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Main Articles

Histochemical, immunohistological and scanning electron microscope analysis of tissue retained on spontaneously extruded ventilation tubes

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Abstract

The aim of this study was to analyse the tissue surrounding prematurely extruded ventilation tubes (grommets). Thirty-one ventilation tubes, including 21 Shah and six Shepherd tubes, that had been extruded naturally into the ear canal were examined. After formalin fixation, material adhering to the tubes was retrieved and processed to paraffin wax. Five tubes were processed for scanning electron microscope (SEM) analysis. Sections were stained using histochemical methods for collagen, keratin and keratohyaline. In addition, the presence of collagen (type I and III), keratin, vimentin, fibronectin, tenascin, factor VIII-related antigen, CD31 and CD45 was tested for by immunohistochemistry. Results showed that all specimens consisted of an acellular fibrous material, oriented in concentric rings parallel to the tube surface that was often associated with small collections of $CD45^+$ inflammatory cells. Two specimens contained collagen that was detectable by histochemical and immunohistological methods. Twelve specimens contained identifiable desquamated epithelial cells containing keratin and keratohyaline. Only one specimen stained positively for connective tissue markers (vimentin, fibrous fibronectin, tenascin) or showed the presence of vascular epithelium. SEM revealed adherent clusters or sheets of plate-like structures, consistent with the presence of epithelial squames, on three of the five ventilation tubes examined. It is concluded that tissue retained on extruded grommets consists of orthokeratinitizing epithelium infiltrated by inflammatory cells, a finding that is consistent with the theory that tube extrusion occurs as a result of squamous epithelial proliferation, medial migration, differentiation and desquamation.

Key words: Middle Ear Ventilation; Epithelial Cells; Immunohistochemistry; Microscopy, Electron, Scanning, Transmission

Introduction

The insertion of ventilation tubes (VT) or grommets in the treatment of otitis media with effusion is one of the most common surgical procedures in children.[1] However, frequently, the tubes are spontaneously extruded into the ear canal before they have fulfilled their useful function and need to be replaced. The length of time the tube remains *in situ* depends on a number of factors including insertion technique, the presence of intercurrent infection and tube design features[2] including tube flange depth and sharpness of angle, and tube length.[3–5] The material used is also important although its influence cannot readily be separated from that of design.[1] Dingle *et al.*,[6] comparing polytetrafluorethylene (PTFE) and titanium tubes of identical dimensions and design, found that PTFE tubes were extruded slightly more quickly than titanium ones. However, it is not clear whether the most important factor influencing duration of intubation is the material itself or its weight. Prendergast *et al.*[7] used computer simulations and finite element analysis to determine the vibrational stresses exerted by titanium and lighter PTFE tubes in the tympanic membrane. The authors predicted that heavier titanium tubes would move less and suggested that they are less likely to stretch the tympanic membrane than PTFE tubes, consistent with their slower extrusion rate.

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The mechanism of extrusion of grommets is still not well understood. Gibb[8] suggested that intubation disturbed the normal pattern of epithelial migration, the tube behaving like a foreign body, interrupting the stream of migratory cells from the centre of the tympanic membrane to the periphery. He suggested that epithelial cells pile up at the side of the obstruction, exerting pressure on it, causing it to tilt and then extrude as epithelial cells advance beneath the inner flange easing it outwards like a foreign body. However O'Donahue[9] showed, by tracking the migration of ink dots placed at different sites on the membrane, that the tubes did not affect the pattern of epithelial migration. He proposed that extrusion occurs as a result of forces exerted on the tube either by the collagen or the elastic fibres of the connective tissue layer of the pars tensa. Bingham and Milroy[10] found that the edges of perforations residual to surgical ventilation tube removal in humans were characteristically hyperplastic and hyperkeratotic with a prominent granular layer, consistent with the observations of Stenfors and Winblad,[11] and proposed that extrusion occurs by chronic hyperplastic and inflammatory processes at the tympanic membrane edge next to the VT, which elevate the tube above the tympanic membrane. Epithelial cell migratory processes may then carry the elevated grommet away.

