An assessment of ADAMs in bone cells: absence of TACE activity prevents osteoclast recruitment and the formation of the marrow cavity in developing long bones

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Abstract ADAMs (A Disintegrin And Metalloprotease domain) are metalloprotease–disintegrin proteins that have been implicated in cell adhesion, protein ectodomain shedding, matrix protein degradation and cell fusion. Since such events are critical for bone resorption and osteoclast recruitment, we investigated whether they require ADAMs. We report here which ADAMs we have identified in bone cells, as well as our analysis of the generation, migration and resorptive activity of osteoclasts in developing metatarsals of mouse embryos lacking catalytically active ADAM 17 [TNFα converting enzyme (TACE)]. The absence of TACE activity still allowed the generation of cells showing an osteoclastic phenotype, but prevented their migration into the core of the diaphysis and the subsequent formation of marrow cavity. This suggests a role of TACE in the recruitment of osteoclasts to future resorption sites.

Key words: Bone; Osteoclast recruitment; A disintegrin and metalloprotease domain; Metalloprotease; TNFα converting enzyme

1. Introduction

ADAMs (A Disintegrin And Metalloprotease domain) constitute a family of glycoproteins that are primarily identifiable by their disintegrin and metalloprotease domains [1,2]. To date, 34 ADAMs with a transmembrane domain have been cloned and sequenced. An additional subset of 21 ADAMs named ADAM TS are not anchored to the cell membrane and contain additional thrombospondin motifs. The metalloprotease domain of ADAMs is also found in matrix metalloproteinases (MMPs), and has been implicated in processing or degradation of extracellular matrix (ECM) proteins as well as in *cis*-shedding of membrane-anchored protein ectodomains. The sheddase activity has been extensively studied for ADAM 17 [TNFα converting enzyme (TACE)], which can release various membrane proteins such as TNFα, TGFα, p75 TNFR or the L-selectin adhesion molecule [3]. In addition, the disintegrin domain of several ADAMs has been reported to mediate cell–cell interaction by binding integrins. Finally, ADAM 1 and 12 show a putative fusion peptide sequence located downstream of the disintegrin domain, and promote cell fusion during fertilization and myogenesis, respectively [4,5].

Because ECM degradation, factor ectodomain shedding, cell binding and cell fusion are all important events in bone, the hypothesis that ADAMs play a role in bone tissue receives increasing attention. Bone turnover is controlled by osteoclasts and osteoblasts. The bone resorbing osteoclasts are multinucleated cells easily detected through tartrate resistant acid phosphatase (TRAP) activity. They are formed by fusion of hematopoietic mononucleated precursors belonging to the myeloid lineage [6]. Osteoclast differentiation is triggered by RANKL, a membrane-anchored member of TNFα family expressed in bone by stromal/osteoblastic cells, which binds to its receptor RANK on the surface of osteoclast precursors [7,8]. Osteoblastic cells express ADAM 9, 10, 12, 15, 19 and ADAM TS-1 [9–11]. In addition, the latter two are up-regulated by osteotropic agents like 1,25-$(OH)_2$ vitamin D_3 and PTH [9,11]. In osteoclasts, transcripts of ADAM 8, 9 and 15 have been detected whereas expression of ADAM 12 is still questioned [9,12,13]. In vitro studies using soluble recombinant ADAMs, antisense oligonucleotides or neutralizing antibodies suggest the involvement of ADAM 8, 9 and 12 in osteoclast differentiation but their mechanism of action is still unclear [12–14]. Finally, the inhibition of osteoclast recruitment in developing metatarsals by metalloproteinase inhibitors demonstrated that activity of metalloproteinases such as MMPs and ADAMs is needed for the generation of osteoclastic activity [15].

