Tympanic membrane organ culture using cell culture well inserts engrafted with tympanic membrane tissue explants

Lawrence J Liew [a, b], Richard M Day [c], Rodney J Dilley [a, b, d, e]

Affiliations

[a] Ear Sciences Centre, School of Surgery, University of Western Australia, Perth, Australia.

[b] Ear Science Institute Australia, Perth, Australia.

[c] Division of Medicine, University College London, UK.

[d] Centre for Cell Therapy and Regenerative Medicine, University of Western Australia, Perth, Australia.

[e] Corresponding author.

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Abstract

Tissue engineering treatments to repair chronic perforations of the tympanic membrane are being developed with growth factors and materials, but there are surprisingly few relevant tissue culture models available to test these new solutions. In this study we developed a simple three-dimensional model system based on micro-dissecting the rat tympanic membrane umbo and grafting it into a culture well insert membrane. We found that cell outgrowth from the graft produced sufficient cells to populate the membrane of similar surface area to the human tympanic membrane within two weeks. Tissue grafts from the annulus region showed cell outgrowth but were not as productive. The umbo organoid supported substantial cell proliferation and migration under the influence of keratinocyte growth medium. Cells from umbo grafts were enzymatically harvested from the polyethylene terephthalate (PET) membrane for further expansion in routine culture and were harvested consecutively from the same graft over multiple cycles. We also used the harvested cells to test cell migration properties and to engraft a porous silk scaffold material as proof-of-principle for tissue engineering. The model is simple enough to be widely adopted for tympanic membrane regeneration studies and has further promise as a tissue equivalent model to reduce animal testing.

Method summary

Here we present a tympanic membrane organoid explant culture using tissue culture insert membrane which allows rapid isolation of tympanic membrane primary cells for biocompatibility testing and tissue engineering. The model is simple enough to be widely adopted for tympanic membrane regeneration studies and has further promise as a tissue equivalent model that may help reduce animal testing.

Introduction

The rapid spontaneous healing of injuries to the tympanic membrane (TM) gives rise to the concept of a robust regenerative mechanism. Studies have localized areas of high cell turnover in the TM to the centre of the membrane (the umbo region) and the outer rim (annulus region) and these have been suggested to contain stem cells (1,2) or regenerative centres (3,4). However, there is limited understanding on the biology of cells responsible or their niche within the TM and *in vitro* models of TM are currently unavailable.

Tympanic membrane cell cultures have been isolated (2,5,6) using trypsin digestion or TM tissue explants in conjunction with selective collagen IV adhesion *in vitro*. We previously showed that TM cells can be isolated via tissue explant culture from humans (5) however these methods can be inconsistent. An opportunity to generate consistent cell cultures arises from a unique aspect of TM biology whereby they produce large numbers of cells at the umbo, which then migrate *en masse* across the TM surface and along the ear canal, to a point where they are ultimately shed externally. An ink dot placed on the keratinized surface at the TM umbo is known to migrate to the peripheral margin of the membrane and then along the ear canal, so that in various species the pattern and rate of migration *in vivo* has been described (7-10). This TM property helps to maintain a clear ear canal and minimal TM keratin burden for sensitive transmission of sound through the middle ear. This also has a profound effect on repair mechanisms, which in distinction to other skin-like tissues, is led by keratinocyte layer responses rather than granulation tissue formation (11-13).

We have used this property of TM epidermis to generate cell cultures from umbo organoids placed in a collecting membrane. Here we describe the method and its use to generate a TM

'tissue equivalent' model and a tissue engineered TM construct *in vitro* using state of the art scaffolds.

Materials and Methods

Animals

Male Sprague-Dawley rats, weighing 250-300 g, were obtained from Animal Resources Centre (Murdoch, Western Australia, Australia). This study was approved by the University of Western Australia Animal Ethics Committee (No. 100/1249). The experiments were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Extraction of tympanic membrane

The animals (n=4) were euthanized by intraperitoneal administration of pentobarbitone (160 mg/kg). Using *en bloc* excision, both left and right external ears were removed at the osteocartilaginous junctions, and the tympanic bulla was isolated. The TM was then dissected free. The harvested TMs were rinsed in phosphate buffered saline pH 7.2 (PBS) plus 2 % antibiotic-antimycotic solution (Gibco, Grand Island, New York, USA)) for 2 min prior to processing.

Enzymatic digestion

For tissue culture, freshly extracted temporal bones were rinsed thoroughly in 2 mL of PBS and transferred to a 35 mm diameter culture dish containing 2 mL of 0.25% Dispase II

solution (Gibco). Following overnight incubation at 4° C, the TMs were peeled from the bony bulla and separated from the ear canal skin using forceps.

