The metalloproteinase MT1-MMP is required for normal development and maintenance of osteocyte processes in bone

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Summary

The osteocyte is the terminally differentiated state of the osteogenic mesenchymal progenitor immobilized in the bone matrix. Despite their numerical prominence, little is known about osteocytes and their formation. Osteocytes are physically separated in the bone matrix but seemingly compensate for their seclusion from other cells by maintaining an elaborate network of cell processes through which they interact with other osteocytes and bone-lining cells at the periosteal and endosteal surfaces of the bone. This highly organized architecture suggests that osteocytes make an active contribution to the structure and maintenance of their environment rather than passively submitting to random embedding during bone growth or repair. The most abundant matrix protein in the osteocyte environment is type-I collagen and we demonstrate here that, in the mouse, osteocyte phenotype and the formation of osteocyte processes is highly dependent on continuous cleavage of type-I collagen. This collagenolytic activity and formation of osteocyte processes is dependent on matrix metalloproteinase activity. Specifically, a deficiency of membrane type-1 matrix metalloproteinase leads to disruption of collagen cleavage in osteocytes and ultimately to the loss of formation of osteocyte processes. Osteocytogenesis is thus an active invasive process requiring cleavage of collagen for maintenance of the osteocyte phenotype.

Key words: Bone, Osteocytes processes, Membrane-type matrix metalloproteinase 1, MMP-13, Collagen cleavage

Introduction

In all land-dwelling vertebrates, bone is a cellular tissue, as opposed to the acellular bone of some fish species including those with the presumed ancestral forms of modern bone such as that found in the aspidin tissue of jawless Heterostraci (Tarlo, 1964). Bone remains a living tissue for as long as osteocytes, the cells embedded in its mineralized matrix, remain alive. Osteocytes represent the ultimate differentiation of cells formerly involved in bone deposition as osteoblasts (Knothe-Tate et al., 2004). Upon converting from osteoblast to osteocyte, the cell progressively reduces its biosynthesis of collagen type I, the major extracellular protein of bone as well as that of the lesser bone-enriched non-collagenous proteins such as alkaline phosphatase, osteocalcin and bone sialoprotein (Robey, 2002; Sasano et al., 2000; Zhu et al., 2001). Osteocytes thus appear to function as quiescent or 'retired' osteoblasts, which occupy lacunae in the mineralized bone matrix. Mature osteocytes communicate with each other and with osteoblasts lining the surfaces of the bone through an extensive network of dendritic cellular processes that permeate the bone matrix and connect via gap junctions (Donahue, 2000; Shapiro, 1997; Yellowley et al., 2000). Neither the ultimate function of this remarkable system of direct cell-to-cell communication across bone (Kamioka et al., 2001) nor the mechanisms leading to its establishment and maintenance during bone formation has been elucidated. Many roles have been proposed for osteocytes and their network of processes (Aarden et al., 1994), including calcium sensing (Kamioka et al., 1995), mechano-sensing (Ajubi et al., 1996; Burger and Klein-Nulen, 1999a; Burger and Klein-Nulend, 1999b; Klein-Nulend et al., 1995b; Miyauchi et al., 2000) and influencing osteoid maturation and subsequent calcification (Mikuni-Takagaki et al., 1995). The observation that a proportion of osteocytes within these networks are normally lost with age raises the question of whether cellular communication through the osteocyte network is absolutely necessary for maintenance of bone (Mullender et al., 1996). 'Osteocytogenesis', the process leading to the entombment of living cells in growing mineralizing bone, is mostly thought of as a passive process governed principally by the rate and duration of matrix deposition. As individual bone-lining osteoblasts cease matrix production, they fail to keep up with more productive and faster

moving neighboring cells and thus become entombed. This is perhaps an overly simplistic view at odds with the distinctive pattern created by the network of osteocyte processes in the osteon and the survival and maintenance of the osteocyte network, which suggest that a more precisely regulated process governs osteocytogenesis.

Type-I collagen is the most abundant matrix protein in bone and we and others have previously demonstrated the indispensable role of cell-mediated 'collagenolysis' in bone formation and growth of the skeleton (Holmbeck et al., 1999; Zhou et al., 2000). Moreover, we have shown that remodeling of the unmineralized collagenous matrices of periskeletal soft tissues (which is a requisite for the growth and normal function of a bone and its appendages as an 'organ') is also critically dependent on membrane-type matrix metalloproteinase 1 (MT1-MMP) (Holmbeck et al., 1999; Zhou et al., 2000). The involvement of matrix remodeling in bone and other related skeletal and periskeletal tissues is well documented, and the evidence for specific functional roles of MMPs is mounting (Holmbeck et al., 1999; Vu et al., 1998; Zhou et al., 2000). MT1-MMP is a membrane-anchored proteinase that is thought to mediate pericellular proteolysis of a wide range of extracellular-matrix substrates. Acting as a collagenase, it can cleave native type-I, -II and -III collagens (Hotary et al., 2003; Ohuchi et al., 1997). Moreover, it is capable of cleaving fibrin (Hiraoka et al., 1998), gelatin (denatured type-I collagen) (d'Ortho et al., 1998), aggrecan, fibronectin, vitronectin, laminin-1 (Ohuchi et al., 1997) and laminin-5 (Koshikawa et al., 2000).

In this study, we set out to determine the extent to which collagenolytic activity is also important to the establishment and/or maintenance of the communicating network of osteocyte processes. We show here that unmineralized collagen fibrils are cleaved along the entire system of osteocyte processes in bone from wild-type mice. The degradation of collagen is specifically mediated by an MMP-type 'collagenase' and coincides with the spatial expression of MT1-MMP. We further demonstrate that bones of MT1-MMP-deficient mice lack this specific cleavage of collagen and have a greatly reduced or absent osteocyte process network, whereas the osteocytes are equally abundant irrespective of genotype. With age, this disparity in osteocyte phenotype between mutant and wild-type mice becomes increasingly evident as lack of matrix cleavage ultimately leads to the involution of cell processes. Collectively, these observations demonstrate that osteocytogenesis is not merely a passive process of cell embedding. The osteocyte phenotype is attained by an active invasive process and maintained and extended over time by continuous dissolution of cell-associated matrix mediated by MT1-MMP.

Materials and Methods

Generation of MT1-MMP-deficient mice

The generation of MT1-MMP-deficient mice is described elsewhere (Holmbeck et al., 1999). Animals used in this study were housed and handled according to guidelines approved by the NIDCR Animal Use and Care Committee.

