Identification of Human Asparaginyl Endopeptidase (Legumain) as an Inhibitor of Osteoclast Formation and Bone Resorption*

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The osteoclast (OCL),¹ the primary bone-resorbing cell, is derived from cells in the monocyte-macrophage lineage (1). Osteoclast activity and formation are regulated by both systemic hormones and locally produced factors. We have used an expression cloning approach with a cDNA expression library prepared from highly purified human OCL-like cells formed in vitro to identify local factors that modulate OCL formation. We have recently identified two novel stimulators of OCL formation, and report here the identification and characterization of a previously unknown inhibitor of the OCL, legumain. Legumain inhibited OCL formation in both long-term human marrow cultures in the absence of any added stimulator of OCL formation.

EXPERIMENTAL PROCEDURES

Materials—DNA transfection kits were obtained from Stratagene (La Jolla, CA), and cDNA synthesis kits were purchased from Amersham Pharmacia Biotech. PCR kits were obtained from Perkin-Elmer. All restriction enzymes used were from New England Biolabs (Beverly, MA) and Life Technologies, Inc., and chemicals were from Sigma. The 23G6 monoclonal antibody was generously provided by Dr. Michael Horton (St. Bartholomew’s Hospital, London, United Kingdom).

Construction and Screening of a Mammalian cDNA Expression Library from Highly Purified Human Osteoclast-like Multinucleated Cells—The construction and screening of the human cDNA expression library prepared from OCL-like multinucleated cells formed in vitro were done as described previously (2). Pools, whose conditioned media significantly and reproducibly inhibited tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cell formation in murine marrow cultures, were progressively subfractionated until the subpool contained only one cDNA that inhibited OCL-like cell formation in both murine and human systems. The cDNA, initially named “osteoclast inhibitory peptide-2,” was then sequenced by standard techniques, compared with cDNA sequences in the GenBank data base and was found to be identical to human legumain (GenBank accession no. Y09862).

Murine and Human Bone Marrow Cultures—The marrow cells (1 × 10⁶ cells/culture) from C57Bl mice were isolated and cultured for OCL-like multinucleated cell formation in the presence or absence of 1,25-(OH)₂D₃ (10⁻¹⁰ M) (day 0, 2, 4, or 6 of culture). The cultures were continued for a total of 7 days, and the number of TRAP-positive multinucleated cells determined. In experiments to assess the effects of legumain on OCL apoptosis, conditioned media from 293 cells transfected with the legumain cDNA, as described by Takahashi and co-workers (5). The cultures were then stained for TRAP using an acid phosphatase staining kit (Sigma). The TRAP-positive multinucleated cells containing three or more nuclei were counted with an inverted microscope. In selected experiments, conditioned media from 293 cells transfected with the legumain cDNA were added at various times after the initiation of murine marrow cultures stimulated with 1,25-(OH)₂D₃, and varying concentrations of conditioned media from 293 cells transfected with the legumain cDNA, as described by Takahashi and co-workers (5).

In experiments to assess the effects of osteotropic factors on expres-
sion of legumain, human bone marrow was cultured with IL-3 (3 ng/ml), stem cell factor (15 ng/ml), and granulocyte/macrophage colony-stimulating factor (200 ng/ml) for 10 days to induce CFU-GM colony formation, and the CFU-GM-derived colonies were isolated with a pipette. One hundred thousand cells/ml were then cultured in α-minimum essential medium supplemented with 10% fetal calf serum (Hyclone, UT), as well as RNA from human bone marrow and MG63 cells.

The legumain cDNA expression library was screened by testing the effects of crude legumain from an E. coli strain expressing a recombinant legumain protein on osteoclasts. Purified legumain recombinant protein, or concentrated conditioned medium from 293 cells transfected with the legumain cDNA were boiled in SDS sample buffer and loaded onto an 8% PAGE slab gel. To detect the protein profile, the PAGE gel was stained with Coomassie Blue. Electrophoretic transfer of proteins from polyacrylamide to nitrocellulose (Schleicher & Schuell) was performed using a semi-dry blotting unit (Fisher) at 20 V for 45 min. After transfer, the nitrocellulose membrane was blocked with 5% skim milk and then blotted with the legumain polyclonal antibody. The nitrocellulose membrane was then washed and reacted with horseradish peroxidase-labeled anti-rabbit IgG and visualized with the ECL system (Amersham Pharmacia Biotech) on the Kodak X-AR5, according to manufacturer’s protocol.

