Regeneration of Chronic Tympanic Membrane Perforation Using an EGF-Releasing Chitosan Patch

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Most chronic tympanic membrane (TM) perforations require surgical interventions such as tympanoplasty because, unlike with acute perforations, it is very difficult for the perforations to heal spontaneously. The purpose of this study was to develop novel therapeutic techniques and scaffolds that release growth factors to treat chronic TM perforations. We evaluated the cell proliferation effects of the epidermal growth factor (EGF) and fibroblast growth factor (FGF) on *in vitro* cultures of TM cells using an MTT assay. They both showed similar efficacy, so we used EGF because of its lower cost. We then constructed an EGF-releasing chitosan patch scaffold (EGF-CPS) based on previous studies. We analyzed its toxicity and strength, and we studied it using scanning electron microscopy. EGF was released from the EGF-CPS for 8 weeks in an *in vitro* system. In animal studies, the EGF group, which was treated with EGF-CPS, showed healing in 56.5% of the animals (13/23), while the control group, which did not receive any treatment, revealed 20.8% healing (4/24) ($p=0.04$). Transmission electron microscopic studies of regenerated eardrums in the EGF group showed much greater preservation of histological features, and TMs of the EGF group were thinner than spontaneously healed TMs. In conclusion, this novel EGF-CPS can be used as a nonsurgical intervention technique for treatment of chronic TM perforations.

Introduction

CHRONIC TYMPANIC MEMBRANE (TM) perforation is defined as stable maintenance of perforations over 3 months, in contrast to acute TM perforations, which heal after 7–10 days.^{1,2} Due to the continuous opening in the TM, chronic perforation is usually attributed to chronic inflammation in the middle ear resulting in suppurative otitis media.³ The main problem in chronic TM perforations is the lack of spontaneous healing capability, thereby causing failure of epithelial growth across the perforation. Surgical repairs, such as myringoplasty or tympanoplasty, are usually employed for patients who are suffering from chronic TM perforation. Caye-Thomasen *et al.* reported that myringoplasty healed 94%, while Onal *et al.* reported that temporalis fascia healed 65.9% and cartilage tympanoplasty healed 92.3% of bilateral chronic otitis media cases, respectively.^{4,5} However, the surgical procedures have disadvantages, including anesthesia-associated risks, high cost, and

the inconvenience of surgery. Hence, methods that are more convenient and inexpensive are desirable, and many methods have been tested. However, none has been generally accepted because of the absence of appropriate chronic TM perforation models that can be used in preclinical research.⁶ Local application of compounds, including hydrocortisone,⁷ glutaraldehyde,⁸ and mitomycin C,⁹ have been tested as ways of generating a suitable chronic TM perforation model, but the success rate and the accuracy of these methods were unacceptable.¹⁰ Recently, Choi *et al.* reported two new models for chronic TM using heat and chemical injuries that retain perforations for at least 8 weeks. These models showed a 67%–71% success rate and higher accuracy than other methods.¹¹

For the nonsurgical regeneration of TM perforations, a paper patch technique is frequently used.¹² Many studies have reported that this technique aids the migration of epithelial cells at the border into the perforation area. However, the paper patch technique has many disadvantages,

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including inflexibility, nontransparency, easy detachment, and nonresistance to infection, therefore disrupting the complete regeneration of TM perforations.^{13,14} To overcome these problems, various biomaterials such as collagen,¹⁵ calcium alginate,¹⁶ silk,¹⁷ and chitosan^{18,19} have been assessed. Among these materials, methods using chitosan in patch scaffolds and three-dimensional scaffolds²⁰ showed effective healing in acute TM perforations because of their high biocompatibility, mechanical properties, cell adhesion, antibacterial functions, and wound-healing ability.²¹ Therefore, chitosan patch scaffolds (CPSs) were expected to show good results in chronic TM perforations. However, CPSs showed a 33.3% rate of healing in a chronic TM perforation study, similar to the 25% healing rate of the paper patch technique, but failing to meet the expectations of otolaryngologists.¹¹ Hence, there is an urgent need to investigate new methods for the regeneration of chronic TM perforations.