Immunolocalization of MMPs and their cleavage products from type-I collagen

Knees and associated tibiae/femora from mice ranging in postnatal age from 20 days to 70 days were harvested, trimmed and fixed for 60 minutes in 4% vol/vol formaldehyde in PBS at room temperature, rinsed in PBS twice for 30 minutes each and kept overnight at 4°C in 0.1 M Tris-HCl, pH 7.2 (to limit cross-linking) and decalcified in 0.2 M EDTA, 50 mM Tris-HCl, pH 7.4 at 4°C. Sagittal frozen sections (8 μm) were prepared on a Bright's cryostat. Immunolocalization of the primary collagenase cleavage site [C-terminus of the Tc^A or three-quarters piece of the α1(I) chain] was performed with a rabbit polyclonal antibody (Billinghurst et al., 1997) using diaminobenzedine (DAB) and H_2O_2 with methodology (Hollander et al., 1995). Sections were counterstained with Fast Green. MMP-13 was localized with an anti-rabbit peptide antibody (Wu et al., 2002) or a commercially available antibody (Neomarkers, Freemont, CA) (data not shown) using the same method. MT1-MMP was localized as above with a rabbit polyclonal antibody to the hinge region (Triple Point Biologics, Portland, OR). Controls were prepared as described (Hollander et al., 1995) by pre-absorption of the specific antibody with the synthetic peptide containing the unique MMP-13 epitope sequence against which the antibody was prepared (Wu et al., 2002).

Electron microscopy

Fresh tissue obtained from 48-day-old wild-type and MT1-MMP-deficient mice were fixed overnight in 3% vol/vol glutaraldehyde in 0.2 M sodium cacodylate, buffer, pH 7.2 at 4°C, rinsed and decalcified in 0.2 M EDTA, 50 mM Tris-HCl, pH 7.4 at 4°C. The diaphysis was cut into small cubes, post-fixed in osmium tetroxide, dehydrated in acetone and embedded in Epon.

Osteocyte analysis

The density of osteocytes in bone was determined by counting of 0.1 mm^2 bone surface fields in quadruplicate counts per slide. Four individual slides were counted for each animal and the corresponding control littermate at ages 10 days, 20 days, 32 days, 40 days and 47 days. Statistical significance was assessed with Student's *t*-test.

Morphometric analysis of the length and number of osteocyte processes was determined from Bodian silver stain sections of control and MT1-MMP mutant mice at 28 days and 40 days of age. Random sections from cortical bone (eight sections per mouse at 28 days and six sections per mouse at 40 days) were imaged and manually thresholded, and measured in NIH Image. Because the osteocyte process network in wild-type mice is continuous throughout the bone, process length was defined as the distance from the lacunar wall to the first major branching point of the process.

TUNEL, Giemsa and silver staining, and in situ hybridization

Tissues from mutant animals and littermate controls were fixed overnight at room temperature with 4% vol/vol formaldehyde in PBS, washed briefly in PBS and decalcified exhaustively in PBS containing 0.25 M EDTA at room temperature, embedded in paraffin and sectioned at 6 μm. Slides were either processed for Giemsa staining or hybridized to [^{33}P]-labeled riboprobes corresponding to nucleotides 291-902 of the mouse *mt1-mmp* cDNA (GenBank X83536) as described previously (Blavier et al., 2001). Cells undergoing apoptosis were detected using the Apoptag in situ kit (Intergen, Gaithersburg, MD). Randomly selected 0.1 mm^2 areas of either parietal bone or femoral cortex were counted on five control sections and five sections from MT1-MMP-deficient animals. TUNEL-positive cells were scored from 30 fields per genotype as a function of total osteocyte number in the field and subjected to Student's *t*-test. Before counting, slide identity was obscured to eliminate any bias. For silver staining, a previously published method was used (Kageyama et al., 1991). Frozen sections were washed in 1% potassium dichromate for 50 minutes and then rinsed in distilled water and stained in the dark for 18-24 hours at 37°C in a solution prepared by combining 100 ml 1%

weight/vol Protargol (silver protein) (Rowley Biochemical Institute, Danvers, MA) with 10 g copper shot. Sections were rinsed in distilled water at 10-15°C for 30 seconds and then immersed in 30 ml distilled water plus 15 ml 10% vol/vol buffered formaldehyde containing 3 g hydroquinone for 10 minutes at 10-15°C. Slides were washed in distilled water and subsequently reacted with 0.2% gold chloride for 60 minutes, washed in distilled water and finally reacted with 2% weight/vol oxalic acid for 10 minutes. Photomicrographs were captured with either a DMC 2 (Polaroid) or Coolsnap v1.1 (Photometrics) camera and assembled using Photoshop 5.0 (Adobe Systems)

Results

Collagen is cleaved along osteocyte processes in bone

Osteocytes communicate through an extensive network of processes, which can be visualized either by Giemsa stain or by the more specific modification of the Bodian silver stain (Kageyama et al., 1991). This network is seen both around mature osteocytes fully embedded in mineralized bone (Fig. 1A-D) and beneath bone cells lining the osteoid/periosteal interface (data not shown). Because fibrils of type-I collagen constitute the predominant structural elements of the organic matrix of bone, we sought to determine whether the formation and/or maintenance of osteocyte processes was dependent on collagen remodeling along the canalicular wall or resulted simply from passive embedding of bone cells in osteoid/bone tissue. We examined the distribution of collagen cleavage products in bones of wild-type mice using antibodies to the neo-epitope generated in the α1(I) chain as a result of 'collagenase-type' cleavage of type I collagen at the characteristic 3/4-1/4 site. Intense staining for collagen cleavage was associated with the fine cellular processes of osteocytes throughout the cortical bone matrix. This was most pronounced at 40 days (Fig. 1F,H,I) and somewhat less evident at 30 days (Fig. 1E). Although staining varied in intensity from site to site, collagen cleavage products

