TREATMENT OF CHRONIC POSTOPERATIVE OTORRHEA WITH CULTURED KERATINOCYTE SHEETS

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Cultured allogeneic ear keratinocyte sheets were used to treat 26 ears presenting with long-standing (average 37 months) chronic otorrhea, resistant to regular treatment, long after surgery for atresia (n = 8), cholesteatoma (n = 10), and chronic otitis media (n = 8). Complete epithelial healing and cessation of otorrhea were obtained in 18 cases (69%), following an average of 2.2 weekly applications. Temporary epithelial healing lasting at least 3 months was observed in 3 patients (12%) subsequently needing repeated applications. Lack of complete epithelialization was documented in 5 cases (19%). In 3 of those 5 cases, a reason could be determined. The authors speculate that the allocultured keratinocytes are able to promote migration and proliferation of resident cells at the wound edges, despite their short survival time, by release of keratinocyte-stimulating factors.

KEY WORDS — cytokine, growth factor, keratinocyte, middle ear surgery, otorrhea, tympanoplasty.

INTRODUCTION

Chronic otorrhea from operated ears remains a troublesome complication, most often seen after radical mastoid surgery[1] or surgery for atresia,[2] but occasionally also after routine tympanoplasty.[3]

Epithelialization of the tympanic graft and (if opened) of the mastoid cavity is usually observed within the first 2 to 3 months after surgery, antibiotic ear drops being given to prevent secondary infection of the exudate and of the epithelial and mesenchymal outgrowths originating from the wound edges. A delayed epithelialization can lead to superinfection and granulation formation, which further prevents epithelial healing. Those ears are most often treated on an outpatient basis by regular otomicroscopic suction cleaning and instillation of topical ear drops after removal or cautery of granulation tissue. This treatment has a high rate of controlling otorrhea; only resistant cases require additional modes of treatment.

Autologous split-thickness skin grafts have been most often used as they are readily available, eg, behind the ears, and give immediate coverage. However, they present major disadvantages: the thickness of the graft prevents good sound transmission, the graft removal requires a small intervention under local anesthesia, and above all, the grafts present a tendency to excessive desquamation. In addition, the loss of the unique property of self-cleansing by migration of the epithelial cells in the depth of the outer ear canal can lead to the accumulation of debris and enhances the risk for epidermoid cyst formation.

The use of cultured keratinocyte layers in the treatment of severe burn wounds and of chronic leg ulcers has been extensively investigated by several centers.[4-9] At the Brussels Burn Center,[4] large burn wounds are now routinely covered not only with autologous meshed split-thickness skin, but also with superimposed sheets of autologous or allogeneic cultured keratinocytes. The epithelialization of the interstices of the meshed skin was thereby found to be accelerated twofold as compared with wound beds covered only with meshed autograft. This twofold stimulation of epithelialization was documented with both autologous and allogeneic keratinocyte cultures.[4]

In 1991 and 1992 we treated ears presenting continuous and resistant otorrhea with autologous cultured skin transplants taken from the depth of the contralateral ear canal. However, culture proved successful in only 2 of the 11 selected patients. The constant fear of damaging the healthy ear often resulted in specimens too small for culture, and they were often contaminated. Also, the long waiting period (3 to 4 weeks) required for expansion of

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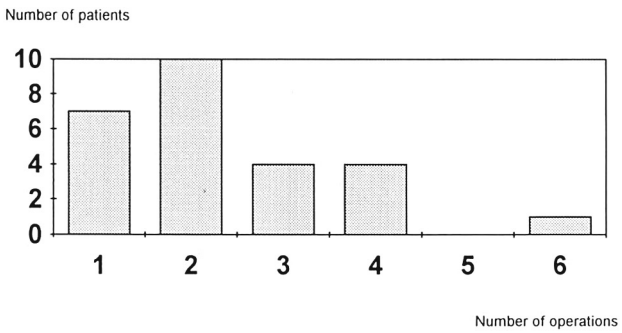

Number of operations undergone by patients prior to keratinocyte treatment.

keratinocytes to obtain multilayer keratinocyte sheets ruled out efficient treatment. In case the graft did not take, the procedure was also difficult to repeat. Since the results of burn wounds treated with allograft cultures were similar to those of wounds treated with autograft cultures, we decided in 1993 to switch to allogeneic keratinocyte cultures. For practical reasons also, allografts are preferred: no waiting period is required for culture, and the cryopreserved grafts are available at all times.

