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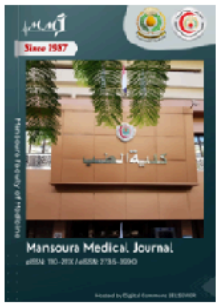

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ULTRASTRUCTURAL STUDY AND CYTOKERATIN EXPRESSION OF NORMAL TYMPANIC MEMBRANE AND AFTER EUSTACHIAN TUBE OBSTRUCTION : AN EXPERIMENTAL STUDY

By

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ABSTRACT

The main target of the present work is to study the ultrastructure and expression of cytokeratin 13/16(CK13/16) , as a marker of cell proliferation, of tympanic membrane (TM) of normal guinea pigs and after Eustachian tube (ET) obstruction. The present study was conducted on 24 healthy guinea pigs. The right ET nasopharyngeal orifice of all animals was obstructed by electrocauterization while the contralateral left ear served as controls. The animals were classified into three equal groups according to the time interval following ET obstruction either after two, four or eight weeks. The TMs of 12 animals were stained immunohistochemically for detection of CK13/16 and that of other 12 animals were subjected for electron microscopic study. The results showed that the pars tensa of control group is formed of outer epithelial layer, lamina propria and inner mucosal layer. Obstruction of ET led to inflammatory changes in the form of hemorrhage and infilteration of the subepithelial and submucosal layers of the lamina propria with inflammatory cells. After eight weeks of ET obstruction, the epithelium showed increase thickness and hyperproliferation as detected by increase the expression CK13/16 that was not restricted to the basal cell layer but found in many suprabasal layer. Also, the dense network of collagen fibers found in the normal TM was destroyed with few fibers remaining. From the present work it can be concluded that the patency of ET is essential for the integrity of TM. Also, the increase of CK 13/16 pattern and

the destruction of the dense collagen network after ET obstruction may explain the retraction pocket cholesteatoma theory.

INTRODUCTION

The external auditory canal (EAC) is a special anatomic region, forming a "cul- de– sac" with the thin tympanic membrane (TM) separating the EAC from the sterile environment of the middle-ear (ME) cavity. The external surface of the intact TM is covered with keratinized stratified epithelium which participates in preventing EAC micro-organisms from reaching the ME cavity [1]. Also, it is well known that the TM is a key element in the transduction of air vibration (tone) into mechanical movement of the ossicular chain [2]. The mammalian TM has two distinct parts; the larger pars tensa (PT) and smaller pars flaccida (PF). The PT is important for sound conduction but the function of PF is not clear [3]. In guinea pigs the area of the tympanic membrane above the lateral process of the malleus is considered as being the pars flaccida and is found to be very small and negligible . The remaining part of the TM is considered as pars tensa that is formed of two portions, inferior larger and superior smaller [4]

The main function of the ET is ventilation of the tympanic cavity to equalize the ME pressure with the atmospheric pressure and cleansing of the tympanic cavity by clearance of the fluid that has accumulated in the ME[5&6]. There is a growing interest in the relationship between impaired function of the ET and various pathological conditions of the middle ear as well as the tympanic membrane. This relationship is well documented in otitis media with effusion (OME) and in many cases of chronic otitis media (COM) and diseases related to the TM such as the presence of keratinizing squamous epithelium in the middle ear (cholesteatoma) and tympanosclerosis [7]. Many investigators have discussed the origin of cholesteatoma. The epidermis of either the external meatal wall or the TM has been considered the most likely source of cholesteatoma .The latter is called retraction pocket cholesteatoma theory [8].

The main target of the present work was to study the ultrastructure and cytokeratin expression of tympanic membrane of normal guinea pigs. Also, to clarify the effect of ET obstruction, for various periods, on the ultrastructure and on the cytokeratin

expression of TM.

MATERIALS AND METHODS

The present study was conducted on 24 healthy guinea pigs weighting 250-300 grams. All animals exhibited healthy tympanic membrane and clean external auditory meatus.

