Cathepsin-L, a Key Molecule in the Pathogenesis of Drug-Induced and I-Cell Disease-Mediated Gingival Overgrowth

A Study with Cathepsin-L-Deficient Mice

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Drug-induced gingival overgrowth, the chronic side effect of calcium antagonists, is frequently seen due to the increase in patients with hypertension, although the etiology of the disease is largely unknown. I-cell disease, which accompanies gingival overgrowth, is characterized by a deficiency in UDP-N-acetyl-glucosamine and is classified as one of the lysosomal storage diseases. Here, we hypothesized that a common mechanism may underlie the etiology of gingival overgrowth seen in patients treated with calcium antagonist and in patients with I-cell disease. A calcium antagonist, nifedipine, specifically suppressed cathepsin-L activity and mRNA expression, but not that of cathepsin-B in cultured gingival fibroblasts. The activity of cathepsin-L was suppressed up to 50% at 24 hours after treatment of the cells with the reagent. The selective suppression of cathepsin-L activity appeared not to be dependent on Ca^{2+} , since treatment of the cells with thapsigargin suppressed both cathepsin-B and -L activity. Mice deficient in the cathepsin-L gene manifested enlarged gingivae. Histological observation of the gingivae demonstrated typical features of acanthosis, a phenotype very similar to that of experimentally induced gingival overgrowth. Since cathepsin-L deficiency was reported to be associated with thickening of the skin, impaired cathepsin-L activity may play a key role in the establishment of skin and gingival abnormalities seen in I-cell disease. In addition, reduced cathepsin-L activ-

ity may play an important role in inducing drug-induced gingival overgrowth. (Am J Pathol 2002, 161:2047–2052)

Calcium antagonists, such as nifedipine, diltiazem, and verapamil, are frequently used as a first choice drug for treating patients with hypertension due to the relatively low incidence of acute side effects. Since the number of patients with hypertension is increasing world-wide due to the increase in the overweight and/or diabetic population, the need for this drug is estimated to grow rapidly not only for the purpose of treating hypertension but for reducing the future risk of cardiovascular events.¹ One of the well-known chronic side effects of calcium antagonists is gingival thickening designated as gingival overgrowth.² All types of calcium antagonists are more or less reported to cause gingival overgrowth in differing frequencies that vary between 19 and 38%.³ Although gingival overgrowth itself does not induce serious problems except for esthetic stress, it frequently complicates pre-existing periodontal disease, leading to the harboring of more bacteria in the gingival sulcus. Recently, attention was paid to such subclinical inflammations that may act to increase the future risk for developing vascular events such as cardiovascular or cerebrovascular diseases.^{4,5} Although it is commonly accepted that the nature of gingival overgrowth is an excess accumulation of extracellular matrix in gingival connective tissues, the pathogenesis of the disease is largely unknown.²

Several organs and/or tissues undergo progressive fibrosis under pathological conditions such as liver cirrhosis, chronic renal diseases, lung fibrosis, and dilated cardiomyopathy. Among these, suppressed cathepsin-L and -B activity has been suggested to be involved in the establishment of diabetic nephropathy⁶ and chemically

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induced experimental nephrosis.^{7,8} In addition, mice deficient in the cathepsin-L gene were recently reported to develop dilated cardiomyopathy,⁹ another fibrotic disease with high mortality. Cathepsins are lysosomal cysteine proteinases responsible for the digestion of up to 90% of long-lived cellular proteins.¹⁰ Thus, impaired cathepsin activity may lead to the excess accumulation of extracellular matrix in such fibrotic diseases.

I-cell disease (mucopolipidosis II) is a rare congenital lysosomal storage disease characterized by a defect in N-acetylglucosamine-1-phosphotransferase (UDP-N-acetyl-glucosamine), which is involved in the processing and maturation of various lysosomal enzymes.^{11,12} As a result, the activity of almost all lysosomal enzymes including cathepsins is impaired.¹³ Patients with I-cell disease manifest several characteristic features including thickening of the skin, gingival overgrowth, as well as low body height and mental retardation.^{14,15} Thus, it can be hypothesized that one or several lysosomal enzymes with lowered activity are responsible for the establishment of such features seen in I-cell diseases. Since lysosomal cysteine proteinases, mainly cathepsin-L and -B, are responsible for the digestion of long-lived cellular proteins, we hypothesized that the gingival overgrowth seen in patients with hypertension treated with calcium antagonist and that seen in I-cell disease was mediated by impaired cathepsin activity and subsequent matrix accumulation. Therefore, we examined the effects of calcium antagonists on cathepsin activity, especially that of cathepsin-L and -B *in vitro*. We then phenotypically confirmed the *in vivo* effects of such enzyme deficiency by investigating the mice lacking such enzymes.

