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MAIN ARTICLE

Utilising silk fibroin membranes as scaffolds for the growth of tympanic membrane keratinocytes, and application to myringoplasty surgery

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Abstract

Background: Chronic tympanic membrane perforations can cause significant morbidity. The term myringoplasty describes the operation used to close such perforations. A variety of graft materials are available for use in myringoplasty, but all have limitations and few studies report post-operative hearing outcomes. Recently, the biomedical applications of silk fibroin protein have been studied. This material's biocompatibility, biodegradability and ability to act as a scaffold to support cell growth prompted an investigation of its interaction with human tympanic membrane keratinocytes.

Methods and materials: Silk fibroin membranes were prepared and human tympanic membrane keratinocytes cultured. Keratinocytes were seeded onto the membranes and immunostained for a number of relevant protein markers relating to cell proliferation, adhesion and specific epithelial differentiation.

Results: The silk fibroin scaffolds successfully supported the growth and adhesion of keratinocytes, whilst also maintaining their cell lineage.

Conclusion: The properties of silk fibroin make it an attractive option for further research, as a potential alternative graft in myringoplasty.

Key words: Tympanic Membrane Perforation; Myringoplasty; Silk Fibroin; Graft; Keratinocyte

Introduction

Otitis media is defined as infection of the middle-ear cleft, and is the most common reason for children to seek paediatric care.[1] It is also the most common childhood otological condition which compromises sound conduction in the middle ear and can adversely affect long-term hearing.[2] Chronic suppurative otitis media describes a stage of ear disease in which there is chronic infection of the middle-ear cleft, and in which a non-intact tympanic membrane (i.e. a perforation) and discharge are present.[3]

Despite advances in medical and surgical treatment, as well as increased public health awareness, chronic suppurative otitis media is still highly prevalent worldwide, and may be the most important cause of hearing impairment in some developing countries.[3,4] Possible sequelae include speech and language deficits as well as developmental problems in children.[5] The magnitude of hearing loss is proportional to the size of the perforation and the degree of damage caused to middle-ear structures.[2,6]

Although hearing loss is the most common complication of chronic suppurative otitis media, chronic tympanic membrane perforations are also associated with many other extracranial and intracranial complications, and have a significant mortality rate.[4,7] The most frequent extracranial complications include mastoiditis, facial paralysis, subperiosteal abscess formation and labyrinthitis; the most frequent intracranial complications include meningitis, cerebral abscess formation, lateral sinus thrombosis, extradural abscess formation, otic hydrocephalus and encephalitis.[4,7] It is of particular concern that although antibiotic therapy has reduced the incidence of chronic suppurative otitis media, its complication rate has not altered in the past 10 years.[8]

Myringoplasty (also known as type one tympanoplasty) is the term used to describe the surgical repair of a perforated tympanic membrane by utilising a graft to close the perforation. It is one of the most common otological procedures performed in adults and children, and generally has a very high success

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rate with respect to perforation closure, although this is dependent on surgeon and patient factors.[9,10]

However, myringoplasty does have a number of limitations. Few studies have reported hearing outcomes after surgery, and documented success rates often refer solely to perforation closure and the cessation of aural discharge.[9] Some of the studies which did examine post-operative hearing observed a considerable discrepancy between pure tone audiogram results and the subjective hearing benefit reported by the patient, describing only a 55 per cent improvement in subjective hearing after surgery.[11] Whilst many papers refer to the high success of myringoplasty in terms of perforation closure rates, there is evidence to suggest that post-myringoplasty hearing may be improved but not fully restored.[12–14]

Multiple factors affect the success of myringoplasty, including graft type.[15] Although multiple autologous grafts, allografts and synthetic grafts are currently used in myringoplasty, all have documented limitations.[9] Current materials used as grafts by different surgeons include temporalis fascia, cartilage, perichondrium, fat, paper and alloderm.[15–18]

Ideally, the material used to surgically repair a perforated tympanic membrane would satisfy all of the following criteria: safety, ease of purchase, unlimited availability, low cost, resistance against negative ear pressures, and lack of interference with hearing.[19] A transparent material would also be preferable as this would more closely mimic the native tympanic membrane and would allow otoscopic visualisation of middle-ear structures during patient follow up.

