PROTEOLYSIS OF NATIVE BONE COLLAGEN BY CATHEPSIN K: QUANTITATIVE RELEASE OF THE CROSS-LINKED N-TELOPEPTIDE NEOEPITOPE (NTX).

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Introduction

The immunoassay of cross-linked N-telopeptides of type I collagen (NTx) in urine provides a sensitive index of osteoclast mediated bone resorption (1). Our studies have focused on identifying the osteoclast-specific protease(s) that cleave bone collagen to generate immunoreactive NTx (Figure 1). Of particular interest has been the role of the cysteine proteinase, cathepsin K, since there is growing evidence that cathepsin K is specific to osteoclasts and is essential for mineralized collagen degradation during bone resorption (2-4).

Using synthetic substrates, we have shown that cathepsin K cleaves a G-L peptide bond in the human α2(I) N-telopeptide sequence to release latent NTx epitope (5). We have also shown that cathepsin K rapidly and quantitatively releases immunoreactive NTx from denatured extracts of human bone collagen *in vitro* (5). The aim of this study was to determine if recombinant cathepsin K, without the cooperation of other proteases, was able to degrade demineralized bone matrix and release the NTx epitope.

Methods

The cDNA for human procathepsin K was expressed in the methylotropic yeast *Pichia pastoris* as an alpha-factor fusion construct. On methanol induction, the proenzyme was secreted into the culture medium. Autoprocessing of the proenzyme was initiated by reducing the pH of the medium to 5.0 by dialysis. The mature active enzyme was purified by anion exchange chromatography and its homogeneity demonstrated by SDS/polyacrylaminde gel electrophoresis (PAGE).

Decalcified matrix was prepared from mid-shaft, adult human cortical bone. Bone pieces were defatted in methanol/chloroform and powdered under liquid nitrogen in a SPEX mill. The bone powder was demineralized in three changes of 0.1 M HCl at 4°C, washed with distilled water and freeze-dried.

Demineralized bone was incubated with recombinant cathepsin K (100 substrate : 1 enzyme, w/w) in 50 mM sodium acetate, 2 mM EDTA, 2 mM DTT, pH 5.0. Immunoreactive NTx was measured using the Osteomark® assay kit (Ostex International, Inc., Seattle, WA), a competition ELISA based on a previously described monoclonal antibody 1H11 (1).

Results and Discussion

The core peptide recognized by monoclonal antibody 1H11 is the α2(I) N-telopeptide sequence QYDGKGVG where Q is pyroglutamic acid and K is part of a complex cross-link (Figure 1). The C-terminal GVG is an essential feature of the epitope, and results from proteolytic cleavage of a G-L peptide bond.

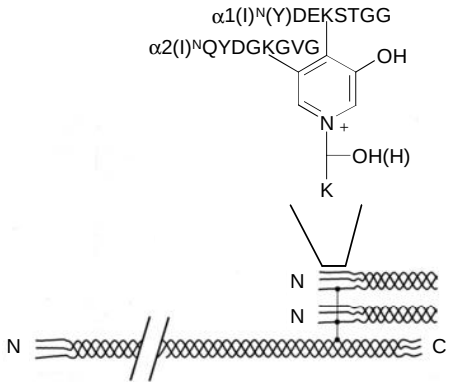

Figure 1: Molecular origin of the NTx analyte in type I collagen fibrils. Urinary NTx consists of telopeptide fragments still attached to a pyridinoline cross-linking residue.

Aliquots of demineralized bone powder were incubated with cathepsin K at two temperatures, 25°C and 37°C. At 25°C initial triple-helical fragments will remain in a native conformation whereas at 37°C they will be denatured. Bacterial collagenase which quantitatively generates immunoreactive NTx (1) was run as a positive control. At 37°C, cathepsin K released latent NTx epitope from the bone collagen (Figure 2) and completely dissolved the bone particles in 48 h. The yield of immunoreactive NTx and the timecourse for its release were similar to those generated by bacterial collagenase. At 25°C, cathepsin K released about 20% of the epitope generated at 37°C or by bacterial collagenase.

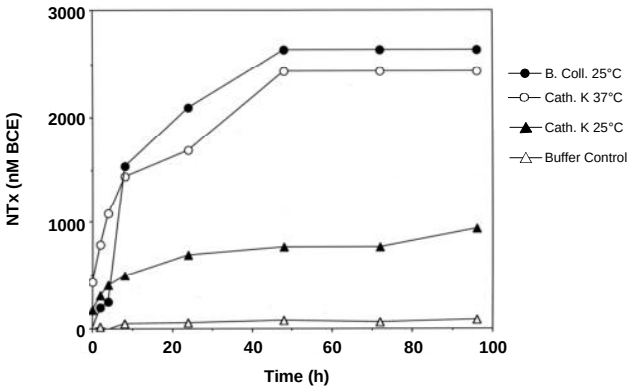

Figure 2: Timecourse for the release of immunoreactive NTx from bone collagen by cathepsin K or bacterial collagenase.

We have shown that cathepsin K, without the activity of other proteases, can solubilize human bone matrix and quantitatively release the NTx epitope. Given the specificity of cathepsin K to osteoclasts (4), this observation provides a molecular explanation for the responsiveness and specificity of NTx as a clinical marker of bone resorption, and for the observed ability of osteoclasts to generate NTx quantitatively when resorbing bone *in vitro* (6). Further work is underway to purify immunoreactive NTx from cathepsin K digests of bone collagen and to identify the key cleavage sites in the telopeptide and helical domains. Preliminary sequence data suggest that the main 1H11-immunoreactive peptides comprise the α2(I) chain octapeptide linked to a short segment of the α1(I) N-telopeptide and a fragment of the α1(I) or α2(I) helix. The finding that cathepsin K alone can degrade cross-linked, fibrillar bone collagen to low molecular weight peptides was confirmed by SDS/PAGE and reverse-phase-HPLC analysis of the digestion products. The findings support the concept that cathepsin K is the critical protease involved in osteoclast-mediated bone resorption.

References

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