Cystatin 10, a Novel Chondrocyte-specific Protein, May Promote the Last Steps of the Chondrocyte Differentiation Pathway*

Yu Koshizuka‡, Takashi Yamada‡, Kazuto Hoshi‡, Toru Ogawara‡, Ung-il Chung§, Hirotaka Kawanô‡, Yusuke Nakamura‡, Kozo Nakamura‡, Shiro Ikegawa‡, and Hiroshi Kawaguchi‡**

From the Departments of *Orthopedic Surgery and ‡Tissue Engineering, Faculty of Medicine, and the ¶Laboratory of Molecular Medicine, Institute of Medical Science, University of Tokyo, Tokyo 113-8655 and the ¶Laboratory of Bone and Joint Diseases, SNP Research Center, Institute of Physical and Chemical Research, Tokyo 198-8639, Japan

Received for publication, November 14, 2002, and in revised form, September 15, 2003 Published, JBC Papers in Press, September 17, 2003, DOI 10.1074/jbc.M211639200

Endochondral ossification is an essential process for skeletal development, bone growth, and fracture healing and is implicated in pathological conditions such as osteoarthritis and ectopic ossification. During this process, chondrocytes first proliferate and then progressively differentiate into mature hypertrophic chondrocytes. Once fully matured, these hypertrophic cells mineralize the surrounding matrix and undergo apoptosis. This is followed by a local recruitment of blood vessels and osteoclasts, leading to progressive replacement of cartilage by bone. Thus, in this process of endochondral bone formation, proliferation, maturation, mineralization, and apoptosis of chondrocytes must be properly coordinated. To elucidate the molecular mechanisms of endochondral ossification, we have been attempting to isolate novel genes implicated in this process (1–4). For this study, we took advantage of the naturally occurring mouse mutant ttw (tiptoe walking), which exhibits ectopic ossification in various soft tissues such as tendons, cartilage, and ligaments of the extremities and the spine (5). We previously found that ttw is caused by a nonsense mutation of the nucleotide pyrophosphatase gene encoding an ectoenzyme generating phosphate and pyrophosphate (4). Based on the fact that a high phosphate diet accelerates ectopic ossification of ttw, using a differential display method, we identified nine mouse genes whose expression is regulated by a high phosphate diet (1). Six of the nine genes were novel; and among them, we isolated one, termed cystatin 10 (Cst10),† that is up-regulated by a high phosphate diet and is expressed exclusively in cartilage, suggesting its specific role in endochondral bone formation.

In this study, we first characterized temporal and spatial expression patterns of Cst10, a novel member of the cystatin superfamily. The cystatin superfamily is known to inhibit the papain-like cysteine proteinases cathepsins B, H, and L by the formation of a tight reversible complex (6). These cysteine proteinases are thought to be associated with terminal degradation of proteins in lysosomes, so the cystatin superfamily is ubiquitously expressed and exhibits various biological functions (7). However, the present study reveals that Cst10 is expressed exclusively in mature chondrocytes. In addition, overexpression of the Cst10 gene accelerates hypertrophic maturation, mineralization, and apoptosis of chondrocytes. These data suggest a crucial and specific role of Cst10 in the later stage of endochondral ossification, implying a physiological role distinct from those other members of the cystatin superfamily.

EXPERIMENTAL PROCEDURES

Determination of the Genomic Structure of the Mouse Cst10 Gene—

Bacterial artificial chromosome (BAC) clones containing the mouse Cst10 gene were isolated using a BAC PCR screening system (Genome Systems, St. Louis, MO) according to the manufacturer’s protocol. The set of primers used for screening was Cst10/BAC/R (5′-TCCTGAG-GATATATGTCAGGC-3′) and Cst10/BAC/F (5′-ATCTCTGTCTGAG-GAAAGGAC-3′). To determine the size of introns of the Cst10 gene, interexon PCRs were carried out with primers designed according to the cDNA sequence we determined in this study. The BAC clones and PCR products were sequenced directly, and the exon-intron junctions were