Materials and Methods

Reagents

The blocker of L-type Ca (2+) channels, nifedipine, was from Bayer AG Pharma Research Center (Wuppertal, Germany). The Ca²⁺ mobilizer, thapsigargin (TG), was obtained from Calbiochem (Darmstadt, Germany). Z-Phe-Arg-MCA and Z-Arg-Arg-MCA were obtained from Peptide Institute Inc. (Osaka, Japan), and pepsin was obtained from Sigma (St. Louis, MO).

Cells and Cell Culture

Human gingival fibroblasts were obtained from patients with nifedipine-induced gingival overgrowth and from patients with chronic adult periodontitis. In cases of chronic adult periodontitis, a healthy part of the gingivae was obtained during periodontal surgery. Before tissue resection, informed consent was obtained from each volunteer (approved by the Review Board of Human Tissues and Cells for Research Use of Okayama University Graduate School of Medicine and Dentistry). Gingival fibroblasts outgrown from explanted tissues were expanded and maintained as previously described.¹⁶ The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY) supplemented with

10% fetal bovine serum (ICN, CA), and 10 mg/ml of gentamicin (Life Technologies). Nifedipine and TG were dissolved in DMSO and diluted with culture medium. Nifedipine was used at a concentration of 0.1 to 1000 ng/ml, while TG was used at a concentration of 10 nmol/L.

Measurement of Cathepsin Activity

The activity of the active form of cathepsin-(B+L), cathepsin-B in gingival fibroblasts was determined fluorometrically using Z-Phe-Arg-MCA or Z-Arg-Arg-MCA as the substrates. Gingival fibroblasts cultured with or without nifedipine in combination with TG for the indicated time period were washed twice with phosphate-buffered saline and scraped into 2 ml of ice-cold 50 mmol/L sodium acetate buffer (pH 5.2) containing 1 mmol/L EDTA and 0.1 mol/L NaCl with a rubber policeman. Then, the cells were permeabilized by sonication. Soluble fractions were obtained by centrifugation and the protein concentration was determined by protein assay (Bio-Rad, Hercules, CA). The activity of cathepsin-(B+L) and cathepsin-B was assayed as described by Barrett and Kirschke.¹⁷ Briefly, 1 μ g of each cell lysate was incubated at 37°C for 10 minutes with the substrates. The reaction was stopped by adding 100 mmol/L monochloroacetic acid in sodium acetate buffer. The standard curve was generated with 7-amino-4-methyl-coumarin (AMC) under identical conditions. The fluorescence of the free AMC released was determined by excitation at 380 nm and emission at 460 nm using a fluorescence spectrophotometer (F-2000; Hitachi, Tokyo, Japan). The activity was calculated as released AMC/ μ g cellular proteins. The activity of the latent form of cathepsin-(B+L), or that of cathepsin-B was determined following activation by limited proteolysis with pepsin at a final concentration of 50 ng/ml as described previously.¹⁸

Reverse Transcription Polymerase Chain Reaction

Detection of the mRNA encoding cathepsin-B, -D, -L, β -D galactosidase, and β -N-acetylglucosaminidase in gingival fibroblasts was performed by reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated using TRIZOL LS reagent (Gibco BRL, Grand Island, NY) from the cells cultured with or without nifedipine for the indicated time period, followed by the synthesis of cDNA by reverse transcription with oligo (dT)₁₂₋₁₈ primer. The sets of primers used were as follows; cathepsin-L (5'-agtgtggctcttgttgggct-3' for forward, 5'-gcccaaccacagcatagcat-3' for reverse), cathepsin-B (5'-gttacagtgcagacaggcca-3' for forward, 5'-gttcctttgagccgcgtc-3' for reverse), cathepsin-D (5'-ctgtctgtctctccatctgt-3' for forward, 5'-tttgtcccctctcactcct-3' for reverse), β -D galactosidase (5'-ttaacctggccgctattgg-3' for forward, 5'-ttgttttttgcgggggtgg-3' for reverse), β -N-acetylglucosaminidase (5'-actatgaggaggcaagaagc-3' for forward, 5'-gccctggaattagcggaaaa-3' for reverse), and β -actin (5'-atgacccagatcatgtttgag-3' for forward, 5'-aggagcaatgatcttgatcttca-3' for reverse). The annealing temperature was

calculated using OLIGO 4.0 software (National Biosciences, Plymouth, MN). About one-tenth of the cDNA sample was used for amplification for 35 cycles with DNA polymerase (Ampli-Taq Gold, Perkin Elmer Cetus, Norwalk, CT) in a thermal cycler (Program Temp Control System PC-700; ASTEC, Fukuoka, Japan) according to the manufacturer's instructions. One-fifth of the amplified products were subjected to agarose gel electrophoresis and the DNA was visualized by staining the gel with ethidium bromide.