Rat TM explant culture on culture well insert membrane

A culture well insert with polyethylene terephthalate (PET) membrane (0.4 µm pore size, 12 well Thincert, Greiner Bio One, Frickenhausen Germany) was rinsed in PBS and a 1 mm scalpel incision was made in the centre. The malleus bone attached to the TM umbo was then partially inserted into the incision with the outer (lateral) surface of the umbo facing vertically and close to the level of the upper surface of the PET membrane. A length of annulus tissue was dissected from the pars tensa and similarly placed through a PET membrane incision. The inserts were then mounted into 12 well plates and filled with 1 mL of serum-free keratinocyte medium (KSFM), supplemented with human recombinant Epidermal Growth Factor 1-53, Bovine Pituitary Extract, 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco,). Explant cultures were incubated at 37°C in 5% CO_2 with a twice weekly media change for up to 2 weeks.

Culture expansion

Cell outgrowth from the explant onto the upper surface of the PET membrane occurred and was allowed to continue until a monolayer of cells approached the periphery of the culture well insert membrane, upon which the cells were passaged by enzymatic digestion into fresh culture dishes. Briefly, the cell monolayer was rinsed thoroughly with PBS and digested with 250 µL of pre-warmed Tryple Express (Gibco) for 15 min at 37°C. Digestion was stopped by adding 2 mL of KSFM containing 10% FBS. Detached cells were collected as supernatant and centrifuged at 600 g for 5 min and resuspended in 5mL of medium before replating in a

25 cm^2 cell culture flask (Greiner Bio-One). These cells were cultured in KSFM supplemented with 10% FBS and fed with fresh medium twice weekly, then upon reaching 60-70% confluence passaged into fresh dishes at a 1:3 ratio. The explant and membrane construct was kept intact and fresh culture medium applied to investigate continued outgrowth.

Immunofluorescent staining for characterization of the primary cells

Cells were cultured on a glass-bottom 35 mm dishes (Greiner Bio-One) for 2-3 days at 37°C before staining. The cells were rinsed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min at RT. The cells were incubated with ice-cold 100% methanol for 10 min at -20°C before blocking with 5% bovine serum albumin in PBS for 30 min at RT. Primary antibodies used were mouse anti-pancytokeratin (Biocare, Concord, California, USA) and anti-vimentin (Abcam Cambridge, UK). Following overnight incubation at RT, the cells were rinsed three times with PBS +0.01% Tween 20 before the addition of secondary antibodies: anti-rabbit IgG Alexafluor 555 (Abcam) or anti-mouse IgG Alexafluor 488 (Molecular Probes, Eugene, Oregon USA). The cells were incubated for 1h at RT and then rinsed in PBS + 0.01% Tween 20 and counterstained with DAPI (0.05 mg/mL, Molecular Probes,)) for 20 min. The cells were mounted in PBS:Glycerol (1:1) before viewing with an Olympus BX60 fluorescent microscope with appropriate fluorescence filters.

Epidermal cell enrichment using timed typsinisation

Homogeneous epidermal cell culture was obtained by carefully timed trypsinisation. Confluent cells in a 25 cm^2 cell culture flask (Greiner Bio-One) were rinsed once in PBS and incubated with 2 mL of pre-warmed Tryple Express (Gibco,). Cells were incubated at 37°C for 5-10 min and flasks gently tapped to detach the fibroblasts. Enzyme solution and

suspended fibroblasts were discarded or cryopreserved for later use. The remaining epidermal colonies were fed with fresh KSFM with 10% FBS and passaged as previously described upon reaching 70-80% confluent.

Wound healing migration assay

Epidermal cells (p4-5) were seeded at $5x10^4$ cells into the reservoirs of a two-well culture insert (Ibidi, Martinsried, Germany), adhered to a 35 mm glass bottom dish (Greiner Bio-One) and grown overnight at 37°C. On the day of assay, the insert was removed using a pair of forceps and dish was briefly rinsed with PBS to remove cell debris. Dish was then filled with 1mL of KSFM containing 10% FBS and placed on a stage top incubator (Tokai Hit, Fujinomiya, Japan). Phase contrast images were viewed and captured at 10x objective magnification using an Olympus IX-81 microscope system at 15 min intervals over 18 h.

