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Role of *N*-acetyl-β-D-hexosaminidase in cholesteatoma tissue

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Cholesteatoma is a destructive disease characterized by the progressive expansion of keratinizing squamous epithelium in the middle ear and mastoid, and chronic inflammatory reaction of the subepithelial connective tissue. *N*-Acetyl-β-D-hexosaminidase (HEX) catalyzes the release of terminal non-reducing *N*-acetyl-D-hexosamine residues acting on glucosides and galactosides in glycoproteins, G_{M2}-gangliosides and glycosaminoglycans (GAGs). In this study the activities of HEX were measured in cholesteatoma tissue and in normal skin to demonstrate a possible role of HEX in bone resorption in the area adjacent to cholesteatoma. Cholesteatomas (n=21) and normal adult retroauricular skin (controls, n=21), were collected from patients during surgery due to chronic otitis media. In 20 of 21 specimens a significantly higher activity of HEX was observed in cholesteatoma tissue compared with that in normal skin. Mean release of HEX from the activated cells was 68.55±30.77 nkat/g wet tissue in cholesteatoma and 31.79±10.02 nkat/g wet tissue in skin specimens. It may explain the process of bone resorption in the area adjacent to cholesteatoma, i.e. ossicles or temporal bone. This study suggests that drugs inhibiting HEX activity, such as iminocyclitols, may be useful in cholesteatoma treatment.

Keywords: hexosaminidase, HEX activity, cholesteatoma, normal retroauricular skin

INTRODUCTION

Middle ear cholesteatoma has been known since 1683, when du Verney (1683) gave the first description of a cholesteatoma-like mass, which he called "steatoma". However, the discussion on its epidemiology, etiology and pathogenesis is still open. Cholesteatoma is a destructive middle ear disease characterized by the progressive expansion of keratinizing squamous epithelium in the middle ear and mastoid and a chronic inflammatory reaction of the subepithelial connective tissue, the so-called perimatrix (Chole *et al.*, 2001). Its exact pathogenesis remains unknown. We have reported the invasive and hyperproliferative behavior of cholesteatoma epithelium as well as altered differentiation, aggressiveness and recidivism of this lesion (Olszewska *et al.*, 2003; 2004; 2005). We have also shown some characteristic processes occurring in cholesteatoma tissue, e.g. angiogenesis, apoptosis and bone resorption (Chodynicki *et al.*, 2002; Olszewska *et al.*, 2003; 2005). The aggressiveness of cholesteatoma is strictly related to the resorption of bone in the area adjacent to cholesteatoma perimatrix (Cynamon *et al.*, 2000). The erosion caused by bone resorption of the ossicular chain and otic capsule may result in hearing loss, vestibular dysfunction, facial paralysis and intracranial complications. Bone resorption is stimulated by inflammation and a variety of factors, including keratin, cytokines, such as interleukins (IL-1α, IL-1β, IL-6) and interferon (INFβ), all known to be released by cholesteatoma. A crucial group of enzymes that include lysosomal glycosidases play an important role in bone resorption.

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Abbreviation: HEX, *N*-Acetyl-β-D-hexosaminidase.

