Expression of Matrix-Degrading Cysteine Proteinase Cathepsin K in Cholesteatoma

Torsten Hansen, M.D., Ronald E. Unger, Ph.D., Andreas Gaumann, M.D., Inga Hundorf, M.D., Jan Maurer, M.D., C. James Kirkpatrick, M.D., Ph.D., D.Sc., Jörg Kriegsmann, M.D., Ph.D.

Institute of Pathology (TH, REU, AG, IH, CJK, JK) and Department of Oto-Rhino-Laryngology (JM), Johannes Gutenberg-University, Mainz, Germany

Cholesteatoma is a nonneoplastic lesion of the middle ear space or mastoid that is histologically characterized by a progressive bone erosion of the ossicles and surrounding bone. Several matrix-degrading enzymes have been implicated as mediators of this bone erosion. Because the novel cysteine proteinase cathepsin K has been shown to play a central role in bone resorption, we examined the expression of this enzyme in tissue specimens of cholesteatoma. Tissue specimens of 9 patients with cholesteatoma were obtained during middle-ear surgery. Expression of cathepsin K mRNA was determined by RT-PCR using specific primers. Immunohistochemical analysis of cathepsin K protein expression in tissue sections was performed by using the streptavidin–alkaline phosphatase technique. Expression of both cathepsin K mRNA and protein was detected in areas affected by cholesteatoma, whereas specimens of nonaffected ear cartilage and surrounding tissue were not positive. In addition, cathepsin K was detected in numerous multinucleated giant cells, particularly osteoclasts at the site of bone degradation. In contrast, keratinized squamous epithelium was negative for cathepsin K. These data demonstrate that the matrix-degrading cysteine proteinase cathepsin K may be involved in bone erosion in cholesteatoma. Strong expression of this collagenolytic enzyme in osteoclasts suggests that these cells are mainly involved in cathepsin K–mediated bone destruction.

KEY WORDS: Bone degradation, Cathepsin K, Cholesteatoma, Osteoclasts.

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Address reprint requests to: Torsten Hansen, M.D., Institute of Pathology, University of Mainz, Langenbeckstrasse 1, D-55101 Mainz, Germany; e-mail: hansen@pathologie.klinik.uni-mainz.de; fax: 49-6131-176604.

Cholesteatoma is a nonneoplastic but potentially aggressive lesion characterized by the presence of keratin-producing squamous epithelium in the middle ear space or mastoid. Cholesteatoma may be congenital or acquired. Congenital cholesteatomas have been attributed to the proliferation of aberrant epithelial remnants. They are commonly found in children with no history of ear disease. They are localized behind an intact tympanic membrane. In contrast, most acquired cholesteatomas are associated with a perforated eardrum and a history of chronic otitis media, trauma, or surgical manipulation (1). Regardless of its origin, cholesteatoma is histologically composed of keratinaceous debris in a concentric fashion surrounded by keratinizing squamous epithelium that is also called matrix (1, 2). The underlining subepithelial inflammatory tissue (*i.e.*, perimatrix) consists of mononuclear and multinucleated macrophages, lymphocytes, neutrophils, fibroblasts, and endothelial cells (2, 3).

Progressive bone erosion of the ossicles and surrounding bone is one of the hallmarks of cholesteatomas. Originally, it was believed that this bone erosion was due to the pressure from within the tumor mass (1, 2). However, numerous studies have shown that collagenolytic enzymes are involved in the destruction of the bone matrix as well. A variety of matrix-degrading proteinases have been found, such as several types of matrix metalloproteinases (MMP), acid phosphatase, calpain Types I and II, and the cysteine proteinase cathepsin B (3–8). The recently discovered cysteine proteinase cathepsin K may play a crucial role in bone resorption because it degrades large amounts of the major bone collagen Type I as well as the bone matrix protein osteonectin (9, 10). Furthermore, it has been shown to be unique among mammalian proteinases because it is able to cleave the collagen molecule both inside and outside of the collagen helix (10). Deficiency of cathepsin K results in pyknodysostosis, an autosomal recessive osteochondrodysplasia (11). Expression of cathepsin K has been demonstrated in sev-

eral chronic inflammatory lesions associated with bone destruction such as osteomyelitis (12), rheumatoid arthritis (13), osteoarthritis (14), nodular tenosynovitis (15), and aseptic hip prosthesis loosening (16). In addition, specific exogenous inhibitors of cathepsin K have been shown to decrease the destruction of bone significantly (17). To provide new insights in the pathogenesis of bone destruction by cholesteatoma, we investigated the expression of cathepsin K mRNA and protein in tissue specimens of patients with cholesteatoma.