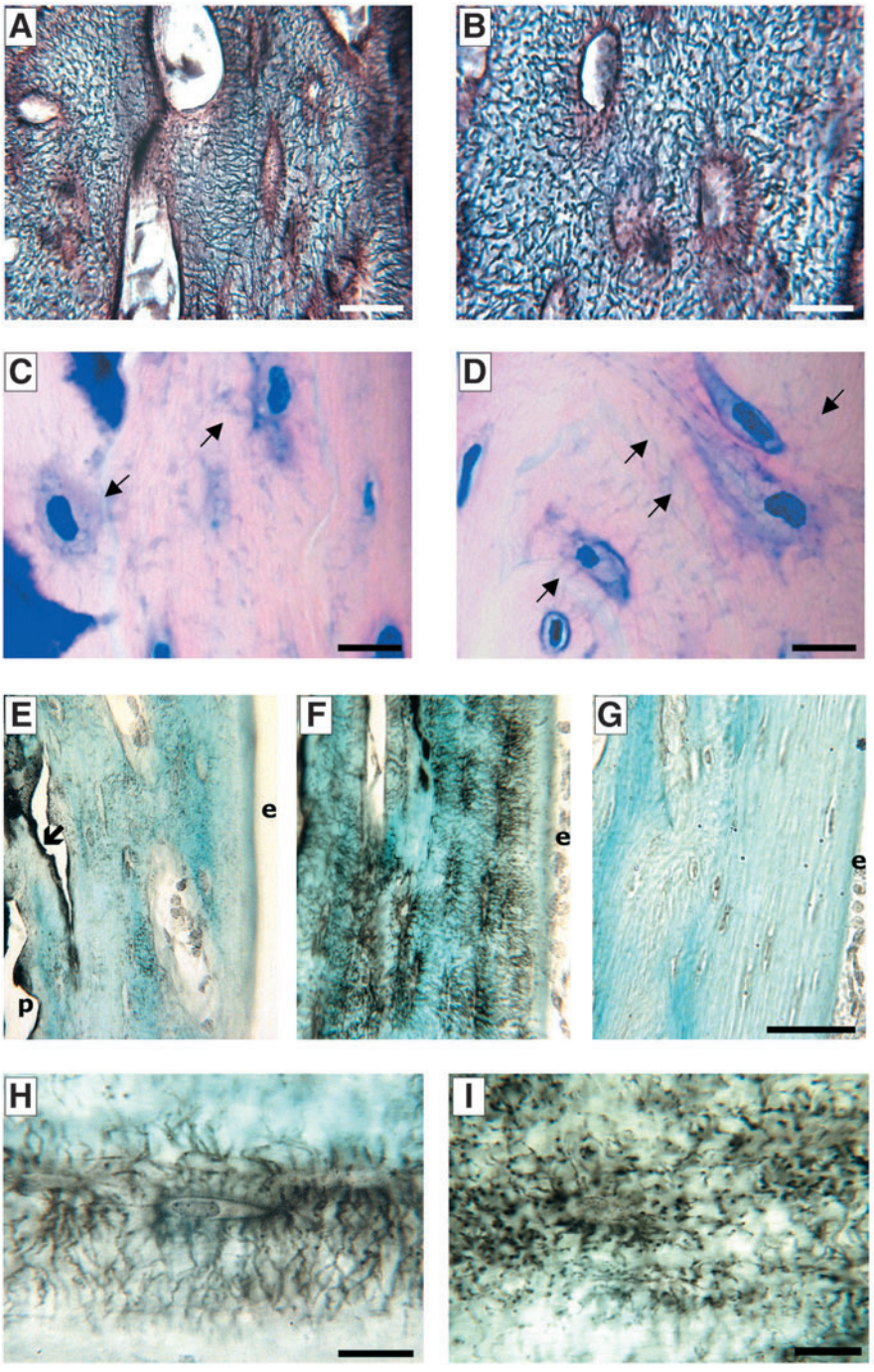

Fig. 1. Osteocyte processes are associated with collagen cleavage. Cortical bone sections stained with Bodian silver stain (A,B), Giemsa (C,D), and collagen-cleavage-specific antibody (E-I). (A) Osteocytes are surrounded by many well-defined cell processes emanating from the lacunae and extending into the pericellular bone matrix to generate a mesh-like appearance in this 30-day-old wild-type mouse. (B) At 40 days of age, the network is even more highly developed. The progressive development of osteocyte processes can be appreciated more easily in sections from wild-type mice stained with Giemsa (C,D). (C) Bone section from a 20-day-old wild-type mouse demonstrating weakly outlined osteocyte processes (arrows). (D) In bone from a 40-day-old wild-type mouse, processes now appear more defined as canal-like structures radiating from the osteocyte lacuna (arrows). (E) Collagen cleavage is modest in the cortex of 30-day-old wild-type mouse and more obvious at the periosteal surface (p and arrow). The endosteal surface (e) in this section displays no staining. (F) Conspicuous staining of the osteocyte processes in a 40-day-old wild-type mouse. Clearly outlined processes are radiating from the osteocytes. (H) High-power magnification of osteocytes and their associated processes radiating from lacunae (H). (I) Punctate staining demonstrates areas cross-sectioned osteocyte processes. (G) Negative control stained with peptide-absorbed antiserum. Bars, 10 μm (A-D,H,I), 30 μm (E-G).

were observed ubiquitously in all mice at four different ages (n=4 per time point). Collagen cleavage products were also seen in cortical bone at periosteal surfaces (Fig. 1E). Pre-absorption of the immune serum with the peptide epitope against which the antibody is directed abolished staining (Fig. 1G).

Expression of MT1-MMP and MMP-13

We have previously shown that osteogenic cells from MT1-MMP-deficient mice possess a collagenolytic defect in vivo that results in a severe bone phenotype, and that fibroblasts from mutant mice fail to degrade type-I collagen fibrils in vitro (Holmbeck et al., 1999). These observations raised the question of whether the collagenolytic activity associated with osteocyte processes could also be dependent on MT1-MMP. In wild-type mice, *mt1-mmp* transcripts were expressed in cells of the periosteum of long bones in areas that co-localized with collagen cleavage products, and in mature osteocytes embedded in bone matrix (Fig. 2A,B). Moreover, osteocyte bodies and the immediate pericellular matrix were also consistently stained with antibodies to MT1-MMP (Fig. 2C,D). MT1-MMP staining also extended further into the bone matrix, where the outline of osteocyte processes was clearly visualized in a pattern similar to that revealed by staining for collagen type-I cleavage products, including the punctate staining associated with cross-sectioned osteocyte processes (Fig. 1E,F, Fig. 2C,D).

Although MT1-MMP is indispensable for collagen cleavage in some tissues of the mouse, other enzymes posses potent collagenolytic activity and could be involved either cooperatively or independently in the process of collagen fibril degradation in bone. One of the most likely candidates in the mouse is the interstitial-type collagenase MMP-13. Immunostaining for MMP-l3 produced results very similar to those obtained for collagen cleavage and MT1-MMP protein. Osteocyte-associated staining was clearly seen at 30 days (Fig. 3A) and even more so at 40 days (Fig. 3B,D,E); punctate staining of the osteocytic processes was also seen at this time point (Fig. 3E). Periosteal surfaces also stained strongly for MMP-13 in a manner identical to that observed for collagen cleavage products and MT1-MMP protein. Collectively, the detection of collagen cleavage, MMP-13 and MT1-MMP protein demonstrated complete overlap between protein expression and collagen degradation, and suggested that one or both of these MMPs could be instrumental in mediating the cleavage of collagen associated with osteocyte processes.

