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The behaviour of tympanic membrane perforations in tissue culture: a scanning electron microscopic study

ERLING GRØNVOLD OLSEN, M.D., PH.D.,* LARS-ERIC STENFORS, M.D., PH.D.† (Tromsö, Norway)

Abstract

The effects of keeping rat tympanic membranes with an artificially made pars tensa perforation in tissue culture were observed under a scanning electron microscope. After one day and onwards, spreading and thickening of the keratinizing, outer squamous epithelium (OE) was noted. In addition, ballooning of the innermost cells of the outer epithelium apposing the inner tympanic epithelium (IE) was seen. No appreciable reaction was noted in the connective tissue layer of the drum. The inner tympanic epithelium appeared to be swollen, containing spherical structures in the cytoplasm, especially close to the area of contact with the outer meatal epithelium. No complete cover of the drum defect was seen after 14 days of tissue culture. Hyperplasia and spreading of the keratinizing, outer squamous epithelium of the drum is not sufficient to achieve covering of a drum perforation and complete healing cannot take place unless supported by granulation tissue formation.

Key words: Tissue culture; Tympanic membrane; Wound healing; Ear, middle

Introduction

Both the macroscopic and the microscopic anatomy of the rat tympanic membrane (TM) is well documented (Shrapnell, 1832; Stenfors *et al.*, 1984; Albiin *et al.*, 1985). The principal, conically-shaped, structure – the pars tensa – consists of five different layers. On the outer meatal side there is an epidermal layer of keratinizing, stratified squamous epithelium, two to three cells in thickness (outer epithelium: OE). Underlying the epithelium there is a thin layer of connective tissue. Thereafter follows a dense layer consisting predominantly of collagen fibres arranged in a typical pattern of inner circular and outer radical fibres. Finally, facing the middle ear cavity there is a thin layer of loose connective tissue covered by a single layer of flattened epithelial cells (inner epithelium: IE).

Although the manner of healing of traumatically induced pars tensa perforations has been extensively studied (Taylor and McMinn, 1965; McMinn and Taylor, 1966; McIntire and Benitez, 1970; Reijnen and Kuijpers, 1971; Clawson and Litton, 1971; Boedts, 1978; Stenfors *et al.*, 1980), the opinions differ about the cytological healing pattern. Thus some authors suggest primary epithelial closure, whereas others (Taylor and McMinn, 1965) have described a primary covering of the defect by granulation tissue, followed by epithelial migration.

In a recent study concerning the healing of pars flaccida perforations in tissue culture (Stenfors *et al.*, 1990), we found that no complete covering of a pars flaccida perforation took place unless accompanied by granulation tissue formation.

The purpose of the present study was to evaluate whether or not pars tensa perforations in tissue culture could heal as observed by scanning electron microscopy (SEM). Particular attention was paid to the interaction between the two epithelia of the drum, which meet at the edge of the perforation. The findings are discussed in relation to earlier *in vivo* studies on healing mechanisms of experimentally induced drum perforations.

Materials and methods

Thirty-six healthy, male Sprague-Dawley rats were used, and sacrificed by means of CO_2 inhalation then the temporal bones were removed. The tympanic membranes with the bony rim, were excised and, using a myringotomy lancet, a small perforation was made in the upper posterior quadrant. The drums were immediately placed in tissue culture dishes containing 10 ml of tissue culture medium: RPMI 1640, supplemented with penicillin (100 IU/ml), streptomycin (500 µg/ml), and 10 per cent inactivated foetal calf serum (GIBCO, Renfrewshire, Scotland). The dishes were incubated at 37°C, in an atmosphere of five per cent CO_2 and air in a moist environment. The tissue culture medium was changed every third day. After various intervals (0, 1, 2, 3, 5, 7, 10, 12 and 14 days) four randomly chosen drums were examined under the operating microscope, fixed in McDowell's solution (one per cent glutaraldehyde and four per cent formaldehyde in phosphate buffer) for 18–24 hours, and then post-fixed for 1.5 h in one per cent OsO_4.

