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MOLECULAR CLONING OF HUMAN cDNA FOR CATHEPSIN K: NOVEL CYSTEINE PROTEINASE PREDOMINANTLY EXPRESSED IN BONE+

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SUMMARY We have previously cloned a rabbit cDNA clone (OC-2) from an osteoclast cDNA library by the differential screening. OC-2 was found to encode a novel cysteine proteinase, tentatively called cathepsin K, which is predominantly expressed in osteoclasts. By use of a rabbit OC-2 fragment as a probe, its human counterpart was cloned from a cDNA library of osteoarthritic hip bone. The cloned human cDNA (hOC-2) encoded a protein of 329 amino acid residues and its deduced amino acid sequence showed 94% homology to rabbit cathepsin K. Multiple alignment of amino acid sequences of human cathepsins B, H, L, S and K showed the highest homology of cathepsin K to cathepsin S 48%. Northern blot analysis showed that cathepsin K mRNA is expressed at high levels in some osteoarthritic hip bones and at a very high level in osteoclastoma compared to very low levels in other tissues. These results suggest that cathepsin K is closely involved in human osteoclastic bone resorption.

Bone formation and bone resorption are coupled under strict control in bone metabolism. In bone resorption osteoclasts degrade mineralized bone matrix. Bone resorption takes place upon contact of the ruffled border of osteoclasts with bone, which results in a sealed extracellular acidified microenvironment (1, 2). This acidified environment is thought to be necessary for activation of lysosomal enzymes which have acidic pH optima, as well as for solubilization of bone minerals. It has been shown by *in vitro* studies that at acidic pH lysosomal cysteine proteinases, cathepsins B, H, L and S efficiently degrade type I collagen, the most abundant extracellular protein in bone matrix (3-6). Immunohistochemical studies revealed intense staining and localization of cysteine proteinases, including cathepsin B, H and L, in osteoclasts, suggesting a significant contribution of the cysteine proteinases to the degradation of bone matrix proteins.

+The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X82153 HSOC2RNA for human cathepsin K.

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The importance of the cathepsins in osteoclastic bone resorption was further confirmed by inhibitor studies demonstrating that several inhibitors of cysteine proteinases, *e.g.*, leupeptin and E-64, block bone resorption both *in vitro* and *in vivo* (7-10). Despite these studies, it has not been conclusively determined which cysteine proteinases play the most important role in the degradation of bone matrix proteins.

Recently we succeeded in establishing a convenient method to isolate a large number of relatively homogenous osteoclasts from rabbits (11), from which we extracted a sufficient amount of osteoclast mRNA to construct a cDNA library. By differential screening of the osteoclast cDNA library with a reference cDNA library derived from spleen cells or from alveolar macrophages, we isolated three clones that were predominantly expressed in rabbit osteoclasts. A homology search of nucleotide sequences of the three cDNAs revealed two of them to be known proteins, *i.e.*, osteopontin and matrix metalloproteinase 9 (11, 12). The third clone, OC-2, was found to encode a novel protein that shows homology ranging from 20 to 60% to cathepsins B, H, L and S (13). The predominant expression of this cathepsin, tentatively called cathepsin K, in osteoclasts strongly suggested its close involvement in the degradation of bone matrix proteins.

It has been thought for some time that a unique cysteine proteinase closely associated with osteoclastic bone resorption may have pathogenic significance in human disorders related to bone, *e.g.*, osteoporosis and osteoarthritis (14). We, therefore, undertook cDNA cloning of human cathepsin K for further studies concerning its physiological function and pathological relevance in osteoclastic bone resorption. We report herein the complete nucleotide sequence of a cDNA coding for human cathepsin K and its tissue distribution, confirming a predominant expression in osteoclasts from giant cell tumor of bone.

