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Matrix metalloproteinases are obligatory for the migration of preosteoclasts to the developing marrow cavity of primitive long bones

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SUMMARY

A key event in bone resorption is the recruitment of osteoclasts to future resorption sites. We follow here the migration of preosteoclasts from the periosteum to the developing marrow cavity of fetal mouse metatarsals in culture, and investigate the role of proteinases and demineralization in this migration. Our approach consisted in testing inhibitors of proteinases and demineralization on the migration kinetics. Migration was monitored by histomorphometry and the (pre)osteoclasts were identified by their tartrate resistant acid phosphatase (TRAP) activity. At the time of explantation, TRAP+ cells (all mononucleated) are detected only in the periosteum, and the core of the diaphysis (future marrow cavity) consists of calcified cartilage. Upon culture, TRAP+ cells (differentiating progressively into multinucleated osteoclasts) migrate through a seam of osteoid and a very thin and discontinuous layer of mineral, invade the calcified cartilage and transform it into a 'marrow' cavity; despite the passage of maturing osteoclasts, the osteoid develops into a bone collar. The migration of TRAP+ cells is completely prevented by matrix metalloproteinase (MMP) inhibitors, but not by a cysteine proteinase inhibitor, an inhibitor of carbonic anhydrase, or a bisphosphonate. The latter three drugs inhibit, however, the resorptive activity of mature osteoclasts at least as efficiently as do the MMP inhibitors, as assessed in cultures of calvariae and radii. Furthermore, in situ hybridizations reveal the expression of 2 MMPs, gelatinase B (MMP-9 or 92 kDa type IV collagenase) in (pre)osteoclasts, and interstitial collagenase (MMP-13) in hypertrophic chondrocytes. It is concluded that only MMPs appear obligatory for the migration of (pre)osteoclasts, and that this role is distinct from the one MMPs may play in the subosteoclastic resorption compartment. We propose that this new role of MMPs is a major component of the mechanism that determines where and when the osteoclasts will attack the bone.

Key words: bone resorption, bone development, osteoclast recruitment, cathepsin, MMP-9, MMP-13, interstitial collagenase, 92 kDa type IV collagenase

INTRODUCTION

Bone development depends on the precise regulation of a series of bone resorbing and bone forming events. During adult life, this mechanism is still essential for maintaining, adapting and repairing the bone matrix. Improper regulation may lead to bone loss and results in pathological situations. The central step of bone resorption is the removal of bone matrix and the cell responsible for this is the osteoclast. During the last decade, there have been major advances in the understanding of the enzymatic systems used by osteoclasts to exert this activity (Baron et al., 1993).

It must be emphasized, however, that this resorbing activity is determined by the recruitment of new osteoclasts, which are targeted at specific times to specific sites of the bone matrix. The osteoclasts are generated from progenitor cells of the mononuclear phagocyte system (Suda et al., 1992). At an early stage of embryonic life, the mononucleated precursors of the osteoclasts are disseminated via the blood stream and deposited in the mesenchyme surrounding the bone rudiments. There, they proliferate and differentiate into TRAP+ cells (Scheven et al., 1986). The switch to an invasive behavior and the migration mechanism of the TRAP+ cells from the mesenchyme to the future resorption sites is raising much interest. There is evidence for a control of their migration by TGF-β (Dieudonné et al., 1991), TNF-α (Van der Pluijm et al., 1991), LIF (Van Beek et al., 1993), M-CSF (Hofstetter et al., 1992; Fuller et al., 1993) and the C3 component of the complement (Mangham et al., 1993); integrins appear to be involved (Van der Pluijm et al., 1994); osteocalcin (Glowacki et al., 1989; Mundy and Poser, 1983), α2-HS-glycoprotein and collagen fragments (Malone et al., 1982) have been proposed as chemoattractants. Nothing is known, however, about the enzymatic system(s) mediating this migration.