* This work was supported by Grant-in-aid for Scientific Research 14370454 from the Japanese Ministry of Education, Culture, Sports, and Technology and by a grant-in-aid from the Investigation Committee on the Ossification of Spinal Ligaments, Japanese Ministry of Public Health and Welfare. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The abbreviations used are: Cst10, cystatin 10; BAC, bacterial artificial chromosome; FITC, fluorescein isothiocyanate; RT, reverse transcription; CstC, cystatin C; PBS, phosphate-buffered saline; PI, propidium iodide; pNA, p-nitroanilide; BMP, bone morphogenetic protein; PTHrP, parathyroid hormone-related protein.
Cystatin 10 in Chondrocyte Differentiation

Chromosomal Localization—To determine the chromosomal localization of the mouse Cst10 gene, we performed fluorescence in situ hybridization as described previously (8). A BAC clone containing the mouse Cst10 gene was labeled and hybridized to the mouse metaphase chromosome. Hybridization signals were rendered visible with fluorescein isothiocyanate (FITC)-avidin. Precise assignments of the signals were determined by visualization of the replicated G-bands.

Animal—The dd/y and ttw mice were purchased from Shizuka Laboratories Animal Center (Shizuoka, Japan) and the Central Institute for Experimental Animals (Kanagawa, Japan), respectively. All animal experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee for the Study of Pain (8) and the Central Institute for Experimental Animals (10, 11).

Cell Culture—Primary mesenchymal cells (osteoblasts, chondrocytes, and fibroblasts) were extracted from the calvariae, cartilage, and skin, respectively, of neonatal dd/y mice as described previously (10, 11). Cells were cultured in α-modified minimal essential medium (Invitrogen) containing 5% fetal bovine serum (Invitrogen) at 37°C. Mouse chondrogenic ATDC5 cells were obtained from the RIKEN Cell Bank (Saitama, Japan). The cells were cultured in medium consisting of a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (Invitrogen) containing 5% fetal bovine serum, 10 μg/ml human transferrin (Roche Applied Science, Mannheim, Germany), and 3 × 10⁻³ M sodium selenite (Sigma) as described previously (12). The inoculum density of the cells was 4 × 10⁴ cells/ml in 12-mmwell plates (Corning Inc., New York). For induction of chondrogenesis, the cells were cultured in medium supplemented with 10 μg/ml bovine insulin (Wako Pure Chemicals, Osaka, Japan). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. The medium was replaced every other day.

Expression of the Cst10 Transcript—Expression of the Cst10 mRNA was examined by semiquantitative reverse transcription (RT)-PCR, followed by Southern blotting using auricular cartilage from dd/y mice, mesenchymal cells from neonatal dd/y mice, and cultured mouse chondrogenic ATDC5 cells. For the experiments with dd/y mice, the mice were divided into two groups according to the content of phosphate in the diet, i.e. high (90.87%) and low (9%) phosphate groups after weaning at 3 weeks of age. The animals were killed 0, 1, 3, 5, 7, 10, and 14 days after the start of the diet, and the auricular cartilage was resected en bloc. Total RNAs were extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. RT-PCR was done using the following set of primers: Cst10/3'-CACAGTTCCTGCTGTGAATGTTAGTATGCAATATGCT-3' and Cst10/5'-5'-GAA CAG TGG GCC TTT GGA AA-3'. The following amplification cycle was used: 2 min of initial denaturation at 94°C, followed by 35 cycles at 94, 60, and 72°C for 30 s each plus extension at 72°C for 4 min. The primers and RT-PCR conditions used for type II and X collagens were as described previously (13). PCR products were electrophoresed and detected by Southern hybridization. PCR products were then excised, purified, and subcloned into the vector pBluescript. The subcloned cDNA fragment was confirmed by sequencing. ATDC5 cells (2 × 10⁵) were plated in a 6-cm culture dish 24 h before transfection. pcMV-Cst10 (4 mg/6-cm culture dish) or the mock vector (pcMV) was transfected into ATDC5 cells by lipofection using SuperFect transfection reagent (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s instructions. Two days later, the cells were diluted 10-fold and incubated in a serum-free maintenance medium containing 400 μg/ml Geneticin (Invitrogen). After 2 weeks, we isolated drug-resistant colonies, each of which was derived from a single clone, and cultured them separately. To confirm the reproducibility of the effects of Cst10 overexpression, we performed two experiments with independent transfection procedures. For the first experiment, we established 18 stable clones from 18 different colonies of ATDC5 cells transfected with pcMV-Cst10 and selected three clones (clones 3, 8, and 12) with the highest expression of Cst10 (pcMV-Cst10/ATDC5) by RT-PCR analysis. Three mock vector-transfected clones (clones 1–3) that were confirmed by RT-PCR not to express Cst10 were also selected as negative controls (pcMV/ATDC5). For the second experiment, we randomly divided the stable clones into two sets; pcMV-Cst10/ATDC5 is the set transfected above, randomly established four stable clones, and examined the relationship of expressions between Cst10 and collagen expressions by RT-PCR.