To date, none of the above frequently used graft materials have met all of these requirements. Moreover, Kaftan *et al.* noted that a high proportion of patients found to have a chronic perforation did not want surgical treatment, further suggesting the need for novel therapeutic materials and/or procedures.[20]

Silks are naturally occurring polymers that can be extruded by insects or worms.[21] They are composed of a filament core protein (fibroin) and a glue-like coating protein (sericin). Silk from the silkworm *Bombyx mori* has been used clinically for centuries in the form of sutures. Over the last 25 years, the development of wider clinical applications was initially hindered by problems with biocompatibility; however, contamination from sericin was thought to be responsible for this, and the isolation of silk fibroin has largely eliminated these concerns.[22]

In recent years, silk fibroin has been studied with biomedical applications in mind due its remarkable mechanical properties, including biocompatibility and biodegradability. It has been shown to support cell adhesion, proliferation and differentiation *in vitro* and to promote tissue repair *in vivo*. Moreover, its versatile processing and surface modification options have enabled its clinical utility to be expanded, and it can now be made readily available in various forms such as films, fibres, nets, meshes, membranes, yarns and sponges.[21]

In recent times, designing scaffolds for tissue engineering purposes has become particularly relevant.[22] Studies have shown that the behaviour of cells on three-dimensional silk fibroin scaffolds can be related to the structural differences resulting from different scaffold preparation processes.[23] Investigators can therefore be guided to use processing scenarios that match the tissue specifications and clinical applications required.

In order to assess the application of silk fibroin scaffolds to myringoplasty surgery, an animal model of chronic tympanic membrane perforation is ideally required. This is because the majority of acute perforations heal spontaneously.[24] An inexpensive and valid animal model would provide insights into the mechanisms of tympanic membrane wound healing, as well as allow various treatment options to be evaluated.[25] However, although multiple attempts have been made, the ideal animal model for chronic tympanic membrane perforation is yet to be created.[26]

Amoils *et al.* had some success in developing a model in chinchillas, which was permanent, well epithelialised and free from infection.[24] Chronic perforations were achieved in a significant percentage of animals, but persistent infection, or tympanic membrane regeneration despite re-perforation, was problematic. Writing 20 years ago, Amoils *et al.* envisioned that if a completely successful model could be developed, it could be used to assess the utility of various biomembrane scaffolds (impregnated with various growth-promoting substances) in the regeneration of perforated tympanic membranes. They predicted that a membranous disc with biorecombinant growth factors could provide a simple technique for the repair of chronic tympanic membrane perforations, which could be performed in the clinic and which avoided the potential morbidity and prolonged time associated with myringoplasty surgery.[24]

Although the lack of an animal model for chronic tympanic membrane perforation has somewhat hindered *in vivo* research, investigation of membranous scaffolds and their interaction with tympanic membrane keratinocytes has been undertaken *in vitro*.[27,28]

It is important to note that the tympanic membrane has a specific, multi-layered microstructure which is responsible for its unique function.[29] It is a unique membrane suspended entirely in air. From lateral to medial, it comprises 5 layers: an epidermal layer of keratinised squamous epithelium; a thin connective tissue layer; a dense connective tissue layer; a thin layer of connective tissue; and an inner single layer of epithelial cells.[30] This microstructure is vastly different from the various grafts that are commonly used to repair tympanic membrane perforations.[9]

The specific distribution of various types of collagen in both healthy and perforated tympanic membranes has also been studied.[31] Interestingly, the pars tensa

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and pars flaccida of the tympanic membrane are composed of different types of collagen, reflecting the different physiological properties of these tissues. Collagen types I and III are present in the acute phase after perforation, and this collagen content is modified during the inflammatory and healing processes.[31]

It is clear that the tympanic membrane is anatomically and functionally a very complex membrane. It is doubtful that any of the available autologous grafts, homografts or synthetic grafts will be able to mimic both its structure and function. Perforations may be successfully closed, but the difficulty remains in regaining premorbid hearing. Perhaps this is only feasible with a scaffold that supports the overgrowth of native tympanic membrane cells and biodegrades with time. This could potentially result in the formation of a healed tympanic membrane with no foreign graft material and improved hearing outcomes.