Biological molecules and biomaterials have also been used for the regeneration of chronic TM perforations. Hyaluronic acids, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF),²² and pentoxifylline (Trental)²³ have been tested for TM regeneration. Among them, EGF, the growth factor secreted by platelets, was reported to be very effective for the recovery of skin wounds, helping the migration of fibroblasts, endothelial cells, and vascular cells, and promoting their growth and recovery.²⁴ EGF could also promote the synthesis of DNA, RNA, proteins, hyaluronic acids,²⁵ and fibronectin.²⁶ Because of the similarities between skin tissue and TMs, EGF has been frequently used for TM regeneration. EGF has also been used for regeneration of chronic TM perforations. Lee *et al.*²⁷ reported that EGF was as much as 80% effective in a study of chronic TM perforations in chinchillas. However, the study did not test verified animal models with chronic TM perforations. Moreover,

until now, controlled release of biological molecules for the regeneration of chronic TM perforations has not been tested. Teh *et al.* insisted the limitation of previous research only delivering biomolecules in the form of eardrops onto a scaffold or a scaffold impregnated with a growth factor before insertion and claimed new paradigm of biomolecule delivery.²⁸ Controlled release of biomolecules such as growth factors would be expected to stimulate the healing capacity of the remaining cells in chronic perforated eardrums without the need for a continuous supply of medicine, thereby resulting in easier treatment. Our group has developed many methodologies that replace surgical methods and has studied biomaterial compounds for the regeneration of TMs. Among them, we introduce here the most efficient method using new CPSs that not only have good mechanical properties and higher cell viability, but also continuously emit EGF. These EGF-releasing CPSs were evaluated for their mechanical properties, release of EGF (Fig. 1). An *in vitro* study, including a cell viability test, and *in vivo* studies were performed to verify the efficacy of EGF-CPSs.

Materials and Methods

Materials

Chitosan (molecular weight 200,000, deacetylation degree 89%) was purchased from Taehoon Company, and glycerol (0854-1L) was purchased from AMRESCO. Acetic acid (96A521; Duksan) and WST-1 (EZ-Cytox Cell Viability Assay Kit; EZ3000 Daeillab Service Co.) were both used in this study. The Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone. EGF (E4127; Sigma-Aldrich), the Quantikine[®] Mouse EGF Immunoassay (MEG00; R&D systems) and the CytoSelect[™]

FIG. 1. Schematic of strategy. (a) Basic concept of EGF-CPSs. When the EGF-CPSs are in an aqueous environment, they swell and release EGF. (b) Application of EGF-CPSs in a chronic TM perforation model (Choung's COM 1). Discharges caused by inflammation provide an aqueous environment for EGF-CPSs, making the chitosan swell, and therefore release EGF to the site of the chronic TM perforations. Therefore, the EGF promotes the regeneration of chronic TM perforations. EGF-CPSs, epidermal growth factor-releasing chitosan patch scaffolds; COM, chronic otitis media; TM, tympanic membrane. Color images available online at www.liebertpub.com/tea

24-well Wound Healing Assay (CBA-120; Cell Biolabs, Inc.) were used in the present study.

Choice of EGF concentration

Primary TM cells were obtained from the TMs of 5-day-old Sprague-Dawley rats under anesthesia with intraperitoneal injection of Zoletil50 (Virbac Laboratories) and 2% Rompun (Bayer Korea). For *in vitro* tests, TM cells were cultured and maintained at 37°C in the DMEM supplemented with 10% FBS in a humidified atmosphere with 5% CO₂ for 2 weeks.