Among them the highest activity is demonstrated by *N*-acetyl-β-D-hexosaminidase (EC 3.2.1.52) (HEX) (Zwierz *et al.*, 1996). HEX catalyzes the release of terminal sugar moieties from the non-reducing ends of oligosacharide chains of *N*-acetyl-β-D-glucosamine and *N*-acetyl-β-D-galactosamine in glycoproteins, G_{M2}-gangliosides, and glycosaminoglycans (GAGs), including chondroitin 4-sulfate, chondroitin 6-sulfate, hyaluronic acid, keratan sulfate and dermatan sulfate (Winchester, 1996). During the course of catalysis, an oxonium ion-like transition state is thought to be generated, which is stabilized by a deprotonated carboxyl group of the enzyme (Sinnot, 1990; Rye & Withers, 2000). Human hexosaminidase has two major isoenzymes, A and B. Hexosaminidase A (HEXA) is a heterodimer composed of α and β subunits, whereas hexosaminidase B (HEXB) is a homodimer composed of only β subunits. Both isoenzymes recognize terminal *N*-acetylglucosamine and N-acetylgalactosamine, but only isoenzyme A recognizes 6-sulfated residues of these sugars (Winchester, 1996). HEX is active in most tissues and organs such as kidney (Skalova, 2005), spleen (Emiliani *et al.*, 1999), liver (Elsafi *et al.*, 1994), mucous membrane of stomach and intestine (Gil-Martin *et al.*, 1999; Michaels & Hellequist, 2001), cortex (Hammarsund *et al.*, 2004), lung (Baritussio *et al.*, 2001; Minami *et al.*, 1985), epidermal fibroblasts (Ichisaka *et al.*, 1998), placenta (Arciuch *et al.*, 1999) and neoplastic tissues (Bhuvarahamurthy & Govindsamy, 1996; Lerner, 1996; Matsuura *et al.*, 2004). HEX is used as a marker for monitoring the course of sereval diseases like chronic glomerulonephritis (Bazzi *et al.*, 2002), urinaemia (Linko-Lopponen, 1986; Stabellini *et al.*, 2005), arterial hypertension (Perez-Blanco, 1996), Sjogren's syndrom (Sohar *et al.*, 2005), advanced stadium of diabetes (Nakazawa & Tamai, 1991), rheumatoid arthritis (Popko *et al.*, 2005), idiopathic juvenile arthritis (Popko *et al.*, 2003), osteoarthritis (Liu *et al.*, 2004) and for evaluation of kindney function after transplantation (Kotanko *et al.*, 1996). Our previous studies and reports in the literature revealed that HEX is produced by chondrocytes (Lerner, 1996), neutrophil granulocytes, macrophages (Blair *et al.*, 1981), mast cells, leukocytes (Casal *et al.*, 2005) and cells of the synovial membrane (Popko *et al.*, 2003).

Although hexosaminidase was shown in different diseases, its activity in cholesteatoma has never been assessed before. We investigated the activities of HEX in cholesteatoma tissue compared with that in normal skin. We also demonstrated a correlation between HEX activity and cholesteatoma bone resorption.

MATERIAL AND METHODS

Human cholesteatoma (n=21) and normal retroauricular skin (n=21) were taken from the same patients during surgical procedures due to chronic otitis media. The age of patients ranged between 38 and 72 years (mean age: 45.7). The history of chronic otitis media ranged from 2 months to 7 years. Three cholesteatomas were classified as primary acquired cholesteatoma and 18 as secondary acquired cholesteatoma. Granulation tissue and foul-smelling otorrhea were present in 17 cases. A subsequent classification was also made, according to Tos (1988) based on the site of cholesteatoma origination: attic cholesteatoma (n=2), sinus cholesteatoma (n=17) and tensa cholesteatoma (n=1). Specimens were immediately frozen at –80°C.

Preparation of homogenate. Cholesteatoma and skin specimens were thawed and weighed. Specimens were suspended in 0.05 M citrate buffer at a 1:9 ratio (w/v) and homogenized for 2 min using an ultra-turrax T8 homogenizer. Homogenates were then centrifuged for 30 min (12000×*g*) at 4°C. Supernatant was stored at –70°C for further studies.

Reagents. *p*-Nitrophenol-β-D-*N*-acetyl-glucosaminide was purchased from Sigma (St. Louuis, MO, USA) and other reagents from Polish Chemical Reagents (Gliwice, Poland).

***N*-Acetyl-β-D-hexosaminidase release and assay.** Activity of the secretory granule-associated enzyme β-hexosaminidase in cholesteatoma and skin homogenates was determined by the method of Chatterjee *et al.* (1975) with the modification of Zwierz *et al.* (1999).

Statistics. Statistical analysis was conducted using STATISTICA StatSoft program. As data have normal distribution, Student's *t*-test was used to determine the significance of difference; $P<0.05$ was regarded as significant. When significant differences were detected, test groups were compared to controls using multiple comparison test. *T*-test was used to perform pairwise comparisons where indicated.