Obstruction of ET by electro cauterization (9) :

The right ET nasopharyngeal orifice of all animals was cauterized and the contra lateral left ears served as controls. Under Phenobarbital anesthesia and after fixing an earth electrode to the animal shaved back, an endoscope of 2.7 mm and 30 degree was introduced through a hole in the hard palate near the caudal end to visualize the lateral nasopharyngeal wall and to detect the two lip like mucosal swellings of nasopharyngeal opening in the lateral nasopharyngeal wall. A thin isolated electrode bared only at the tip was introduced through the same opening and cauterization of the ET nasopharyngeal orifice was effected under endoscopic vision. No antibiotics were given postoperatively. The operated animals were classified into three groups (eight in each) according to time intervals following the ET obstruction two, four or eight weeks.

Immunohistochemical and ultrastructural study : The animals were anesthetized (two, four and eight weeks after electro cauterization of the ET) then were perfused through the left ventricle first with saline and then with a fixative contained 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The temporal bones were removed and the tympanic membranes together with the surrounding bone were immersed in the same fixative for 48 hours, decalcified in 0.1M EDTA (ethylene diamine tetraacetic acid) in 0.15 M sodium phosphate buffer in a microwave oven(10).

For immunohistochemical study; after decalcification of the temporal bones the TM of 12 animals (four in each group) were dehydrated, embedded in paraffin, sectioned (6 µm thickness) on poly-L-lysine coated slides and underwent haematoxylin and eosin (H & E) staining(11) and immunohistochemical study for cytokeratin 13/16 (K8.12) (Sigma Chemical Company, ST Louis, M.O) as following(12&13) : After dewaxing,

endogenous peroxidase activity was blocked by incubating in 0.3% H2O2 in methanol for 30 minutes .Prior to incubation with keratin antibody, the antigen was retrieved in a microwave oven for 10 minutes. The sections were incubated in 0.5% normal rabbit serum for 20 minutes to reduce non-specific binding of the antibody. Then were incubated for one hour with primary antibody antikeratin 13/16 at dilution of 1:10 in phosphate buffer saline (PBS). The secondary antibody (antirabbit immunoglobulin) at dilution 1: 20 in PBS were used for one hour and then apply peroxidase antiperoxidase (PAP) for 20 minutes. The reaction products in the sections were visualized with freshly prepared chromogen (0.01% 3,3` - diaminobenzidine tetrahydrochloride) and were counter stained with hematoxylin. Substituting the primary monoclonal antibody with PBS produced negative control specimens. Also, sections with known positive reaction for CK 13/16 monoclonal antibody (obtained from Sigma Chemical Company) were used in the same run as a positive control.

Interpretation of the results : Brown diffuse cytoplasmic stains were seen in positive cases .

Electron microcopic study :

The TM of other 12 animals (four in each group)were postfixed in osmium tetroxide, dehydrated, and embedded in epoxy resin. For orientation purpose semithin sections were stained with toluidine blue. The ultrathin sections were cut and double stained with uranyl acetate and lead citrate and examined under electromicroscope(4).

RESULTS

Control group :

The pars tensa is formed of two portions, inferior larger and superior smaller. The two portions is composed of outer epithelial layer (the epidermis), the lamina propria subdivided into subepitheial layer composed of loose connective tissue (CT), a middle fibrous and very thin submucosal CT layer and thin inner mucosal layer (Figs.1&2). The epidermis (of the two portions) was composed of stratified epithelium formed of few layers (three to four). The cells of the epidermis, the keratinocytes, were polyhedral, and separated by intercellular spaces and were connected with each other by many desmosomes. The cytoplasm contained tonofilaments and the nuclei of the cells were rounded (Fig.3).The contact between

the basal cell layer and the underlying subepithelial layer of lamina propria was secured by dermoepidermal junction that appeared rather smooth, without the papillae usually seen in the skin (Fig.4).

The middle fibrous layer of lamina propria of the inferior portion consisted of two layers of fibers bundles, outer radial and inner rounded (Fig. 5).In superior portion of pars tensa the middle fibrous layer of lamina propria consisted of several layers of fibers ran in different directions (Fig.6).