Periosteocytic collagen cleavage is absolutely dependent on MT1-MMP

MT1-MMP-deficient mice showed no evidence of collagen

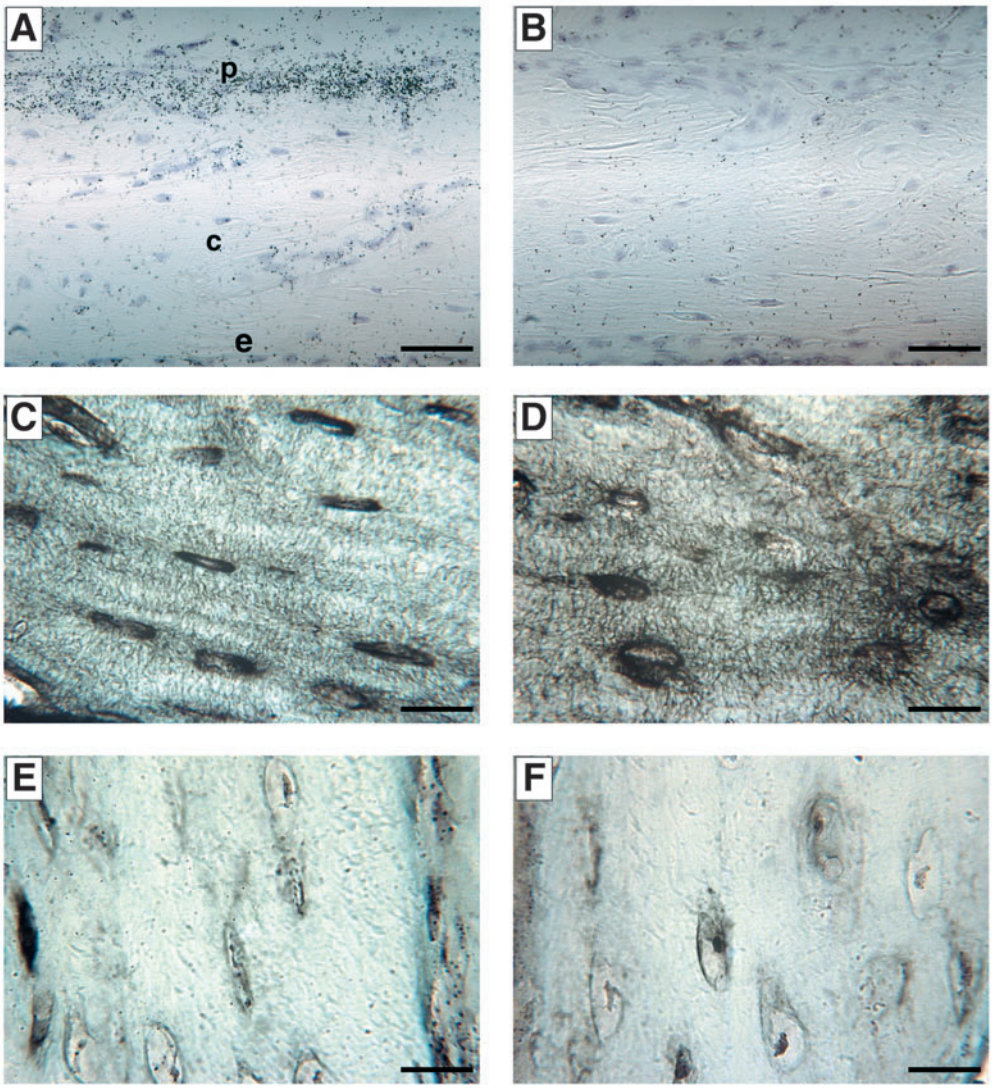

Fig. 2. Localization of MT1-MMP in bone. (A) Bright-field image of a bone section from a 20-day-old wild-type mouse hybridized to MT1-MMP-antisense RNA probe. Staining is intense in the periosteum (p), with multiple cells expressing MT1-MMP-encoding mRNA. Isolated osteocytes in the bone cortex (c) likewise display signal indicating expression of MT1-MMP. e, endosteum. (B) Serial section from the same animal hybridized with MT1-MMP-sense RNA probe. (C) Immunolocalization of MT1-MMP in cortical bone from a 30-day-old wild-type mouse demonstrating osteocytes and their cell processes expressing MT1-MMP. (D) Bone section from 40-day-old wild-type mouse demonstrating even more intense stain than that seen at day 30. (E,F) Negative controls. Bone sections from 30-day-old MT1-MMP-deficient mouse stained for MT1-MMP (E) and 40-day-old MT1-MMP-deficient mouse stained for MT1-MMP (F). Bars, 40 μm (A,B), 10 μm (C-F).

cleavage along osteocyte lacunae or processes. As demonstrated in Fig. 4A,B, bone from 30-day- and 40-day-old mice were devoid of collagen cleavage products and resembled the absorption control in Fig. 4C,H. Examination at higher magnification likewise failed to reveal staining for collagen cleavage around osteocytes (Fig. 4D,E). Interestingly, periosteal surfaces in deficient mice still showed evidence of 'collagenase-type' collagen cleavage (Fig. 4D). In addition, these surfaces also reacted strongly with antibody to MMP-13 (Fig. 4F) in a manner similar to what was observed in wild-type mice (Fig. 3A). However, although osteocyte bodies of MT1-MMP-deficient mice stained for MMP-13, the pericellular area displayed little or no evidence for staining of osteocytic processes (Fig. 4F,G,I,J). The MMP-13 peptide controls for the deficient tissues (Fig. 4H) were negative demonstrating the specificity of the staining.

Osteocytes in MT1-MMP-deficient mice lack a network of processes

These observations raised the question of whether osteocytes of MT1-MMP-deficient mice were lacking in cellular processes or merely in collagen cleavage. Giemsa or Bodian silver stain demonstrated that osteocyte processes were largely absent from bone irrespective of the age of the animals from 20 days to 70 days (Fig. 5A-D; Table 1 for animals 28 days and 40 days of age). Most osteocytes in MT1-MMP-deficient mice contained less than one osteocyte process per 6 μm section. These processes were significantly shorter and moreover failed to communicate with other processes or osteocytes. By contrast, wild-type littermates displayed an extensive network of processes, with prominent intercellular process communication throughout the bone (Fig. 1A-D). This nearly complete lack of osteocyte processes in mutant mice, however, did not affect osteocyte viability or osteocyte density. Enumeration of osteocytes in cortical bone from wild-type mice and mutant littermates showed differences of less than 15% in either direction in five different age groups (Table 2) and no significant difference could be documented. Based on these observations, it was concluded that loss of MT1-MMP did not significantly affect the number of osteocytes in bone. The osteocyte counts are further consistent with the additional observation that apoptosis was not a prominent feature in either genotype as determined by TUNEL staining. Enumeration of TUNEL-positive osteocytes demonstrated 1±1.12 cell in 144 for control animals versus 1±0.92 in 130 for MT1-MMP-deficient mice (P=0.707).

Ultrastructural analyses

Transmission electron microscopy (TEM) confirmed the observation made in Bodian-silver- or Giemsa-stained sections. Long, thin osteocytic processes were clearly less abundant in the MTl-MMP-deficient mice (Fig. 6A-D) but were not completely absent in the vicinity of osteocyte cell bodies (Fig. 6C,D). We also noted that lacunae walls surrounding osteocytes of deficient mice (Fig. 7B), but not wild-type mice (Fig. 7A), contained intact and banded collagen fibrils (Fig. 7B). In wild-type mice, the cross-striated fibril pattern was replaced with fine granular and occasionally fibrillar staining. This observation is consistent with lack of degradation of the collagen fibrils in deficient mice, as previously indicated by the lack of staining for the collagenase cleavage site in type-I collagen. The TEM pictures, like the TUNEL staining, provided no evidence of apoptosis in the form of nuclear condensation of chromatin, cellular vacuolization and nuclear disintegration among osteocytes (Fig. 6A-D).