To our knowledge, our group is the first to apply the use of silk fibroin biomaterials to the field of otology, with the aim of alleviating some of the limitations of the various grafts currently used in myringoplasty.[9,27,28] We report the growth and proliferation of human tympanic membrane keratinocytes on silk fibroin scaffolds, with a view to their potential future use in the repair of tympanic membrane perforations.

Materials and methods

See Appendix 1 for a detailed description of the scientific methods and materials used.

Results and discussion

Light microscopy images confirmed that the silk fibroin scaffolds successfully supported the growth of human tympanic membrane keratinocytes. In order for these scaffolds to be considered as grafts for myringoplasty surgery, we needed to investigate various characteristics, interactions and functions of the cells growing upon them. This was achieved using immunofluorescent cell staining and laser-scanning confocal microscopy to verify the expression of key cellular proteins expressed by human tympanic membrane keratinocytes.

Figure 1 shows human tympanic membrane primary keratinocytes spreading circumferentially outwards from the tympanic membrane explant (day 14). The typical, cobblestone appearance of keratinocytes is visible.

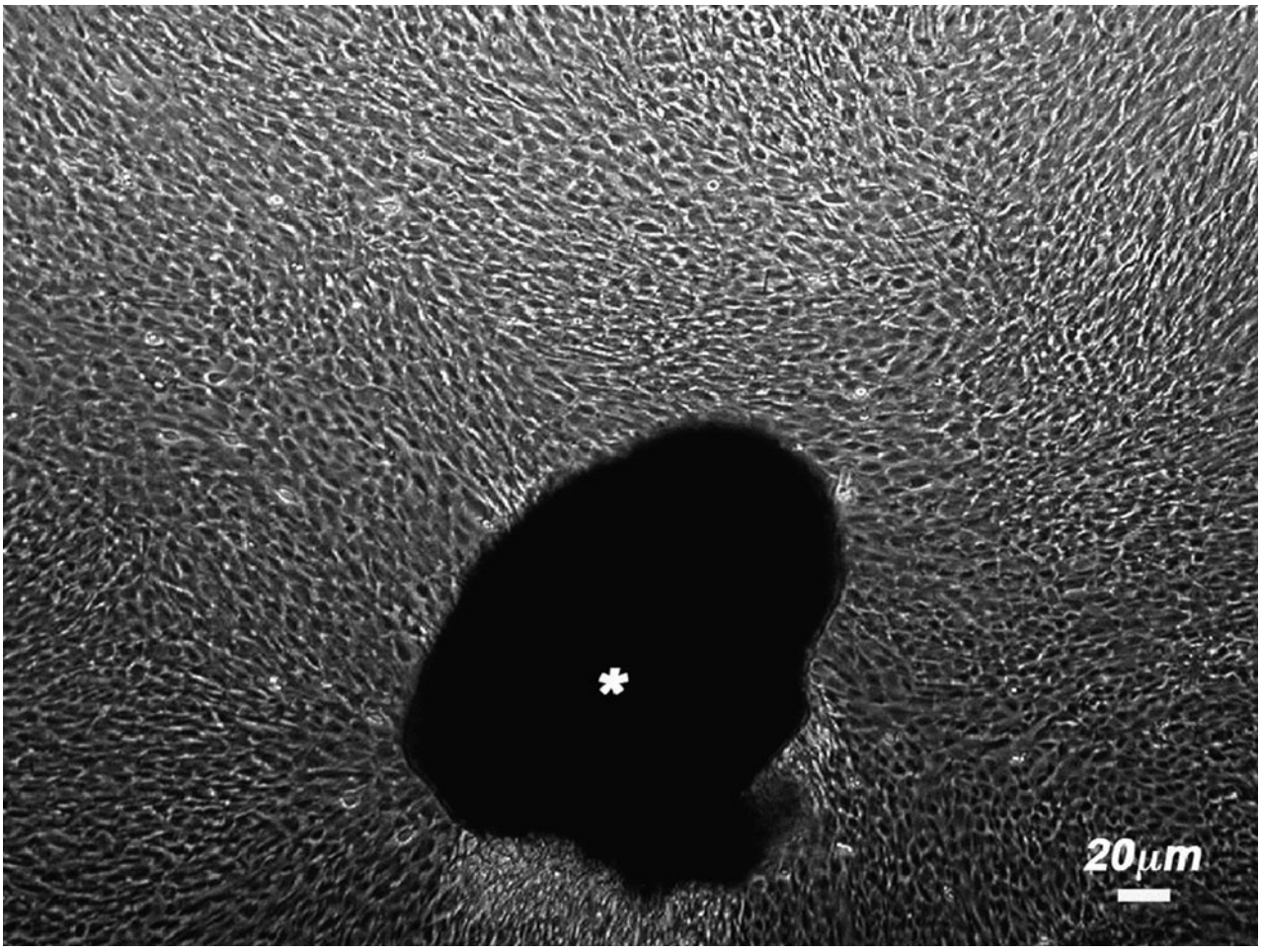

FIG. 1

Photomicrograph of human tympanic membrane cells spreading circumferentially from the explant (asterisk) in culture media, on day 14 of culture. (Greyscale light microscopy; ×4, scale bar = 20 μm)

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Figure 2 shows human tympanic membrane keratinocytes growing and proliferating on the silk fibroin membrane, 8 days (Figure 2a; 90 per cent confluence) and 14 days (Figure 2b) after seeding onto the membrane. In Figure 2(c), 18 days after seeding on the scaffold, the interface between the tympanic membrane cells on the silk fibroin scaffold and the culture plate surface is clearly visible, and the cells are confluent on the scaffold.

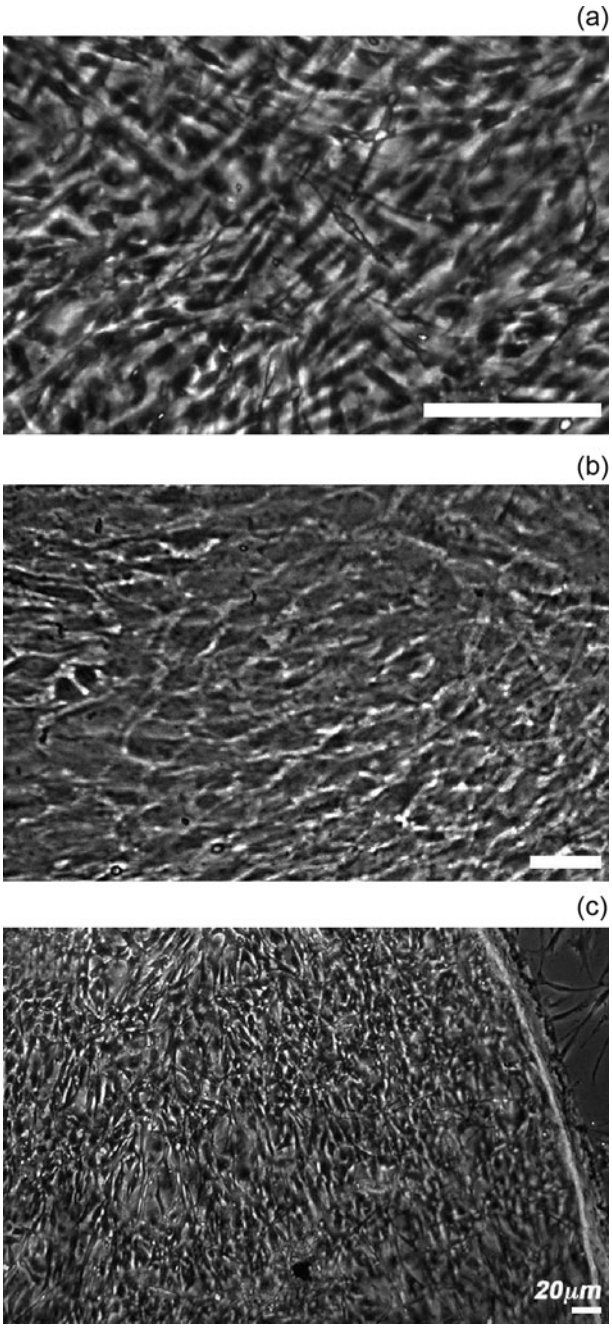

FIG. 2

Photomicrographs of human tympanic membrane keratinocyte cell growth on a silk fibroin membrane after (a) 8 days (×4), (b) 14 days (×20) and (c) 18 days (×4). The interface between the cells on the membrane and the culture media can be seen in part (c). (Greyscale light microscopy; scale bar = 20 μm in all parts)