For SEM studies, the fixed specimens were dehydrated in increasing concentrations of ethanol, and thereafter dried by the 'critical-point' drying procedure using CO_2. The specimens were mounted on blocks, conventionally

From the Department of Ophthalmology,* University of Tromsö, Tromsö, Norway and the Department of Otolaryngology,† Central Hospital of Keski-Pohjanmaa, Kokkola, Finland.
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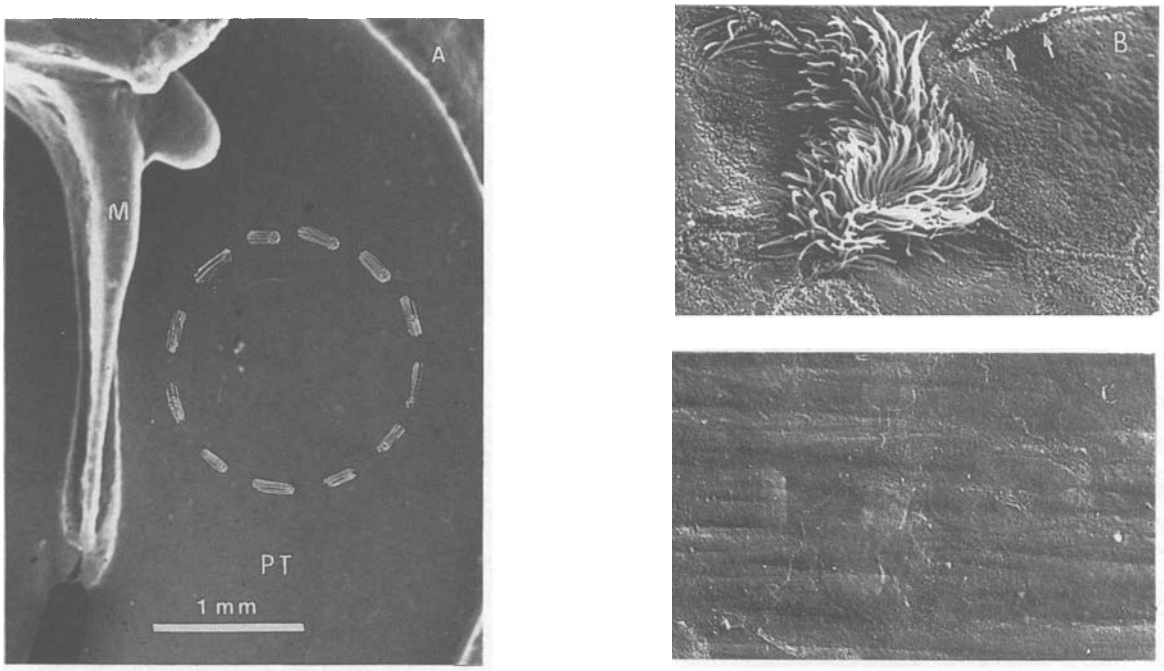

FIG. 1

Scanning electromicroscopic (SEM) pictures showing: (A) the tympanic side of the upper, posterior quadrant of a normal untreated pars tensa (PT) and handle of the malleus (M). Dotted line shows the extent of the perforation. (× 25); (B) at the rim of the pars tensa, scattered ciliated cells can be seen among the nonciliated epithelial cells. Abundant microvilli (arrows) are evident at the cell borders. (× 3055); (C) central portion of the keratinizing, outer squamous epithelium where radiating collagen fibres are visible. (× 975).

splutter-coated with gold, and studied in a JEOL JSM-840 scanning electron microscope.

Results

Under the scanning electron microscope (SEM) the tympanal side of the drum appeared to be covered by flat, predominantly non-ciliated cells with a distinct cell border (Figure 1 a, b). There were, along the cell borders, numerous microvilli present. The nucleus was protruding. Close to the bony rim, scattered ciliated cells were visible (Figure 1b). The outer, meatal side of the TM was covered by an irregularly-arranged keratin layer. No distinct cell borders could be seen. The keratin layer of the central portions of the pars tensa was so thin that the radiating collagen fibres of the drum could be discerned (Figure 1c). The smooth collagen fibres sloped steeply from the umbo towards the periphery of the drum. The keratin layers increased in thickness and irregularity towards the periphery of the drum.

After *one day* in tissue culture, the drum showed a distinct reaction pattern close to the perforation. The outer meatal side of the drum had thickened noticeably and the outermost keratin layer displayed crater-like structures. In some areas the OE had overlapped the edge forming, even at this stage, contact with the inner tympanic epithelium (Figure 2a). In the tympanic epithelial cells several bleb formations had appeared in the cytoplasm, especially in those epithelial cells that were in contact with the outer, meatal epithelium (Figure 2b).