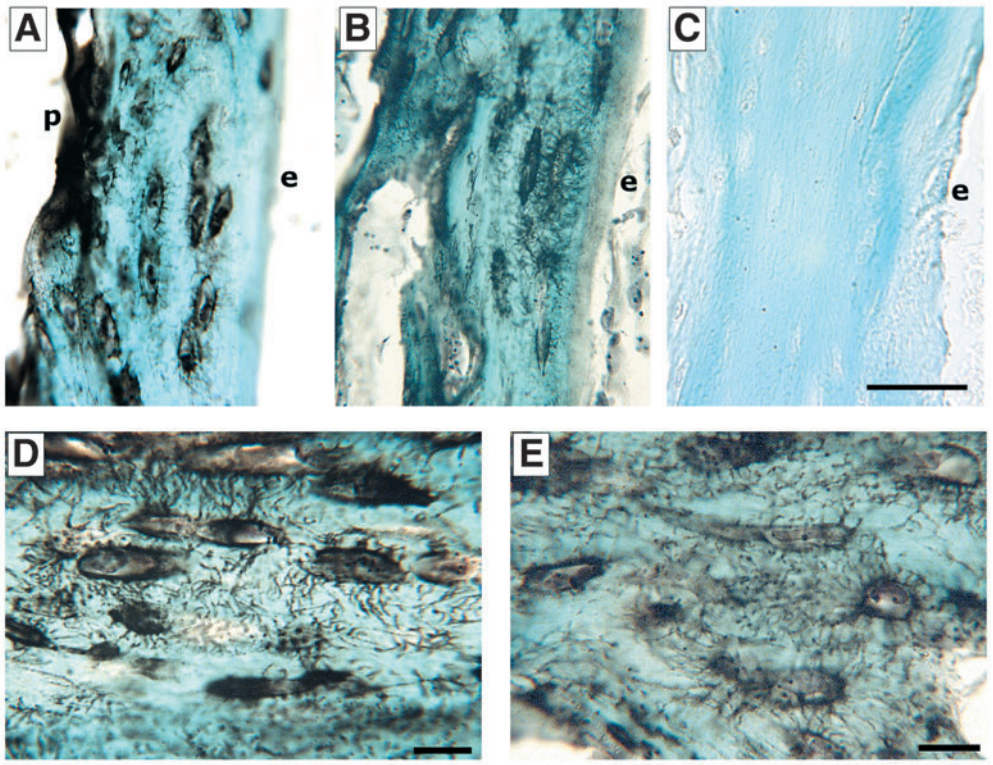

Fig. 3. Immunolocalization of MMP-13 in bone. (A-E) Bone sections from 30- and 40-day-old wild-type mice stained with antibody specific for MMP-13. (A) Section of cortical bone from 30-day-old wild-type mouse demonstrating staining of the periosteal surface (p) as well as of osteocyte lacunae and cell processes radiating into the bone matrix. e, endosteal surface. (B) Bone section from 40-day-old wild-type mouse demonstrating intense staining for MMP-13 associated with both osteocytes and their processes. e, endosteal surface. (D,E) High-power magnification of bone from 40-day-old wild-type mouse demonstrating osteocytes and associated processes staining intensely for MMP-13. Notice the punctate staining pattern in E, which indicates cross-sectioned cell processes. (C) Negative control. Bars, 30 μm (A-C), 10 μm (D,E).

