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Localisation of basic fibroblast growth factor in cholesteatoma matrix: an immunochemical study

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Abstract

Objective. To investigate the expression of basic fibroblast growth factor in the matrix of human acquired cholesteatoma compared to the deep meatal skin. This topic does not appear to have been fully investigated before.

Methods. An immunochemical study was conducted. Cholesteatoma tissues from adult patients were collected during surgery ($n = 19$). Control specimens were taken from the deep meatal skin ($n = 8$) and compared.

Results. A highly significant difference in basic fibroblast growth factor expression was identified between cholesteatoma and skin (mean ± standard error = 58.53 ± 3.6 per cent in cholesteatoma *vs* 40.6 ± 3.5 per cent in skin; $p = 0.005$). Both basal and parabasal keratinocytes were stained positive with basic fibroblast growth factor. Additionally, there was specific staining in the basal columnar middle-ear epithelium and mast cell membrane.

Conclusion. Basic fibroblast growth factor plays an active role in proliferative activity of cholesteatoma through its overexpression in basal and parabasal layers of cholesteatoma matrix. Moreover, its expression in the mast cell membrane supports its role in bone resorption activity.

Introduction

The pathogenesis of acquired cholesteatoma is still unclear, and this has been the subject of scientific inquiry for many otologists and pathologists.[1,2] A complex variety of cellular and molecular mechanisms co-ordinate together and orchestrate its activity.[2,3]

Angiogenesis is a crucial factor for cholesteatoma growth and expansion. This process is initiated and maintained via different cytokines and growth factors. One of these molecules is basic fibroblast growth factor ('b-FGF').[4] Investigators previously showed that basic fibroblast growth factor was overexpressed in the perimatrix (subepithelial connective tissue) of cholesteatoma. Therefore, it has a robust action in growth, survival and bone destruction associated with this serious illness.[4,5] However, previous reports have provided no clear evidence about the expression of basic fibroblast growth factor in the matrix (keratinised epithelium) of cholesteatoma. Such expression, if present, might reflect an additional proliferative function for this growth factor.

In this study, we aimed to investigate both the intensity and pattern of basic fibroblast growth factor expression in the matrix of acquired cholesteatoma compared to that in the deep meatal skin, in an attempt to aid our understanding of the aetiopathogenesis of human acquired cholesteatoma.

Materials and methods

A prospective immunochemical study was conducted. This study conformed with the ethical guidelines on human studies and the Declaration of Helsinki. All patients or their caregivers provided written informed consent for participation.

Cholesteatoma tissues were collected during cholesteatoma surgery performed at a university affiliated hospital for adult patients with newly acquired cholesteatoma ($n = 19$). Control tissues were sampled from the deep bony portion of the external auditory canal skin from the same patients ($n = 8$). Congenital, paediatric and recurrent cases were excluded. Tissues were immediately fixed in formaldehyde 10 per cent and then embedded in paraffin.

Immunochemistry

Paraffin-embedded tissues were cut into slices of 3 μm thickness and mounted. They were incubated in a paraffin oven overnight at a temperature of 60 degrees, and then left to cool at room temperature.

Main Article

Dr M A Hamed takes responsibility for the integrity of the content of the paper

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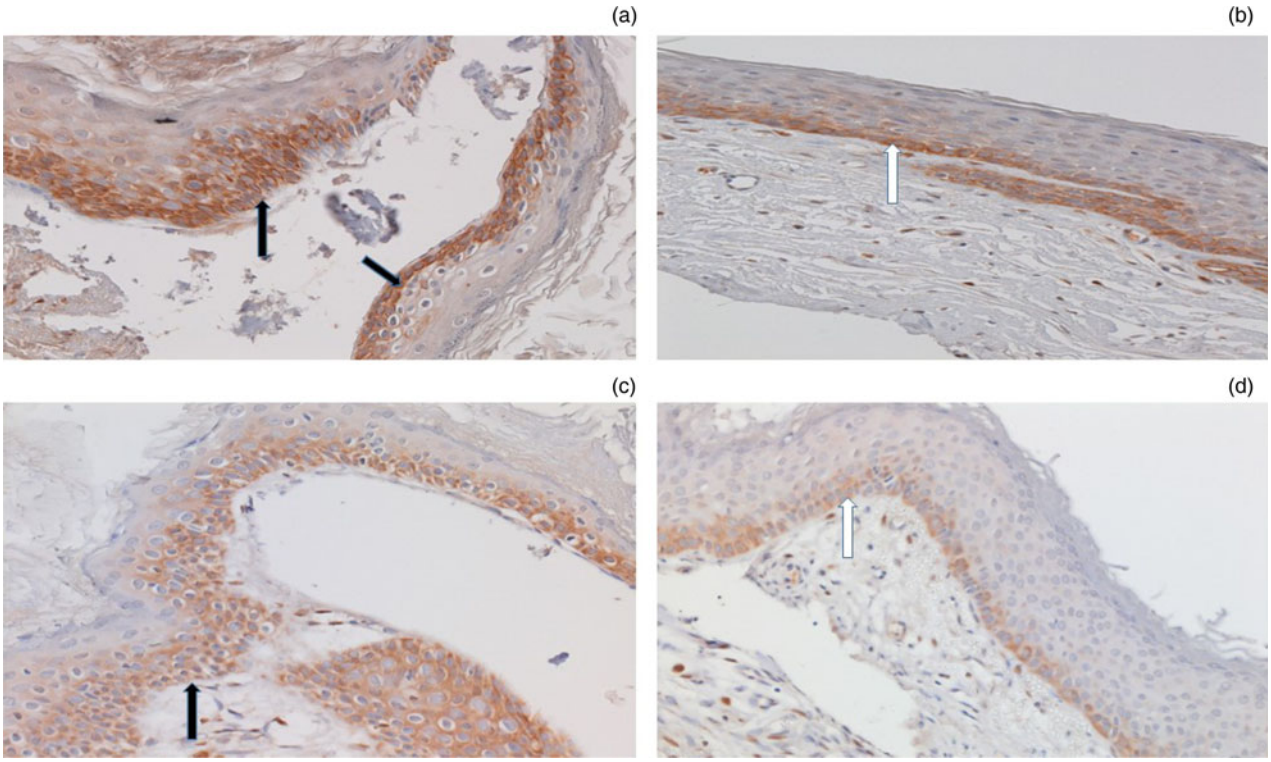

