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Heparin Binding Epidermal Growth Factor Like Growth Factor Heals Chronic Tympanic Membrane Perforations With Advantage Over Fibroblast Growth Factor 2 and Epidermal Growth Factor in an Animal Model

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Abstract

Hypothesis—That heparin binding epidermal growth factor like growth factor (HB-EGF) heals chronic tympanic membrane (TM) perforations at higher rates than fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF) in an animal model.

Background—A non-surgical treatment for chronic TM perforation would benefit those unable to access surgery or those unable to have surgery, as well as reducing the cost of tympanoplasty. Growth factor (GF) treatments have been reported in the literature with variable success with the lack of a suitable animal providing a major obstacle.

Methods—The GFs were tested in a validated mouse model of chronic TM perforation. A bio absorbable hydrogel polymer was used to deliver the GF at a steady concentration as it dissolved over four weeks. A control (polymer only, n=18) was compared to polymer loaded with HB-EGF (5ug/ml, n=18), FGF2 (100ug/ml, n=19) and EGF (250ug/ml, n=19). Perforations were inspected at four weeks.

Results—The healing rates, as defined as one hundred percent perforation closure, were control (5/18, 27.8%), HB-EGF (15/18, 83.3%), FGF2 (6/19, 31.6%) and EGF (3/19, 15.8%). There were no differences between FGF2 (p=0.80) and EGF (p=0.31) with control healing rates. HB-EGF (p=0.000001) showed a significant difference for healing. The HB-EGF healed TMs showed layers similar to a normal TM, whilst the other groups showed a lack of epithelial migration.

Conclusion—This study confirms the advantage of HB-EGF over two other commonly used growth factors and is a promising non-surgical treatment of chronic TM perforations.

Introduction

Chronic tympanic membrane perforation results in hearing loss in more than 50 percent of cases hearing loss is associated with poor language and cognitive development in children

and social isolation in patients of all ages.(1–3) Currently the gold standard is type 1 tympanoplasty (myringoplasty) with over one hundred thousand cases being performed in the United States every year. (4) However, initial graft take rates can be as low as sixty five percent. (5,6) In the developed world, a non-surgical treatment would reduce the need for a general anesthetic and outpatient admission for treatment. In the developing world, the problem is larger however access to treatment and surgery contributes to a larger prevalence. Here a non-surgical treatment would provide a treatment option in an area of unmet need.

So far non-surgical treatments have failed to be superior to surgery or be available for the developing world (7) (8). Two other growth factor treatments, fibroblast growth factor (FGF) 2 and epidermal growth factor (EGF) have claimed efficacy in the literature. (9–14) These studies are difficult to interpret considering many of the treatments have been tried in acute, not chronic, perforations and the FGF 2 human studies have not been performed without excising the perforation margin. Excising the perforation margin is a confounding factor for the true healing ability of FGF2 and also requires surgical skill. This makes its use in the developing world limited. After identifying that heparin binding epidermal growth factor like growth factor (HB-EGF) may have a critical role in keratinocyte proliferation and migration in acute TM wound healing, it was shown that HB-EGF has efficacy in healing chronic TM perforations without excising the perforation margin in animal models of chronic TM perforation (15,16). We aim to test the efficacy of HB-EGF against both FGF2 and EGF in a validated mouse model of chronic tympanic membrane perforation.

Materials and Methods

All animal work was approved by Stanford's Administrative Panel on Laboratory Animal Care. All mice used for all experiments were 6–10 week old male CBA/CAJ (15–25g) mice purchased from Jackson Laboratories (Florida, USA). All otoscopy and surgical interventions were performed using inhaled isoflurane at 3–4% for induction and 1–2% for maintenance. Determination of animal numbers was performed using STATA version 13 aiming for an α of 0.05, a minimum β of 0.8.

Mouse model of chronic tympanic membrane perforation

A previously validated mouse model of dry chronic TM perforation was used to compare the treatment groups.(16) Briefly, this animal model was developed bilaterally using a curved needle to create subtotal perforations in the pars tensa. KB-R7785 (10mM), a metallomatrix proteinase inhibitor to inhibit wound healing, was applied trans canal to gelfoam placed through the perforation over a week. The perforation was left untouched for three months and allowed to become chronic. The KB-R7785 used in this experiment was synthesized by the Department of Chemistry at Stanford University.