After *3 to 5 days*, the OE was seen to have increased in thickness at some distance from the perforation. At the periphery, near the bony rim, there was no visible cellular reaction. Several ballooned cells belonging to the outer keratinizing epithelium were seen, apposing the inner tympanic cells (Figure 3). The inner epithelial cells harboured spherical structures in the cytoplasm.

After *7 to 9 days* the proliferation and thickening of the OE layer has reached its peak (Figure 4a). The keratin produced had spread some distance onto the inner tympanic side of the drum (Figure 4b). Several ballooned cells were noted between the outer and the inner epithelia (Figure 4c). Cells completely filled with spherical structures were

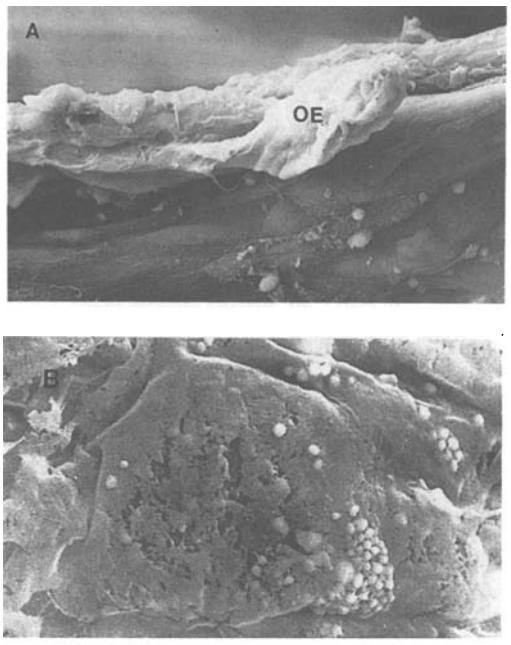

FIG. 2

(A) Perforated drum after 1 day in tissue culture. The outer meatal epithelium (OE) is appreciably thickened and overlaps the perforation edge. (× 265); (B) tympanic epithelial cell harbouring numerous spherical structures. (× 1.625).

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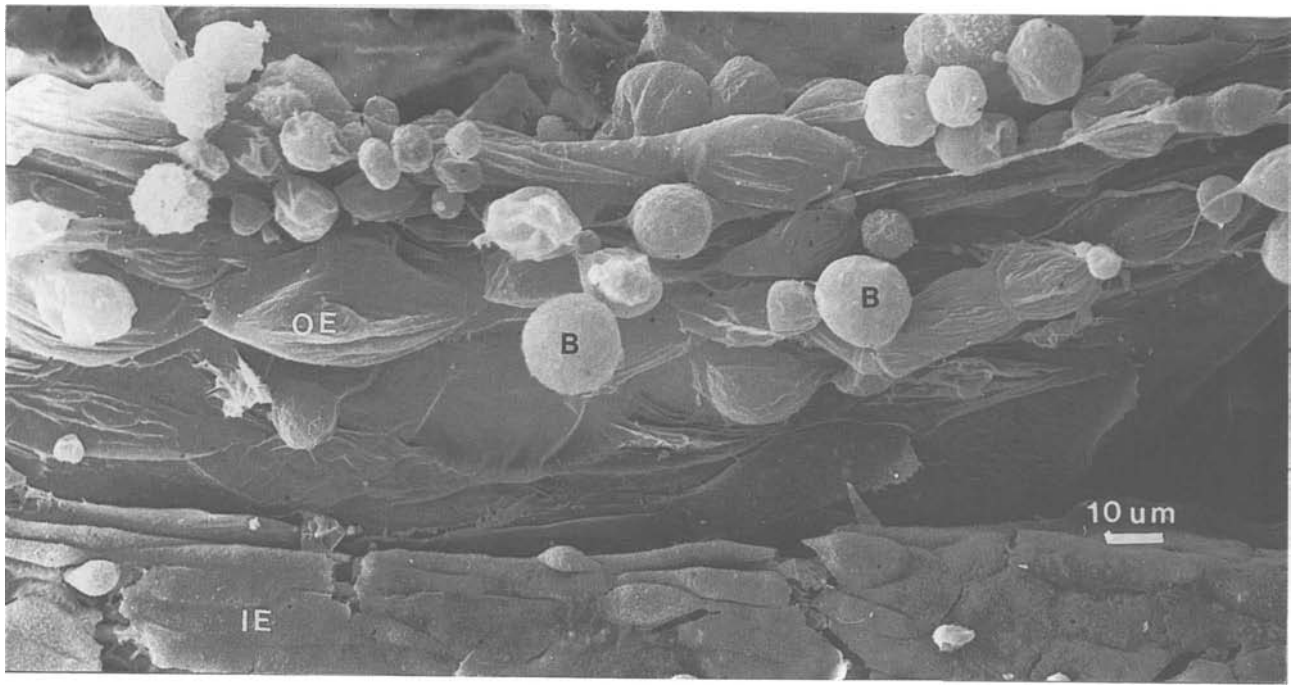