Fig. 1. Basic fibroblast growth factor is overexpressed in cholesteatoma tissues (a & c) (black arrows), compared to meatal skin tissues (b & d) (white arrows). (immunostaining; 200×)

The universal immunoperoxidase polymer technique of immunostaining was conducted. The entire procedure was performed in a humidified chamber at room temperature. Tissue sections were deparaffinised with xylene, and rehydrated in graded series of ethanol solution (four times each). In order to block endogenous peroxidase activity, sections were incubated with 0.3 per cent hydrogen peroxide in methanol for 30 minutes at room temperature, and then washed with tap water. Heat-induced antigen retrieval was performed using a pressure cooker for 10 minutes. Citrate buffer pH 6.0 is the optimal soaking solution for basic fibroblast growth factor. After pressure pan treatment, the sections were left to cool at room temperature for 30 minutes, washed in phosphate buffer saline, and then incubated with primary rabbit polyclonal antibody for basic fibroblast growth factor (dilution 1:1000; Lifespan Biosciences, Seattle, Washington, USA) overnight at room temperature. On the following day, the sections were rinsed in phosphate buffer saline, incubated with Histofine Simple Stain Max Peroxidase (Nichirei Biosciences, Tokyo, Japan) for 30 minutes at room temperature and then washed in phosphate buffer saline.

The reaction products were visualised by incubating the sections in a 3,3′-diaminobenzidine solution ('DAB') containing 0.006 per cent hydrogen peroxide for 5 minutes. The nuclei were lightly counterstained with Mayer's haematoxylin. Finally, specimens were dehydrated in alcohol followed by xylene, mounted and examined.

Immune absorption test OR absorption test

In order to confirm the specificity of immunostaining for basic fibroblast growth factor, we conducted an absorption test,[6] in which the primary antibody is mixed with different concentrations of the antigen in question.

Prior to immunostaining, the diluted antibody was admixed with recombinant human fibroblast growth factor antigen (Peprotech, Rocky Hill, New Jersey, USA) at concentrations of 0.01, 0.1, 1 and 10 ug/ml (four tubes) for 1 hour at 37°C. Fibroblast growth factor immunoreactivity was considerably weakened at the lowest concentration (0.01 ug/ml) and completely abolished at the highest one (10 ug/ml). The fifth tube contained only primary antibody, with no added antigen (0 ug/ml). Centrifugation was conducted at 100 000 turns for 1 hour. Subsequently, only the supernatant (containing the immune complexes) were added over the tissue slides as usual, followed by the same immunostaining steps previously described.

Immunostaining evaluation

Evaluation was carried out independently by two examiners: the researcher and a pathologist. The cytoplasmic staining for basic fibroblast growth factor was considered positive. The percentage of positive cells was calculated in three different areas at 200× magnification under a Zeiss light microscope, and then the average percentage of the three areas was calculated for each patient. Counting was conducted using ImageJ cell software programme.

Statistical analysis

Patients' demographics were expressed as median, range and percentage. Measurable variables were compared using the student's *t*-test for independent samples, after checking the data were normally distributed. All statistical analyses were performed using SPSS Software for Windows, version 16.0 (SPSS, Chicago, Illinois, USA). *P*-values of less than 0.05 were considered significant, with a confidence interval of 95 per cent.

