Title: REPAIR OF TYMPANIC MEMBRANE PERFORATION

Abstract: The present invention relates to methods and compositions for repair of tympanic membrane perforations. The compositions of the invention comprise one or more growth factors such as EGF, TGF-α and PDGF, where the growth factor is present in amounts effective for treatment of tympanic membrane perforations. The compositions of the invention further comprise biocompatible scaffolds which are used as a matrix onto which growth factors such as EGF, TGF-α and PDGF can be incorporated. The methods of the invention comprise placing such biocompatible scaffolds, containing growth factors, over the tympanic membrane perforation thereby stimulating healing of the perforation.
REPAIR OF TYMPANIC MEMBRANE PERFORATION

SPECIFICATION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application No. 60/580,642, filed June 16, 2004, entitled “Repair of Tympanic Membrane Perforation” and U.S. Provisional Patent Application No. 60/611,201, filed September 17, 2004, entitled “Repair of Tympanic Membrane Perforation,” both of which are incorporated by reference in their entireties herein.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to methods and compositions for repair of tympanic membrane perforations.

Background

Tympanic membrane (eardrum) perforations are a common consequence of infection or trauma, afflicting approximately 1-3% of the American population (Gladstone et al. Tympanic Membrane Wound Healing. Wound Healing for the Otolaryngologist-Head and Neck Surgeon. N. Am Clin ORL Oct 1995;28/5:913-32). Although 88% of acute perforations heal spontaneously, the remainder become chronic and require treatment (Amoils et al. Repair of Chronic Tympanic Membrane Perforations using Epidermal Growth Factor. Otolaryngol Head Neck Surg 1992; 1 07:669-83). The persistence of such perforations have been attributed to such circumstances as, for example, the size of the perforation, which may be too large to be bridged by epithelial proliferation; infection which may impair the normal tissue response; insufficient blood supply; lining of the perforated margin with outer epithelium causing closure of the middle fibrous layer with its vascular network; and eustachian tube dysfunction (abnormal middle ear pressure).

Few options are currently available to patients with chronic tympanic membrane perforations, i.e., perforations which do not heal within about six weeks. The use of scaffolding to support the regenerating membrane with materials such as
rice paper, fat, or gelfoam after de-epithelialization of the perforation margins to close chronic perforations have high failure rates. For example, the closure rate of perforations greater than 5mm using paper patches is 12.5% in human patients and 63% for perforations that are less than 3mm (Golz et al. Paper patching for chronic tympanic membrane perforations, Otolaryngol Head Neck Surg. 2003 Apr;128(4):565-70). Tympanoplasty with autologous fascia, i.e., microsurgery that uses a patient's own tissues to reconstruct the tympanic membrane, is successful in more than 90% of cases, but is costly, requires surgical skill for successful outcome, and carries the risk of general surgery/general anesthesia (Sheehy et al. Myringoplasty. A review of 472 cases, Ann Otol Rhinol Laryngol, 1980;89:331-34). In addition, there are potential complications like cholesteatoma (benign tumor) and persistent perforation. Without full closure, patients may suffer from speech delay, chronic otorrhea (ear drainage), cholesteatoma formation, and chronic hearing loss.

Growth factors are cytokines that regulate cell proliferation and cell differentiation. They were initially discovered because of their ability to stimulate continuous mitosis of quiescent cells in a nutritionally complete medium that lacked serum. An immense amount of knowledge has been gained about growth factors through advancements in genetic engineering and recombinant DNA techniques which has led to sequencing of these genes and subsequent mass production, enabling scientists to investigate their biologic activities, chemical structures, and target receptors. They are synthesized and secreted by many types of cells involved in tissue repair including platelets, inflammatory cells, fibroblasts, epithelial cells, and vascular endothelial cells.

Mechanisms through which growth factors activate wound healing are not fully understood, although phosphorylation of proteins on tyrosine is highly favored. There are five major growth factor families that appear to contribute significantly to the wound healing process: platelet derived growth factor (PDGF), transforming growth factor (TGF), epidermal growth factor (EGF), fibroblast growth factor (FGF) and insulin growth factor (IGF) (See, Table 1; Bennett, N.T., Schultz, G.S. Growth Factors and Wound Healing: Biochemical,Properties of Growth Factors and Their Receptors. Am J Surgery 1993;165:728-37; McGrath, M.H. Peptide Growth Factors and Wound Healing. Clin Plastic Surgery, 1990; 17(3):421-32).
<table>
<thead>
<tr>
<th>Growth Factors</th>
<th>Source</th>
<th>Target Cells / Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermal growth factor (EGF)</td>
<td>Macrophages, platelets, epithelium</td>
<td>Mitogenic for epithelial tissues, fibroblasts, endothelial cells</td>
</tr>
<tr>
<td>Fibroblast growth factor (FGF)</td>
<td>Fibroblasts, endothelial cells, bone cells, macrophages</td>
<td>Endothelial cells, fibroblasts</td>
</tr>
<tr>
<td>Transforming growth factor (TGF-alpha)</td>
<td>Macrophages, eosinophils, keratinocytes, epithelial cells, platelets</td>
<td>Similar to EGF, but more potent angiogenesis factor</td>
</tr>
<tr>
<td>Transforming growth factor (TGF-beta)</td>
<td>Macrophages, lymphocytes, fibroblasts, keratinocytes, platelets, bone</td>
<td>Inhibits replication of most cells in vitro.</td>
</tr>
<tr>
<td>Platelet derived growth factor (PDGF)</td>
<td>Endothelial cells, platelets, macrophages, fibroblasts</td>
<td>Mitogenic for vascular smooth muscles, fibroblasts, macrophages</td>
</tr>
<tr>
<td>Insulin-like growth factor (IGF-I)</td>
<td>plasma, liver, fibroblasts</td>
<td>Mitogenic for fibroblasts, endothelial cells, fibroblasts, fetal tissues</td>
</tr>
</tbody>
</table>

