Communication

Cathepsin K Antisense Oligodeoxynucleotide Inhibits Osteoclastic Bone Resorption*

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Cathepsin K is a recently identified cysteine protease which is abundantly and selectively expressed in osteoclasts. To evaluate the contribution of cathepsin K to bone resorption processes, we investigated the effect of cathepsin K antisense phosphorothiate oligodeoxynucleotide (S-ODN) on the bone-resorbing activity of osteoclasts. Rabbit osteoclasts were cultured on dentine slices for 24 h in the presence or absence of antisense S-ODN on the bone-resorbing activity of osteoclasts. Rabbit osteoclasts were cultured on dentine slices for 24 h in the presence or absence of antisense S-ODN in a medium containing 100 nM Tfx™-50, polycationic liposome, as a carrier of the S-ODN. Uptake of the S-ODN by osteoclasts was confirmed microscopically using fluorescein-labeled S-ODN. The treatment with antisense significantly decreased the amount of cathepsin K protein in osteoclasts. The antisense inhibited the osteoclastic pit formation in a concentration-dependent fashion. At 10 μM the antisense reduced the total pit number and area and average pit depth by 46, 52, and 30%, respectively. The sense and mismatch S-ODNs, which were used as negative controls, had no effect on either the cathepsin K protein level or the pit formation. A nonspecific cysteine protease inhibitor, E-64, also reduced pit formation in a concentration-dependent manner with maximum reductions at 1 μM of 46, 48, and 35% in the above pit parameters. The inhibitory effect of the antisense almost equal to that of E-64 demonstrates that cathepsin K is a cysteine protease playing a crucial role in osteoclastic bone resorption.

Bone tissue is a composite matrix comprising of hydroxyapatite and fibrous proteins (mainly Type I collagen) and is constantly subjected to a cycle of bone resorption and bone formation (1). Bone resorption is mainly carried out by osteoclasts which are multinucleate giant cells. In osteoclastic bone resorption, demineralization, in which osteoclasts release protons to solubilize the inorganic salt (2), is followed by the degradation of the protein fibers with cysteine proteases (1, 3). The involvement of the cysteine proteases has been verified in both in vitro and in vivo studies showing that various types of cysteine protease inhibitors reduce bone resorption (1, 3–10). From studies based on substrate preference, inhibitor preference, and immunoreactivity, the cathepsins L and B were suggested to be responsible for osteoclastic bone resorption processes (8–14).

Recently, several research laboratories (including our own) have successfully cloned cDNAs for a novel cysteine protease, namely cathepsin K, from rabbit and human cDNA libraries (15–19), and its role in bone resorption has been the focus of recent attention. Human cathepsin K is highly and selectively expressed in osteoclasts (16–21); in fact its expression level is much greater than those of cathepsins B, L, and S (20, 21). Brümme et al. (22) and Bossard et al. (23) showed that cathepsin K expresses a potent proteolytic activity against Type I collagen. Sanneshige et al. (24) demonstrated that retinoic acid, a vitamin A metabolite, both up-regulates the gene expression of cathepsin K in osteoclasts and increases osteoclastic bone resorption. Moreover, Gelb et al. (25) reported that a deficiency of cathepsin K causes pyknodysostosis, which is an inherited sclerosing skeletal dysplasia. These findings strongly suggest that cathepsin K is critically involved in bone resorption, but the direct evidence to prove the role of cathepsin K in bone resorption is not provided.

For the first time, based on the investigation of the inhibitory effect of cathepsin K antisense S-ODN on osteoclast-mediated pit formation, we herein describe the significant role of cathepsin K in osteoclastic bone resorption.

EXPERIMENTAL PROCEDURES

Materials—Acid hematinolyn and E-64 (trans-epoxyoxysucinyl-l-leucylamido(4-guanidino)butane) were purchased from Sigma. α-Minimum essential medium (α-MEM) was obtained from Flow Laboratories (McLean, VA), and fetal bovine serum (FBS) was purchased from Life Technologies, Inc. Tfx™-50, a mixture of cationic lipid (N,N',N''-tetramethyl-N,N''-bis(2-hydroxyethyl)-2,3-dioleoyloxy-1,4-butanediammonium iodide) and 1-dioleoylphosphatidylethanolamine was purchased from Promega.

Oligodeoxynucleotide Synthesis—The cathepsin K antisense S-ODN was designed to be composed of 20 bases and targeted to the region that spans the translation start codon of the rabbit cathepsin K mRNA (Table I). The sense and three 4-base mismatches (MS1, MS2, and MS3) to the antisense S-ODN were also designed as negative controls (Table I). All these S-ODNs were synthesized (Sawaday, Inc., Tokyo, Japan) on an automated solid-phase nucleotide synthesizer and subsequently filtered-sterilized. The GC content was the same among these S-ODNs. A fraction of the synthesized S-ODNs were labeled with fluorescein at the 5'-ends.

