·Review·

Evaluation of corneal cell growth on tissue engineering materials as artificial cornea scaffolds

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Foundation items: National Natural Science Foundation of China (No.50973082)

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Received: 2012-06-05 Accepted: 2013-09-05

Abstract

• The keratoprosthesis (KPro; artificial cornea) is a special refractive device to replace human cornea by using heterogeneous forming materials for the implantation into the damaged eyes in order to obtain a certain vision. The main problems of artificial cornea are the biocompatibility and stability of the tissue particularly in penetrating keratoplasty. The current studies of tissue-engineered scaffold materials through comprising composites of natural and synthetic biopolymers together have developed a new way to artificial cornea. Although a wide agreement that the long-term stability of these devices would be greatly improved by the presence of cornea cells, modification of keratoprosthesis to support cornea cells remains elusive. Most of the studies on corneal substrate materials and surface modification of composites have tried to improve the growth and biocompatibility of cornea cells which can not only reduce the stimulus of heterogeneous materials, but also more importantly continuous and stable cornea cells can prevent the destruction of collagenase. The necrosis of stroma and spontaneous extrusion of the device, allow for maintenance of a precorneal tear layer, and play the role of ensuring a good optical surface and resisting bacterial infection. As a result, improvement in corneal cells has been the main aim of several recent investigations; some effort has focused on biomaterial for its well biological properties such as promoting the growth of cornea cells. The purpose of this review is to summary the growth status of the corneal cells after the implantation of several artificial corneas.

• KEYWORDS: artificial cornea; keratoprosthesis; tissue-engineered scaffold; corneal cells collagen; fibrin; amniotic membrane; biomaterial

DOI:10.3980/j.issn.2222-3959.2013.06.23

Wang HY, Wei RH, Zhao SZ. Evaluation of corneal cell growth on tissue engineering materials as artificial cornea scaffolds. *Int J Ophthalmol* 2013;6(6):873-878

INTRODUCTION

The World Health Organization estimates that 50 million people worldwide are bilaterally blind, and at least 150 million people have impaired vision in both eyes. Corneal diseases are a major cause of vision loss and blindness, irreversible loss of optical quality of the cornea due to disease or damage results in permanent vision loss or blindness [1,2]. Penetrating keratoplasty, as the main treatment by using allograft, has its defects such as poor rehabilitation due to severe dry eye, cornea neovascularization, as well as graft rejection and donor shortage. Recently, the development in bioengineered corneal substitutes designed to replace the full or partial thickness of damaged or diseased corneas have been reported [3,4]. Results have demonstrated that tissue-engineered materials and hydrogels could permit the integration of the implant and the host tissues [5-8]. The applications of artificial cornea along with the studies of corneal tissue engineering have brought a hope to patients with corneal blindness, because of the biocompatibility and stability of the tissue, materials of the cornea remain an important factor in the development of the artificial cornea especially in full thickness replacement.

NATURAL MATERIALS

Acellular Cornea Stroma Acellular cornea stroma has been derived from the acellular allogeneic or autologous graft. Because of the deficiency of lipid membrane, membrane-associated antigen and soluble protein, immunogenicity decline significantly, especially promote the natural corneal extracellular matrix (ECM) retaining many types of cytokines and chemical signals, it is suitable as a scaffold for artificial cornea, its micro-environment close to the physiological status and conduct to cell adhesion, migration, proliferation, and promote tissue regeneration. Research also found that acellular corneal stroma has a complete ECM membrane, the basement membrane, Bowman layer and stroma, which can benefit stem cells and epithelial cells to be attached to each other firmly[9].

While attempting to develop a successful material for an

artificial cornea like other acellular biologic materials, Xu *et al* [10] used the acellular corneal matrix from porcine (ACMP) as a potential corneal cell sheet frame. They have investigated the biocompatibility of the ACMP for one month through using rabbit corneal stroma and three types of cultured corneal cells. *In vivo* study in rabbit showed that an intact epithelium and keratocytes in the implant region and three types of cultured rabbit corneal cells were positive for K3, vimentin, and aquaporin A, and the cultured rabbit corneal cells formed a confluent cell sheet on the ACMP. They concluded that the corneal scaffold made by the ACMP as a frame with three types of allogeneic rabbit corneal cells may be a new concept in treating injured corneas [10]. The study develop a method using phospholipase A_2 (PLA_2) to prepare acellular porcine corneal stroma (APCS) from native porcine cornea (NPC) for tissue engineering. This method displayed that there were no significant differences in the areal modulus, curvature variation and epithelialization between APCS and NPC after rabbit lamellar keratoplasty[11]. It also used various concentrations of sodium dodecylsulfate to treat porcine corneas for different lengths of time, corneal acellular matrix (ACM) showed similar biomechanical properties and biocompatibility as natural ones after transplantation into the interlaminar stroma of rabbit corneas. These acellular matrices (which are similar to natural corneas in structure, strength, and transparency) have tremendous potential for corneal transplantation as ideal implants for donors and as suitable scaffolds for tissue engineering applications[12].