In order to identify further which ADAMs are expressed in bone cells, we performed reverse transcription-polymerase chain reaction (RT-PCR) on osteoblastic and osteoclast precursor cell lines, on osteoclasts isolated from rabbit long bones, as well as Northern blots on bone tissue. Furthermore, we examined whether inactivation of the proteolytic domain of TACE would affect the recruitment and the resorptive activity of osteoclasts in developing metatarsals.

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Abbreviations: ADAM, a disintegrin and metalloprotease domain; MMP, matrix metalloproteinase; TRAP, tartrate resistant acid phosphatase; ECM, extracellular matrix; TACE, TNFα converting enzyme

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2. Materials and methods

2.1. Knockout mice and cell lines

Mice from DBA/1 background, homozygous for a targeted mutation in TACE metalloprotease active site ($TACE^{\Delta Zn/\Delta Zn}$) were generated as described previously [3]. For histological studies, limbs were taken from $TACE^{\Delta Zn/\Delta Zn}$ 17.5- and 18.5-day-old embryos whereas controls were from wild-type or heterozygous embryos of the same litters in order to have animals exactly at the same age. Mouse MOCP-5 cell line was a gift from Dr. Y.-P. Li (Forsyth Dental Center, Boston, MA, USA). MC-3T3-E1 cell line was from ECACC (Wiltshire, UK).

2.2. Preparation of rabbit osteoclasts

For RT-PCR, primary osteoclasts were prepared from long bones of 10-day-old rabbits according to Sato et al. [16]. Briefly, cells isolated from bones were incubated for 2 h at 37°C in αMEM complemented with 5% FBS to allow osteoclasts to attach on plastic, and then non-adherent cells were removed. The day after, cells were washed and treated with pronase E (0.001%) and EDTA (0.02%) to discard contaminating stromal cells and adherent osteoclasts were washed and used for RNA extraction. These cells have been characterized as a population containing at least 95% primary osteoclasts [16].

2.3. Amplification of ADAM cDNA fragments

Total RNA was extracted from rabbit osteoclasts and mouse cells by guanidinium/acid-phenol extraction [17]. Single strand cDNA was synthesized from total RNA by using a cDNA synthesis kit (Pharmacia). cDNAs from MOCP-5 and MC-3T3 cells were submitted to PCR amplification using gene-specific primers (see Table 1). cDNA from rabbit osteoclasts was amplified by PCR using degenerate primers corresponding to conserved amino acid sequences in the metalloprotease catalytic site, HEXGHX and the disintegrin domain, CGNXXV. The reactions were cycled 45 times through the following steps: 94°C, 1 min; 55°C, 1 min; 72°C, 1 min. cDNA fragments were then purified, subcloned and sequenced. The cDNA sequences were compared with DNA sequences in GenBank and EMBL.

2.4. Northern blot analysis

The DNA probes were made from the PCR fragments generated with primers specific for ADAM 1, 8, 9, 12, 15, 19, TACE and ADAM TS-1. They were labelled with ^{32}P by using the Amersham rediprime kit protocol (Amersham Pharmacia Biotech). Ten micrograms of total RNA isolated from calvariae of E18 mice was blotted on nylon membranes after formaldehyde agarose gel electrophoresis and hybridized with radioactive probes in ultrahyb ultrasensitive hybridization buffer (Ambion). After hybridization, membranes were washed and exposed to Biomax MS X-ray film (Kodak).

2.5. Histology

For analysis of osteoclasts in metatarsals, hindlimbs of mouse embryos were processed, stained for TRAP, and counterstained with Ehrlich's hematoxylin according to [15]. For immunohistochemistry, TRAP staining was omitted. Sections were treated with 0.45% H_2O_2 in ethanol, 0.1% trypsin, and blocked with casein. Next, they were incubated overnight at 4°C with a primary anti-RANKL ectodomain antibody (C-20, Santa Cruz) diluted at 1:200, washed, incubated for 30 min at room temperature with a secondary biotinylated anti-goat antibody (Sigma) diluted at 1:20, washed again, and treated with peroxidase-conjugated extravidin (ABC, Vector Elite). RANKL immunoreactivity was visualized by developing the slides in DAB and H_2O_2. Counterstaining was performed with Ehrlich's hemeatoxylin.