RESULTS

In 20 of 21 specimens we observed a significantly higher activity of HEX in cholesteatoma tissue compared with that in normal retroauricular skin. The release of HEX from the activated cells was approx. 1.08 to 5.57-fold higher as compared to controls. In one cholesteatoma specimen, the activity of HEX was 5.57-fold higher than in the skin. In four cholesteatoma specimens, the activity of HEX was 3.02 to 3.34-fold higher than in the skin and in four — 2.01 to 2.94. The remaining nine cholesteato-

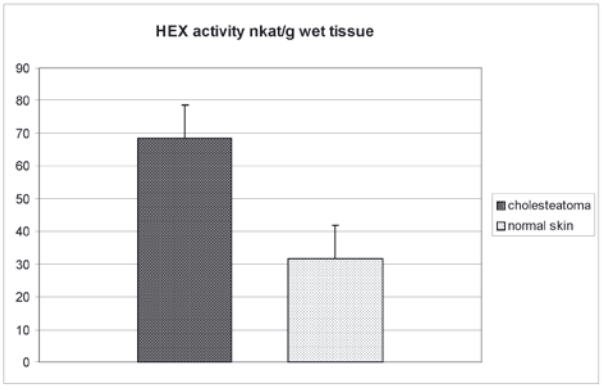

Figure 1. Parametrical statistics using Studen's *t*-test.

ma specimens had a 1.08 to 1.89-fold higher HEX activity compared with the skin. In only one cholesteatoma specimen the activity of HEX was a little decreased relative to the control specimen (21.53 nkat/g wet tissue in cholesteatoma, and 23.40 nkat/g wet tissue in the skin). The mean release of HEX from activated cells was 68.55±30.77 nkat/g wet tissue in cholesteatoma and 31.79±10.02 nkat/g wet tissue in skin specimens.

The descriptive statistics of cholesteatoma is shown in Fig. 1.

Pearson's coefficient of variation is shown in Fig. 2.

DISCUSSION

In the pathogenesis of cholesteatoma**,** numerous pathogenetic factors occur. The aggressiveness of cholesteatoma behavior is related to the process of bone destruction which is stimulated by a variety of factors, including inflammatory cells, e.g. macrophages, lymphocytes, epithelial cells and fibroblasts which release cytokines, e.g. IL-1α, IL-1β, IL-6, TNF-α, TNF-β. IL-1α and IL-1β stimulate the resorption of bone and induce synthesis of prostaglandins that play an important role in the process of bone destruction (Chole *et al.*, 1994; Dinarello, 1996; Kim *et al.*, 1996; Fox *et al.*, 2000). IL-6, TNF-α and TNF-β are elevated in cholesteatoma tissue and stimulate osteoclastic bone resorption *in vivo* and *in vitro* (Bujia *et al.*, 1996; Kato *et al.*, 1998; Shiwa *et al.*, 1998; Lam *et al.*, 2000). Lerner (1996) examined the relationship between bone resorption and cell proliferation after stimulation with TGF-β in a murine model. The author proved that TGF-β enhanced the release of HEX, however IL-1β is the most important for HEX activation (Berenbaum *et al.*, 2000). An increase in HEX activity was observed in several inflammatory diseases, such as rheumatoid arthritis, idiopathic juvenile arthritis, osteoarthritis and chronic glomerulonephritis (Bazzi *et al.*, 2002; Popko *et al.*, 2003; 2005; Liu *et al.*, 2004). Inflammation is always observed in the microenvironment of cholesteatoma. Owing to the essential role of *N*-acetyl-β-D-hexosaminidase (HEX) in inflammatory diseases it may be assumed that HEX is also crucial in the pathogenesis of cholesteatoma. In a preliminary study conducted in a smaller group (n=15 cholesteatomas and n=15 retroauricular skin specimens) we demonstrated that HEX was in the cholesteatoma specimens 1.5- to 5.5-fold higher than in the controls (Olszewska *et al.*, 2006). In the present study we enlarged the study and the control groups. The level of HEX was substantially increased compared to that in normal skin. However, we did not observe a correlation between the level of HEX activity and the site of cholesteatoma origin, the severity of inflammation or the length of disease history.