Tissue engineering using cells isolated from membrane cultures

TM cells at passage 2 were resuspended in KSFM ($5x10^5$ per mL) and seeded onto silk fibroin coated coverslips in a 12 well culture plate. The scaffold was produced by immersing 13 mm borosilicate coverslips in 50 mg/ml silk fibroin solution (Advanced Biomatrix, Carlsbad, California, USA). The coated coverslips were immediately immersed into liquid nitrogen to achieve thermally induced phase separation. The frozen coverslips were placed into an Edwards Modulyo freeze drier and lyophilized for 24 h. The porous silk films were sterilised in 70% ethanol overnight before conditioning for 2 h in growth medium containing 10% FBS. Once seeded with cells, scaffolds were incubated at 37°C /5% CO_2 for 24 h, then rinsed twice in PBS, fixed in 4% paraformaldehyde for 10 min and stained with DAPI for 20 min before visualising with an epifluorescence microscope as described.

Results and Discussion

TM regeneration is of considerable general interest due to its robust and relatively scarless healing compared to skin; however, it is most specifically relevant in otology to chronic failure of wound healing after TM perforation, and to the serious consequence of invasive growth of TM cells into the middle ear and mastoid cholesteatoma (14,15).

There are few models that explore the biology of TM cells (2,6) and those available suffer from the very small amount of tissue and cells available. One avenue explored here was to establish a cell culture model that provides access to cells of the membrane and allows exploration of their properties in relation to growth and use for tissue engineering. A flow diagram for this rat TM umbo explant culture method on culture well insert membranes is shown in Supplementary Figure 1. Rat TM was dissected as a thin sheet suspended in a shell-like bony ring structure, the tympanic ring. Figure 1A shows a TM peeled whole off the bone and still attached to the malleus upon overnight digestion in 0.25% Dispase II. The annulus of the membrane was retained as a prominent fibrous ring and the thin and transparent pars tensa intact within it. As shown in Figure 1B, the umbo could be dissected free from the whole TM and the handle of the malleus removed, leaving only a remnant at the umbo. This method of rat TM extraction is superior in comparison to *in situ* harvest or other previously described method (2,6) as it preserves the normal anatomical relationships of TM and more importantly, it enables cell isolation from specific regional areas of TM (umbo and annulus).

The excised umbo was inserted in the slit (black arrow heads) of the PET membrane and the umbo surface and pars tensa remnant positioned on top of the membrane upper surface to create a PET membrane/explant construct that mimicked the structural arrangement of the umbo in its native TM (Figure 1C). As comparison, a separate explant using TM annulus

tissue was also placed through a similar slit in the membrane, shown in Figure 1D. The appearance of a culture well insert and its porous and semitransparent PET membrane is depicted in Supplementary Figure 2.

Figure 1E shows outgrowth of cells from the umbo explant onto the PET membrane at 48-72 h. Cells continued to grow peripherally until confluent across the membrane surface (~3 cm^2) at 12-14 days, as depicted in Figure 1F. Explant cultures fixed in 4% paraformaldehyde were stained with DAPI to visualize the presence and organization of cells. In Figure 2A, a substantial cell population could be visualised in the umbo explant and also grew as a monolayer over the entire culture well insert membrane surface, indicating that the model likely supports a strong source of cell proliferation in the explant rather than just a founding population of TM cells which migrated out over the surface. Fewer cells were observed to grow out onto the membrane from the annulus explant, shown in Figure 2B. The TM annulus is thought to contain regenerative centres (1,16,17) however, when we attempted to graft annulus tissue into the membrane the grafts were not as successful. Orienting the annulus tissue in the PET membrane was not as straightforward as the umbo grafts had been with the malleus handle. Outgrowth from an anchored annulus tissue explant did produce cells on the membrane but the area covered in the same time frame was not as extensive. Nevertheless we continue to pursue this avenue as the annulus provides more opportunity to source autograft tissue for myringoplasty in patients who may have lost their umbo regenerative centres in subtotal perforation.

In Figure 2C, cell nuclei on the culture well insert membrane were identified in mitosis showing proliferative expansion on the membrane. The cell nucleus orientation in intact TM

is tightly regulated in radial and circumferential directions, shown in Figure 2D (whole mount DAPI staining), but this same arrangement was not apparent on the PET membrane.