In vitro studies using TM cells, EGF, and FGF were conducted to find the optimum concentration of EGF and FGF for incorporation into CPSs. TM cells were seeded on 24-well plates at 5×10^4 cells/well. Next, 0, 0.01, 0.05, 0.1, or 0.2 µg/mL of the EGF or 0, 0.002, 0.01, 0.02, or 0.04 µg/mL of the FGF were added to the wells. After 2 days, cell viability was measured using water-soluble tetrazolium salt (WST-1). For the WST-1 assay, the WST solution was added to each well, and the plates were incubated at 37°C and 5% CO₂ for 4 h. The samples were then gently agitated for 1 h. Absorbance was measured at 450 nm using a microplate reader (Versamax Reader; Molecular Devices).

Preparation of EGF-CPSs

EGF-CPSs were prepared as previously reported, except with a different concentration of glycerol and with EGF incorporation. Briefly, 3% (w/v) chitosan and 3.5% (w/v) glycerol were added to a 2% acetic acid solution and stirred for 12 h. After stirring, the mixture was filtered through a 1-µm pore-size filter, a 0.1-µg/mL EGF was added, and the solution was stirred for 15 min. Finally, 7-mL aliquots of the mixture were poured into 100-mm-diameter Petri dishes and dried at 36.5°C for 12 h. CPSs fabricated without the EGF were used as a control.

Morphological analysis of the EGF-CPSs

External and cross-sectional morphologies of the EGF-CPSs were observed by field-emission scanning electron microscopy (FESEM; JEOL, JSM-5410LV) at 2-kV acceleration voltage. A micrometer (Mitutoyo) with ± 1 µm precision was used for determining the thickness of CPSs and EGF-CPSs. Each measurement was repeated five times for each sample. The measured thickness was then used for calculating the mechanical properties of the patch scaffolds.

Mechanical properties of the EGF-CPSs

EGF-CPSs with 10×30-mm dimensions were cut from the full-sized samples. Their tensile strength and elongation were measured using a texture analyzer (Stable Micro Systems Ltd.). The cross-head speed was set at 500 mm/min. The elastic modulus of patch scaffolds was calculated using the stress-strain graph measured by the texture analyzer. Each test was repeated five times for each sample.

In vitro EGF release test

EGF-CPSs were cut to 5×5 mm² and placed into wells of a 24-well plate along with 1 mL phosphate-buffered saline (PBS). The 24-well plate was stored at 36.5°C. After the

designated time, the samples were collected, and 1 mL of fresh PBS was poured into each well. The EGF in each collected sample was quantified using an enzyme-linked immunosorbent assay (ELISA; Quantikine[®] Mouse EGF Immunoassay).

Cell viability test

Before the test, a 24-well plate was coated with chitosan mixtures. The CPS and EGF-CPS solutions (0.15 mL) were poured into the 24-well plates and flattened into the wells; the plates were then dried at 36.5°C for 12 h. After plate preparation, the samples were neutralized with ethanol for 10 min and washed three times with PBS. TM cells were then seeded on the plates at a density of 5×10^4 cells/well. Cell viability was determined by WST-1 assays at 0, 0.25, 1, 3, and 5 days following TM cell seeding.

Wound-healing assay

The effect of EGF-CPSs on the migration of TM cells was assessed with the CytoSelect[™] 24-well Wound-Healing Assay. Briefly, inserts were put in the 24-well plate wells and aligned in a single direction, 500 µL of cell suspension containing 0.5×10^6 cells/mL was added to each well, and the well plate was incubated at 37.0°C overnight. The insert was then removed from each well, and CPSs and EGF-CPSs were placed on the wound area. The wound sites were observed each day until the wounds were entirely closed; their healing ratio was then measured.

In vivo animal tests

Twenty five Sprague-Dawley female rats (8 weeks, 200–250 g) were used in this study. Ajou University School of Medicine-Institutional Animal Care and Use Committee (AUSM-IACUC) approved the surgical procedures in accordance with the guidelines regarding the care and use of animals for experimental procedures (AMC 94). All efforts were made to minimize the number of animals used and their suffering.