Amniotic Membrane Amniotic membrane (AM) is situated in the inner membrane of fetal membranes, including the monolayer of epithelial cells, thick basement membrane and avascular stroma. As a part of placenta, AM usually has the properties of immunologic inertia, promoting the growth of corneal epithelial cells, inhibiting proliferation of vascular endothelial cell, inflammatory response, scarring and angiogenesis[13].

The effect of amniotic membrane as corneal scaffolds on epithelial growth and attachment to synthetic polymers has been evaluated by several researchers. Human amniotic membrane was used as substrate for culturing corneal epithelial cells and transplanting them onto severely injured rabbit eyes. A confluent primary culture of limbal corneal epithelial cells was established on acellular human amniotic membrane after 14d. Cells were partially stratified and fairly well attached to the underlying amniotic membrane, but a fully formed basement membrane was not evident[14].

Uchino *et al* [15], took advantage of both the stability of artificial polymers with the biocompatibility of natural materials, and designed a hybrid polymer composed of an amniotic membrane (AM) immobilized polyvinyl alcohol hydrogel (PVA-AM) for using as an artificial cornea material. When comparing on PVA-AM and collagen immobilized polyvinyl alcohol hydrogel (PVA-COL)in Air-lift Cell Culture, a fully stratified rabbit corneal epithelium was successfully engineered using PVA-AM while defects in the epithelial sheet observed in approximately half of the PVA-COL polymers. In immunohistochemistry, rabbit corneal epithelium cultivated on PVA-AM expressed collagen type IV which was positive in normal rabbit cornea, while epithelium cultivated on PVA-COL was negative. When transplanted into the rabbit stroma, hematoxylin-eosinstaining (HE) staining of PVA-COL at 2 weeks showed a defect in the stratified epithelium, while epithelialization on PVA-AM was intact, and the epithelium that overlying the AM was normal in morphology, but stabilizing of the AM component of PVA-AM is still an issue remaining to be resolved. The cornea epithelium's growth on polymers with various surface properties was measured quantitatively *in vitro* by Baharvand and colleagues. It found that the denuded-human AM increased epithelial cell growth better when compared with control of matrigel and collagen on the expansion of limbal stem cells (SCs). Two dimensional electrophoresis (2-DE) coupled with mass spectrometry (MS) identification analyzing the protein pattern showed that epithelial outgrowth of limbal explants on AM expressed more p63 and K19 (SC markers) and less K3 and connexin 43 (corneal differentiation markers) in comparison with other extracellular matrices (ECMs). Results showed that epithelium-denuded AM provides a superior niche for limbal SC proliferation and phenotype maintenance *in vitro*[16]. Similarly, Ahn *et al* [17] also demonstrated that corneal epithelium reconstruction on a lyophilized amniotic membrane (LAM) is a good model *in vitro* for autologous or allogeneic transplantation of corneal epithelium and skin epidermis in patients with damaged epithelia.

SYNTHETIC BIOPOLYMERS

Collagen Collagen is a natural protein material that commonly used in tissue engineering. Human have at least 22 types of collagen，type I collagen is the major composition of human cornea. The environment of the cell metabolism provided by collagen scaffold is close to physiological conditions, conducive to the growth of corneal cells [18]. Collagen fibrils and their networks function as the extracellular matrix ECM provide physical support to tissues by occupying the intercellular space, acting not only as benign native scaffolds for arranging cells within cornea, but also as a dynamic, mobile, and flexible substance defining cellular behaviors and tissue function [19]. As natural polymers, they can promote cell adhesion and proliferation better than synthetic polymers. They have been used successfully in a wide variety of tissue-engineering applications, such as skin, cartilage, bone, and nerve tissue

engineering[20,21].