MATERIALS AND METHODS

Patient Selection and Tissue Preparation

Tissue samples of nine patients (all male; age range: 9–71 years) with an acquired cholesteatoma were obtained during middle-ear surgery. Examination of cathepsin K mRNA expression by RT-PCR was performed on six cholesteatoma specimens and on 2 specimens of ear cartilage and surrounding external ear canal skin that were macroscopically not affected by cholesteatoma. These samples were rapidly frozen in liquid nitrogen without prior fixation and were stored at −80° C. For immunohistochemical analysis of cathepsin K protein expression, cholesteatoma specimens ($n = 9$) and nonaffected tissue probes ($n = 2$) were fixed in 4% PBS-buffered formaldehyde and embedded in paraffin according to standard protocols.

RNA Isolation

Frozen tissue specimens were disrupted using a Polytron homogenizer (Kinematica, Littau, Switzerland). Total RNA was then isolated from homogenized specimens using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with the additional incubation step, using RNase-free DNase (Qiagen) for 15 minutes at room temperature, according to the manufacturer's instructions. RNA samples were stored at −80° C until used.

cDNA Synthesis (Reverse Transcription)

The RNA templates were incubated with a master mix (Qiagen) including RNase-free water, 1× reaction buffer, 0.5 mM dNTP Mix, 1.0 μM oligo-dT primer, RNase inhibitor (30 units/μL), and 4 U per reaction of Omniscript reverse transcriptase in a 1:2 dilution for 60 minutes at 37° C. After reverse transcription samples were stored at −20° C.

Polymerase Chain Reaction

The following specific primer pairs were applied for the subsequent PCR. β-actin: forward, 5' gac ctg act gac tac ctc atg a 3'; reverse, 5' agc att tgc cgt gga cga tgg ag 3'. Cathepsin K (13): forward, 5' ggc caa ctc aag aag aaa ac 3'; reverse, 5' gtg ctt gtt tcc ctt ctg g 3'. cDNA was amplified by PCR under standard conditions using *Taq* DNA-polymerase (Qiagen) and 5 pmol of both β-actin primers or 1 pm of both cathepsin K primers. In the case of PCR for β-actin, a 3-step protocol was performed with 35 cycles and an annealing temperature of 65° C, denaturation at 94° C for 2 minutes, extension at 72° C for 30 seconds, and a final extension at 72° C for 10 minutes (Gene Amplification System 2400; PE Applied Biosystems, Weiterstadt, Germany). Water was used instead of the cDNA as a negative control.

In the case of PCR for cathepsin K, a 3-step protocol was applied with 40 cycles and an annealing temperature of 60° C, denaturation at 94° C for 2 minutes, extension at 72° C for 30 seconds, and final extension at 72° C for 10 minutes. As a positive control for cathepsin K, we used cDNA of cathepsin K (13) that were cloned in an LXSN vector. Water was applied instead of the cDNA for negative control.

Reaction products were analyzed by running PCR samples on a 0.8% agarose gel (Life Technologies, Paisley, UK) and staining with ethidium bromide. The size of the amplification products was confirmed by a commercial molecular weight marker (100-bp DNA ladder; New England BioLab, Beverly, MA).

Immunohistochemistry

Four-micrometer paraffin sections were mounted on silane-coated slides, dewaxed, and rehydrated through alcohol gradients. Staining with monoclonal antibodies against human cathepsin K (clone 182–12G5; Chemicon, Temecula, CA) was performed using the streptavidine-alkaline phosphatase technique. As control, mouse immunoglobulins (Cymbus Biotechnology, Chandlers Ford, UK) were used to replace the primary antibodies. All steps were performed at room temperature. Slides were treated with an avidin–biotin blocking kit (Vector, Burlingame, CA), after which nonspecific binding of immunoglobulins was blocked by incubation with 4% nonfat dried bovine milk (Sigma, St. Louis, MO) and 2% normal horse serum (Vector) in Tris buffer (pH 7.6). Primary antibodies diluted 1:2000 and negative control (diluted 1:200) in Tris buffer (pH 7.6) were then incubated for 1 hour, followed by a 30-minute incubation with biotinylated horse anti-mouse IgG (Vector). Slides were then covered with streptavidine-conjugated alkaline phosphatase (1:50 dilution; DAKO, Glostrup, Denmark) for 30 minutes. The color reaction was performed using the new fuchsin method. Endogenous alkaline phosphatase was blocked by adding levamisole (Sigma) to the substrate solution. Color development was stopped by immersing the slides in Tris

buffer (pH 7.6). Finally, slides were counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany) and mounted in Kaiser's glycerol gelatin (Merck).