Immunohistochemistry—The samples harvested from embryonic mice were fixed in 4% paraformaldehyde, cryosectioned at a thickness of 10 μm by cryomicrotome. The cryosections were treated following methods similar to those used for immunohistochemical investigations, being post-fixed in 1% OsO₄ in 0.1 M cacodylate buffer at 4°C for 1 h. The specimens were dehydrated in a graded ethanol series and embedded in PolyBed 812 resin (Polysciences, Warrington, PA). Ultrathin sections stained with lead citrate were observed under a transmission electron microscope (H-7100, Hitachi, Tokyo) following the pre-embedding method described previously (8). A BAC clone containing the mouse Cst10 for 24 h at 4°C and then with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Dakopatts, Glostrup, Denmark) at a dilution of 1:500 for 1 h at room temperature. After washing with PBS, the sections were immersed in diaminobenzidine solution for 10 min at room temperature for visualization and counterstained with hematoxylin. Immunohistochemistry was performed using an FITC-labeled annexin V apoptosis detection kit (Medical and Biological Laboratories) was used (18). ATDC5 cells harvested 7 days

Available at rsb.info.nih.gov/nih-image/download.html.
after induction were incubated with MitoCapture solution for 15 min at 37 °C and viewed under a fluorescent microscope using a band-pass filter (detects FITC and rhodamine). In healthy cells, MitoCapture accumulates and aggregates in the mitochondria, giving off a bright red fluorescence. In apoptotic cells, MitoCapture cannot aggregate in the mitochondria due to the altered mitochondrial membrane potential and thus remains in the cytoplasm in its monomer form, fluorescing green. For flow cytometric analysis, ATDC5 cells were harvested 0, 1, 3, 5, 7, 10, and 14 days after induction by insulin and fixed with 75% ethanol and PBS at 4 °C for 1 h. After rinsing twice with PBS, cells were incubated for 30 min with 1 ml of PBS containing 1 mg of boiled RNase at 37 °C and then stained with 1 ml of PBS containing 10 μg of PI. A total of 2 × 10^4 cells were analyzed with a flow cytometer (FACSCaliber, BD Biosciences). To determine whether caspase-3 is activated in pCMV-Cst10/ATDC5, a PhiLux kit (Medical and Biological Laboratories) was used according to the manufacturer’s protocol (19). Briefly, ATDC5 cells harvested 7 days after induction were incubated with 10 mM GDEVDGI and labeled with two molecules of rhodamine, which was selectively cut by caspase-3. After incubation, cells were viewed under a fluorescent microscope. Caspase-3 activity was determined with a caspase-3/CPP32 colorimetric protease assay kit (Medical and Biological Laboratories) according to the manufacturer’s protocol. In brief, ATDC5 cells were harvested 7 days after induction with insulin and lysed in lysis buffer. Cell lysate (150 μg of protein in 50 μl of lysis buffer) was incubated with DEVD-p-nitroanilide (pNA) as a substrate for 1 h at 37 °C, and the amount of pNA generated was determined spectrophotometrically at 405 nm. Caspase-8 and caspase-9 activities were determined with caspase-8/FLICE and caspase-9/Mch6 colorimetric assay kits, respectively (Medical and Biological Laboratories), according to the manufacturer’s protocols. IETD-p-nitroanilide for caspase-8 and LEHD-p-nitroanilide for caspase-9 were used as substrates. After incubation for 1 h at 37 °C, the amount of p-nitroanilide generated was determined spectrophotometrically at 405 nm (20–22).