After Eustachian tube obstruction:

Two weeks after ET obstruction: The epidermis was not affected and the lamina propria disclosed distended capillaries and infiltrated with inflammatory cells within the subepithelial (Figs.7-9) and submucosal connective tissue layer (Fig.10). The collagen fibers of the middle fibrous layer of both inferior (Fig.11) and superior (Fig.12) portions of pars tensa were not affected.

Four weeks after ET obstruction: There was increase of the thickness of the epidermis with thick keratin (hyperkeratosis). The subepithelial and the submucosal CT layers contained distended capillaries and infiltrated with inflammatory cells (Figs.13-15).The collagen fibers of the middle fibrous layer of both inferior(Figs.16) and superior(Fig17) portions of pars tensa were decreased mildly.

Eight weeks after ET obstruction: There was increase of the thickness of the epidermis with papillary down growth formation and decrease of the thickness of the lamina propria (Figs.18&19). The basal surface of keratinocytes made projection inside the lamina propria and the dermoepidermal junction appeared irregular (Fig.20). Some keratinocytes showed degenerative changes in the form of degenerative vacuoles and presence of lysosomes (Fig.21). The subepithelial and submucosal CT layer contained dilated blood vessels and inflammatory cells (Figs. 18, 19 &22). There was decrease of thickness of the lamina propria (Fig.19) and decrease of collagen fibers and remnant of collagen fibers were seen in the middle fibrous layer of both inferior (Fig.23) and superior portions of pars tensa (Fig 24).

Immunohistochemical result :

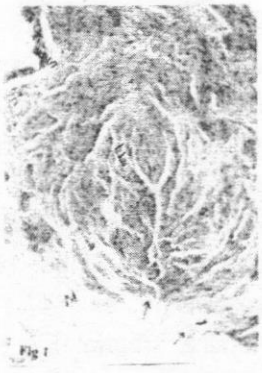
Control group: The CK 13/16 was

expressed in the basal epithelial cells only (Fig.25).

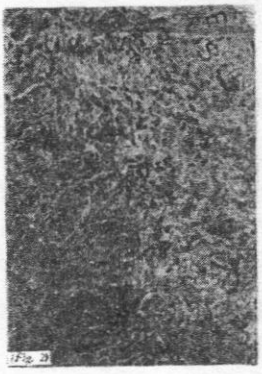
Two weeks after ET obstruction the expression of CK 13/16 was not differ from that of control.

Four weeks after ET obstruction the expression of CK 13/16 expression was not restricted to the basal layer and more than one layer of the epidermis were stained (Fig.26).

After eight weeks of ET obstruction CK 13/16 was not restricted to the basal cells but several suprabasal cells showed staining reaction (Figs .27 &28).



(Fig. 1) : A photomicrograph of the pars tensa of control group showing the epidermis (E),the lamina propria formed of sub epithelial connective tissue layer (S), middle fibrous (F) and very thin sub mucosal (M)connective tissue layer and inner mucosal layer (Arrows).(H&E X100)

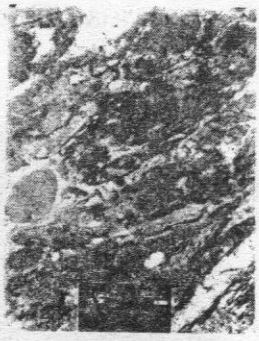


(Fig. 2) : A photomicrograph of semi thin section of pars tensa of control group showing the epidermis (E), sub epithelial connective tissue (S) and middle fibrous layer (F). (Toluidine blue stain X1000)

(Fig. 3) : Electron micrograph of pars tensa of control group showing the outer epithelium (epidermis) consists of keratinocytes separated by intercellular spaces (arrows) and joined by desmosomes (double arrows). The cytoplasm contain tonofilaments (T) and the nuclei (K).(X15500)

(Fig. 5) : Electron micrograph of inferior portion of the pars tensa of control group. The middle fibrous layer of the lamina propria consists of outer fiber bundles (OF) run radially and the inner (IF) is rounded. (X15500)