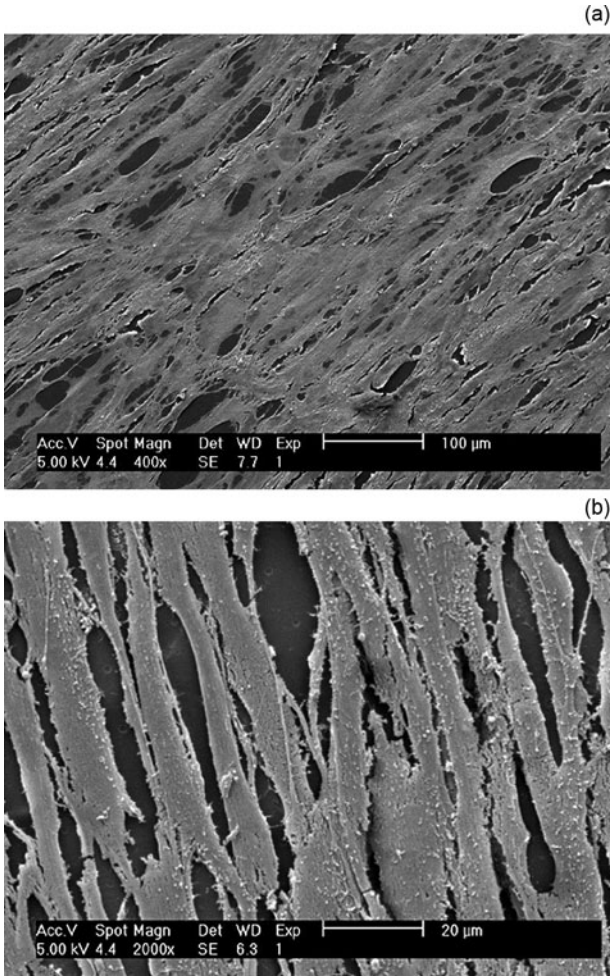

FIG. 3

Scanning electron micrographs of keratinocytes after (a) 5 days and (b) 15 days of growth on a silk fibroin membrane

Figure 3 shows a scanning electron micrograph of the same cells after 5 days (Figure 3a) and 15 days (Figure 3b) of growth on the silk fibroin membrane.

It was important to verify the keratinocytes' cell phenotype as epithelial, and to confirm that these cells did not transform into other cell types while growing on the silk fibroin membrane.[32] Epithelium-specific Ets-1 is a highly tissue-specific member of the Ets transcription factor and oncogene family, and is expressed exclusively in epithelial cells. This protein is induced during terminal differentiation of the epidermis and during primary human keratinocyte differentiation.[33] Figure 4 shows strong expression of epithelium-specific Ets-1 in human tympanic membrane keratinocytes grown on the silk fibroin membrane. The green fluorescence indicates that epithelium-specific Ets-1 expression is located in both the cell cytoplasm and cytoplasmic membrane. The red fluorescence indicates cell nuclei labelled with 4',6-diamidino-2-phenylindole.

Occludin is an integral plasma membrane protein located specifically at tight junctions. Zona occludens protein 1 is a protein located on the cytoplasmic membrane surface of intercellular tight junctions, which is

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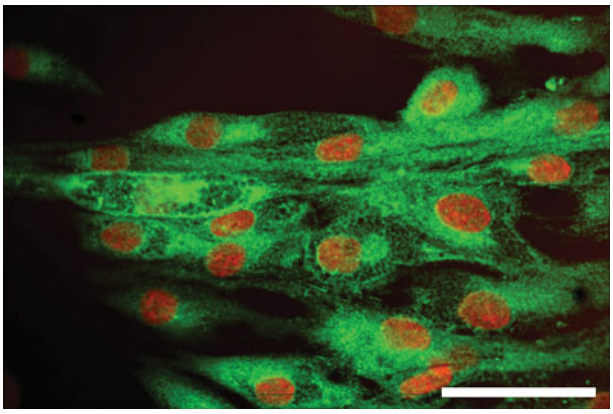

FIG. 4

Photomicrograph of human tympanic membrane keratinocytes on a silk fibroin membrane, with immunofluorescent staining showing expression of epithelium-specific Ets-1 protein (green fluorescence). (Scale bar = 20 μm)