Slides were examined and photographed with a Leica Microscope DMRX (Leitz, Wetzlar, Germany). Microscopic evaluation of the slides was performed according to previously described methods (15). Cell types were monitored as nonaffected chondrocytes, surrounding connective tissue, and external ear canal skin (NT); keratinized squamous cholesteatoma epithelium, *i.e.*, matrix (MA); mononuclear cells of the perimatrix (MC); multinucleated giant cells of the perimatrix (MGC); and osteoclasts (OCL). The extent of binding was estimated by counting the number of positive cells per 5 high-power fields. Results were classified according to 4 categories: −, no staining; +, less than 20% of cells positive; ++, between 20 and 60% of cells positive; +++, more than 60% of cells positive.

RESULTS

Reverse Transcription Polymerase Chain Reaction

Gel electrophoresis revealed distinct bands for β-actin in all preparations of both cholesteatoma and nonaffected tissue specimens (data not shown). Four of six cholesteatoma mRNA samples were PCR-positive for cathepsin K. In contrast, both ear cartilage and surrounding external ear canal skin that were not affected by cholesteatoma showed no cathepsin K reaction (Fig. 1).

Immunohistochemistry

The results of the microscopic evaluation of immunohistochemistry for cathepsin K are summarized in Table 1. Cathepsin K could be detected in many multinucleated giant cells (MGC) of the perimatrix, whereas mononuclear cells (MC) in this area exhibited only moderate staining (Fig. 2). Furthermore, cathepsin K was not found in keratinized squamous epithelium of the matrix (Fig. 2). This proteinase was abundantly expressed in numerous osteoclasts at sites of bone destruction (Fig. 3). Nonaffected ear cartilage and surrounding external ear skin did not reveal cathepsin K expression (Fig. 4). Negative controls did not exhibit staining.

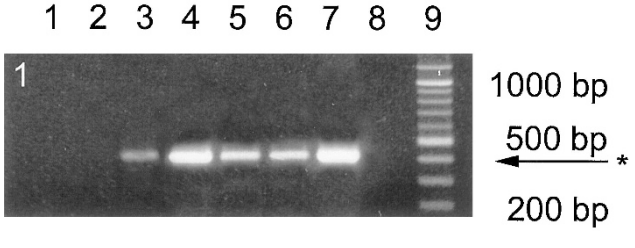

FIGURE 1. RT-PCR of cathepsin K of the cholesteatoma tissue probes with positive signals for cathepsin K in 4/6 probes (***Lanes 3–6***). Nonaffected tissue probes are negative (***Lanes 1*** and ***2***). **Lane 7** shows the positive control; **Lane 8** exhibits the negative control. **Lane 9,** 100-bp marker demonstrating the positive signals running at about 430 bp (***asterisk***).

DISCUSSION

Regardless of its origin, cholesteatoma is characterized by the destruction of ossicles and surrounding temporal bone (1, 2, 18). It has been previously believed that this bone erosion occurs because of the pressure from within the tumor mass (1, 2). However, many recent studies showed that a local inflammatory reaction, combined with proteolytic activity of numerous matrix-degrading enzymes, plays a major role (2–4, 6–8, 19). Among these enzymes, matrix metalloproteinases (MMP) including gelatinase A and B (MMP-2 and MMP-9), stromelysin-1 (MMP-3), and neutrophil collagenase (MMP-8) are of special interest (3, 7, 8). However, the cysteine proteinases are now considered to be critically involved in bone matrix degradation of several skeletal disorders (12–16, 20, 21). Moreover, collagenolytic cysteine proteinase cathepsin B has previously been shown in cholesteatoma (5, 6). Since the discovery of the cysteine proteinase cathepsin K, many studies have shown that this enzyme is of importance in bone erosion. To provide new insights into the pathogenesis of bone erosion in cholesteatoma, we investigated the expression of both cathepsin K mRNA and protein in tissue specimens of patients with cholesteatoma.