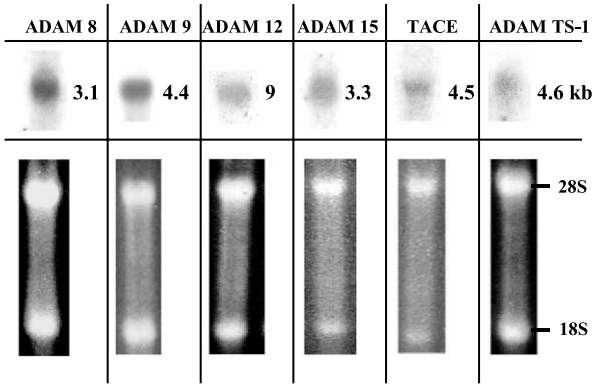

Fig. 1. Northern blot analysis of ADAMs in mouse bone. Total RNA from calvarium of 18-day old mouse embryos were analyzed by Northern blot for ADAMs. Lower pictures show the corresponding ethidium bromide gels.

3. Results

3.1. Expression of ADAMs in osteoblastic and osteoclastic cells and bone tissue

To get insight into the potential role of ADAMs in bone, we first analyzed which ADAMs are expressed in bone cells (Table 2). We performed RT-PCR with specific primers on transcripts of MOCP-5, a mouse osteoclast precursor cell line reported to form 95% TRAP positive multinucleated cells when cocultured with osteoclast-inductive MS12 stromal cells in presence of 1,25-$(OH)_2$ vitamin D_3 [18] and MC-3T3-E1, a mouse osteoblastic cell line that develops into mature osteoblasts [19]. We found that ADAM 1, 8, 9, 12, 15, 19, TS-1 and TACE were all expressed in osteoblasts and osteoclast precursors. We then examined ADAMs expression in osteoclasts. As cell differentiation in cultures can induce non-specific gene regulation, we used primary osteoclasts from rabbit long bones that can be obtained in high yield and purity. Using degenerate primers designed from consensus sequences in the metalloprotease and disintegrin domains, we found that cDNA fragments amplified by RT-PCR from RNA of mature rabbit osteoclasts corresponded to ADAM 1, 9 and TACE sequences. We could however not exclude that osteoclasts expressed more ADAMs, since using degenerate primers may favor the amplification of only some of them.

To verify that ADAMs identified by RT-PCR are expressed in bone tissue, we performed Northern blot analysis with RNAs extracted from calvaria of E18 mice, a developmental stage where the calvarium undergoes important bone turn-

Table 1
Primer sequences for RT-PCR of mouse ADAMs

Target mRNA	Primer sequences
ADAM 1	sense: 5′-CTAATGCTGATCGCCTAC-3′; antisense: 5′-CAGGAGCTGACAGGGGC-3′
ADAM 8	sense: 5′-GACTGGAGGGACGGTGC-3′; antisense: 5′-CTGGTTCATGAGGGCATC-3′
ADAM 9	sense: 5′-AGCAGCCTGCGCAGTTC-3′; antisense: 5′-CCACTAGGCTCCTGTGT-3′
ADAM 12	sense: 5′-CAGTGTCCACTAAGGATGC-3′; antisense: 5′-CATGCTGGCTATTGGGTC-3′
ADAM 15	sense: 5′-CTGCAGCATCTTCGCTC-3′; antisense: 5′-CAGCATCCCGCTGCAGC-3′
TACE	sense: 5′-AGCTGCAGCGTCAGAGC-3′; antisense: 5′-CAGCACTGTCACCAGGAAC-3′
ADAM 19	sense: 5′-CTAGCCAAGAGCTACCAGC-3′; antisense: 5′-CTGCTGTCTATGCTACTTAC-3′
ADAM TS-1	sense: 5′-TGCACACTGACACAGTGC-3′; antisense: 5′-CAGATGCTACTGTGCCTC-3′

