneal-type of differentiation.[51-53,56,57]

Subsequently, Ebato and coworkers[88,89] showed that under explant culture conditions, human corneal limbal epithelial cells grow much better than peripheral and central corneal epithelial cells. This work suggested that the central corneal epithelium was not the source of the cellular engines that would complete the re-epithelialization of wound healing, but that the limbal epithelium was probably the source of such cells.

In 1989, Cotsarelis and associates[6] established the existence of a population of corneal limbal basal cells that are normally slow to cycle but that can be preferentially stimulated to proliferate by a tumor promoter (TPA) or by the physical removal of the central corneal epithelium. No such slow-cycling cells were detected in the corneal epithelium. These data suggest that corneal epithelial stem cells are located preferentially, if not exclusively, in the corneal limbus. These results also suggest that some of the limbal basal epithelial cells are the stem cells for the corneal epithelial cellular proliferation and differentiation. These limbal cells fulfill the kinetic criteria of stem cells and seem to be located preferentially at the limbus. Furthermore, Tsai and colleagues,[90] in an animal model, demonstrated that re-epithelialization of a damaged corneal surface with conjunctival cells did not provide a normal corneal phenotype but did produce a conjunctival phenotype including goblet cells. These investigators used monoclonal antibodies, of their own making, for conjunctival cells and mucin-producing cells.[90,91] In the same model, presumed corneal epithelial stem cells taken from the contralateral limbus, in the form of a limbal conjunctival autograft, provided more phenotypically normal corneal epithelial cells, and decreased vascularization.[90] This work further suggested the presence and importance of the limbal corneal epithelial stem cells and, incidentally, added further weight to the conclusion that conjunctival cells do not transdifferentiate into corneal epithelial cells.[90]

Additional circumstantial evidence for limbal corneal epithelial stem cells came from clinical work by Kenyon and Tseng.[92] They reported that when limbal epithelium was included in conjunctival transplantation for ocular surface disorders, the chance of successful re-epithelializaion was greater. Other clinical hints for the presence of corneal stem cells in the limbus can also be found in the older literature. In 1965, Roper-Hall[93] provided clinical evidence by showing that an important prognostic factor in

alkali burns was the extent of damage to the limbus.

In 1991, Wiley and associates[94] demonstrated the topographic distribution of presumed corneal epithelial stem cells with immunohistochemical staining mechanisms. These investigators used the antibody to recognize the previously mentioned CK3 (recognized by antibody AE5) and an antibody (AE1) to recognize a subset of acidic keratins complementary to the basic cytokeratin CK3.[95,96] The antibody AE1 recognizes a 48-kD cytokeratin present in a variety of hyperproliferative human diseases, suggesting that AE1 reactivity seen in corneal basal epithelial cells may be related to their proliferative state.[97] The work by Wiley and associates[94] found that the limbal basal cells (presumed corneal stem cells) were AE1-positive and AE5-negative. Using these antibodies, they described the topographic distribution of the presumed stem cells, suggesting that such cells were highly concentrated at the superior limbus, less concentrated along the inferior limbus, and almost absent in the palpebral aperture. They also illustrated that such cells extended deeply into the basal cell region of the peripheral cornea in the superior and inferior sectors. They and others also evaluated the importance of a basement membrane component recognized by a monoclonal antibody, AE27.[94,98] They confirmed that AE27-positive regions along Bowman's layer correlated with overlying basal epithelial cells that expressed AE5. This correlation did not hold beyond Bowman's layer, in that the basal cells on AE27-positive basement membrane beyond Bowman's layer did not stain for AE5. Nevertheless, these studies provide further circumstantial evidence that the extracellular matrix and/or microenvironments of the subepithelial tissues play an important role in determining the cellular phenotype and cellular metabolic state.[94,98]

In 1993, Zeiske and colleagues[99] identified a 50-kD protein (with 4G10.3 antibody) believed to be a biochemical and immunologic marker of limbal basal cells, and hypothesized that cells containing this marker would be the corneal epithelial stem cells. Lauweryns and coworkers[100] later that year documented additional cellular characteristics of the presumed stem cell. They found that CK19 would stain basal limbal cells as well as clusters of peripheral corneal basal cells and some suprabasal cells. Interestingly, the cells that were positive were found to be smaller cells having crowded nuclei and prominent nucleoli. Vimentin staining was only seen in the transition zone of peripheral cornea between the peripheral cornea and the limbus in the same clusters of cells that express CK19. The vimentin-positive cells were not seen in the basal layer of the limbus, however. According to the particular distribution of the staining patterns-including the positivity for CK19 and vimentin and the morphology of these cells—the investigators speculated that these cells represented stem cells or possibly transitional cells of the human cornea. These investigators

were not the first to recognize vimentin positive cells in the transition zone, but did unite the additional immunohistologic characteristics of these cells.[100-102] In a companion work, these same investigators found that the transitional cells and the basal cells of the limbus (believed to be corneal epithelial stem cells) shared expression of α6β4-integrin, metallothionein, AE1, and transferrin receptor.[101] These two works taken together suggest that there is a population of limbal stem cells that represent the progenitor cells of the corneal epithelium. Cellular proliferation of all self-renewing epithelia originates from stem cells that are undifferentiated and mitotically quiescent under normal conditions.[103-105] Furthermore, such work suggests that upon demand for tissue regeneration, stem cells differentiate into transient amplifying cells which cycle rapidly and can amplify the total cell number before they become postmitotic and eventually become terminally differentiated themselves.[105] These nests of highly active cells in the peripheral corneal epithelium are rapidly dividing vimentin-positive and CK12-negative cells that provide the necessary mass of cells for normal corneal epithelial development.[100-102]

In 1993, Wei and associates[5] provided strong evidence that limbal stem cells produce a lineage of corneal cells that is distinct from the lineage produced by conjunctival cells. They confirmed that corneal cells and certain limbal cells produce large amounts of CK3 and CK12 believed to be markers for corneal-type differentiation. Conjunctival cells, however, had only minimal amounts of CK3 and CK12, but expressed other cytokeratin pairs including CK5/CK14, CK6/CK16 and CK8/CK18 and CK19. This study also suggested that there were at least three different sets of conjunctival epithelia and that some conjunctival epithelial stem cells are probably located in the fornix of the eye.[5] In 1996, Wei, Sun and Lavker[9] used an athymic mouse to show that cultured corneal stem cells produced a distinct lineage apart from conjunctival epithelial cells. They implanted rabbit corneal, limbal, and conjunctival epithelial cells into athymic mice and produced epithelial cysts. Each cyst maintained the epithelial phenotype from which it had been drawn. This result, and other portions of the investigation, provided strong evidence for precursor cells such as corneal epithelial stem cells. These studies provided stronger evidence that corneal limbal basal epithelium was indeed the stem cell for corneal epithelium. They also documented that transdifferentiation did not occur satisfactorily, and what did occur was easily reversible.[5,9]

We now believe, and substantial evidence suggests, that epithelial stem cells do indeed reside at the limbus and these cells produce daughter cells that migrate toward the central cornea and mature. These supporting observations include (1) these cells are present in self-renewing tissues; (2) these cells are long- lived; (3) these cells are relatively undifferentiated

and have few or no differentiation products in the cytoplasm; and (4) the cells are located in extremely protected positions such as the bone marrow, and the crypts of the intestinal epithelium.[106] Additional evidence suggests that there may be a transitional cell that divides frequently and provides the daughter cells necessary for the corneal epithelium, and that these cells are separate from the stem cells.[5,9]

Various investigators, then, have determined the keratin markers of the epithelial cells that compose the limbus and the corneal epithelium (Table I). With these markers we are now able to construct a model of where such cells are normally found and the replicative potential for each cell (Figs 1 and 2). The model is still speculative, but the above evidence suggests the model and confirms certain components of it.[10]

Corneal epithelial repair, then, is believed to be generated through a division of specialized stem cells located in the basal epithelial layer of the peripheral cornea and corneal limbus.[106] These presumed limbal stem cells are crucial for maintaining the cell mass of the corneal epithelium under normal circumstances, and they play an important role in corneal epithelial wound healing. These putative stem cells are similar to other epithelial

TABLE I: OCULAR SURFACE IMMUNOHISTOCHEMICAL COMPONENTS

CELL TYPE	ANTIBODIES	KERATINS/PROTEIN STAINED	REFERENCE
Cornea cells	AE5	Epithelial basic keratin	84
Central cornea cells		64 kD keratin - K3	
(mature cells)	K12	Corneal differentiation Acidic keratin	84
Peripheral corneal cells			
Mature surface cells	AE5	64 kD keratin	84
Basal cells	CK19	Keratin 19	100
(Probably transit amplifying cells)		(Anticytokeratin 4.62)	
or possibly stem cells		Vimentin	100,101, 102
	AE1	Acidic keratins	94
Conjunctival cells			
limbal basal cells	CK19	Cytokeratin19	100, 101
(Presumed stem cells)	$\alpha^6\beta^1$ integrins	$\alpha^6\beta^1$ integrins	
	AE1		94
Suprabasal limbal conjunctival cells	AE5	64 kD cytokeratin	94
Goblet cells	AM3	Ocular mucin	91
Basement membrane			
Corneal basement Membrane	AE27	Supports K3 positive cells	94, 98

CK, cytokeratin; kD kilodalton;

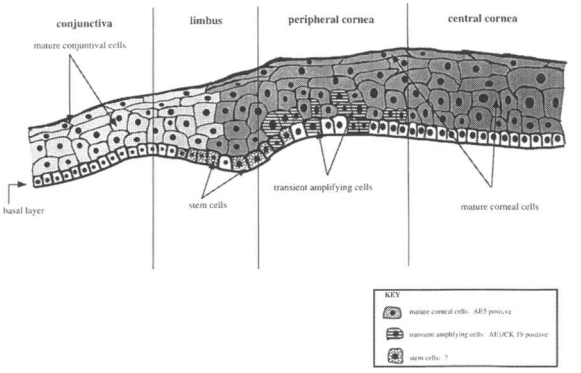

FIGURE 1
Diagrammatic representation of limbal and peripheral corneal epithelium in profile. Different epithelia are marked with shading.

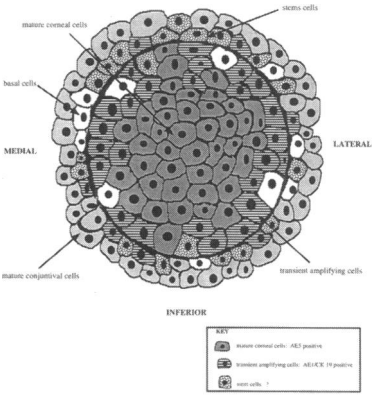

FIGURE 2
Anterior view of corneal epithelia in diagrammatic fashion.

stem cells and reveal a common set of features, including preferred location, pigment protection and growth properties, all of which presumably play a crucial role in epithelial stem cell functions.[6] Regeneration occurs by centripetal migration of differentiated cells (derived from stem cells) from the periphery to the central cornea.[107] Failure of limbal stem cells can cause ocular surface disease that may result in an unstable epithelium, pain, and reduced visual acuity.[10] Deficiency or absence of limbal stem cells can explain the pathogenesis of several ocular surface disorders characterized by defective conjunctival transdifferentiation or conjunctivalization of the cornea.[10]

The Extracellular Aspects of Corneal Epithelial Homeostasis

We are just beginning to understand the importance of the extracellular matrix, and this may play as important a role as, if not a more important role than, the epithelial cells themselves in wound healing and normal homeostasis of the corneal epithelium.

Although prompt restoration of the corneal epithelial surface is essential to visual recovery, and integrity of the eye, we have incomplete knowledge of the multiple physical and biochemical factors that are involved in the process of corneal re-epithelialization following injury.[108]

Although we understand that corneal epithelial cells migrate centripetally, the driving force is unknown. The epithelial cells move horizontally from the periphery to the center of the cornea, at least after experimental wounding, as mentioned above.[43,109] It has been suggested that limbal epithelium proliferates at a higher rate than central corneal epithelium and that this creates a population and tissue pressure toward the central cornea. However, Lavker and associates[56] proved that in animal models, at least, the driving force cannot be only population pressure. These investigators suggest that preferential desquamation of central corneal epithelium may "draw" peripheral cells toward the central cornea. They also documented a second-tier basal layer or suprabasal layer of DNA-synthesizing cells, but they found that these cells had a limited, but nevertheless direct, connection to the basement membrane through a thin stalk of cytoplasm. This may actually create a "second-tier" of basal cells that still have basement membrane connections. These observations suggest that the extracellular matrix plays an important role in cellular maturation and perhaps cellular migration.

Stable attachment of external epithelia to the basement membrane and underlying stroma is mediated by transmembrane proteins within the hemidesmosomes.[110] It is known that differentiation of epithelial cells and growth of cultured corneal cells can be influenced and modulated by the extracellular environment (eg, the basement membrane).[111,112] There is even some suggestion that cell phenotypes can be modulated by manipulation of the cells' basement membrane component.[113] Kurpakus and colleagues[114] reported that cultured conjunctival epithelial cells can express a corneal-type keratin pair when grown on the corneal basement membrane substrate, suggesting that conjunctival epithelium, at least in a unique in vitro condition might adopt a phenotypic change. Tseng and coworkers[115,116] have demonstrated that an extracellular factor (namely, retinoic acid) is of great importance in the modulation of ocular surface differentiation. Kruse and associates[117] raised questions about this work, however, as these investigators review the relationship of the animal model to the rate of transdifferentiation. According to these investigators, it is extreme-

ly difficult to remove all of the limbal stem cells with the chemical damage models currently employed. Not surprisingly, these investigators found that the duration of exposure of n-heptanol and the extent of corneal and limbal epithelial removal were related. They found that exposure of the limbal epithelium to n-heptanol resulted in incomplete removal of the basal layer even when the duration of treatment was extended to 180 seconds. From this work, he and his coworkers believe that if any of the basal limbal epithelial cells (the presumed stem cells) remain, they will eventually produce a more normal corneal epithelium, even after such a chemical injury, and interfere with the interpretation of the transdifferentiation evaluation. Nevertheless, retinoic acid is an important modulator of epithelial proliferation and differentiation, and retinoic acid is present in serum in biologically active concentrations.[118,119] Kruse and Tseng[120] expanded this work to suggest that retinoic acid acts on a special subpopulation of progenitor cells in the limbal epithelium, believed to be the limbal corneal epithelial stem cells.

These studies suggest that the extracellular matrix and/or the local microenvironments may play a significant role in the regulation of corneal and conjunctival epithelium.

With this understanding, we believe that corneal epithelial stem cells could be harvested, isolated and preferentially stimulated for growth in vitro. These cells could then be grown on an appropriate substrate or extracellular matrix and be returned to the original donor as an autologous transplantation or returned to a related or eventually an unrelated recipient for successful repair of ocular surface disease.

Management of Ocular Surface Disease

The management of ocular surface disease–including such variable diseases as Stevens-Johnson's syndrome, toxic epidermal necrolysis, chemical and thermal burns, congenital abnormalities, cicatrizing conjunctival conditions, and ocular surface tumors—has been a challenging problem. Many different methods have been tried with varying degrees of success.

There has been a long history of surgical attempts to treat such ocular surface diseases, and all of these procedures were developed with the aim of restoring the morphology, and to some extent the physiology, of the ocular surface.[121-123] Most of these procedures have been based on the use of mucous membrane, or even skin, collected from autologous sites or with the addition of artificially constructed tissues.

Mucous Membrane Grafts

Mucous membrane grafting has been used since 1944, when Siegel[124] first described obtaining the superficial epithelial layer from the lower lip to be

used in the treatment of severe foreshortening of the fornix. This technique is somewhat effective, but oral mucosa is not truly conjunctival and certainly not corneal epithelium. Although these grafts can be effective in certain situations, they are used infrequently. They never have a normal appearance and do not heal the cornea properly. These cells do not develop a normal corneal phenotype or physiology, at least in part, because mucous membrane grafts neither generate corneal stem cells nor originate from them. These grafts often suffer the same fate of foreshortening that the tissues were harvested to cure. Other tissues, including nasal and vaginal mucosa, have been used as conjunctival replacements, but these have had cosmetic, infectious, or scarcity problems.[125,126]

Conjunctival Flaps

One of the first attempts at ocular surface repair was with a conjunctival flap described by Scholer in 1877.[127] Occasional reports of the use of conjunctival flaps continued over the next 80 years.[128,129] This procedure was not widely used, however, until Gunderson's description popularized the technique.[130,131] In fact, the procedure is now commonly referred to as a "Gunderson flap," although it has been changed, especially with the advent of modern microsurgery.[132,133] Although a conjunctival flap is often successful, it does not attempt to restore the phenotypically normal cell to the corneal surface, but it does at least help resurface corneae with particularly vexing problems.

Conjunctival Grafts

Perhaps the first published account of a free conjunctival graft was published in 1951 by Hartman.[134] He described the use of free grafts in the correction of recurrent pterygia, pseudopterygia, and symblepharon. He suggested that conjunctiva would be the best tissue to use for grafting and that this tissue might be harvested and used to solve ipsilateral or contralateral conjunctival epithelial abnormalities. This idea gained little enthusiasm until Thoft[135,136] described the technique in 1977 and again in 1979 as he began conjunctival grafting for ocular surface disease. This proved to be a relatively successful technique and was recognized as a novel approach for certain problems. Vastine and others advanced this technique to treat pterygia, and it has proven effective.[137,138] Conjunctival grafting was believed to be effective, in part at least, because of transdifferentiation, but as discussed above, transdifferentiation probably does not occur. Nonetheless, conjunctival transplantation remains a powerful tool for restoration of a damaged ocular surface, especially if some normal corneal epithelial stem cells remain in the recipient eye.[139,140] Although these techniques require conjunctival epithelium taken from the normal

bulbar surface, the presence of normal corneal epithelial stem cells in the recipient eye will help prevent complete conjunctivalization of the ocular surface from the free conjunctival graft. The required conjunctival graft can be harvested from the ipsilateral eye, but it must be healthy tissue. Surgeons often recommend the bulbar surface beneath the upper temporal lid. In patients with severe uniocular disease, the normal ocular surface can often be restored by autologous conjunctival transplantation of donor tissue taken from the contralateral eye, presumably as long as some corneal epithelial stem cells exist on the surface of the damaged eye. When successful, this procedure results in corneal re-epithelialization and an improvement in symptoms, but less commonly an improvement in visual acuity.

Lamellar Keratoplasty

Lamellar keratoplasty has been used with some success for recurrent pterygia, suggesting that restoration of a normal Bowman's layer or normal basment membrane plays an important role in normal corneal epithelial metabolism.[141-143] These techniques have not been championed recently.

Keratoepithelioplasty

The evolution of epithelial transplantation and lamellar keratoplasty took a propitious turn in 1984 when Thoft[144] published his work on keratopeithelioplasty. This remarkable idea included the transplantation of cadavaric corneal tissues to include lenticules of peripheral cornea and limbus with a thin stromal carrier. Thoft advocated it as an alternative to conjunctival transplantation in patients with severe bilateral chemical injuries to the ocular surface. This appeared to be a satisfactory alternative for patients who had little or no normal corneal epithelial surface, but investigators soon learned that these grafts were difficult to obtain and perform, readily rejected, and failed to produce convincing results.[145] Human limbal lenticules include the epithelial cells expressing class I human leukocyte antigens (class I HLA) and are subject to rejection.[145] Nevertheless, this procedure was perhaps the first attempt at the transplantation of corneal epithelial stem cells, although it was not understood as such. Thoft did not have the benefit of our current understanding of the presumed limbal stem cell population, but he did appreciate the potential of the limbus as an engine for epithelial cellular growth. Later investigators attempted to treat severe chemical burns with large-diameter penetrating keratoplasty, and actually did perform the equivalent of an enlarged keratoepithelioplasty.[146,147] Although these procedures probably did transplant limbal corneal epithelial stem cells, many of these patients had problems with eventual stem cell rejection.[146,147] Occasionally, some of these large

corneal transplants were reported to be successful in the treatment of certain severe alkali burns.[16] Although unsuccessful, Thoft's work heralded a new age in ocular resurfacing.[16]

Limbal Conjunctival Grafts

With the accumulating evidence of the 1980s and 1990s suggesting that corneal epithelial stem cells resided at the limbus of each cornea, investigators began to consider that complete autologous limbal transplantation could be used to resurface eyes in patients with unilateral surface problems. Armed with this knowledge, surgeons began transplanting autologous limbal tissues from a healthy eye to a diseased contralateral eye in victims who had only unilateral ocular surface injury or disease.[92,148-155] Interestingly, this procedure was probably done previously under an entirely different pretense. Herman and associates[156] modified Thoft's original conjunctival grafting procedure, and may have inadvertently stumbled on the limbal conjunctival autograft, although little attention was given this publication. In his original description of conjunctival grafting from a healthy eye to a damaged contralateral eye, Thoft[144] described taking small (3 to 4 mm) circular grafts from the normal eye on the bulbar conjunctiva sparing the limbus. Herman and associates modified the procedure by using a Flieringa ring to act as support for the donor graft. The donor material was then secured to the ring and transported to the recipient eye. In this case, the limbus could be transplanted, and if this was done, the recipient eye would have received nearly a full complement of corneal epithelial stem cells. This would have been helpful for the recipient eye but may have been hazardous for the donor eye.[156]

Limbal conjunctival autografts have shown dramatic success in patients with severe and difficult problems.[92,148-154] Various investigators began using these techniques and getting similar results in other forms of stem cell injury or other cases requiring corneal surface reconstruction.[155] These limbal conjunctival autografts were used for acute and chronic chemical injury, thermal burns, contact lens–induced keratopathy, and ocular surface failure after multiple surgical procedures. Most patients showed consistent visual acuity improvement, rapid surface healing, stable epithelial adhesions, and no recurrent erosion or persistent epithelial defects. Corneal neovascularization stopped or regressed.[92,148-155] In these studies, some investigators, using impression cytology, immunopathology, and light microscopy, showed restoration of the corneal epithelial phenotype and regression of the goblet cells from the recipient corneal.[92,148-155] These grafts showed definite improvement over free conjunctival grafts for conditions requiring the regrowth of corneal epithelium. This simple fact offered further clinical evidence of the authenticity of the limbal stem

cell theory. These limbal conjunctival autografts proved to be able to provide corneal epithelial stem cells without the attraction of neovascularization that the conjunctival grafts would often exhibit.