Treatment groups

After creation of the chronic perforations, mice (original total of 40 mice with creation of 80 perforations) were assigned to one of four groups. Ears which did not have a chronic perforation after three months (total of six) were excluded from the study. This left a total of 74 perforations left for use in the study. Each group received either recombinant mouse

Proheparin-Binding EGF-like Growth Factor (HB-EGF) purchased from Prospec (New Jersey, USA, catalogue number CYT-068) at 5ug/ml (n=18 perforations), recombinant mouse fibroblast growth factor-basic (FGF 2) purchased from Prospec (New Jersey, USA, catalogue number CYT-386) at 100ug/ml (n=19 perforations), recombinant mouse epidermal growth factor (EGF) purchased from Prospec (New Jersey, USA, catalogue number CYT-326) at 250ug/ml (n=19 perforations) or no growth factor (control) (n=18 perforations).

Hydrogel polymer delivery

Each growth factor was delivered by a previously described hydrogel polymer. (16) Briefly, this polymer was developed in and provided by the Department of Orthopedic Surgery at Stanford University and contains a novel combination of chitosan, polylactide and fibrinogen with sodium metabisulfide as a crosslinking agent. (16) The treatment was injected via the external auditory canal, using a syringe and 27 gauge needle, through and onto and around the TM perforation. The total volume delivered was approximately 0.4ml in each case. In control ears, only the hydrogel polymer was injected. TMs were evaluated at four weeks, when the hydrogel was no longer visible in the ear canal.

Histology

Histology using hematoxylin and eosin was performed on half the treated perforations in each group according to a previously published technique.(16,17) The other half of perforations were subjected to cytokeratin staining described below. Briefly in addition, due to the fragility of the mouse TM, a section of the tympanic bulla was harvested around the TM under microscopic assistance. The intact TM along with a portion of the tympanic bulla was then embedded in optimal cutting temperature compound (OCT). Sections were cut perpendicular to the plane of the tympanic annulus.

Immunohistochemistry

To observe the keratin and keratinocytes of the TMs, cytokeratin staining was performed in half the treated perforations using Monoclonal Mouse Anti-Human Cytokeratin Clone MNF116 (Dako Cat #M 0821) using a previously published technique. (18)

Photographic recording

All perforations were assed under the microscope but also trans canal photographs were taken with a Digital MacroView Otoscope (Welch Allyn). The images were used to calculate the size of the residual perforations in those that did not heal completely. ImageJ (19) software was used to trace around the residual perforation and present it as a percentage of the pars tensa.

Statistical analysis

STATA version 13.0 was used for analysis. For statistical analysis of the rate of healing, a Pearson's chi squared test for goodness of fit with Bonferroni correction was performed. For statistical analysis of the size of the perforations in those samples that did not show complete

healing, a one way ANOVA with Bonferroni correction was performed. A significance level of 0.05 was used for the null hypothesis.

Results

The healing of the chronic TM perforations by treatment group, as defined by total closure of perforation as observed under the microscope immediately post mortem after transection of the external ear canal at the bony cartilage junction. This is shown in table 1. The healing rates of the TMs after treatment were 83.3% (n = 15/18), with HB-EGF, 15.8% (n = 3/19) with EGF, 31.6% (n = 6/19) with FGF2 and 27.8% (n = 5/18) with control. HB-EGF is the only growth factor tested with significant efficacy over control, EGF and FGF2 (control vs HB-EGF $\chi^2 = 11.25$, $p = 0.003$, control vs EGF $\chi^2 = 0.78$, $p = 1.00$, control vs FGF2 $\chi^2 = 0.06$, $p = 1.00$, HB-EGF vs EGF $\chi^2 = 16.88$, $p = < 0.001$, HB-EGF vs FGF2 $\chi^2 = 10.09$, $p = 0.003$, EGF vs FGF2 $\chi^2 = 1.31$, $p = 0.75$)