Tissue retained on prematurely extruded grommets may provide further clues as to the mechanism of extrusion. The aim of this study was to investigate the nature of this tissue using a combination of histochemical, immunocytochemical and SEM techniques.

Materials and methods

Specimens

Naturally extruded VTs (23 Shah, six Shephard, two mini Shah, two Long Armstrong and three T-tube) were obtained from four Birmingham Hospitals. Tubes, including any attached soft tissue, were removed from the external ear in theatre and immediately fixed in neutral buffered formalin for 18–48 hours. Soft tissue, present on 31 VTs, was removed from the tubes and bisected at 90° to the long axis of the tube. Tissues were routinely processed to paraffin and blocked out on their cut surfaces.

Histology, histochemistry and immunohistology

Five micron sections of paraffin-embedded specimens were stained using haematoxylin and eosin, Gram's stain, van Gieson, and phloxine tartrate using standard protocols.[12] Immunohistochemistry was performed using a biotin-streptavidin immunoperoxidase technique (StrAviGen: Biogenex) as previously described.[13,14] Freshly cut 5 µm thick paraffin sections were flattened onto poly-L-lysine-coated glass slides at 56°C for two hours and then left at room temperature overnight. After dewaxing, sections were immersed in 0.1 per cent trypsin (Difco: 1:250 grade, in phosphate buffered saline (PBS)) and agitated for 30 minutes at room temperature. After rinsing in tap water for five minutes the sections were covered with 0.3 per cent H_2O_2 in PBS for 10 minutes to abolish endogenous peroxidase activity. All subsequent procedures, until the overlaying with diaminobenzidine (DAB) reagent, were carried out at room temperature in a humidity box. Sections were then overlaid with normal goat serum (diluted 1:5) for 30 minutes. Excess goat serum was tipped off and the sections were overlaid with optimally diluted primary antibodies (two hours at room temperature; sources, dilutions and specificities of primary antibodies are shown in Table I). After washing, the sections were overlaid with biotinylated goat anti-rabbit/mouse immunoglobulin (diluted 1:75 in buffer containing 10 per cent human plasma; one hour; Multilink, Biogenex), washed and overlaid with peroxidase labelled streptavidin (1:75, one hour; Biogenex). Bound peroxidase was visualized by immersing washed sections in 3,3'-diaminobenzidine reagent (five minutes). Stained sections were lightly counterstained in Meyer's haematoxylin and mounted in

TABLE I
SPECIFICITY, DILUTIONS AND SOURCES OF PRIMARY ANTIBODIES

Antibody/Clone	Specificity	Tissue reactivity	Dilution	Source
MNF116	45–65.5kD keratins inc. 5, 6, 8, 17	Epithelial cells	1/200	Dako
C-11 + PCK-26 + CY 90 + Ks-1A3 + M20 + A53-B/A2	Pan keratin	Epithelial cells	1/1000	Dako
Rabbit antibody (2150–0100)	Collagen I		1/600	Biogenesis Ltd
HWD1.1	Collagen III	Connective tissue extracellular matrix proteins	1/600	Biogenex
FN568	Fibronectin		1/100	Novocastra
BC-24	Tenascin		1/2000	Sigma
3B4	Vimentin	Mesenchymal cells	1/100	Boehringer Mannheim Biochemica
Rabbit antibody	Factory VIII-related antigen	Endothelial cells	1/2000	Dako
JC/70A	CD31		1/40	Dako
2B11 + PD7/26	CD45	Leukocyte common antigen (LCA). Inflammatory cells, including neutrophils, macrophages and lymphocytes	1/50	Dako

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XAM. All reagent dilutions and washings were performed in 0.01M phosphate buffered saline (PBS), pH 7.6.

Scanning electron microscopy

Five formalin-fixed tubes (one of each type), after having macroscopic soft tissue removed, were processed for scanning electron microscopy (SEM) to assess the presence of any adherent cells or debris. Tubes were dehydrated through a graded series of ethanol and covered with hexamethyldisilizane that was allowed to evaporate. After air-drying overnight, they were gold sputter coated (Denton Vac Desk II) for 120 seconds before being examined using a JEOL JSM-5300LV SEM operating in high vacuum mode at 30Kv (magnifications between × 35 and × 7500).