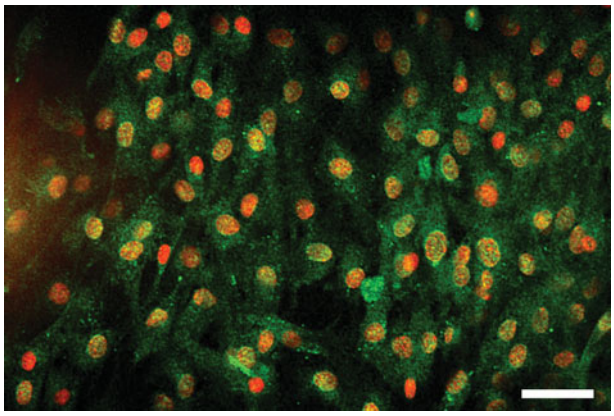

FIG. 6

Photomicrograph of human tympanic membrane keratinocytes on a silk fibroin membrane, with immunofluorescent staining showing expression of E-cadherin protein (green fluorescence). (Scale bar = 20 μm)

thought to be involved in signal transduction between cells at these junctions. Occludin and zona occludens protein 1 both play key roles in cell adhesion.[34,35] Figure 5 shows strong expression of both occludin (green fluorescence) and zona occludens protein 1 (red fluorescence) in the tympanic membrane cells. Strong expression of these 2 integral proteins indicates that the important function of cell–cell adhesion between human tympanic membrane keratinocytes is maintained on the silk fibroin scaffold. Blue fluorescence indicates cell nuclei labelled with 4',6-diamidino-2-phenylindole. Yellow fluorescence is a result of the co-localisation of the two tight junction proteins.

Figure 6 shows expression of E-cadherin in the human tympanic membrane keratinocytes. E-cadherins are transmembrane proteins that also function in intercellular adhesion between epithelial cells.[36,37] Strong expression of these important proteins is evident (indicated by green fluorescence). Red fluorescence indicates cell nuclei stained with 4',6-diamidino-2-phenylindole.

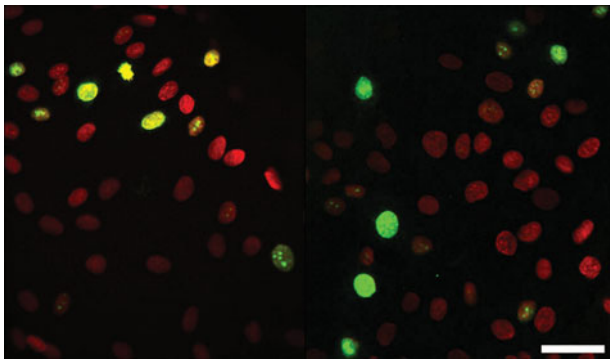

FIG. 7

Photomicrograph of human tympanic membrane keratinocytes on a silk fibroin membrane, with immunofluorescent staining showing expression of MIB-1 protein (green fluorescence). (Scale bar = 20 μm)

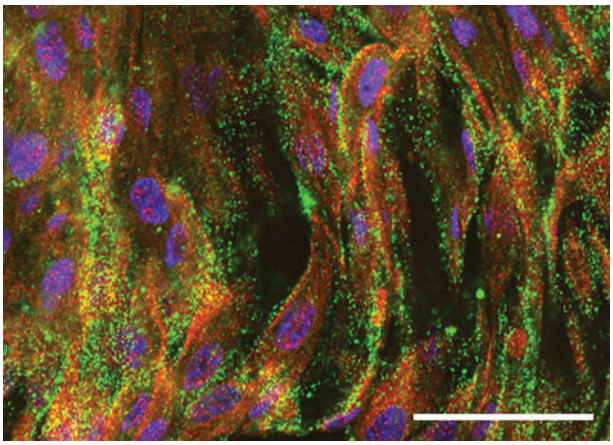

FIG. 5

Photomicrograph of human tympanic membrane keratinocytes growing on a silk fibroin membrane, with immunofluorescent staining showing expression of the tight junction proteins occludin (green fluorescence) and zona occludens protein 1 (red fluorescence). (Scale bar = 20 μm)

- **Chronic tympanic membrane perforations can cause significant morbidity**
- **Many graft types are used for myringoplasty; all have limitations**
- **The properties of silk fibroin protein suggest its utility for myringoplasty grafting**
- **These include high tensile strength, biocompatability, slow biodegradability, and capacity for diverse morphologies**
- **In this study, silk fibroin scaffolds successfully supported human keratinocyte growth, adhesion and cell lineage integrity**

The proliferation marker MIB-1 is a commonly used monoclonal antibody that detects the Ki-67 antigen. This nuclear protein is associated with cellular proliferation and is present in all active phases of the cell cycle.