**TABLE 1**

Wound healing in general involves the following steps: clot formation and platelet degranulation (the “hemostasis phase”); recruitment of inflammatory cells to the wound and release of cytokines / growth factors (the “inflammatory phase”); migration of fibroblasts and epithelial cells to the wound area; division and repopulation of the wound area with the fibroblasts and epithelial cells, vascular endothelial cells extending new capillaries into the wound site to establish a new blood supply, synthesis by the fibroblasts of an extracellular matrix that forms a scar to replace damaged tissue (the “proliferative phase”); and, later, the cellularity of the wound decreases, and the extracellular matrix is remodeled (the “remodeling phase”) (See, e.g., Horn, D.E., Maisel, R.H. Angiogenic Growth Factors: Their Effects and Potential in Soft Tissue Wound Healing. Ann OtolRhinol Laryngol, 1992;101:349-54; Grotendorst, G.R. Growth Factors as Regulators of Wound Repair.Int J Tissue React 1988; 6:337-44). Growth factors play key roles in initiating and sustaining these phases of tissue repair. Injury induces blood clotting and platelet degranulation. Contained within platelet granules are several growth factors, including, inter alia, PDGF, TGF, EGF, and IGF. The growth factors released from platelets appear to initiate the cascade of events that lead to healing. Chemotaxis of inflammatory cells,
fibroblasts, epithelial cells, and vascular endothelial cells to the wound site is followed by release of growth factors from platelets rapidly diffusing from the site of injury and are degraded by proteases. To continue healing, additional or new growth factors are synthesized by the inflammatory cells, fibroblasts, and epithelial cells initially drawn to the wound by the platelet growth factors. (See, Figure 1)

The normal healing pattern of acute tympanic membrane perforations has been well described by several authors (Boedts, D. The Healing Mechanism in Eardrum Perforations. The influence of the Squamous Epithelium. Acta Otorhinolaryngol1978;32:329-55; Dunlap, A.M., Schuknecht, H.F. Closure of Perforations of the Tympanic Membrane. Laryngoscope 1947;57:479-90; See Figure 2). The wound margins initially retract, which is followed by increased epithelial cell mitotic activity. The epithelial layer then migrates over the perforation, narrowing the defect. Lagging behind the epithelial layer, the lamina propria layer moves forward, followed by the mucosal layer. Unlike soft tissue healing, the epithelium, rather than the connective tissue, initially closes the defect (Johnson, A., Hawke, M. The Function of Migratory Epidermis in the Healing of Tympanic Membrane Perforations in the Guinea Pig. Acta Otolaryngol, 1987;130:81-6). Although the histological events are well-mapped, insight into the complex cascade of biologic process occurring at the molecular level as well as its regulation is absent. Several hypotheses have been proposed for the impaired healing process: growth inhibition due to epithelialization at the perforation edge (Spandow et al. M. Structural Characterization of Persistent Tympanic Membrane Perforations in Man. Laryngoscope, 1996; 106:346-52; Stinson, W. Reparative Processes in the Membrane Typani. Arch Otolaryngol, 1936;24:600-06), insufficient blood supply in the central tympanic membrane areas (Masutani et al. Microvasculature of the Tympanic Membrane. Acta Otolaryngol, 1991;486(suppl):99-104), connective tissue hyperproliferation (Reeve, D. Repair of Large Experimental Perforations of the Tympanic Membrane. J Laryngol, 1977;91:767-78), or insufficient supply of growth factors at the proliferation border (Somers. Histology of the Perforated Tympanic Membrane and its Mucoepithelial Junction. Clin Otolaryngol, 1997;22:162-66).

Mondain and Ryan show that expression of EGF and FGF-2 increased after acute perforations in guinea pig tympanic membranes, whereas EGF was observed near the annulus tympanic area but FGF-2 was not present in intact tympanic membranes (Mondain, M., Ryan, A. Epidermal Growth Factor and the basic
Fibroblast Growth Factor are Induced in Guinea Pig Tympanic Membrane Following Traumatic Perforation. Acta Otolaryngol, 1995;115:50-54). After perforation, EGF expression was detected in polymuclear cells, pericytes, and basal epithelial cells, mainly in the area adjacent to the perforation, which peaked in three days after the perforation.

Koba and Kawabata also show that cells staining for TGF-alpha were present in the epidermal layer around the perforation margin and scattered throughout the membrane in freshly perforated rabbit tympanic membranes (Koba et al. Immunohistochemical Study of Transforming Growth Factor alpha Expression in Normal and Perforated Tympanic Membrane. Ann Otol Rhinol Laryngol, 1995;104:793-97). After the healing was complete, the expression of TGF-alpha subsided. The authors also found no expression in normal tympanic membranes.

O'Daniel used radio-labeled EGF to detect EGF receptors in porcine tympanic membranes (O'Daniel et al. Epidermal Growth Factor Binding and Action on Tympanic Membranes. Ann Otol Rhinol Laryngol, 1990;99:80-84). The cells of the stratified squamous epithelial layer and in the stromal/mucoepithelial layer expressed the EGF receptor. Approximately twice as much receptor expression in the epithelial layer was detected, in comparison to receptor expression in the stromal and mucosal layers.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Control</th>
<th>TM Perforation</th>
</tr>
</thead>
<tbody>
<tr>
<td>O'Daniel et al. (1990)</td>
<td>Porcine (acute)</td>
<td>EGF+</td>
</tr>
<tr>
<td>Koba et al. (1995)</td>
<td>Rabbit (acute)</td>
<td>TGF alpha -</td>
</tr>
<tr>
<td>Mondain et al. (1995)</td>
<td>Guinea Pig (acute)</td>
<td>FGF -</td>
</tr>
<tr>
<td>Somers et al. (1998)</td>
<td>Human (chronic)</td>
<td>EGF rec+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FGF +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGF alpha +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGF beta -</td>
</tr>
</tbody>
</table>

TABLE 2

The studies cited above, summarized in Table 2, demonstrate increased expression of individual growth factor proteins in acute tympanic membrane perforations, as well as presence of growth factor receptors in quiescent, healthy cells.