Orwin and Hubel [22] investigated the effectiveness of a tissue-engineered collagen sponge as a substrate for the culture of human corneal cells. Human epithelial cells were cultured on collagen sponges that was composed of native fibrillar collagen with a pore size of approximately 0.1mm. Histological sections stained with HE showed that epithelial cells form a monolayer of cells on the sponge surface; images of the surface in these cultures obtained using scanning electron microscope showed that the cells have migrated from the center to the entire peripheral area that are smooth in appearance. Additional analysis of the smooth areas showed that epithelial cells have formed confluent layers. Co-culture of epithelial cells and endothelial cells also exhibited an increased number of epithelial cell layers on the sponge surface compared to epithelial cell cultures on the collagen sponge alone. Histological sections of these cultures showed three to four layers of epithelial cells covering the entire surface of the sponge by day 14 of culture[22].

Collagen-based composites with synthetic acrylamide-based polymers [poly (N-isopropylacrylamide), pNIPAAm] were first studied in the eyes of white rabbits when compared with human amniotic membranes (AMs) and sham-operated corneas served as controls, it found the regrowth of the epithelium over the central corneal defects was achieved by 3d in 21 of 24 eyes (87.5%) in the polymer group. When type I collagen was immobilized onto poly (vinyl alcohol) (PVA-COL) as a possible artificial cornea scaffold, it can sustain a functional corneal epithelium. Thus, the permeability of PVA-COL was shown to support a stratified epithelium *in vitro* as well. In this study, 6 out of 9 trials resulted in the stratification of human epithelium on PVA-COL following air-lift cultures, stratified human epithelium on PVA-COL expressed the cornea-specific differentiation marker keratin 3/12 and the tight-junction-associated protein, occludin. Inhibition of horseradish peroxidase intrusion showed the superficial cell tight junctions to be intact, electron microscope showed that the superficial cells were equipped with microvilli-like structures observed in healthy corneal epithelia. These results indicated that the stratified epithelium on PVA-COL has the same histological and functional characteristics of the healthy epithelial surface[23,24].

Hoping to better mimic the natural ECM and to increase functionality and mechanical properties, Vrana *et al*[25] combined collagen with glycosaminoglycan (GAG) molecules and chondroitin sulfate, by using N-ethyl-N_-[3-dimethylaminopropyl] carbodiimide (EDC) as coupling reagent to react with carboxyl group and amine group. Histology demonstrated the epithelial layer appeared as a thin, differentiated, and stratified on the upper surface. In immunofluorescence and immunohistochemistry, differentiated epithelial cells showed the classical cytokeratin 3 staining for corneal epithelium, basal lamina deposition was shown by the collagen type IV-positive staining between the epithelial and stromal layers[25].

Collagen vitrigel (CV) also has the potential suitability as a substrate for the separate reconstruction of the three main cellular layers of the cornea. Ponce Márquez *et al*[9] cultured keratocytes and endothelial cells on transparent membranes made of type I collagen. RT-PCR, light and electron microscopy indicated partially stratified epithelial sheets with upregulation of the putative stem cell marker p63. Keratocytes cultured in serum on CV exhibited stellate morphology along with a marked increase in expression of corneal crystallin aldehyde dehydogenases (ALDH) and keratocan compared to identical cultures on tissue culture plastic. Epithelial and endothelial cells also exhibited adhesive structures (desmosomes and hemidesmosomes) and evidence of apical specialization (microplicae), these results indicated that CV holds promise as a substrate for corneal reconstruction[26].

Synthetic collagen-based corneal scaffolds still have some barriers to overcome, the researcher Griffith from Canada modified collagen gels with Tyr-Ile-Gly-Ser-Arg (YIGSR) as a model cell adhesion peptide. It was found that YIGSR incorporation into the bulk and YIGSR modification of surface promoted the adhesion and proliferation of human corneal epithelial cells as well as neurite extension from dorsal root ganglia. They crosslinked Porcine type I collagen and 2-methacryloyloxyethyl phosphorylcholine (MPC) by using 1-ethyl-3- (3-dimethyl aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) as coupling reagents, then moulded them to appropriate corneal dimensions to serve as substitutes for natural corneal ECM. These materials were implanted by penetrating keratoplasty into the corneas of guinea pigs after removal of the host tissue. Histopathology and ex *vivo* nerve terminal impulse recordings indicated that the implants promoted regeneration of corneal cells, nerves and the tear film, while retaining optical clarity at three months and among eight months.