**Statistical Analysis**—Means of groups were compared by analysis of variance, and significance of differences was determined by post-hoc testing using Bonferroni’s method.

**RESULTS**

**Characterization of the Cst10 Gene**—In a previous study, using differential display analysis, we identified nine genes, including Cst10, whose expression is regulated in the auricular cartilage of ttu mice fed a high phosphate diet (1). The full-length cDNA sequence of the mouse Cst10 gene determined by the manufacturer’s protocols. IETD-p-nitroanilide for caspase-8 and LEHD-p-nitroanilide for caspase-9 were used as substrates. After incubation for 1 h at 37 °C, the amount of p-nitroanilide generated was determined spectrophotometrically at 405 nm (20–22).
cating a novel member of the type II cystatin superfamily with 40.5 and 39.0% homologies to mouse and human CstC (Cst3), respectively, the closest cystatin family member (Fig. 1A). Its homologies to other mouse cystatins were around or less than 30%: 31.5% to Cst9, 31.4% to Cst7, 27.0% to CstEM, 28.6% to CstSC, and 26.7% to CstTE. To investigate the localization of the Cst10 gene in the mouse chromosome, 50 metaphase cells were examined by fluorescence in situ hybridization using a BAC clone containing the mouse Cst10 gene as a probe. Specific hybridization signals were identified on chromosome 2 in almost all cells, and no significant background was observed at any other chromosomal sites (Fig. 1B).

Temporal and Spatial Expression of Cst10 in Vivo and in Vitro—We first examined the temporal expression pattern of Cst10 mRNA levels in the auricular cartilage of ttw mice whose endochondral ossification was enhanced with a high phosphate diet. Expression appeared 3 days after weaning and was upregulated by a high phosphate diet at 5 days and thereafter (Fig. 2A). Our previous study on the tissue distribution of Cst10 expression in a variety of mouse tissues showed that this gene is expressed exclusively in cartilage (1). We therefore examined the expression pattern of Cst10 using cell cultures. Among three cultured mesenchymal cells from neonatal ddY mice (primary osteoblasts from calvariae, chondrocytes from costal cartilage, and fibroblasts from skin), Cst10 expression was confirmed to be specific to chondrocytes (Fig. 2B). To characterize the expression pattern during differentiation of chondrocytes, we used the mouse chondrogenic cell line ATDC5, which can be induced to differentiate into mature chondrocytes in the presence of insulin (12). During induction of differentiation with insulin, expression of type II collagen remained unchanged throughout the culture period up to 14 days, whereas that of type X collagen, a marker for hypertrophic chondrocytes, appeared 7 days after induction (Fig. 2C). Expression of the Cst10 gene appeared at 3 days and increased thereafter, indicating that Cst10 expression is in synchrony with the maturation of chondrocytes.

To examine the localization of Cst10 in cartilage, we first confirmed by Western blot analysis the specificity of a polyclonal antibody against Cst10 without cross-reactivity with CstC, the closest member of the cystatin superfamily (Fig. 2D). Using this antibody, we performed immunohistochemical analysis on the growth plates of embryonic ddY mice and found that Cst10 was expressed mainly in mature chondrocytes, including...
prehypertrophic and hypertrophic cells (Fig. 2E). Electron microscopic examination of a hypertrophic chondrocyte revealed that Cst10 was immunolocalized in the cytosolic areas, but was not found in the nucleus or within the lumen of the rough endoplasmic reticulum (Fig. 2F). These findings suggest that Cst10 is not transported into the Golgi-endoplasmic reticulum system, but acts as an intracellular protein in the cytosol.