Several studies have shown that topical application of growth factor to the tympanic membrane increases the closure rates of perforations in animal models.
Fina et al. created 1-2 mm tympanic membrane perforations in guinea pigs, followed by FGF application onto gelfoam pledgets for several days. By day 8, all FGF-treated perforations closed, in comparison to 74% of the controls (Fina et al. A Improved Healing of Tympanic Membrane Perforations with basic Fibroblast Growth Factor, Growth Factors, 1991;5:265-72). Mondain et al. observed 12 out of 15 rats with growth factor treatment achieved closure of 1mm perforations before that of the untreated control ear, with an average time to closure of 6.1 days in the treated ears in comparison to 8.9 days in the untreated control ears (Mondain et al. A Fibroblast Growth Factor Improves the Healing of Experimental Tympanic Membrane Perforations. Acta Otolaryngol, 1991; 111:337-41). Both studies show modest increases in the closure rates when growth factors are applied. However, only acute perforation model systems were used. It is well known in the art that the majority of acute perforations are capable of healing spontaneously.

Persistent, or chronic, tympanic membrane perforations, however, require continuous treatment and remain difficult to heal. As discussed above, the difficulties may arise from the larger size of the perforation, insufficient blood supply to promote closure, infection that may disrupt the healing process, or the blockage of perforation model systems were used. It is well known in the art that the majority of acute perforations are capable of healing spontaneously.

Models of chronic tympanic membrane perforations have also been disclosed. Amoils et al. teach a model of chronic tympanic membrane perforation in chinchillas by using thermal myringectomy with medial inrolling of the wound edges, followed by a 6-8 week observation period to ensure a stable chronic perforation (Amoils et al. An Animal Model of Chronic Tympanic Membrane Perforation. Otolaryngol Head Neck Surg, 1992; 106:47-55). In one scenario, the chronic perforation edges were de-epithelialized and covered with a rice paper patch and a gelfoam pledget, or a small wound dressing. The pledget was saturated with EGF solution at the time of the initial procedure and every day thereafter for 6 days. Closure of the perforations was achieved in 81% of EGF-treated ears in comparison to 25% of control ears. Significantly, the time to heal was not listed (Amoils, et al. Repair of Chronic Tympanic Membrane Perforations using Epidermal Growth Factor. Otolaryngol Head Neck Surg, 1992; 107:669-83). In a subsequent study, a gelfoam pledget was applied to the chronic perforations, with no other surgical manipulation. EGF was applied to the pledget every other day for a total of three doses. Complete
closure was achieved in 80% of treated ears in comparison to 20% of ears treated with buffered saline solution. Most of the EGF-treated membranes healed over a period of 3 to 5 weeks (Lee et al. Repair of Chronic Tympanic Membrane Perforations Using Epidermal Growth Factor: Progress Toward Clinical Application. Am J Otol, 1994;15:10-18). The published studies were silent on the healing rate, lacking a measure of the decrease in size of the perforations over time. Such an analysis could have shown whether any healing had occurred, even if closure was not obtained.

Despite the studies discussed above (summarized in Table 3), a recent prospective double-blind study of 17 adults with chronic tympanic membrane perforations showed no benefit to topical application of EGF (Ramsay et al. Effect of Epidermal Growth Factor on Tympanic Membranes with Chronic Perforations: a Clinical Trial. Otolaryngol Head Neck Surg, 1995;113:375-79). This study reinforces the limited understanding of the role of growth factors in wound healing.

<table>
<thead>
<tr>
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<th>Animal Model</th>
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<th>+FGF</th>
<th>+PDGF</th>
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<tr>
<td>O'Daniel et al.</td>
<td>Cats (acute)</td>
<td>Not closed by day 6</td>
<td>Not closed by day 6</td>
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<td>Fina et al. (1991)</td>
<td>Guinea pig (acute)</td>
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<td>100</td>
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<tr>
<td>Amoils et al.</td>
<td>Chinchilla (chronic)</td>
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<td>81</td>
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<tr>
<td>Jackler et al.</td>
<td>Chinchilla (chronic)</td>
<td>20</td>
<td>80</td>
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<tr>
<td>Ramsay et al.</td>
<td>Human (chronic)</td>
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<td>No change</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kato et al.</td>
<td>Chinchilla (chronic)</td>
<td>41</td>
<td>81</td>
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<td></td>
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<tr>
<td>Soumekh et al.</td>
<td>Chinchilla (chronic)</td>
<td>NSSD</td>
<td>NSSD</td>
<td>NSSD</td>
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<tr>
<td>Friedman et al.</td>
<td>Chinchilla (acute)</td>
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<td>NSSD</td>
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<tr>
<td>Chavin et al.</td>
<td>Guinea pig (acute)</td>
<td>63.6</td>
<td>100</td>
<td>85.7</td>
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<tr>
<td>Yeo et al. (2000)</td>
<td>Rat (acute)</td>
<td>75</td>
<td></td>
<td>100</td>
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</tbody>
</table>

**TABLE 3**

There is a compelling need to develop a treatment for chronic tympanic membrane perforations that is effective, easy to administer, and cost-effective.
SUMMARY OF THE INVENTION

The present invention relates to methods and compositions for repair of tympanic membrane perforations.

The compositions of the invention comprise one or more growth factors, such as EGF, TGF-α and PDGF, used in combination for the treatment of tympanic membrane perforation. In particular, the compositions of the invention are enhanced by the presence of two or more of these growth factors present in amounts effective for treatment of tympanic membrane perforations. The compositions of the invention also comprise biocompatible scaffolds which are used as a matrix onto which growth factors, such as EGF, TGF-α and PDGF, can be incorporated.

The methods of the invention comprise administering to a subject having a tympanic membrane perforation a composition comprising one or more growth factors, such as EGF, TGF-α and PDGF. In a specific embodiment of the invention, biocompatible scaffolds, containing growth factors, are placed over the tympanic membrane perforation thereby stimulating healing of the perforation.

In an embodiment of the invention, ear drops are placed on the scaffold to solubilize the growth factors embedded on the matrix. Through capillary action, the growth factors will be absorbed by the leading edges of the perforated membrane while minimizing contamination of the middle ear cleft with the growth factors thereby reducing undesirable middle ear mucosa proliferation.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the steps involved in wound healing.

Figure 2 shows a schematic of the method utilized to analyze growth factor expression during tympanic membrane perforation repair in accordance with the present invention.

Figure 3 shows the tympanic membrane of a mouse before and after subtotal tympanic membrane perforation in accordance with the present invention.