PATIENTS AND METHODS

Patient Selection and Population. A total of 26 patients were admitted to the study. The age of the patients averaged 33.4 years, ranging from 7 to 60 years. All 26 patients had undergone previous surgery for cholesteatoma (n = 10), noncholesteatomatous otitis media (n = 8), or atresia (n = 8). The number of operations per patient varied from 1 to 6 (see Figure).

The technique used at the last surgical intervention to try to definitively solve the problem of otorrhea was either a closed one (20 cases) or an open one (6 cases). The mean duration between the onset of otorrhea after the last surgery and the new treatment modality averaged 37 months (range 3 months to 22 years). Fourteen patients had previously been followed for more than 1 year in our outpatient clinic, and many treatment modalities had been tried to stop the otorrhea and to improve the epithelialization.

To make sure the continuous otorrhea was not due to improper, unsatisfactory, or irregular treatment, we accepted only ears that for 3 months had been intensively treated by thorough suction cleaning, removal of granulation tissue, and application of trichloroacetic acid (15%) every 1 or 2 weeks according to the severity of the discharge. Daily instillation of antibiotic ear drops was carried out by the patients. Only ears that failed to heal after this intensive treatment were enrolled in the study and served as internal controls to be compared (after the additional 3 months of intensive therapy) with the same ears treated with use of keratinocyte sheets.

The authors excluded all ears in which the ear canal or mastoid cavity was not well isolated from the mesotympanum: tympanic perforations, uncontrollable retraction pockets, or active cholesteatomatous disease. Although the application of epidermal growth factor on perforations does not seem to induce cholesteatoma formation in animal studies,[10] we decided for safety reasons to exclude ears presenting a communication with the mesotympanum. We excluded also those ears in which a bony bare surface was present in the mastoid cavity or ear canal, because a vital wound bed is essential for epithelial overgrowth.

In Vitro Culture of Keratinocytes. Keratinocyte cultures were established from myringectomy specimens obtained from donor patients who needed a tympanoplasty procedure because of noncholesteatomatous chronic otitis media. At the time of tissue donation, blood was tested and was found negative for hepatitis B virus, hepatitis C virus, human immunodeficiency virus 1 and 2, syphilis, and cytomegalovirus. Since, in our department, tympanoplasty is usually performed with tympano-ossicular homografts, a total myringectomy is carried out with conservation of the epithelial lining in the depth of the ear canal.[3] The eardrum and the annulus were immersed in a transport medium containing penicillin G sodium and streptomycin sulfate and sent within 12 hours to the laboratory. Keratinocyte culturing was based on the technique of Rheinwald and Green.[11] The dermis was separated from the epidermis by incubation of the specimen in trypsin and collagenase in order to remove the fibrous elements of the eardrum. The obtained suspension of primary keratinocytes was centrifuged and the pellet was brought again into suspension with 10 mL of culturing medium. Those primary keratinocytes were seeded in a 25-cm^2 tissue culture flask on a feeder layer of 300,000 mitomycin C–treated Swiss 3T3-fibroblasts. The culture medium was changed every 2 days and the culture was observed daily for the appearance of keratinocyte clones. Fibroblasts were washed away with a solution of 0.2% trypsin (Life Technologie, Belgium) and 0.02% ethylenediaminetetraacetic acid (Sigma, NTL, Belgium) in phosphate-buffered saline. After confluence the cells can be picked up for serial subculture up to passage 4 (with a 1:3 split ratio at each passage). Of the 49 tympanic membranes brought in for culture, only in 3 cases could subculture be reached up to passage 3 (n = 2) or 4 (n = 1). In 34 cases the culture became contaminated. In 5 cases no growth was observed and in 6 cases the human fibroblasts overgrew the keratinocyte cells. A total of 48 passage 3 and 23 passage 4 sheets (each 25 cm^2) were produced

PATIENT DATA

Patient No.	*Age (y)*	*No. of Operations*	*Indication for Surgery*	*Last Technique Used*	*Duration of Otorrhea (mo)*	*No. of Applications*	*Follow-up Duration (mo)*	*Result*
1	7	1	Atresia	C	3	4	11	±
2	33	4	COM	C	6	1	11	+
3	38	2	COM	C	60	4	22	±
4	52	1	COM	C	6	1	8	+
5	25	1	Atresia	C	9	6	11	–
6	32	3	Cholest	C	12	4	11	+
7	47	4	Cholest	C	12	3	6	+
8	9	2	Atresia	C	48	2	10	+
9	34	2	Cholest	C	8	2	6	+
10	53	2	Cholest	O	72	11	14	–
11	16	2	COM	C	14	2	14	+
12	19	2	Atresia	C	9	2	12	+
13	32	1	Atresia	C	4	10	14	±
14	38	6	Atresia	O	260	2	10	–
15	15	2	Cholest	O	9	1	12	+
16	36	3	Cholest	O	6	2	7	+
17	52	3	Cholest	C	21	2	11	+
18	58	4	COM	C	60	3	9	+
19	43	3	COM	C	132	2	12	–
20	60	2	Cholest	O	5	3	15	+
21	44	1	COM	C	4	2	6	+
22	21	2	Atresia	C	48	2	9	+
23	33	4	Cholest	O	84	3	5	+
24	9	1	Atresia	C	3	2	11	+
25	23	1	COM	C	38	5	12	–
26	40	2	Cholest	O	36	3	7	+