Results

Histology

Review of haematoxylin and eosin stained sections showed that 25 specimens were composed of cellular and/or fibrous tissue (Figure 1) while six specimens appeared to consist entirely of fibrin clots (ie. structureless eosinophilic material containing a few inflammatory cells). All the VT-derived specimens could be oriented according to where the VT had been situated because of the presence of a well-defined smooth, curved edge or circular hole. Inflammatory cells, present in 10 of the tissue specimens, were most often found close to where the VT had been, either as focal collections of cells sandwiched between fibrous strands or at the edge of the tissue adjacent to the VT surface (Figure 1(a)). The majority of inflammatory cells were polymorphonuclear leukocytes with only a few mononuclear cells present that were mostly lymphocytes. In a few specimens, most of the inflammatory cells were not within fibrous tissue but were surrounded by eosinophilic fluid exudate.

The fibrous material that made up the bulk of the specimens showed a variable staining reaction with haematoxylin and eosin appearing pink, purple and blue. This variation occurred within, and between, specimens and was not related to the presence of inflammatory cells or proximity to the VT. The fibrous strands were often oriented parallel to the VT surface forming concentric rings and sometimes appeared as an ordered meshwork of fibres. The majority of the VT-associated tissues appeared acellular in that there was little, or no evidence for, the presence of cell nuclei other than those of inflammatory cells. However, many specimens contained flattened cell-like structures that took the

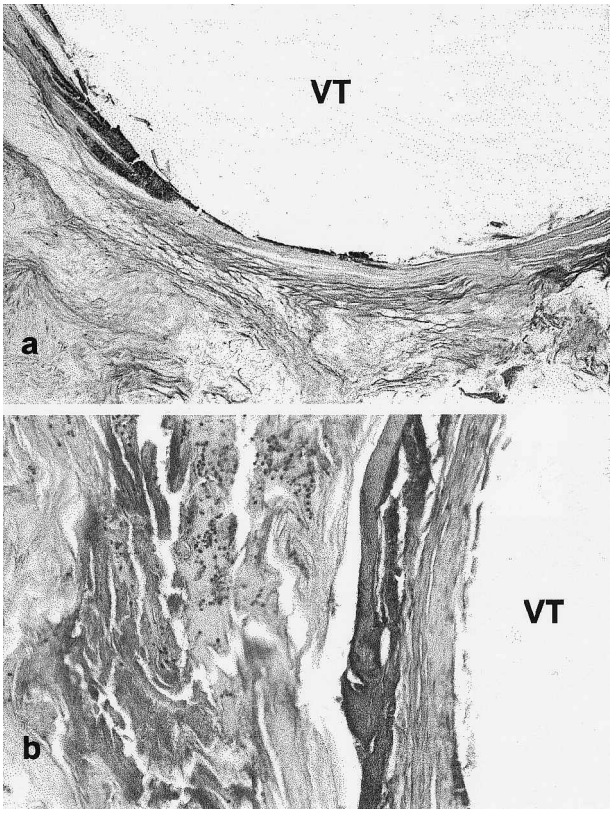

FIG. 1

Histological features of the tissue attached to ventilation tubes. Typical appearance showing a compressed layer of inflammatory cells at the VT margin (dark staining material) and the variably stained fibrous material forming the bulk of the tissue. A specimen showing a compressed cellular layer adjacent to the VT with a less obvious fibrillar structure containing inflammatory cells some distance from the VT margin (H&E; (a) × 80; (b) × 200)