Figure 4 shows the external auditory canal of a mouse with a soft tissue incision before and after the incision in accordance with the present invention.
Figure 5 shows a bar graph of the percentage of mice with complete tympanic membrane closure after 3, 7, and 28 days after perforation of tympanic membrane in accordance with the present invention.

Figures 6A-6F show the healing of soft tissue incision on the external auditory canal (A-C) and detached temporal bone tissue, including the tympanic membrane, (D-F) at 3, 7, and 28 days post-surgery in accordance with the present invention.

Figures 7A-7D show isolated temporal bone and attached tympanic membrane (lateral view, 7A; medial view, 7B) and other ear structures (ossicles, 7C; annulus, 7D).

Figures 8A-8F show hematoxylin and eosin stained and anti-EGF stained sections collected from perforated tympanic membrane at 0, 3 and 7 days after surgery in accordance with the present invention.

Figures 9A-9F show hematoxylin and eosin stained and anti-EGF stained sections collected from external auditory canal soft tissue in accordance with the present invention.

Figure 10 shows a schematic representation of RNAase protection assay in accordance with the present invention.

Figure 11 shows the expected probe size for various growth factors used in the RNAase protection assays in accordance with the present invention.

Figure 12 shows the expression of various growth factors in perforated tympanic membrane tissue in comparison to intact skin tissue at 0, 3, 7 and 28 days after surgery obtained using RNase protection assay in accordance with the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The compositions of the invention comprise one or more growth factors, such as EGF, TGF-α and PDGF, used in combination for the treatment of tympanic membrane perforation. The present invention is based on the observation that expression of specific growth factors are upregulated upon acute trauma to the tympanic membrane tissue.

In an embodiment of the invention, the tympanic membrane perforation is a chronic wound. A perforation that is chronic is permanent, well-
epithelialized (having a mature stratified squamous epithelium), and free from infection. In humans patients, a tympanic membrane perforation develops a chronic nature at greater than six weeks after the injury has occurred. Accelerating wound healing in a human patient having a chronic tympanic membrane perforation comprises administering to the patient a composition containing an effective amount of growth factor to promote healing of the wound.

In another embodiment of the invention, the tympanic membrane perforation is an acute wound and can be healed within a short period of time, usually within two weeks. The rate of healing is dependent upon the size of the perforation. Usually, the healing occurs at approximately 1 mm in diameter per day. Accelerating wound healing in a human patient having an acute tympanic membrane perforation comprises administering to the patient a composition containing an effective amount of growth factor to promote healing of the wound.

Appropriate growth factors include, but are not limited to, *inter alia*, EGF, TGF-α, PDGF, FGF-2, IGF, TNF-α, VEGF (vascular epidermal growth factor), KGF (keratinocyte growth factor) and angiopoietin. The composition of the present invention may also comprise neurotrophins selected from the group consisting of NGF, BDNF, NT-3 and NT-4/5. Preferably, the present composition comprises EGF, PDGF and/or TGF-α.

In a specific embodiment of the invention, the compositions incorporate one or more of the EGF, TGF-α and PDGF growth factors. Experiments presented in the Examples section identify growth factors, such as EGF, TGF-α and PDGF, which are involved in repair of the wounded tympanic membrane. These observations demonstrate that more than one growth factor is involved in healing and multiple growth factors may act together to promote healing.

The administered composition may comprise from about 1 microgram to about 50 microgram of growth factor. For EGF, the composition comprises preferably from about 10 microgram to about 50 microgram of growth factor administered per day. For TGF and PDGF, the composition comprises preferably from about 1 microgram to about 5 microgram of growth factor administered per day.

The compositions may further comprise a pharmaceutically acceptable carrier, and can comprise agents in the form of an aqueous solution, a gel, a lotion, a balm, a powder, a paste. In a specific embodiment, the term "pharmaceutically
acceptable" means approved by a regulatory agency of the Federal or a state
government or listed in the U.S. Pharmacopeia or other generally recognized
pharmacopeia for use in animals, and more particularly in humans. The term "carrier"
refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is
administered. Examples of suitable pharmaceutical carriers are described in
"Remington's Pharmaceutical sciences" by E.W. Martin.

The compositions of the invention may further comprise a
biocompatible scaffold which is used as a matrix onto which growth factors can be
incorporated.

The scaffold will have all the features commonly associated with being
biocompatible, in that it is in a form that does not produce an adverse, or allergic
reaction when administered to the recipient host. Such matrices may be formed from
both natural or synthetic materials and may be designed to allow for sustained release
of growth factors over prolonged periods of time. Thus, appropriate matrices will
both provide growth factors and also act as an in situ scaffolding. In preferred
embodiments, it is contemplated that a biodegradable matrix that is capable of being
reabsorbed into the body will likely be most useful. The scaffolds of the invention
further comprise growth factors capable of stimulating the growth and regeneration of
the tympanic membrane tissue.

The biocompatible scaffolds may comprise from about 100
micrograms to about 5 milligrams growth factor. For EGF, the biocompatible
scaffolds comprises preferably from about 1-5 milligrams of growth factor. For TGF
and PDGF, the biocompatible scaffolds comprises preferably from about 100-500
micrograms of growth factor.

The invention also provides a method of promoting healing of
tympanic membrane perforation in a subject in need of such treatment, which
comprises administering to the subject a composition of the present invention
containing an effective amount of one or more growth factor for promoting healing of
tympanic membrane perforation. In particular, the invention is directed to treatment
of chronic tympanic membrane perforations. The compositions may also be used to
accelerate healing of acute tympanic membrane perforations. Preferably, the subject
is a human.
In particular, the methods of the present invention comprise administering compositions to promote cell growth, proliferation, or regeneration of various cell types, such as those found in the tympanic membrane.

The composition can be administered as ear drops to the ear. Preferably, three drops of the composition are administered at one time. In one embodiment, the composition administration occurs everyday, twice a day, for 7-14 days. The healing will be monitored periodically, for example at day 10, and the treatment regimen may be amended in accordance with the progress of the healing. The treatment continues until complete tympanic membrane closure is achieved or unless hypertrophic scarring is observed.

Alternatively, the present invention provides a method of promoting healing of tympanic membrane perforation in a subject in need of such treatment, which comprises administering to the subject a composition of the present invention comprising a biocompatible scaffold containing an effective amount of one or more growth factors for promoting healing of tympanic membrane perforation.