These findings showed much promise with regard to biointegration and biocompatibility of the tissue-engineered cornea [27,28]. Based on the idea that corneal ECM is largely collagenous and the collagen is a biopolymer that is amenable to modification, Fagerholm *et al* [29] implanted a 500-μm-thick biosynthetic corneal substitutes, composed of 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide (EDC) cross-linked recombinant human collagen into ten patients as anterior lamellar keratoplasty (ALK). At the 24th month, the regenerated epithelium was morphologically normal in all patients. Although only oversimplified mimics can be reached currently, they did represent advances in materials engineering and cell biology. However, current application is

still in the beginning, hoping that it may initiate development in tissue regeneration of the human artificial cornea.

Fibrin Fibrin is a another commonly used protein material. With thrombin, fibrinogen completes the final step of the natural coagulation. Using these two components can synthesized fibrin gel that is similar to physiological blood clots, cross-linking can enhance the adhesion and migration of the epithelial cells on the gel [30]. Fibrin gel can be degraded completely after the implantation into the body, eventually replaced by the autologous collagen. Because both fibrinogen and thrombin can be obtained from autologous plasma, so it is an ideal tissue engineering. Human fibrin has been proposed as a stromal substitute to construct different tissue substitutes, also it has the advantages of low price, availability and good tolerance to cells.

The corneal epithelium's growth on a fibronectin/fibrin gel was measured quantitatively *in vitro* by Han *et al*[31]. Suspended cells proliferated in the fibrin gel, giving rise to colonies that eventually coalesced to near confluence over the 15 days of cultivation. The gels were sectioned and immunostained for keratin 3 (AE5) and keratin 19, all of the cells cultivated in the fibrin gel stained positively for keratin 3 (AE5), indicating differentiation along the corneal epithelium lineage. Cells located in the center the colonies were keratin 19-positive, suggesting a more primitive cell type. Similarly, Talbot *et al* [32] evaluated the restoration of the corneal epithelium by grafting autologous limbal cells cultured on fibrin gels onto denuded corneas. Rabbit limbal epithelial cells (RLECs) were cultured on a fibrin gel matrix, the cultured epithelia grew into confluence after 2 weeks.

In order to acknowledge fibrin further, Alaminos *et al*[33,34] quantitatively measured the epithelial cell seeded on the scaffold and found that the scaffold mixed with fibrin and agarose increased epithelial cell growth *in vitro* when compared with controls. Three main cell types of the cornea (epithelial, stromal, and endothelial cells) obtained from ten rabbit corneas, epithelial cells exhibited typical cobblestone morphology, tended to migrate from the corneal explant around the fifth day of culture (5.1±2.4d), reaching confluence and forming a monolayer of cells after 11 days of culture (11.1±5.6d) in the absence of 3T3 feeder layers, compared with around day 6 of culture (6.3±2.3d), reaching confluence in 13 days (13.6±5.2d) when the feeder layer of inactivated 3T3 cells was used. Nevertheless, use of the feeder layer prevented stromal contamination. Microscopic evaluation of the corneal constructs revealed that epithelial cells tended to form a normal, stratified, tightly packed epithelium with several layers of cells, and the superficial cells were flattened after exposure to air. In addition, immunohistochemical analysis of the constructed corneas demonstrated phenotypic similarities with normal, native rabbit corneas, with high expression of cytokeratin 3 in the epithelial cell layer. Although a stromal substitute was synthesized by a mixture of fibrin and 0.1% agarose, the use of agarose demonstrated better consistency and good transparency than fibrin alone. Alaminos *et al* [33] investigated intercellular junction formation and differentiation on human corneal substitutes. To generate these artificial human corneas, they continued using fibrin and agarose as scaffolds and immersed them within human keratocytes, then cultured the human corneal epithelium on the top. Electron microscopy and immunofluorescence analyses revealed that several types of cell-cell junction, especially desmosomes, were found in multilayered mature corneal substitutes. Concomitantly, the expression of genes encoding for plakoglobin 3 (PKG3), desmoglein 3 (DSG3), desmoplakin (DSP), zonula occludens 1 (ZO-1), 2 (ZO-2) and connexin 37 (Cx37) was higher in multilayered artificial corneas than in immature artificial corneas. They also demonstrated that cultured corneal substitutes submitted to air-liquid culture technique tend to form a well-developed epithelium that is very similar to the epithelium of human native corneas.