MATERIALS AND METHODS

cDNA cloning. Total RNA was isolated from osteoarthritic hip bones by the acidic guanidium isothiocyanate-phenol-chloroform extraction method (15). The poly(A)$^+$ RNA was isolated by batch purification on oligo(dT)-cellulose type 77F (Pharmacia) (16). A cDNA library of human bone was constructed using oligo(dT) priming technique in λgt11 as described previously (17). The 1.2 kb *Eco*RI fragment containing the open reading frame and 200 bp of 3'-noncoding region of rabbit OC-2 cDNA was used as a DNA probe to screen the human bone cDNA library. The rabbit OC-2 cDNA fragment was radiolabeled by a Random Primer DNA Labeling kit (Boehringer Mannheim) and [α-^{32}P]dCTP (NEN-DuPont, 3000 Ci/mmol). The radiolabeled probe was used to screen 2.5 x 10^5 plaques from the human bone cDNA library by the standard plaque hybridization procedure (16). The replica filters were washed three times with 0.2 x SSC, 1% SDS for 20 min at 50°C and exposed to X-OMAT AR films (Kodak) for over night at -70°C with intensifying screens. Twenty-three positive clones were isolated and λDNA was prepared from them. A 1.6 kb cDNA insert from the longest recombinant phage DNA was subcloned into the *Eco*RI site of pBluescript SK$^-$ (Stratagene). The plasmid was designated as pHC-9.

DNA sequencing and homology analyses. The plasmid pHC-9 was purified with a plasmid purification kit (Qiagen). The cDNA insert was sequenced in both directions using a Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems Inc.). Nucleotide and amino acid sequences were analyzed with the DNASIS software (Hitachi Software Engineering). Homology search was carried out against the GenBank, EMBL, Swiss Prot and PIR data bases. Multiple alignment of amino acid sequences was manually carried out by the aid of the DNASIS software.

Northern hybridization analysis. Poly(A)$^+$ RNAs from human osteoarthritic hip bones and human osteoclastoma were isolated by the same method as described above. Two μg each of the

poly(A)$^+$ RNA was separated on a 1.2% formamide gel and transferred to the Byodyne A transfer membrane (Pall) (16). A Northern blot with 2 μg of poly(A)$^+$ RNA from eight different human tissues (MTN blot) was purchased from Clonetech. The cDNA insert of pHC-9 was ^{32}P-labeled by the same method as described above. Hybridization was performed by the standard procedure (16). The blots were washed three times with 0.2 x SSC, 1% SDS for 20 min at 60°C and analyzed quantitatively with a BAS-2000 Bioimage Analyzer (Fuji film). Signal intensities for the cloned cDNA were normalized with signals obtained in the corresponding samples with a cDNA probe of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) to analyze the expression of the cloned cDNA in human tissues.

RESULTS AND DISCUSSION

The full length cDNA encoding human cathepsin K, which was designated as hOC-2, was obtained by screening of a human osteoarthritic hip bone cDNA library with a probe of the 1.2 kb *Eco*RI fragment of rabbit OC-2. The nucleotide and deduced amino acid sequences of hOC-2 are shown in Fig. 1. The longest open reading frame identified in this cDNA encodes a protein of the identical number of amino acid residues, 329 amino acids, to rabbit cathepsin K and a predicted molecular weight of 37,000. All cathepsins so far structurally well characterized have been found to be glycosylated. Human cathepsin K also has two putative N-glycosylation sites, which are indicated by arrowhead in Fig. 1, in the middle of the coding region. It is well known that two highly conserved regions are present in the N-terminal and C-terminal regions of all mature cysteine proteinases among mammalian cathepsins and cathepsin-like cysteine proteinases in plants and fungi (18, 19). Two amino acid sequences, CGSCWAF at the amino acid positions 136-142 and HWIIKNSW at the positions 291-298 (underlined in Fig. 1) show good agreements with the consensus sequences of these conserved regions, CGSCWAF (the N-terminal region) and YWLVKNSW (the C-terminal region), respectively. The 3'-untranslated region is 550 nucleotides long and has a canonical polyadenylation signal AATAAA (underlined in Fig. 1), located near the 3'-terminus of the cDNA.

The deduced amino acid sequence of human cathepsin K shows 94% homology to its rabbit counterpart, indicating a very high conservation of the primary structure between human and rabbit. In nucleotide sequence, the coding region of hOC-2 shares also a very high identity of 93% with rabbit OC-2. The 3'-noncoding region of hOC-2 shows a slightly lower but still high homology of 77% with that of OC-2.

Searching the Swiss Prot and PIR data base with the deduced amino acid sequence of human cathepsin K revealed significant sequence identity with members of cysteine proteinase family including other cathepsins. No amino acid sequence of a cysteine proteinase derived from rabbit, except for our rabbit cathepsin K (OC-2), was detected in the search. On the other hand, four deduced amino acid sequences of human cathepsins (20-23) were found to show moderate to high homology to human cathepsin K, which allowed us to compare five sequences of the human cathepsins, *i.e.*, cathepsins B, H, L, S and K, in multiple alignment (Fig. 2). According to this multiple alignment, the sequence of human cathepsin K was found to be 25%, 35%, 42% and 48% identical with those of human cathepsin B, H, L and S, respectively. Human cathepsin S shares the highest sequence identity with human cathepsin K over the entire deduced amino acid sequence.