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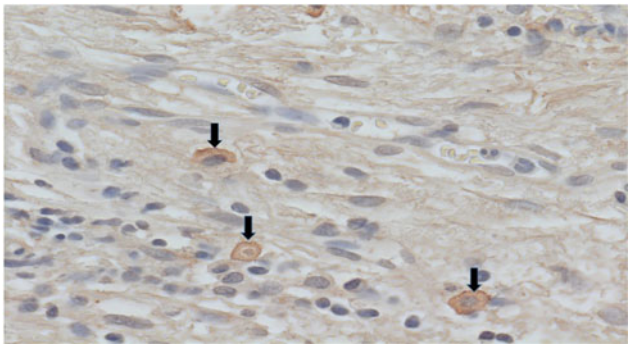

Fig. 2. Mast cell membrane shows positive staining for basic fibroblast growth factor (black arrows). (immunostaining; 400×)

Results

The median age of the 19 patients in our study was 54 years (range, 16–80 years); 11 of the patients were male (57.9 per cent) and 8 were female (42.1 per cent). Cholesteatoma involved the right ear in seven patients (36.84 per cent), the left ear in seven patients (36.84 per cent) and was bilateral in the remaining five patients (26.31 per cent). Intact canal wall mastoidectomy was performed in 7 patients (36.84 per cent) and canal wall down was performed in 12 patients (63.16 per cent).

A highly significant difference in basic fibroblast growth factor expression was identified between cholesteatoma and skin tissues (mean ± standard error = 58.53 ± 3.6 per cent in cholesteatoma *vs* 40.6 ± 3.5 per cent in skin; $p = 0.005$). Consequently, basic fibroblast growth factor was found to be overexpressed in cholesteatoma tissues compared to that in deep meatal skin (Figure 1).

Regarding the pattern of basic fibroblast growth factor expression, and as confirmed by an absorption test for immunofluorescence staining, both basal and parabasal keratinocytes, and to lesser extent the suprabasal layer, were specifically stained. In the context of stromal cells, there was specific staining mainly in the basal columnar cell layer (middle-ear epithelium), in addition to mast cell membrane positivity (Figure 2).

Discussion

Angiogenesis is a mandatory requirement for the survival of proliferating tissues in both physiological and pathological conditions including cholesteatoma. It maintains the continuous migration of keratinocytes in the middle-ear cavity, with resultant expansion of cholesteatoma. This process is mediated through a variety of cytokines and growth factors such as basic fibroblast growth factor, vascular endothelial growth factor, and transforming growth factors α and β.[4]

Owing to its role in wound repair and its angiogenic power, basic fibroblast growth factor has been used by many researchers in the repair of traumatic tympanic membrane perforations.[7–9] In addition, many reports have demonstrated an important role for basic fibroblast growth factor in growth and bone destruction associated with acquired cholesteatoma, through its expression in the cholesteatoma perimatrix (subepithelial connective tissue) and inflamed middle-ear mucosa nearby to blood vessels, with overexpression found close to areas with histological signs of inflammation. Basic fibroblast growth factor exerts its action by energising a wide variety of target cells, including fibroblasts, endothelial cells and keratinocytes.[4,5,10,11]

- Acquired cholesteatoma pathogenesis is still a subject of scientific inquiry for many otologists and pathologists
- Basic fibroblast growth factor is overexpressed in cholesteatoma perimatrix and claimed to have an important role in cholesteatoma pathogenesis
- This study investigated the intensity and pattern of basic fibroblast growth factor expression in acquired cholesteatoma matrix versus deep meatal skin
- Basic fibroblast growth factor was overexpressed in cholesteatoma matrix
- This finding indicates an additional proliferative function for fibroblast growth factor in cholesteatoma growth and expansion through an alternative route
- The positivity for basic fibroblast growth factor in mast cell membrane suggests a role for these cells in cholesteatoma pathogenesis

Our results are in line with previous reports regarding the expression of basic fibroblast growth factor in the perimatrix.[4,5] However, this cytokine has not been fully studied in the keratinised epithelium of the cholesteatoma matrix. The current study specifically assessed the pattern and intensity of basic fibroblast growth factor expression in the cholesteatoma matrix. We found a unique strong positive signal for fibroblast growth factor in the basal and parabasal layers of the cholesteatoma matrix. Occasionally, suprabasal layers were stained positive too. Moreover, basic fibroblast growth factor was overexpressed in the cholesteatoma compared with meatal skin ($p = 0.005$). An absorption test was performed to validate the specificity of immunostaining, which confirmed our findings.[6] These findings might support an additional proliferative function for fibroblast growth factor in the growth and expansion of cholesteatoma through an alternative route. Another finding is the positivity for basic fibroblast growth factor in the membrane of mast cells, which suggests a possible mechanism through which these cells play an active role in pathogenesis and bone resorption in cholesteatoma.[12,13]

Our results emphasise the role of basic fibroblast growth factor in cholesteatoma, and add new information that can enhance our understanding of the pathogenesis of acquired cholesteatoma. This knowledge could be helpful in the future management of this serious disease.

Conclusion

In addition to its role in angiogenesis, basic fibroblast growth factor plays an active role in the proliferative activity of cholesteatoma, through its overexpression in the basal and parabasal layers of the cholesteatoma matrix. Moreover, its expression in the mast cell membrane supports its role in bone resorption activity.

Competing interests. None declared

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