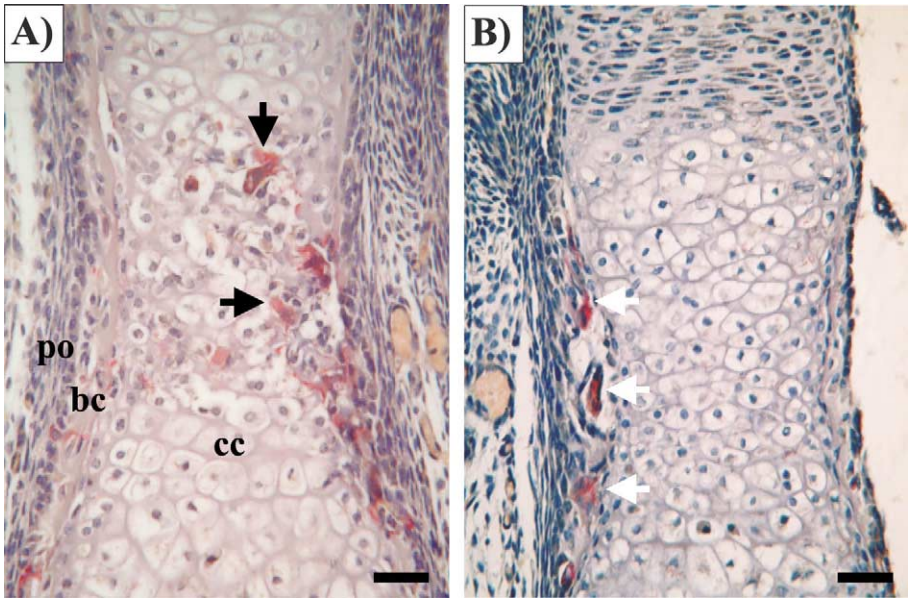

Fig. 2. Osteoclast distribution in sections through the diaphysis of metatarsals of $TACE^{\Delta Zn/\Delta Zn}$ and control embryos. Sections were obtained in metatarsals of E18.5 $TACE^{\Delta Zn/\Delta Zn}$ mice (B), and of control littermates (A); po, periosteum; bc, bone collar; cc, calcified cartilage. Osteoclasts were detected by TRAP straining (red). In controls (A), osteoclasts (black arrows) have invaded the core of the diaphysis and resorb the septae. In $TACE^{\Delta Zn/\Delta Zn}$ (B), osteoclasts (white arrows) remained at the vicinity of the bone collar. Bar: 40 μm.

over (Fig. 1). ADAM 8, 9, 12, 15, TS-1 and TACE were all found whereas expression of ADAM 1 and 19 could not be confirmed .

Overall our study thus strengthens previous evidence for ADAM 8, 9, 12, 15, TS-1 in bone tissue, and identifies TACE for the first time in bone tissue cells.

3.2. *Role of TACE in osteoclast recruitment and activity during development of long bones*