We address this question in the present work, using metatarsals of 17-day-old fetal mice. The interest in using these bones for investigating early events of the bone resorption processes was first emphasized by Burger et al. (1982). At this

age, these bones have no mature osteoclasts, but only pre-osteoclasts that are differentiating into TRAP+ cells. These cells are located at the level of the periosteum (po). Later, they invade the calcified cartilage (cc) while maturing into multi-nucleated osteoclasts, that generate the primitive marrow cavity. Thus we characterize here the route of TRAP+ cells from the po to the future marrow cavity and investigate the enzymatic mechanism of their migration, by analysing whether it is affected by inhibitors of the enzymatic systems used by mature osteoclasts to resorb bone (Delaissé and Vaes, 1992). We show by a histomorphometric approach that inhibitors of MMPs completely block this migration, whereas an inhibitor of cysteine proteinases and inhibitors of demineralization merely slow down the rate of migration into the cc. This demonstrates a specific and obligatory role of MMPs at an early stage of the resorption processes. In situ hybridizations suggest a possible involvement of gelatinase B (MMP-9 or 92 kDa type IV collagenase) and interstitial collagenase (collagenase-3 or MMP-13 and previously known as 'rodent MMP-1').

MATERIALS AND METHODS

Materials

The MMP inhibitor RP59794 (Lelièvre et al., 1990) was a gift from Dr B. Terlain (Rhone-Poulenc Santé, Vitry-sur-Seine, France); tiludronate from Dr C. Barbier (Sanofi Recherche, Montpellier, France); cDNA encoding mouse gelatinase B (Masure et al., 1993) from Dr G. Opdenakker (Katholieke Universiteit, Leuven, Belgium); cDNA encoding mouse interstitial collagenase (Henriet et al., 1992) from Dr P. Henriet (Université de Louvain, Bruxelles, Belgium). Ethoxyzolamide, E-64 and cytosine-β-D-arabinofuranoside were from Sigma (St Louis, MO). BGJb culture medium was from Gibco-BRL (Paisley, Scotland). [^{35}S]UTP, ^{45}Ca and [^{3}H]thymidine were from Amersham (Gent, Belgium). Z-Phe-Ala-CHN_2 was from Bachem (Bubendorf, Switzerland). The glycolmethacrylate 70-2218-500 Historesin Embedding Kit (Jung) was from H. Kulzer Gmbh (Heidelberg, Germany). Other reagents were from suppliers previously mentioned (Delaissé et al., 1985; Leloup et al., 1994).

Cultures

The 3 middle metatarsals of each hindlimb of 17-day-old mouse embryos (considering the day of vaginal plug discovery as day 0 of gestation) were dissected as a triad. One triad of each pair was cultured as a control, the other as a test. The culture conditions that we set up for this work were reported earlier (Leloup et al., 1994). Each triad was cultured in 700 μl BGJb medium, supplemented with $NaHCO_3$ (2,200 mg/l), NaCl (900 mg/l), ascorbate (50 mg/l), glutamine (200 mg/l), bovine serum albumin (1 g/l), $1,25(OH)_2D_3$ (3×10^{-8} M) and the inhibitors as indicated. Media were renewed every day (tiludronate was added to the medium only during the first day of culture, because of its high and irreversible binding to bone). Radii were sometimes isolated from the same embryos and cultured in the same way. Calvariae of 18-day-old mouse embryos were cultured in medium 199 with or without PTH (2×10^{-8} M), and with or without inhibitor (Delaissé et al., 1985).

Release of ^{45}Ca and [^{3}H]thymidine incorporation

Long bones and calvariae were labelled by injecting the pregnant mice with 100 μCi ^{45}Ca, respectively, 24 hours (Burger et al., 1982) and 64 hours (Vaes, 1968) before their explantation. Demineralization of the explants during culture was monitored by determining the amount of ^{45}Ca released into the media harvested at successive days, and that remaining in the bones at the end of the cultures. The release of ^{45}Ca during the cultures was expressed cumulatively as the percentage of the total amount of radioactivity recovered. The amount of ^{45}Ca released during the first day of culture of metatarsals and radii corresponds to passive exchange and was subtracted in order to obtain a value representative of cell mediated resorption. For calvariae, it is the ^{45}Ca release that occurs in the absence of PTH that was subtracted, in order to determine the effect of the drugs on the PTH stimulated resorption. Autoradiographs of paraffin sections show that the ^{45}Ca injections result in labelling mainly the cc of the metatarsals and the innerside of the bone collar of the radii. Thus the release of ^{45}Ca from metatarsals is mainly due to resorption of cc, and that from radii is mainly due to resorption of bone collar at its endosteal surface. [^{3}H]Thymidine incorporation into the metatarsals was determined according to the method of Bagi and Miller (1992).