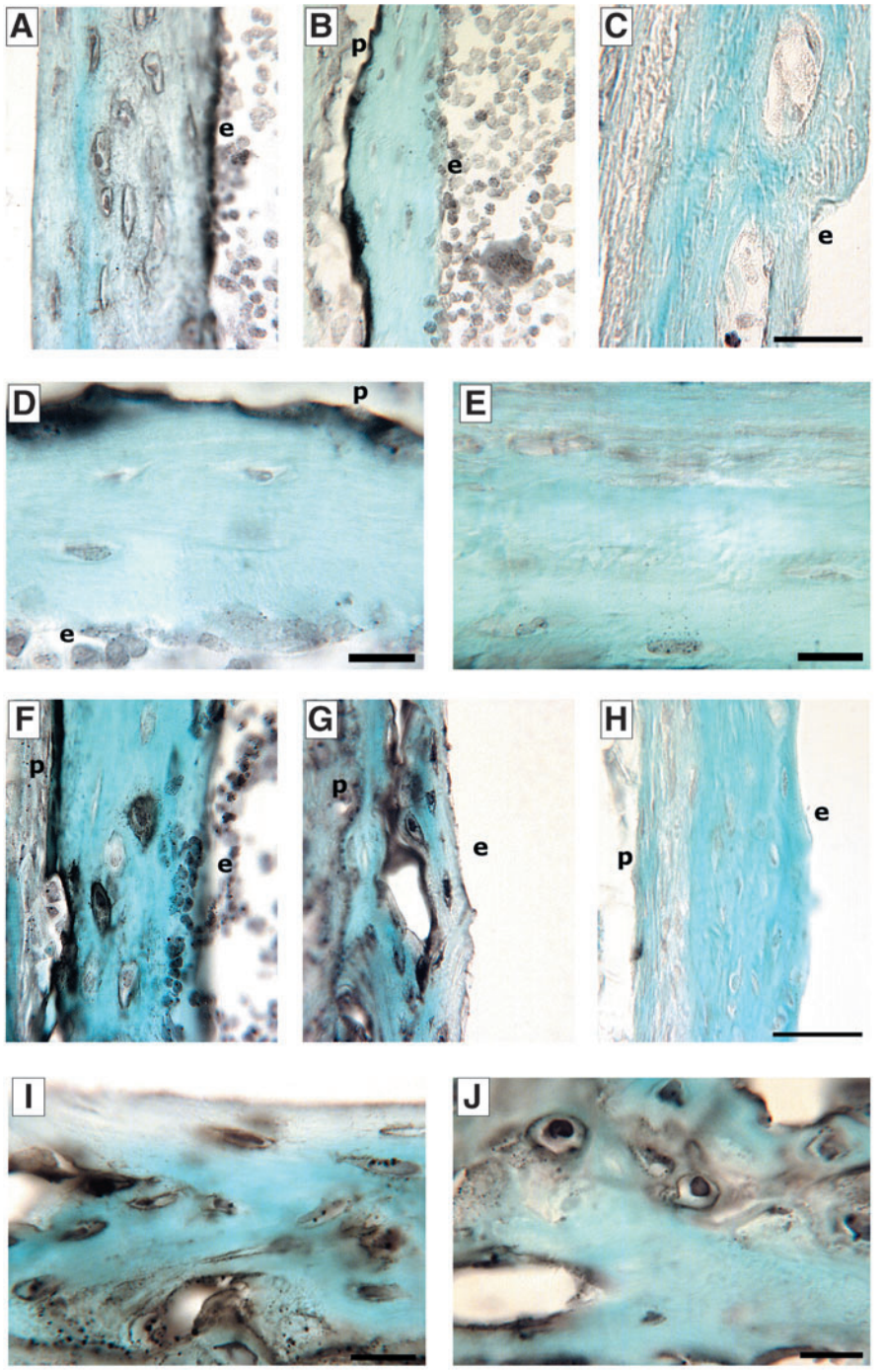

Fig. 4. Localization of collagen cleavage and MMP-13 in MT1-MMP-deficient mice. (A-E) Immunolocalization of collagen cleavage in cortical bone. (F-J) Immunolocalization of MMP-13 antigen in cortical bone. Bone sections from MT1-MMP-deficient mice at 30 days (A,F) and 40 days (B-E,G-J). (A) Collagen cleavage is absent from the pericellular area of osteocytes in a 30-day-old MT1-MMP-deficient mouse but is obvious at the endosteal surface (e). (B) 40-day-old MT1-MMP-deficient mouse displaying a complete absence of osteocyte process staining but with collagen cleavage at the periosteal surface (p). (C) Negative control for collagen cleavage. (D) High-power magnification from a 40-day-old MT1-MMP deficient mouse displaying a complete absence of cell process staining but with staining of the periosteal surface (p). (E) High-power magnification of an additional section from the same animal demonstrating in detail the lack of collagen cleavage in osteocyte processes. (F) 30-day-old MT1-MMP-deficient mouse demonstrating MMP-13 reactivity in osteocytes and periosteal (p) and endosteal surfaces (e). Notice the absence of staining associated with osteocyte processes, as seen in wild-type animals (Fig. 3). (G) 40-day-old MT1-MMP-deficient mouse displaying staining of osteocytes for MMP-13 but no staining of cell processes. e, endosteum; p, periosteum. (H) Negative control for MMP-13 antigen. (I,J) High-power magnification from 40-day-old MT1-MMP-deficient mouse demonstrating the absence of cell-process-associated MMP-13 staining. Only osteocyte cell bodies display reactivity for MMP-13. Bars, 30 μm (A-C,F-H), 10 μm (D,E,I,J).

Discussion

In this study, we show that the development and/or maintenance of osteocyte processes and their canaliculi are associated with matrix degradation that requires the activity of MT1-MMP. The emergence of osteocyte processes is an early event in the transition of osteoblasts to osteocytes (Palumbo et al., 1990). It is often thought that their presence merely reflects a passive embedding of cellular junctions as a result of deposition of bone matrix by osteogenic cells derived from the periosteum and/or from the endosteum. Yet the continuous remodeling of matrix described here suggests that the network is actively maintained in all stages of life, presumably for specific metabolic or communication purposes. Our findings therefore raise the interesting question of whether the formation of osteocyte processes is an active invasive process.

Previous results, as well as those presented here, suggest that MT1-MMP plays an essential role in the pericellular cleavage of type-I collagen (the fibrillar collagen found in bone), acting either directly as a collagenase or as an obligate activator of other collagen-cleaving enzymes such as, but not necessarily limited to, MMP-13 and MMP-2 (Hotary et al., 2003; Aimes and Quigley, 1995; Cowell et al., 1998; Knauper et al., 1996; Seltzer, 1999). The co-localization of collagen cleavage, MT1-MMP and MMP-13 in the osteocyte pericellular environment suggests that these two proteinases might work together in a cooperative manner to establish or maintain the network of processes, although at present the specific substrate specificity for each proteinase is only partially uncovered. The marked reduction in osteocyte processes in MT1-MMP-deficient mice does, however, demonstrate that MT1-MMP plays an indispensable role in the formation of these networks. We note that, although

pericellular collagen cleavage was not detected near osteocytes of MT1-MMP-deficient mice, osteoid on periosteal surfaces of cortical bone did show collagen-cleaving activity at the 3/4-1/4 site, which was independent of MT1-MMP. Other MMPs, such as the recently characterized murine orthologs of human *MMP-1* or *MMP-8* (Balbin et al., 1998; Balbin et al., 2000) could be candidates responsible for this activity.

Collagen remodeling takes place not only at the periosteal

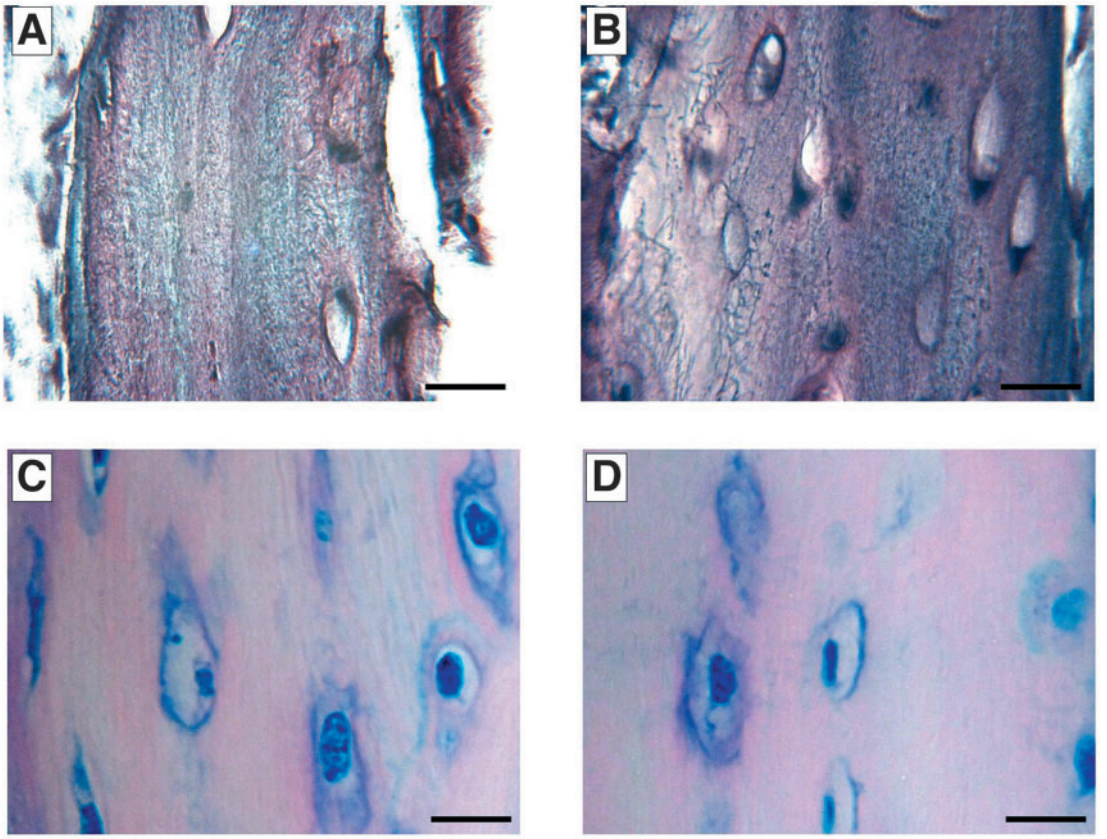

Fig. 5. Osteocyte process formation in cortical bone is dependent on MT1-MMP. Cortical bone sections stained with Bodian silver stain (A,B) or Giemsa stain (C,D). (A) Bone from 30-day-old MT1-MMP-deficient mouse displaying the absence of clearly defined osteocyte processes. The conspicuous mesh-like appearance of the bone matrix seen in wild-type mice is absent from this sample (Fig. 1). (B) Bone from a 40-day-old MT1-MMP-deficient mouse displaying a similar absence of osteocyte processes despite more advanced age. (C,D) Osteocyte processes are absent from these Giemsa-stained bones from a 20-day-old (C) and a 40-day-old (D) MT1-MMP-deficient mouse. Bars, 10 μm (A-C).