The methods of the invention comprise placing such biocompatible scaffolds, containing growth factors, over the tympanic membrane perforation, thereby stimulating healing of the perforation. An example of such a method applies the use of paper tape patches (Camnitz et al. Traumatic perforation of the tympanic membrane, early closure with paper tape patching. Otolarynol Head Neck Surg, 1985, 93(2) 220-3).

In an embodiment of the invention, the growth factors embedded in the scaffold are released using release drug delivery systems, such as nanoparticles, matrices, such as controlled-release polymers and hydrogels.

In another embodiment of the invention, the growth factors embedded on the scaffold covering the perforation are released upon contact with an agent. Compositions comprising such agents may be applied to the scaffold through ear drops. Through capillary action, the growth factors will be absorbed by the leading edges of the perforated membrane while minimizing contamination of the middle ear cleft with the growth factors thereby reducing undesirable middle ear mucosa proliferation. Either the clinician or the patient can administer the ear drops to release the active growth factors. Upon release by the ear drops, the scaffold produces physiologically active amounts of growth factor to promote healing. Released growth factor amounts are from 10 micrograms to about 5 milligrams growth factor.
Preferably, the amount of EGF is from about 10-50 micrograms of growth factor. Preferably, the amount of TGF and PDGF is from about 1-5 micrograms of growth factor. The ear drops are administered twice a day, everyday, for a period of 7-14 days, weeks, or months. Commercially available ear drops are suitable for dissolving and releasing the growth factor from the scaffold. For example, such ear drops as ofloxacin (FloxinTM), ciprofloxacin (CiproTM), or sterile water are useful as wetting agents.

EXAMPLES

EXAMPLE 1: TYMPANIC MEMBRANE PERFORATION ANIMAL MODEL

The ideal animal model must have a tympanic membrane perforation that free from infection. The perforation must also be subtotal (close to but not including the tympanic rim) to preserve a rim of membrane for experimental manipulations. The external auditory canal must be wide with relatively large tympanic membrane for easy visualization. The animal must also have its genome sequenced to design polymerase chain reaction primers for growth factors, EGF, FGF, and PDGF. These considerations are met in the mouse model used for the examples discussed in this section. This example is related to the preparation, gross anatomical characterization of the animal model.

A schematic of an exemplary method utilized to analyze growth factor expression during tympanic membrane perforation repair is illustrated in Figure 2. Healthy adult Swiss Webster male mice weighing 50-60 grams (Charles River Lab; Wilmington, MA) were used in accordance with the National Institutes of Health and the New York Medical College IACUC guidelines. The animals were anesthetized with intraperitoneal injections of ketamine/acepromazine mixture (75mg/kg). The ear canal was sterilized with a povidone iodine solution, and using an operating microscope, subtotal tympanic membrane perforations were created in the pars tensa portion of the tympanic membrane on both ears with a 28g ophthalmic knife (Xomed Corporation, Jacksonville, Fla). Only the rim of the annulus in the pars tensa remain behind. See Figure 3.
Skin and soft tissue at the entrance into the external ear canal was incised to serve as a comparative control of soft tissue wound healing (Figure 4). No perioperative antibiotics were administered.

Four groups of thirteen mice were prepared. The first group was set aside as controls and represent baseline at day 0, without any surgical intervention. Subtotal tympanic membrane perforations were created in the remaining three groups of mice for a total of thirty-nine mice.

At certain times, animals were sacrificed by lethal injection of ketamine and decapitated to isolate the temporal bone and the soft tissue wound site for histological studies. The time required for complete closure was noted, and the remaining defect estimated on each inspection. Photographs were taken with a 3 mega pixel digital cameral (Canon; Tokyo, Japan) attached to the operating microscope.

The second group of mice was observed daily to monitor the progression of tympanic membrane regeneration. At day 3, only one out of thirteen mice had complete closure of the perforation, while the remaining mice had ~50% persistent TM perforations (Figure 5). The remaining twelve mice of the second group with incomplete closure were sacrificed and the temporal bone, including the tympanic membrane, was harvested for histology and RNA extraction, as discussed in Examples 2 and 3, respectively.

The third group was also observed daily to monitor the progression of tympanic membrane regeneration. At day 7, twelve out of the thirteen mice achieved complete tympanic membrane closure (Figure 5), while one had residual (~5%) TM perforation. The twelve mice with complete closure were sacrificed and samples harvested for histology and RNA extraction, as discussed in Examples 2 and 3, respectively.

The fourth group was also observed daily to monitor the progression of tympanic membrane regeneration. By day 28, twelve out of thirteen mice had complete tympanic membrane closure (Figure 5), while one developed otitis media, which is an inflammation of the area behind the eardrum. The twelve mice were sacrificed and samples harvested and processed for histology and RNA extraction, as discussed in Examples 2 and 3, respectively.

Healing of soft tissue wound created at the entrance into the external auditory canal progressed as expected. Figures 6 A-C show the soft tissue healing
progress at 3 (Figure 6A), 7 (Figure 6B), and 28 days (Figure 6C) post-surgery. By day 3, there is scab formation and granulation tissue (Figure 6A). By day 7, there is minimal granulation (Figure 6B). By day 28, there is complete healing with regeneration of skin (Figure 6C). Therefore, any evidence of healing process is resolved by day 28.

Figures 6D-6F show examples of detached temporal bone, including the tympanic membrane at 3 (Figure 6D), 7 (Figure 6E) and 28 (Figure 6F) days post-surgery. There was incomplete healing or persistence of the tympanic membrane perforation to ~50% by day 3 from the initial subtotal perforation created on day 0 to ~90% leaving only the annulus intact. By day 7, complete tympanic membrane closure was observed. Evidence of myringosclerosis, a thickening and calcification of the tympanic membrane, was observed on the completely healed tympanic membrane on day 28.

EXAMPLE 2: HISTOLOGY OF HEALED TYMPANIC MEMBRANE TISSUES

This example presents the histological and immunohistological analysis of healed tympanic membrane tissues. Surgical procedures were performed as discussed in Example 1.