Oligodeoxynucleotide Uptake by Osteoclasts—Long bones were taken from 10-day-old rabbits. After careful removal of the adhered soft tissue, the bones were minced with scissors for 10 min in a medium containing S-ODN, and the incubation continued for an additional 16 h.

1 The abbreviations used are: S-ODN, phosphorothiate oligodeoxynucleotide; MS, mismatched antisense; PBS, phosphate-buffered saline; FBS, fetal bovine serum; α-MEM, α-minimum essential medium; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.

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For the experiments examining the effect of E-64, the cells were incubated on dentine slices in α-MEM containing 5% FBS and various concentrations of E-64 with 10% CO₂ at 37 °C for 24 h.

**Measurement of Resorption Pits**—After the 24-h cultivation period, the cells were washed off the dentine slices with a rubber policeman, and the slices were washed in distilled water and stained with acid hematoxylin for 2 min. Total number and area of pits on each dentine slice were counted manually under a light microscope (Nikon, Tokyo, Japan) equipped with a micrometer (giving 625 μm² for each square) on the eyepiece (objective lens, ×20). For depth measurement, we selected 10 pits/slice having approximately the same area of 1000–1500 μm². A scanning surface profiler (TENCOR Instruments) was used to determine the deepest point of each pit. In this study, the pits that were produced by osteoclasts on dentine slices in the absence of S-ODNs or E-64 had an average area of 1460 ± 200 μm² (total pit area/pit number, n = 7) and average depth of 5.95 ± 0.16 μm (n = 70).

**Fluorescence Microscopy**—Fluorescence microscopy was used to detect the uptake of fluorescein-labeled cathepsin K antisense S-ODN by osteoclasts. Rabbit osteoclasts isolated according to the method of Tsuchiya et al. (26) were cultured in α-MEM containing 5% FBS, 100 nM TfxTM-50, and fluorescein-labeled cathepsin K antisense S-ODN for 24 h. After removing the incubation media from the culture plates at the end of cultivation period, the cells were washed three times with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde in PBS for 10 min at room temperature. The cells were dehydration in ethanol (40, 70, and then 100%) for 10 min on ice, rinsed in PBS, and photographed with a camera fitted to a X2F-EFD2 fluorescence microscope (Nikon, Japan).

**Western Blot Analysis**—The osteoclasts were cultured for 8 h in α-MEM containing 5% FBS and 100 nM TfxTM-50, and 24 h. After removing the incubation media from the culture plates at the end of cultivation period, the cells were washed three times with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde in PBS for 10 min at room temperature. The cells were dehydrated in ethanol (40, 70, and then 100%) for 10 min on ice, rinsed in PBS, and photographed with a camera fitted to a X2F-EFD2 fluorescence microscope (Nikon, Japan).

**Uptake of S-ODNs and Viability**—The uptake of the cathepsin K antisense S-ODN by osteoclasts was confirmed by microscopic. Fig. 1A shows a phase contrast photograph of osteoclasts loaded with fluorescein-labeled antisense S-ODN (10 μM). The intense fluorescence in both the cytoplasm and nuclei, as shown in Fig. 1B, indicates that the antisense S-ODN entered the osteoclasts and reached the inside of the nuclei by liposome-mediated transfection. The sense S-ODN and the three 4-base mismatches to the antisense S-ODN (MS1 S-ODN to MS3 S-ODN) were also introduced in a similar fashion (data not shown). At concentrations of S-ODNs up to 10 μM there were no cytotoxic effects on osteoclasts as determined by trypan blue staining (data not shown).

**Western Blot Analysis of Cathepsin K**—The antisense S-ODN-treated osteoclasts showed a marked decrease in the protein level of both the pro form (38 kDa) and the mature form (27 kDa) of cathepsin K, compared with untreated osteoclasts (Fig. 2A). On the other hand, the MS1 S-ODN treatment did not alter the protein levels of cathepsin K (Fig. 2B). No change of the protein levels of cathepsin K was observed with sense S-ODN, MS2 S-ODN, and MS3 S-ODN treatment (data not shown). These results clearly indicate that the antisense S-ODN was targeted to cathepsin K mRNA and inhibited the synthesis of cathepsin K protein.

**Pit Formation Assay**—The effect of the cathepsin K antisense S-ODN on osteoclastic bone resorption was investigated by the pit formation assay. By detailed examination of the pit...
number, area, and depth, the antisense S-ODN was found to decrease osteoclastic pit formation in a concentration-dependent manner (Fig. 3A). In a comparison with control values, the antisense S-ODN (10 μM) caused a maximum reduction of 46% (p < 0.05), 52% (p < 0.01), and 30% (p < 0.01) in total pit number and area and average depth, respectively. The effect of E-64, a potent and non-selective cysteine protease inhibitor, was also investigated by the pit formation assay. As shown in Fig. 3B, E-64 caused a concentration-dependent inhibition of the pit formation in the range of 10 nm to 1 μM. At 1 μM E-64 reduced the pit number, area, and depth by 46% (p < 0.05), 48% (p < 0.05), and 35% (p < 0.01), respectively. A higher concentration of E-64, 10 μM, did not show further inhibition in these pit parameters. Consequently, these results indicate that the inhibitory effect of the cathepsin K antisense S-ODN on osteoclastic bone resorption is almost equal to that of E-64.