Autologous limbal conjunctival autografts offer improved prognosis over previous reconstruction techniques. Unfortunately, there are problems with this approach. The technique is restricted to uniocular disease, and the donor eye must be completely normal. Failure to notice corneal disease can result in a severe decrease of vision in the donor eye.[157] Furthermore, not all patients may be willing to risk their uninvolved and healthy eye. In such cases, as well as those with bilateral injuries, it becomes necessary to consider other alternatives, including the use of allografts.[153,158]

Bilateral severe ocular surface injury or disease, as is usually seen in alkali or thermal burns, probably is more common than unilateral disease. The success seen with autologous limbal conjunctival autograft led investigators to consider the treatment of bilateral disease with the use of allogeneic limbal conjunctival epithelial grafts using tissue from siblings or related donors. The success of these allogeneic grafts has led many investigators to advocate such sibling transplants for bilateral injuries.[152,158] Curiously, the fate of limbal conjunctival allografts in these circumstances is unclear. One would expect the grafts to be rejected, but this does not invariably occur.[159] Allografts may survive in the absence of immunosuppression, but the prognosis improves for patients in whom systemic immunosuppression is utilized.[159] In several well-documented cases, patients who have received allografts have improved dramatically with better corneal epithelium.[159,160] This may suggest rejection, yet in each case clinical improvement remained. This may suggest that extracellular matrix may play more of a role than first thought.

Unfortunately for allogeneic transplants, the donor must provide as much as half of his or her limbal tissues. This may represent a majority of corneal epithelial stem cells, because the advocates of this procedure suggest using the superior and inferior limbus where we believe the largest number of stem cells are concentrated.[153,157,158] This may leave the donor at higher risk of future epithelial surface disease because much of the limbal epithelial stem cell complement may be removed. This may limit future donors and the acceptance of such procedures.

The major limitation for both autologous and allogeneic limbal conjunctival epithelial stem cell grafts, then, is the availability of normal healthy limbal conjunctival epithelium from the contralateral eye or from related donors, and the potential threat to the contralateral or donor eye when such limbal cells are removed. Moreover, such severe burns are difficult to treat with any modality, and it is doubtful that any surface repair

consisting of epithelium alone will be sufficient for many of these problems. Extensive damage to the ocular surface causes mucus deficiency and persistent subconjunctival inflammation leading to severe dry eye and fibrosis of the subconjunctival tissue. These are very complicated conditions requiring more complex reconstruction. Some investigators believe that contact with healthy basement membrane is essential and pivotal for the normal epithelialization.[98] If healthy basement membrane is necessary, and that seems likely, transplantation of epithelial cells alone will probably not be sufficient.

Amniotic Membrane Grafts

Other investigators have more recently used amniotic membrane as an organic device to promote the resurfacing of the ocular surface.[161-166] Amniotic membrane is a thin semitransparent tissue forming the innermost layer of the fetal membranes. This remarkable membrane has a single layer of epithelial cells bound to a thick and continuous basement membrane with a full complement of certain subtypes of type IV and V collagen, laminin, fibronectin, elastin, and various integrins, which are principal basement membrane components.[167-170] Interestingly, certain subtypes of type IV collagen have been recognized histochemically in conjunctival but not in corneal epithelial basement membrane.[171] This suggests that collagen in the amniotic membrane could serve as a suitable substrate for conjunctival re-epithelialization and would be considered substrate for transplantation, especially for corneal epithelial stem cells traditionally found at the limbus. The various laminins known to be present in amniotic membrane could provide signals for hemidesmosomal attachment of epithelium, which could help adherence.[110] Amniotic membrane is known to have a thick basement membrane and has been used successfully for other epithelial cell growth.[172] Moreover, there is good evidence that amniotic membrane and amniotic epithelium do not express HLA-A,B,C, or DR antigens and, as a result, should not be rejected by the immune system of the host.[173,174]

Amniotic membrane has a long, if irregular, history of use for repairing ulcerated epithlelial surfaces. Davis, in 1910, was perhaps the first to report the use of amniotic membrane in skin transplantation, and it has been used subsequently in this role.[175,176] Amniotic membrane has been used intermittently in the first half of this century in the management of ulcerated skin defects found in burns and other forms of skin ulcerations.[177]

De Rotth,[178] in 1940, was probably the first to use amniotic membrane for the ocular surface when he reported successful use of amniotic membrane as a conjunctival graft in the repair of a symblepharon. Other

reports followed with the next decade.[179,180] Curiously, in 1962, Forgacs[181] demonstrated that placental extract hastened the repair of superficial corneal lesions, but little attention was given this work, and it was largely ignored.

Amniotic membrane has been used intermittently for other purposes in the last few decades, but ocular surface disease was not considered until 1995 when Kim and Tseng revived the idea.[176,182] They reported that the transplantation of amniotic membrane to the corneal surface in a rabbit chemical burn model caused epithelialization of the corneal surface with cells expressing a corneal-type keratin.[182] Their work suggested that amniotic membrane alone could be sufficient to allow for re-epithelialization of a chemically damaged cornea with conjunctival cells that would express a corneal phenotype. They raised the possibility that the presence of certain basement membrane factors may cause or allow for conjunctival transdifferentiation.[182] Recently, however, other investigators have found that amniotic membrane is of little clinical use in the treatment of chemical burns or corneal abscesses,[183] and we have seen earlier that true conjunctival transdifferentiation does not occur.

Other investigators have used amniotic membrane for the treatment of end-stage Stevens-Johnson's syndrome and ocular cicatricial pemphigoid, persistent epithelial defects with ulceration, and pterygia.[149,161-164,184] More recently, amniotic membrane has been shown to reduce keratocyte proliferation and corneal haze during corneal wound healing following photorefractive keratotectomy. It may act by reducing the infiltration of inflammatory cells and loss of keratocytes in the ablation area during the early postoperative period.[185] This same study suggested that the amniotic membrane prevented the influx of inflammatory cells and reduced inflammatory damage to the underlying stroma.[185]

Transplanted amniotic membrane seems to promote normal conjunctival re-epithelialization while preventing excessive subconjunctival fibrosis formation. As mentioned above, certain type IV collagen subtypes have been recognized histochemically in conjunctival but not in corneal epithelial basement membrane, and type IV collagen has been recognized in amniotic membrane.[167-171] This suggests that the collagen in the amniotic membrane probably serves as a substrate suitable for conjunctival epithelialization and would be suitable for transplantation, as other investigators working with pneumocytes and endometrial cells have suggested.[165,186] Using damaged rabbit corneae as a model, Kim and Tseng[182] showed that the various components of basement membrane mentioned above may well play a role in epithelial healing after de-epithelialization, illustrating the role of the extracellular matrix in wound healing.

Prabhasawat and Tseng[166] performed impression cytology on eyes of

patients with ocular surface disease from acquired melanosis, conjunctival intraepithelial neoplasia, inferior conjunctival chalasis, aniridia, toxic epidermal necrolysis, and a chemical burn. They used this technique to show that conjunctival transdifferentiation does not occur in vivo with amniotic membrane transplantation alone and that active stem cell transplantation is needed for defective corneal surface reconstruction when stem damage or deficiency is encountered. The only mechanism for providing these limbal stem cells, however, has been the harvesting of 90° or more of healthy limbal tissues from the contralateral eye or allogeneic sources.

Cultured Corneal Epithelial Grafts

Cultured corneal epithelial stem cell transplants have been considered since as early as 1982 when Friend and associates[187] sought to use in vitro epithelial stem cell cultures on stromal carriers. Unfortunately, this did not meet with much success, possibly because stem cells were not included in the cell cultures. In 1985, Gipson and colleagues[188] even attempted direct transplantation of corneal epithelium to rabbit corneal wounds in vivo. They reported that the adhesion of freshly dissected rabbit corneal basal epithelial cells to denuded basal lamina of corneae can take place within 60 to 90 minutes in vitro or within 6 hours in vivo.[189] However, these investigators also observed that these epithelial sheets failed to remain adherent to rabbit corneal stroma in vivo after 24 hours.[188] In 1985, Geggel and coworkers[190] applied corneal epithelial cell sheets (obtained by applying dispase grade II to donor rabbit corneae) to a collagen gel (Vitrogen) and created a safe and nontoxic substrate that allowed for epithelial adherence for up to 13 days in vitro. They also discovered that the gel, without the epithelial cells, remained on the rabbit eye and was well tolerated for at least 6 weeks until the end of the animal investigation. Both investigations used epithelium dissected from the cornea and probably did not include corneal epithelial stem cells.[188-190]

Friend and associates[191] later suggested that epithelial sheets obtained from rabbits adhered to stroma in vitro within 24 to 72 hours and hemidesmosomes formed with host basement membrane at the same time. Additional attempts at in vitro culture and re-implantation continued but were not successful. Nevertheless, the potential for this work was suggested.[192]

Little additional investigation was done until work by He and McCulley[193] documented that limbal epithelial stem cells could be grown in vitro and would become stratified on type IV collagen-coated collagen shields. These shields could subsequently be transferred to denuded ex vivo human corneal stromal in organ culture. Histologic examination revealed that the epithelial cells had attached tightly to the recipient stromal surface even after the removal of the collagen shield.

Torfi and colleagues[194] reported (in abstract form) the application of cultured autologous grafts in 4 patients with apparent success in 3 of 4 of these patients. More recently, this procedure has been replicated and reported by a European group.[195] To document the corneal phenotype of the transplanted cells, Pellegrini and colleagues[195] documented that the cultured epithelia were CK3-positive and represented cells of a corneal lineage. Both groups documented that sufficient corneal epithelial cells to cover the entire corneal-limbal surface can be obtained from a 1 to 2 mm^2 limbal biopsy sample, allowing for minimal stem-cell depletion from the healthy eye.[194,195] In both investigations, however, one cannot be absolutely certain of the long-term fate of the transplanted autologous cells or, for that matter, that the tranplanted cells were responsible for the improvement in the ocular surface. CK3-positive staining does suggest that these cells were of corneal lineage, but this does not document the source. Do the donated cells persist and proliferate in the recipient eye, or do they stimulate a repair response process and are they then gradually replaced by the recipient ocular surface cells?

Investigators had been unable to use cultured corneal epithelium on amniotic membrane before Tsai[196] presented (in abstract form) 3 cases of autologous stem cell transplantation grown on amniotic membrane. Tsai documented the epithelial cell growth by using cytokeratin markers that stained positive with AE5 immunoperoxidase stain to document the multicellular layers of cells on the amniotic membrane, but he did not present a control to document that these cells were not the original amniotic epithelium. He also did not present any evidence that the amniotic epithelium had been removed. Nevertheless, he reported prompt re-epithelialization of the corneal surface in unilateral alkali burns.

The amniotic membrane is a basement membrane that serves as a barrier against damaging cytokines and may prevent fibrotic scarring.[161,162,185] Tsai suggested that this technique may provide an alternative to complete limbal autografts. There is, however, no proof that these are indeed stem cells, because these cells cannot be confirmed in vivo even in the normal eye. Moreover, no documentation showed that the transplanted cells remained after transplantation.[196] Other investigators have provided an in vitro model for tracheal epithelial growth on amniotic membrane, but no in vivo work has yet been documented with this method.[197]

As mentioned above, investigators have proven that complete autologous limbal transplants have resurfaced eyes with unilateral surface problems.[92,148-155] Unfortunately, this leaves the donor eye at some risk to future surface problems because of the depletion of stem cells from the donor eye. Additionally, this technique does not address bilateral ocular surface injury. The techniques of cultured corneal epithelial cell transplants offer

an unrealized potential for successful ocular resurfacing without significant threat to the donor eye. We propose using the techniques we have developed to grow and expand a limited population of limbal corneal epithelial stem cells in vitro and reimplant these expanded epithelial cells on a suitable carrier, such as amniotic membrane. This composite graft can then be used to manage the ocular surface of damaged or diseased eyes.

MATERIALS AND METHODS

The 3 sections of this investigation are each described separately: (1) a human trial using cultured corneal epithelium to treat ocular surface abnormalities, (2) amniotic membrane preparation, and (3) an animal model for composite epithelial graft transplantation.

HUMAN CULTURED EPITHELIAL TRANSPLANTATION

Patients with ocular surface problems that had not been managed successfully with currently available techniques were selected for expanded corneal epithelial cell transplants. These ocular surface problems included 2 patients with primary pterygia or pseudopterygia, 9 patients (including 10 eyes) with recurrent pterygia or pseudopterygia (frequently with restriction of ocular motility), 2 patients with extensive ocular surface malignant or premalignant conditions (1 patient had 2 procedures on the same eye), 1 patient with an unresponsive neurotrophic ulcer (including previous tarsorrhaphy), 3 patients with ocular surface thermal or chemical burns, and 1 patient with stem cell failure secondary to pseudopemphigoid. The 2 patients receiving the allogeneic transplants included 1 of the patients in the thermal or chemical burns group and the single patient with epithelial stem cell failure. The remaining 16 patients (1 operated on twice and 1 operated on both eyes, for a total of 18 procedures) were selected for autologous transplants (Table II).

Institutional review board approval was sought and secured for each individual portion of the human investigation including (1) the autologous transplantation, (2) the implantation of donor amniotic membrane, (3) the harvest of sibling cells to be cultured and expanded for transplantation, and (4) the re-implantation of allogeneic sibling cells from sibling subject to patient. Informed consent was obtained from patients and donors, and all human subjects were treated according to the Helsinki Accord. Both donor siblings were anonymously tested for human immunodeficiency virus 1 and 2 antibody (HIV 1 and 2), hepatitis B virus surface antigen, human T-lymphocyte virus 1 antibody, and syphilis.

TABLE II: PATIENTS WITH OCULAR SURFACE CONDITIONS TREATED WITH CULTURAL EPITHELIUM

OCULAR SURFACE DISEASE	AUTOGRAFT	ALLOGRAFT
Primary pterygia or pseudopterygia	2	
Recurrent pterygia or pseudopterygia	9 (10 eyes)	
Premalignant state or malignancy	2 (1 patient twice)	
Neurotrophic ulcer	1	
Thermal or chemical burn	2	1
Stem cell failure (pseudopemphigoid)		1

Surgical Technique for Autologous and Allogeneic Transplantation

The surgical repair techniques differed depending on the carrier, and they are summarized below. Eight patients (9 procedures) received expanded epithelial cell cultures that were placed atop corneal stromal lamella. Each of these patients had severe primary or recurrent pterygia or pseudopterygia approaching the visual axis or pseudopterygia with motility restriction. The pterygia or pseudopterygia were removed, and hemostasis was maintained. A lamellar dissection was performed to encompass the damaged sclera and cornea where the previously removed growth had been found. A donor corneal lamella without the original epithelium was sewn onto the host defect. The expanded epithelial cell culture was placed on the bare sclera and the bare corneal stroma of the lamellar graft and sewn over the defect with 10-0 nylon by attaching the anterior edge of the expanded epithelial cell graft to clear corneal stroma along the anterior edge of the lamellar graft, and the posterior edge of the expanded epithelial graft was sewn to the resected/recessed conjunctiva as in the repair of a conjunctival autograft described previously.[92,137,138]

The 2 patients who had malignant or premalignant conditions (conjunctival intraepithelial neoplasia and acquired ocular melanosis) had similar procedures. The entire ocular surface that appeared abnormal or atypical was removed, including the bulbar and palpebral surfaces, and hemostasis was maintained. The expanded epithelial cell cultures were positioned, and the anterior edge was sewn to the peripheral corneal stroma with 10-0 nylon. The posterior margin of the expanded epithelial cell graft was sewn to the recessed/resected edge of conjunctiva with 8-0 Vicryl. Both of these cases required removal of abnormal conjunctiva from the bulbar and palpebral surface.

The expanded corneal epithelial cell graft was applied to the palpebral surface in case 3 (see "Results" section for description of case) in order to prevent symblephara. This patient with conjunctival intraepithelial neoplasia (CIN) received an expanded corneal epithelial cell graft during the

early course of the investigation, was free of tumor for 16 months, and had only one symblepharon. He was seen again with a recurrence of CIN and was advised to undergo surgery again. His second procedure included an amniotic membrane transplant with expanded autologous epithelial cells as described below. The second patient with a premalignant condition (case 9) received an expanded epithelial cell graft on only the bulbar surface and suffered some foreshortening of the superior fornix to the posterior edge of the expanded epithelial graft.

The patient with an unresponsive neurotrophic ulcer received an expanded autologous epithelial cell transplant from the ipsilateral eye placed atop a plano therapeutic contact lens. These cells were grown into a sheet and grown across the contact lens. The contact lens and expanded epithelial cell culture were placed atop the recipient cornea, which had been cleaned of debris and mucus.

The patients receiving autologous and allogeneic expanded epithelial cell transplants atop amniotic membrane carriers had similar procedures. All of the abnormal tissue was removed, and the conjunctiva was resected and recessed. The amniotic membrane with expanded corneal epithelial cells was placed atop the defect, and the corneal edge was sewn onto the peripheral cornea with 10-0 nylon. The posterior peripheral edge of the amniotic membrane was sewn to the peripheral recessed/resected conjunctiva with 10-0 nylon, and a bandage contact lens was placed to prevent lid trauma. The contact lens was left for approximately 2 to 3 months. During this time, the amniotic membrane gradually dissolved, and the peripheral conjunctival sutures were removed. Once the bandage contact lens was removed, the corneal sutures were also removed.

Representative portions of the transplanted amniotic membrane were studied histologically. After fixation, specimens were stained with hematoxylin and eosin (H and E), immunohistochemical localization staining for cytokeratins AE5, (ICN Biomedicals, Inc, Aurora, Ohio).

Epithelial Cell Harvest

Epithelial cell harvest was performed in a similar manner whether cells were taken from a patient (autologous) or donor sibling (allogeneic). Following informed consent, and a sterile preparation and draping of the eye, a lid speculum was placed. Approximately 0.2 cc of 1% xylocaine was injected beneath the conjunctiva at the superior temporal limbus. A 2 mm^2 biopsy to include the limbal conjunctiva was harvested and placed in a cellular transport medium for transportation to the laboratory. The limbal conjunctiva was removed as closely to the reflection of the adherent corneal epithelium as possible. Antibiotic ointment was placed, and the eye was covered with a patch for 12 to 24 hours. No complications were

encountered from this procedure with the exception of minor brief irritation.

Preparation of Cultured Corneal Epithelial Cell Grafts
The preparation of the cultured corneal epithelial cell grafts has two steps depending on the carrier. The first step is similar for all carriers of the epithelial cell grafts. The second step is different, depending on the material used for the carrier.