Generation of Stably Transfected 293 Cell Lines That Constitutively Express Legumain (293-Legumain Cells)—293 cells (2 x 10⁶) were stably transfected with the legumain cDNA in the pIREs/neo vector (293-legumain cells) or the empty vector (293 cells) (CLONTECH) and then cultured in media containing 400 µg of G418. Every 4 days, the selection media containing G418 was changed, and after 2 weeks of culture in selection media, single colonies were isolated using cloning rings. Each colony was tested for legumain expression by Western blot analysis. The biological activity of legumain produced by these colonies was determined by treating fetal rat long bone cultures with serum-free media lacking G418 that has been conditioned by the cells for 48 h, and determining ⁴⁵Ca release as described above.

Effect of Human Legumain on Bone Resorption in Vivo Using the 293-Legumain Cell Line—Four-week-old nude mice (4 mice/group) were injected in the thigh muscles with 5 x 10⁶ 293 cells or 293-legumain cells in 100 µl of PBS 3 weeks before treating the mice with PTHrP. Mice with palpable tumors were injected with 2 µg of PTHrP in 100 µl of phosphate-buffered saline four times per day subcutaneously for 5 days using a protocol that we have shown previously causes hypercalcemia. 31P extinctions were increased above baseline at baseline. The left tibia was then harvested, and the amount of bone mineral density from each calvaria using a digitizing table and the Bioquant (Schleicher & Schuell) was performed using a semi-dry blotting unit (CLONTECH), as well as RNA from human bone marrow and MG63 cells.

Expression of Recombinant Legumain—From the pcDNA1 mammalian expression clone, the coding region of the legumain cDNA was generated by PCR amplification with two primers, GCC ATATA TGG TTT GGA AAG TAG CTC T GAG AAT TCT C CG AAT AAG GAC TCC T. The DNA fragment generated by PCR was digested with NdeI (recognition sequence underlined) and BamHI (to cleave the cloning site in the vector) and ligated into the PET 1b vector. Twelve transformants were isolated and confirmed by sequence analysis. The Escherichia coli recombinant protein was produced and purified on His-Bind resin (Novagen Co., Madison, WI) as described previously (4). The recombinant legumain fusion protein was dissolved in 10 mM NaOH. This recombinant legumain was used for the generation of a rabbit polyclonal antibody, and for testing in mouse and human bone marrow cultures and fetal rat organ culture assays.

Generation of Polyclonal Anti-legumain Antiserum—The recombinant legumain protein was emulsified in PBS containing complete Freund’s adjuvant, and a rabbit was immunized with 200 µg of emulsified protein in a total volume of 0.2 ml. After 14 days, the animal was hyper-immunized by injection of 200 µg/0.2 ml of the recombinant legumain preparation. At least three repeat challenges were performed once every 14 days. The specific reactivity of the legumain antiserum was determined by testing its capacity to detect legumain in Western blots.

SDS-PAGE and Western Immunoblotting—Crude legumain from an E. coli strain expressing a recombinant legumain protein was concentrated using an Amicon Ultra-15 microconcentrator, purified legumain recombinant protein, or concentrated conditioned medium from 293 cells transfected with the legumain cDNA were boiled in SDS sample buffer and loaded onto an 8% PAGE slab gel. To detect the protein profile, the PAGE gel was stained with Coomassie Blue. Electrophoretic transfer of proteins from polyacrylamide to nitrocellulose (Schleicher & Schuell) was performed using a semi-dry blotting unit (Fisher) at 20 V for 45 min. After transfer, the nitrocellulose membrane was blocked with 5% skim milk and then blotted with the legumain polyclonal antibody. The nitrocellulose membrane was then washed and reacted with horseradish peroxidase-labeled anti-rabbit IgG and visualized with the ECL system (Amersham Pharmacia Biotech) on the Kodak X-AR5, according to manufacturer’s protocol.

Statistical Analysis—In vitro results are reported as the mean ± S.E. for five replicate samples and were compared by Student’s t test. Results were considered significantly different for p < 0.05. The in vivo results were compared using Student’s Neuman-Keuls test and are representative of the findings in three to five separate experiments.

RESULTS

Screening of the Osteoclast cDNA Expression Library—The cDNA expression library was screened by testing the effects of conditioned media from 293 cells transfected with cDNA from each pool for their capacity to inhibit OCL-like multinucleated cell formation in mouse bone marrow cultures. Conditioned media (1–10%) were added to mouse bone marrow cultures in the presence of 10⁻⁴ M 1,25-(OH)₂D₃. The number of TRAP-positive multinucleated cells was counted and compared with the control cultures treated with conditioned media from 293 cells transfected with the empty pcDNA1 vector. A total of five pools that reproducibly inhibited multinucleated cell formation in murine marrow cultures treated with 10⁻⁴ M 1,25-(OH)₂D₃
were detected from the original 200 pools. The positive pools were then screened by PCR for the presence of transforming growth factor β or γ-interferon mRNA, factors known to inhibit OCL formation. γ-Interferon cDNA was detected in one of the pools (data not shown), which was not screened further.