Once fully confluent, the cells were passaged into fresh culture dishes and explored for phenotype and tissue engineering. Passaging was performed up to 5 times from a single explant and the same explant continued to produce cells over 2 months. Aside from this capacity to harvest multiple cultures continuously from the same explant, TM explant cultures on well inserts were superior to conventional tissue culture plastic cultures as the porous PET membrane provides a supportive microenvironment for rapid outgrowth onto the membrane while also maintaining the 3D structure of the malleus and umbo. It seems reasonable to speculate that the device maintains an anatomy similar to the native TM through multiple cell harvesting cycles, rather than the disrupted structures used for TM explants. Moreover, the malleus handle was securely held within the PET membrane and hence eliminated the problem with conventional explant methods where tissue is prone to lift off the culture dish when cell culture medium is added.

The model has two major benefits in regard to reducing animal use: firstly it allows the efficient recovery and expansion of cells from a small sample of tissue, reducing the number of animals required to create a usable culture within a short time frame. Secondly, creation of an epithelialised membrane may enable in vitro testing of novel materials and factors for TM perforation healing that would normally be performed in vivo (for example see refs 18, 19, 20). Multiple samples can be generated from each umbo and will provide a new method for short to medium term screening that may approach high throughput methods.

An attempt to transplant an umbo from one membrane to another after cell harvesting did not produce any cell outgrowth. The explant most likely carries a durable niche for the regenerative centre and deserves further exploration to understand what might constitute a supportive environment for long-term grafts in TM tissue repair. Trans-well membranes are attractive for this purpose and the various membrane varieties available may give further opportunity to optimise aspects of the system, such as migration rate, proliferation or differentiation of cells. Activity of various agents (ECM, growth factors) on cell proliferation and migration mechanisms may be tested by coating the PET membrane or delivering in the culture medium, or even by co-culture in the underlying well for testing of paracrine activity from cells in the underlying well.

The phenotype of cells on the culture well insert PET membrane was difficult to ascertain from their phase contrast appearance, where resolution was reduced by the membrane pores. The cells grew as monolayers but were best observed for phenotype after replating in plastic cell culture dishes. In Figure 3A, cells were grown in keratinocyte growth media to form clumps of epithelial-like cells. Cells proliferated further to form confluent layers of cells, some of which developed into tightly packed cobble-stone like morphology and a fibroblast–like shape.

A 2-day-old mixed culture, grown on glass coverslip showed sparse epithelial cells positive for pancytokeratin and a large number of vimentin positive fibroblast-like cells as depicted in Figure 3B.When confluent, the mixed population can be enzymatically sorted to generate homogenous epidermal and fibroblast cultures. As epidermal cells and fibroblasts respond to trypsin differently, a carefully timed trypsinisation is easy and sufficient to enrich/isolate

homogeneous cell cultures and is particularly useful when low cell numbers were inadequate for FACS or magnetic sorting.

A time lapse wound healing migration assay was performed to assess epidermal cells functionality. As depicted in Figure 4, epidermal cells isolated from rat TM migrated to close the gap created by the two-well culture insert over 18 h. Nevertheless, rat TM epidermal cells appeared to migrate and respond to serum differently to human TM keratinocytes previously isolated in our laboratory (5) (data not shown) which, may be due to cross-species variation and different culture conditions.

Silk fibroin materials have effective mechanical properties for TM tissue engineering (21) and provide a suitable substrate for human TM keratinocytes (18). In order to test whether these TM cells would support a tissue engineering approach, we cultured explant-derived cells on porous silk membrane scaffolds (Supplementary Figure 3A).. Primary cultures were replated directly from the PET membrane onto silk-coated coverslips. Cells adhered and engrafted on the silk within 6 h producing clusters and sheets of cells covering the surface and the adjacent culture dish Supplementary figure 3B. Nuclear staining confirmed that the adherent cells formed contiguous patches of cells and in some areas confluent monolayers (Supplementary figure 3C). Over time, sheets of confluent epithelia developed on the silk scaffold to form tissue-engineered membranes.

Evidence of proliferation on the silk scaffold indicates that this mechanism of expansion occurs on the scaffold in an acute time frame. Tissue engineering will benefit from a more controlled analysis, seeding specific cell cultures rather than mixed cultures, but proof of principle that the explant-sourced cells can be suitable for tissue engineering is established

here. These TM cells have a unique biology that cannot be simply modelled using cells from other skin sources, which don't have such robust and specific migratory behaviour. A simple direct measure of a scaffolds engraftment potential might be gained by placing the PET membrane in contact with the scaffold material (22) replacing the PET membrane with scaffold material or even, based on the cell behaviour principles established here, implanting the umbo explant as a graft directly into the scaffold material and evaluating the migration of cells from the explant onto the scaffold surface. The approach may also prove useful in exploring other tissue explants with a defined stem cell niche, such as hair follicle or corneal limbus explants.