**Overexpression of the Cst10 Gene Accelerates Maturation of ATDC5 Cells**—To elucidate the function of Cst10 in chondrocytes, we established stable clones of ATDC5 cells overexpressing the Cst10 gene (pCMV-Cst10/ATDC5). We first compared by RT-PCR the differentiation of pCMV-Cst10/ATDC5 cells with that of control clones of ATDC5 cells transfected with the mock vector (pCMV/ATDC5) upon induction with insulin (Fig. 3A). In pCMV-Cst10/ATDC5 cells, Cst10 mRNA expression was clearly seen not only after, but also before induction (time 0), whereas in pCMV/ATDC5 cells, expression was faintly seen 7 days after induction and increased moderately thereafter. Western blot analysis revealed that the Cst10 protein was localized in the cell lysate, but not in the culture medium of pCMV-Cst10/ATDC5 cells (Fig. 3B), indicating that Cst10 is not a secreted protein. Expression of type II collagen, which is known to be produced by chondrocytes from their early phase of differentiation, was constitutively seen before and after induction, and expression was not different between pCMV-Cst10/ATDC5 and pCMV/ATDC5 cells (Fig. 3A). However, expression of type X collagen, a marker of hypertrophic chondrocytes, was observed earlier and was stronger in pCMV-Cst10/ATDC5 cells than in pCMV/ATDC5 cells. We also compared the cartilage nodule formation and mineralization between cultured pCMV-Cst10/ATDC5 and pCMV/ATDC5 cells using Alcian blue and alizarin red staining, respectively. No difference was seen in the Alcian blue staining between the two cells; however, alizarin red staining was stronger in cultured pCMV-Cst10/ATDC5 cells than in pCMV/ATDC5 cells (Fig. 3C).

To confirm the reproducibility of the effects of Cst10 overexpression, we performed another experiment using ATDC5 cell clones that were independently transfected with pCMV-Cst10, independent of the first experiment, and four stable clones (clones 1–4) were randomly established. Expression of Cst10 and type II and X collagens was compared among the clones by semiquantitative RT-PCR before (time 0) and 5 days after induction with insulin. Gapdh, glyceraldehyde-3-phosphate dehydrogenase.

---

**Fig. 3. Effect of Cst10 overexpression on maturation of ATDC5 cells.** We performed two experiments with independent transfection of the Cst10 gene. For the first experiment (A–C), 18 stable clones of ATDC5 cells transfected with pCMV-Cst10 were established as described under “Experimental Procedures,” and three clones (clones 3, 8, 12) with the highest expression of Cst10 (pCMV-Cst10/ATDC5) were selected by RT-PCR analysis. Three clones (clones 1–3) transfected with the mock vector (pCMV/ATDC5) were also selected as negative controls. Although the data shown in A–C are for representative clones (clone 3 for pCMV-Cst10/ATDC5 and clone 1 for pCMV/ATDC5), the results were reproducible when other clones in each group (clones 8 and 12 for pCMV-Cst10/ATDC5 and clones 2 and 3 for pCMV/ATDC5) were used. A, shown are the temporal expression patterns of Cst10, type II collagen (Col II), and type X collagen (Col X) during differentiation of pCMV-Cst10/ATDC5 and pCMV/ATDC5 cells cultured in the presence of insulin. At the indicated days after induction with insulin, cells were harvested, and the mRNA levels were examined by semiquantitative RT-PCR. B, Cst10 protein levels in the cell lysate and medium of pCMV-Cst10/ATDC5 cells cultured for 5 days in the presence of insulin were determined by Western blot analysis. The Cst10 protein could not be detected in the culture medium even if it was lyophilized and condensed (data not shown). C, pCMV-Cst10/ATDC5 and pCMV/ATDC5 cells were stained with Alcian blue and alizarin red at 21 and 28 days of culture, respectively, in the presence of insulin. D, for the second experiment, ATDC5 cells were transfected with pCMV-Cst10, independent of the first experiment, and four stable clones (clones 1–4) were randomly established. Expression of Cst10 and type II and X collagens was compared among the clones by semiquantitative RT-PCR before (time 0) and 5 days after induction with insulin. Gapdh, glyceraldehyde-3-phosphate dehydrogenase.
the Cst10 gene accelerates the later (but not earlier) stage of chondrocyte differentiation and mineralization.