The remaining four positive cDNA pools were separated into 12 subpools each, containing 100-200 clones/pool. The cDNAs from the individual pools were transfected into 293 cells, and the conditioned media tested for their effects on TRAP-positive multinucleated cell formation in mouse bone marrow cultures.

One of the positive subpools containing 200 clones was fractionated into two 96-well plates, with one clone per well. Conditioned medium from each row and column pool of these matrices was tested for its capacity to inhibit multinucleated cell formation in mouse marrow cultures.

Identification of Inhibitory Clone as the cDNA for Legumain—The inhibitory cDNA was approximately 1.9 kilobase pairs in length. The cDNA sequence of the clone was determined, and it encoded a 1299-base pair open reading frame and one RGD sequence.

Tissue Distribution of Legumain—Legumain mRNA was expressed in all tissues, and very low levels were detectable in brain compared with the other tissues, similar to results reported by Chen et al. (13, 14). Significant expression levels of legumain mRNA were also present in bone marrow, but MG63 osteosarcoma cells did not express legumain (data not shown).

Immunocytochemical analysis using our polyclonal antibody to legumain of cells in human marrow cultures confirmed that OCL-like multinucleated cells and mononuclear cells expressed legumain (data not shown).

Effects of Legumain Conditioned Media on OCL-like Multinucleated Cell Formation—We then tested the effects of conditioned media from 293 cells transfected with the legumain cDNA on OCL-like multinucleated cell formation in mouse and human bone marrow cultures. In the mouse bone marrow cultures, legumain conditioned medium inhibited TRAP-positive multinucleated cell formation in the presence of 1,25-(OH)2D3 in a dose-dependent fashion at concentrations from 1% to 10% (v/v) (Fig. 1A) and maximally decreased TRAP-positive multinucleated cell formation approximately 60% at a concentration of 10% (v/v). Addition of legumain conditioned media for only the first 2 days of culture did not significantly inhibit TRAP-positive multinucleated cell formation in the cultures. Addition of legumain between days 2 and 6 of cultures markedly inhibited multinucleated cell formation (Fig. 1B).

The level of inhibition of OCL formation when legumain was added during the second phase of the cultures was similar to that when legumain was present for the entire culture period. Legumain conditioned media also decreased 23c6-positive multinucleated cell formation in human marrow cultures induced by 10−9 M 1,25-(OH)2D3, approximately 80% at a concentration of 5% (v/v) compared with the control cultures (Fig. 1A). Legumain conditioned media also inhibited multinucleated cell formation in murine and human marrow cultures treated with PTHrP (data not shown). However, although legumain conditioned media inhibited OCL formation (Fig. 1B), it did not significantly increase the percentage of apoptotic OCLs in

Fig. 1. Effects of legumain on osteoclast formation in murine and human marrow cultures. A, human and murine marrow cultures were treated with conditioned media from 293 cells transfected with human legumain cDNA as described under "Experimental Procedures." Legumain conditioned media inhibited mouse TRAP(+) multinucleated cell formation and human 23c6(+) multinucleated cell formation stimulated with 10−9 M 1,25-(OH)2D3 in a dose-dependent manner, compared with media from 293 cells transfected with the empty vector. Results are expressed as the mean ± S.E. for four determinations in a typical experiment. A similar pattern of results was seen in three independent experiments.
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Effects of Legumain Conditioned Media on Bone Resorption in the Fetal Rat Long Bone Organ Cultures—$^{45}$Ca-labeled fetal rat long bones were stimulated with $10^{-9} \text{ M} 1,25-(OH)_2\text{D}_3$ and treated with varying concentrations of legumain conditioned media. Legumain inhibited $^{45}$Ca release about 70% at 32% (v/v) compared with conditioned media from 293 cells transfected with the empty pcDNA1 vector (Fig. 2A). The legumain conditioned media also inhibited PTHrP-stimulated bone resorption in a dose-dependent manner and inhibited $^{45}$Ca release by about 70% at a concentration of 32% (v/v) compared with conditioned media from 293 cells transfected with the empty pcDNA1 vector (Fig. 2B).