In those samples that did not show complete healing, the sizes of the residual perforations, as a percentage of the total pars tensa area, were 28.8% for HB-EGF (3 samples, stdev 12.9), 38.0% for EGF (13 samples, stdev 20.4), 25.1% for FGF2 (13 samples, stdev 20.4), 41.7% for control (13 samples, stdev 18.0). According to a one way ANOVA test, there was no significant difference between the residual perforation size in the treatment groups ($F(3,40) = 1.55$, $p = 0.216$). Post hoc Bonferroni adjustments confirmed no individual treatment group differences (HB-EGF vs control $p = 1.00$, HB-EGF vs EGF $p = 1.00$, HB-EGF vs FGF2 $p = 1.00$, EGF vs FGF2 $p = 0.66$, EGF vs control $p = 1.00$, FGF2 vs control $p = 0.32$).

Figure 1 demonstrates representative images of the cytokeratin staining of the TMs after treatment. Figure 2 demonstrates representative images of the hemotoxylin and eosin staining of the TMs after treatment.

The HB-EGF treated groups show epithelial layers with greater thickness than in comparable areas of the healed groups in the other treatment or control groups. Some areas of epithelialization in the HB-EGF treated groups appear with differing degrees of thickness (Observed range was 0.009 to 0.033mm). Some areas are comparable to the normal TM keratinocyte layers where others were increased. Compared to the HB-EGF treated groups (Figure 1), the FGF2, EGF2 showed a lack of keratin staining even when macroscopically healed.

The chronic perforation edges (Figure 2) in the FGF2, EGF and control groups demonstrate thickening of the connective tissue layer and in some cases show the mucosal layer joining directly adjacent to the keratinocyte layer. No discernable histological difference could be observed between FGF2, EGF and control groups.

Discussion

When interpreting the literature it is important to recognize the difference between acute wounds (less than three months) and chronic wounds. Laboratory created acute perforations heal spontaneously and devalues any intervention used in such a model. (17,20). The model used in this study has been validated as mimicking the human condition in histological

appearance, making it useful for testing potential treatments for chronic TM perforations. (16)

The efficacy of HB-EGF as a treatment for chronic TM perforations has been previously shown in animal models. (16) HB-EGF has a wide array of pathological and physiological functions, and has been shown to play a role in wound healing, cardiac development and vasculature. (21) It is the predominant growth factor involved in cutaneous wound healing, and is a major component of wound fluids. (22) (23). HB-EGF is from the EGF family of growth factors, but unlike EGF has specific binding to the HER1 and HER4 receptors in the EGFR family (HER1, HER2, HER3 and HER4). HB-EGF then acts via both EGFR dependent and EGFR independent mechanisms. (23–25) HB-EGF is non ototoxic, however further animal studies including understanding the dose response and systemic safety need to be completed before moving to clinical trials.

Some studies involving FGF2 and EGF have suggested efficacy in healing chronic TM perforations, however most of the animal studies have been done in acute perforations and the only FGF2 human studies have also included excision of the perforation edge.. (9–14) Using a validated animal model it is possible to compare the efficacy of all three growth factors in the same conditions. A review of the literature was used to determine the likely effective doses for comparison of FGF2 and EGF in healing chronic TM perforations. Four prior studies utilized EGF to treat chronic TM perforations. One of these studies claimed efficacy of 250ug/ml of EGF but was tested in acute perforations rather than chronic perforations. (26) One study, testing in a chinchilla model, demonstrated 81% efficacy of 100ug/ml compared to 25% in controls. Another study in chinchillas applied 50 μ L EGF at 250 μ g/mL via gel foam. 100% of EGF-treated TMs healed compared to 80% of control TMs. (27) This study also discovered re-perforations and cholesteatomas forming in a small group lowering the long term success rate of EGF treated TMs to 71%. Another study with 250 μ g/mL documented partial healing in 100% although these were subacute perforations. (28) Unfortunately these results did not translate to success in human chronic TM perforations with 0% efficacy seen in humans. (14). Based on the apparent success of EGF in chinchillas we decided to use a dosage of 250 μ g/mL EGF in our study.