COM — chronic otitis media, Cholest — cholesteatoma, C — closed, O — open, ± — temporary success, + — success, – — failure.

with the 3 successful subcultures. Upon confluence of the final passage, cultures were allowed to stratify into multilayered sheets for an additional week, in order to allow easier manipulation of the keratinocyte sheets. The flask was opened by the use of a heating wire and the confluent keratinocyte layer was detached by means of 10 mL of Dispase (2.5 μg/mL, Boehringer Mannheim, Belgium). A carrier gauze (N-terface, Hospithera, Belgium) was put on top of the keratinocytes and the borders were folded back onto this plastic gauze. The culture could then be taken out of the tissue flask for storage in an aluminum bag and was processed through a cryopreservation program and stored in liquid nitrogen. This ensures permanent access to frozen sheets in the outpatient otolaryngology clinic. After thorough suction cleaning of the ear, the keratinocyte sheet was thawed and applied in one or several pieces into the depth of the ear canal or mastoid cavity. On an irregular mastoid surface, small pieces were cut and arranged as a patchwork to obtain a close contact with the surface to be covered. The patient was sent home with Bacicoline-B (Chibret) ear drops containing colistimethate sodium 20 mg/mL and bacitracin 8 mg/mL, which were found to be noncytotoxic for the keratinocyte cultures. One week later, the plastic carrier sheet was removed and a new keratinocyte layer was applied if healing was not complete. The ear was controlled every week and reapplication was performed until complete epithelialization and cessation of otorrhea were reached. Follow-up was thereafter secured by monthly assessment. The maximum number of applications was fixed at 6. Persisting otorrhea was considered a failure.

RESULTS

A total of 84 applications were performed in our 26 patients (see Table). The average number of previous operations (mostly elsewhere) was 2.4. Eight ears had previously been operated on for chronic otitis media, 8 for atresia, and 10 for cholesteatomatous disease. The last technique used was a closed one in 19 cases and an open radical cavity in 7. Six of the 10 cholesteatomatous ears had been treated by the open technique. The duration of chronic otorrhea before the keratinocyte grafting averaged 37 months (range 3 months to 22 years). The average number of applications per patient was 3.2 (range 1 to 11). The av-

erage follow-up time was 9.8 months (range 6 to 22 months). Complete epithelialization and cessation of discharge was obtained in 18 of the 26 cases (69%). In those successful cases, an average of 2.2 applications proved necessary to dry the ear. In those ears progression of healing in the depth of the outer ear canal, eardrum, and/or mastoid cavity was observed by weekly otomicroscopic examination and was characterized by a progression of the epithelial front, with simultaneous regression of the exudating granulation area. The otorrhea most often disappeared after 2 applications, requiring in some cases a third one to obtain a totally closed and epithelialized surface. In the first weeks, neovascularization by capillary sprouting was often observed under the new epithelial layer and was gradually followed by a decrease in capillary proliferation and an increase in subepithelial (probably fibroblastic) organization.

A temporary epithelialization with repeated need for reapplication after a symptom-free period of 3 months was observed in 3 patients (12%). Absence of epithelialization with uninterrupted otorrhea meant failure: this was found in 5 patients (19%). An explanation for partial failure with temporary cessation of otorrhea could be found in 2 of the 3 cases. Case 1 was a bilateral atresia patient wearing, all day long, an air conducting hearing aid closing off the ear canal, thereby causing maceration and superinfection. Case 13, also an atresia patient, presented a too-narrow, almost stenotic, meatoplasty.