CHITIN AND CHITOSAN

Chitin is a linear polysaccharide composed of N-acetyl-D-glucosamine, and it can be used for artificial skin, cartilage tissue engineering, drug delivery carriers, artificial liver, artificial cornea because of the characteristics of non-toxic, no stimulation, no antigenicity and biodegradation, along with the ability to promote the growth factors production and epithelium differentiation, also as the carrier for the release of growth factors and inhibition of fibroblast growth[35-38].

Various methods have been used for reconstruction of the artificial cornea such as the use of complexes of collagen-chitosan-sodium hyaluronate (Col-Chi-NaHA). The results showed that rabbit corneal epithelial cells, endothelial cells and stromal cells cultured on Col-Chi-NaHA complexes had biological characteristics because of good biocompatibility with cornea [39]. Zhu Y and his colleagues[40] found that collagen-chitosan scaffolds suitable for the proliferation of adipose tissue-derived stem cells (ADSCs). Chitosan films has good light transmittance and mechanical strength, it also has high water absorption, but a large number of amino groups on the surface of chitosan films that may inhibit the proliferation of cells, how to improve the biocompatibility is the current research focus[41].

SILK FIBROIN

Silk fibroin extracted from natural silk protein polymer fibers contains 18 kinds of amino acids, it has good biocompatibility, mechanical properties and physicochemical properties, such as good flexibility and tensile strength, air permeability, and after different treatment can forms fibers, solution, powder, gel film, *etc*.

Chirila *et al* [42] taked advantage of its biocompatibility, they prepared membranes from fibroin, a protein isolated from the domesticated silkworm (Bombyx mori) silk cuture corneal limbal epithelial cells. The membranes supported levels of human limbal epithelial (HLE) cell growth comparable to that observed on tissue culture plastic. Currently, Higa and Shimazaki [43] have developed a technique for generating carrier-free sheets using fibrin sealants compared with AM, these sheets seem to contain more differentiated epithelium while retaining similar levels of colony-forming progenitor cells. They have also generated epithelial sheets a clinical trial using as biological carrier silk fibroin film, which offers a more transparent medium than conventional sheets. This technique may help maintain progenitor cells during *ex vivo* expansion of epithelial cells. Although these advances are expected to improve clinical outcomes in patients with ocular surface disorders, further improvements, such as the development of cultivation methods that do not require 3T3 feeder cells or real-time assessment of cultivated sheets, are required in the near future. Liu *et al* [44] also evaluate a silk fibroin (SF) biomaterial as a substrate for corneal epithelial cell proliferation, differentiation, and stratification *in vitro* compared with denuded human AM. Silk fibroin a novel biomaterial, could support corneal epithelial cells to proliferate, differentiate, and stratify, retaining the normal characteristic epithelium phenotype. Compared with AM, its unique features, including the transparency, ease of handling, and transfer, and inherent freedom from disease transmission, make it a promising substrate for corneal wound repair and tissue-engineering purposes.Madden *et al* [45] grow an endothelial layer on silkworm (Bombyx mori) fibroin membranes coatings of collagen IV, FNC Coating Mix® and a chondroitin sulphate-laminin mixture. All the coatings improved the final mean cell count comparable to uncoated tissue-culture plastic. It is the first successful growth of primary human corneal endothelial cells on coated fibroin as a step in evaluating fibroin as a substratum for the transplantation of tissue-constructs for endothelial keratoplasty.

In conclusion, it is possible to fabricate synthetic or composite naturall/synthetic polymeric scaffolds that can be used in the development of artificial corneas. Now more commonly used tissue-engineered artificial cornea compared to other non-tissue artificial cornea have the following advantages: first of all, it is more close to the physiological characteristics of the normal cornea, and it can avoid serious complications after implantation of non-tissue materials. Secondly, bioscaffolds can well support the proliferation and secretion of corneal epithelium and stem cells, they also can accelerate healing. Finally, it is convenient for surgical operation, overcoming the traditional complex immobilization problem of artificial cornea [46,47]. Although models using tissue engineering cornea vary and showed a different levels of promise, the methods of forming composite scaffolds are all critical to achieving an artificial cornea or replacements for different portions of corneas for transplantation. The perfect KPro has yet to be developed; the current researches which use biologic materials are promising and may eventually offer sight to millions of people impaired by corneal blindness around the world.

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