The reductions of the total pit area by the antisense and E-64 were greatly attributed to the decrease in pit number, as the average area of each pit for the treatment with the antisense (1150 ± 70 μm²) or E-64 (1300 ± 170 μm²) was not significantly different from that in the control cultures (1460 ± 200 μm²). This result showed that the cathepsin K antisense and E-64 reduced the resorbed pit number and depth, but did not intrinsically affect the surface area of individual pits.

As a series of control experiments, we investigated the effects of the sense and mismatch (MS1, MS2, MS3) S-ODNs on the pit formation. All these S-ODNs did not show any inhibitory effects on pit formation at concentrations up to 10 μM (see Fig. 3C for a representative example). These results confirm the specificity of the cathepsin K antisense to the inhibition of pit formation.

**DISCUSSION**

Cathepsin K is a cysteine protease expressed abundantly and selectively in osteoclasts (15–21). Despite data detailing the potent proteolytic activity of cathepsin K for Type I collagen (22, 23), there has been no direct evidence to prove the function of cathepsin K in the osteoclastic bone resorption process. We have utilized antisense S-ODN to cathepsin K mRNA to demonstrate that cathepsin K plays a critical role in bone resorption.

The present study employed S-ODNs, which are more resistant to nucleases as compared with phosphodiesters (28), and the polycationic liposome, Tfx™-50, as a carrier. Employing 100 nM Tfx™-50, a concentration which did not manifest cytotoxicity, the uptake of S-ODNs by osteoclasts was microscopically confirmed using fluorescein-labeled S-ODNs (Fig. 1). In
the absence of Tfx™-50, the cathepsin K antisense S-ODN did not significantly inhibit osteoclastic bone resorption (data not shown). Therefore, the liposomal carrier system, employing Tfx™-50, functions effectively to introduce S-ODNs into the osteoclasts.

The cathepsin K antisense S-ODN caused a reduction in the cathepsin K protein levels in osteoclasts, which was correlated with a reduction in the pit formation, reaching a maximum inhibition of total pit number and area of approximately 50% and average pit depth of 30%. These effects were not observed for the sense S-ODN or the S-ODNs having 20% mismatch sequences. The cDNA sequences between rabbit cathepsin K and rabbit cathepsins L, B, and S share only 56, 51, and 56% homology, respectively, and the cathepsin K antisense used for the present study was found to be less than 70% homologous to any region on the antisense strands of the cDNAs for rabbit cathepsins L, B, and S. These results suggest that the antisense suppressed osteoclastic bone resorption by specifically blocking the expression of cathepsin K, while having no influence on the expression of cathepsins L, B, or S in osteoclasts. The nonspecific and potent inhibitor of cysteine proteases E-64 similarly reduced the osteoclastic pit formation by a maximum of approximately 50% in total pit number and area and of 35% in average pit depth. The present results are in good agreement with the previous reports of Delaisse et al. (6) and Kakegawa et al. (8) examining the effect of E-64 on pit formation (approximately 50% reduction in total pit number (6) and area (8)).

These results suggest that cysteine protease inhibitors or the reduction of cathepsin K cannot completely inhibit pit formation. Since initial pit formation is thought to be mediated by the demineralization of bone by acidification, cysteine protease most likely contributes to later stages of resorption. Indeed, Sundquist et al. (29) and Ohba et al. (30) observed that optimum concentrations of bafilomycin A₁, a specific vacuolar H⁺-ATPase inhibitor, almost completely inhibited bone resorption by blocking osteoclast proton transport. In this study, by the treatment with cathepsin K antisense or E-64, the surface area of bone resorbed was not intrinsically affected, while the pit depth was significantly reduced. This suggests that the inhibition of cysteine protease impairs continuation of the process of cavitation, without significantly affecting the initial pit formation. This suggestion is supported by the fact previously reported by Delaisse et al. (3, 4) that E-64 or another cysteine protease inhibitor, leupeptin, showed more inhibition of collagen degradation than calcium loss in resorbing mouse calvaria. However, both cathepsin K antisense and E-64 also reduced the pit number. It was confirmed that the present experimental conditions did not affect the viability of osteoclasts. One possible explanation for the reduction of the pit number is that there might be a relative increase in the number of undetectable indentations, where the initial process of demineralization has occurred, but true cavity formation has been constrained. Alternatively, a subpopulation of osteoclasts might enter a resting state by the interference of the collagen degradation.

In conclusion, cathepsin K antisense effectively inhibited the osteoclast-mediated pit formation by the selective suppression of the protein synthesis of cathepsin K. Although cathepsins L and B have been proposed to be important in osteoclastic bone resorption, it has recently been demonstrated that they are expressed at much lower levels in osteoclasts than cathepsin K

2 T. Inaoka, unpublished observations.