In 3 of the 5 cases a reason for total failure could be found. Case 15, with a large mastoid cavity, presented uninterrupted granulation formation, despite thorough, repeated granulation removal, cleaning, and several keratinocyte applications. During subsequent revision surgery, a large foreign body granuloma was found overlying a piece of the surgical packing left behind by the previous surgeon. Case 10, with an open cavity, presented residual mastoid cells with areas covered by respiratory mucosa (as proven by biopsy) causing recurrent discharge during upper respiratory tract infections. This patient is waiting for revision surgery. In case 23, a closed technique for chronic otitis media had been used, but after a trouble-free healing period the graft started to present a chronic granulomatous myringitis in the anterior half of the eardrum. After unsuccessful treatment including allograft sheets, the ear was reoperated and an edematous "sick" mucosa was found filling the whole tympanic cavity. After thorough removal of this hyperplastic mucosa and myringoplasty with allograft tympanic material, a dry eardrum was finally achieved. In 2 of the 5 failed cases no reason for lack of epithelial healing could be disclosed.

DISCUSSION AND CONCLUSION

The three subgroups in our study population are mastoid cavities, tympanoplasties, and operated atretic ears. All the studied ears had in common the absence of epithelial coverage in the depth of the outer ear canal or mastoid cavity. It has long been known that the epithelium of the ear canal presents the unique ability to migrate.[12-14] The absence of this self-cleaning mechanism and the permanent fluid exudation from uncovered areas leads to fluid stagnation and accumulation of debris, which form the ideal culture medium for bacteria. Once infection sets in, granulation tissue develops, which further impedes epithelial healing. By suction cleaning and instillation of antibiotic ear drops, this vicious circle can often be interrupted and spontaneous epithelial healing can take place. However, in some cases the epithelial repair mechanism seems to be defective or too slow.

The reported incidence of intermittent or persistent otorrhea following open cavity mastoidectomy varies from 20% to 60%.[1] This is one of the reasons why, in our department, we seldom perform an open technique for eradication of cholesteatoma, the open cavity technique being reserved for giant primary cholesteatoma or recurrent, rapidly extensive cholesteatoma.

All 6 discharging cavities presented a proper low facial ridge, a large meatus, and a middle ear isolated from the cavity. In 5 cases a dry cavity was obtained after an average of 2.4 applications. In 1 case, uninterrupted otorrhea compelled us to perform revision surgery, which revealed some of the surgical packing left behind by the previous surgeon. Cell grafting seems, therefore, to be a good treatment modality of last resort before revision tympanoplasty or mastoid surgery is undertaken.

Reconstructive surgery for atresia remains a much-debated subject — even more so with the advent of bone-anchored hearing devices.[15] We now operate only on cases with favorable anatomy as revealed by computed tomography scan, where after creation of a new ear canal and tympanic membrane a good hearing result can be expected. In those cases, a canalplasty with tympano-ossicular allografting is preferred because of the proven better long-term results as compared with the classic large cavity techniques, which often present a granulomatous covering of the cavity. But even with the allograft technique, when the extraconchal nonmigratory skin does not grow over the graft, the tympanic membrane and the depth of the newly created canal may be covered, in 14% of the cases by a granulomatous and often suppurative layer, as reported by Marquet and Declau.[2] In 4 of our 7 granulating ears operated on by the Marquet tech-

nique,[2] complete healing was reached. Two were temporarily dry; 1 continued to discharge.

In our experience, chronic granulomatous myringitis rarely complicates treatment of chronic otitis media by closed technique tympanoplasty: between 1% and 2% of cases.[3] After every myringotympanoplasty, a competition sets in between the natural tendency of a graft to necrosis and the epithelial coverage and fibrosis of the autologous or allogeneic scaffold material.[16] All factors delaying the natural epithelial coverage can lead to troublesome granulomatous myringitis. It is generally due to excessive removal of skin from the depth of the external auditory canal, to improper postoperative follow-up, or to premature postoperative contact with water, such as by swimming.

Although most cases studied here have been followed and treated for months or even years, only ears that were intensively treated in the outpatient department for another 3 additional months and failed to heal were brought into the study.

With regard to the long-standing and often crippling otorrhea that all 26 patients presented prior to grafting cultured keratinocytes, these allografts did provide a long-term epithelial repair in a high percentage of ears (69%). Similar results were obtained by Premachandra et al[17] using keratinocyte autografts. They reported in 17 of their 26 cases (65%) a complete epithelial healing without evidence of recurrence of granulation tissue after a follow-up period ranging from 10 to 18 months. All were mastoid cavities. In their study population, partial success with only temporary improvement in aural discharge was found in 7 cases (27%), whereas in 3 patients (8%) the discharge continued unabated after grafting. They used for culture a full-thickness retro-auricular autologous skin biopsy. This technique has the disadvantage that the original migratory epithelium is replaced by nonmigratory cells, which can prevent migration of surrounding migratory cells and in the long run lead to accumulation of debris. On the other hand, however, there is a real take and survival of autologous cells, which is not the case for allogeneic cells.