Effects of Osteotropic Factors on Expression of Legumain mRNA in Human OCL Precursors—Treatment of highly purified human OCL precursors (CFU-GM-derived cells) with $1,25-(OH)_2\text{D}_3$ or IL-1, factors that stimulate OCL formation, decreased the relative levels of legumain mRNA compared with control cultures. Comparison of the relative amounts of legumain mRNA to that of GAPDH (a housekeeping gene used to control for mRNA loading) demonstrated that the ratio of legumain mRNA to GAPDH mRNA for IL-1 and $1,25-(OH)_2\text{D}_3$-treated cultures was 0.4 or 1.04, respectively, compared with 3.05 and 4.0 for control or calcitonin-treated cultures (Fig. 3).

Isolation of Recombinant Legumain—Recombinant legumain was expressed in E. coli BL21 by induction with 1 mM isopropyl-$\beta$-d-thiogalactoside using the PET14b system. The cell pellet was solubilized with 6 M GnHCl and purified with a His-Bind resin column as described above. Recombinant legumain was eluted with a 50–80 M imidazole gradient in 6 M GnHCl and precipitated by double-distilled H$_2$O dialysis. After re-chromatography in the presence of 6 M GnHCl on the His-Bind column, about 1 mg of recombinant legumain was obtained from 1 liter of E. coli culture with more than 95% purity.

Detection of Legumain in Conditioned Media of 293 Cells and Human Bone Marrow Supernatants and Cultures—Legumain cDNA was transfected into 293 cells, and the conditioned media were harvested from these cells (control: 13 ± 1% versus legumain-treated: 8.6 ± 0.6%) when legumain was added for the last 48 h of the cultures.

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Legumain is present in significant amounts in normal human marrow and in human marrow cultures, and neutralization of legumain in human marrow cultures, in the absence of any added stimulator of OCL formation, induced OCL formation to levels similar to those induced by 1,25-(OH)₂D₃, one of the most potent inducers of OCL formation in vitro. Treatment of mice

**DISCUSSION**

We have cloned and characterized a previously unknown inhibitor of OCL activity, legumain, which inhibits OCL formation in both murine and human cultures in vitro and PTH-stimulated bone resorption in vivo. Legumain is a member of the mammalian cysteine protease family, the asparaginyl endopeptidases, which have been identified recently (13, 14). Legumain is a potent inducer of OCL formation, and PTH treatment in mice bearing control tumors and was inhibited by legumain (p < 0.05). The results represent the mean ± S.E. for six determinations for four independent experiments. **, p < 0.01 compared with the other three treatment groups. †, p < 0.05 compared with animals bearing 293 control tumors and treated with PTHrP.

**FIG. 6.** Effects of legumain on whole blood ionized calcium levels in mice treated with PTHrP. Mice bearing control or legumain-producing tumors were injected with PTHrP (2 μg four times per day for 5 days or vehicle (PBS) in a volume of 100 μl as described under "Experimental Procedures." Data represent the mean ± S.E. of whole blood ionized calcium levels for 4 mice at each time point. Signiﬁcant hypercalcemia occurred by days 4 or 5 of PTH treatment in mice bearing control tumors and was inhibited by legumain (p < 0.05). The results represent the mean ± S.E. for four determinations for four independent experiments. **, p < 0.01 compared with the other three treatment groups. †, p < 0.05 compared with animals bearing 293 control tumors and treated with PTHrP.

**FIG. 5.** Effects of an antibody to human legumain on osteoclast formation. A, anti-human legumain was added to human marrow cultures that did not contain any exogenous stimulator of osteoclast formation. Anti-legumain enhanced conditioned basal 23c6(+) multinucleated cell formation in human bone marrow cultures at a dilution of 1:2500. Multinucleated cell formation in the presence of anti-legumain was almost to the same level as that seen in cultures stimulated with 10⁻¹⁰ M 1,25-OH)₂D₃. B, polyclonal antibody generated against human legumain. Conditioned medium from 293 cells transfected with human legumain cDNA showed a 50-kDa band that was larger than recombinant human legumain expressed in E. coli. Control medium from 293 cells transfected with the empty vector (Vector CM) and untransfected 293 cell conditioned media also expressed a 50-kDa legumain band. Human bone marrow plasma and conditioned media from human bone marrow cultures showed two bands for legumain, 50 and 30 kDa, respectively. Detection of both bands was competed by addition of recombinant legumain. The expression level of human legumain in human bone marrow plasma was more than 50 ng/ml.