Step I: The 2 mm^2 biopsy was transferred to the epithelial autograft laboratory in transport medium. In the laboratory, the epithelium was removed aseptically and transferred to a 60 mm petri dish. The tissue was washed 3 times for 5 minutes each with 5 mL of Dulbecco's Phosphate Buffered Saline-Calcium Magnesium Free (DPBS-CMF) (Life Technologies)/5% antibiotic-antimycotic: 10,000 U penicillin-G and 10,000 μg/mL streptomycin with 25 μg/mL fungizone (ABAM) (Gemini Gio-Products Inc), transferring the tissue to a new dish with each wash. The tissue was incubated in a solution of trypsin/edetate disodium (EDTA) solution for 30 minutes at 37° C in a 5% CO^2 incubator. The action of the trypsin was inhibited by adding an equal volume of medium that contained 10% fetal bovine serum. The sample was minced with a scalpel blade and centrifuged at 3,200 revolutions per minute for 5 to 7 minutes. The cells were plated at approximately $1.0x10^6$ cells/mL Growth Medium (GM; consisting of Dulbecco's Modified Eagle's Medium, Fetal Calf Serum glutamine, ABAM, Epidermal Growth Factor, hydrocortisone, and cholera toxin) on two 100 mm dishes with mitomycin C-treated 3T3 cells. The 3T3 cells had been treated and trypsinized. The dishes containing the corneal cells and the 3T3 feeder cells were placed into a 37° C/5% CO^2 incubator. Within 3 days, small colonies of cells formed. At that time, the growth medium was replaced with Keratinocyte Growth Medium (Medium 154 +ABAM and human keratocyte growth supplement, KGM). When the primary dish was at 40% to 50% confluence, the cells were passed into 4x100 mm dishes (passage 1). These cells were then allowed to reach 40% to 50% confluence.

Step II With Collagen Gel as a Carrier. The collagen gel was prepared as follows: Eight cc of chilled Vitrogen 100R Collagen was mixed with 1.0 mL of 10xGM (final collagen concentration 0.5 mg/mL). One mL of 0.1 M NaOH was added. The pH was adjusted to 7.4 by adding a few drops of 0.1M HCL or 0.1 M NaOH. Two milliliters of collagen was added to 35 mm dishes. These dishes were stored at 37° C in a 5% CO^2 incubator until needed. When the corneal epithelial cells were 40% to 50% confluent, the

cells were trypsinized and plated at 3.0x 10^6 corneal epithelial cells resuspended in 2 to 3 mL of Dulbecco's Modified Eagle's Medium (DMEM), into a prepared collagen gel in a 35 mm dish. Because of the plating density, the cells were postconfluent in 4 to 6 hours. The graft was ready for transplantation after 2 days and available up to 7 days, allowing for the cells to adhere to the collagen gel. Cells will peel off of the collagen gel if they remain in vitro more than 1 week. The medium should be changed every 1 to 3 hours before delivery of the corneal graft.

Step II With a Collagen Shield or Contact Lens as a Carrier. When the corneal epithelial cells were 40% to 50% confluent, they were trypsinized and plated 1x10^6 onto a 35 mm dish; 2 cc of GM was added. The cells were grown to confluency for 48 to 72 hours. The culture dish containing the postconfluent corneal epithelial cells was rinsed 3 times with 2 cc of DPBS-CMF; 2 cc of thermolysin solution (150 μg/mL) was added into the dish. This dish is incubated at 37° in 5% CO^2 incubator for approximately 20 minutes. The cells were removed from the incubator when the edges of the corneal epithelial sheet started to separate from the dish. Thermolysin was removed from the dish. The cells were rinsed 3 times with 2 cc of DPBS-CMF, and 2 cc of DMEM without supplements was added to the side wall of the dish. The remaining attached edges and corners of the corneal epithelial sheet were loosened. A corneal collagen shield or contact was placed onto a 60 mm dish with the concave side up. The corneal epithelial sheet was slid from the 35 mm dish onto the 60 mm dish over the collagen shield or contact lens keeping the basement membrane down. One milliliter of unsupplemented DMEM was added to the dish. The collagen shield or contact lens was ready for placement after 2 to 3 hours.

Step II With Amniotic Membrane as a Carrier. The preparation of the amniotic membrane played an important role in this investigation. This preparation is described below followed by the technique for application of the expanded epithelial cell cultures.

AMNIOTIC MEMBRANE PREPARATION

Acquisition of Amniotic Membrane for Animal Investigation

Amniotic membrane was secured from fresh placentae from the our university hospital 3 to 4 days following delivery of a healthy infant. Institutional Review Board approval was obtained for the harvest of these placental tissues, and informed consent was obtained from each postpartum mother shortly after birth. Each mother who donated the amniotic

membrane had been cleared for HIV-1 and -2, hepatitis B virus surface antigen, and syphilis, even though this protocol was only for rabbit investigational purposes. The placentae were kept at 4°c for 3 to 4 days to be certain that the infant was healthy and no further pathologic examination was required of the placenta.

The amniotic membrane was harvested in the following manner. The amnion was dissected from the placenta in a sterile environment with blunt dissection only. Once the single layer had been dissected from the placenta, it was cleaned and rinsed 3 times in normal saline. The amniotic membrane was cut into squares approximately 40 mm^2 and placed in storage medium. The membrane was then transferred to the autograft laboratory and rapidly frozen to -80°. As amniotic membrane was needed, it was individually thawed.

Preparation of Amniotic Membrane

Untreated amniotic membrane has epithelium, which we believed would interfere with the potential adherence of expanded corneal epithelial cells to the amniotic membrane. Initially, amniotic membrane epithelium could not be removed by trypsinization alone. Previous attempts by other investigators suggested that sonification would be necessary to remove this epithelium.[198] The following protocol was established to discover the most effective method of removing amniotic epithelium.

We reviewed 17 techniques to confirm the best method for removing the amniotic epithelium while maintaining, as much as possible, the histologic health and appearance of the amniotic basement membrane.

These techniques included sonification for 15, 30, 45, or 90 minutes followed by gentle scraping of the epithelial surface; sonification for 15, 30, 45, or 90 minutes with trypsinization for 15 minutes, followed by gentle scraping of the epithelial surface; trypsinization for 15 minutes, followed by gentle scraping of the epithelial surface, followed by 15, 30, 45, and 90 minutes of sonification; and, trypsinization for 15, 30, 60, 90, and 120 minutes followed by gentle scraping (Table III). The exact method of trypsinization and scraping used is as follows: 3 mL DPBS with PBS/1%ABAM was placed in a 60 mm tissue culture dish. Using forceps, a 1 x 1-inch amniotic membrane was placed into each dish. The PBS was gently aspirated, taking care not to aspirate the amniotic membrane. The membrane was washed twice more with PBS/1%ABAM. Then, 3 mL of 0.25% trypsin/0.01mM EDTA was placed in the culture dish. The dish was placed in an incubator at 37° for the specified time as described above. Control amniotic membrane underwent the same procedure covered only with PBS. After the chosen time, the trypsin was neutralized with 3 mL DMEM with 10% FCS. The amniotic membrane was again rinsed with

TABLE III: PREPARATION OF AMNIOTIC MEMBRANE

MINUTES	AMNIOTIC EPITHELIUM°	AMNIOTIC BASEMENT MEMBRANE°
Sonification/scraping†		
15	I	I
30	I-II	II
45	I-II	III
90	I-II	II-III
Sonification/scraping/trypsin‡		
15	IV	I
30	IV	II
45	IV	II-III
90	IV	II-III
Trypsin/scraping/sonification§		
15	IV	II-III
30	IV	II
45	IV	II
90	IV	III
Trypsin/scraping¶		
15	IV	I
30	IV	II
45	IV	III
90	IV	III
120	IV	III

° See Tables IV and V for explanation of grading scales.

† Sonification for set minutes followed by scraping.

‡ Sonification for designated minutes followed by 15 minutes of trypsinization.

§ Trypsinization for 15 minutes followed by scraping followed by sonification for designated minutes.

¶ Trypsinization for designated minutes followed by scraping.

PBS/1%ABAM. The epithelial layer was scraped off using blunt forceps. The membrane was then washed twice with PBS/1%ABAM and fixed with Streck Tissue Fixative. These portions of amniotic membrane were fixed and stained with H and E and reviewed in a masked fashion by 2 observers with agreement between the 2 observers. In each of these combinations, the presence or absence of amniotic epithelium and the quality of the underlying basement membrane were assessed by histology to determine the best method for removing the amniotic epithelium and preserving of the basement membrane of the amnion. Comparison was made with the normal nontrypsinized amnion (Fig 3). The grading scale for the removal of amniotic epithelium is summarized in Table IV. At the same time, the amniotic membrane was also evaluated by the same 2 observers in a masked fashion (masked as to technique of treatment of amniotic mem-

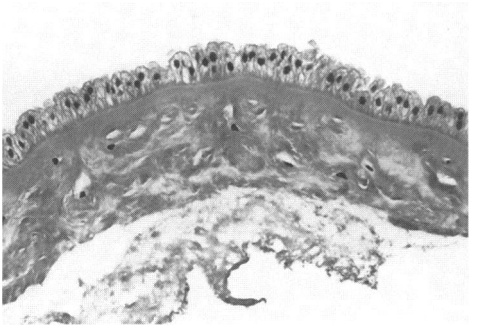

FIGURE 3
Normal amnion with epithelium. Epithelial cells are tightly adherent with normal basement membrane.

brane) for the histologic appearance of the basement membrane. The grading scale of the basement membrane is summarized in Table V. The technique that successfully removed all amniotic epithelium, yet main-

TABLE IV: GRADING SCALE FOR AMNIOTIC MEMBRANE EPITHELIUM

I	Normal amniotic epithelium
II	Majority of amniotic epithelial cells pynotic flattened and containing vacuoles
III	Few amniotic membrane cells remaining and those remaining being flattened and pynotic
IV	No amniotic epithelium seen

tained the best basement membrane of the amnion, was chosen for subsequent investigations. (Fig 4)

Rabbit stem cell epithelium was then grown on the best amniotic basement membrane following the removal of the amniotic epithelium in the same fashion that the human stem cell composites were grown. Once confluent epithelial growth was achieved, these were stored and used for the

TABLE V: AMNIOTIC MEMBRANE BASEMENT MEMBRANE HISTOLOGIC EVALUATION

I	Normal appearance compared to nontreated basement membrane
II	Somewhat thinned basement membrane with minimal or mild disruption of basement lamella
III	Moderately to markedly thinned basement membrane with disrupted and fragmented lamella

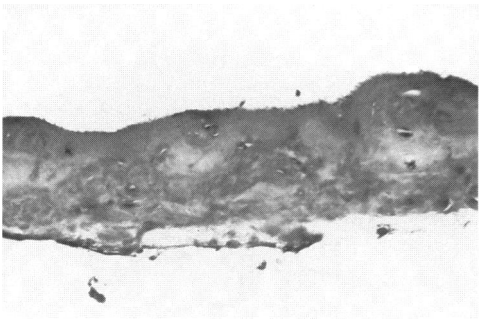

FIGURE 4
Amniotic membrane stripped of epithelium. Basement membrane is minimally disturbed by histologic examination. Grade IV for epithelium and grade I for basement membrane.

rabbit investigation.

Representative portions of the amniotic membrane with rabbit epithelium were studied histogically.

Acquisition and Preparation of Amniotic Membrane for Human Investigation

Amniotic membrane for human use was obtained from Bio-Tissue, Miami, Florida, and was stored at -80°C until use. The membrane was obtained from donor mothers who had been screened at delivery and again at 3 months for HIV-1, HIV-2, HTLV-1, and 2, HBsAg, HBcAb, HCV, and syphilis, as performed by BioTissue.

The human amniotic membrane (HAM) from BioTissue was thawed in 37°C water bath. The HAM was rolled onto a sterile 100 mm petri dish containing 15 mL PBS/1%ABAM. The filter paper was removed, keeping the epithelial side up.

The HAM from Bio-Tissue was prepared as described above to remove the amniotic epithelium. After the aforementioned investigations it was determined that trysinization for 15 minutes followed by gentle scraping was satisfactory for removing the amniotic membrane epithelium with the least damage to the underlying basement membrane (Figs 2 and 3). Following removal of the amniotic epithelium, the expanded corneal epithelial cell population for rabbit or human transplantation was grown onto the amniotic membrane as follows: The HAM was rinsed 3 times with PBS/1% ABAM. In the fourth rinse bath, the HAM was applied to a circular sterile stainless steel mesh with a 1.5 x 1.5 cm square cut into the center of the mesh. The center of the membrane was placed over the center of the mesh.

The cell growth techniques were identical through Step I as detailed

in the above section on the preparation of cultured corneal epithelial grafts. Then, the corneal cells, suspended in 0.5 mL GM, were inoculated onto the center of the HAM. The optimal number of corneal cells was 1.5-3 x10^6 cells. Two mL of GM was added to the dish. The dish with the HAM is then placed into a CO_2 incubator at 37°C. The HAM was kept covered with GM to prevent drying. More cells were plated if necessary. The medium was changed every 2 days. One or 2 days before grafting, the medium was changed to one without ABAM or cholera toxin. The cells were allowed to attach before human transplantation for 10 to 14 days. Before grafting, the medium was aspirated and the graft washed 3 times with 7 mL PBS, aspirating between washes. After the final wash, 7 mL of unsupplemented DMEM was added to the dish. The dish was placed in an incubation chamber and purged with 95%O_2/5%CO_2. The graft is then ready for transplant.

ANIMAL MODEL

The entire animal investigation was approved and supervised by the university experimental animal control committee. All experiments satisfied the ARVO recommendations for the humane treatment of animals.

Six male and 6 female unrelated nonpigmented New Zealand adult rabbits (2.8-3.2 kg) were anesthetized with ketamine/xylazine/buprenorphine following the ARVO recommendations for the ethical treatment of animals. Once satisfactory anesthesia had been obtained, each rabbit received a drop of half-strength betadine to the conjunctival sac of each eye for antibiosis. Each rabbit underwent a procedure similar to the following: Balanced salt solution was injected beneath the superior conjunctiva of the right eye (OD) adjacent to the limbus to elevate the conjunctiva in order to allow dissection of the limbal epithelium, which was removed and saved for later growth. The remainder of the entire limbal tissues extending 3 mm posteriorly from the limbus 360° was excised to remove, as much as possible, the putative stem cells. All corneal epithelium was removed by scraping. Following removal of the corneal and limbal epithelia and associated limbal tissues, hemostasis was maintained. N-Hepanol was then applied for 60 seconds to the entire limbus, with the treatment beginning at the superior limbus with the application of a moistened applicator stick and circling the limbus 360°. All limbal stem tissues were marked according to the rabbit number. The postoperative course was benign, although the animals did require and receive anagelsia for 48 hours.

Presumed rabbit corneal epithelial stem cell harvests were grown in the laboratory with the same techniques discussed above for human cells. Expanded corneal epithelial cells were grown onto amniotic membrane as

described above. Sufficient rabbit cells were obtained to create an allogeneic opposite-sex graft for each rabbit.

Amniotic membrane was harvested and depleted of amniotic epithelium in our laboratory as described above. This stored amniotic membrane was divided into squares measuring 25 mm along each edge. Expanded corneal epithelial cells were grown onto the basement membrane side or the smooth surface of the amniotic membrane where the amniotic epithelium had been previously.

Six weeks after the initial removal of the limbal epithelial stem cells and unilateral ocular surface injury, a second procedure was performed on these 12 rabbits. The animals were successfully anesthetized according to the ARVO recommendations for the ethical treatment of animals using ketamine, xylazine, and buprenorphine. Once successful anesthesia had been obtained, all rabbits were examined. One male and 1 female rabbit were euthanized as controls. The right and left corneal caps were enucleated and fixed. The right eyes were used control for the injury model, and the left eye of one rabbit was used as a normal control.

The remaining 10 rabbits (5 male and 5 female rabbits) were anesthetized, and each right eye received 1 drop of half-strength betadine for antisepsis. Each rabbit then had all ocular surface tissues again removed from the previously injured right cornea and limbus of the right eye. All rabbits had moderate to severe ocular surface injury. All epithelial tissues were removed to a point 4 mm behind the limbus. Hemostasis was maintained. Each rabbit then received an amniotic membrane transplant with cultured rabbit corneal epithelium adhering to the surface of the human amniotic membrane. The procedure was performed in the following manner.

The composite amniotic membrane with expanded allogeneic rabbit corneal epithelial cells lining the surface was transferred to the bare sclera and corneal stroma on a wire mesh with a central square opening measuring 15 x 15 mm. The edge of the membrane was gently swept from the periphery of the wire mesh to the bare stroma, leaving the membrane directly covering the stroma with the epithelium up. Because the epithelial cells initially had been placed centrally, the center of the membrane was aligned with the centre of the cornea. The membrane was divided in half horizontally and gently pulled superiorly and inferiorly, leaving a 6 to 8 mm opening within the palpebral aperture. The anterior edge of the superior portion of the composite graft was sewn to the superior peripheral corneal stroma. The posterior edge of the superior portion of the composite graft was trimmed to fit the resected and recessed conjunctiva and sewn to the conjunctival rim approximately 6 to 8 mm posterior to the superior limbus with 10-0 nylon. The grafts were sutured into place, leav-

ing the knots exposed because of the potential for tearing the membrane if the knots were turned beneath the stroma.

The anterior edge of the inferior half of the membrane was sewn to the cornea approximately 2 to 3 mm anterior to the inferior limbus, and the inferior edge of the inferior graft was sewn to the bare sclera because of the difficulty of reaching the recessed conjunctiva. The composite graft was sewn in place with 10-0 nylon, leaving the knots exposed. Each male rabbit received amniotic membrane with an epithelial cell culture of female cells from an unrelated donor, and each female rabbit received amniotic membrane with an epithelial cell culture of male cells from an unrelated donor. A contact lens was placed to help prevent the nictitans from damaging the composite graft. Subconjunctival Kenalog (20 mg) was given inferiorly to all rabbits.

On day 14 following the procedure, each rabbit was anesthetized for a complete ocular surface examination of the right eye. The results of this examination are summarized in Table VIII, and in the "Results" section. Five of the rabbits did not have contact lenses remaining in the operated eyes (1 male and 4 females), and 1 of these 5 appeared to have a corneal infection. These eyes all appeared to be, and were classified as, more inflamed than those of the other 5 rabbits. Because of these changes and the need for understanding the biology of the composite transplants, these rabbits were anesthetized and euthanized 2 days later (day 16) and are defined as Group I. The right eyes of these rabbits were enucleated and fixed. On day 14 the remaining 5 rabbits had their original contact lenses in place. Each had a clear cornea and no discernible epithelial defects, although the contact lenses were not removed; the eyes were not stained with fluorescein. All had dissolving, but still visible, composite grafts. These 5 rabbits (1 female and 4 males) were followed for an additional 14 days (until day 28 following the composite graft implantation), and then anesthetized, examined, and euthanized; they are defined as Group II. The right corneal caps were removed and fixed. The clinical examination is summarized in Table VII in the results.

These ocular tissues were examined histologically with H and E staining and with immunohistochemical staining for AE5, (ICN Biomedicals, Inc, Aurora, Ohio) and vimentin (Sigma, St Louis, Missouri).

RESULTS

HUMAN OCULAR SURFACE RECONSTRUCTION WITH CULTURED CORNEAL EPITHELIUM

The investigation involves 19 patients. The limbal corneal epithelial cells of 1 patient did not grow. We have performed cultured corneal epithelial

cell transplants on the 18 remaining patients to include 20 procedures using different carriers for different conditions as the surface disease required, and as the technique evolved. All 20 procedures were initially successful with no surgical complications. Three patients had unsuccessful results, 1 allogeneic patient had a partially successful procedure, and 1 allogeneic patient had a procedure with an undetermined result, as yet. These are summarized in Table VI.