The animal models for chronic TM perforations were prepared by the previously reported method.¹⁰ Fifteen Sprague-Dawley rats (4 weeks, 200–250 g) were anesthetized with an intraperitoneal injection of Zoletil50 (Virbac Laboratories) and 2% Rompun (Bayer Korea). Eardrums were mechanically perforated with a heated micropick on the anterior half of the TM, resulting in a perforation size of approximately 50% of the TM as observed with a surgical microscope (Carl Zeiss). Precautions were taken to prevent injury of the malleus handles and annulus during this procedure. Gelfoam soaked with mitomycin C (0.5 mg/mL; Kyowa) was applied to the perforation site for 10 min, and then gelfoam with dexamethasone disodium phosphate (5 mg/mL; Ilsung) was placed on the perforation site for 1 week. EGF-CPSs were applied to left ears of 25 rats, and the right ears were used as the control group. The healing state of the perforated eardrums was checked once a week for 10 weeks. In cases where the perforation was not clearly observed through a surgical microscope, the patches were lifted off of the TMs.

For evaluating the healing state, we imaged the perforated eardrums with an endoscopic camera every week for 10

weeks. For histological analysis, TMs were obtained from rats 10 weeks after applying CPSs and analyzed with a transmission electron microscope (TEM; EM 902A; Jeiss).

Statistical analysis

Statistical analyses were performed using the Statistical Analysis System (SAS) for Windows Ver. 9.1.2 (SAS Institute). The least significant difference (LSD) method and unpaired Student's *t*-tests were used to compare the means of the properties of CPSs and EGF-CPSs. The level of significance was $p < 0.05$.

Results

Determination of EGF concentration for EGF-CPSs

As a preliminary test, the optimum EGF and FGF concentrations were determined. Figure 2a, b represent the result of MTT assays performed with different concentrations of EGF and FGF. Cell viability of TM cells was maximal at $0.1 \mu\text{g/mL}$ -EGF ($117.2\% \pm 5.2\%$) and $0.2 \mu\text{g/mL}$ -FGF ($126.7\% \pm 7.8\%$). Despite the greater cell viability displayed with the FGF, the costs of the EGF and FGF (expressed in cell viability percentage per dollar) were $34.4\%/\$$ and $2.66\%/\$$, respectively, because of the tenfold higher price of the FGF. Therefore, to maximize the commercialization potential of EGF-CPSs, $0.1 \mu\text{g/mL}$ of EGF was chosen as the optimum for EGF-CPS fabrication.

Characteristics of EGF-CPSs

Figure 3 shows the morphology of CPSs and EGF-CPSs. The patch scaffolds have the same surface and cross-section morphology regardless of the incorporation of the EGF (Fig. 3a, b). The thickness of EGF-CPSs was $61.8 \pm 3.95 \mu\text{m}$, not significantly different from control CPSs (Fig. 3c).

Similar results were also observed with regard to mechanical properties. The elastic modulus of EGF-CPSs was $13.1 \pm 2.94 \text{ MPa}$ (Fig. 3d), with maximum elongation of 33.4 ± 4.73 (Fig. 3e) and maximum tensile strength of

$7.58 \pm 2.82 \text{ MPa}$ (Fig. 3f). Most mechanical properties of EGF-CPSs were not significantly different from control CPSs; the exception was maximum elongation ($p < 0.01$). As with morphological properties, the mechanical properties of EGF-CPSs were not changed by EGF incorporation.

The EGF release ability of EGF-CPSs was investigated using ELISA. Figure 4 shows the accumulated release of EGF for 56 days. This result confirmed that the EGF was not denatured in the fabricating process and that the EGF-CPSs successfully filled the role of a drug carrier and release agent. When used *in vivo*, EGF-CPSs are expected to release the EGF for a longer duration than *in vitro* as the environment around the TM is much drier.