Histology. Baseline control samples were obtained without any surgical manipulations on day 0 from the first group discussed in Example 1. Mice with surgical interventions were sacrificed on day 3, 7, and 28 days post-surgery. En bloc temporal bone and the soft tissue wound site at the entrance into the external ear canal were fixed in 4% paraformaldehyde and decalcified in 8% ethylenediamine tetraacetic acid. After the decalcification, specimen were dehydrated with graded alcohol baths, embedded in paraffin, sectioned in a 4 um microtome (LKB Pharmacia; Uppsala, Sweden), and stained with hematoxylin and eosin. Each specimen was examined under a photomicroscope (Zeiss Photomicroscope Co; Oberkochen, Germany) and photodocumented.

Immunohistochemistry. Sections from collected temporal bone and the soft tissue wound site at the entrance into the external ear canal were incubated with an antibody specific for murine epidermal growth factor antibody (Accurate Chemical; Westbury, NY). Sections were deparaffinized, permeabilized with 0.2% Triton X-100/PBS for 30 min, blocked with 1% normal rabbit serum for 1 hr, and
incubated with murine epidermal growth factor antibody diluted 1:20 in blocking solution for 2 hours. A biotinylated rabbit anti-murine secondary antibody was applied for 30 min, washed with PBS, incubated with ABC reagent (Vector Laboratories) for 30 min, washed with PBS, and subjected to DAB reaction for 5-10 min with microscopic guidance. Staining was recorded as either positively or negatively immunoreactive under a photomicroscope (Zeiss Photomicroscope Co; Oberkochen, Germany) and photodocumented.

Routine hematoxylin and eosin stain of mouse tympanic membrane sections collected at day 0 show that the tissue is composed of a thin epithelial outer layer, a middle fibrous layer and an inner mucosal layer (Figure 8A). The malleus is also visible (Figure 8A). Immunostained sections show the absence of epidermal growth factor (EGF) expression (Figure 8D).

Figure 8B shows a much thinner tympanic membrane collected at day 3, in comparison to the tympanic membrane collected at day 0. The hematoxylin and eosin stained section also shows the perforation in the tympanic membrane. The leading edge of the persistent tympanic membrane perforation demonstrates positive immunostaining with anti-EGF antibody (Figure 8E).

The tympanic membrane achieved complete closure by day 7, as evidence by the hematoxylin and eosin stained section (Figure 8C). Furthermore, there was negligible detection of EGF (Figure 8F). Specimens collected at 28 days post-surgery show a histological and immunohistological profile similar to specimen collected at day 7.

In comparison to the perforated tympanic membrane tissues, similar histology and expression of EGF was observed in healing soft tissue wound site at the entrance into the external ear canal. Figure 9A is a hematoxylin and eosin stained section from the soft tissue site, showing a thick epidermal and submucosal layer and Figure 9D shows the absence of EGF immunostaining.

Scab formation representative of wound healing is shown in a soft tissue section collected at day 3 and stained with hematoxylin and eosin Figure 9B. The corresponding immunostained section illustrate positive EGF staining (Figure 9E). By day 7, the epidermal and mucosal layer appear fully healed (Figure 9C), with the absence of EGF expression (Figure 9F). Sections prepared from day 28 samples show a similar morphological and immunohistological pattern similar to section prepared from day 7 samples.
EXAMPLE 3: RNA EXPRESSION OF GROWTH FACTORS IN HEALED TYPANIC MEMBRANE TISSUES

This example shows the expression of various growth factors implicated in general wound healing in an acute tympanic membrane perforation mouse model.

RNA Extraction. Tympanic membrane was dissected from its annular groove and stored at −70°C until ready for RNA extraction. Soft tissue wound site at the entrance into the external auditory canal was also isolated and stored at −70°C. Tissue was homogenized in TRI Reagent at 1 ml/50 - 100 mg tissue (Molecular Research Center; Cincinnati, OH). Homogenate was stored for 5 minutes at room temperature to permit complete dissociation of nucleoprotein complexes. Next, the homogenate was supplemented with 0.2 ml chloroform per 1 ml of TRI Reagent and shaken vigorously for 15 seconds. The resulting mixture was stored at room temperature for 15 minutes and centrifuged at 12,000 g for 15 minutes at 4°C. Following centrifugation, the mixture separated into a lower red phenol-chloroform phase, interphase and the colorless upper aqueous phase. RNA remained exclusively in the aqueous phase and was harvested, whereas DNA and proteins were in the interphase and organic phase. The aqueous phase was transferred to a fresh tube and RNA precipitated from the aqueous phase by mixing with 0.5 ml of isopropanol per 1 ml of TRI Reagent used for the initial homogenization. The samples were stored at room temperature for 10 minutes and centrifuged at 12,000 g for 8 minutes at 4°C. The supernatant was removed, and the RNA pellet was washed by vortexing with 75% ethanol and subsequent centrifugation at 7,500 g for 5 minutes at 4°C. The ethanol wash was removed and the RNA pellet air dried for 5 minutes. RNA was dissolved in diethyl pyrocarbonate (DEPC) treated water and incubated for 10-15 minutes at 65°C for 15 minutes. Samples were then stored at -20°C.

Multiprobe Mouse Growth Factor DNA Template Set. Through BD Biosciences (San Diego, CA), a mouse template set was custom made containing 10 various growth factor DNA templates (Table 4) plus one house keeping gene, L32, for the purpose of assessing total RNA levels for normalizing samples and technique errors. These templates were primed to be compatible with T7 RNA polymerase-directed synthesis of 32P-labeled anti-sense RNA probe that can then be used to
hybridize against target mRNAs. Each probe has at the minimum 20 base pair difference in size to allow for separate bands when ran on an electrophoresis gel. Figure 11 shows the expected probe size for various growth factors used in the RNAase protection assays in accordance with the present invention.