Patient Reports

Case 1, AR. A 68-year-old man was referred in October 1994 with a large and severe recurrent pseudopterygium on his right eye (Fig 5). This lesion extended 5 mm onto the cornea from the temporal limbus, and the cord length along the temporal limbus was 10 mm. The lesion covered nearly

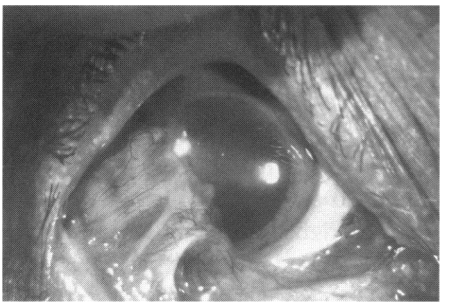

FIGURE 5
Case 1. Preoperative view of large pseudopterygium and symblepharon.

half of the corneal surface. He stated that he had had 10 operations on this eye, most recently 5 months before his initial visit. His best corrected vision was 20/60 with a nuclear cataract. He had moderate restriction of adduction (2 of 4 on a graded scale). He had an ipsilateral superior nasal limbal biopsy, which was expanded in culture. In November 1994, he underwent removal of this pseudopterygium, including a lamellar dissection of cornea and sclera. He received an 8 mm corneal lamellar graft straddling the limbus. An expanded corneal epithelial autograft was sewn over this lamellar carrier, with the posterior edge of the graft sewn into the resected edge of the conjunctiva, and the corneal edge sewn into the corneal stroma adjacent to the edge of the lamellar graft without directly involving the visual axis. (Figs 5 and 6) A therapeutic contact lens placed over the graft was removed at 2 months. At 6 months he, had some mild recurrence inferiorly along the edge of the graft extending 2 mm toward

TABLE VI: SUMMARY OF HUMAN AUTOLOGOUS TRANSPLANTATION

CASE NO.	TYPE OF GRAFT SOURCE OF CELLS	CARRIER	INDICATION	PRE OP VISION	POST OP VISION	LENGTH OF FOLLOW-UP (MO)	RESULT
AR 1	Autologous ——— Ipsilateral eye	Donor corneal lamellar graft Collagen gel	Recurrent pseudopterygium with restriction of adduction	20/60	20/400 13 (cataract)	13	Minimal recurrence Smooth surface No restriction
AR 2	Autologous ——— Ipsilateral eye	Donor corneal lamellar graft Collagen gel	Recurrent pseudopterygium, symblepharon,restriction	20/70	20/40	24	No recurrence Smooth graft No restriction
VH 3	Autologous ——— Contralateral eye	Collagen gel	Bulbar and palpebral CIN	20/30	20/25	16 (See No.14)	Healed epithelium Recurrence of CIN 2 yrs later One symblepharon (See No. 14)
MH 4	Autologous ——— Contralateral eye	Collagen gel	Alkali burn	HM	HM failed graft	12	Phthisis; failed graft 2nd to entropion / trichiasis
CV 5	Autologous ——— Ipsilateral eye	Donor corneal lamellar graft Collagen gel	Bilateral pterygia	20/25	20/25	5	No recurrence No restriction
DO 6	Autologous ——— Ipsilateral eye	Donor corneal lamellar graft Collagen gel	Recurrent pterygium with motility restriction, astigmatism	20/20	20/20	24	No recurrence
EA-OD 7	Autologous ——— Ipsilateral eye	Donor corneal lamellar graft Collagen gel	Recurrent pterygium into visual axis with motility restriction	20/400	20/30	10	No recurrence Smooth surface No restriction
EA-OS 8	Autologous	Donor corneal	Recurrrent pterygium into	20/400	20/30	6	No recurrence

TABLE VI (CONTINUED): SUMMARY OF HUMAN AUTOLOGOUS TRANSPLANTATION

CASE NO.	TYPE OF GRAFT SOURCE OF CELLS	CARRIER	INDICATION	PRE OP VISION	POST OP VISION	LENGTH OF FOLLOW-UP (MO)	RESULT
	lamellar graft Contralateral eye	visual axis Collagen gel				No restriction	
BW 9	Autologous ——— Contralateral eye	Collagen shield	Primary acquired melanosis, bulbar and palpebral surface of upper tarsus	20/25	20/25	18	Complete resurfacing No symblepharon Foreshortening of superior cul-de-sac
AD 10	Autologous ——— Ipsilateral eye	Donor corneal lamellar graft Collagen shield	Recurrent pterygium (multiple recurrence) Motility restriction to midline, diplopia	20/40	20/25	14	No recurrence Clean cornea No restriction No diplopia
DG 11	Autologous ——— Ipsilateral eye	Donor corneal lamellar graft Collagen shield	Recurrent pterygium Motility restriction	20/50	20/60	12	Recurrence of 3 mm No restriction, but considered unsuccessful
AB 12	Autologous ——— Ipsilateral eye	Donor corneal lamellar graft Collagen shield	Recurrent pterygium into visual axis Motility restriction	20/80	20/30	12	Improved vision No recurrence No restriction
HL 13	Autologous ——— Ipsilateral eye	Therapeutic contact lens	HZO neurotrophic ulcer, tarsorrhophy	CF 2 ft.	20/100 (Retinal damage)	9	Healed defect
VH #2 14	Autologous ——— Contralateral eye	Amniotic membrane	Bulbar CIN recurrence	20/200	20/100	8	Smooth surface No recurrence Complete
VO 15	Autologous	Amniotic membrane	Recurrent pterygium Motility restriction	20/20	20/20	8	Recurrence of ptergium but considered

TABLE VI (CONTINUED): SUMMARY OF HUMAN AUTOLOGOUS TRANSPLANTATION

CASE NO.	TYPE OF GRAFT SOURCE OF CELLS	CARRIER	INDICATION	PRE OP VISION	POST OP VISION	LENGTH OF FOLLOW-UP (MO)	RESULT
	Ipsilateral eye						unsuccessful No restriction
JB 16	Sibling (female) allograft Left eye	Amniotic membrane	Bilateral pseudopemphigoid and stem cell failure	CF	20/200	5	Epithelial resurfacing Infectious crystalline keratopathy Healed epithelium Partially successful
MS 17	Autologous Ipsilateral eye	Cells did not grow	Recurrent ptergium	20/20	--	--	Cells did not grow
JR 18	Autologous Contralateral eye	Amniotic membrane	Thermal chemical burn Pseudopterygium	Count fingers	20/30	3	No recurrence No symblephara Completely resurfaced
RM 19	Autologous Ipsilateral eye	Amniotic membrane Clear cornea	Pseudopterygium	20/25	20/25	2	Completely resurfaced No recurrence
LB 20	Sibling (female) allograft Left eye	Amniotic membrane	Alkali burn 1986,dense superficial vascularization	Hand motion	Hand motion	3	Lost contact lens Lost epithelim reapplication of cells with contact lens Undetermined
MJ 21	Autologous Ipsilateral eye	Amniotic membrane	Pseudopterygia	20/20	20/20	2	Successfully reepithelized, quiet eye Clear cornea

CF, count fingers; CIN, corneal intraepithelial neoplasia; HM, hand motion vision; HZO, herpes zoster ophthalmicus.

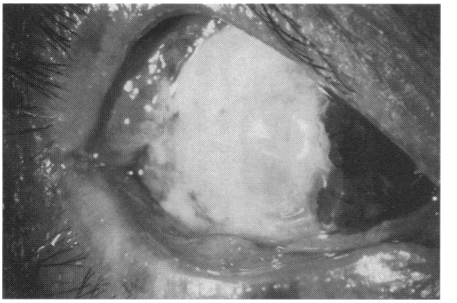

FIGURE 6
Case 1. Seven days postoperatively. Note edge of lamellar stromal graft and edge of composite graft.

the visual axis, but he had a clear visual axis and no restriction. His vision had worsened to 20/400 because of his cataract.

Case 2, AR. In January 1995, a 73-year-old Filipino man was referred for a large pterygium extending 5 mm toward the visual axis from the nasal limbus of his right eye. This had been previously resected. He had mild limitation of abduction (1 of 4 on a graded scale). His visual acuity was 20/70. In March 1995, he had resection of his pterygium and adjoining conjunctival tissues. He received a lamellar corneal stromal graft followed by a 10 mm expanded corneal epithelial autologous graft, which was sewn into place over the stromal graft. The posterior edges were sewn to the resected conjunctival edges near the nasal canthus, and the anterior edge was sewn into the cornea approximately 4 mm from the nasal limbus (Figs 7,8, and 9). A contact lens was placed over the epithelium. The contact lens was removed and the corticosteroids were stopped at 2 months. At 3 months, his vision was 20/40 with a cortical cataract believed to be responsible for the remaining visual loss (Fig 10). At 24 months, he had no recurrence and no motility restriction.

Case 3 and 14,VH. In March 1995, a 49-year-old Caucasian man presented with 270° of limbal involvement with conjunctival intraepithelial neoplasia (known postoperatively by pathological diagnosis) on the surface of the left eye (Fig 11). The limbal region between 12 and 3 o'clock appeared free of tumor. He had extension onto the lower palpebral surface to include the lower lid margin. His tumor extended nasally and superiorly approximately 6 mm behind the limbus. He had a history of a previously resected cutaneous squamous cell carcinoma on the lower lid of the same eye, as well as multiple squamous cell carcinomas on the facial skin. His vision was 20/30. We obtained a limbal biopsy from the superior lim-

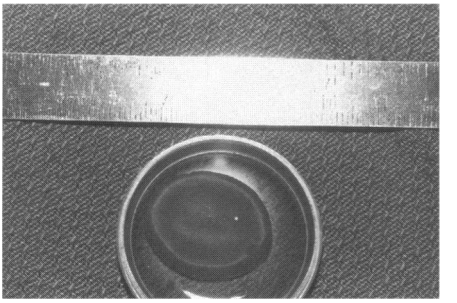

FIGURE 7
Composite graft used in case 2. Graft is slightly curled in small sterile plastic dish. Graft is approximately 25 x 20 mm.

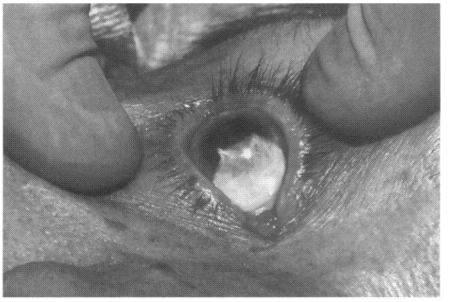

FIGURE 8
External intraoperative photograph of case 2 taken after composite graft sewn into place.

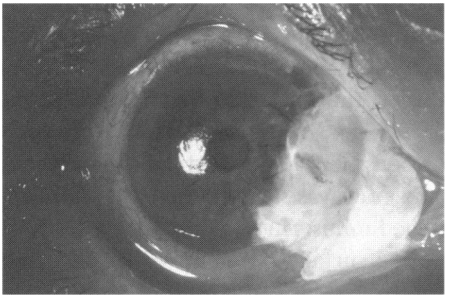

FIGURE 9
Case 2. Seven days postoperatively. Note edge of lamellar graft and edge of composite graft.

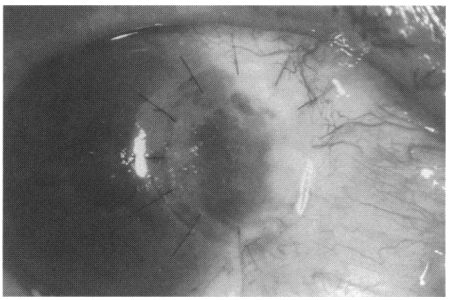

FIGURE 10
Case 2. Three months postoperatively. Lamellar stromal graft in place with unintentional sublamellar hemorrhage. Composite graft has dissolved, presumably leaving epithelial cells.

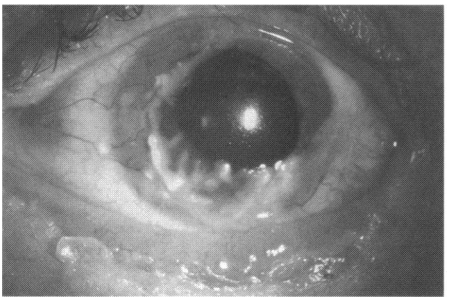

FIGURE 11
Case 3. Preoperative view. Note limbal involvement from 3 to 6 o'clock positions. Lower lid lash loss was due to previous excision of cutaneous squamous cell carcinoma. Conjunctival intraepithelial neoplasia involved palpebral surface and lower lid margin.

bus of the OD and expanded these tissues in vitro as discussed above. Four weeks later, in April 1995, we removed his bulbar and palpebral tumor and applied light cryotherapy and placed the expanded epithelial graft onto the bulbar and palpebral surfaces. An epithelial graft was placed to cover the denuded bulbar surface and sewn to the resected conjunctival edge superiorly, nasally, and temporally (Fig 12). The anterior edge of the superior portion of the graft was sewn to the superior peripheral cornea stroma. The anterior edge of the inferior portion of the graft was sewn to the inferior peripheral corneal stroma. The posterior edge of the inferior portion of the graft was sewn to the bare sclera approximately 9 to 10 mm posterior to the limbus. An additional expanded epithelial graft was placed on the palpebral surface of the lower lid and was sewn to the lid margin as well as approximately 7 mm inferior to the posterior edge of the lower lid margin on the palpebral surface. A therapeutic contact lens was placed on the cornea. The contact lens and corneal sutures were removed

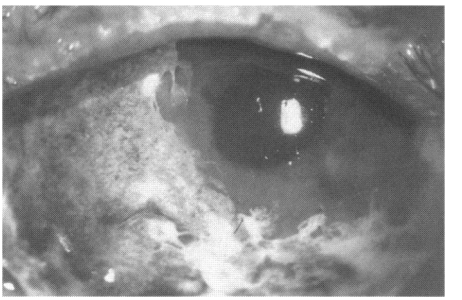

FIGURE 12

Case 3. One day postoperatively. Note composite graft straddling limbus (graft is somewhat hemorrhagic). Composite graft extends into cul-de-sac and onto palpebral surface of lower lids.

2 months later. His vision was 20/25. He had a smooth corneal surface with a mildly injected conjunctival surface but only one deep inferior temporal symblepharon (Fig 13). By April 1996, he had 20/25 vision with a clear smooth corneal surface and mildly injected conjunctiva and no additional symblephara. He had also developed peripheral nodules in the superior nasal peripheral cornea suggestive of Salzmann's nodular degeneration.

He did well until April 1998, when he presented with what appeared to be a recurrence of his conjunctival intraepithelial neoplasia 270° from 6 to 3 o'clock in a clockwise fashion. The previously uninvolved sector between 12 and 3 o'clock now appeared to have tumor involvement. Vision was 20/200. He had a biopsy of the right superior limbus and expansion of his limbal corneal epithelial stem cells. He had a repeat epithelial autograft atop amniotic membrane in May 1998. The limbal tumor and associated epithelial tissues were removed with excision to approximately 5 mm posterior to the limbus 360°. Additional suspicious conjunctival tis-

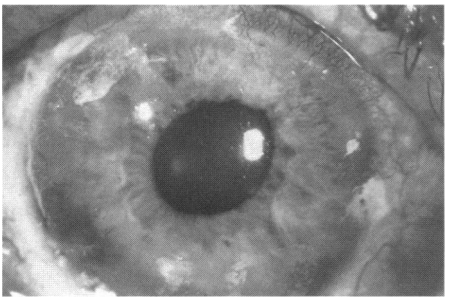

FIGURE 13

Case 3. Two to 3 months after surgery. Note that most of composite graft has dissolved, although remnants remain.

sue was removed from the inferior palpebral surface but was found to be only inflammatory tissue. Light cryotherapy was placed 360°. The epithelial graft, in a "doughnut" shape, was sewn to the edge of resected conjunctiva approximately 5 to 6 mm posterior to the limbus. The anterior edge of the amniotic membrane composite graft was sewn to the peripheral corneal stroma. A therapeutic contact lens was placed. Postoperatively, he was treated with mild corticosteroids. His epithelial surface remained epithelialized as the amniotic membrane gradually dissolved. Five months later, he appeared to be free of tumor and had complete re-epithelialization with no defect and no evidence of amniotic membrane. There was a distinct line of demarcation where the amniotic membrane had been sewn to the posterior edge of the conjunctiva. There remained only a single deep temporal symblepharon. Vision was 20/100.

Case 4 MH. A 38-year-old man sustained a grade 4 alkali burn to his left eye in May 1995. He developed multiple problems associated with this injury, including a chronic corneal epithelial defect. In June 1995, he had a conjunctival biopsy of the superior limbus of the contralateral eye as described above. The expanded epithelial autograft was sewn into place 4 weeks later after extensive removal of injured bulbar tissues. The graft was placed to include the area of the damaged epithelium. A therapeutic contact lens was placed. Over the next 3 months, he developed trichiasis and entropion, lost his therapeutic lens, and lost the epithelial graft. He subsequently developed phthisis and lost all vision in the eye. This was classified as unsuccessful.

Case 5, CV. In December 1995, a 39-year-old Asian man was referred with bilateral nasal pterygia. Both pterygia extended 4 mm toward the visual axis from the nasal limbus and appeared injected. By May 1996, growth was documented from the pterygium on his right eye (Fig 14). He underwent a biopsy with a harvest of 2 mm of limbal conjunctival tissues to include the presumed corneal epithelial stem cells for expansion in vitro. In June 1998, he underwent resection of his pterygium on the right eye with a lamellar corneal stromal graft placed astride the limbus. The anterior edge of the expanded autologous epithelial graft was sewn onto the clear corneal stroma in advance of the edge of the lamellar graft. The posterior edge of the graft was sewn into the resected edge of conjunctiva approximately 8 to 9 mm from the nasal limbus (Fig 15). A therapeutic contact lens was placed. The contact lens was removed 2 months later, and examination 3 months after surgery showed that the eye was quiet with mild injection of the conjunctiva posterior to the resected edge but no evidence of recurrence of the pterygium. He was lost to follow up after that visit.

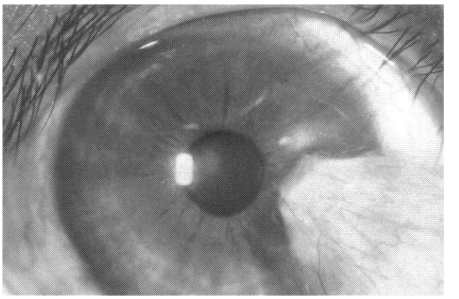

FIGURE 14
Case 5. Preoperative view of nasal pterygium.

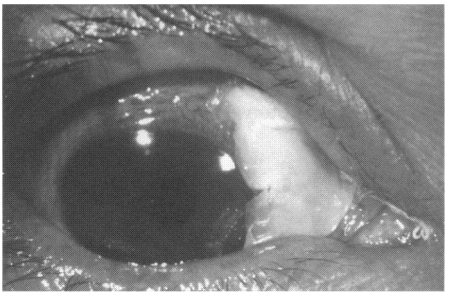

FIGURE 15
Case 5. One week postoperatively with composite graft in place.

Case 6 DO. In April 1996, a 45-year-old Caucasian man was referred for a rapidly recurring (second recurrence) pterygium on his left eye extending 4 mm toward the visual axis from the nasal limbus with an 8 mm base at the limbus. His visual acuity was 20/20. In May 1996, after successful growth of his ipsilateral limbal stem cell biopsy, he underwent resection of his pterygium and implantation of a corneal stromal lamellar graft straddling the nasal limbus with an expanded corneal epithelial stem cell graft overlay. This epithelial autograft measured about 9 mm and was sewn into the resected edge of the conjunctiva approximately over the insertion of the medial rectus and into the corneal stroma at about 4 mm toward the visual axis from the nasal limbus at the edge of the lamellar graft. A therapeutic contact lens was placed. In 2 months, his contact lens was removed and the corticosteroids were discontinued. At 3 months and at 2 years, he had no sign of recurrence. He did have injection over the medial rectus nasal to the conjunctival wound edge. Vision remained 20/20.

Cases 7 and 8, EA. In May 1996, a 57-year-old Hispanic woman was seen

with recurrent bilateral pterygia (2 pterygia on OD and 1 pterygium on OS) (Fig 16). The nasal pterygia in each eye had been resected previously with prompt recurrence and obstruction of the visual axis to the 20/400

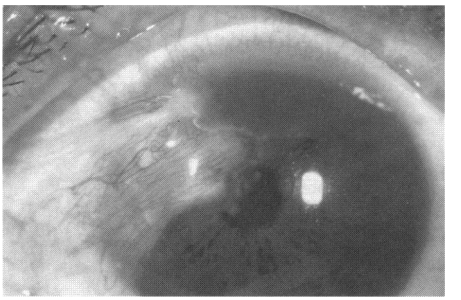

FIGURE 16
Case 7. Preoperative view of recurrent pterygium.

level. Each eye had moderate (2 of 4 on evenly divided scale) restriction of abduction. The limbal conjunctiva was harvested from the right eye and expanded in vitro. In June 1996, (4 weeks later) she had a resection of the nasal pterygium, and a lamellar corneal graft (as a stromal carrier) was placed with an expanded corneal epithelial autograft overlay sewn atop the carrier and bare sclera of left eye (Fig 17). A therapeutic soft contact lens was placed. In 6 weeks, the contact lens was removed, and her topical corticosteroids were tapered. In September 1996, she had a similar procedure performed on the left eye, but this eye had 2 simultaneous procedures with an expanded epithelial autograft sewn in place over the carrier on both the nasal and temporal horizontal limbus. At 6 weeks following the second set of procedures, the second therapeutic lens was removed. Within 5 months her vision had improved to 20/30 in each eye with a clear visual axis and clear intact nonstaining corneal epithelium (Fig 18). There were no recurrences of the pterygia, although injection persisted in the palpebral aperture posterior to the lamellar graft. She was lost to follow-up.