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   1 GAAACAAGCACTGGATTCCATATCCCACTGCCAAAACCGCATGGTTCAGATTATCGCTAT
  61 TGCAGCTTTCATCATAATACACACCTTTGCTGCCGAAACGAAGCCAGACAACAGATTTCC

 121 ATCAGCAGGATGTGGGGGCTCAAGGTTCTGCTGCTACCTGTGGTGAGCTTTGCTCTGTAC
   1           M  W  G  L  K  V  L  L  L  P  V  V  S  F  A  L  Y

 181 CCTGAGGAGATACTGGACACCCACTGGGAGCTATGGAAGAAGACCCACAGGAAGCAATAT
  18  P  E  E  I  L  D  T  H  W  E  L  W  K  K  T  H  R  K  Q  Y

 241 AACAACAAGGTGGATGAAATCTCTCGGCGTTTAATTTGGGAAAAAAACCTGAAGTATATT
  38  N  N  K  V  D  E  I  S  R  R  L  I  W  E  K  N  L  K  Y  I

 301 TCCATCCATAACCTTGAGGCTTCTCTTGGTGTCCATACATATGAACTGGCTATGAACCAC
  58  S  I  H  N  L  E  A  S  L  G  V  H  T  Y  E  L  A  M  N  H

 361 CTGGGGGACATGACCAGTGAAGAGGTGGTTCAGAAGATGACTGGACTCAAAGTACCCCTG
  78  L  G  D  M  T  S  E  E  V  V  Q  K  M  T  G  L  K  V  P  L

 421 TCTCATTCCCGCAGTAATGACACCCTTTATATCCCAGAATGGGAAGGTAGAGCCCCAGAC
  98  S  H  S  R  S  N  D  T  L  Y  I  P  E  W  E  G  R  A  P  D
                    ▲

 481 TCTGTCGACTATCGAAAGAAAGGATATGTTACTCCTGTCAAAAATCAGGGTCAGTGTGGT
 118  S  V  D  Y  R  K  K  G  Y  V  T  P  V  K  N  Q  G  Q  C  G

 541 TCCTGTTGGGCTTTTAGCTCTGTGGGTGCCCTGGAGGGCCAACTCAAGAAGAAAACTGGC
 138  S  C  W  A  F  S  S  V  G  A  L  E  G  Q  L  K  K  K  T  G

 601 AAACTCTTAAATCTGAGTCCCCAGAACCTAGTGGATTGTGTGTCTGAGAATGATGGCTGT
 158  K  L  L  N  L  S  P  Q  N  L  V  D  C  V  S  E  N  D  G  C
                ▲

 661 GGAGGGGGCTACATGACCAATGCCTTCCAATATGTGCAGAAGAACCGGGGTATTGACTCT
 178  G  G  G  Y  M  T  N  A  F  Q  Y  V  Q  K  N  R  G  I  D  S

 721 GAAGATGCCTACCCATATGTGGGACAGGAAGAGAGTTGTATGTACAACCCAACAGGCAAG
 198  E  D  A  Y  P  Y  V  G  Q  E  E  S  C  M  Y  N  P  T  G  K

 781 GCAGCTAAATGCAGAGGGTACAGAGAGATCCCCGAGGGGAATGAGAAAGCCCTGAAGAGG
 218  A  A  K  C  R  G  Y  R  E  I  P  E  G  N  E  K  A  L  K  R

 841 GCAGTGGCCCGAGTGGGACCTGTCTCTGTGGCCATTGATGCAAGCCTGACCTCCTTCCAG
 238  A  V  A  R  V  G  P  V  S  V  A  I  D  A  S  L  T  S  F  Q

 901 TTTTACAGCAAAGGTGTGTATTATGATGAAAGCTGCAATAGCGATAATCTGAACCATGCG
 258  F  Y  S  K  G  V  Y  Y  D  E  S  C  N  S  D  N  L  N  H  A

 961 GTTTTGGCAGTGGGATATGGAATCCAGAAGGGAAACAAGCACTGGATAATTAAAAACAGC
 278  V  L  A  V  G  Y  G  I  Q  K  G  N  K  H  W  I  I  K  N  S