In summary, this model shows it is possible with a simple culture device to recapitulate the *in vivo* condition of a TM, where the umbo tissues serve as a regenerative centre and the cells produced will actively migrate along the lateral surface of the lamina propria to contribute to the cell populations of the membrane and ear canal. The TM organoid or tissue equivalent models produced may be suitable to explore in more detail the biology of TM cells and their unique migratory capacities, including mechanisms of disruption during wound healing in chronic perforations or invasiveness in cholesteatoma (21). The model is simple enough to be widely adopted for TM regeneration studies and has further promise as a tissue equivalent model that may help to reduce animal testing.

Authors contributions

L.J.L carried out the experiment and data analysis. R.J.D and R.M.D carried out part of the data analysis. L.J.L and R.J.D designed and supervised the experiments. R.M.D prepared the silk fibroin scaffold. L.J.L, R.M.D. and R.J.D wrote and approved the manuscript.

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Competing Interest

The authors declare no competing interests.

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Figure Legends

Figure 1

A. A tympanic membrane after dissection from the tympanic ring. The annulus (AN), malleus (M) and pars tensa (PT) can be seen. Scale bar = 1 mm

B. An umbo explant, showing the remnant malleus (M) and transparent pars tensa (PT) around it. The umbo (U) is positioned under the tip of the malleus bone. Scale bar = 500 µm

C. The umbo explant with malleus handle inserted through a slit in the PET membrane. Scale bar = 500 µm

D. The annulus explant inserted through the slit (black arrow heads) in the PET membrane. Scale bar = 500 µm

E. Cells have migrated out from the explant and cover the PET membrane surface. Scale bar = 500 µm

F. Cells migrating across the surface of the PET membrane (black dashed line indicates the migration front, above the black arrows) and approaching the periphery of the insert (bottom). Scale bar = 500 µm

Figure 2

A. Fluorescence micrograph showing DAPI stained cell nuclei in the 10 day umbo explant, and cells covering the entire surface of the PET membrane surrounding. Scale bar = 1 mm

B. Fluorescence micrograph showing DAPI stained cell nuclei in the 10 day annulus explant, and cells partially covering the surface of the PET membrane. Scale bar = 1mm

C. DAPI stained cell nuclei in 10 day cell outgrowth showing mitotic bodies and dividing cells (white arrows). Scale bar = 100 µm

D. Rat tympanic membrane whole mount showing radial and circumferential arrangement of nuclei. DAPI, Scale bar = 200 µm

Figure 3

A. Cells passaged from the umbo explant cultures into plastic culture flasks.. The cells were heterogeneous in morphology and at day one after passaging had spindle shaped fibroblasts mixed amongst epitheloid phenotypes. By day 10, the cells had proliferated in culture to form confluent monolayers and in some areas had developed the tightly packed cobblestone morphology of epithelial cells. Scale bar = 500 µm

B. Immunofluorescence staining of rat TM primary cells. Images in the first row show staining for nuclei (DAPI), pancytokeratin (immunofluorescence) and merged image respectively. In the bottom row images show staining for nuclei (DAPI), vimentin (immunofluorescence) and merged image. Scale bars = 200 µm

Figure 4

Passaged cells were subject to a wound healing assay were they were found to migrate to cover the exposed surface over 18 h. Scale bar = 200 µm

Supplementary Figure 1

Schematic for rat TM umbo explant culture method on culture well insert membrane. Whole TM was dissected free from the temporal bone of a SD rat. TM (bony bulla intact) was digested in 0.25% Dispase overnight. TM was peeled off the bony bulla and the umbo region was dissected free. The malleus handle was inserted into an incision in the culture well insert

membrane with the lateral surface of the umbo facing up. Insert was mounted into a 12 well plate filled with 1mL of keratinocyte growth medium.

Supplementary Figure 2

Culture well insert (A) and phase contrast microscopy showing the porous PET membrane before (B) and after cutting (C) to make an insertion point for the TM tissue explant. Scale bars = 500 µm

Supplementary Figure 3

A. Scanning electron micrograph of porous silk film scaffolds used to demonstrate tissue engineering with explant-derived cells. Scale bar =10 µm

B. Cells from primary cultures were replated onto silk-coated coverslips showed engraftment on the silk surface after 24h in culture. The margin of the coverslip is imaged here to show cells adherent to the plastic dish and to the coverslip. Scale bar = 500 µm

C. DAPI labelled nuclei of adherent cells show contiguous epithelial patches and sheets. Scale bar = 1 mm