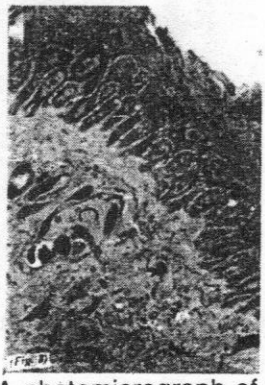
(Fig. 4) : Electron micrograph of pars tensa of control group showing the region of contact between basal cells (BC) and the sub epitheiial layer of the lamina propria (asters) is composed of loose connective tissue. The dermoepidermal junction (arrows) appear smooth .(X 15500)



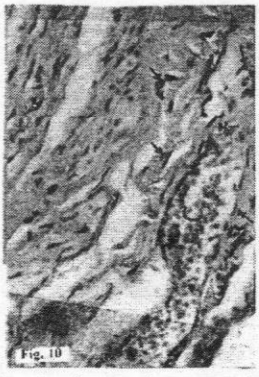
(Fig. 6) : Electron micrograph of superior portion of the pars tensa of control group. The middle fibrous layer of the lamina propria consists of several layers of fiber bundles run in different directions. (X15500)

(Fig.7) : A photomicrograph of pars tensa two weeks after Eustachian tube obstruction showing the epidermis (E) , distended capillary (C) and infiltration with inflammatory cells (arrows) within the sub epithelial connective tissue layer.(H&E X400)

(Fig. 9) : Electron micrograph of pars tensa two weeks after ET obstruction showing inflammatory cell (arrow) within the sub epithelial CT layer. Note the nucleus of inflammatory cell (N) and RBCs (R) within the capillary . (X8900)

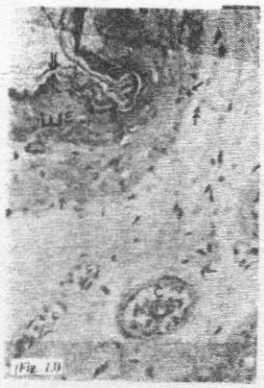


(Fig. 8) : A photomicrograph of semi thin section of pars tensa two weeks after Eustachian tube obstruction showing the epidermis(E) , distended capillary(C)and infiltration with inflammatory cells (arrows) within the sub epithelial connective tissue layer .(Toluidine blue X1000)



(Fig. 10) : A photomicrograph of pars tensa two weeks after Eustachian tube obstruction showing distended capillary (C) and infiltration with inflammatory cells (arrows) within the sub mucosal CT layer.(H&E X400)

(Fig. 11) : Electron micrograph of inferior portion of pars tensa two weeks after ET obstruction. The middle fibrous layer consists of outer radial (OF) and inner rounded fiber (IF) layers.(X21000)



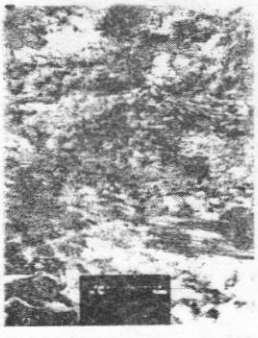
(Fig. 13) : A photomicrograph of pars tensa four weeks after ET obstruction showing increase of the thickness of the epidermis(E) with thick keratin (double arrows) and presence of inflammatory cells (arrows) and distended capillaries (C) in subepithelial CT layer .(H&E X400)

(Fig. 12) : Electron micrograph of superior portion of pars tensa two weeks after ET obstruction. The middle fibrous layer consists of several layers of fiber bundles run in different direction.(X15500)

(Fig. 14) : A photomicrograph of semi thin section of pars tensa four weeks after ET obstruction showing increase of the thickness of the epidermis (E) with thick keratin (K) and presence of inflammatory cells (arrows) and distended capillary (C) in subepithelial CT layer .(Toluidine blue X1000)