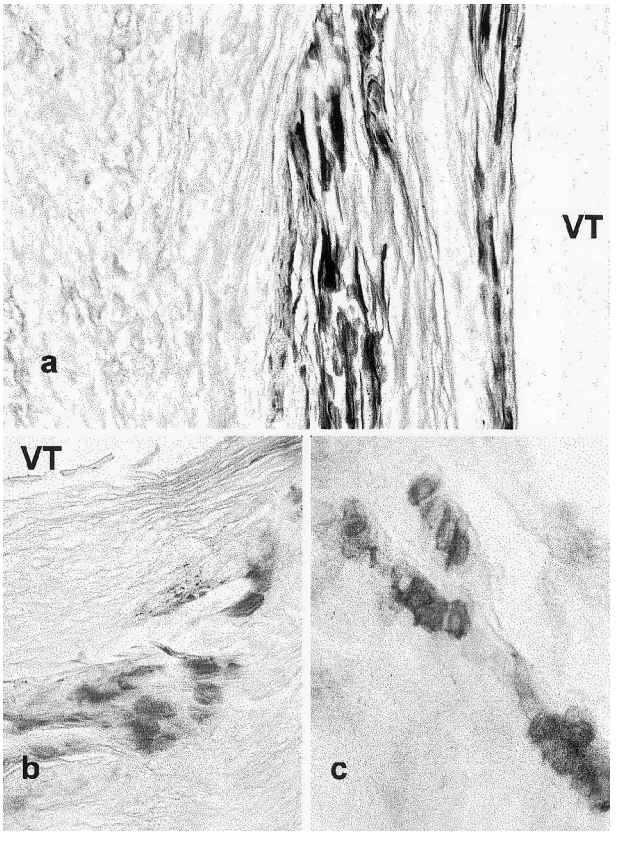

FIG. 2

Gram positive cells and fibres within tissue near to the VT margin (× 200). (a) Darkly stained, flattened superficial cells and (b and c) less intensely reactive cells showing the granular nature of the staining.

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form of compressed layers close to the VT margin (Figure 1(b)) as pink, plate-like discs similar to desquamated epithelial cells at the edges of the tissue. Many of the latter contained identifiable nuclei.

Histochemistry

Sixteen specimens of VT tissue were investigated using stains showing some specificity for either collagen (Van Gieson) or keratin/keratohyaline (Gram's stain, phloxine-tartrate). Only two specimens contained fibres with the deep pink/red reaction consistent with the presence of collagen. Gram-positive structures, similar to epithelial cells, were found in 11/16 specimens tested. Many of these positive cells contained darkly staining granules consistent with the presence of keratohyalin granules (Figure 2). Those close to the VT margin were flattened and generally stained more darkly than those in deeper areas. In addition, some of the fibrous material in most specimens stained Gram positive, indicating the presence of keratin. Similarly, phloxine-tartrate staining of six of the Gram positive specimens showed the presence of dark pink/red fibres throughout the tissues that was consistent with the presence of keratin (Figure 3). Three also contained positively stained flattened squames.

Immunohistochemistry

Twelve specimens containing sufficient material were further characterized using immunohistochemical methods to investigate further the nature of the tissue adhering to the VTs.

Keratin. All specimens showed positive reactivity with both anti-keratin antibodies tested but the strongest and most consistent staining was obtained with the mixture of anti-keratin monoclonal antibodies. Although the staining intensity was variable between, and within, specimens positive reactivity was consistently expressed by fibrous strands and cell-like structures (Figure 4), indicating the epithelial nature and derivation of the material attached to the tubes. In most cases however, the phloxine-tartrate positive hyaline material adjacent to the VT margin (Figure 3a) did not stain for keratin (Figure