Table 1. Osteocyte processes per osteocyte and process length at 28 days (A) and 40 days (B)

Osteocyte processes per cell, MT1-MMP$^{-/-}$	Process length (μm), MT1-MMP$^{-/-}$	Osteocyte processes per cell, control	Process length (μm), control
(A)			
1±1.5	1.5±1.66	18±3.60	5.83±2.73
1.66±0.81	2.993±0.98	26±2.82	6.51±2.66
0±0	0±0	20.66±3.51	6.66±2.18
0±0	0±0	19.5±19.5	4.18±1.64
0.14±0.375	0.6685±1.80	19.5±1.5	5.81±2.84
0.167±0.57	0.47±1.14	17±2.82	6.98±3.79
0.7±0.68	1.26±1.38	20.5±4.94	4.43±3.56
0.625±0.25	4.23±0	26±0	6.86±3.69
(B)			
0.33±0.49	4.11±1.60	9.6±3.04	5.995±2.68
1.33±1.37	1.92±1.38	17±0	5.54±1.74
0.833±1.60	3.54±0.67	12±0	5.6±1.97
0.25±0.5	5.5±0	16±4.24	6.18±2.57
0.18±0.4	4.57±1.30	17±0.7	6.74±2.44
0.25±0.45	2.94±0.95	19±0	8.06±2.38

P values for process number: (A) 5.4539×10^{-10}; (B) 1.64229×10^{-9}.
P values for process length: (A) 6.28783×10^{-9}; (B) 2.386381×10^{-3}.

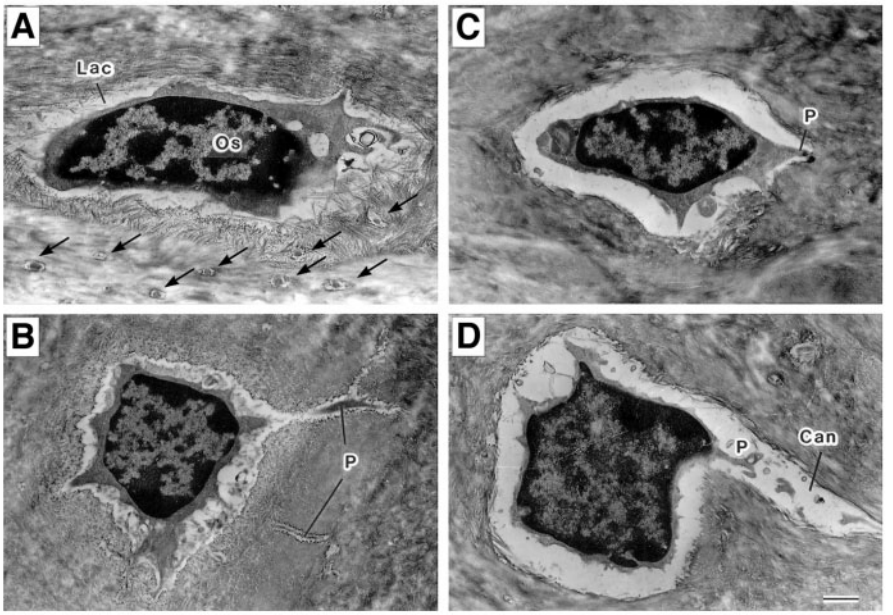

Fig. 6. Imaging of osteocytes by transmission electron microscopy. (A-D) 48-day-old wild-type (A,B) and MT1-MMP-deficient (C,D) littermates. (A) Osteocyte (os) residing in lacuna (Lac). Notice the well-defined cell processes and canaliculi seen in cross section (arrows). (B) Section of osteocyte intersecting two well-defined processes (P). The matrix around the processes is electron dense and granular in appearance. (C,D) Osteocytes from MT1-MMP-deficient littermate displaying canaliculus (Can). Bar, 1 μm.

surfaces but also in the periosteal soft connective tissues covering the bone. We have previously demonstrated an absolute requirement for MT1-MMP in these locations (Holmbeck et al., 1999). In its absence, severe fibrosis develops and recruitment of osteoblasts is impaired. We also demonstrated the presence at these sites of *mt1-mmp* transcripts in wild-type mice. Proteolysis in this location might not be required for the actual secretion of bone matrix per se but rather to facilitate migration of osteogenic cells through the collagenous soft tissue, where they differentiate and associate with the bone/osteoid surface. It is noteworthy that proteolysis is required for mineralization in cartilage (Wu et al., 2002) and that this requirement might extend to the mineralization of osteoid as well.

Osteogenic cells establish processes and canaliculi before becoming fully embedded in the unmineralized osteoid matrix (Menton et al., 1984; Palumbo et al., 1990), presumably by a process that depends on limited but highly focused proteolysis. Our previous studies have shown that a related cell invasion of the unmineralized (type-II) collagenous matrix of hyaline cartilage requires MT1-MMP. This is clearly observed in formation of the epiphyseal secondary ossification centers of long bones, which is completely blocked in MT1-MMP-deficient mice (Holmbeck et al., 1999).

At the ultrastructural level, we noted that some processes do exist in the MT1-MMP deficient animals, but only very close to the osteocyte cell bodies. We speculate that osteocytes in MT1-

