Chondromodulin I Is a Bone Remodeling Factor

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Chondromodulin I (ChM-I) was supposed from its limited expression in cartilage and its functions in cultured chondrocytes as a major regulator in cartilage development. Here, we generated mice deficient in ChM-I by targeted disruption of the Chm-I gene. No overt abnormality was detected in endochondral bone formation during embryogenesis and cartilage development during growth stages of Chm-I−/− mice. However, a significant increase in bone mineral density with lowered bone resorption with respect to formation was unexpectedly found in adult Chm-I−/− mice. Thus, the present study established that Chm-I is a bone remodeling factor.

Endochondral bone development during embryogenesis and longitudinal bone growth in growing vertebrates require continuous cartilage growth (18). Proliferating chondrocytes originate from a region of resting chondrocytes, differentiate first into prehypertrophic chondrocytes and then into hypertrophic chondrocytes able to secrete the cartilage matrix. Through invasion by blood vessels, the calcified cartilage and vascular matrix are gradually replaced by bone matrix with the recruitment of osteoclasts and osteoblasts that mediate bone resorption and formation and eventual bone remodeling (1, 30). Thus, in bone growth, blood vessel invasion into cartilage is pivotal to the process of endochondral bone formation.

Distinct classes of factors are thought to play cognate roles in the spatiotemporal regulation of the complicated yet sequential processes of cartilage differentiation and bone formation, particularly in angiogenic events. Fibroblast growth factor-2 (5, 31), transforming growth factor β (3), and vascular endothelial growth factor (4) are expressed in cartilage and have been identified as strong angiogenic agents. However, these factors are also present in avascular cartilage and in surrounding vascular regions. These findings raise the possibility that the actions of angiogenic factors may be suppressed by the inhibitory action of a specific factor in avascular cartilage. While tissue inhibitors of matrix metalloproteinase 1 and 2 have been identified from cartilage as possible angiogenesis inhibitors, they are also expressed in other tissues (20). The search for a cartilage-specific inhibitor of angiogenesis led to the identification of chondromodulin I (Chm-I), initially isolated from bovine epiphyseal cartilage as a factor with growth-promoting activity on cultured chondrocytes (10). Chm-I was found to be a potent stimulator of proteoglycan synthesis in growth plate chondrocytes and of chondrocyte colony formation in agarose (12). However, Chm-I inhibited cultured vascular endothelial cell tube morphogenesis and growth (8, 9). Thus, the physiologival significance of Chm-I during endochondral bone formation as a bifunctional factor of chondrocyte growth and angiogenesis inhibition was suggested from distinct lines of evidence in vitro (27). However, due to the lack of mice deficient in Chm-I, there has been no information regarding the physiological role of Chm-I.

In the present study, we disrupted the murine Chm-I gene by homologous recombination to generate Chm-I knockout (Chm-I−/−) mice. Homozygous Chm-I−/− mice were born without overt abnormalities and grew normally. Unexpectedly, Chm-I−/− mice exhibited no aberrations in endochondral bone formation during embryogenesis or in cartilage development during growth stages. However, a significant increase in bone mineral density was observed in 12-week-old Chm-I−/− mice. Analyses of bone formation and resorption indicators revealed that bone minerals accumulated in Chm-I−/− mice due to lowered bone resorption with respect to formation. Thus, our study revealed that the physiological role of Chm-I appears to be involved in the stimulation of bone remodeling through control of osteoclast and osteoblast functions rather than in cartilage development in intact animals.