Preparation of tissue sections

Paraffin sections for histomorphometry were prepared according to the method of Scheven et al. (1986). Briefly, metatarsals were fixed in 4% neutral buffered formalin for 3 hours at 4°C, decalcified in 5% formic acid and 5% formalin for 3 hours at 4°C, stained for TRAP by the 'en bloc' procedure (using Naphtol AS-B1 phosphate as substrate, pararosanilin as coupler and 10 mM L(+)-tartaric acid as inhibitor), dehydrated in a graded series of ethanol and embedded in paraffin. Serial sections of 5 μm were obtained and counterstained with Ehrlich's hematoxylin.

Paraffin sections for in situ hybridization were prepared similarly, but omitting decalcification and staining for TRAP from the hereabove described procedure. They were collected on 3-amino-propyltriethoxysilane coated slides.

Plastic sections for a detailed characterization of the migration pathway were also prepared. Metatarsals were fixed in 4% neutral buffered formalin for 18 hours at 4°C, and embedded in glycol-methacrylate according to the instructions of the 'Historesin Embedding Kit'. Sections of 3 μm were cut with glass knives on a Reichert microtome. Depending on the experiments, the sections were stained for TRAP (van de Wijngaert and Burger, 1986), with methylene blue (Beertsen et al., 1985), with Masson-Goldner's (Gruber, 1992) or with von Kossa's stain.

Histomorphometry

The cultures for histomorphometry were set up so as to test the drugs on paired triads after 1, 2 and 3 days culture, within the same litter. This allowed usually a number of 3 triads (i.e. 9 metatarsals) per experimental condition. The number of TRAP+ cells and of their nuclei were determined in the serial sections, at the level of the periosteum (po), of the calcified cartilage (cc) and of the intermediary zone corresponding to the mineralizing collar of osteoid (bc). A cell (and its nuclei) was scored 'cc' as soon as part of it had invaded the cc, thus indicating its ability to reach the cc. Cells at the interface of the po and of the bc were scored 'po', because in paraffin sections, it was sometimes difficult to distinguish clearly the limit between cells of the po and osteoblasts layering the osteoid. The analysis of both cells and nuclei counts was performed. They lead to the same conclusions concerning migration; however, mainly nuclei counts are shown since they are judged more representative of migration of cells undergoing fusion. Each drug was tested in at least 2 experiments. In most experiments, noncultured metatarsals were analysed in the same way, so as to make sure that the cultures were initiated with metatarsals with noninvaded cc.

Statistical evaluations

The release of ^{45}Ca from paired experimental groups was compared by the analysis of variance for multiple comparisons. The counts of nuclei of TRAP+ cells in the po, bc and cc of paired metatarsals were compared using the Wilcoxon matched pairs signed ranks test. The number of nuclei of TRAP+ cells in paired experimental groups were compared by the χ^2 test.

In situ hybridization

A 2 kb fragment of the mouse gelatinase B cDNA, cloned in a pBlue-

script plasmid (Masure et al., 1993) was digested with *Bam*HI restriction enzyme. A 421 bp fragment was subcloned into a pGEM3 plasmid (Promega). A clone was selected, linearized with *Ava*I and transcribed with SP6 RNA-polymerase to generate an antisense probe, or linearized with *Sal*I and transcribed with T7 RNA-polymerase to generate a sense probe.

A 2.7 kb mouse collagenase cDNA cloned in a pBluescript plasmid (Henriet et al., 1992) was digested with *Hpa*I. A 666 bp fragment was subcloned into the *Eco*RV site of a pBluescript plasmid. A clone was selected, linearized with *Sph*I and transcribed with T3 RNA-polymerase to generate a 186 bp antisense probe. The sense probe was generated by cutting directly the first plasmid vector with *Fok*I and transcribing with T3 RNA-polymerase.