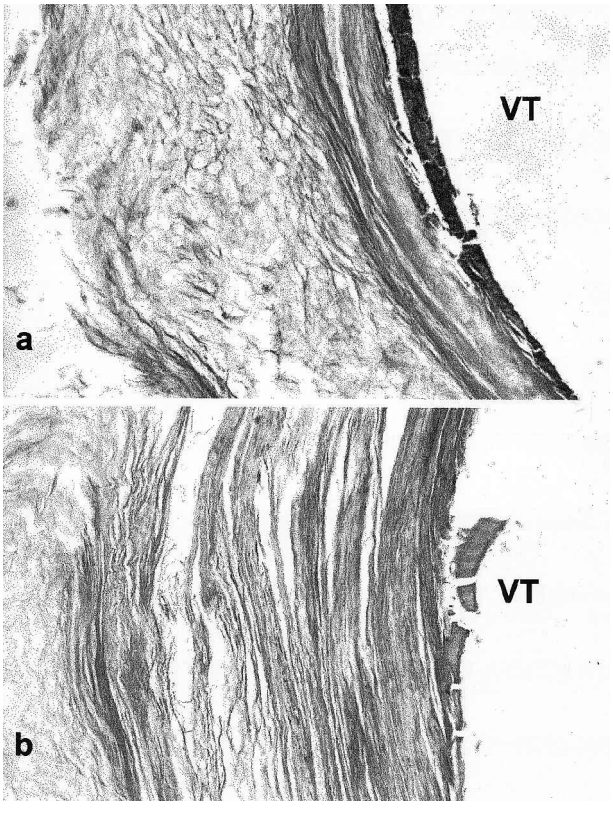

FIG. 3

Phloxine-tartrate staining of the hyaline material adjacent to the VT margin and fibres throughout the specimens (× 200).

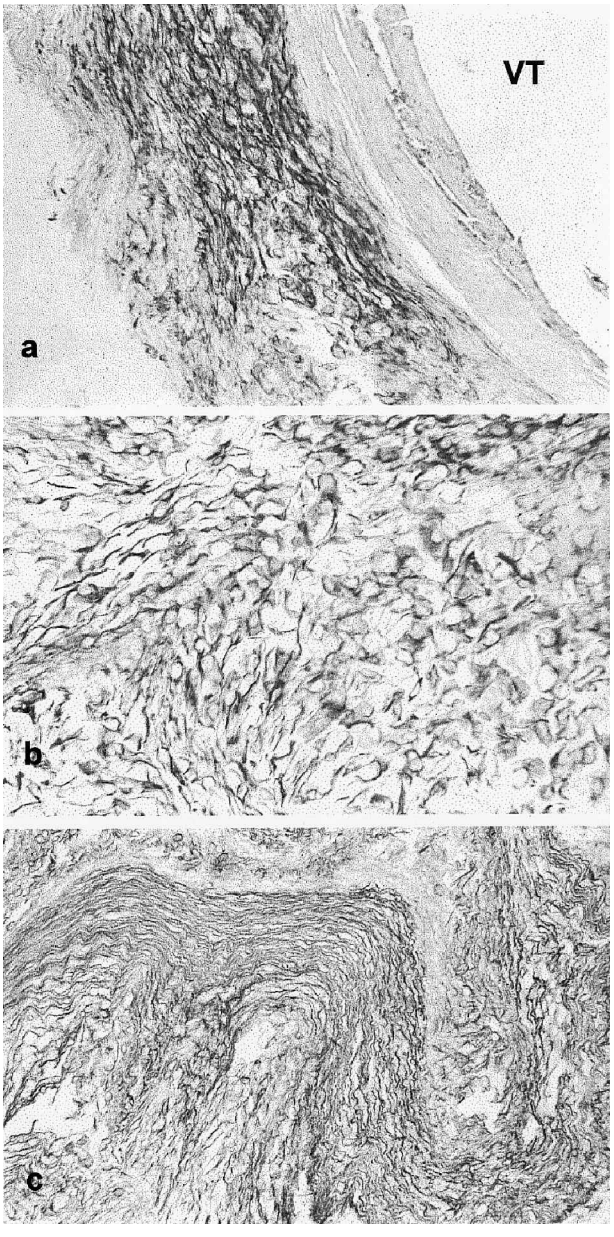

FIG. 4

Keratin staining using the mixture of anti-keratin monoclonal antibodies (× 200). (a) Section adjacent to that shown in Figure 3(a) showing positive staining of fibres except those adjacent to the VT margin. (b) Keratin-positive meshwork giving the impression of cellular structure. (c) Extensive area of keratin-positive fibres.