FIG. 3

Perforated drum after 5 days in tissue culture showing ballooning of the cells belonging to the outer meatal epithelium (OE) close to the inner tympanic epithelium (IE). Ballooned epithelial cells (B). (× 580).

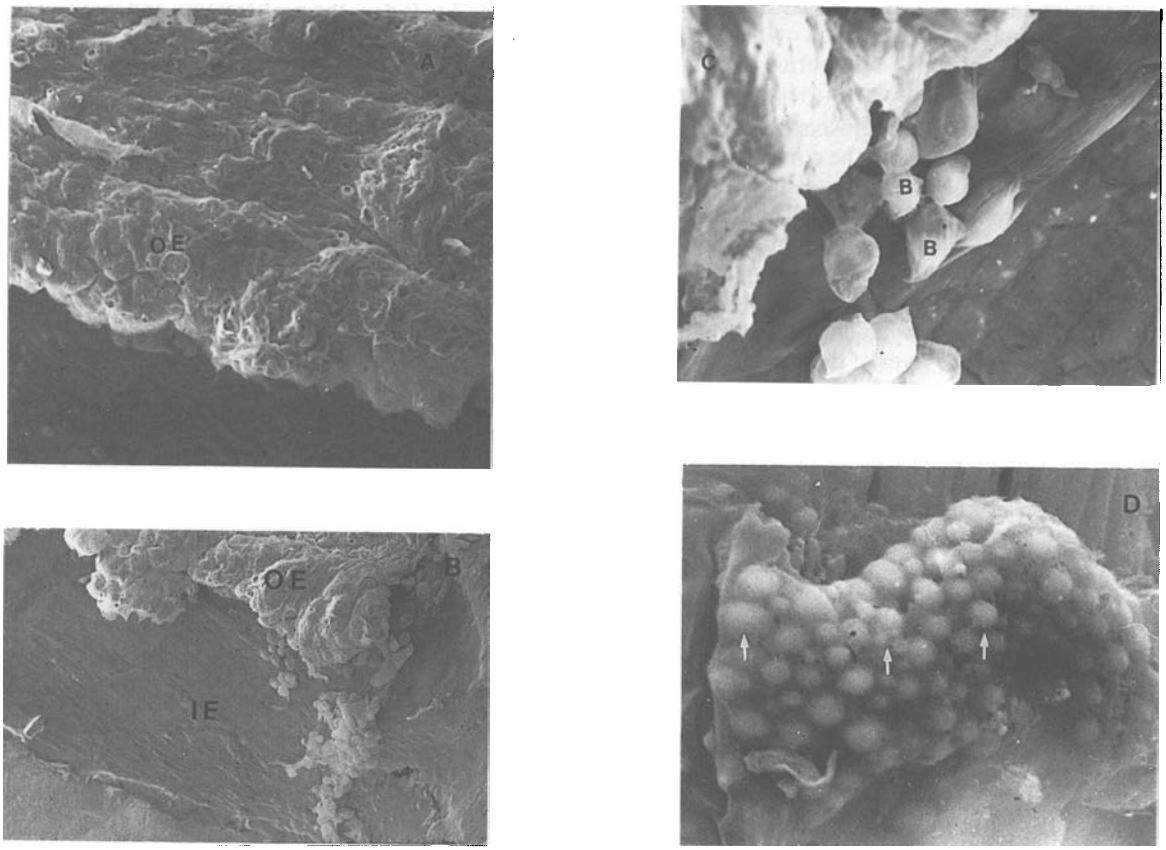

FIG. 4

(A) Perforated drum after 9 days in tissue culture. The outer keratinizing epithelium (OE) is noticeably hyperplastic and thickened. (× 120); (B) The outer keratinizing epithelium (OE) has overgrown the inner tympanic epithelium (IE). (× 112); (C) Ballooned cells (B) belonging to the outer meatal epithelium. (× 560); (D) tympanic epithelial cell with rounded structures (arrows). (× 1.625).