Despite a lack of efficacy demonstrated in animal models (9), FGF2 has also received attention in the literature and the human results are encouraging in terms of closure rates (9–12) but there are some concerns about epidermal hyperplasia, serous otorrhea and patient groups have been highly selective. So far efficacy has only been demonstrated when excising the perforation edge in dry perforations without infection for a long period. Based on the reported efficacy in humans a dosage of 100 μ g/ml FGF2 was chosen for this study.

In this study, using a validated animal model of chronic TM perforation, only HB-EGF had efficacy over control. In those perforations that did not heal, there was no difference in the healing area between groups. As the initial chronic perforation size in this model can be variable, it would have been useful to measure the healing area as a proportion of the size of the initial chronic perforation used in each sample. Unfortunately an accurate estimate is only able to be measured post mortem when debris in the canal can be cleaned allowing the perforation margin to be examined without injury. (16) HB-EGF was also previously

identified to be significant in acute wound healing of the TM, whereas FGF2 and EGF did not show significantly different change during the week of wound healing. (15,29,30). Despite this lack of significant role in acute wound healing of the TM, benefits have been suggested in chronic TM wound healing for FGF2 and EGF. FGF2 has is yet to show efficacy without excising the perforation margin in chronic TM perforations. Excising the perforation margin is a confounding factor as it induces an acute inflammatory response potentially restarting the acute wound healing process. Excising the margin also requires surgical skill, a microscope and a cooperative patient. This excludes the use of this technique as an in office procedure in the majority of patients who have chronic TM perforations where a large proportion are pediatric or in countries with limited resources. If the lack of keratinization seen in the healing of chronic TMs by FGF2 in our model translates to poor keratinization in humans there may be a high risk of re-perforation in FGF2 treated TMs. HB-EGF is also the only growth factor to demonstrate efficacy in chronic TM perforations with Eustachian tube dysfunction or CSOM. (16) Given the lack of efficacy of EGF and FGF2 in our study, these growth factors would be unlikely to have efficacy in the animal models of chronic TM perforations with Eustachian tube dysfunction or CSOM. A limitation of this research is that bias is possible as the results were not blinded from the treatment arms. Blinding would have been difficult considering the animal model, treatments and histological assessment requires experience and training. This study also must be interpreted with consideration of the limitations of animal models used in mimicking the human condition.

In summary, HB-EGF as shown in this animal model is promising as a treatment for chronic TM perforation and, unlike other tested growth factors, may be effective without excising the perforation margin.

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Figure 1. HB-EGF, FGF2 and EGF treatment of chronic TM perforations

Figure 1 demonstrates the tympanic membrane after treatment with (a) HB-EGF (b) FGF2 (c) EGF and (d) control. The cytokeratin (brown) staining is used to identify the keratinocytes and keratin layers in the TM. The TM in the HB-EGF groups show healed TMs with thick keratin layers compared to FGF2, EGF and control which demonstrate a thickened connective tissue layer and lack of keratin staining. Scale bar =10µm (Magnification 20x)

Figure 2. The perforation edge of chronic TM perforations compared after treatment

Figure 2 demonstrates the tympanic membrane after treatment with (a) HB-EGF (b) FGF2 (c) EGF and (d) control. The HB-EGF group shows a healed TM compared to the thickened connective tissue layer seen at the chronic perforation edges (arrows) with the FGF2, EGF and control groups. Scale bar =10μm (Magnification 20x)

Table 1
Efficacy of Growth Factor Treatments in the Mouse Model Of Chronic TM Perforation

Table 1 shows the efficacy of HB-EGF, EGF and FGF compared to control. Only HB-EGF showed significant benefit over control and other treatments (bolded).

Treatment	Outcome (Complete closure of perforation)	Difference
HB-EGF (5ug/ml)	15/18 (83.3%)	vs control $\chi^2 = 11.25$, p = 0.003 vs EGF $\chi^2 = 16.88$, p = < 0.001 vs FGF2 $\chi^2 = 10.09$, p = 0.003
EGF (250ug/ml)	3/19 (15.8%)	vs control $\chi^2 = 0.78$, p = 1.00 vs FGF2 $\chi^2 = 1.31$, p = 0.75
FGF2 (100ug/ml)	6/19 (31.6%)	vs control $\chi^2 = 0.06$, p = 1.00
Control (polymer only)	5/18 (27.8%)	