1021 TGGGGAGAAAACTGGGGAAACAAAGGATATATCCTCATGGCTCGAAATAAGAACAACGCC
 298  W  G  E  N  W  G  N  K  G  Y  I  L  M  A  R  N  K  N  N  A

1081 TGTGGCATTGCCAACCTGGCCAGCTTCCCCAAGATGTGACTCCAGCCAGCCAAATCCATC
 318  C  G  I  A  N  L  A  S  F  P  K  M  *

1141 CTGCTCTTCCATTTCTTCCACGATGGTGCAGTGTAACGATGCACTTTGGAAGGGAGTTGG
1201 TGTGCTATTTTTGAAGCAGATGTGGTGATACTGAGATTGTCTGTTCAGTTTCCCCATTTG
1261 TTTGTGCTTCAAATGATCCTTCCTACTTTGCTTCTCTCCACCCATGACCTTTTTCACTGT
1321 GGCCATCAGGACTTTCCCTGACAGCTGTGTACTCTTAGGCTAAGAGATGTGACTACAGCC
1381 TGCCCCTGACTGTGTTGTCCCAGGGCTGATGCTGTACAGGTACAGGCTGGAGATTTTCAC
1441 ATAGGTTAGATTCTCATTCACGGGACTAGTTAGCTTTAAGCACCCTAGAGGACTAGGGTA
1501 ATCTGACTTCTCACTTCCTAAGTTCCCTTCTATATCCTCAAGGTAGAAATGTCTATGTTT
1561 TCTACTCCAATTCATAAATCTATTCATAAGTCTTTGGTACAAGTTTACATGATAAAAAGA
1621 AATGTGATTTGTCTTCCCTTCTTTGCACTTTTGAAATAAAGTATTTATC
```

Fig. 1. Nucleotide sequence and deduced amino acid sequence of hOC-2. The numbers indicate the positions of the first nucleotide and amino acid in each line. An asterisk denotes the stop codon and putative N-glycosylation sites (N-X-S/T) are indicated by arrowheads. Two consensus sequences among cysteine proteinases and poly(A) adenylation signal are underlined.

The multiple alignment allowed identification of the putative cleavage sites of the signal peptide and of the pro-form of cathepsin K to give the mature proteinase, which are indicated by arrowheads in Fig. 2. The putative signal sequence consisting of 15 amino acid residues contains a positively charged amino acid (Lys^5) close to the initial methionine and a stretch of hydrophobic

```
                                        ▼
cathepsin B       1 MWQLWASLCC LLVL-----A NA-------R SRPSFHPVSD ELVNYVNKRN
cathepsin H       1 MWATLPLLCA GAWLLGVPVC GA-----AEL SVNSLEKFHF KSWMSKHRKT
cathepsin L       1 MNPTLILAAF CLGI-----A SATLTFDHSL EAQWTKW-KA MHNRLY-GMN
cathepsin S       1 MKRLVCVLLV CSS------A VAQLHKDPTL DHHWHLW-KK TYGKQYKEKN
cathepsin K       1 MWG-LKVLLL PVV------S FA-LYPEEIL DTHWELWKKT HRKQYNNKVD
consensus           M                     A

cathepsin B      39 TTWQAGHNFY NVDMSYLKRL CGTFLGGPKP PQRVMFTEDL K---------
cathepsin H      46 YSTEEYHHRL QTFASNWRKI NAHNNGNHTF KMALNQFSDM SFAEIKHKYL
cathepsin L      44 EEGWRRAVWE KNMKMIELHN QEYREGKHSF TMAMNAFGDM TSEEFRQVMN
cathepsin S      44 EEAVRRLIWE KNLKFVMLHN LEHSMGMHSY DLGMNHLGDM TSEEVMSLTS
cathepsin K      43 EI-SRRLIWE KNLKYISIHN LEASLGVHTY ELAMNHLGDM TSEEVVQKMT
consensus                                    G           D
                                        ▼
cathepsin B      80 ---------- ---------- ---LPASFDA REQWPQCPTI K-EIRDQGSC
cathepsin H      96 WSE-PQNCSA TKSN-YL-RG TGPYPPSVD- ---WRKKGNF VSPVKNQGAC
cathepsin L      94 G---FQNRKP RKGKVFQEPL FYEAPRSVD- ---WREKGYV T-PVKNQGQC
cathepsin S      94 SL--RVPSQW QRNITYKSNP NRILPDSVD- ---WREKGCV T-EVKYQGSC
cathepsin K      92 GLKVPLSHSR SNDTLYIPEW EGRAPDSVD- ---YRKKGYV T-PVKNQGQC
consensus                                  P S D                 QG C
                 ▼
cathepsin B     103 GSCWAFGAVE AISDRICIHT NAHVSV-EVS AEDLLTCCGS MCGDGCNGGY
cathepsin H     139 GSCWTFSTTG ALESAIAIAT GKMLSLAEQQ LVDCA-QDFN NYGCQGGLPS
cathepsin L     136 GSCWAFSATG ALEGQMFRKT GRLISLSEQN LVDCS-GPQG NEGCNGGLMD
cathepsin S     137 GACWAFSAVG ALEAQLKLKT GKLVTLSAQN LVDCSTEKYG NKGCNGGFMT
cathepsin K     137 GSCWAFSSVG ALEGQLKKKT GKLLNLSPQN LVDCV---SE NDGCGGGYMT
consensus           G CW F     A        T              D          G