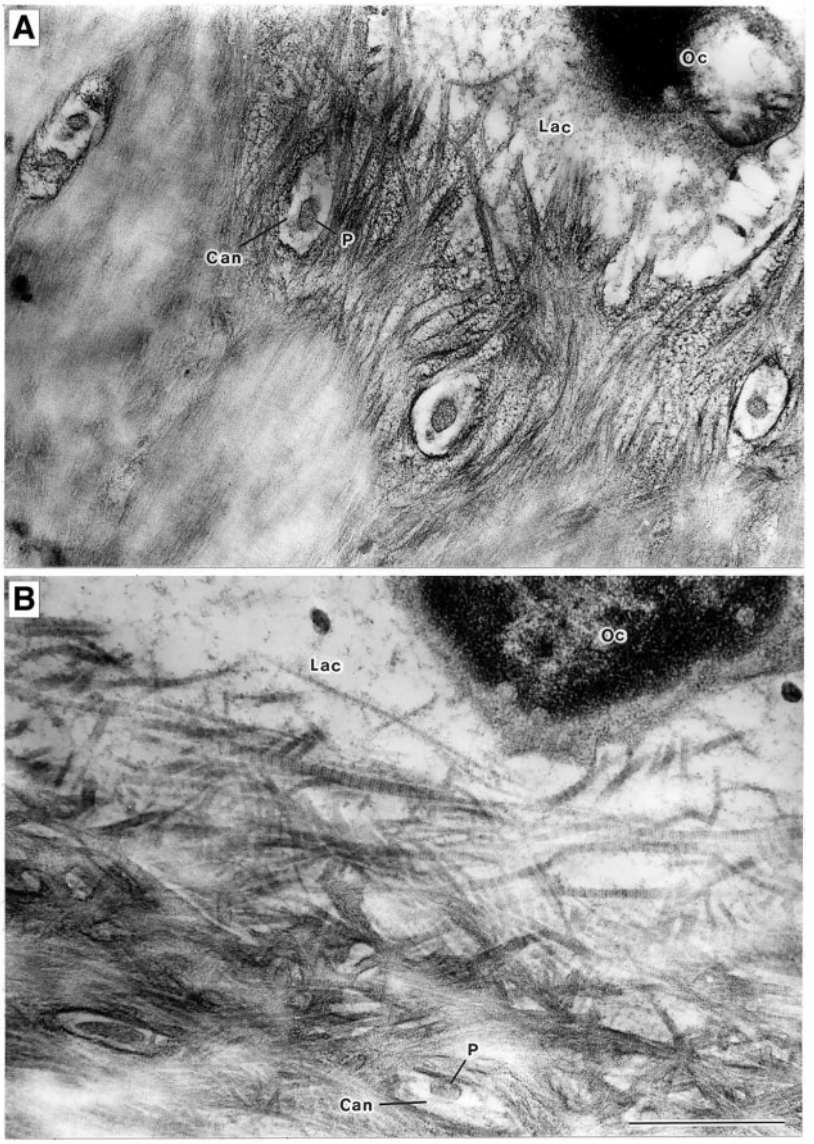

Fig. 7. High-power transmission electron microscopy images of osteocytes. Electron micrographs show higher magnification of perilacunar matrix organization of osteocytes in (A) wild-type and (B) MT1-MMP-deficient 48-day-old mice. Canaliculi (can), fine osteocytic processes (p) and lacunae (Lac) are indicated. (A) Collagen fibrils have lost their banded pattern and granular staining in the lacuna wall. (B) The collagen fibrils display characteristic cross-striated banding pattern and no evidence of fine granular material. Lacunae and fine cellular processes are more obvious in the wild-type than in the deficient mouse. Bar, 1 μm.

Table 2. Osteocyte density in control and MT1-MMP-deficient mice (cells/0.1 mm^2)

Age	Control	MT1-MMP$^{-/-}$
Day 10	69.75±14.87	68±12.33
Day 20	69.68±7.54	76.12±15.44
Day 32	72.75±11.65	77.18±13.56
Day 40	53.56±13.66	51.25±14.32
Day 47	62.75±9.13	70.06±11.66
P value	0.63911548	

Mean value cell number±s.d. from sixteen 0.1 mm^2 bone sections per time point per mouse. Counts were obtained from four individual slides per animal.

MMP-deficient mice might compensate to a limited degree for loss of proteolytic capacity by extending short processes in their immediate vicinity by proteolysis-independent ameboid motility by a mechanism similar to that previously described by the Friedl laboratory (Friedl et al., 2001; Friedl and Brocker, 2000). However, because osteocytes also maintain some, albeit minor, collagen-secreting activity, a need eventually and inevitably arises for matrix degradation (Aarden et al., 1996; Sun et al., 1995). Not only is the maintenance of an equilibrium by deposition and subsequent remodeling of matrix consistent with our observation of collagen cleavage associated with osteocytes but it also explains the lack of osteocyte processes in mutant mice and the presence of coarse banded collagen fibrils in the lacunar spaces, reflecting a lack of collagen degradation. We have previously shown that the appearance of cross-striated collagen fibrils in arthritic articular cartilage is indicative of a lack of degradation (Dodge et al., 1991). These observations are also consistent with the fact that membrane-type MMPs, in particular MT1-MMP, endow otherwise non-invasive cells with invasive properties for the penetration of matrix and the formation of invasive cellular processes (Hotary et al., 2000). Importantly, these properties cannot be attained by cells expressing activated forms of MMP-2, MMP-9 and MMP-13, suggesting that (in relation to maintenance of osteocyte processes) MMP-13 might target a substrate in bone matrix other than type-I collagen (Hotary et al., 2003).

Although osteocyte processes are one of the more prominent structural features of bone, their role in bone homeostasis, growth and adaptation to external load changes is not well understood. The uniquely adaptable properties of bone certainly suggest that some entity of the bone, whether it be the cells in the mineralized hard matrix or in the soft connective tissues associated with osteogenesis, harbors sensory functions that enable bone to respond anabolically and catabolically to external and internal stimuli. In this context, the osteocyte network and its extension into the Haversian system has been proposed to function in several ways (Aarden et al., 1994; Ajubi et al., 1996; Burger and Klein-Nulen, 1999a; Burger and Klein-Nulend, 1999b; Kamioka et al., 1995; Klein-Nulend et al., 1995a; Miyauchi et al., 2000), although providing unequivocal proof of this in vivo is hampered by our inability at this time to segregate the functions of the hard skeleton from its periskeletal soft connective tissue. We note that impairment of osteocyte process formation in MT1-MMP-deficient mice in and of itself does not adversely affect osteocyte viability or induce apoptosis. This is in contrast to the extensive onset of apoptosis seen in mice in which the primary collagenase cleavage site in type-I collagen has been mutated to render it resistant to collagenases (Zhao et al., 2000). Many questions pertaining to the function of the osteocyte process network and its role in bone formation, maintenance and turnover therefore remain unresolved, and we cannot at present predict with any degree of certainty the direct consequences of loss of this network. Although a deficiency of MT1-MMP most profoundly affects bone formation and skeletal growth, the specific contribution of dysfunctional osteocytes to these changes cannot easily be evaluated given the complexity of all phenotypic changes associated with MT1-MMP deficiency (Holmbeck et al., 1999; Holmbeck et al., 2003). The observations made here do, however, provide evidence that collagen remodeling governed by the proteolytic activity of MT1-MMP is a molecular tool associated with osteogenic cells in all stages of their life cycle. These findings support the notion that osteogenic cells even in the mature state engage in both matrix secretion and its subsequent trimming. Given the inherent ability of osteoid to calcify, a limited but continuous turnover of uncalcified matrix would be one mechanism by which cells in an otherwise calcifying environment could maintain a three-dimensional space for their expanse of body and processes. Osteocytogenesis can therefore be thought of as an active invasive process dependent on continued proteolytic activity and is thus not merely a passive process leading to the seclusion of former osteoblasts in the calcified bone matrix.

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