Recently, there is a lot of attention for the sheddase activity of TACE towards membrane proteins, including cytokine, growth factor, cytokine receptor and cell adhesion molecule [3]. Our present discovery of TACE in osteoblasts, osteoclastic cells and bone tissue, prompted us to investigate its role in developing metatarsals. Interestingly, between E16 and E18.5, metatarsals show cellular activities where ADAMs could be involved, and at this stage, metalloproteinase activity proved indispensable for their development [15]. During this period, osteoclast precursors located outside the diaphysis differentiate into TRAP+ mononucleated cells, fuse to form TRAP+ multinucleated osteoclasts, migrate into the future bone marrow cavity, and start digesting the calcified matrix [15]. We therefore investigated the effect of TACE inactivation on osteoclast recruitment by comparing the distribution of TRAP+ cells in metatarsals of mouse embryos genetically generated with a catalytically inactive TACE and of control littermates. E18.5 metatarsals of control littermates showed the typical features of this developmental stage: multinucleated TRAP+ osteoclasts had crossed the bone collar and were mainly inside the core of the diaphysis (Fig. 2A, arrows). Septae of calcified matrix were partially degraded in some regions next to osteoclasts indicating the beginning of the formation of the future marrow cavity. In sections of E18.5 $TACE^{\Delta Zn/\Delta Zn}$ metatarsals, multinucleated TRAP+ cells were also found (Fig. 2B, arrows) but only within the bone collar, not further inside the core of the diaphysis. This peculiar distribution of TRAP positive cells was observed in all $TACE^{\Delta Zn/\Delta Zn}$ mice used for this study (percentage of TRAP positive cells in the core of the diaphysis: 79.68% ± 11.4 in control embryos versus 0% in $TACE^{\Delta Zn/\Delta Zn}$ littermates; mean ± S.D. representative of six animals of each group taken from three different litters). These observations indicate that proteinase activity of TACE is not required for the generation of multinucleated TRAP+ cells but contributes significantly to the migration of these cells through the core of the diaphysis as is required for the development of the marrow cavity.

Because it was recently suggested that TACE could shed biologically active RANKL [20] and RANKL has deep effects on osteoclasts, including chemotaxis [21], it was of interest to investigate whether there is a relation between the impaired migration of osteoclasts and possible effects of TACE on the tissue distribution of RANKL. Therefore, we immunostained

Table 2
RT-PCR analysis of ADAMs in bone cells

	ADAM							
	1	8	9	12	15	TACE	19	TS-1
Rabbit primary OC	+	ND	+	ND	ND	+	ND	ND
MOCP-5 (mouse OC precursors)	+	+	+	+	+	+	+	+
MC-3T3 (mouse OB)	+	+	+	+	+	+	+	+

RT-PCR amplifications of RNA from rabbit osteoclasts and from mouse cells were performed with degenerated and specific primers, respectively. PCR products were sequenced for verification. ND: Not determined.

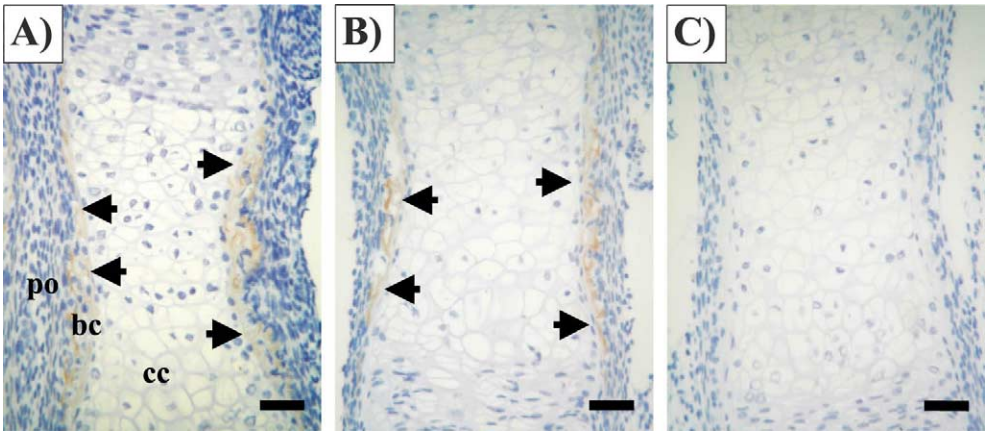

Fig. 3. Immunostaining of RANKL in TACE$^{\Delta Zn/\Delta Zn}$ and control metatarsals. RANKL distribution was examined by immunohistochemistry in the diaphysis of metatarsals of E17.5 TACE$^{\Delta Zn/\Delta Zn}$ mice (B) and control littermates (A). Negative controls were performed in control metatarsals sections with a primary non-immune antibody (C). RANKL immunoreactivity showed an intense signal (brown color) restricted to bone collar whether in controls (black arrows in A) or in TACE$^{\Delta Zn/\Delta Zn}$ (black arrows in B). Bar: 40 μm.