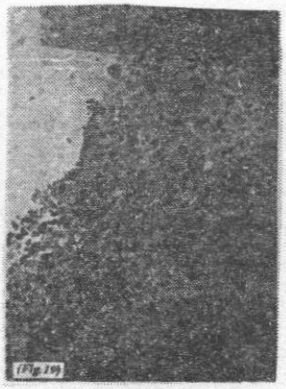
(Fig. 15) : Electron micrograph of pars tensa four weeks after ET obstruction showing inflammatory cell (IC) and RBC in capillary within the sub epithelial CT layer. (X15500)

(Fig. 17) : Electron micrograph of the superior portion of pars tensa four weeks after ET obstruction. There is decrease of collagen fiber of the middle fibrous layer.(X28500)

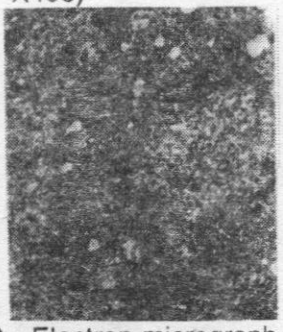


(Fig. 16) : Electron micrograph of the inferior portion of pars tensa four weeks after ET obstruction. There is decrease of the collagen fibers of middle fibrous layer (X15500)

(Fig. 18) : A Photomicrograph of pars tensa eight weeks after ET obstruction showing i-crease the thickness of the epidermis (E) with papillary downgrowth formation (double arrows) and inflammatory cells (arrows) within the subepithelial connective tissue



(Fig.19) : A photomicrograph of semithin section of pars tensa eight weeks after ET obstruction showing increase the thickness of the epidermis (E) with papillary downgrowth formation (double arrows). The lamina propria discloses inflammatory cells (arrows) and dilated capillary(C) . Note decrease of the thickness of the lamina propria. (Toluidine blue X400)



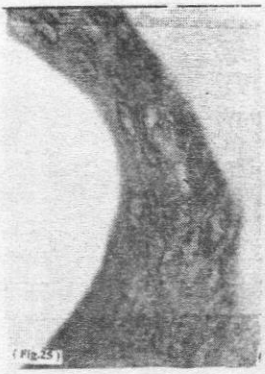
(Fig. 20) : Electron micrograph of the pars tensa eight weeks after ET obstruction showing the keratinocyte of the epidermis. There is projection of its basal surface inside the lamina propria (arrow) and the dermoepidermal junction appears irregular. The nucleus of the keratinocyte (K). (X15500)

(Fig. 21) : Electron micrograph of pars tensa eight weeks after ET obstruction showing keratioincyte with degenerative changes in the form of intracytoplasmic vacuoles (V) and lysosomes (arrows). (X8900)

(Fig. 22) : Electronmicrograph of pars tensa eight weeks after ET obstruction showing dilated blood vessels contains RBCS. (X8900)

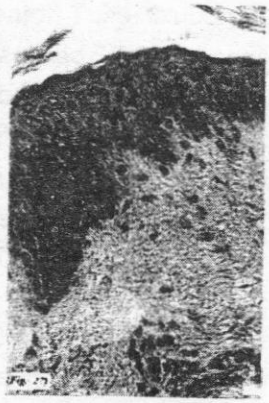
(Fig. 23) : Electron micrograph of inferior portion of pars tensa eight weeks after ET obstruction .There is marked decrease of collagen fibers and remnant of collagen fibers (arrows) isseen. Note degenerated cell (D).(X21000)

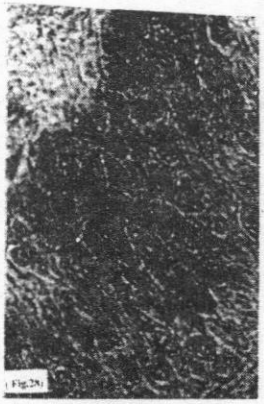
(Fig. 24) : Electron micrograph of superior portion of pars tensa eight weeks after ET obstruction .There is marked decrease of collagen fibers and remnant of collagen fibers (arrows) is seen. (X21000)