[^{35}S]UTP-labeled-single-stranded RNA was prepared using a Boehringer SP6T7 transription kit. In situ hybridization, the subsequent washings and autoradiography were performed as described by Shinar et al. (1993). The sections were stained with hematoxylin and eosin.

RESULTS

Morphological aspects of the migration of TRAP+ cells

The route of TRAP+ cells through the extracellular matrix was characterized using plastic sections and multiple stains (Fig. 1). Further to earlier reports (e.g. Burger and Van Delft, 1976), our observations show that the diaphysis of metatarsals of 17-day-old embryos (Fig. 1A,D,G) consists of: (1) a core of calcified cartilage (cc), with typical hypertrophic chondrocytes in lacunae whose septae are heavily but discontinuously mineralized and that do not show any resorption; (2) a collar of osteoid (bc) whose thickness is fairly variable (15-25 μm) and whose inner edge is slightly and discontinuously mineralized; (3) a periosteum (po) consisting of several layers of elongated mononucleated cells surrounding the collar of osteoid. At this age, TRAP+ cells are not detected in the cc (Fig. 1D). Most TRAP+ cells are next to the inner cell layer of the po, just at the periphery of the osteoid (Fig. 1J), an area where osteoblasts are particularly numerous. The TRAP+ cells are mononucleated.

Metatarsals of 10 to 24 hours older mice have TRAP+ cells in the whole thickness of the osteoid (Fig. 1K,L) and in the cc (as in Fig. 1E). Many TRAP+ cells of these older bones are multinucleated. The TRAP+ cells that reach the level of the nascent mineralization line of the bone collar are either in non-mineralized spaces of this line (i.e. between mineral clusters) (Fig. 1L), or adjacent to the mineral layer (Fig. 1K). The osteoid collar through which the cells migrate, is left intact. Actually, it even further differentiates; the mineral layer becomes thicker but remains discontinuous. In contrast, the cc is progressively resorbed and replaced by the marrow cavity (Fig. 1B).

Metatarsals of 17-day-old embryos, cultured for 1 or 2 days (Fig. 1E,C,I), show similar features to those described above in vivo. Thus in our culture conditions, as in vivo, the tissue that the differentiating preosteoclasts must cross to reach the cc is a 15-25 μm seam of mineralizing osteoid and this osteoid further develops into a bone collar while only cc is removed.

Evaluation of the relative potency of inhibitors of osteoclastic activity on bone resorption

Prior to testing a series of inhibitors of mature osteoclasts on the migration kinetics of preosteoclasts, we established their relative inhibitory potencies against bone resorption itself. Therefore, we performed cultures of bone tissues where mature osteoclasts are abundant and present from the beginning of the culture, i.e. calvariae of 18-day and radii of 17-day-old fetal mice. The inhibitors that we tested are the general MMP inhibitors CI-1 (Delaissé et al., 1985) and RP59794 (Lelièvre et al., 1990); the cysteine proteinase inhibitor E-64 (Barrett et al., 1982); inhibitors acting on the demineralizing processes, i.e. ethoxyzolamide, which inhibits carbonic anhydrase (Raisz et al., 1988) and tiludronate, a bisphophonate. All inhibit rapidly and strongly the demineralization of calvariae (Fig. 2A) and of radii (Fig. 2B), as expected from previous data (Delaissé and Vaes, 1992). The inhibitors of proteinases (E-64, CI-1, RP59794) produce weaker inhibitions of the Ca losses than do the inhibitors that act directly on the demineralizing processes (ethoxyzolamide, tiludronate), as previously discussed (Delaissé et al., 1985; Everts et al., 1992).