**Overexpression of Cst10 Leads to Apoptosis of ATDC5 Cells**—Staining with Hoechst 33342 revealed the existence of cells with fragmented and condensed nuclei with increased fluorescence, suggesting apoptotic cell death in pCMV-Cst10/ATDC5 cells, but not in pCMV/ATDC5 cells (Fig. 4A). To distinguish between apoptosis and necrosis in these cells, the kinetics of loss of membrane integrity was examined by double staining with annexin V and PI. Annexin V is known to stain positive in the early stage of apoptotic cells that retain the ability to exclude vital dyes, whereas PI becomes positive in necrotic cells that have lost membrane integrity and that have undergone rapid swelling and lysis (15–17). Most pCMV-Cst10/ATDC5 cells were stained green, indicating that annexin V was positive; however, pCMV/ATDC5 cells were stained red, indicating that PI was positive (Fig. 4B). In the analysis of apoptosis through the mitochondrial pathway, many pCMV-Cst10/ATDC5 cells were stained green, indicating the change in the mitochondrial membrane potential, whereas there were no positively stained cells in the pCMV/ATDC5 cell culture (Fig. 4C). We further examined the involvement of caspases in the stimulation of chondrocyte apoptosis by Cst10. Staining of substrates specific to caspase-3 revealed that there were many cells with a high activity of this caspase in pCMV-Cst10/ATDC5 cells, but not in pCMV/ATDC5 cells (Fig. 4D). Furthermore, we examined the activities of caspase-3, -8, and -9 by measuring pNA free of each substrate. pCMV-Cst10/ATDC5 cells exhibited significantly higher activities of all these caspases compared with pCMV/ATDC5 cells (Fig. 4E). Caspase-3 and caspase-9, which are known to be associated mainly with the mitochondrial pathway of apoptosis, were strongly activated by Cst10 gene overexpression. These results imply the importance of the mitochondrial pathway in the induction of chondrocyte apoptosis by Cst10.

To determine whether the induction of apoptosis by Cst10 overexpression is a direct action or secondary to that of maturation, we examined the time course of hypertrophic matura-
tion and apoptosis determined by the type X collagen mRNA level shown in Fig. 3A and the percentage of cells in the sub-G1 population by flow cytometric analysis, respectively (Fig. 4F).

In pCMV-Cst10/ATDC5 cells, significant induction of hypertrophy was seen at 3 days and that of apoptosis at 10 days, whereas in pCMV/ATDC5 cells, hypertrophy was at 7 days and apoptosis at 14 days. Hence, the time gaps between hypertrophy and apoptosis were similar (~7 days) in both cells, suggesting that induction of apoptosis by overexpression of the Cst10 gene may not be direct, but is secondary to that of hypertrophic maturation.

DISCUSSION

Based on our previous study in which we identified a novel gene (Cst10) whose expression is up-regulated during ossification of auricular cartilage by a high phosphate diet in ttw mice (1), in the present study, we investigated the possible role of Cst10 in endochondral ossification. Expression was seen exclusively in differentiated chondrocytes in both in vivo and in vitro mouse models. Overexpression of the Cst10 gene in ATDC5 cells induced their maturation, followed by apoptosis, suggesting an important role of Cst10 in the last steps of chondrocyte differentiation.

Cst10 is a novel member of the cystatin superfamily and shows ~40% homology to both mouse and human CstC. CstC is an abundant extracellular inhibitor of all cysteine proteinases of the papain superfamily (23) and is related to several human disorders such as atherosclerosis and aortic aneurysms (24) and hereditary amyloid angiopathy of the brain (25, 26). CstC also down-regulates bone resorption by inhibiting osteoclastic proteolytic enzymes released into the resorption lacunae in skeletal tissues (27-29). Unlike other members of the cystatin superfamily such as CstC, expression of Cst10 is limited to chondrocytes, implying some specific role of Cst10 in cartilage homeostasis. In addition, electron microscopic examination (Fig. 2F) and Western blot analysis (Fig. 3B) of cultured pCMV-Cst10/ATDC5 cells indicated that Cst10 is not a secreted protein, but is an intracellular enzyme localized in the cytosol. It is therefore possible that Cst10 has a function distinct from those of other cystatin members that act as extracellular inhibitors of proteinases. Investigation of the physiological role of Cst10 in cartilage in vivo is now under way by creating a Cst10 gene-deficient mouse line using homologous recombination in embryonic stem cells.