MATERIALS AND METHODS

Gene targeting. A T72 embryonic stem (ES) cell (34) genomic library was screened with a mouse Chm-I cDNA probe (24). A 9-kb fragment of mouse Chm-I containing the coding exons 1 to 3 was used to construct a targeting vector. A stop mutation was introduced at the beginning of the Chm-I coding region, and 3.5-kb fragment containing exon 3 was replaced with a phosphoglyceraldehyde kinase-neomycin cassette. TT2 ES cells were transfected with a linearized targeting vector (25 V and 500 μA per 1.0×107 cells) by using a Bio-Rad Gene Pulser II at 250 V and 500 μA and grown under G418 selection as described previously (23, 36). Targeted ES cell clones were identified by Southern blot analysis with probe A and probe NEO (Fig. 1A) and were aggregated with CD-1 single 8-cell embryos to generate chimeras as described previously (23, 36). Chimeras were crossed with C57BL/6 female mice to produce germ line transmission of the targeted allele. Offspring were genotyped either by Southern blotting with probe A or by PCR with the three specific primers P1 (5′-TGTTTGATGCTTCAG
FIG. 1. Disruption of the mouse ChM-I gene. (A) Schematic representation of the ChM-I gene locus (top), gene targeting vector (middle), and the recombinated locus (bottom). The digested fragments detected by probe A, probe B, and probe NEO are indicated by bars. (B) Southern blot analysis of targeted ES clones. The targeting frequency was 0.3%. The presence of the 4.5-kb BamHI fragment indicates proper targeting of the ChM-I locus, and the 3-kb EcoRV fragment indicates the introduction of the stop mutation. (C) Southern blot analysis of tail DNA from the offspring of heterozygous mates with probe A as described for panel B. (D) PCR genotyping of embryos at e13.5 of heterozygous matings with three primers (P1, P2, Pneo) as indicated in panel A. Primer 1 and primer 2 were used to detect the wild-type (WT) allele (amplification of a 452-bp fragment). Primer 2 and primer NEO were used to detect the targeted allele (amplification of a 351-bp fragment). (E) Northern blot analysis of RNA from ChM-I++ and ChM-I−/− mice in the top panel. The bottom panel shows ethidium bromide (EtBr) staining of the RNA used. (F) Western blot analysis confirming the absence of ChM-I protein with an anti-rhChM-I polyclonal antibody. The top panel shows the immunoreactivity after hybridization with anti-ChM-I antibody. The bottom panel shows Coomassie brilliant blue staining of the SDS-polyacrylamide gel electrophoresis gel.
TGTTG-3', P2 (5'-CTTGTGCACAGCAGGAAACA-3'), and Pneo (5'-CCG CTTCTCTGTGCTTACGG-3'). Temperature cycling conditions were as follows: denaturation at 95°C for 2 min followed by 35 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 1.5 min.

Neonatal Mammalian assay. Total RNA was prepared from rib cartilage of 3-to-4-week-old ChM-I+/− or ChM-I−/− mice by a single-step method (35). Total RNA (15 μg) was separated by electrophoresis on a 1% agarose formaldehyde gel and transferred onto a Nytran filter with TurboBlotter (Schleicher and Schuell). The filter was then hybridized with a 32P-labeled probe that is an 822-bp EcoRI fragment from nucleotides 628 to 1449 of the ChM-I cDNA. After hybridization, the filter was washed at 55°C for 30 min in 1×SSPE (1×SSPE is 0.18 M NaCl, 10 mM Na2HPO4, and 1 mM EDTA [pH 7.4])–0.1% sodium dodecyl sulfate (SDS) once, then washed at 55°C for 30 min in 0.1×SSPE–0.1% SDS twice. The filter was exposed to BIOMAX film (Eastman Kodak) at −80°C.

Western blot analysis. Whole ribs from 3-week-old mice were homogenized in extraction buffer (20 mM MES [2-(N-morpholino)ethanesulfonic acid]–NaOH [pH 6.0], 0.1 M aminocapric acid, 6 M guanidinium chloride) at 4°C and centrifuged at 10,000 × g. The supernatant was diluted with 2 volumes of distilled water and applied to a butyl-toyopearl 650 affinity column (OSOM). The column was washed three times with distilled water. Bound proteins were eluted by 70% ethanol and dried. The dried materials (8 μg) were dissolved in Laemmli buffer, electrophoresed by SDS–15%polyacrylamide gel electrophoresis, and blotted onto an Immobilon-P membrane (Millipore). Bands that were immunoreactive with an epitope of human ChM-I, corresponding to the Asp252 to Val334 residues, which is a semiautomated system for bone analysis (Osteoplan II; Carl Zeiss) at 200-fold magnification, tibiae were embedded in glycolmethacrylate without decalcification. Bones were prepared with a microtome (model 2050; Reichert Jung). The sections were formed for at least eight optical links, a marker of bone resorption (32), was measured in urine samples by using the Pyrllks-D enzyme-linked immunosorbent assay (Metra Biosystems). Results were expressed as nanomoles per milliliter of urine creatinine (Cr), as measured by a standard colorimetric method with an autoanalyzer (type 7170, Hitachi).

RESULTS

Generation of targeted ChM-I-null mice. We disrupted the murine Chm-I gene in ES cells by homologous recombination to generate Chm-I−/− mice. The targeting vector (Fig. 1A) was constructed to introduce a stop codon at Cys-21, and a 3.5-kb genomic fragment containing exom 3 was replaced by a phosphoglycerate kinase-neomycin cassette. No overt abnormalities were found in Chm-I−/− mice, and crossingbreed of Chm-I−/− mice produced normal numbers of pups of all three possible genotypes (Fig. 1C) with the expected Mendelian distribution (245 offspring) (Fig. 1B and C). Northern blot analysis of rib cartilage from normal mice with a cDNA probe encoding the mature form of ChM-I protein detected a single 1.7-kb transcript. No transcripts were found in Chm-I−/− mice, confirming the disruption of the Chm-I gene (Fig. 1E). Western blot analysis of whole-rib extracts from Chm-I−/− mice with an antibody against the mature Chm-I protein also confirmed the absence of the Chm-I protein (Fig. 1F).