cathepsin B     155 PAEAWNFWTR KGLVSGGLYE SHVGCRPYSI PPCEHHVNGS RPPCTGEGDT
cathepsin H     188 QAFEYILYNK -GIMGEDTY- ------PYQ- --------GK DGYCKFQPGK
cathepsin L     185 YAFQYVQDNG -GLDSEESY- ------PYE- --------AT EESCKYNPKY
cathepsin S     187 TAFQYIIDNK -GIDSDASY- ------PYK- --------AM DQKCQYDSKY
cathepsin K     184 NAFQYVQKNR -GIDSEDAY- ------PYV- --------GQ EESCMYNPTG
consensus            A          G      Y        PY                C

cathepsin B     205 PKCSKICEPG YSPTYKQDKH YGYNSYSVSN SEKDIMAEIY KNGPVEGAFS
cathepsin H     221 A-------IG FVKDVANITI Y--------D EE-AMVEAVA LYNPVSFAFE
cathepsin L     218 SVA---NDTG FVDIPKQE-- ---------- --KALMKAVA TVGPISVAID
cathepsin S     220 RAA------- -TCSKYTELP YGREDVLKEA VANKGPVSVG VDARHPSFFL
cathepsin K     217 KAA------- -KCRGYREIP EGNEKALKRA VARVGPVSVA IDASLTSFQF
consensus                                          ▼
cathepsin B     255 VY-SDFLLYK SGVYQHVTGE ---MMGGHAI RILGWGVE-- --NGTPYWLV
cathepsin H     255 VT-QDFMMYR TGIYSSTSCH KTPDKVNHAV LAVGYG-E-- -KNGIPYWIV
cathepsin L     251 AGHESFLFYK EGIYFEPDCS --SEDMDHGV LVVGYGFEST ESDNNKYWLV
cathepsin S     262 YRSGVYY-EP SCTQ------ ----NVNHGV LVVGYGDL-- --NGKEYWLV
cathepsin K     259 YSKGVYYDES CNSD------ ----NLNHAV LAVGYGIQ-- -KGNK-HWII
consensus                                        H      G G           W

cathepsin B     297 ANSWNTDWGD NGFFKILRG- QDHCGIESEV VAGIPRTDQY WEKI
cathepsin H     300 KNSWGPQWGM NGYFLIERGK -NMCGLAACA SYPIPLV
cathepsin L     299 KNSWGEEWGM GGYVKMAKDR RNHCGIASAA SYPTV
cathepsin S     297 KNSWGHNFGE EGYIRMARNK GNHCGIASFP SYPEI
cathepsin K     295 KNSWGENWGN KGYILMARNK NNACGIANLA SFPKM
consensus            NSW    G   G            CG
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Fig. 2. Multiple alignment of amino acid sequences of human cathepsins. The sequences of human cathepsins are derived from the Swiss Prot and PIR data bases. The sequences were aligned to achieve maximal homology. Arrowheads indicate the putative cleavage sites to release the signal peptide and mature enzyme. The arrows indicate active site residues, cysteine and histidine. Numbers indicate the position of the first amino acid in each line.