In vitro study

The effect of EGF-CPSs on cell viability was studied by WST-1. Figure 5a shows cell viability with EGF-CPSs and CPSs. The cell viabilities of both patch scaffolds increased steadily until 5 days, and the cell viability with EGF-CPSs showed significant differences from CPSs after 3 to 5 days. The EGF regulates cell growth, cell recovery, and the synthesis of DNA, RNA, and proteins composing the extracellular matrix. Therefore, it is likely that the EGF incorporated into the EGF-CPSs and released to TM cells enhances cell viability. In this study, both CPSs and EGF-CPSs showed a lower cell viability than tissue culture polystyrene (TCPS). The result is attributed to the swelling and gelation of the chitosan film in an aqueous environment. When the chitosan film swells, cells cultured on it cannot adhere on the chitosan substrate tightly, rather aggregate with each other, and finally form spheroids, causing delaying of proliferation, whereas cells cultured on TCPS retain its monolayer, and have a higher proliferation ability.^{29–31} Although the interrupted proliferation could hamper *in vitro* tissue regeneration, the problem does not exist in the biological system due to the lack of enough water around the eardrum as seen in the *in vivo* experiment.

A wound-healing assay was conducted to determine the effect of patch scaffolds on the growth and migration of TM cells. Figure 6b shows the closure ratio with CPSs and EGF-

FIG. 2. *In vitro* optimization of EGF and FGF concentrations. (a, b) MTT assay results with TM cells. (a) EGF was dissolved at 0, 0.01, 0.05, 0.1, or $0.2 \mu\text{g/mL}$ in culture media. The group with $0.1 \mu\text{g/mL}$ of the EGF had the highest absorbance. (b) FGF was dissolved at 0, 0.002, 0.01, 0.02, 0.2, or $0.04 \mu\text{g/mL}$ in culture media. The group with $0.2 \mu\text{g/mL}$ of the FGF had the highest absorbance. Error bars in (a, b) represent the standard deviation from the mean. FGF, fibroblast growth factor.

FIG. 3. Properties of EGF-CPSs. (a–c) Morphological properties of EGF-CPSs; FESEM images of (a) CPSs and (b) EGF-CPSs. The main figures represent cross-sectional images ($\times 400$); insets show the surface images of the respective patch scaffolds ($\times 1000$). (c) Thickness of CPSs and EGF-CPSs. No significant difference was observed ($n=5$, t -test for each group). (d–g) Mechanical properties of EGF-CPSs. (d) Representative stress–strain curves, (e) elastic modulus, (f) maximum elongation, and (g) maximum tensile strength of CPSs and EGF-CPSs ($n=3$, t -test for each group). The two types of patch scaffolds were not significantly different in the elastic modulus and maximum tensile strength and showed a significant difference only in maximum elongation, indicating that the patch scaffolds have similar mechanical properties regardless of whether the EGF is incorporated. Error bars in (c–g) represent the standard deviation from the mean. Scale bars of inset = 20 μm. FESEM, field-emission scanning electron microscope. **, $p < 0.01$. Color images available online at www.liebertpub.com/tea

CPSs, and Figure 5c–j show representative images from the wound-healing assay for 3 days. As shown in Figure 5j, wounds were entirely closed on day 3 in the presence of EGF-CPSs, showing better results than with control CPSs. It is thus likely that the EGF in the EGF-CPSs successfully stimulated the wound healing of TM cells. Moreover, the wounds with control CPSs were nearly healed after 3 days. It

is widely known that chitosan promotes skin wound closure. It has also been reported that CPSs healed TM wounds better than paper patches, confirming the effect of chitosan on TM cells.¹⁶ Therefore, the combinational use of chitosan and EGF may be expected to have a synergistic effect on chronic TM perforations in comparison to the use of chitosan or EGF separately.

FIG. 4. Cumulative release of the EGF from EGF-CPSs for 56 days as measured by ELISA. The EGF was continuously released for 56 days. Error bars represent the standard deviation from the mean. ELISA, enzyme-linked immunosorbent assay.