Using RT-PCR, we found positive signals for cathepsin K in 4 of 6 cholesteatoma tissues examined, whereas nonaffected tissue samples did not result in any signals. This may reflect the expression of cathepsin K mRNA in cholesteatoma, but not in the nonaffected areas. Furthermore, immunohistochemical staining demonstrated strong expression of cathepsin K protein in cholesteatoma specimens, particularly at sites of bone destruction. In contrast, nonaffected regions of ear cartilage and surrounding tissue were not positive for cathepsin K protein. Compared with the case of nonaffected tissues in cholesteatoma, both cathepsin K mRNA and protein were elevated. Therefore, we suggest that in cholesteatoma, expression of cathepsin K is up-regulated.

Several previous studies examined the role of inflammatory cytokines in the pathogenesis of cholesteatoma, including the osteoclastogenic tumor necrosis factor-alpha (TNF-α; 6, 8, 19, 22, 23). Serum levels of TNF-α were higher in patients who had more than 2 ossicles destroyed (22). TNF-α could be detected in the perimatrix as well as in osteoclasts at sites of bone degradation (23). More-

TABLE 1. Summary of Cathepsin K Expression by Immunohistochemistry

Patient No.	Age (y)	NT	MA	MC	MGC	OCL
1	9	∅	−	+	+++	+++
2	29	−	−	+	∅	∅
3	30	∅	−	+	++	++
4	30	∅	−	+	∅	∅
5	37	∅	−	+	+++	∅
6	45	∅	−	−	∅	+++
7	50	−	−	+	∅	∅
8	56	∅	−	++	+++	+++
9	72	∅	−	+	+++	+++

MA, matrix; MC, mononuclear cells of the perimatrix; MGC, multinucleated giant cells of the perimatrix; NT, nonaffected tissue; OCL, osteoclasts; ∅, cell type not present; −, no staining; +, <20% of cells positive; ++, between 20 and 60% of cells positive; +++, >60% of cells positive.

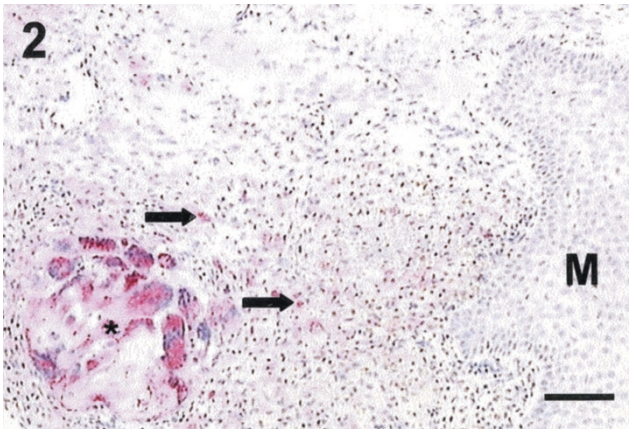

FIGURE 2. Strong expression of cathepsin K (***red***) in numerous MGC (***asterisk***) and in several MC (***arrows***) of the perimatrix. Squamous epithelium of the matrix (***M***) without staining for cathepsin K (original magnification, 200×; bar, 100 μm).

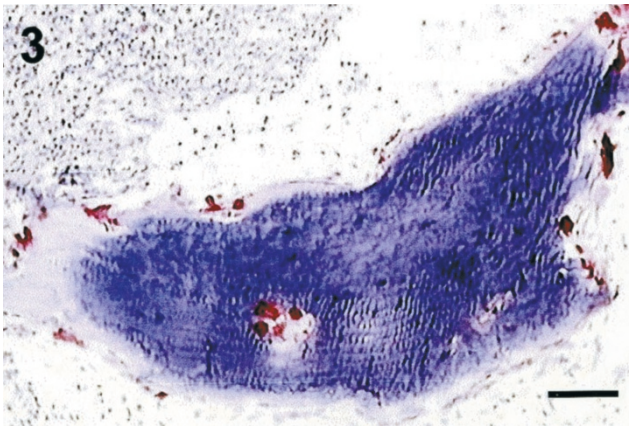

FIGURE 3. Strong expression of cathepsin K (***red***) in osteoclasts at the site of bone degradation (original magnification, 200×; bar, 100 μm).