Chm-I is not required for cartilage development and endochondral bone formation. Chm-I−/− mice grew normally without any discernible physical defects and with normal fertility. As Chm-I had been implicated in cartilage development, endochondral bone formation, and morphogenesis of the eye, careful histological examination of these tissues from Chm-I−/− mouse embryos and mice at various growth stages was performed. However, we failed to detect any abnormalities in cartilage formation (Fig. 2A), first ossification (Fig. 2B) in Chm-I−/− fetuses, and secondary ossification in Chm-I−/−
FIG. 2. No overt difference was detected between ChM-I^{-/-} and ChM-I^{+/+} mice. (A) Alizarin red and Alcian blue staining of e13.5 embryos. Bar, 0.5 mm. In all panels, osteo-chondroprogenitors staining of epiphysis from 3-week-old mice. Bar, 0.5 mm. In all panels, osteo-chondroprogenitors staining of epiphysis from 1-week-old mice. Bar, 0.5 mm. (B) Safranin O-fast green-hematoxylin staining of proximal growth plate of tibiae from e18.5 embryos. Bar, 0.1 mm. h, hypertrophic zone; p, proliferating zone; r, resting zone. (C) Safranin O-fast green-hematoxylin staining of epiphysis from 1-week old mice. Bar, 0.1 mm. (D) Safranin O-fast green-hematoxylin staining of epiphysis from 3-week old mice. Bar, 0.5 mm. In all panels, no overt difference was detected between ChM-I^{-/-} and ChM-I^{+/+} mice.
mice at 1 and 3 weeks old (Fig. 2C and D), in agreement with normal growth of Chm-I−/− mice.

**Mice homozygous for the Chm-I mutation exhibit increased bone mineral density.** Unexpectedly, a significant increase in bone mineral density was observed in 12-week-old Chm-I−/− mice but not in Chm-I+/− mice. Also, the radiographic mineral density of the femur in mutant mice was approximately 10% higher than in wild-type mice (Fig. 3A and B). Histomorphometric analyses confirmed that trabecular bone volumes (bone volume per tissue volume) in mutant mice were 2.5-fold higher than in wild-type mice (Fig. 3C and E). However, no significant differences in bone or body size and shape were observed between Chm-I−/− and wild-type mice. Osteoid surfaces (osteoid surface per bone surface) in mutant mice were 54% lower than in wild-type mice (Fig. 3F), but the osteoid thickness value in mutant mice was equivalent to that in wild-type mice (Fig. 3G). Indeed, the expression of the Chm-I gene was detected in the primary culture osteoblasts and total bone, though their expression levels appear to be much lower than those in cartilage (Fig. 4).

As it was possible that the observed increase in bone mineral density was related to bone remodeling (2), we studied bone formation and resorption in terms of osteoblast and osteoclast function. TRAP-positive mature osteoclast and chondroclast numbers were reduced in Chm-I−/− mice (Fig. 3D). This was in agreement with the results of histomorphometry analyses that showed that the numbers of bone osteoclasts (osteoclast number per bone perimeter) and surface osteoclasts (osteoclast surface per bone surface) in Chm-I−/− mice were 33 and 34% lower than in wild-type mice, respectively (Fig. 3H and I). Eroded surface (eroded surface per bone surface) values, which represent osteoclast activity, were also significantly decreased in Chm-I−/− mice (Fig. 3J). In addition, osteoblast surface (osteoblast surface per bone surface) values, a reliable histomorphometric indicator of active osteoblast numbers, were significantly reduced to approximately 60% by Chm-I inactivation (Fig. 3K). Reflecting the reduced osteoclast and/or chondroclast activity in Chm-I−/− mice, more cartilaginous matrix remained in the first spongiosa of tibias in the Chm-I−/− mice than in wild-type mice (Fig. 3C, right).