Up to now, tympanic and annular keratinocyte culture has been mainly used as a research tool in order to compare the normal in vitro keratinocyte migration pattern with that of cholesteatomatous keratinocytes.[18] In this study, cultured "ear" keratinocytes were used for the first time for therapeutic means.

The observed enhancement of epithelial coverage by these cultured allograft keratinocytes is not due to a permanent take of the grafts, as shown by several studies on burns and chronic ulcers, but has generally been attributed to an indirect stimulation of the recipient's repair mechanisms.[4-9,19,20] Several groups have shown that in burn wounds the survival time of these allocultured grafts is limited, and that they are gradually replaced by autologous cells.[19,21] Initially, some authors claimed that these cultured allografted cells were not rejected, because Langerhans cells, regarded as critical in the process of skin graft rejection, are lost after 7 or more days in keratinocyte cultures.[19] More recent studies using human leukocyte antigen analysis,[22] Y chromosome analysis[21] in sex-mismatched grafts, and DNA fingerprinting techniques[19] have shown that host keratinocytes replace the allograft. This suggests that these viable allografts do not remain in situ permanently. Therefore, the observed wound repair–promoting effects are explained not by a grafting process per se, but by the stimulation of the endogenous wound repair mechanisms by the allografted cells. The release of growth factors during autolysis of the cells probably induces wound closure by stimulating the migration and proliferation of resident epithelial cells present at the wound edges.

Indeed, numerous growth factors and cytokines have been reported to be synthesized by epidermal cells. These include TGF-α, a-FGF, b-FGF, PDGF, TGF-β, amphiregulin, HB-EGF, NGF, TNF-α, IL-1α, IL-1β, IL-6, IL-8, GM-CSF, G-CSF, and M-CSF.[4,10,18-20,23-25] Many of these factors have important effects on one or more stages of the wound healing process. TGF-β, the interleukins, and the colony stimulating factors are powerful modulators of the postwounding immune response. Angiogenesis is stimulated by the fibroblast growth factors. Fibroblast proliferation is promoted by PDGF, FGFs, and EGF-like factors, and the deposition of extracellular matrix is promoted by TGF-β.[4] Both FGFs and EGF-like growth factors are important keratinocyte mitogens, and may promote wound closure by stimulation of keratinocyte proliferation and migration.[25] Several animal studies have shown the effect of these two keratinocyte stimulating factors on acute or chronic tympanic membrane perforations. Animal studies by Lee et al[10] with recombinant epidermal growth factor (EGF) showed a significantly higher rate of closure of induced chronic perforations as compared to controls. Also, those EGF-healed tympanic membranes were found to be histologically similar to normal tympanic membranes, as opposed to the few spontaneously healed tympanic membranes from the control groups, where the thickness was only half that of normal tympanic membranes.

In acute perforation in rats, Mondain and Ryan[23]

also showed similar accelerated healing, but with basic fibroblast growth factor (b-FGF). The histologic results of these healed eardrums demonstrated that b-FGF can produce a tympanic membrane scar containing more connective tissue, which may be of benefit in the prevention of atrophic tympanic membranes. Histologic studies of neomembranes in human eardrums by Govaerts et al[26] have clearly shown that the lamina propria can be atrophic, with fibroblasts absent, and with disoriented fibers.

In conclusion, our study confirms some of the current views on the biologic mechanism by which allocultured keratinocytes, despite their short postgrafting survival time, are able to promote the wound healing process, most probably by the release of growth factors. It is, however, still unclear which growth factors are involved, although we have detected b-FGF in keratinocyte extracts (unpublished results).

What is the level of activity of these cultured keratinocyte sheets compared with growth factors? Recently, Duinslaeger et al[4] showed that the total amount of mitogenic activity in the keratinocyte cell extract from a 175-cm^2 culture is equivalent to the activity produced by 40 to 80 μg of EGF. This implies that the amount of activity used in the present study (about 2 cm^2 per application) is very low as compared with the microgram amounts usually used in in vivo studies. This may support the growing awareness that growth factor therapy will probably only be maximally effective if multiple factors, which are only marginally effective upon isolated use, are combined. Since the different factors may have different activities or target cell specificities, such combinations may be able to modulate the different stages of the healing process more effectively than any single factor.

The future will tell us which of the options contains the greatest therapeutic value: the bulk administration of all intracellular components (as with cultured cells), or the selective, but possibly marginally effective, application of a single growth factor.

ACKNOWLEDGMENT — We thank Dr Alain Vanderkelen, Head of the Burn Center, Military Hospital, Brussels, for encouraging the collaboration between the two centers, and for his advice.

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