Regarding the regulation of chondrocyte differentiation and maturation, several secreted molecules have been identified. Bone morphogenetic proteins (BMPs) are reported to be positive regulators, whereas parathyroid hormone-related protein (PTHrP) acts to slow the rate of chondrocyte maturation and to maintain chondrocytes in a proliferative state. Analyses of PTHrP knockout (30, 31) and transgenic (32, 33) mice point to there being a direct function of those of other cystatin members that act as extracellular inhibitors of proteinases. Investigation of the physiological role of Cst10 in cartilage in vivo is now under way by creating a Cst10 gene-deficient mouse line using homologous recombination in embryonic stem cells.

Bone morphogenetic proteins (BMPs) are reported to be positive regulators, whereas parathyroid hormone-related protein (PTHrP) acts to slow the rate of chondrocyte maturation and to maintain chondrocytes in a proliferative state. Analyses of PTHrP knockout (30, 31) and transgenic (32, 33) mice point to there being a direct function of those of other cystatin members that act as extracellular inhibitors of proteinases. Investigation of the physiological role of Cst10 in cartilage in vivo is now under way by creating a Cst10 gene-deficient mouse line using homologous recombination in embryonic stem cells.

Cystatin 10 in Chondrocyte Differentiation

Cystatin 10 is a novel member of the cystatin superfamily and shows ~40% homology to both mouse and human CstC. CstC is an abundant extracellular inhibitor of all cysteine proteinases of the papain superfamily (23) and is related to several human disorders such as atherosclerosis and aortic aneurysms (24) and hereditary amyloid angiopathy of the brain (25, 26). CstC also down-regulates bone resorption by inhibiting osteoclastic proteolytic enzymes released into the resorption lacunae in skeletal tissues (27-29). Unlike other members of the cystatin superfamily such as CstC, expression of Cst10 is limited to chondrocytes, implying some specific role of Cst10 in cartilage homeostasis. In addition, electron microscopic examination (Fig. 2F) and Western blot analysis (Fig. 3B) of cultured pCMV-Cst10/ATDC5 cells indicated that Cst10 is not a secreted protein, but is an intracellular enzyme localized in the cytosol. It is therefore possible that Cst10 has a function distinct from those of other cystatin members that act as extracellular inhibitors of proteinases. Investigation of the physiological role of Cst10 in cartilage in vivo is now under way by creating a Cst10 gene-deficient mouse line using homologous recombination in embryonic stem cells.

Regarding the regulation of chondrocyte differentiation and maturation, several secreted molecules have been identified. Bone morphogenetic proteins (BMPs) are reported to be positive regulators, whereas parathyroid hormone-related protein (PTHrP) acts to slow the rate of chondrocyte maturation and to maintain chondrocytes in a proliferative state. Analyses of PTHrP knockout (30, 31) and transgenic (32, 33) mice point to there being a direct function of those of other cystatin members that act as extracellular inhibitors of proteinases. Investigation of the physiological role of Cst10 in cartilage in vivo is now under way by creating a Cst10 gene-deficient mouse line using homologous recombination in embryonic stem cells.

Bone morphogenetic proteins (BMPs) are reported to be positive regulators, whereas parathyroid hormone-related protein (PTHrP) acts to slow the rate of chondrocyte maturation and to maintain chondrocytes in a proliferative state. Analyses of PTHrP knockout (30, 31) and transgenic (32, 33) mice point to there being a direct function of those of other cystatin members that act as extracellular inhibitors of proteinases. Investigation of the physiological role of Cst10 in cartilage in vivo is now under way by creating a Cst10 gene-deficient mouse line using homologous recombination in embryonic stem cells.