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FIG. 8
Silk fibroin membranes prior to keratinocyte seeding

Consequently, MIB-1 is used as a marker of cellular proliferation.[38] Figure 7 demonstrates strong expression of the MIB-1 protein in human tympanic membrane keratinocytes, visualised as green fluorescence. However, co-localisation with 4',6-diamidino-2-phenylindole nuclear expression (yellow fluorescence) is also seen. No expression of MIB-1 is seen in cells in the resting phase of their cell cycle (red fluorescence).

Conclusion

The present study investigated the growth of human tympanic membrane keratinocytes on a scaffold composed of silk fibroin. The results indicated that the scaffold supported the growth and proliferation of these keratinocytes. Immunofluorescent protein expression confirmed that human tympanic membrane keratinocytes maintained their cell lineage on the scaffold, and that the production of integral cellular proteins essential for proliferation, differentiation and adhesion was preserved.

The biocompatibility, biodegradability and transparency of silk fibroin are major advantages of its use as a tissue engineering scaffold. The findings of the present study are important for further investigation of silk fibroin scaffolds and their application to myringoplasty surgery. To date, no scaffold with the properties of silk fibroin (i.e. facilitation of human tympanic membrane keratinocyte overgrowth, and biodegradability) has been used in clinical myringoplasty. The use of silk fibroin scaffolds may enable otological surgeons to successfully close chronic tympanic membrane perforations; furthermore, this material has the additional advantages of permitting post-operative inspection of middle-ear structures, being transparent, and of potentially facilitating improved hearing outcomes.

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Appendix 1. Materials and methods

Preparation of silk fibroin membranes

Cocoon pieces from *Bombyx mori* silkworms were boiled in 2 g/l sodium carbonate and 0.6 g/l sodium dodecyl sulphate solution. The resultant fibrous material was washed thoroughly in hot and cold water. After drying, the fibres were dissolved in a concentrated solution (9.3 M) of lithium bromide.

The resultant solution was dialysed for four days at 4°C using dialysis sacks (molecular weight threshold 12 kDa) against Milli-Q® water, which was changed every 6 to 12 hours. The concentration of silk solution was adjusted to 3 per cent (weight by volume) by diluting with Milli-Q water. A portion of silk solution was retained for the preparation of water-based films, while the remaining solution was frozen at −80°C overnight and lyophilised. The lyophilised silk solids were dissolved separately in 98 per cent formic acid and ≥98 per cent trifluoroacetic acid to obtain 3 per cent (weight by volume) trifluoroacetic acid and formic acid fibroin solutions.

The silk solution was poured into polyethylene discs and dried inside fume hoods to obtain dry films (approximately 33 μm in thickness). The films were stored inside the fume hood for a further 24 hours to evaporate residual solvents completely.

For structural stability (i.e. conversion into the 'silk II' conformer which is insoluble in water), the membranes were treated with 75 per cent aqueous solution of ethanol for 6 hours. After hydration to equilibrium and extensive rinsing in water exchanges, the *Bombyx mori* silk fibroin membranes were stored at 4°C.

Prior to experimentation, discs were cut from membranes using an 8 mm biopsy punch (Stiefel Laboratory, Hesse, Germany). Figure 8 shows the raw silk fibroin membranes after preparation.

Primary culture of keratinocytes

Ethics approval was obtained from the St John of God Hospital care ethics committee (approval number 193) to collect excess tympanic membrane tissue from patients undergoing relevant otological procedures at that hospital, Perth, Western Australia.