Next, we tested the effect of these agents on the demineralization of the cc of the metatarsals. Fig. 2C shows that in contrast with the situation in calvariae and radii, MMP inhibitors are as efficient as agents acting directly on the demineralizing processes. E-64 displays, however, lower inhibitory activities than in the former experimental models. Higher inhibitions are not obtained (not shown) with other inhibitors of cysteine proteinases such as leupeptin or Z-Phe-Ala-CHN2, which penetrates into the cells more readily than E-64 (Wilcox and Mason, 1992). CI-2, a stereoisomer of CI-1, inhibits ^{45}Ca loss to a smaller degree than does CI-1, as observed in calvariae (Delaissé et al., 1985). This fits the relative inhibitory activities of CI-1 and CI-2 towards collagenase. The inhibitions by all these agents are reversible (not shown), except that by tiludronate, as expected from the mechanism of action of bisphosphonates (Sato et al., 1991).

Moreover, it was checked that cytosine-β-D-arabinofuranoside, an antimitotic agent that produces a 90% inhibition of [^{3}H]thymidine incorporation in the metatarsals, does not inhibit at all the loss of ^{45}Ca (not shown). This indicates that the osteoclast precursors, which are responsible for resorption later during culture, are at a postmitotic stage (Scheven et al., 1986) when these cultures are initiated.

Effect of inhibitors of osteoclast resorbing activity on the migration kinetics of preosteoclasts

Fig. 3 shows the migration kinetics of the maturing osteoclasts to the cc in control conditions, as evaluated from the counts of TRAP+ cells and their nuclei in serial sections of a number of metatarsals. When the cultures are initiated, cell and nuclei counts are almost identical, showing that almost all TRAP+ cells are mononucleated. Moreover, all are found at the level of the po+bc, and the cc is not invaded. Upon culture, there is a progressive increase in the number of cells and nuclei per metatarsal as described by others (Dieudonné et al., 1991; Van der Pluijm et al., 1994). The increase is larger for nuclei than for cell numbers, showing that the TRAP+ cells become multinucleated. These observations are compatible with a continuous differentiation of TRAP-negative precursors into TRAP+ cells, and with the concept that multinucleated osteoclasts are generated by fusion of TRAP+ cells (Baron et al., 1986). The culture leads also to increasing numbers of TRAP+ cells and of nuclei in the cc; the proportion of nuclei in the cc increases from 0 to 44±11%, 79±8% and 81±7% (means ± s.d.), respec-