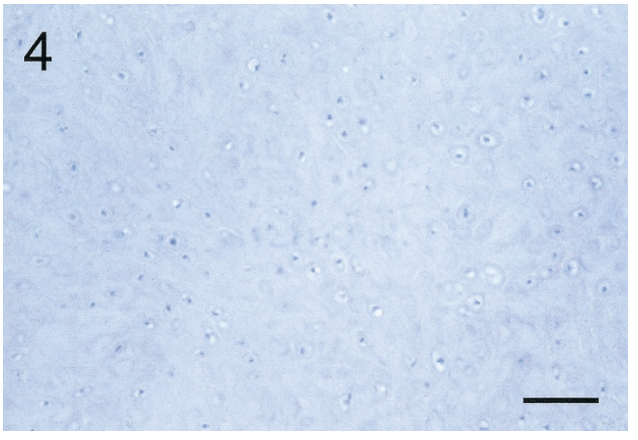

FIGURE 4. Tissue section of unaffected ear cartilage without any positivity for cathepsin K (original magnification, 200×; bar, 100 μm).

over, this cytokine was capable of stimulating the synthesis of several MMPs (8, 23). *In vitro*, TNF-α increased the levels of the osteoprotegerin ligand (OPGL), which has been strongly considered as the essential factor required for osteoclastogenesis (24, 25). Because Shalhoub *et al.* (26) found that in peripheral mononuclear osteoclast precursors, cathepsin K mRNA levels are elevated by treatment with OPGL, further studies should elucidate whether there might be interactions between cathepsin K and TNF-α and/or OPGL in cholesteatoma that might influence the up-regulation of cathepsin K.

Expression of cathepsin K has been shown in several cell populations like synovial fibroblasts (13), macrophages and smooth muscle cells in atheroma (27), various cell types of the lung (28), thyroid epithelial cells (29), and breast cancer cells (30). However, several studies indicate a predominant expression of cathepsin K in osteoclasts and osteoclast-like giant cells (12, 14, 15, 31–34). In the present study, staining of this proteinase was strong in osteoclasts at sites of bone destruction and in multinucleated giant cells of the perimatrix, whereas only few mononuclear cells in this region were detected. In contrast to studies investigating the MMPs (3, 7, 8), we did not find expression of cathepsin K in the keratinocytes of the matrix. Therefore, we conclude that in cholesteatoma, cathepsin K is predominantly expressed in multinucleated giant cells, mainly in osteoclasts at sites of bone degradation. These cells, but not the remaining mononuclear cells of the perimatrix and keratinocytes of the matrix, may be involved in the cathepsin K–mediated bone degradation in cholesteatoma.

In summary, our experiments demonstrate for the first time that both cathepsin K mRNA and protein are expressed in cholesteatoma tissue specimens, but not in nonaffected regions. Immunohistochemistry revealed that this proteinase was strongly expressed in multinucleated giant cells, particularly in osteoclasts at sites of bone destruction. We suggest that in cholesteatoma, cathepsin K is involved in bone destruction; that cathepsin K may be up-regulated; and that osteoclasts at sites of bone erosion may be predominantly involved in cathepsin K–mediated bone degradation. Finally, these data confirm the view that bone destruction in cholesteatoma is not only caused by the pressure from within the tumor mass but is particularly mediated by matrix-degrading proteinases.

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Book Review

Mendelson J, Howley PM, Israel MA, Liotta LA: Molecular Basis of Cancer, Second Edition, 752 pp, Philadelphia, W.B. Saunders ($150.00).

The first edition of this outstanding book was greeted in this journal, to quote, as "a clearly written text with many excellent tables, diagrams, illustrations, and photographs. . .An outstanding reference." The same statement could be applied to the second edition. A very good book was made even better through a thorough revision and by adding 11 new chapters.

With contributions from more than 50 leading cancer researchers from the best known American medical centers, the book covers the basic aspects of cancer. It is divided into four sections. The first section deals with cellular aspects of malignant transformation and related cell biology, such as cell cycle regulation or apoptosis; genetic aspects of cell function and growth; oncogenes; and signal transduction. The second section deals with the growth and spread of cancer. The third section deals with molecular pathogenesis of specific malignancies, usually subdivided by the organ system in which they arose. The fourth section deals with the molecular basis of cancer therapy.

All chapters are up-to-date, well-edited, and informative. The text is well-referenced and quite detailed but written so that it can be read meaningfully even by those who have a limited knowledge of the basic biology of cancer. Researchers who work in this field will find the chapters as a good summary and review of the current state of affairs.

This book will be most useful to clinicians in need of a good review of the basic biology of cancer, but it could be recommended to graduate and medical students as well as basic researchers.

Hazem Hallak
Thomas Jefferson University
Philadelphia, Pennsylvania