(Fig. 25) : Immunohistochemical staining of pars tensa of control group with monoclonal antibody to cytokeratin 13/16 appears in the basal cell layer of the epidermis only (arrows).(PAP X1000)

(Fig. 26) : Immunohistochemical staining of pars tensa four weeks after ET obstruction with monoclonal antibody to cytokeratin 13/16 .More than one layer of the epidermis showing positive reaction (arrows).(PAP X1000)





(Figs. 27&28) : Immunohistochemical staining of pars tensa eight weeks after ET obstruction. The cytokeratin 13/16 is not restricted to the basal cell but several suprabasal cells (arrows) showing reaction. (PAP X400 &X1000)

DISCUSSION

In the present study the ultrastructure of the tympanic membrane of normal guinea pig and after ET obstruction was studied. Almost all of the tympanic membrane of the guinea pig is composed of the pars tensa while the pars flaccida is very small and negligible [4].The pars tensa is formed of outer epithelial layer (the epidermis), the lamina propria and the inner mucosal layer .The epidermis is consisted of three to four layers only and the cellular contact was established with many desmosomes. The outer epithelial layer participates in preventing EAC microorganisms from reaching the middle ear cavity[14].In this concept, Boe et al. (1999)[15] had been proved that the surface epithelium of TM produced potent antimicrobial peptide human B-defensin-1 (HBD-1). This HBD-1 participates in the innate antimicrobial defense of the EAC and middle ear cavity. In inferior portion of pars tensa the middle fibrous layer of lamina propria consists of collagen fibers arranged into two layers the outer fiber bundles are radial and inner are rounded. Narita et al.

(1989)[16] reported that the size of the outer radial fibers is twice as thick as that of the inner circular fibers in the inferior larger portion of the guinea pig tympanic membrane. They also, observed that the TM resists tension from a radial direction. It was concluded that the outer fiber layer is the main skeleton of the tympanic membrane, at least at the inferior larger portion of the pars tensa. In the human tympanic membrane as well, the outer radial fibers seem to play a major structural role since this layer is thicker than the inner circular layer (3).

The ET provides ventilation and clearance of the tympanic cavity and is a mechanical, immunological and biochemical barrier against ascending infection[17].In the present study the endoscopic method for achieving ET obstruction was used to avoid the effect of cutting the palatal muscles by the palate splitting incision used by Hellstrom and Stenfors (1984)[18]. Therefore, it becomes possible to isolate the effect of tubal obstruction per se on the tympanic membrane. Moreover, this endoscopic method has the advantage of avoiding the need to fully open the mouth all the time of the operation and it saves the time needed for cutting and stitching the palatal incision in such small area. Clinically the obstruction of ET may be either functional or mechanical. Functional obstruction could result from persistent collapse of the ET because of increased tubal compliance, and inadequate active opening mechanisms, or both. Mechanical obstruction may be the result of either intrinsic or extrinsic factors. Intrinsic mechanical obstruction of ET may be caused by inflammation and extrinsic mechanical obstruction may be the result of extrinsic compression by nasopharyngeal tumors or adenoids [19]. The results of the present work showed that two weeks after ET obstruction there was distended capillaries and infiltration of inflammatory cells within the subepithelial and submucosal connective tissue layer of the lamina propria. Four and eight weeks after ET obstruction in addition to previous findings there were increase of the thickness of both the keratin and the epidermis. In this context, Yamaguchi et al., (1990)[20] found proliferation and dilatation of blood vessels in the lamina propria adjacent to the epithelial layer of the pars tensa in otitis media with effusion (OME) in patients with head and neck tumors. Also, Berger (1996)[21] found proliferation of dilated capillar-

ies in the sub epithelial and sub mucosal layers of the lamina propria of the pars tensa in patients with acute and secretory otitis media (AOM and SOM). The author explained this observation due to engorgement of the capillary bed secondary to inflammation. Also, the infiltration of polymorphonuclear leucocytes in AOM and of mononuclear cells in SOM could be attributed to the presence of inflammatory mediators, including chemotactic factor in the exudates that attract leucocytes to the site of inflammation[22].