<table>
<thead>
<tr>
<th>GENES</th>
<th>PROBE SIZE (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-alpha B</td>
<td>407</td>
</tr>
<tr>
<td>IGF-1A</td>
<td>358</td>
</tr>
<tr>
<td>IL-1RII</td>
<td>320</td>
</tr>
<tr>
<td>TNF-alpha A</td>
<td>287</td>
</tr>
<tr>
<td>EGF</td>
<td>256</td>
</tr>
<tr>
<td>FGF-2</td>
<td>226</td>
</tr>
<tr>
<td>TGF-alpha</td>
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</tr>
<tr>
<td>PDGF-A</td>
<td>160</td>
</tr>
<tr>
<td>VEGF</td>
<td>148</td>
</tr>
<tr>
<td>Angiopoietin-I</td>
<td>136</td>
</tr>
<tr>
<td>L32</td>
<td>113</td>
</tr>
</tbody>
</table>

**TABLE 4**

RNAse Protection Assay. - $^{32}$P-labeled anti-sense RNA probe generation. The RNAse Protection Assay is a highly sensitive and specific method for detecting and quantitating mRNA of interest. This assay can quantify several mRNA species in a single sample of total RNA. In the exemplary embodiment, the MAXIscript transcription kit (Ambion; Austin, TX) was used to generate $^{32}$P-UTP labeled anti-sense RNA probes. Briefly, the following ingredients were added to a sterile 1.5ml eppendorf tube: 7ul DEPC water, 2ul 10x Transcription buffer, 1ul 10mM ATP, 1ul 10mM CTP, 1ul 10mM GTP, 1ul mouse growth factor DNA template set, 5ul $^{32}$P-UTP (Perkin Elmer; Boston, MA), 2ul Rnase inhibitor/T7 RNA polymerase. The mixture was vortexed, incubated at 37°C water bath for 45 minutes. The reaction was stopped by adding 1ul DNase to the mixture, vortexed, and placed at 37°C water bath for 15 minutes. The anti-sense RNA probe was then precipitated by adding 8ul 5M Ammonium acetate, 60ul 100% ETOH, 0.5ul glycol blue at -70°C for 45 minutes. Mixture was then spun at 12,000g for 15 minutes at 4°C. Supernatant was carefully discarded, and the pellet washed with 200ul 75% ETOH. Mixture was again
spun at 12,000g for 5 minutes at 4°C. After the supernatant was discarded, and the pellet suspended in 50ul hybridization solution. Radiolabeled probe was quantitated by diluting 1ul of the probe to 1ml scintillation fluid and using the following formula: 
\[
\text{radiolabel count/ul} \times 49ul \text{ divided by 3.33 counts/ul} - 49ul = \_
\]
ul hybridization solution to add to the probe. The radiolabeled probe was be stored at -70°C.

RNA hybridization. 5ul RNA from tympanic membrane samples and soft tissue/skin samples isolated, as previously described above, were mixed with 20ul hybridization solution. Yeast RNA was used as a positive control. 3ul radiolabeled probe (10^6 counts) was added to each RNA sample. Yeast RNA, without the addition of the radiolabeled probe, served as a negative control. Samples were heated in 93°C heating block for 4 minutes, and then incubated overnight at 42°C for the hybridization reaction.

RNAse digestion and precipitation of protected fragments. Any single stranded RNA which represents untargeted RNA were digested by adding 198ul RNase digestion buffer and 2ul RNase A/RNase T1. RNA representing the growth factors of interest were protected from digestion by hybridizing with the ^{32}P-UTP labeled anti-sense RNA probes, thereby remaining in a double stranded state (Figure 10). Samples were incubated at 37°C for 30 minutes. Reaction was stopped and the protected double stranded RNA precipitated by adding 300ul of RNase PPT to each sample, vortexing, and placed at -70°C for 45 minutes. Samples were then spun at 12,000g for 15 minutes at 4°C. Supernatant was discarded, and the pellet washed with 200ul 75% ETOH. The final pellet was resuspended in 7ul loading buffer, heated at 95°C heating block for 4 minutes, and placed on ice until loaded onto the gel.

Gel electrophoresis and probe detection. A 40cm gel plate was assembled with a 0.4mm spacer. 5% acrylamide gel was prepared by combining 8.85ml 40% acrylamide, 9.31ml 2% bis acrylamide, 7.45ml 10xTBE, 35.82gm Urea, 450ul 10% ammonium sulfate, 60ul TEMED. Gel was run at 250 volts for ~4 hours until the leading edge of the bromophenol blue reached the bottom of the gel. The gel was placed on a 3mm Whatman paper and dried on a gel dryer (BioRad) for 2 hour. The dried gel was placed in a Kodak cassette with an intensifying screen and film, and developed at -70°C. Exposure time varied with the intensity of each probe signal from 2 hours for the yeast positive control to 1 week for growth factor genes. The differences in intensity at different time points were graded by two investigators without knowledge of sample lanes.
Statistical Analysis. All statistical analysis involving the comparison of
data from two groups were computed by using the Student’s t-test (paired). A p value
of <0.05 was considered statistically significant.

Figure 12 shows a blot exhibiting expression of various growth factors
in perforated tympanic membrane tissue, in comparison to intact skin tissue at 0, 3, 7
and 28 days after surgery obtained using the above-described RNase protection assay.
Table 5 summarizes the results of various growth factor gene expression obtained
through the RNase protection assay. The data in Table 5 was based on a scale of + for
faint expression to +++ for strong expression. Absence of signal was represented by a
minus sign. The even intensity of the house keeping gene, L32, in samples collected
from day 0, 3, 7, and 28 ensures that the differences in signal intensity of the growth
factor genes was not due to sampling error.

<table>
<thead>
<tr>
<th></th>
<th>SKIN</th>
<th>TM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DAY #</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>TNF-alpha B</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IGF-1A</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>IL-1RII</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>TNF-alpha A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EGF</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>FGF-2</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>TGF-alpha</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PDGF-A</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>VEGF</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Angiopoetin-I</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L32</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

**TABLE 5**

TNF-α, TNF-β, and VEGF were not detected in any of the samples
collected from day 0-28. Several growth factors, e.g. IGF, IL-1, FGF, and
angiopoetin were detected, but did not display any significant change in expression,
*i.e.*, up or down regulation of its RNA.
Three growth factors, EGF, TGF-α, and PDGF-A, demonstrated some fluctuations during the healing process. EGF expression was absent at day 0, strongly positive by day 3, returned to its negative baseline level by days 7 and 28. TGF-α, expression also followed a similar trend, being strongly present at day 3, and falling to negligible levels by days 7 and 28. PDGF-A was also upregulated by day 3, maintained its expression level at day 7, and exhibited a reduced signal intensity at day 28.