Case 9, BW. In June 1997, a 70-year-old Caucasian woman presented with extensive primary acquired melanosis (PAM) extending across 80% of the superior bulbar surface into the superior cul-de-sac and extending onto the palpebral surface of the upper tarsus, and even onto the margin of the upper lid. A 2 mm^2 limbal biopsy was obtained as described above. Four weeks later, after successful expansion of the corneal epithelial cell culture, she underwent removal of the bulbar conjunctiva, including much

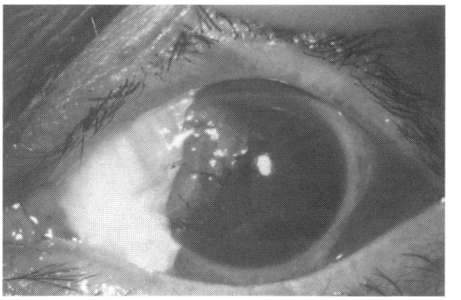

FIGURE 17
Case 7. One day postoperatively. Lamellar graft edge and composite graft edge both visible.

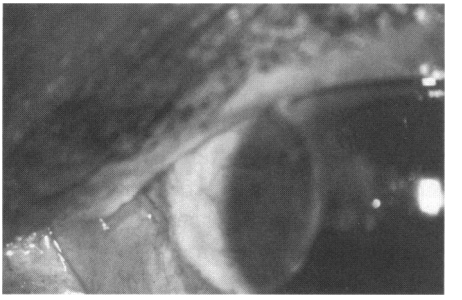

FIGURE 18
Case 7. At about 5 months postoperatively, nasal limbus is clear.

of the superior limbus and palpebral surface of the upper tarsus to remove the epithelium with acquired melanosis. The expanded epithelial autograft on a collagen shield was placed atop the denuded bulbar, but not palpebral, surface and sewn into place using sutures into the upper edge of the superior cul-de-sac. A therapeutic contact lens was placed. Postoperatively, she was treated with topical steroids, and the therapeutic contact lens remained in place for 6 weeks. At that time, all sutures were removed. By 3 months, her operated left eye was quiet with normal-appearing conjunctival and corneal epithelium. Vision had returned to her preoperative level of 20/25. She had some foreshortening of the upper cul-de-sac. By 18 months, she had some bulbar recurrence of the PAM, which has been subsequently resected, but the cornea remains clear without staining, and the superior cul-de-sac has remained unchanged.

Case 10, AD. In August 1997, a 64-year-old Caucasian woman was seen with a severe recurrent nasal pterygium on the right eye extending 4.5 mm onto the cornea with restriction of abduction (3 of 4 on an equally divided scale) (Fig 19). She had diplopia in abduction from her pterygium but had

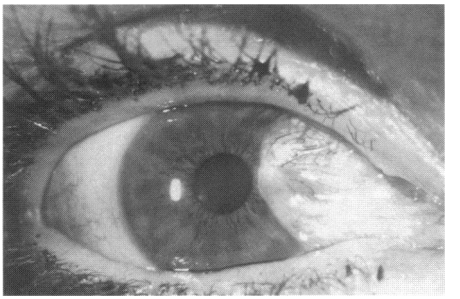

FIGURE 19
Case 10. Preoperative view of recurrent nasal pterygium (with significant motility restriction).

20/40 vision. She underwent a superior limbal conjunctival biospy from the OD which was expanded in vitro. Four weeks later, she underwent resection of the pterygium with a lamellar corneal graft placed astride the limbus. A corneal epithelial autograft grown on a collagen shield was sewn into place to cover the nasal cornea to the edge of the resected conjunctiva. A therapeutic contact lens was placed, and she had been treated with mild corticosteroids for 6 weeks when the contact lens was removed. Within 3 months, she had 20/25 vision and full motility (Fig 20). At 1 year, she continued to have a clear cornea with no recurrence of the pterygium,

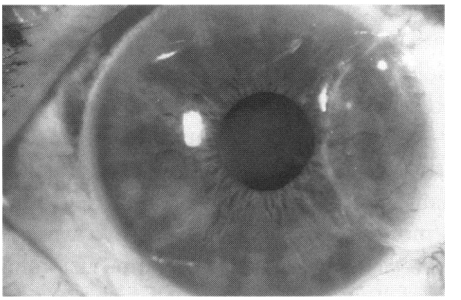

FIGURE 20
Case 10. Postoperative view at about 4 months. Note edge of lamellar stromal graft with sutures removed. Composite graft has dissolved.

and full motility (4 of 4 on a divided scale), but she did have distortion of the nasal conjunctiva where the edge of the resected conjunctiva had been.

Case 11, DG. In November 1997, a 37-year-old black man was referred for a recurrent pterygium on the nasal aspect of his left eye. This had been removed 2 years earlier, and he had beta irradiation. The recurrence extended 4 mm onto the cornea toward the visual axis from the nasal limbus. His best-corrected visual acuity was 20/50, but motility was full. After biopsy of his ipsilateral superior temporal limbus, his limbal corneal epithelial stem cells were grown in vitro. In January 1998, he had resection of his pterygium and placement of a lamellar stromal graft straddling the nasal limbus. He received an expanded corneal epithelial graft approximately 9 mm in diameter grown on a collagen shield. This graft extended from the resected edge of the conjunctiva medially to the leading edge of the lamellar stromal graft in the peripheral cornea and was sewn into place. The collagen shield had melted to a large degree and the remaining tissues were very difficult to apply. A therapeutic contact lens was placed. At approximately 2 months the therapeutic contact lens was removed but the corticosteroids were continued for an additional 2 months because of chronic inflammation. Within 4 months he had a recurrence of 3 mm onto the cornea over the lamellar graft but no restriction. He maintained the conjunctival injection in that quadrant. He has had no further change in his recurrence for 1 year. This procedure was deemed unsuccessful because of a recurrence of the pterygium. The collagen shield was nearly melted at the time of surgery, and it is believed that the cells did not adhere.

Case 12, AB. In October 1997, a 71-year-old Filipino woman was referred with a severe recurrent pseudopterygium on her left eye. She had had multiple procedures with at least 3 attempts at removal in the United States. She had dense scarring of the medial half of the cornea, including a large pseudopterygium that extended 6 mm onto the cornea from the nasal limbus, involving nearly the entire medial half of the cornea (Fig 21). She had diplopia in primary gaze with an left esotropia of 4 to 5 prism diopters. She had marked restriction of abduction of the left eye (3 of 4 on a graded scale). Her best-corrected visual acuity in the left eye was 20/80. In January 1998, after ipsilateral superior limbal biopsy and in vitro expansion of the putative stem cell population, she had complete removal of her pseudopterygium with an 8 mm lamellar corneal stromal graft placed astride the nasal limbus. An expanded corneal epithelial autograft was placed atop the lamellar stromal graft on a collagen shield carrier and sewn into place directly over the cornea. A therapeutic contact lens was placed. After 2 months, her therapeutic contact lens was removed. Corticosteroids were continued for 5 months because of chronic injection, but there was

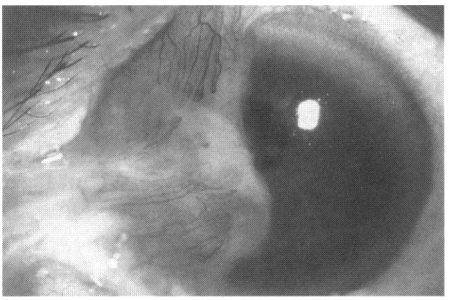

FIGURE 21
Case 12. Preoperative view of large pseudopterygium.

no recurrence. By 7 months, her best-corrected visual acuity was 20/30, although she did have irregular astigmatism secondary to the lamellar graft. Nevertheless, at 1 year she had no signs of corneal recurrence and she had complete return of normal abduction. The nasal aspect of her conjunctiva remains injected beyond the edge of the lamellar graft.

Case 13, HL. A 91-year-old man was referred in January 1998 with a neurotrophic ulcer in the left eye secondary to previous herpes zoster ophthalmicus. He had been struggling with this ulcer intermittently for 4 years. He had had multiple medications, lubricants, and a 40% tarsorrhaphy, all of which had helped for different periods of time. When he was seen, he had hand-motion vision with a large neurotrophic ulcer measuring 4 to 5 mm horizontally and 3 mm vertically (Fig 22). He was treated with a variety of agents, including a therapeutic contact lens, without success. Prior to a conjunctival flap, he was offered an expanded epithelial autograft. He consented and had a 2 mm^2 biospy of his ipsilateral superi-

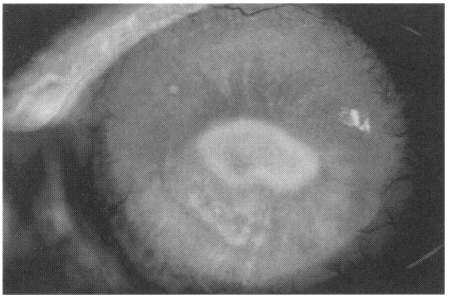

FIGURE 22
Case 13. Neurotrophic ulcer with fluorescein stain just prior to application of contact lens.

or temporal limbal conjunctiva. His presumed corneal epithelial stem cells were grown and placed on the concave surface of a therapeutic contact lens, which was placed on the surface of his eye 4 weeks later. His neurotrophic ulcer healed within 24 hours. The contact lens was left in place. His contact lens spontaneously came out 2 months later, leaving him with a 2 mm oval epithelial defect (less than half the original size). A replacement contact lens provided prompt healing of his neurotrophic ulcer. His contact lens was removed at 6 months with no epithelial defect, and at 9 months, his epithelium remained healed and his visual acuity was 20/100.

Case 14. See Case 3 for details.

Case 15, VO. A 42-year-old Hispanic woman was referred in July 1998 with a recurrent pterygium on her left eye. She had had 2 previous procedures, in April 1996 and again in March 1998 with prompt recurrence. She had diplopia in her left gaze and moderate restriction of abduction (2 of 4 on a graded scale). Her nasal pterygium measured 3 mm onto the cornea toward the visual axis but was quite taut in abduction. She underwent a biopsy of her superior temporal limbus with growth of her presumed limbal corneal epithelial stem cells. Four weeks later, she underwent resection of her pterygium to approximately 6 mm posterior to the limbus. She had an amniotic membrane graft with expanded corneal epithelial cells placed over the corneal stroma and the exposed sclera (Fig 23). The amniotic membrane was sewn to the resected conjunctival edge posteriorly and onto the peripheral cornea at the nasal limbus. A therapeutic contact lens was placed. At the 1-month visit, the therapeutic contact lens was not present, although the duration of wear is unknown. At that time, her vision was 20/20 with no restriction, and complete epithelialization with no signs

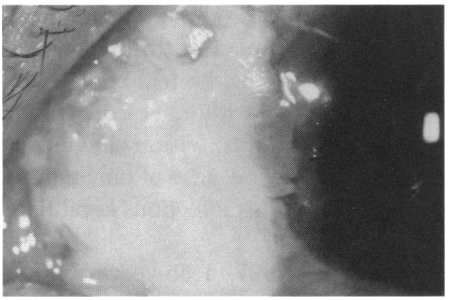

FIGURE 23

Case 15. One day postoperatively with composite graft of amniotic membrane and expanded autologous epithelial cells in place.

of recurrence. At 4 months, she had vascularization of the amniotic membrane graft and recurrence of the pterygium, although there was no restriction of motility at this time. This was judged to be unsuccessful with recurrence of the pterygium, although she did not have any restriction of motility.

Case 16, JB. A 73-year-old Caucasian man was referred in October 1995 with pseudo-pemphigoid and stem cell failure believed to be secondary to chronic glaucoma medications. His ocular surface was worse on the right eye than the left eye with count fingers and 20/60 visual acuity, respectively. The surface of his right eye showed conjunctivization and vascularization of the surface. In July 1998, his sister had a biopsy of her superior temporal limbus of the superior temporal limbus of her left eye. Her presumed corneal epithelial stem cells were grown atop amniotic membrane, and 5 weeks later in August 1998, they were transferred to the patient. At that time, the patient had complete resection of the conjunctiva of the right eye to 4 mm posterior to the limbus 360°. The amniotic membrane was sewn onto the resected edge of the conjunctiva 4 mm behind the limbus, and a 6 mm circular opening was cut into the center of the amniotic membrane. The corneal or anterior edge of the composite graft was sewn into place with sutures to the corneal stroma at the anterior edge, leaving a 6 mm clear visual axis and bare stroma. A therapeutic contact lens was placed. At 24 hours, he had begun to re-epithelialize, and his vision was 20/100. Topical corticosteroids, topical cyclosporin A, and oral cyclosporin A treatment was begun.

At 4 weeks, he developed infectious crystalline keratopathy in the central 3 mm zone, but the epithelium surrounding this area was clear and intact. The amniotic membrane had retracted, leaving an 8 mm clear zone. Cultures of the central cornea yielded *Staphylococcus* species, *Streptococcus virdans,* and *Cornynebacterium* species, and he was treated appropriately. Cyclosporin A was discontinued, and the corticosteroids were reduced. Over the next 3 months, this corneal defect slowly healed and re-epithelialized. At 5 months, he had completely re-epithelialized. He had clear corneal epithelium without vascularization, and a hazy stromal scar from the infectious keratitis. Vision had improved to 20/200. He is considered partially successful because of only minimal visual improvement and the corneal scar from his infectious keratitis.

Case 17, MS. In novenber 1997, a 55-year-old Hispanic woman was referred because of a recurrent pterygium on the left eye. This pterygium had been resected twice previously, and on the second excision had had β irradiation. The pterygium extended 4 mm onto the cornea from the nasal

limbus. Her visual acuity was 20/20. She had an ipsilateral superior temporal limbal biopsy in the standard manner of previous patients for an eventual expanded epithelial cell transplant. Her limbal stem cells did not grow in vitro, however. While waiting for her cells to grow, she lost funding for her procedure and was lost to follow-up. No procedure was performed.

Case 18, JR. A 40-year-old Caucasian man was referred in July 1998 with a severe prolonged thermal burn of his right eye caused by molten metal. He had ischemia of the nasal, inferior, and superior limbus and sclera with evidence of ischemia extending from 12 to 8 o'clock. He had marked corneal stromal edema and a small hyphema. His wound was treated as an alkali burn and retained an epithelial defect for 6 weeks, with evidence of stem cell failure. By 3 months, he had a marked pseudopterygium to include the nasal 180° of his cornea. He had inferior and superior symblephara with marked restriction of adduction (3 of 4 on a graded scale) (Fig 24). His visual acuity was count fingers. His eye gradually quieted, and in September 1998, he had a 2 mm^2 limbal conjunctival biopsy of the contralateral eye to include the presumed stem cells. These were grown in vitro and placed atop the amniotic membrane. In October 1998, he underwent a procedure to remove the pseudopterygium and symblephara, and to clear the bulbar and palpebral surfaces of scar tissue. A large amniotic membrane graft with expanded epithelial stem cells was placed and sewn posteriorly to the resected edge of the conjunctiva at approximately 11 mm from the nasal limbus and 8 to 9 mm from the limbus superiorly and inferiorly. The anterior edge was sewn onto the peripheral corneal (Fig 25). A therapeutic contact lens was placed. At 3 months, his contact was removed and he had a visual acuity of 20/30 with no restriction and no

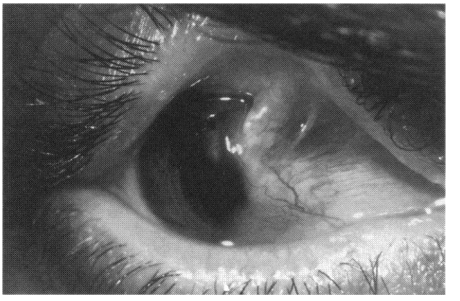

FIGURE 24

Case 18. Preoperative appearance of severe pseudopterygium and upper-lid ankyloblepharon following sustained thermal burn.

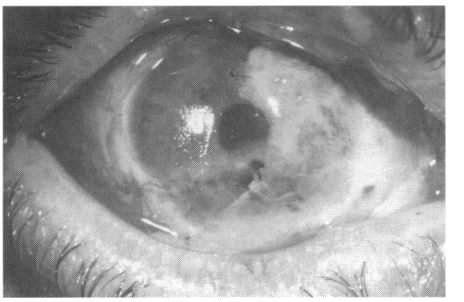

FIGURE 25
Case 18. One day postoperatively. Edge of composite graft at edge of nasal pupillary border can be seen.

sign of recurrence. He had some amniotic membrane remaining on the corneal surface and complete re-epithelialization (Fig 26). He still had some persistent injection restricted mostly to the conjunctiva posterior to the amniotic membrane graft.

Case 19, RM. In July 1998, a 33-year-old Hispanic man was referred with a recurrent pseudopterygium on his left eye, extending from his inferior nasal cul-de-sac with a symblepharon extending across the bulbar conjunctiva and 4 mm onto his cornea toward his visual axis. He had restriction of adduction (3 of 4 on a graded scale) in his left eye (Fig 27). He had a 2 mm^2 limbal conjunctival biopsy to include his presumed stem cells in September 1998. These cells were grown atop amniotic membrane in vitro. In October 1998, he had a procedure to remove his pseudopterygium and symblepharon and place the composite graft over the bare stro-

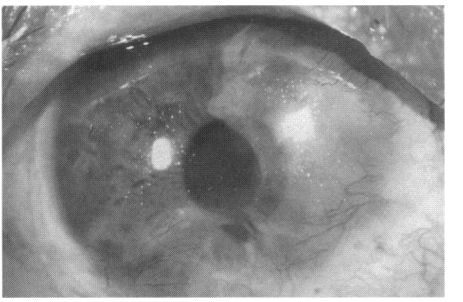

FIGURE 26
Case 18. Three months postoperatively with sutures removed. Edges of dissolving amniotic membrane can be seen. Peripheral corneal vascularization is seen in mid stroma and could not be removed with superficial dissection. Note that amblyoblepharon has been relieved.

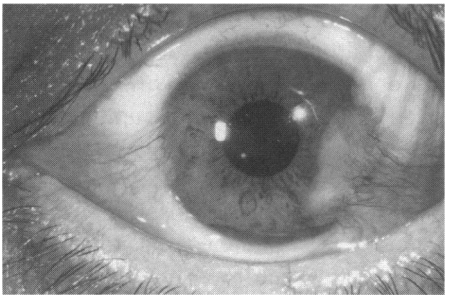

FIGURE 27

Case 19. Preoperative appearance of left eye. Note mild nasal pterygium and moderate temporal pseudopterygium with symblepharon.

ma. The posterior edge was sewn into place approximately 10 mm posterior to the limbus, and the anterior edge was sewn into place onto the corneal stroma in the peripheral cornea (Fig 28). A therapeutic contact lens was placed. Two months later, the contact lens was removed, and 3 months later, he had a clear cornea with no sign of recurrence or restriction and his 20/25 preoperative vision had returned. His left eye was relatively quiet, but there was persistent injection in the inferior cul-de-sac and in the temporal palpebral aperture. The injection was especially noted posterior to the edge of the previously placed amniotic membrane graft. Most of the amniotic membrane had dissolved, and his cornea was completely re-epithelialized.