In vivo study

Table 1 shows the healing rate of chronic TM perforations at weekly intervals. Figure 6a–d are representative serial

images of the eardrums with chronic perforations for 10 weeks, and Figure 6e–h show the changes in the individual eardrum perforation size for 10 weeks. With application of EGF-CPSs, 56.5% of chronic TM perforations completely healed over 10 weeks, whereas the control showed a 33.3%; this difference was significant ($p < 0.05$). In our previous study, CPSs showed a 33.3% healing rate with no significant difference with spontaneously healed chronic TM perforation of 28.4%.¹¹ In contrast, EGF-CPSs showed a much higher healing rate than CPSs. In cases with small perforations (less than 30% of the TM), complete healing usually occurred within 4 weeks. However, perforations larger than 30% seemed to need at least 7 weeks. In addition, the initial perforation size of the EGF-CPS-healed eardrums was as large as 50%, while the corresponding number for control eardrums was 20%.

Histological results are shown in Figure 7. Transmission electron microscope images are shown of (Fig. 7a) a TM spontaneously healed from chronic TM perforation, (Fig. 7b) a TM healed from chronic TM perforations with EGF-CPSs, and (Fig. 7c) a normal, unperforated TM. As shown in Figure 7c, the unperforated TM has three layers: an outer epidermal layer, radiate and circular fibrous layers of lamina propria, and an inner mucosal layer. When chronic TM

FIG. 5. *In vitro* tests of EGF-CPSs. (a) WST-1 assay of EGF-CPSs for 5 days. Cell viabilities were significantly different on days 3 and 5 ($n=3$, t -test analysis for each group). (b) Migration assay of EGF-CPSs ($n=10$, t -test analysis for each group). EGF-CPSs showed complete TM cell migration by day 3. (c–j) Representative images of migration assay ($\times 100$). (c–f) Migration assay in which CPSs were used and (g–j) migration assay in which EGF-CPSs were used. The experiment was conducted for 3 days; (c, g) 0 day, (d, h) 1 day, (e, i) 2 days, and (f, j) 3 days. Both CPSs and ECPSs promoted gap narrowing compared to paper patches.¹⁵ Error bars in (a, b) represent the standard deviation from the mean. Scale bars of (c–j) = 200 μm . WST, water-soluble tetrazolium salt. *, $p < 0.05$; **, $p < 0.01$. Color images available online at www.liebertpub.com/tea

FIG. 6. *In vivo* studies of EGF-CPSs. (a–d) Serial images of chronic TM perforations: (a) chronic TM perforation that is not healed, (b) chronic TM perforation that healed spontaneously, and (c, d) chronic TM perforations healed by EGF-CPSs. The EGF-CPSs healed chronic TM perforations for 4 weeks and 7 weeks, in contrast to the CPSs, which only healed for 3 weeks (red circle). (e–h) Continuous changes of perforation sizes in eardrums. (e, g) Cases of healing chronic TM perforations; (f, h) cases of nonhealing chronic TM perforations; (e, f) cases in which chronic TM perforations were not induced and (g, h) cases where chronic TM perforations were induced. Cases in which EGF-CPSs were applied showed a higher healing rate and a longer healing period than spontaneous healing. Color images available online at www.liebertpub.com/tea

TABLE 1. *In Vivo* Success Ratio of Control and EGF-CPSs Over 10 Weeks

Week	1	2	3	4	5	6	7	8	9	10
Control (24 ears)	1/24 (4.2%)	1/24 (4.2%)	3/24 (12.5%)	4/24 (16.7%)	4/24 (16.7%)	5/24 (20.8%)	5/24 (20.8%)	5/24 (20.8%)	5/24 (20.8%)	5/24 (20.8%)
EGF-CPSs (23 ears)	4/23 (17.4%)	7/23 (30.4%)	8/23 (34.8%)	10/23 (43.5%)	10/23 (43.5%)	10/23 (43.5%)	11/23 (43.5%)	12/23 (52.2%)	13/23 (56.5%)	*13/23 (56.5%)

EGF, epidermal growth factor; CPS, chitosan patch scaffold.