amino acids that is terminated with the consensus alanine residue (Ala^{15}). The N-termini of the mature forms of the cathepsins are precisely aligned with the consensus proline, serine and aspartate residues, as shown in Fig. 2. The pro-form of human cathepsin K, therefore, is most likely cleaved at the peptide bond between Arg^{114} and Ala^{115} to render 215 residues of the mature enzyme. The molecular weights of the putative pro- and mature forms of human cathepsin K are calculated, without sugar chains, to be 35,300 and 23,500, respectively. One of two putative N-linked glycosylation sites discussed above is to be located in the region of the pro-sequence and the other in the mature proteinase. Two amino acids, cysteine and histidine, known to be in the active sites of cysteine proteinase were also identified by the multiple alignment as Cys^{139} and His^{276}, which are shown by arrows in Fig 2. The highest degree of homology among the human cathepsins was observed in the vicinity of Cys^{139}, which is identical to the N-terminal region highly conserved in the cysteine proteinase family discussed previously.

To investigate the expression of the cathepsin K gene in human tissues, Northern blots, which contained 2 μg each of poly(A)$^+$ RNA from heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, osteoarthritic hip bones and osteoclastoma, were hybridized with the full-length hOC-2 cDNA (Fig. 3). The Northern hybridization revealed the presence of a single transcript of about 2.0 kb at very low levels in many tissues. The osteoclastoma, however, showed an extremely high expression of cathepsin K (Fig. 4), probably reflecting a high population of osteoclast in the tumor tissue. The predominant occurrence of cathepsin K in human osteoclasts seems to indicate a close involvement of this enzyme in human osteoclastic bone resorption. It should be also noted that two samples of human osteoarthritic bone exhibited a very high expression of the cathepsin K gene (Fig. 4), suggesting that cathepsin K participates, at least partially, in the disorder of bone remodeling.

We have started the study to characterize the enzyme protein of cathepsin K. The presence of this enzyme in osteoclasts has been already confirmed by a Western blotting analysis and results of the study will be reported elsewhere.

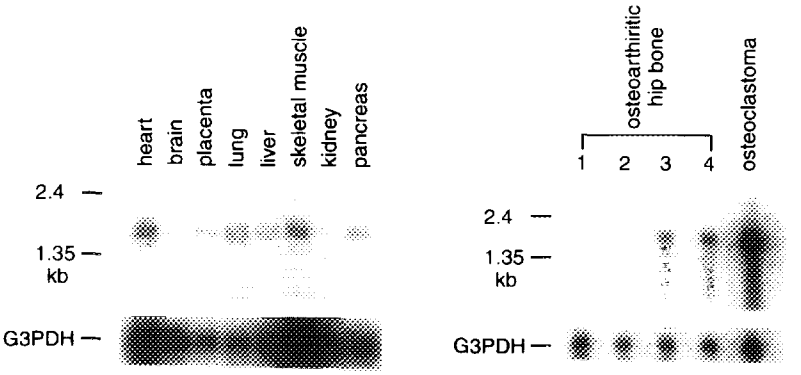

Fig. 3. Northern blot analysis of cathepsin K. Two μg each of the poly(A)$^+$ RNA from osteoarthritic hip bones from four different patients and osteoclastoma was electrophoresed, blotted onto a nylon membrane and hybridized with ^{32}P-labeled hOC-2 cDNA or human G3PDH cDNA fragment. Positions of the molecular markers are indicated on the left.

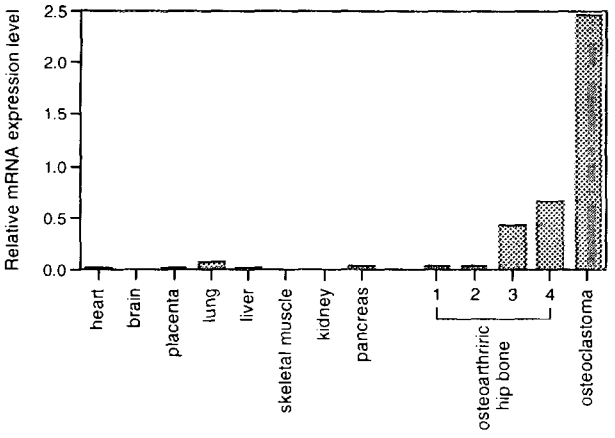

Fig. 4. Quantitative analysis of cathepsin K mRNA expression. Signal intensities of cathepsin K mRNA on the Northern filters in Fig. 3 were measured with a BAS-2000 Bioimage Analyzer. The expression level of cathepsin K mRNA was normalized by the signal intensity of human G3PDH mRNA on the same lane.

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