Case 20, LB. A 46-year-old Caucasian man was referred in September 1998 with a history of a bilateral alkali burn. He had had multiple surgeries on each eye, with the right eye retaining a successful corneal transplant

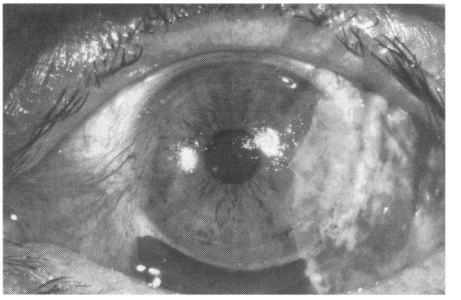

FIGURE 28

Case 19. Postoperative appearance at 1 week. Composite graft is somewhat hemorrhagic. Restriction and symblepharon are relieved.

and 20/30 vision with marked conjunctival distortion and limbal damage. His left eye had had multiple corneal transplants that had failed, leaving him with complete opacification of the cornea and hand-motion vision. One week later, a 2 mm^2 limbal conjunctival epithelial biopsy was obtained from his sister and expanded in vitro. Four weeks later, after successful expansion of his sister's presumed limbal corneal epithelial stem cells, he had a surgical procedure to remove all surface epithelium and surface vascularization. An amniotic membrane composite graft with epithelial stem cell overlay was sewn into place to cover his entire cornea and limbal region to 5 mm behind the limbus 360°. His cornea was completely opacified, and because the intent was to create a normal surface for later corneal transplantation, no central opening for the visual axis was made. This composite graft was sewn onto the resected conjunctival edge posteriorly, and a therapeutic contact lens was placed. At 24 hours, the contact lens and the epithelial cells were in place. Treatment with topical corticosteroids and topical cyclosporin A was begun. Oral cyclosporin A was to begin at 48 hours, but the patient neglected to fill the prescription. Between days 1 and 4 the contact lens was lost, because on his day 4 visit, he had only a peripheral rim of epithelium on the amniotic membrane and no contact lens. His sister's cells were regrown in the laboratory, placed onto a collagen shield, and 6 weeks later placed onto his ocular surface, including the amniotic membrane. A therapeutic contact lens was placed. At his 1-month examination, the therapeutic contact lens remained, and he was completely epithelialized. If the epithelium is retained, a corneal transplant may be planned in 6 months. This procedure is deemed a partial failure, but the ultimate fate of his epithelial graft and the epithelial cells is still undetermined.

Case 21, MJ. A 46-year-old Caucasian man was referred in July 1998 with pseudopterygia on the nasal and temporal aspect of his right globe with extension inferior temporally. The entire inferior 180° had some degree of peripheral vascularization and pannus. These pseudopterygia extended 3 to 4 mm toward the visual axis from the limbus and were injected. He was treated with doxycycline, bacitracin, and topical corticosteroids, which did not improve the pseudopterygia, although injection decreased. In October 1998, he had a biopsy of his superior temporal limbus of his contralateral eye. This 2 mm^2 biopsy was cultured and the presumed corneal epithelial stem cell population expanded. The corneal epithelial cells were grown onto amniotic membrane. Four weeks later, he underwent an excision of the nasal and temporal pseudopteryia with implantation of amniotic membrane with expanded autologous corneal epithelial cells. The posterior edge of the graft was sewn onto the resected edge of the conjunctiva for 180° of

the nasal, inferior, and temporal quadrants approximately 6 to 7 mm behind the limbus circumferentially. The anterior margin was sewn into place onto the corneal stroma in the peripheral cornea. A therapeutic contact lens was placed. The lens and sutures were removed at 2 months, and at that time his vision remained 20/20 with no signs of recurrence and mild injection over the host conjunctiva peripherally (Fig 29).

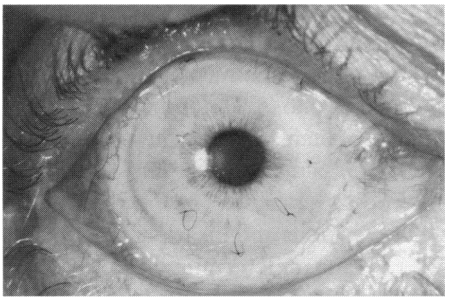

FIGURE 29

Case 21. Postoperative appearance at 2 months showing distribution of nasal, inferior, and temporal application of composite graft. Most of composite graft has dissolved, but anterior corneal edge can be seen at the sutures and posterior edges can be seen approximately 4.5 mm posterior to limbus.

Summary of Human Results

Growth of cultured corneal epithelial stem cells was successful in 18 of 19 patients included in the investigation (18 patients, 19 eyes, and 20 transplantation procedures). In these 18 patients, the limbal epithelial cells (presumed corneal epithelial stem cells) grew rapidly, and within 4 to 5 weeks, produced enough corneal epithelial cells for an expanded autologous epithelial transplant of approximately 25 to 35 mm in diameter with 3 to 5 epithelial cell thickness. This size graft was sufficient to cover most of the bulbar conjunctival surface, especially that adjacent to the corneal limbus of the operated eye. There were no complications at the biopsy site of any autologous or allogeneic donor.

One of the patients had a biopsy that did not produce expanded epithelial cells, although there was no known laboratory problem with these cells. All other autologous and allogeneic biopsies grew well.

The ocular surface appeared to benefit in 15 of the 20 transplantation procedures. No permanent complication appeared because of the procedure although one patient considered partially successful had infectious keratitis which delayed healing. Three procedures were unsuccessful,

one was partially successful, and one remains undetermined.

Nine of these 20 procedures (8 patients) used a lamellar corneal stroma as a carrier, and these almost could be considered as autologous keratoepithelioplasties. These lamellar grafts were 6 to 8 mm and were placed onto a dissected lamellar bed. The autologous expanded epithelial graft was placed on top of bare corneal stroma/sclera and sewn into place much like a free conjunctival graft. One of the pterygia (case 11) recurred across this lamellar graft and was considered unsuccessful. This was believed to be due to melting of the collagen shield, which was the carrier of the epithelium. The remaining 8 were successful and, in most cases, had full return of motility. One of the 7 amniotic membrane/expanded epithelial cell grafts was unsuccessful, one of the amniotic membrane grafts was partially successful, and one remains undetermined. In the unsuccessful procedure (case 15), the therapeutic contact lens was lost between the second and fourth weeks postoperatively. In the second procedure (case 20), the therapeutic soft contact lens and his expanded epithelium were lost by day 4, and his result remains undetermined.

There were 2 expanded epithelial grafts using only collagen gel as a carrier, and 1 of these failed because of mechanical reasons (case 5). There were 4 grafts using a collagen shield as the carrier with 1 of these considered unsuccessful because of the collagen shield. There was 1 composite graft using a therapeutic contact lens, believed to be successful (case 13). There were 7 composite graft procedures using amniotic membrane, and 4 were believed to be successful. One procedure was considered unsuccessful (case 15), one composite amniotic membrane graft was partially successful, and the remaining composite amniotic membrane graft is undetermined at present (case 20) (summarized in Table VI) (unsuccessful patients summarized in Table VII).

TABLE VII: PRESUMED CAUSE FOR FAILURE°

Stem cell growth failure in vitro	1
Lost contact lens/lost epithelium/melted carrier	3
Mechanical loss of cells (trichiasis/entropion)	1

°Causes of failure from all 19 cases admitted to study. Cells failed to grow in 1 patient and were unsuccessful, partially successful, or undetermined in 4 patients.

PREPARATION OF AMNIOTIC MEMBRANE

The results indicated that 15 minutes of sonification followed by trypsinization followed by scraping removed all epithelium (score IV for epithelium) yet left the basement membrane appearing histologically nor-

mal (score I). Trypsinization at 15 minutes followed by scraping also provided the same score and result (Table III) and was much simpler. The simplest, most effective method was trypsinization for 15 minutes followed by gentle scraping. This represents the best basement membrane preservation with complete removal of amniotic membrane (Tables III, IV, V) (Figs 3, 4, 30, 31). Following confirmation of the removal of amniotic epithelium and preservation of basement membrane, expanded epithelial cells were grown atop the bare amniotic membrane. Selected portions of

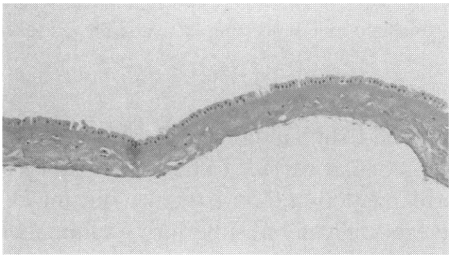

FIGURE 30
Normal amniotic membrane with amniotic epithelium present (hematoxylin and eosin, x110).

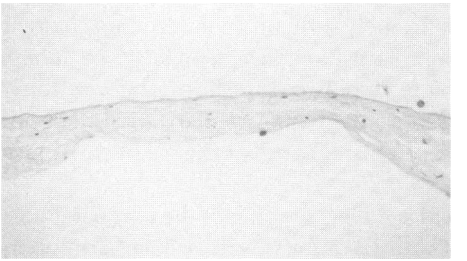

FIGURE 31
Amniotic membrane with epithelium having been removed by 15 minutes of trypsinization (hematoxylin and eosin, x110).

amniotic membrane with expanded epithelial cells were examined histologically to confirm that the expanded epithelium had become adherent to the amniotic membrane (Fig 32).

ANIMAL MODEL

All rabbits underwent similar surgery with removal of the limbal tissues and application of n-heptanol as discussed above. Clinically, all 6 male and

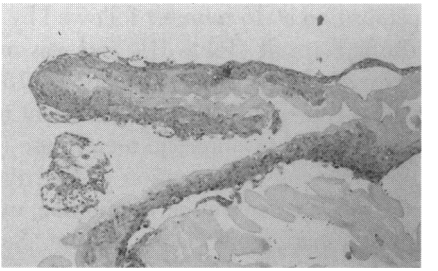

FIGURE 32
Amniotic membrane with adherent human corneal epithelium. This representative piece was taken from composite graft used for case 15. Note the multilayered epithelium that has not been dislodged by the folding during processing (hematoxylin and eosin, x60).

all 6 female rabbits had similar injuries, with evidence of moderate to severe damage to the ocular surface of the right eye, including superficial and deep neovascularization and an irregular but intact epithelium with subepithelial haze especially noted superiorly, (summarized in Table VIII; scoring system in IX, X, and XI).

Clinical Evaluation

Some variability was noted, and a scoring system was devised to assess each cornea (Table IX and X). Clinically, the stromal level of the neovascularization was extremely difficult to determine, so no attempt was made to do so. All neovascularization was assumed to be subepithelial. In retrospect, after review of the histologic slides, this was found not to be true. Some rabbits had more deep neovascularization than others, but this was difficult to assess even histologically because of variability of different sections.

Each rabbit had a clinical grading of the corneal injury with differences noted (Table VIII). All 12 rabbits were evaluated preoperatively, and all 10 rabbits that received a composite graft were evaluated postoperatively, on this scale. As mentioned above, the group enucleated on day 16 is defined as Group I, and the group enucleated on day 28 is defined as Group II. Each rabbit in Group I was re-evaluated on day 14 and again before enucleation on day 16. Each rabbit in Group II was evaluated at day 14 and on day 28 at sacrifice (Table VIII).

No rabbit had significant (greater than 4 mm toward the visual axis) neovascularization of all 4 quadrants, but all rabbits had some degree of vascularization of all 4 quadrants. In each rabbit, the most intense damage in terms of vascularization and corneal haze occurred superiorly.

In Group I, on evaluation on day 14, all 5 rabbits had lost their thera-

TABLE VIII: EVALUATION OF RABBIT MODEL

RABBIT	PREOP°	POSTOP DAY 14†	POSTOP DAY 28‡	AMNIOTIC MEMBRANE IN PLACE	CONTACT LENS IN PLACE	IMMUNOLOGIC STAINING AE5§	H&E HISTOPATH¶	SUMMARY
1	D3	Control	Control	Control	Control	5%	III-IV	Control††
2	C3	Control	Control	Control	Control	5%	IV	Control††
3	C2	X	-	No	No	0%	IV	Complication
4	D3	C3	-	Yes	No	5%	II-III	Failure
5	D3	C3	-	Yes	No	10%	III-IV	Failure
6	C3	B2	-	Yes	No	50%	I-III	Partial failure
7	C3	B3	-	Yes	No	50%	II	Partial failure
8	C3	B2	B2	Yes	Yes°° No	60%	II	Partial failure
9	C3	A1	A1	Yes	Yes	90%	II	Partial success
10	C3	A1	A1	Yes	Yes	90%	I-II	Partial success
11	B2	A1	A1	Yes	Yes	95%	I-II	Partial success
12	C2	A1	A1	Yes	Yes	100%	I	Success

°° Examination of injured eye at 6 weeks immediately before composite transplant.
† Examination of damaged eyes on day 14 before before sacrifice of Group I rabbits.
‡ Examination of damaged eyes on day 28 before sacrifice of group II rabbits.
§ Immunoperoxidase staining for cytokeratin 3 with antibody AE5. (See Table I for explanation of cytokeratins).
¶ Hematoxylin and eosin. (See Table X for explaination of grading of corneal histopathology).
†† Rabbit that had similiar ocular injury but no composite transplant performed.
° In place at 14 days, but not at 28 days.

TABLE IX: EVALUATION OF RABBIT CORNEA*

A	No neovasculatization beyond 3 mm in any quadrant
B	Neovascularization of 4 mm, or more, in one quadrant
C	Neovascularization of 4 mm, or more, in two quadrants
D	Neovascularization of 4 mm, or more, in three quadrants
E	Neovasculatization of 4 mm, or more, in four quadrants

*Scale for evaluation of neovascularization found on clinical examination of damaged rabbit corneae. Examinations were performed preoperatively before composite graft, at 14 days following composite graft for Groups I and II, and at 28 days for Group II.

TABLE X: EVALUATION OF RABBIT CORNEA HAZE*

1	No haze: clear cornea
2	Mild haze: visible but no change in visualization of iris details
3	Moderate haze: some change in visualization of iris
4	Severe haze: moderate change in visualization of iris, distortions of details

*Scale for evaluation of haze found on clinical examination of damaged rabbit corneae. These examinations were performed preoperatively before composite graft, at 14 days following the composite graft for Groups I and II, and at 28 days for Group II.

TABLE XI: GRADING SCALE FOR CORNEAL EPITHELIAL ARCHITECTURE BASED ON HEMATOXYLIN AND EOSIN STAIN SEEN WITH HISTOLOGIC EXAMINATION

GRADE	DESCRIPTION
I	Nearly normal with few, if any, goblet cells; basal cells with maturation progression seen; strongly resembles normal control.
II	Abnormal epithelium, but basal cells and some maturation of superficial cells seen; multiple layers present; may have some goblet cells (<10%); resembles normal control.
III	Abnormal epithelium cells with flattened cells; some layering of epithelial cells persists; some normal-appearing basal cells; may have goblet cells (<25%); some resemblance to normal.
IV	Disorganized epithelium with flattened abnormal-appearing cells; minimal, if any, maturation or layering; little resemblance to normal corneal epithelium.

peutic contact lenses. One rabbit had complete opacification of the cornea secondary to infectious keratitis caused by *Pasteurella multocida*. Of the remaining 4 rabbits, all had one-step improvement in grading of neovascularization. Two of the 4 rabbits had one-step improvement in haze, but all 5 had moderately injected conjunctiva, possibly because of the loss of the contact lens and the exposed knots of the sutures.

In Group II, all rabbits retained their therapeutic contact lenses on day 14 and had moderate to marked clinical improvement. On day 14, 2 rabbits had one-step improvement in neovascularization and one-step improvement in corneal haze. The 3 remaining rabbits had 2 steps of improvement in neovascularization and 2 steps of improvement in corneal haze. When re-evaluated on day 28 prior to enucleation, 4 of the 5 retained their contact lenses. Their clinical grading remained the same, although the rabbit that had lost his contact lens had more neovascularization and appeared worse. This did not change his classification on our scale. All had partial remnants of the composite grafts, although an estimated 80% (estimated by author) of the previously grafted amniotic membrane had dissolved. Eyes that retained a contact lens were white and quiet with no neovascularization and minimal, if any, haze. There were no epithelial defects, and there was no fluorescein staining (Table VIII).

Histologic Evaluation

Because of the variability of the sections and the appearance of the histologic and immunohistologic staining patterns, a histologic grading scale was established to evaluate the epithelial and stromal morphology as seen on H and E staining (Table XI). Immunoperoxidase staining for CK3 (with AE5) was graded by evaluation of the percentage of cells positive for AE5 staining.

The eyes of the rabbits including the normal control (Fig 33), the control damaged eyes (ocular surface damage but no subsequent composite graft repair) (Figs 34 and 35), Group I (enucleated at day 16 following composite graft), and Group II (enucleated at day 28 following composite graft) were studied and evaluated with the same histologic scale (Figs 36-45).

The control rabbits that had been enucleated at 6 weeks without placement of a composite graft were used as a baseline (Figs 34 and 35). These eyes showed distinct epithelial abnormalities. Although there were no areas of epithelial loss, there were markedly distorted epithelial cells and areas of only a single epithelial cell layer without cellular maturation. Where there were multiple cell layers, the basal cells were flattened as were the more superficial cells. Multiple goblet cells seen interspersed within the epithelium. There was stromal neovascularization, subepithelial

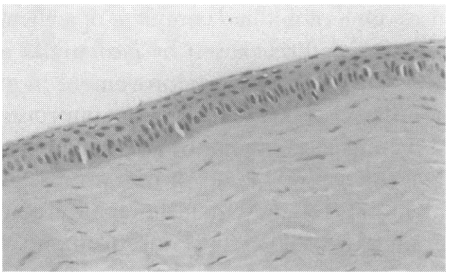

FIGURE 33
Normal rabbit corneal epithelium of untreated control rabbit. (hematoxylin and eosin, x110).

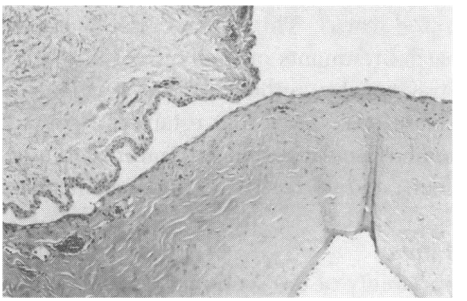

FIGURE 34
Control rabbit 1. Right eye received chemical injury but no composite graft. At 6 weeks, there is markedly abnormal, distorted epithelium, often with a single layer of cells. Multiple goblet cells can be seen with subepithelial and stromal neovascularization. (hematoxylin and eosin, x110).

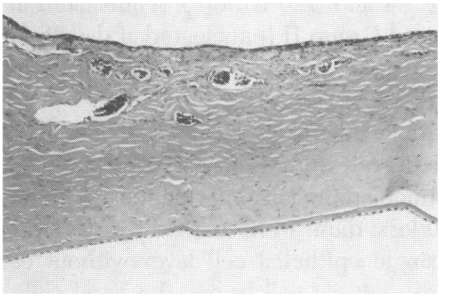

FIGURE 35
Control rabbit 2. Right eye. Similar epithelial changes are seen as noted in Figure 34. (hematoxylin and eosin, x110).

FIGURE 36

Rabbit 3 (group I). treated with composite graft. Note marked inflammatory cell infiltrates, infectious keratitis, and destruction of cornea. (hematoxylin and eosin, x110).

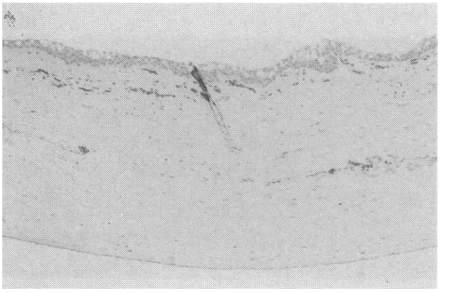

FIGURE 37

Rabbit 4 (group I). Note irregular epithelium, goblet cells, and subepithelial and stromal neovascularization. Graded as II-III histologically (see grading scale in Table XI) (hematoxylin and eosin, x110).

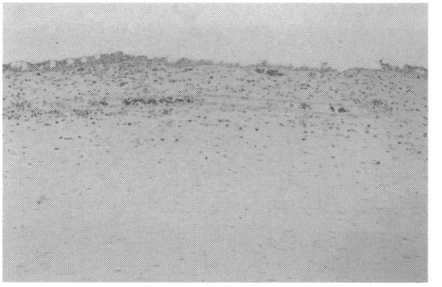

FIGURE 38

Rabbit 5 (group I). Note thinned, irregular epithelium, goblet cells. Stromal neovascularization, and anterior stromal inflammatory cells. Graded as III-IV histologically (see grading scale in Table XI) (hematoxylin and eosin, x110).

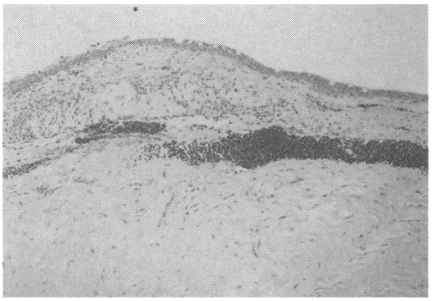

FIGURE 39

Rabbit 6 (group I). Distorted epithelium but some layering and few goblet cells. Note amniotic membrane remnant. (hematoxylin and eosin, x110).