*, $p < 0.05$.

perforations were spontaneously healed, the three layers of the TM were not readily apparent; in particular, the lamina propria layer was frequently disorganized and replaced by a scar tissue such as calcification (tophi or myringosclerosis). The eardrums healed with EGF-CPSs showed relatively well-organized layers with radiate and circular fibrous layers even though the layers were thicker than those of normal eardrums. Additionally, tophi or other scar tissues were not detected in the eardrums, in contrast to spontaneously healed eardrums. The average thicknesses of spontaneously healed TMs, EGF-CPS-treated TMs, and normal TMs were 38.9 ± 4.05 , 27.1 ± 2.20 , and 15.3 ± 2.63 μm , respectively (Fig. 7d). EGF-CPS-treated TMs were thinner than those of spontaneously healed TMs. A thinner ear-

drum vibrates more readily, and thus, EGF-CPS-treated TMs are better sound conductors than thick, spontaneously healed, TMs.

Discussion

Reason why EGF was selected

Thanks to their good morphological, mechanical, and biocompatible properties, CPSs were expected to aid healing of chronic TM perforations, but this expectation was not met. Because cells in the chronic TM perforations lose their spontaneous healing ability, CPSs were not able to effectively promote the healing of chronic TM perforations, in contrast to their effectiveness for acute TM perforations. Therefore, in

FIG. 7. Histological analyses of chronic TM perforations. TEM images ($\times 4400$) of (a) spontaneously healed TM, (b) TM healed of chronic TM perforation by EGF-CPSs, and (c) normal TM. The outer epidermal layer (arrow), radiate (R) and circular (Ci) fibrous layer, and inner mucosal layer (arrowhead) were observed in all TMs. TMs treated with EGF-CPSs had dense and regular circular fibrous layers, while spontaneously healed TMs had disorganized circular fibrous layers with tophi (*). (d) Average thickness of TMs ($n = 10$, LSD). The thickness of TMs treated with EGF-CPSs was less compared with spontaneously healed TMs and was close to normal TM thickness. Scale bars in (a–c) = 2.5 μm . Error bars in (d) represent the standard deviation from the mean.

this study, we addressed the need to use signaling molecules that promote cell division and proliferation in chronic TM perforations.

To date, many signaling molecules have been used for the healing of chronic TM perforations, including hyaluronic acids, EGF, FGF, and pentoxifylline (Trental). Among them, the basic FGF (bFGF) has been most frequently used because the fibrous layer comprises 98% of the TM. FGF-containing gelfoam significantly improved the healing ratio compared to spontaneous healing,³² and bFGF treatment also promoted closure of chronic TM perforations.³³ In this study, the EGF was used for several reasons. First, the EGF can regenerate the outer epidermal layer. Fibroblasts, the constituents of the fibrous layer, grow much faster than epithelial cells and can lead to the formation of granulation tissue, which can prohibit the closure of the epidermal layer. The EGF minimizes this problem as it promotes the regeneration of epithelial cells in the outer epidermal layer. This expectation was confirmed in the histological results by the moderately clear formation of an epidermal layer resulting from the growth of keratinocytes. Second, the EGF is cost effective. In addition to the scientific motivation for this study, we also considered the potential for commercialization of the EGF-CPS. Because the EGF is 10 times cheaper than the FGF and can be used at half the concentration of the FGF, EGF-treated CPS has a 20-fold lower cost as a commercial product. For these reasons, the EGF was chosen as a component of CPSs in this study.

Potential of EGF-CPS as a clinical tool

In our previous study, CPSs accelerated the healing efficiency of acute TM perforation. Accordingly, CPSs were used to regenerate the chronic TM perforation in the same anticipation. However, contrary to our expectation, CPSs resulted in only 33.3% healing rate with no significant difference with spontaneously healed chronic TM perforation (28.4%).¹¹ Due to this inefficiency, the CPSs were discarded among the experimental groups and EGF-incorporated CPSs were studied for the treatment of chronic TM perforations.