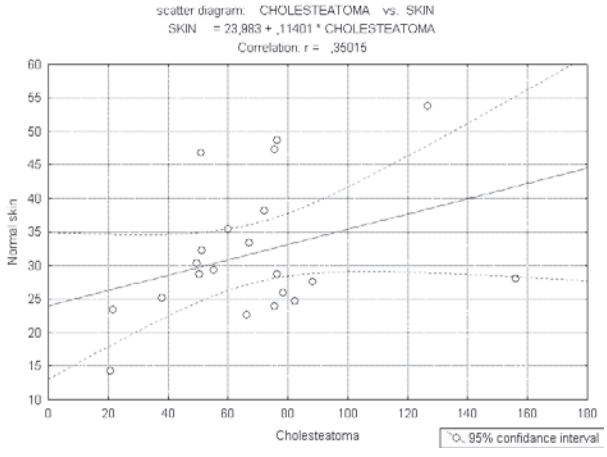

Figure 2. Scatter diagram of cholesteatoma *versus* skin. Pearson's coefficient of variation.

Chronically inflamed tissue adjacent to bone has been observed in several disorders such as rheumatoid arthritis, gingivitis and juvenile idiopathic arthritis (Goldring & Gravallese, 2000; Kinane & Lappin, 2001; Lee & Weinblatt, 2001; Popko *et al.*, 2003). Similarly, bone-resorbing activity was detected in cholesteatoma specimes and granulation tissue with and without middle ear cholesteatoma (Jung & Chole, 2002). However, the bone-resorbing activity was found to be more strongly produced by chronic otitis media with cholesteatoma than without it. Bone destruction is clinically associated with cholesteatoma. Shikhman *et al.* (2000) reported that during inflammation the stimulation of chondrocytes with interleukin-1β results in an increase in secretion of HEX. There are several different cells which are able to release HEX under inflammatory conditions. Baram *et al.* (2001) reported a marked increase in β-hexosaminidase release in response to direct contact of mast cells with activated T cell membranes. Among the several different proteins

released during inflammation, HEX is released after 4 h of incubation with activated T cell membranes and increases over time. In cholesteatoma the presence of a strong immune cell infliltrate releasing different cytokins and growth factors seems to be related with inflammation. HEX is produced in and released from leukocytes, neutral granulocytes, mast cells, synovial cells and chondrocytes (Berenbaum *et al.*, 2000). Most of these cells are found in cholesteatoma subepithelial connective tissue, the so-called perimatrix (Zwierz *et al.*, 1989). Mast cells originated from hematopoetic stem cells are capable of releasing cytokines and proteinases (Hebda *et al.*, 1993; Harvima *et al.*, 1994) and play a direct role in the pathogenesis of cholesteatoma. Albino *et al.* (1998) reported that the concentration of the mast cells in primary acquired cholesteatoma is sevenfold higher, in secondary acquired one — 3.8-fold higher, in congenital cholesteatoma 3.3-fold higher than in normal canal wall skin or postauricular skin. Mast cells have been observed in the pars flaccida throughout the subepithelial layer in acquired cholesteatoma and located within suprabasal layers of cholesteatoma epithelium which is not their normal location (Hochstrasser *et al.*, 1994). Mast cells are also involved in the pathologic events in cholesteatoma by means of releasing potent cytokines, including Il-1, IL-3, IL-4, IL-5, IL-6, TNF-α, bFGF, and also HEX (Elsafi *et al.*, 1992; Galli, 1993; Reed *et al.*, 1995). Inflammatory cells release cytokines involved in the regulation of immune and hematopoetic cells. Among them IL-1 and IL-6 are released which then stimulate the releasing of HEX. Jung and Chole (2002) emphasize that the infiltration of inflammatory cells and secretion of cytokines in cholesteatoma result in osteoclast activation. The inflammatory osteolysis induced by cholesteatoma leads to a common bone resorption pathway. One can expect obvious symptoms such as hearing loss, vestibular disfunction and facial paralysis in patients suffering from chronic otitis media with cholesteatoma.

By inference from the essential role of HEX in a variety of inflammatory diseases it may be assumed that HEX also takes part in cholesteatoma development. The molecular and cellular defects are observed in cholesteatoma. Those defects are manifested in the form of invasion, migration, hyperproliferation, aggressiveness and reccurrence. Our study has demonstrated an increased activity of HEX in cholesteatoma tissue. We will conduct futher studies with an enlarged study group. We will also search for the distribution of HEX isoenzymes and the correlation of the enzyme activity with the severity of inflammation. We believe that HEX may play an important role in this destructive lesion.

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