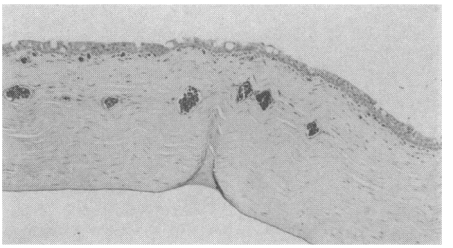

FIGURE 40

Rabbit 7 (group I). Distorted epithelium but some layering and few goblet cells. Grade II histologically (see Table XI for grading scale) (hematoxylin and eosin, x110).

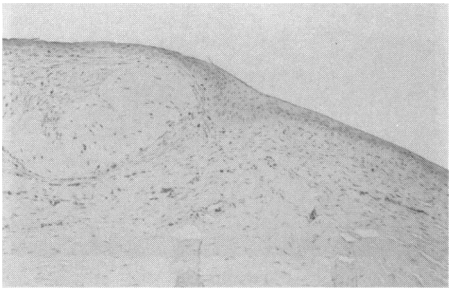

FIGURE 41

Rabbit 8 (group II). Epithelial layering and few goblet cells. Note amniotic membrane remnants. Grade II histologically (see Table XI). (hematoxylin and eosin, x110).

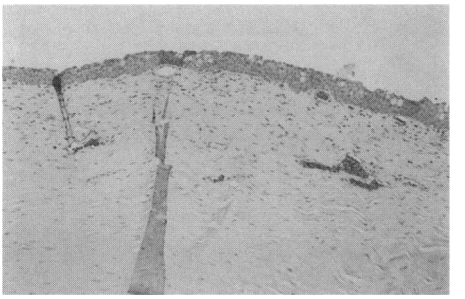

FIGURE 42

Rabbit 9 (group II). Epithelial layering and some goblet cells, with evidence of stromal and subepithelial disruption. (hematoxylin and eosin, x110).

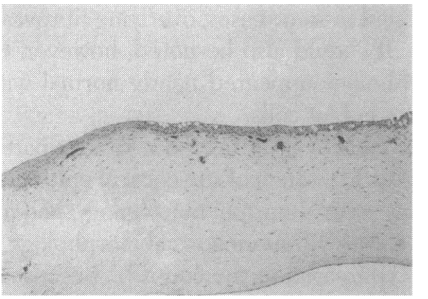

FIGURE 43

Rabbit 10 (group II). Good epithelial morphologic appearance with few goblet cells. Note stromal and subepithelial neovascularization. Graded I-II histologically (see Table XI) (hematoxylin and eosin, x110).

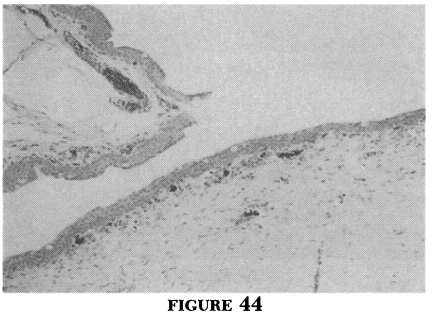

FIGURE 44

Rabbit 11 (group II). Good epithelial morphology with few goblet cells seen. Graded I-II histologically (see Table XI) (hematoxylin and eosin, x110).

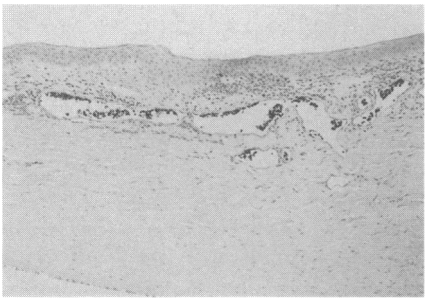

FIGURE 45

Rabbit 12 (group II). Good epithelial architecture despite significant stromal neovascularization. Note few inflammatory cells and no goblet cells in epithelium (hematoxylin and eosin, x110).

neovascularization, and in some cases, overlying fibrovascular tissue on top of the epithelium. It should also be noted, however, that in some areas epithelial cell morphology appeared nearly normal with no evidence of neovascularization or goblet cells.

The AE5 immunohistology reflected a similar pattern in the control rabbit eyes (Figs 46 to 58). Much of the corneal epithelium, especially that portion of the cornea with abnormal morphology, had minimal if any AE5 staining. Yet, some areas with more normal morphology had nearly normal AE5 staining when compared to the controls. Because of the incomplete nature of the clinical appearance of haze and neovascularization, the H and E staining, and the AE5 staining pattern, it appears as if this model is incomplete and does not have complete depletion of the limbal corneal epithelial stem cells of the rabbit.

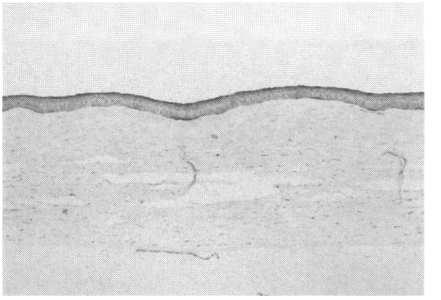

FIGURE 46

Normal rabbit cornea with no damage. Immunoperoxidose staining for CK3 with AE5. Somewhat overstained but all corneal epithelium is positive for AE5 (immunoperoxidase, x110).

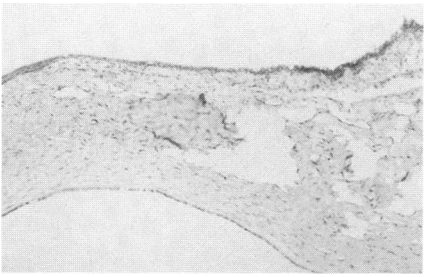

FIGURE 47

Rabbit 1 (control rabbit, damaged eye with no composite graft). Minimal AE5 staining despite overstained slide indicating lack of corneal phenotype in damaged eyes (immunoperoxidase, x110).

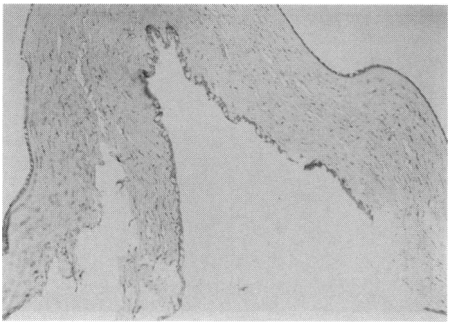

FIGURE 48

Rabbit 2 (control rabbit, damaged eye with no composite graft). Similar to Fig 47 with no AE5 staining of damaged corneal epithelium despite overstained slide (immunoperoxidase, x110).

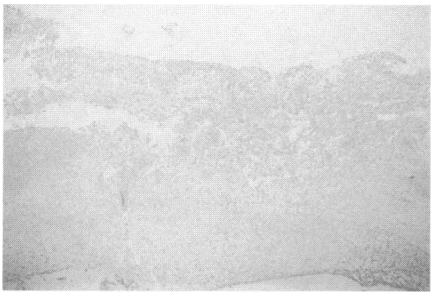

FIGURE 49

Rabbit 3 (group I). Intense inflammatory response and no AE5 staining. This cornea was destroyed by infectious keratitis (immunoperoxidase, x110).

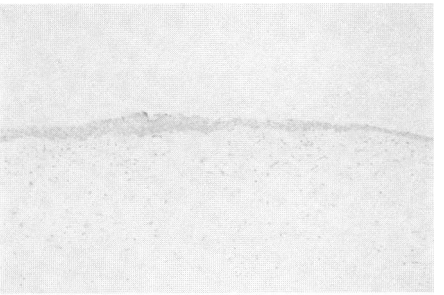

FIGURE 50

Rabbit 4 (group I). Minimal AE5 staining of epithelial cells indicating little, if any, re-epithelialization with cells having a normal corneal phenotype (immunoperoxidase, x110).

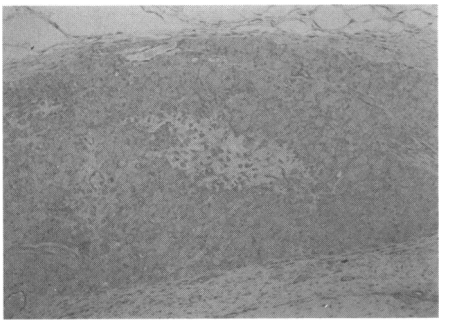

FIGURE 51

Rabbit 5 (group I). Minimal epithelial AE5 staining. Subepithelial tissues beneath remnants of amniotic membrane do stain with AE5, suggesting that amniotic graft and expanded epithelium were accidently inverted at time of surgery (immunoperoxidase, x110).

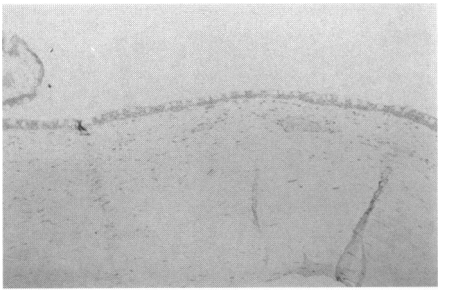

FIGURE 52

Rabbit 6 (group I). Moderate AE5 staining even though histologic appearance is abnormal, suggesting some tendency to corneal phenotype. (immunoperoxidase, x110).

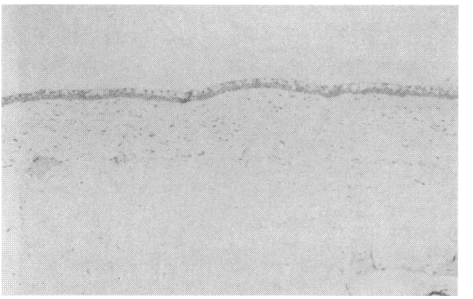

FIGURE 53
Rabbit 7 (group I). Moderate AE5 staining. Similar to Fig 52 (immunoperoxidase, x110).

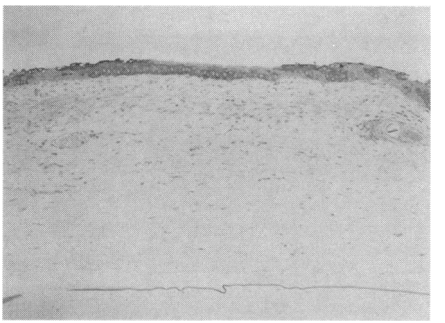

FIGURE 54
Rabbit 8 (group II). Approximately 60% AE5 staining despite better histologic appearance, suggesting that corneal phenotype not completely responsible for re-epithelialization. (immunoperoxidase, x110).

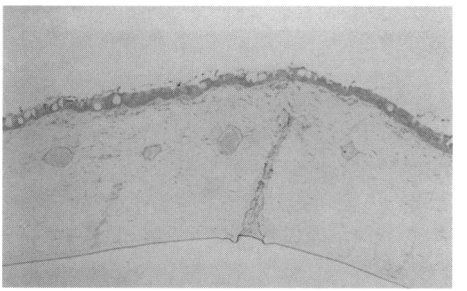

FIGURE 55
Rabbit 9 (group II). Nearly complete AE5 staining of epithelium. High degree of AE5 staining and clinical examination suggest an improved status of ocular surface, but epithelial distortions remain (immunoperoxidase, x110).

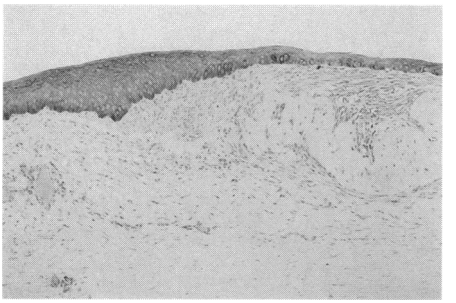

FIGURE 56

Rabbit 10 (group II). Nearly complete AE5 staining of epithelium. Note amniotic membrane remnant (immunoperoxidase, x110).

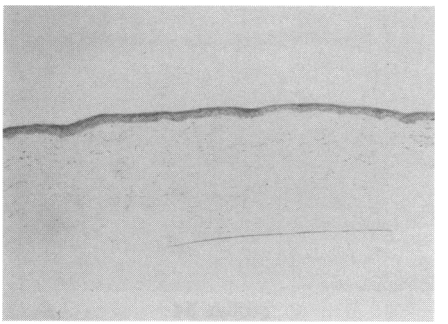

FIGURE 57

Rabbit 11 (group II). Nearly complete AE5 staining of epithelium suggests corneal epithelial phenotype (immunoperoxidase, x110).

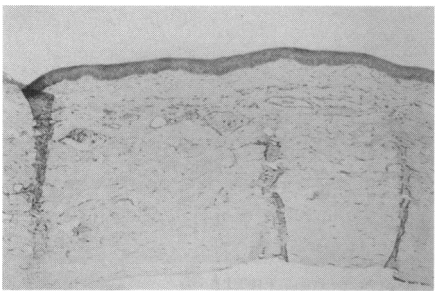

FIGURE 58

Rabbit 12 (group II). Nearly complete AE5 staining of epithelium. Stromal neovascularization and deeper stromal damage remain, however (immunoperoxidase, x110).

In Group I, histologic examination revealed that 1 rabbit had complete loss of epithelium and much of the corneal stroma as a result of bacterial keratitis with *Pasteurella multocida* (Fig 36). Two of the remaining eyes had mild, if any, histologic improvement (Figs 37 and 38) (Table VIII). The other 2 remaining rabbits had moderate improvement with 1 to 2 levels of histologic improvement on these scales (Figs 39 and 40).

In Group II, 1 of the eyes had mild to moderate improvement histologically. Three of the eyes had moderate histologic improvement on these scales, and 1 rabbit had significant improvement with this scoring system (Table VIII) (Figs 41-45).

Immunohistologic staining was graded by the percentage of cells that stained with the AE5 immunoperoxidase stain for CK3. The entire epithelial surface was reviewed. If there was variability, representative fields were chosen, and 200 cells were counted and an approximation of the percentage of AE5 positive cells was obtained (Table VIII).

In Group I, 1 rabbit had no immunoperoxidase staining because all epithelium had been lost to bacterial keratitis as mentioned above (Fig 49). Two of the remaining 4 rabbits had only 5% to 10% of AE5-positive cells. The remaining 2 had approximately 90% of AE5 positive cells (Figs 50 to 53). In Group II, 1 rabbit had only 60% of AE5 positive cells. The remaining 4 rabbits had 95% or more AE5-staining cells (Table VIII) (Figs 54 to 58).

The summary table (Table VIII) describes success as defined by clinical appearance, histologic appearance, and immunohistologic staining. A summary evaluation was given to each rabbit to include the final clinical evaluation, the H and E histologic appearance, and the AE5 staining pattern. These rabbits can be summarized as follows: One rabbit was graded as a failure and a complication by virtue of an untreated suppurative keratitis and loss of the graft. Two rabbits were graded failures by clinical and histologic appearance. Three rabbits were graded partial failures by clinical and histologic grading. Three rabbits were graded partial successes by these criteria, and 1 rabbit was graded successful by these criteria.

At first glance, this summary system may be confusing. The clinical appearance was important because this represents the observable result of the surgery. The 2 rabbits in Group I and the single rabbit in Group II that are classified as partial failures have fair to good histology and AE5 staining. Their clinical appearance lowers their classification. Because there was some variability in the sections of even of the control eyes (eyes damaged without composite graft), it was assumed that the model was incomplete. The sections for H and E histopathology and for AE5 immuoperoxidase may not have been as representative as they should have been in rabbits 6, 7, and 8. Although the sections contained subepithelial and stromal neovascularization, the histologic review may not have included enough of

the damaged surface to discern the full extent of the morphologic damage or decreased AE5 staining. These eyes were still classified as partial failures on the basis of their clinical appearance, despite the histologic appearance of a better result.

DISCUSSION

Severe ocular surface damage causes frustration for the physician and certainly for the patient. Often this ocular surface damage covers an otherwise normal eye. The posterior segment and neurologic mechanisms remain intact only to be defeated by a distorted image, if any image whatsoever is projected onto the retina.

Repair of the ocular surface requires an understanding of anatomy and physiology. Ocular surface reconstruction has been evolving as this understanding improves. Recent work suggests that the corneal epithelial stem cell resides at the limbus and seems to confirm, at least in large part, the "xyz" hypothesis of Thoft and Friend.[68] Nevertheless, problems and barriers remain. Most evidence suggests that corneal epithelial stem cells are necessary to create normal corneal epithelial cells, at least for a prolonged period of time.

SUMMARY HYPOTHESIS OF EPITHELIAL REGENERATION

The summary of current evidence suggests that corneal epithelial maintenance and repair can be described as follows and seems to resemble the model for skin, which is understood in more detail.[199]

This evidence suggests that each stem cell ("grandmother" cells) will spawn a limited number of active ("mother") cells. These transient amplifying cells ("mother cells") will rapidly proliferate for many but not an infinite number of generations of mature corneal epithelial cells ("daughter "cells). Each of the daughter cells will stream toward the center of the cornea and upward from the basal cell layer to the superficial layers. These daughter cells are probably capable of division only while in direct contact with the basement membrane of the cornea, and they lose this ability once that contact is lost. As these daughter cells reach the most superifical layer of the central cornea, they slough, and are continuously replaced. The mother cells eventually can no longer sustain the metabolic activity, and they themselves stream toward the center of the cornea as daughter cells. The grandmother cell is then stimulated to create another mother cell, and the cycle continues. This minimizes the demands on the original stem cell, or grandmother. If this model, or a similar one for stem cell and corneal epithelial maintainance, is true, then corneal epithelial stem cells will be required to re-epithelialize a damaged cornea.

The number of corneal epithelial stem cells is definitely finite, and a donor cannot part with all of these cells, although it is unclear what percentage of the original stem cell population is necessary to maintain a normal ocular surface. If progress is to be made in the resurfacing of eyes with damaged stem cells, donor stem cells must be used. These can be obtained from autologous tissue or potentially from allogeneic donors. However, there may be danger from acquiring autologous donor stem cells if 80% to 90% of the complement of stem cells is transferred from one eye to another. Similarly, if living allogeneic donors, such as siblings, are used, there may be a danger to the donor, and this may not be apparent for several years. Hence, for unilateral ocular stem cell damage, and especially bilateral stem cell failure, other techniques must be considered.

Bioengineered skin substitutes have been used in the field of dermatology, and the techniques applied herein are simply extensions of these dermatologic techniques to the eye.[200] Cultured ocular surface grafting is in its infancy, and this work represents a step toward the goal of bioengineered ocular surface transplantation and reconstruction.

HUMAN SUBJECTS

Of the 19 patients who were admitted to the project, 18 had successful growth of the corneal epithelial stem cells in vitro. One patient admitted to the investigation had a limbal biopsy in standard fashion, but the epithelial cells did not grow. This patient had a recurrent pterygium despite previous β irradiation. Perhaps the previous irradiation damaged the stem cells, so that these cells could not be made to undergo mitosis, but there was no clinical evidence of stem cell failure. She lost her healthcare plan and still awaits more conventional surgery.

One patient had complete failure of the graft because of trichiasis/entropion, and this proved early in the investigation that all lid deformities must be addressed before any ocular surface surgery is attempted. Unfortunately, this patient developed phthisis from the underlying disease (severe alkali burn) before another graft could be performed. This procedure would not have prevented phthisis and would have been destined to fail, in any case.

The remaining 17 patients (19 procedures on 18 eyes) had varying degrees of success associated with autologous or allogeneic transplantation with follow-up from 2 to 24 months. Two patients had no improvement but did not worsen and were relieved of motility restriction. One patient receiving an allogeneic graft had successful re-epithelialization and improvement in vision but sustained a corneal scar from bacterial keratitis. One patient receiving an allogeneic graft has, as yet, and undetermined result.

The technique of re-application of in vitro cultured corneal epithelial stem cells has undergone an evolution of carriers beginning with collagen gel. This gel was technically difficult to handle and appeared to be fragile, although it remained in place on all 8 cases in which it was used successfully. Similarly, a collagen shield seems to be a poor choice as a carrier. These shields melt quickly and may not maintain epithelial contact long enough for adherence. In all cases, a therapeutic contact lens was placed on the graft to help maintain cellular position and adherence following surgery. Contact lenses are difficult to use as substrate, since adherence is a problem for all carriers, and contact lenses were never designed to have cellular adherence.