Animal Study

Generation of cathepsin-L-deficient mice was reported elsewhere.¹⁹ These mice were maintained in animal facilities at Osaka University. The mice deficient in cathepsin-L (–/–) and wild-type littermates were developed and sacrificed at 5 weeks after birth. Gingival appearance was observed by microscopy (magnification, $\times 40$). Gingival tissues with mandibles were then fixed, decalcified, and embedded in paraffin for the histological observation. The tissue sections were stained with hematoxylin and eosin staining.

Statistical Analyses

Statistical analyses to compare the enzyme activity of the cells treated with or without the indicated reagents were performed by the Mann-Whitney *U*-test using a statistical program, Stat View software (Abacus Concepts, Inc., Berkeley, CA).

Results

Calcium Antagonist Suppresses Cathepsin-L Activity in Fibroblasts

We first observed the effects of different doses of nifedipine on cathepsin-L and -B activity in gingival fibroblasts. Gingival fibroblasts obtained from nifedipine-induced gingival overgrowth and from normal gingival tissues were used. Findings obtained from normal gingival fibroblasts are shown in Figure 1. All cells used in the experiments showed similar results. Nifedipine, especially 100 ng/ml or greater, suppressed cathepsin-(B+L) activity in a dose-dependent manner (Figure 1A), while cathepsin-B activity was not apparently influenced (Figure 1B). Based on these results and the usual clinical drug dose, we decided to use 100 ng/ml of nifedipine for subsequent *in vitro* studies. We next examined if suppression of cathepsin-L activity by nifedipine was in the enzymatic conversion from the pro- to active-form. Total cathepsin activity was measured following activation by pepsin. Results are shown in Figure 2. As shown, the activity of both the total and active form of cathepsin-(B+L) was significantly suppressed by nifedipine ($P < 0.05$ at day 1 and day 2 for the latent form, $P < 0.05$ for the active form at day 1 and $P < 0.02$ at day 2, respectively), while that of cathepsin-B was not impaired (no

Figure 1. Dose-dependent suppression of cathepsin-L activity in gingival fibroblasts cultured with nifedipine. Gingival fibroblasts were cultured with or without various doses of nifedipine for the indicated time periods. Cathepsin activity was measured as described in Materials and Methods. Since a specific substrate for cathepsin-L is not available, combined activity of cathepsin-(B+L) (A) and cathepsin-B activity (B) was measured. Data are representative of the results obtained from gingival fibroblasts isolated from normal gingival tissues and nifedipine-induced gingival overgrowth, since gingival fibroblasts obtained from nifedipine-induced gingival overgrowth and from normal tissues were similarly affected by nifedipine. Values are expressed as the % activity against that of controls (day 0).

significant differences). The maximum inhibition was about 50% for both latent and active forms. In this experiment, all cells tested, including the cells from nifedipine-induced gingival overgrowth, showed similar results.

Calcium Antagonist Specifically Suppress Cathepsin-L Gene Expression

We then observed if nifedipine had any influences on gene expression of lysosomal enzymes. The effects of nifedipine on mRNA expression encoding various lyso-

Figure 2. Latent and active cathepsin activity of cells cultured with or without nifedipine. Enzyme activities of active forms of cathepsin-(B+L) (A), active form of cathepsin-B (B), total forms of cathepsin-(B+L) (C), and total form of cathepsin-B (D) were measured in gingival fibroblasts cultured with or without 100 ng/ml of nifedipine. Gingival fibroblasts cultured with or without nifedipine were subjected to measurement of cathepsin activities as described in Materials and Methods. Values are representative of the results obtained from gingival fibroblasts isolated from normal gingival tissues and nifedipine-induced gingival overgrowth, since gingival fibroblasts obtained from nifedipine-induced gingival overgrowth and from normal tissues were similarly affected by nifedipine. Values are expressed as the % activity against that of controls (day 0). Both active and total forms of cathepsin-(B+L) activity were significantly suppressed by nifedipine, while that of cathepsin-B was not influenced.