**Loss of Chm-I affects bone metabolism.** We then estimated the bone formation rate by using calcein double-labeling of the mineralized matrix (22). Both the mineral apposition rate (MAR) and bone formation rate (BFR) (BFR per bone surface) were significantly decreased in Chm-I−/− mice (Fig. 5A, B, and C). Reduced levels in serum of markers for osteoblastic function (alkaline phosphatase activity and osteocalcin) (Fig. 5E and F) and no alteration in serum minerals (Fig. 5G, H) supported the hypothesis that bone formation activity was reduced by Chm-I inactivation. Total urinary deoxypyridinoline levels, a marker of bone resorption, were measured by enzyme-linked immunosorbent assay; however, no statistical difference was observed between wild-type and Chm-I−/− mice (Fig. 5D). Moreover, in vitro osteoclastogenesis with donor bone marrow macrophages from mutant and wild-type mice revealed that Chm-I inactivation appears to cause no abnormality in osteoclastogenesis (Fig. 6). Our results indicated that the increased bone mineral density in Chm-I−/− mice appeared to be due to lowered bone resorption with respect to bone formation. Thus, the present study established that Chm-I is likely to be a bone remodeling factor rather than being involved in chondrocyte development.
DISCUSSION

ChM-I is a 25-kDa glycoprotein generated from a larger transmembrane precursor after posttranslational modification and proteolytic cleavage at a processing signal site (10). ChM-I was originally purified from bovine epiphyseal cartilage as a growth factor that stimulated anchorage-independent growth of chondrocytes in agarose (8) and induced proteoglycan synthesis (8). However, ChM-I also possesses inhibitory activity on the growth and tube morphogenesis of cultured vascular endothelial cells. Due to its bifunctional activities in vitro, it was thought that ChM-I might play a pivotal role in endochondral bone development during embryogenesis and in postnatal cartilage growth in vivo (27). However, our present observations with ChM-I−/− mice showed that ChM-I was not essential for normal cartilage formation and development. Indeed, our study revealed that ChM-I is more likely to be involved in normal bone remodeling, probably through regulating osteoclast and osteoblast numbers and functions. Detailed analysis of bones from ChM-I−/− mice showed that bone resorption was lower in comparison to bone formation, leading to increased bone mineral density and insufficient bone turnover.

Considering the marked cartilage phenotypes of mice deficient for angiogenic factors (6, 11, 33) or their inhibitors (7, 14), with which ChM-I was considered an equally potent factor in the cell culture systems (8, 9), the normal development of cartilage at embryonic and postnatal stages in ChM-I−/− mice was unexpected. Furthermore, no overt abnormalities were found in tests for rib fracture healing in adult ChM-I−/− mice (data not shown). It is possible that a functionally redundant factor may compensate for the lack of ChM-I activity in ChM-I−/− mice.

FIG. 5. Dynamic histomorphometric and serum biochemical parameters of bone metabolism in ChM-I+/+ and ChM-I−/− mice (12 weeks of age). MAR (A) and BFR (B) are using sections from animals that were double-labeled with calcein in vivo. MAR and BFR measure the amount of bone that is mineralized or deposited per time unit and are based on the measurement of the distance between the two fluorescent labels. (C) Two calcin-labeled mineralization fronts of tibial trabecular bones from ChM-I+/+ and ChM-I−/− mice were visualized by fluorescent microscopy. (D) Urinary deoxyriboflavin (DPD) excretion in 12-week-old wild-type and ChM-I−/− mice. (E) Alkaline phosphatase activity in serum. (F) Osteocalcin level in serum. (G) Calcium level in serum. (H) Phosphorus level in serum. Bars represent means ± standard errors for wild-type (black bars) and mutant (white bars) mice. Asterisks indicate statistically significant differences between the two groups (*, P < 0.05; **, P < 0.01; ***, P < 0.005).
$I^{-/-}$ mice. While tenomodulin was once assumed to mimic ChM-I action in cartilage development, it was recently shown that its expression patterns do not overlap with that of ChM-I in cartilage (25). Moreover, tenomodulin expression was not affected by ChM-I inactivation in mice (data not shown). Thus, taken together, our study suggested the possible existence of a functionally redundant factor for ChM-I in chondrocytes. 

As the only reported activity for ChM-I involved the stimulation of osteoclast proliferation and differentiation (19), which does not appear to explain the bone phenotype of the $ChM^{-/-}$ mice, the function of ChM-I in osteoclasts remains elusive. However, given the increased bone mineral density in $ChM^{-/-}$ mice, ChM-I expression in osteoblasts at low levels may regulate the expression of receptors or ligands that control the proliferation and/or differentiation of both osteoblasts and osteoclasts. In this respect, as decreased numbers of TRAP-positive mature osteoclasts were observed in $ChM^{-/-}$ mice, the possibility of the involvement of ChM-I in the RANKL-receptor activator of nuclear factor-$\kappa$B system of osteoclastogenesis (13, 15) is of interest and remains to be tested.

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