The human subjects in this work were not uniformly successful. As the work began, these techniques were applied to the more difficult and recalcitrant problems, such as the more severe restrictive pterygia and pseudopterygia. The technique of using cultured corneal epithelial cells was used in combination with a lamellar corneal donor as a substrate for attachment of the epithelial cells (9 procedures on 8 patients). It is presumed that the combination of a normal substrate and normal corneal epithelial stem cells created a more normal ocular surface and did not attract the recurrence of the pterygium or pseudopterygium. Eight of these 9 procedures were on patients with recalcitrant pterygia or pseudopterygia. These patients had had multiple recurrences, usually with ocular motility restrictions. It could be argued that these patients would have responded and improved with only the corneal lamellar graft, but most surgeons would cover similar lamellar grafts with healthy and previously unaffected conjunctiva such as would be seen with a free conjunctival graft.[137,138] Surgeons generally recommend that a free conjunctival graft be obtained from the superior bulbar surface to help prevent the aggressive regrowth of these tissues atop the new lamellar graft.[137,138] Moreover, it should be noted that one of the patients, who was considered unsuccessful, had recurrence over the lamellar graft. This patient received cultured epithelium applied to a collagen shield, and this shield had nearly melted upon application. The epithelial cells in this case were very difficult to apply and to sew into place with minimal collagen shield support remaining. We believe that these cells were probably not successfully applied.

Pellegrini and associates[195] reported success using autologous expanded corneal epithelial cells in a collagen gel in the treatment of alkali-damaged eyes with a very similar technique. We believe that the patients who were thought to be successful in this investigation maintained the transplanted cells, but this cannot be proven with these patients or in other isolated reports of such work.[194,195] At the very least, we believe, the autologous epithelial cells functioned as a free conjunctival graft in helping to

prevent recurrence of the pterygium.

The patients receiving the amniotic membrane with overlying expanded epithelial grafts may have improved with only the amniotic membrane, as other have suggested.[163] These were not primary pterygia, however, as has been reported previously, and these procedures required some form of epithelial coverage.[163] Additionally, one of the amniotic membrane grafts failed to prevent recurrence of the pterygium (case 15). This patient lost her autologous epithelium and her therapeutic contact lens, suggesting that the epithelial graft may have been lost at the time of, or shortly after, the contact lens loss. This suggests that the amniotic membrane graft alone is not sufficient to prevent recurrence, at least of the more difficult pterygia.

Two of the patients receiving expanded epithelial stem cells for premalignant epithelial conditions had no lamellar carrier and seemed to have a "take" of the autologous donor tissue and prevention of symblephara. These patients with ocular surface premalignant/malignant conditions (conjunctival intraepithelial neoplasia and primary acquired melanosis) both had such extensive disease that symblephara were likely to obscure the bulbar and palpebral surfaces after removal of their tumors. Both of these patients had large autologous cultured epithelial grafts placed over their defects once the suspicious lesions had been removed. Symblephara did not occur where these grafts had been placed, and re-epithelialization spontaneously occurred over the opposing surface. Patient 3 had conjunctival intraepithelial neoplasia on both the bulbar and palpebral surface of the lower lid, and this was resected completely. Both denuded surfaces were covered with the autologous grafts. Only one small symblepharon occurred temporally where the grafts had not fully covered both surfaces. The patient with PAM (case 9) had extensive bulbar and upper palpebral surface involvement. The expanded epithelial tissues were applied and sewn only to the bulbar surface after removal of both the bulbar and palpebral epithelia. This patient developed no symblepharon where the graft had been applied, although she did sustain foreshortening of the culde-sac beyond the point of graft application.

The patient with the corneal/conjunctival intraepithelial neoplasia had a recurrence, possibly from the remaining untreated quadrant of the limbus, and subsequent placement of amniotic membrane graft with expanded corneal epithelial cells with successful re-epithelialization despite the 2 surgical procedures to remove the limbal epithelium with 2 cryotherapy applications.

In both of these cases (patient 3 and 9), however, we cannot be certain that the epithelial cells "took" because there was no tracking of the autologous donor cells. It is notoriously difficult to remove all of the stem cells as seen in the rabbit portion of this work.[201] Perhaps these patients

improved simply because the abnormal cells were removed, and symblephara did not form because of the contact lens placement.

The single patient who received autologous cells plated onto a contact lens had had 9 months of treatment for a neurotrophic ulcer with various agents, appliances, and surgery, including tarsorrhaphy and therapeutic contact lens application. Curiously, limbal stem cells were harvested from the ipsilateral eye, and these cells grew readily in vitro, and yet in vivo could not heal his epithelial defect. He healed promptly with the application of autologous cells, although he, too, lost at least some of these cells 2 months later when the contact was lost. He developed a smaller defect in the same area as the original defect. Curiously, he healed again promptly with re-institution of the contact lens, and 4 months later, we were able to remove the lens without loss of the epithelium. This suggests that additional time may be necessary for these cells to adhere properly. It could be argued that the autologous transplant had little to do with his epithelial healing, although he had had therapeutic contact lenses placed before without success. Neurotrophic ulcers are notoriously quixotic but also very difficult to heal. It is interesting to speculate that the cultured epithelial cells may have produced chemical mediators or extracellular matrix which stimulated healing or that the cells may have adhered themselves. In either case, these techniques represent novel and potentially new avenues to treat neurotrophic ulcers and other stem cell defects.

The 7 patients who received the amniotic membrane, including 4 autologous grafts and 2 allogeneic grafts, had challenging problems. The single patient who received cultured epithelium on amniotic membrane for a recurrent pterygium had a prompt return of good vision, and did well during the first few weeks, but by 1 month she had lost her therapeutic lens and began to have signs of recurrence. Eventually, the pseudopterygium recurred, although she did not have the restriction of motility seen initially. Perhaps the amniotic membrane was sufficient to allow for additional conjunctival regrowth to prevent restriction, but was not sufficient to prevent recurrence of the pterygium without epithelial cells.

One patient receiving allogeneic cells on an amniotic membrane had re-epithelialization off the membrane onto the cornea seen on the first and fourth postoperative day. This re-epithelialization continued until he developed infectious crystalline keratopathy. It is unlikely that this initial re-epithelization came from host conjunctival cells because he had had indolent epithelial defects in the past. His infectious keratitis and treatment may have delayed the continued re-epithelization, but he eventually did completely re-epithelize. He had visual improvement but was left with corneal haze in the area of keratitis. We do not know the fate of the original donor cells, however.

The second allogeneic graft lost his contact lens and overlying epithelial graft at approximately 1 to 4 days. He did epithelialize the amniotic membrane at 5 weeks, just prior to the reapplications of additional allogeneic cells on a collagen shield and a therapeutic contact lens. This would suggest that adherence may be a problem with such composite grafts and that the therapeutic contact lens is essential to maintaining epithelial cell contact long enough for hemidesmosomal attachment to form to amniotic membrane or corneal stroma. Initial work done with cultured corneal epithelial grafts had difficulty with adherence.[18] In this current work, patients who maintained a contact lens for 2 months appeared to have the best chance for success. The fate of this patient's reapplication of cells is still undetermined. As mentioned above, this suggests that there may be an adherence problem between the expanded epithelium and the amniotic membrane.

There were no complications from the harvest of the stem cells of either the autologous or allogeneic biopsies. These sites healed promptly without sequalae. The biospy procedures appear safe. The epithelial growth procedures do not appear to introduce any potential complications. The in vitro growth process, however, was done in a medium with streptomycin and penicillin. Presumably, this composite graft should not be applied to a patient with an allergy to either antibiotic or to any known component of the cell culture medium. If a culture is infected with bacteria or fungus, this is recognizable before implantation. If the procedure is unsuccessful, other options remain open to the surgeon and patient. If a limbal conjunctival autograft is deemed to be essential to the restoration of the damaged eye, this can still be undertaken should the expanded autologous or allogeneic graft fail because only a small biospy of limbal tissue is taken from the contralateral eye.

There were few complications following surgery, and none were believed to be directly due to the cellular transplantation. One graft failed completely as a result of mechanical removal by entropion/trichiasis. One of the allogeneic graft patients with stem cell failure had infectious crystalline keratopathy. Although this was successfully treated, his surface and systemic immunosuppression was discontinued. He did re-epithelialize after 2 months of treatment with appropriate antibiotics. This was not primarily due to the composite graft, but it should be a reminder that a compromised ocular surface, immunosuppression, a therapeutic contact lens, and depletion of the previous barrier through surgery create additional risks for any patient undergoing any similar procedure.

Perhaps the most important evidence for the suggestion of clinical success of this work is that almost all, if not all, of these patients would have received some form of conjunctival graft to help heal their defect. If these

cells in this composite graft "took" as if they were conjunctival grafts, it is logical to assume that they produced corneal epithelial cells.

EVOLUTION OF CARRIER

As the work continued, the search for an improved carrier led us to amniotic membrane as the beginning of composite tissue. Amniotic membrane was used in 7 patients. This tissue has much in common with conjunctival basement membrane and may represent an excellent substrate onto which to plate cells.[110] As others have reported, amniotic membrane may facilitate epithelialization without allowing host fibrovascular ingrowth onto the amniotic membrane, making this tissue ideal for ocular surface reconstruction.[158] Amniotic membrane gradually dissolves in vivo and is nonantigenic.[173,174]

We evaluated several methods for removal of amniotic epithelium and settled on 15 minutes of trypsinization followed by gentle scraping. This technique seems to remove the epithelium and does not damage the basement membrane histologically, but requires great care. This technique may dissolve critical extracellular matrix factors that may not be visible, and therefore should not be considered sufficient without further evidence. We found that the culture of expanded corneal epithelial cells did not adhere quickly to the amniotic membrane and required several days for adherence. Trypinsination may make adherence more difficult. The problems encountered with amniotic membrane as the carrier in humans and in the animal model suggest that adherence may represent a significant barrier to the success of epithelial cell transplantation. It is also doubtful that amniotic epithelium can function like corneal epithlelial stem cells or even normal corneal epithelium. We therefore believe that it is critical to remove the amniotic epithelium if this membrane is to be a carrier.

Furthermore, some of the human subjects and the rabbit model suggest that amniotic membrane alone will not be sufficient for some of these challenging ocular surface problems. As we begin to understand the extracellular mediators, anatomy, and matrix, other tissues, factors, or agents will probably be used as a carrier. Hence, the search for an improved carrier should continue.

ANIMAL MODEL

The laboratory portion of this work provides additional evidence as to the effectiveness of both the model itself and the amniotic membrane grafting with overlying expanded corneal epithelial cells. We find the model to be incomplete, and the procedure of a composite graft composed of expanded corneal epithelium overlying amniotic membrane shows promise but is not uniformly successful.

Effectiveness of Model

The model established for this work was found to be incomplete. After reviewing the clinical, histologic, and immunohistochemical appearance, there is evidence that the full complement of corneal epithelial stem cells was probably not removed. Others have suggested that complete removal is very difficult without keratectomy, sclerectomy and treatment with n-heptanol.[201] We did not wish to perform a keratectomy and sclerectomy and treat with n-heptanol, because it is doubtful that this composite would sufficiently manage such an injury. This would have provided little direction for the continuation of this work.

The incomplete nature of the model illustrates that proving the success or failure of expanded epithelial stem cell grafts or any form of composite grafting may be very difficult. The model was incomplete, probably because of insufficient contact time with n-heptanol or our manner of application. The n-heptanol was applied with an applicator stick, beginning with the superior limbus and proceeding 360°. Contact time with n-heptanol was probably too short at 60 seconds and should have been 120 seconds, as others have suggested.[201] Nonetheless, even at 120 seconds it is difficult to remove all of the stem cells, and this is not surprising.[201] There would be a teleologic imperative to preserve such cells, and to protect them at all costs. Nevertheless, this model can provide some clues for future work.

Evaluation of Composite Transplant

Six of the 10 rabbits had complications, outright failure, or partial failure. The eye with supparative keratitis may be an aberration of the model, but rabbit corneae are otherwise difficult to infect. This cornea reminds us of the immunocompromised nature of the ocular surfaces we are trying to treat. In the 2 rabbits with failure, there was little evidence of improvement despite placement of the amniotic membrane and histologic presence of the membrane. Both of the eyes had lost the contact lens in the early postoperative period, probably because of the nicititans. These eyes did not have clinical, histologic, or immunohistologic evidence of success. Interestingly, in 1 of these rabbits, AE5-positive cells were found beneath the amniotic membrane histologically, but few AE5-positive were found on the surface. This rabbit eye is classified as a failure, and it may have been iatrogenic. It is possible that the graft was inverted at the time of surgical repair.

In the remaining 2 rabbits that had partial failure, both also had lost their contact lens during their early postoperative course. These eyes had some evidence of improvement, but were not convincing in their improvement despite having amniotic membrane grafts present. The

epithelium was probably lost, as was the contact lens in the early postoperative period, but there was a high degree of corneal epithelial antigen present (AE5-positive corneal epithelial cells) with a fair histologic appearance, suggesting that at least some of the grafted epithelium may have remained. There is controversial evidence that conjunctival epithelium can express a corneal phenotype when in contact with the appropriate basement membrane, although most observers believe this is not true.[117,182] We know that AE5-positive cells could represent conjunctival cells that have developed a corneal phenotype when in contact with the appropriate basement membrane.[117,182]

The remaining 4 eyes showed varying degrees of success. All 4 of these rabbits had a contact lens in place at the examination on day 14 and day 28, suggesting that the therapeutic contact lens is important to the health of both the amniotic membrane and the expanded corneal epithelial graft. Three of the 4 remaining rabbit eyes had partial success indicating that the clinical appearance had improved and the histologic appearance was improved and, in these eyes, much closer to normal. The immunohistochemical characteristics suggested that the corneal epithelial phenotype had been achieved. In 1 of these 5 eyes, the procedure was deemed a success because of a substantially improved clinical, histologic, and immunohistologic appearance. The histology closely resembled normal, and the immunohistologic appearance revealed a corneal epithelial phenotype covering the cornea. These last 4 rabbits suggest that the presence of the therapeutic contact lens supports the expanded corneal epithelial cells by keeping them from being mechanically removed by the nictitans. These 4 rabbits also provide evidence that the epithelium was transplanted successfully and retained by the recipient eyes. Some portion of the treatment made a dramatic difference, at least for these 4 eyes. It is not likely to be the amniotic membrane alone, since 5 other eyes were unsuccessful with amniotic membrane whether the epithelial cells were present or not. It was not likely to be the surgery or the postoperative corticosteroids, because 5 rabbits in the surgery groups had unsuccessful grafts. We suspect that the cultured corneal epithelial cell transplants with amniotic membrane as the carrier were the important treatment element. We also suspect that the therapeutic contact lens plays an important role in maintaining adherence until the epithelial cells can adhere on their own.

Interestingly, the human subjects that were unsuccessful also had problems with lost therapeutic lenses and subsequent mechanical loss of expanded corneal epithelial cells, even when the amniotic membrane remained in place.

Our animal investigation provided evidence that suggests that the

donor epithelial cells remain for at least 4 weeks without immune suppression other than corticosteroids. The 5 rabbits that were sacrificed at 28 days had evidence of corneal epithelium (AE5-positive staining) without evidence of rejection. However, we cannot be certain that the original expanded corneal epithelial cells remain even if the cells present on the cornea are corneal cells that were not there previously. It is possible that the expanded epithelial cells or the expanded corneal epithelial cells in combination with the amniotic membrane produced biochemical signals that caused the host to create phenotypically normal cells. It is unlikely to be the amniotic membrane alone, because 3 of the first 5 rabbits that were sacrificed before there was a chance for rejection (at 16 days) did not have many AE5-positive cells.

Previous investigators have tried a similar animal model to evaluate the use of amniotic membrane without any additional cellular elements.[182] Their procedure for complete removal of the limbal stem cells included treatment with n-heptanol (although the time was not stated), followed by surgical dissection of the lamellar limbal tissues at 2 mm within the limbus and a 360° conjunctival peritomy to 3 mm beyond the limbus. The model used in that work may have yielded better success than the model used in this current work, although there are questions when the 2 models are compared. Their model did show extensive epithelial and subepithelial damage, as did ours. The control rabbits that did not receive amniotic membrane transplants had no significant AE5 staining of remaining corneal surface epithelium in either model. In our model, however, the rabbits that lost a contact lens, and presumably their complement of epithelial stem cells, at 14 days, had little, if any, AE5 staining of the epithelial cells covering the cornea, and they had distinct morphologic changes, suggesting that these were not corneal cells. This remained true even when surviving portions of the amniotic membrane were directly visible beneath the epithelium. In contrast, in the aforementioned study, the epithelium covering the cornea atop the amniotic membrane was AE5-positive. These investigators, however, in contrast to our investigation, did not remove the amniotic epithelium, which may have been responsible for this AE5-positive staining.

Our investigation suggests that expanded epithelium was transplanted successfully onto the rabbit eyes. This is perhaps the best, but not the only, explanation for the presence of AE5-positive cells with a nearly normal morphologic appearance at 28 days in those rabbits that maintained their contact lens. There is strong evidence that the contact lens is essential for maintainence of the expanded donor epithelium, at least for 28 days. The eyes that were enucleated at 16 days had lost their contact lens and the

epithelium that covered the cornea. These eyes demonstrated little, if any, AE5 staining of the epithelial cells, and the cells were morphologically abnormal when compared to the control.

SUMMARY OF INVESTIGATIONS

Despite the appearance of some clinical success in humans and cautious optimism regarding the potential for a composite graft with cultured corneal epithelia in the treatment of an animal model of severe stem failure, this work does not provide a definitive product. There were problems as discussed above. The review of the histology of the eyes of the rabbit model provides ample evidence that some injuries, such as chemical burns, damage more than just epithelium. Simply transplanting stem cells, even if successful, will not be the complete answer to these problems. The extracellular matrix will prove to be a very important part of the composite graft, and further attention must be directed to it. Nevertheless, the tantalizing taste of some success suggests the direction for further study.

Cultured corneal epithelial stem cell transplantation is a nascent technology that has shown itself to be a potentially powerful technique to reepithelialize a damaged ocular surface. Our technique has evolved as this work progressed. We discovered that the carrier is important to the success of the procedure, although the early cases using collagen gel appear to have given satisfactory results. However, this material was very difficult to use and apply directly to the surface and maintain the integrity of the surface. Amniotic membrane appears to be the superior carrier currently, and we have shown that the amniotic cells can be removed and replaced by the cultured corneal epithelial stem cells. This provides a suitable carrier and a better subepithelial matrix than other carriers. Lamellar corneal tissue does provide a satisfactory carrier but requires further surgery to produce a lamellar bed. In certain cases, however, the use of lamellar corneal tissue with or without amniotic membrane may still be needed.

This work provides many questions and directions for further progress in the quest for a better composite graft. The longevity of the expanded epithelial grafts would tell us much. Immunologic marking of such cells to tag original cells and progeny for recognition in vivo would allow topographic evaluation of the success of any composite graft that included such cells. Similarly, further work is essential to increase the prompt and vigorous adherence of the expanded epithelial graft to the carrier tissues, such as amniotic membrane. Further work with the microenvironment for cellular attachment will likely provide much needed help for adherence of these epithelial cells.

In vitro engineering of human skin is already being used for the treat-

ment of burns and chronic nonhealing defects, even if problems remain.[202-206] We believe that this form of technology can and will be applied to the artificial resurfacing of the eye through techniques similar those described in this work. Nevertheless, much is to be learned before satisfactory composite grafts can be easily produced and transplanted.

CONCLUSIONS

1. Presumed corneal epithelial stem cells can be harvested safely from the limbus and expanded successfully in vitro.
2. Expanded corneal epithelial cell cultures can be grown onto various carriers, but currently appear best suited to denuded amniotic membrane as a carrier for ocular surface repair.
3. Expanded corneal epithelial cell transplants appear to resurface damaged ocular surfaces successfully, but cellular tracking and further confirmation are required.
4. Expanded allogeneic corneal epithelial cell transplants are technically possible and may represent alternative treatment modalities for selected ocular surface problems.
5. These techniques potentially offer a new method of restoring a normal ocular surface while minimizing the threat of damage to the contralateral or sibling limbal corneal epithelial stem cells.
6. The rabbit model was probably incomplete, and interpertation should be cautious.
7. The rabbit model provided some suggestion that allogeneic grafts may restore a nearly normal ocular epithelial surface to certain ocular surface injuries.

ACKNOWLEDGEMENTS

I would like to thank Rivkah Isseroff, MD, for her help and forbearance throughout this project. Her inspiration and ideas were and continue to be essential. I would also like to thank Merle Reyes and Glenmar Obedenico, who performed much of the laboratory work required throughout the project. Luis Izquerido Jr, MD, was similarly of great help, particularly with the amniotic membrane portion of the work. I would like to thank George Weinstein, MD, and Bruce Spivey, MD, for their support and review of the manuscript.

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