Small pieces of human tympanic membrane (approximately 0.25 mm^2) were placed into BD Falcon six-well culture plates (BD Biosciences, North Ryde, New South Wales, Australia). Dulbecco's modified Eagle's medium with 4500 mg/l D-glucose was supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Mount Waverly, Victoria, Australia) and 10 per cent fetal bovine serum (Invitrogen). Plates were incubated in a humidified cell culture incubator at 37°C with 5 per cent carbon dioxide (CO_2).

Keratinocyte seeding onto silk fibroin membranes

Discs of silk membrane, 8 mm in diameter, were placed into BD Falcon six-well culture plates (BD Biosciences). Discs were disinfected by immersion in 4 ml of 70 per cent ethanol for 2 hours at room temperature, followed by six washes in 4 ml sterile phosphate-buffered saline over a 24-hour period. Prior to use, the discs were briefly rinsed in high-glucose Dulbecco's modified Eagle's medium supplemented with 10 per cent fetal bovine serum and 100 U/ml penicillin.

Human tympanic membrane keratinocytes (passage six) were seeded into each membrane-containing well, at 0.3×10^6 cells/ml. Plates were left for 12 days at 37°C in 5 per cent CO_2. The culture medium was changed every 48 hours.

Antibodies

The primary antibodies used included rabbit polyclonal anti-human epithelium-specific Ets-1 (Novus Biologicals, Littleton, Colorado, USA), mouse monoclonal anti-human E-cadherin (Zymed, Carlsbad, California, USA) and mouse monoclonal anti-human MIB-1 (Dako, Glostrup, Denmark). Secondary antibodies used included Alexa Fluor-488 conjugated

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goat anti-mouse (1:500) and Alexa Fluor-546 conjugated goat anti-rabbit (1:500) (Molecular Probes, Eugene, Oregon, USA). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (Molecular Probes) at 1:500, diluted in one part phosphate-buffered saline.

Immunofluorescent staining

Cultured cells on silk fibroin membranes were washed briefly in phosphate-buffered saline at pH 7.2 (Invitrogen) and then incubated in methanol at −20°C for 10 minutes, followed by three washes with phosphate-buffered saline at pH 7.2 and containing 0.1 per cent Tween® 20.

Incubation with primary antibodies was performed at room temperature for 60 minutes, followed by three washes with the same phosphate-buffered saline Tween solution. Primary antibodies were diluted in phosphate-buffered saline containing 1 per cent bovine serum albumin.

Secondary antibody incubations were performed at room temperature for 60 minutes followed by three washes in the above phosphate-buffered saline Tween solution.

Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole for 20 minutes, washed with the phosphate-buffered saline Tween solution, cover-slipped in anti-fade mounting medium, and sealed with clear nail varnish.

Confocal laser-scanning microscopy

Sequential images were obtained using a Nikon A1Si confocal laser-scanning microscope (Nikon, Tokyo, Japan) on a Nikon TiE (Nikon) with an oil ×40 objective (Nikon Pan Fluor, NA 1.30). The pinhole was set to 1.0 Airy unit.

A 488 nm blue laser was used to excite AF-488 secondary antibodies, and fluorescence emission was detected through a 525/50 nm bandpass filter. A 561 nm green laser was used to excite AF-546 secondary antibodies, and fluorescence emission was detected through a 585/50 nm bandpass emission filter. A violet laser (405 nm) was used for the excitation of 4',6-diamidino-2-phenylindole, and fluorescence emission detected through a 450/50 nm bandpass filter.

Images were saved as 12-bit greyscale images and were subsequently merged using ImageJ (1.42e) software (Wayne Rasband; http://rsb.info.nih.gov/ij/). All images were saved in TIFF format.

Scanning electron microscopy

Human tympanic membrane keratinocytes grown on silk fibroin membranes were fixed in 2.5 per cent glutaraldehyde in one part Tris-buffered saline for 1 hour at room temperature, and washed briefly in one part Tris-buffered saline. A Pelco Biowave microwave processor (Ted Pella, Redding, California, USA) was used to perform the washing and dehydration steps. Samples were subsequently critical point dried and gold coated.

A Phillips XL30 ESEM (FEI-Philips, Eindhoven, The Netherlands) was used in backscatter mode to obtain images of human tympanic membrane keratinocyte growth on the silk membranes, using an accelerating voltage of 5.5 kV and a spot size of 4.4.

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