EXAMPLE 4. TREATMENT OF TYMPANIC MEMBRANE PERFORATION IN AN ANIMAL MODEL USING TWO OR MORE GROWTH FACTORS

The mouse model for use and the preparation of the perforation in this example is prepared as discussed in Example 1.

For acute tympanic membrane perforations, the treatment to be discussed begins on day 2 after the surgery to create the perforation. For chronic tympanic membrane perforation, the treatment to be discussed begins at 6 weeks post-surgery to create the perforation.

These animals are observed weekly for total of 6 weeks to ensure chronicity of the tympanic membrane perforation. Any animal demonstrating signs of otitis externa (inflammation of the ear canal between the ear drum and the outside of the ear) or otitis media (middle ear infection) are discarded. Also, any animal with spontaneous closure of the perforation was recorded and excluded from use as a chronic tympanic membrane perforation model.

After the end of the 6 week observation period status post-myringectomy, the animals are divided into 7 groups, 5 mice per groups as listed below.
Group 1: EGF
Group 2: TGF-α
Group 3: PDGF
Group 4: EFG + TGF-α
Group 5: EGF + PDGF
Group 6: TGF-α + PDGF
Group 7: EGF + TGF-α + PDGF
Prior to the application of growth factor(s) in one ear only for each animal, the tympanic membrane perforation edges are freshened to allow for vascularization and epithelial growth. The contralateral ear for each animal serves as the internal control. The animals are anaesthetized, as previously described in Example 1, before the surgery. The dosage of each growth factor varied based on prior experience and on the manufacturer's recommended dosages, which is 50 microgram/day for EGF, 0.5 microgram/day for TGF-\(\alpha\) and 0.5 microgram/day for PDGF.

Beginning on the day of the surgery, growth factors are applied as three ear drops every day twice a day for three weeks. Ear drops without growth factors are applied to the contralateral ear for each mouse. The animals are examined by visual otologic exam weekly for complete closure of the perforation for a total of one month. Progression of healing is also recorded.

Animals are sacrificed and the tympanic membrane is harvested for histology and mRNA studies.

Animals treated with ear drops containing EFG + TGF-\(\alpha\) appear to heal more quickly than either growth factor applied singly.

**EXAMPLE 5. TREATMENT OF TYMPANIC MEMBRANE PERFORATION IN AN ANIMAL MODEL USING GROWTH FACTORS ON ALLODERM**

A biocompatible scaffold such as allograft can be used as a matrix onto which EGF, TGF-A, and PDGF can be incorporated.

The animals are prepared as discussed in Examples 1 and 4. The animals are anaesthetized, as previously described in Example 1, and the perforation edges are roughened. The patch or allograft comprising growth factors, EGF, TGF-\(\alpha\), PDGF, EFG + TGF-\(\alpha\), EGF + PDGF, TGF-\(\alpha\) + PDGF, or EGF + TGF-\(\alpha\) + PDGF, is placed over the perforation. For treatment of acute tympanic membrane perforation, the patch is applied two days after surgery is performed. For treatment of chronic tympanic membrane perforation, the patch is applied 6 weeks after surgery is performed. Ear drops are applied, which will solubilize the growth factors embedded.
in the matrix of the patch. Through capillary action, these growth factors will be absorbed by the leading edges of the perforated membrane while minimizing contamination of the middle ear cleft with the growth factors possibly leading to middle ear mucosa proliferation. The ear drops are added every day twice a day for a period of up to three weeks. The animals are examined by visual otologic exam weekly for complete closure of the perforation for a total of one month. Progression of healing is also recorded.

Various references are cited herein, the contents of which are hereby incorporated by reference in their entireties:


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It will be understood that the present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying Figures without departing from the scope and spirit of the invention.
What is claimed is:

1. A method of promoting healing of tympanic membrane perforation in a subject in need of such treatment, comprising administering to the subject a composition comprising an effective amount of one or more growth factor for promoting healing of tympanic membrane perforation, wherein the growth factor is selected from the group consisting of EGF, TGF-α and PDGF.

2. The method of claim 1, further comprising, prior to administering the composition, applying a biocompatible scaffold over the tympanic membrane perforation.

3. The method of claim 2, wherein the biocompatible scaffold comprises allograft.

4. The method of claim 1, wherein the step of administering the composition further comprises administering from about 1 microgram to about 50 micrograms growth factor per day.

5. The method of claim 1, wherein the step of administering the composition comprises administering from about 10 micrograms to about 50 micrograms of EGF per day.

6. The method of claim 1, wherein the step of administering the composition comprises administering from about 1 microgram to about 5 micrograms of TGF-α per day.

7. The method of claim 1, wherein the step of administering the composition comprises administering from about 1 microgram to about 5 micrograms of PDGF per day.

8. A method of promoting healing of tympanic membrane perforation in a subject in need of such treatment, comprising applying a biocompatible scaffold over the tympanic membrane perforation, the biocompatible scaffold incorporating a composition comprising an effective amount of one or more growth factor for promoting healing of tympanic membrane perforation, wherein the growth factor is
selected from the group consisting of EGF, TGF-α and PDGF.

9. The method of claim 8, further comprising, applying an agent to the biocompatible scaffold to release the growth factor.

10. The method of claim 9, wherein the agent is selected from the group consisting of water, ofloxacin, and ciprofloxacin.

11. The method of claim 8, further comprising, prior to the step of applying a biocompatible scaffold over the tympanic membrane perforation, roughening the edges of the tympanic membrane perforation.

12. The method of claim 8, wherein the biocompatible scaffold comprises allograft.

13. The method of claim 8, wherein the biocompatible scaffold incorporates a composition comprising from about 10 microgram to about 5 milligrams growth factor.

14. The method of claim 13, wherein the biocompatible scaffold incorporates a composition comprising from about 10 micrograms to about 50 micrograms of EGF.

15. The method of claim 13, wherein the biocompatible scaffold incorporates a composition comprising from about 1 microgram to about 5 micrograms of TGF-α.

16. The method of claim 13, wherein the biocompatible scaffold incorporates a composition comprising from about 1 microgram to about 5 micrograms of PDGF.