EGF-CPSs enhanced not only cell viability, but also the healing rate of chronic TM perforations compared to spontaneous healing. It seemed that the EGF in the EGF-CPSs was crucial for cell growth and TM regeneration. In terms of healing propensity, EGF-CPSs were effective with perforations up to 50% of the TM, while spontaneous healing occurred only with perforations representing less than 20% of the TM. Further, spontaneous healing was only observed within 5 weeks of perforation in nontreated TMs, while with EGF-CPSs, the healing process continued for up to 10 weeks. Histology showed that TMs healed with EGF-CPSs appeared slightly thicker than normal TMs. Douglas *et al.* investigated the long-term effectiveness of the EGF in chronic TM perforations,³⁴ and reported that some TMs cured of chronic TM perforations using EGF treatment became re-perforated because the healed TMs became too thin. During the first 2 weeks, the EGF influenced the proliferation of all three layers. After 2 weeks, long-term treatment with the EGF gradually made the lamina propria thin despite the presence of gelfoam. In contrast, EGF-CPS-treated TMs retained their normal thickness after 10 weeks. Dou-

glas *et al.* applied 0.25 mg/mL of EGF three times each week, while EGF-CPSs release 32.0 ± 11.1 ng/mL per week. Too much stimulation by the EGF leads to catabolism of collagen. However, controlled-release EGF stimulation seems to assist the regeneration of the outer epidermal layer without promoting the elimination of the fibrous layer. Moreover, to our knowledge, EGF-CPS is the first treatment applying the concept of controlled release to the regeneration of chronic TM perforations. Other studies applying growth factors to the TM often used gelfoam or paper patches soaked in the growth factor solution that would be regularly exchanged or refreshed with a growth factor-containing solution. With these methods, a patient suffering from chronic otitis media would require an extended hospital stay. In contrast, EGF-CPS-applied patients would require only a short visit, as EGF-CPSs spontaneously emit a regular quantity of EGF over an 8-week period. Hence, this method would advance the treatment of chronic TM perforation.

Even so, these results might not equal the outcomes that surgical methods such as myringoplasty or tympanoplasty can achieve. Caye-Thomasen *et al.* reported that myringoplasty healed 94%, and Onal *et al.* reported that temporalis fascia and cartilage tympanoplasty healed 65.9% and 92.3% of bilateral chronic otitis media, respectively.^{4,5} Although the healing rate of the EGF-CPS method did not reach those of surgical methods, this treatment can be used for patients suffering less severe chronic TM perforations with sizes up to 50% of TM. With no risk of anesthesia, low cost, and convenient treatment, this method would be a good alternative to surgical methods. It is also meaningful that the efficacy of EGF-CPSs was demonstrated in a verified model. The healing rate of the Choung's model 1 is so low that no acceptable methods show significant results in this model. Therefore, the fact that EGF-CPSs show a distinguishable effect in this model means that EGF-CPSs will be much more effective when applied to chronic TM perforation patients. In other words, the effectiveness of EGF-CPSs on larger perforation sizes than those explored in this experiment and a more successful healing rate may be revealed in further studies. Finally, EGF-CPS treatment could be an adjuvant of surgical methods. In cases where full recovery has not occurred, EGF-CPSs could successfully make the perforations smaller; they might even be expected to completely heal the perforations in some cases. Alternatively, EGF-CPSs could be applied to patients with severe chronic TM perforations to make the perforations smaller, increasing the success rate of surgery. In these patients, EGF-CPSs would represent a preliminary method used before surgery. Collectively, the results and discussion of this study reveal an optimistic picture for the prospects of EGF-CPSs as a treatment method for chronic TM perforation.

Conclusions

In this study, EGF-CPSs were fabricated and evaluated for the regeneration of chronic TM perforations. EGF-CPSs had better mechanical properties and showed greater cell viability than established CPSs. In addition, the EGF-CPSs showed a much higher *in vitro* wound-healing rate than CPSs. Although not as effective as surgical methods, the

application of EGF-CPSS produced much better results than spontaneous healing. With no risk of anesthesia, low cost, and convenient treatment, this method will become a new alternative for patients suffering from less severe chronic otitis media; it may also become a preoperative step to make surgery more effective.

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No competing financial interests exist.

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