Figure 3. mRNA expression of various lysosomal enzymes cultured with or without nifedipine in gingival fibroblasts. The expressions of mRNA encoding various lysosomal enzymes were examined in gingival fibroblasts cultured with (nifedipine) or without (–) 100 ng/ml of nifedipine for the indicated time periods. Total RNA was isolated and was subjected to RT-PCR analysis as described in Materials and Methods. Values are representative of the results obtained from several gingival fibroblasts from different donors, since all cells tested showed similar results. Expression of cathepsin-L mRNA was markedly suppressed in gingival fibroblasts after 1 day of incubation with nifedipine, while that of other lysosomal enzymes was not apparently influenced. HeLa cells served as the positive control.

somal enzymes were examined by RT-PCR analysis. The results are shown in Figure 3. The expression of cathepsin-L mRNA was markedly suppressed at 1 day following stimulation with nifedipine, while that of the other lysosomal enzyme mRNAs (cathepsin-B, -D, β -D galactosidase, and β -N-acetylglucosaminidase) was not apparently impaired.

Suppression of Cathepsin Activity by Calcium Antagonist Does Not Appear to Be Mediated by Ca^{2+} Influx

Since nifedipine antagonizes Ca^{2+} intake into cells, we examined the effects of Ca^{2+} mobilizer, TG, on cathepsin activity. When the cells were treated with 10 nmol/L of TG, both cathepsin-(B+L) and cathepsin-B activities were markedly suppressed (Figure 4), while 100 ng/ml of nifedipine only suppressed cathepsin-(B+L) activity. In addition, co-incubation of the cells with 100 ng/ml of nifedipine and 10 nmol/L of TG further suppressed cathepsin-(B+L) activity, indicating that the inhibitory effects of TG on cathepsin activity was additive.

Figure 4. The effects of TG on the activity of cathepsins. Gingival fibroblasts were cultured with or without 100 ng/ml of nifedipine in the presence or absence of the indicated concentrations of Ca mobilizer TG. Twenty-four hours later, cellular proteins were extracted and were subjected to enzyme assay. TG suppressed both cathepsin-(B+L) and -B activity, while nifedipine alone specifically suppressed cathepsin-(B+L) activity. Values are expressed as the % activity against that of controls (day 0).

Figure 5. The gingival appearances of the mice deficient in cathepsin-L and of control littermates. The morphology of the gingivae of mice deficient in the cathepsin-L gene (b and d) and that of wild-type controls (a and c) are presented. The morphology of incisor lesions of the gingivae (a and b) and of molar lesions of the gingivae (c and d) are shown. Arrows indicate the enlarged gingivae seen in cathepsin-L-deficient mice at both the incisor (b) and molar lesions (d).

Mice Deficient in the Cathepsin-L Gene Develop Gingival Overgrowth

To see the *in vivo* effects of selective suppression of cathepsin-L activity on the gingival tissues, cathepsin-L-deficient mice were prepared. In general, teeth eruption of the mice is completed at 3 weeks after birth. Thus, we compared the appearance of the gingivae between cathepsin-L-deficient and control mice, both of which were 5 weeks old. As expected, the mice deficient in the cathepsin-L gene were characterized by thickened gingival tissues both at the incisor (Figure 5b) and molar (Figure 5d) regions compared with wild-type controls (Figure 5, a and c, respectively). Histological examinations revealed that the thickened gingival tissues were characterized by thickened connective tissues (Figure 6b) and gingival epithelium (acanthosis) (Figure 6d). Elongated rete pegs into connective tissues, typical features of gingival overgrowth, were noted (Figure 6d).

Figure 6. Histological observations of the gingivae of the mice deficient in cathepsin-L and of control littermates. Histological observations of mice deficient in the cathepsin-L gene (**b** and **d**) and that of wild-type controls (**a** and **c**) were performed. Gingival tissues were stained with hematoxylin and eosin. Enlarged connective tissues in cathepsin-L-deficient mice as indicated by the **arrow** (**b**) compared with that of wild-type (**a**). Thickened gingival epithelium as indicated by the **arrow**, and elongated rete pegs into connective tissues, typical features of drug-induced gingival overgrowth, are shown (**d**). Magnification, (**a**) and (**c**) $\times 200$, (**b**) and (**d**) $\times 400$

Discussion

A calcium antagonist, nifedipine, suppressed cathepsin-L mRNA expression and subsequent enzyme activity in gingival fibroblasts *in vitro* regardless of the donors. The drug dose used in the experiment was within the clinical dose range (100 ng/ml). Patients with hypertension are continuously subjected to this drug dose as long as the medication is not changed. Mice deficient in cathepsin-L manifested gingival overgrowth in addition to thickening of the back skin as reported previously,¹⁹ with the morphological phenotype being very similar to that observed in patients with I-cell disease.