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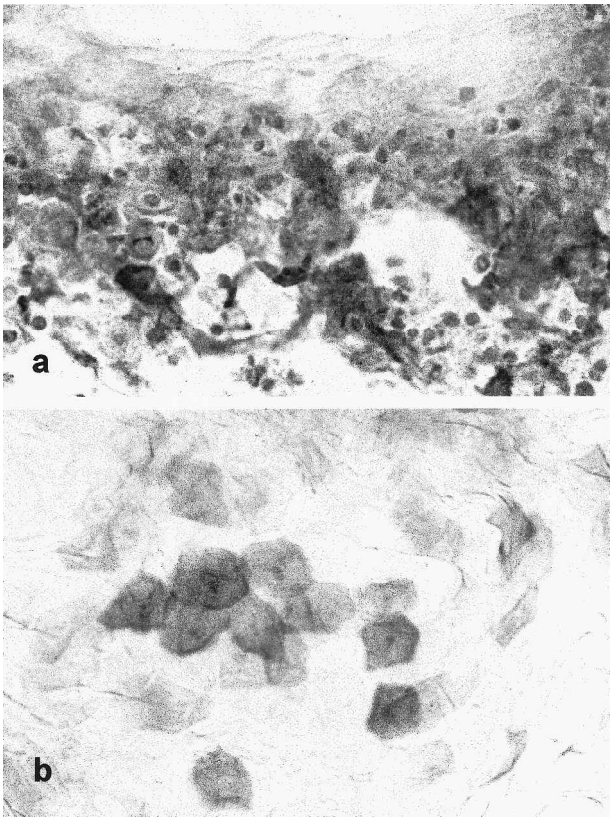

FIG. 5

Epithelial cells at the edge of specimens remote from the VT margin (× 320). (a) Keratin-positive squamous epithelial layer. (b) Desquamating epithelial cells exhibiting positive staining and presence of pyknotic nuclei.

4a), a finding consistent with the known inability of the antibodies used to react with mature stratum corneum keratin. At the edges of the tissues, remote from the VT margin, staining of squamous epithelium (Figure 5a) was detected in three specimens and plate-like epithelial cells (Figure 5b) in 10.

Extracellular connective tissue matrix proteins. The majority of specimens were negative for collage (type I and III), fibronectin and tenascin indicating that they were not composed of fibrous connective tissue. However, fibrillar staining for collagen types I and III was detected in parts of two specimens, both of which had been identified as containing collagen by the van Gieson method. One of these specimens was the only one positive for fibrillar fibronectin and tenascin where deposition appeared to border a blood vessel.

Vimentin, endothelial cell markers and CD45. Essentially, none of the tissues showed positive staining for vimentin or endothelial cell markers except the single specimen that had been shown to contain collagen, fibronectin and tenascin. A number of specimens showed some weak vimentin staining of inflammatory cells that were also shown to be positive for leukocyte common antigen (CD45).

Scanning electron microscopy

In addition to some unidentifiable debris, three tubes (mini-Shah, Shephard and Long Armstrong) exhibited adherent clusters or sheets of plate-like structures consistent with the presence of epithelial cells (Figure 6). SEM analysis of the other two VTs indicated the presence of debris and bacteria only.