The relationship between impaired function of ET and OME and chronic otitis media has been well documented. ET obstruction has been considered the most important single etiologic factor of OME for some time now. However, there has been no conclusion evidence that tubal dysfunction is a primary endogenous factor or only a secondary effect of an infection[23]. Also, ET obstruction may result in persistent high negative ME pressure. If ME pressure equilibration does not occur, persistent ET obstruction could result in a sterile OME. If a large bolus of air enters the ME from the nasopharynx when there is high negative ME pressure, nasopharyngeal secretions could be aspirated into the ME and result in an acute bacterial otitis media[24]. Two weeks after ET obstruction the middle fibrous layer remained relatively intact. Four weeks after ET obstruction the middle fibrous layer of the lamina propria was mild affected. However, eight weeks after ET obstruction there was destruction of the fibrous layer architecture and the dense network of collagen fibers found in a normal TM was destroyed with few fibers remained. This can be explained as ET obstruction can predispose to OME and led to impairment of gas diffusion mechanism in the middle ear cavity resulting in sustenance of the negative pressure. So, over a period time the inflammatory process resulted in destruction of collagen fibers in the lamina propria and weakening of the tympanic membrane[25]. Also, ET obstruction led to lack of ventilation and produced a vacuum within the tympanic cleft that was followed by middle ear effusions. It has been shown that these effusions have high concentration of proteinase, especially collagenase. The prolonged approximation of these enzymes with that aspect of the TM may cause the destruction in the collagenous layer of the TM and resultant weakening in

that part of the TM[26]. Atrophy of TM may be found in children suffering from long-lasting secretory otitis media or chronic tubal dysfunction. In this case there was atrophy of TM showed a thin lamina propria containing just a few fibers or non at all and in severe cases, the lamina propria may be completely lacking. It seemed that a long standing negative middle ear pressure was required to induce atrophy and disappearance of the fibrous layer, and probably inflammation per se did not influence its integrity [20].

In the current work the pattern of cytokeratin (CK) expression in normal TM and after ET obstruction was studied. Cytokeratins are known as the intermediate filament proteins of an epithelial origin [27]. CK have a number of distinct advantages as marker proteins. Keratin polypeptides are the product of different genes and are expressed in different cells at different stages of development and differentiation. Therefore, CK are suitable and specific markers for studies on the classification of epithelial cell types and are useful markers for the identification of epithelial neoplasm [28]. Conveniently in this work a representative CK 13/16 as a marker of cell proliferation was used. The method of antigen retrieval based on microwave heating demonstrated reliable results on paraffin-embedded tissues [12]. The immunohistochemical findings demonstrated that CK 13/16 was normally expressed in basal epithelial cells. Two weeks after ET obstruction the pattern of CK expression appeared as control. Four weeks after ET obstruction the pattern of CK expression was not restricted to the basal layers and more than one layer of the epidermis were stained. Eight weeks after ET obstruction the CK expression was not restricted to the basal cells but many suprabasal cells showed CK expression. These findings suggested that ET obstruction especially for long period could lead to hyperproliferation of the epithelium of the TM. In this filed Kin et al., (2001) [29] had been proved that the pattern of cytokeratin was increased in epithelial neoplasm, cholesteatoma and other proliferative epithelial disorders. The authors found that in keratinocytes from cholesteatoma of tympanic membrane, CK 13/16 were over expressed compared with control specimen, indicating hyperproliferation.

From this work it can be concluded

that the patency of ET is essential for the integrity of TM. The obstruction of ET leads to inflammatory changes in the form of hemorrhage and infiltration of the lamina propria with inflammatory cells. When the obstruction was prolonged the epithelia showed increased thickness and hyperproliferation and the dense network of collagen fibers found in a normal TM was destroyed with few fibers remaining.

Also, the increase of CK 13/16 pattern and the destruction of the dense collagen network after ET obstruction, especially after long periods, may explain the retraction pocket cholesteatoma theory which states that the epidermis of the TM can be considered the most likely source of cholesteatoma.

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