So far, at least three different kinds of drugs have been reported to cause gingival overgrowth as a chronic side effect, the anti-epileptic drug phenytoin, the immunosuppressive drug cyclosporin-A, and calcium antagonists. We previously reported that two other drugs, phenytoin and cyclosporin-A, suppressed cathepsin-L mRNA expression and subsequent enzyme activity *in vitro*.²⁰ Therefore, it is highly likely that these three drugs induce gingival overgrowth in a common pathological mechanism. Moreover, the gingival appearance observed in cathepsin-L-deficient mice resembled that of experimentally induced gingival overgrowth in normal mice, as reported previously.²¹

It is necessary to clarify why three different kinds of medication cause a similar chronic side effect, gingival overgrowth. One of the possible mechanisms is that all these drugs were suggested to influence Ca^{2+} influx. However, as shown in the current study, treatment of the cells with TG, which inhibits cellular uptake of Ca^{2+} , suppressed not only cathepsin-L activity but also cathepsin-B activity. The current study, in addition to our previous study, suggested that all of these drugs selectively suppressed cathepsin-L activity *in vitro*.²⁰ Therefore, it is unlikely that reduced Ca^{2+} intake is a common mechanism involved in the pathogenesis of gingival overgrowth seen in patients taking phenytoin, cyclosporin-A, and/or Ca channel blocker.

Recently, an up-stream regulatory region of the cathepsin-L gene was identified and reported.²² The promoter region of the cathepsin-L gene is TATA-less and GC-rich, supporting the finding that this gene product is widely expressed in various cell types and is expressed at a relatively stable level in such cells for normal tissue turnover. Nevertheless, this gene product is frequently observed to be overexpressed in transformed cells, especially in highly metastatic tumor cells.²³ In addition, the cathepsin-L gene product easily led to overexpression by pharmacological reagents such as phorbol ester (12-O-tetradecanoylphorbol-13-acetate) at the transcriptional level.^{24,25} Therefore, it appears that cathepsin-L gene expression is generally regulated at the basal level for normal tissue turnover, but is regulated at different levels under specific situations such as tumorigenesis and/or the influence of pharmacological reagents.

Complete loss of cathepsin-L function in mice resulted in the development of gingival overgrowth. There are several possibilities accounting for the pathogenesis of gingival overgrowth in these mice models. First, cathepsin-L deficiency might contribute to the hyperproliferation of epidermal cells. A previous study suggested that thickening of the back skin, seen in cathepsin-L-deficient mice, was due to the hyperproliferation of keratinocytes.¹⁹ However, in these mice, not only the epidermis but also the dermis was thickened. Cathepsin-L-deficient mice that lived for up to 12 months were recently reported to develop spontaneous dilated cardiomyopathy,⁹ heart failure characterized by progressive fibrosis. The lesions of cardiomyopathy were characterized by a marked increase in connective tissue in the myocardium and a significant decrease in the volume of cytoplasm per nucleus in cardiomyocytes. Cardiac muscle cells contained multiple large and fused lysosomes, a phenotype characteristic for lysosomal storage diseases. Therefore, another possibility is that intracellular protein digestion was markedly impaired in cathepsin-L-deficient mice, leading to the accumulation of insufficiently digested proteins in

lysosomes. It is also possible that insufficient protein digestion results in the excess accumulation of proteins in intercellular spaces. This can be seen in the case of kidney fibrosis. Impaired cathepsin activity was reported in relation to diabetic nephropathy⁶ as well as experimentally induced mice nephrosis,^{7,8} both of which are characterized by an excess accumulation of extracellular matrices in the kidney, leading to renal failure. In fact, cathepsin-L is involved in the cleavage of a wide range of substances including extracellular matrix such as fibronectin, collagen, and laminin.²⁶ Taken together, all of these possibilities may account for the establishment of fibrotic tissues in the gingival tissues.

In summary, a calcium antagonist, nifedipine, suppresses cathepsin-L gene expression and subsequent enzyme activity. Complete loss of cathepsin-L function results in the development of gingival overgrowth. Thus, impaired cathepsin-L function may lead to the establishment of gingival overgrowth as seen in patients treated with calcium antagonist. Studies investigating the transcriptional regulation of the cathepsin-L gene by various pharmacological reagents will be necessary to fully understand the pathological mechanisms behind these unwanted side effects.

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