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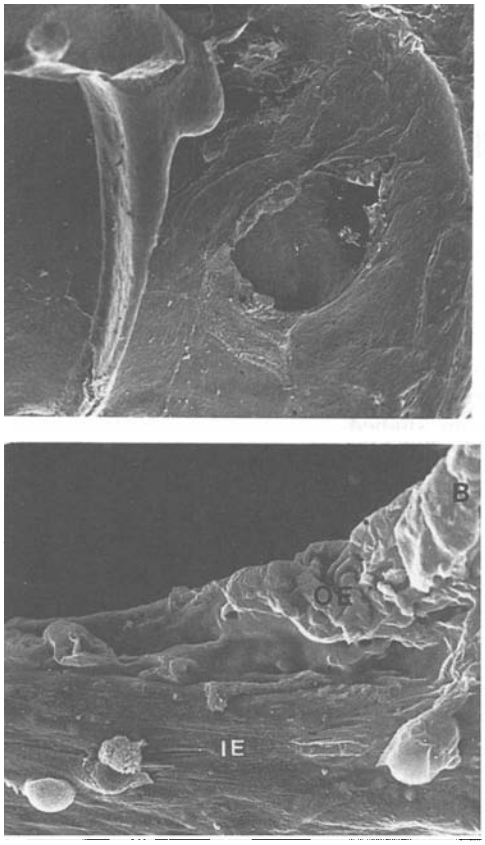

FIG. 5

(A) Perforated drum after 14 days in tissue culture. The perforation (P) is still visible and not completely covered. (× 22); (B) outer keratinizing epithelium (OE) and inner tympanic epithelium (IE). (× 142).

seen in the tympanic epithelium (Figure 4d). All perforations had remained patent.

After *12 to 14 days* in tissue culture, live cells could still be observed at the edges of the perforation. Irregular keratin formation was evident some distance from the perforation, but there was no complete covering of the perforations (Figure 5a, b).

Discussion

A traumatic perforation inflicted to the posterior part of a rat TM, one quadrant in size, heals within 9 to 11 days *in vivo* (Stenfors *et al.*, 1980). Though the perforations in the present *in vitro* study were appreciably smaller, no complete covering of any defect was noted. *In vivo* trials with TM perforations in rats show that initially the gap becomes covered with keratin, which forms a guiding matrix for the healing events (Boedts, 1978; Stenfors *et al.*, 1980). The keratin matrix precedes the formation of granulation tissue, which appears to form a bed for the growing epithelium. The present study has confirmed that the absolute prerequisite for complete covering was the subsequent formation of supporting granulation tissue beneath the proliferating squamous epithelium. Without support from such a bed of granulation tissue, the outer keratinizing epithelium only showed local hyperplasia and thickening in the perforation region, but did not effect complete covering of the perforation.

The present study showed, moreover, that the inner tympanic epithelium reacted by forming distinct spherical structures in the cytoplasm. Such structures were immediately evident in the mucosal cells in direct contact with, or close to, the outer epithelial cells. It cannot be excluded that these cellular reactions represent a significant obstacle to the ingrowth of keratinizing epithelium into the tympanic cavity.

Surgical closure of persistent TM perforations by means of a variety of tissue materials is a generally accepted method. Various materials have been used to cover the TM defect i.e. full-thickness skin, fascia, cartilage with perichondrium, vein, heart valve, hyaluronic acid (Stenfors, 1989; Kristensen, 1992). In addition, trials with artificial collagen for the repair of large drum defects have been performed. However, there is evident difficulty in getting artificial collagen membranes covered with normal epithelium. The present findings indicate that the interaction between epithelium and the underlying connective tissue is of the utmost importance for the healing processes that follow damage to the TM. Tympanic membranes kept in tissue culture induce no inflammatory reactions and no granulation tissue formation can be expected. In consequence, there was only proliferation of the meatal epithelium and some reaction in the tympanic epithelium, but no significant reduction of the defect resulted.

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Address for correspondence:
Professor Lars-Eric Stenfors,
Department of Otolaryngology,
University of Tromsö,
N-9038 Tromsö,
Norway.