There are many reports demonstrating the association of cysteine proteinases and their inhibitors with apoptotic cell death. Disruption of lysosomal membranes and release of lysosomal enzymes, including proteinases such as cathepsins B (36–38), D (39, 40), and L (41, 42), are known to cause apoptosis (43, 44). This pathway is thought to be mediated mainly by cleavage of the Bcl-2 family member Bid (45). Caspases, which play an integral part in the apoptosis pathway, also belong to a family of cysteine proteinases with aspartate specificity. Some cysteine proteinase inhibitors are reported to block apoptosis (41, 46); and among them, leupeptin and E-64 specifically suppress activation of caspase-3-like proteinases (47). Hence, cystatins could also down-regulate apoptosis by inhibiting lysosomal enzymes or caspases. In fact, loss-of-function mutations in the cystatin B gene cause a severe neurological disorder known as Unverricht-Lundborg disease in humans (48, 49) and myoclonic seizures and ataxia in mice (50, 51), in which cerebellar granule cells appear to undergo apoptosis. In contrast, CstC has been reported to induce apoptosis in cultured rat neurons (52) and during mouse embryo implantation and placentation (53). The present study has also shown that Cst10 overexpression led to apoptosis in chondrocytes. A direct contribution of proteinases to chondrocyte apoptosis was actually implied by the finding that matrix metalloproteinase-9-deficient mice exhibit normal hypertrophic maturation, but delayed apoptosis (54). However, the action of Cst10 on chondrocyte apoptosis is not likely to be direct, but secondary to its stimulation of maturation, because the time gaps between hypertrophy and apoptosis were similar between cells with and without Cst10 overexpression (Fig. 4F). Furthermore, in our preliminary experiments, Cst10 gene overexpression failed to promote apoptosis in the non-chondrogenic cell line COS-7 (data not shown). Recent studies on various knockout mice also suggest that there is a direct coupling between maturation and apoptosis in chondrocytes. PTHrP-deficient mice exhibit acceleration of both hypertrophy and apoptosis in growth plate chondrocytes (30, 31). Mice deficient in Bcl-2, an inhibitor of apoptosis, exhibit premature chondrocyte differentiation (33). Although changes in the mitochondrial membrane potential and related caspases were seen in Cst10-expressing chondrocytes, this also may not be a specific action of Cst10 because normal chondrocyte apoptosis is known to involve Bcl-2, mitochondrial integrity, and caspases (33, 55). Further studies on intracellular interactions between Cst10 and signaling molecules of apoptosis such as Bcl-2 will elucidate more precise mechanisms by which Cst10 overexpression leads to chondrocyte apoptosis.

Chondrocyte maturation, mineralization, and apoptosis in the endochondral ossification process are observed in physiological development and growth as well as under pathological conditions such as osteoarthritis and ectopic ossification. Understanding the molecular mechanisms of endochondral ossification through the Cst10 signaling pathway may therefore help elucidate not only the mechanism of skeletal development and growth, but also the pathophysiology of these diseases.

REFERENCES

1. Koshizuka, Y., Ikegawa, S., Sano, M., Nakamura, K., and Nakamura, Y. (2001) Cytogenet. Cell Genet. 94, 163–168
2. Koshizuka, Y., Ikegawa, S., Sano, M., Nakamura, K., and Nakamura, Y. (2001) Genomics 72, 252–259
3. Ikegawa, S., Sano, M., Koshizuka, Y., and Nakamura, Y. (2000) Cytogenet. Cell Genet. 90, 291–297
4. Okawa, A., Nakamura, I., Goto, S., Moriya, H., Nakamura, Y., and Ikegawa, S. (1998) Nat. Genet. 19, 271–273
5. Hosoda, Y., Yoshimura, Y., and Higaki, A. (1981) Ryumachi 39, 271
6. Barrett, A. J., Fritz, H., Grubb, A., Isemura, S., Jarvinen, M., Katunuma, N.,