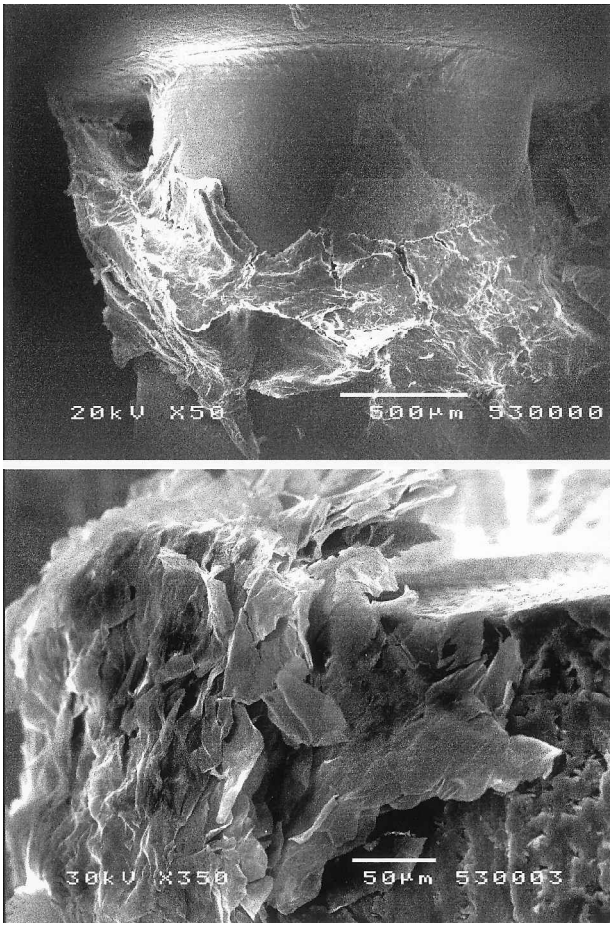

FIG. 6

Scanning electron micrographs of spontaneously extruded ventilation tubes. Upper image: ring of tissue adhering to a tube. Size bar 500 µm. Lower image: adherent cellular tissue with plate-like appearance, resembling epithelial cells. Size bar 50 µm.

Discussion

This is the first study to investigate the nature of tissue adherent to spontaneously extruded ventilation tubes in man. The results demonstrate that in the majority of cases (14/16) the tissue surrounding the VT is essentially epithelial in nature, consisting of concentric layers of apparently acellular (ie. non-nucleated) material showing positive histochemical and immunological reactivity for keratin. Interestingly, the few nucleated, keratin-positive epithelial cells detected were only found at the periphery of the specimens distant from the VT-tissue interface. This, together with the finding that collagen, and other markers of connective tissue were only detected in two specimens, indicates that natural extrusion occurs via a mechanism that leads to the VT being encased in epithelium with the most differentiated elements next to the surface of the implant (Figure 7).

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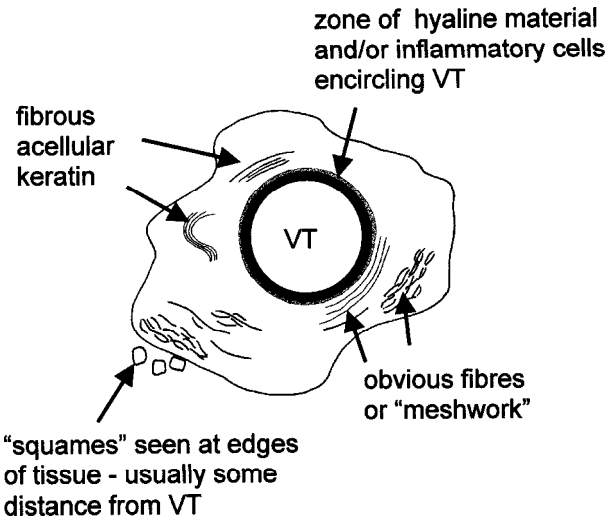

FIG. 7
Diagrammatic representation of tissue attached to naturally extended ventilation tubes.

The implication of these observations is that extrusion involves migration of squamous epithelium along the tube to form a muco-epithelial junction, or near, its medial end, essentially as originally suggested by Wilson.[15] Epithelial hyperplasia then results in the tube being encased in orthokeratinizing epithelium. Extrusion would involve splitting of the epithelial collar in the region of the granular layer caused by forces set up between normal movement of the tympanic membrane and the relatively inflexible whorl of keratin surrounding the ventilation tube. Thus extrusion would be an essentially passive process with the VT simply falling out due to fracture of the keratin ring around the tube. Such a mechanism contrasts with previously proposed theories which all postulate some active force, due to epithelial cells, inflammation and/or connective tissue fibres, that push or elevate the VT out of the tympanic membrane.[9,10,16]

In man there is little clear data on the relationship between VTs and the surrounding tissues of the tympanic membrane or on the material adherent to naturally extruded tubes. From examining haematoxylin and eosin stained sections, Reid *et al.*[17] indicated that the material around the collar and base of extruded tubes consisted of keratin. However, these workers assumed that the material on the base of the tubes probably occurred as a post-extrusion phenomenon. Interestingly, haematoxylin and eosin reactivity of the keratin associated with extruded tubes in this study was very variable and did not allow any definite conclusions to be drawn. This may have been due to the variable time periods between extrusion and removal and fixation of the tubes and attached tissue.