**FIG. 4.** Western blot analysis of human legumain in 293 cells, human bone marrow plasma, and human marrow cultures. A, concentrated conditioned medium (×20) was subjected to Western blot analysis using a polyclonal antibody generated against recombinant human legumain. Conditioned medium from 293 cells transfected with legumain cDNA showed a 50-kDa band that was larger than recombinant human legumain expressed in E. coli. Control medium from 293 cells transfected with the empty vector (Vector CM) and untransfected 293 cell conditioned media also expressed a 50-kDa legumain band. B, human bone marrow plasma and conditioned media from human bone marrow cultures showed two bands for legumain, 50 and 30 kDa, respectively. Detection of both bands was competed by addition of recombinant legumain. The expression level of human legumain in human bone marrow plasma was more than 50 ng/ml.

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Legumain appears to block the later stages of OCL formation. Legumain inhibited OCL formation from mononuclear precursors in murine cultures only when it was added in the second half of the culture period, suggesting that it was inhibiting the differentiation/fusion phase of osteoclastogenesis, rather than the proliferative phase. Biakoning and co-workers (17) have previously shown that osteoclastogenesis occurs in discrete stages in murine marrow cultures. Days 1 through 3 of murine marrow cultures is the phase in which the OCL precursor pool proliferates, and days 4 through 8 is the stage when the cells differentiate to post-mitotic cells and then fuse to form OCLs. Legumain also had no effect on OCL apoptosis. Interestingly, two factors that stimulate OCL formation (IL-1 or 1,25-(OH)2D3) decreased the relative levels of legumain mRNA in human OCL precursors. In contrast, calcitonin, an inhibitor of OCL formation, did not have a significant effect on levels of legumain mRNA. The data suggest that the mechanism of action of legumain is similar to that of another recently described inhibitor of the OCL, osteoprotegerin, a member of the tumor necrosis factor α receptor family (18) that also inhibits the differentiation/fusion stage of OCL formation. However, legumain is structurally unrelated to osteoprotegerin and has different effects in vivo (19). The observations that factors that stimulate OCL formation down-regulate legumain expression while inhibitors of OCL formation do not, also suggest that legumain may be an important regulator of OCL formation. These results further suggest that stimulators of OCL formation may in part increase OCL formation by decreasing production of legumain.

The molecular mechanism responsible for legumain’s inhibition of OCL formation is unknown. A similar activity has been detected in Schistosoma mansoni and acts as a hemoglobinase to degrade host hemoglobin (20, 21). Legumain has an active site highly specific for substrates with asparaginyl bonds and cleaves only some of the asparaginyl bonds in polypeptides (22). However, it is unknown if this enzyme activity plays a role in the inhibitory effects of legumain on OCL formation/activity. It is unlikely that legumain inhibits OCL formation by cleaving PTHrP, since it also inhibits 1,25-(OH)2D3-stimulated OCL formation in murine and human cultures as well as OCL formation stimulated by RANK ligand, a recently described osteoclast differentiation factor (19). Alternatively, legumain also contains an RGD sequence that could participate in the inhibitory activity of legumain. RGD-containing peptides can inhibit OCL bone resorption (23).

Further studies are currently under way to address the potential physiologic role of legumain in bone remodeling and its potential role in pathologic states of bone remodeling.

REFERENCES


Fig. 7. Effects of legumain on bone resorption in calvaria of mice treated for 5 days with PTHrP, as described under “Experimental Procedures.” a, increased numbers of osteoclasts (arrow) are seen resorbing bone along the bone/bone marrow interface in mice treated with PTHrP. Mice bearing legumain producing 293 cell tumors (B) treated with PTHrP had lower numbers of osteoclasts and decreased marrow spaces compared with the mice bearing control 293 cell tumors treated with PTHrP (A). Few osteoclasts were observed in the control mice bearing 293 cells with (C) or without legumain (D), and bone marrow spaces were smaller than those seen in the PTHrP-treated mice. b, Histomorphometric analysis of the effects of legumain on osteoclast activity induced by PTHrP in vivo. Osteoclast number per mm2 bone area and per mm bone/bone marrow interface, and osteoclast surface percentage were significantly reduced in mice bearing legumain producing tumors and treated with PTHrP (p < 0.0001) compared with the control mice treated with PTHrP. However, no difference was observed between vehicle (PBS)-treated mice bearing control or legumain producing tumors. c, Western blot analysis of sera from mice bearing 293-legumain tumors and control 293 cell tumors. Sera were serially diluted and then subjected to Western blot analysis with anti-legumain antibody.

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