RANKL in E17.5 metatarsals, i.e. when differentiation and migration of osteoclasts just start. We found that in TACE$^{\Delta Zn/\Delta Zn}$ and control metatarsals, RANKL showed the same distribution in the bone collar region, (Fig. 3A,B) exactly where osteoclasts are generated. These observations do not support a TACE-shedding-dependent redistribution of RANKL in metatarsals.

4. Discussion

Osteoclasts have a short life, and continuous recruitment of osteoclasts is critical for bone (re)modelling. Metalloproteinases are an important component of the osteoclast recruitment mechanism in primitive long bones [15]. The present study reveals that one of the critical metalloproteinases in this process is TACE.

The biological events required by this recruitment are the generation of preosteoclasts in the soft tissue surrounding the bone, their fusion, and their migration into the core of the diaphysis. It is clear that the role of TACE is related to the latter migration, because the absence of TACE proteolytic activity still allows the generation of multinucleated cells with a typical osteoclast phenotype in the surrounding soft tissue, but prevents their migration into the core of the diaphysis. A contribution of TACE to cell migration and bone development had never been reported before, in contrast, the involvement of TACE in epithelial maturation was stressed at the level of many organs, such as eye, skin, intestine, or lung [3].

The potential direct or indirect molecular mechanism of action of TACE in osteoclast migration is still unknown, as well as its cellular source. TACE is a well-known sheddase, which has been implicated in the release of a series of cytokines and growth factors, including members of the TNFα family [3,20]. RANKL belongs to the TNFα family and there are indications that TACE may also promote RANKL shedding [20]. Furthermore, RANKL exerts chemotaxis on osteoclasts [21]. Taking into account these observations, it may be considered that TACE-induced shedding of RANKL by neighboring cells is important for osteoclast migration into primitive long bones. However, we could not provide evidence supporting this hypothesis, since we found a similar distribution of RANKL immunoreactivity in TACE$^{\Delta Zn/\Delta Zn}$ and control embryos. The absence of changes in RANKL pattern in bone when TACE proteolytic domain is inactivated may indicate that TACE is not the only RANKL sheddase as it has been suggested by other more recent studies [22,23]. Alternatively, one could speculate that TACE promotes migration of osteoclasts by a process of ECM degradation as reported for ADAM 10 and 15, which both can degrade type IV collagen in vitro [24,25]. However, only sheddase activity has been ascribed to TACE so far.

It is of interest that TACE is the third metalloproteinase after MMP9 and MT1-MMP, found limiting for the migration of osteoclasts into primitive long bones [26,27]. This thus stresses the complexity of the proteolytic mechanisms controlling the recruitment process of osteoclasts. Other ADAMs have been implicated in the recruitment of osteoclasts, as based on in vitro observations. ADAM 8 promotes osteoclast differentiation whereas antisense is inhibitory, and this effect was ascribed to its disintegrin domain [13]. ADAM 12 antisense inhibits the differentiation of osteoclast-like cells generated in cocultures of bone marrow cells with osteoblasts [12]. It was speculated that this effect was related to its predicted fusion peptide sequence and to its putative role in formation of myotubes [5]. Finally, anti-ADAM 9 antibody suppressed RANKL-induced formation of multinucleated giant cells from human blood monocytes in vitro, suggesting that ADAM 9 could be involved in osteoclast differentiation [14].

In conclusion, we found that TACE is limiting for osteoclast recruitment in developing bones. Information on the role of other ADAMs found in bone tissue is awaiting more observations especially on the existing ADAM 1, 9, 15, 19, and ADAM TS-1 knockouts, although skeletal phenotypes for these knockouts have not been reported yet.

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