Bingham and Milroy[10] performed a histological study of the human tympanic membranes after removal of indwelling VTs. Unfortunately, their specimens were poorly oriented, making it impossible to identify the position of the muco-epithelial junction. They reported that 18/21 margins examined consisted of hyperplastic and hyperkeratotic epithelium with a prominent granular layer. However, the orientation of the epithelium in respect of the VT is not clear from either the text or photomicrographs. Because all specimens showed some evidence of local inflammation, they suggested that VT extrusion occurred as a result of pressure created by chronic hyperplasia and inflammation causing elevation of the tube. We would contend that the simpler explanation, based on squamous epithelial proliferation, medial migration along the tube, differentiation and desquamation is most likely.

The normal response to an incision and to insertion of polyethylene grommets in the tympanic membrane has been studied in animals. In rats and cats,[11,18] keratin production, epithelial cell hyperplasia and migration at perforation edges is the first stage of the normal healing process. In incisions or VT removal extrusion this is followed by fibroblastic activity and vascular proliferation that restore the three-layered tympanic membrane. Although a similar healing process was sometimes seen in chinchillas,[19] healing of tympanic membranes also appeared to occur after initial bridging of the perforation by hyperplastic connective tissue that was then rapidly covered with epithelium on both sides. The mechanism of healing in humans is not known but is assumed to be similar.

Chronic perforations of the tympanic membrane are thought to be due to a failure in the normal healing process. Although the precise reason for such a failure is still not fully understood it is thought that healing is inhibited by the development of a mature rim around the perforation.[20] In the case of healing of perforations after VT removal it has been known for a long time that the incidence of permanent perforation is significantly reduced if the perforation edge is excised at the time of grommet extraction.[21] A characteristic histological feature of the perforated tympanic membrane is that the rim is lined by epithelium, the nature of which is dependent upon the position of the muco-epithelial junction. Previous studies in humans of persisting perforations indicate that the muco-epithelial junction. Previous studies in humans of persisting perforations indicate that the muco-epithelial junction was at the perforation margin in 50–67 per cent and medial to the rim in 30–33 per cent of cases.[22,23] Thus, at least 30 per cent of chronic, non-healing perforations are lined with squamous epithelium. Interestingly, Yamashita found that the muco-epithelial junction in a membrane with a 'loose fitting' VT *in situ* was medial to the perforation indicating that the tube was contained within a tube of squamous epithelium. This orientation of squamous epithelium is consistent with our data and with a mechanism of extrusion based upon medial epithelial migration along the tube, differentiation and desquamation.

In some patients with a history of otitis media, casts and crusts of the tympanic membrane are observed which arise from the lateral surface of the membrane. Casts are thought to arise because of an inflammation-induced localized inhibition of the

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enzymes steroid sulphatase and aryl sulphatase which normally deactivate cholesterol sulphate, whose function is to maintain cohesion of the keratinocyte layers. The absence of these enzymes results in an abnormal cohesion of the upper layers of the stratum corneum and rapid proliferation of keratinocytes causes the layers to accumulate, forming a scale-like cast that dries hard in air. The underlying migrating epithelial cells are unable to transport layers and the cast is shed entire.[24] Histologically, these casts are remarkably similar to the tissue we observed surrounding extruded VTs and to that on the base of tympanostomy tubes removed during routine treatment of children for otitis media with effusion.[17] One third of our specimens contained identifiable inflammatory cells within the keratin layers and adjacent to the VT surface indicating that the mechanisms involved in cast formation may play a role in extrusion, essentially the tube being shed into the external auditory canal surrounded by a 'cast'.

The present study demonstrates that naturally extruded VTs are usually surrounded by concentric rings of keratin, do not contain connective tissue elements, other than a few inflammatory cells, and have many similarities to casts of the tympanic membrane. These findings indicate that natural extrusion occurs via a mechanism that leads to the VT being encased in squamous epithelium, with the most differentiated elements next to the surface of the implant, followed by normal or enhanced terminal differentiation and desquamation of epithelial cells. As stated above, our results suggest that extrusion involves splitting of the epithelial collar in the region of the granular layer caused by forces set up between normal movement of the tympanic membrane and the relatively inflexible whorl of keratin surrounding the ventilation tube and is thus essentially a passive process with the VT simply falling out due to fracture of the keratin ring around the tube.

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