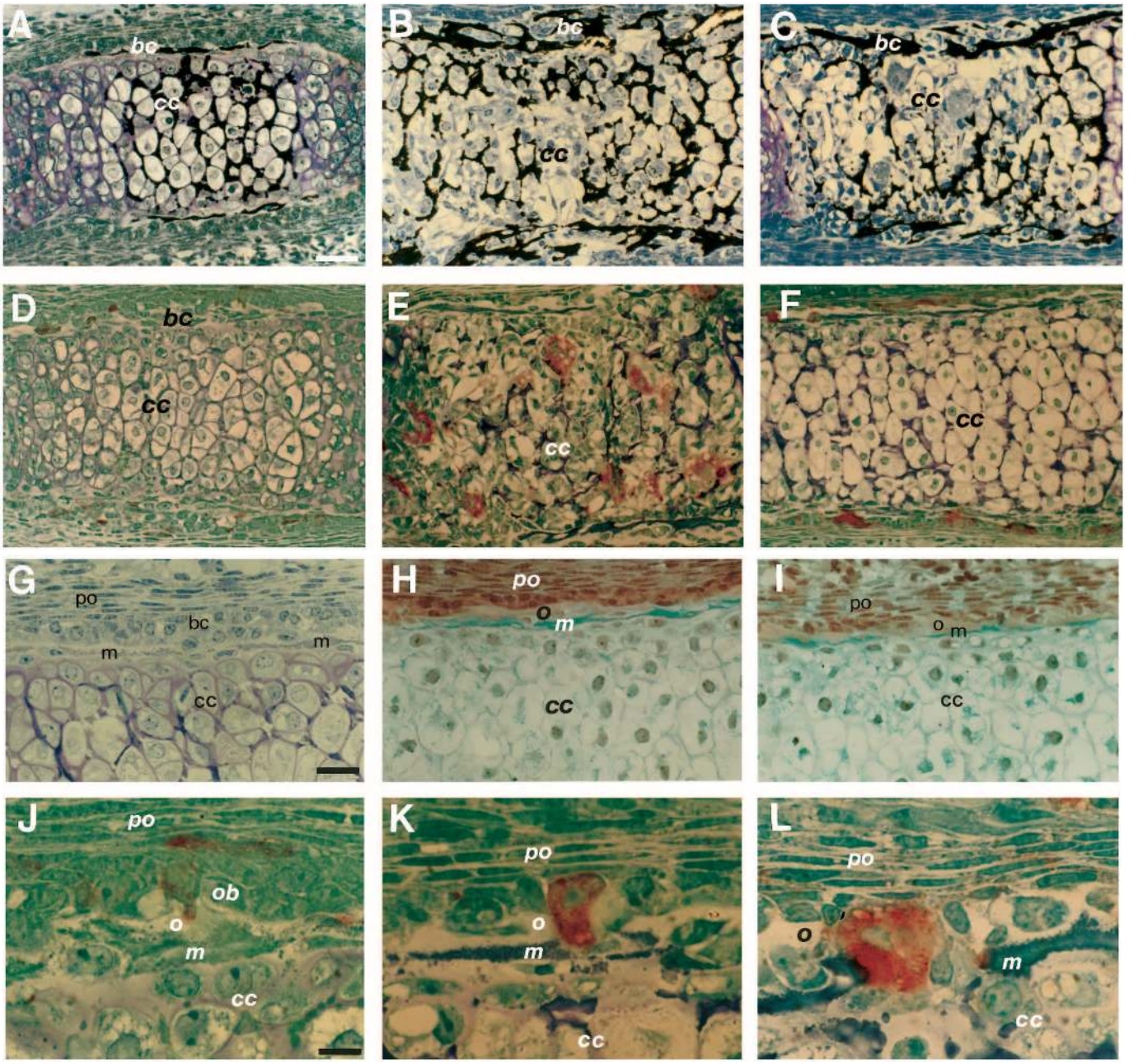

Fig. 1. Ultrastructural features of the diaphysis of metatarsals during their development, either in vivo or in culture. Plastic sections were stained so as to show the mineral, the osteoid and the interactions between (pre)osteoclasts and the extracellular matrix.

(A to F) General view of the diaphysis. Bar in A, 40 µm. Von Kossa staining (A to C) of metatarsals of 17-day-old embryos (A) shows that the mineralization (black) of the septae of the calcified cartilage (cc) is still incomplete and that the mineralization of the future bone collar (bc) is limited to a thin and discontinuous layer of mineral. In 18-day-old fetal metatarsals (B), or after a 2-day culture of 17-day-old fetal metatarsals (C), the cc is partially resorbed whereas the bc has further mineralized. Combined TRAP and methylene blue staining (D to F) of 17-day-old metatarsals (D) reveal TRAP+ cells (red) only outside the intact cc. After a 2-day culture (E), large multinucleated TRAP+ cells (red) have penetrated into the cc and are resorbing it. If CI-1 is present (F), TRAP+ cells are all outside the cc and the cc remains intact.

(G to I) Details of the po, bc and cc. Bar in G, 25 µm. Methylene blue staining of metatarsals of 17-day-old embryos (G) shows several layers of elongated cells forming the periosteum (po) and covering a pale zone that contains osteoblasts and that corresponds to the future bone collar (bc). Areas of small dots layering discontinuously the innerside of this bc reveal the presence of thin mineralized islands (m). The cc has intact septae and typical hypertrophic chondrocytes. Masson-Goldner staining of 17.5-day-old fetal metatarsals (H) and of 17-day-old fetal metatarsals that have been cultured for 1 day (I) shows similar features as in G, but allows a better view of the osteoid (pink) (o) and of the mineralized matrix (green) (m).

(J to L) Migration of TRAP+ cells shown by combined TRAP and methylene blue staining. Bar in J, 10 µm. In metatarsals of 17-day-old embryos (J), TRAP+ cells (red) are against the inner cell layer of the po or at the level of osteoblasts (ob). Already, 7 hours later, TRAP+ cells have enlarged and reach the mineral layering the innerside of the osteoid (o); they are either between (L) or against (K) the mineral clusters (m). TRAP staining appears stronger at the contact points of the cells with the mineral.

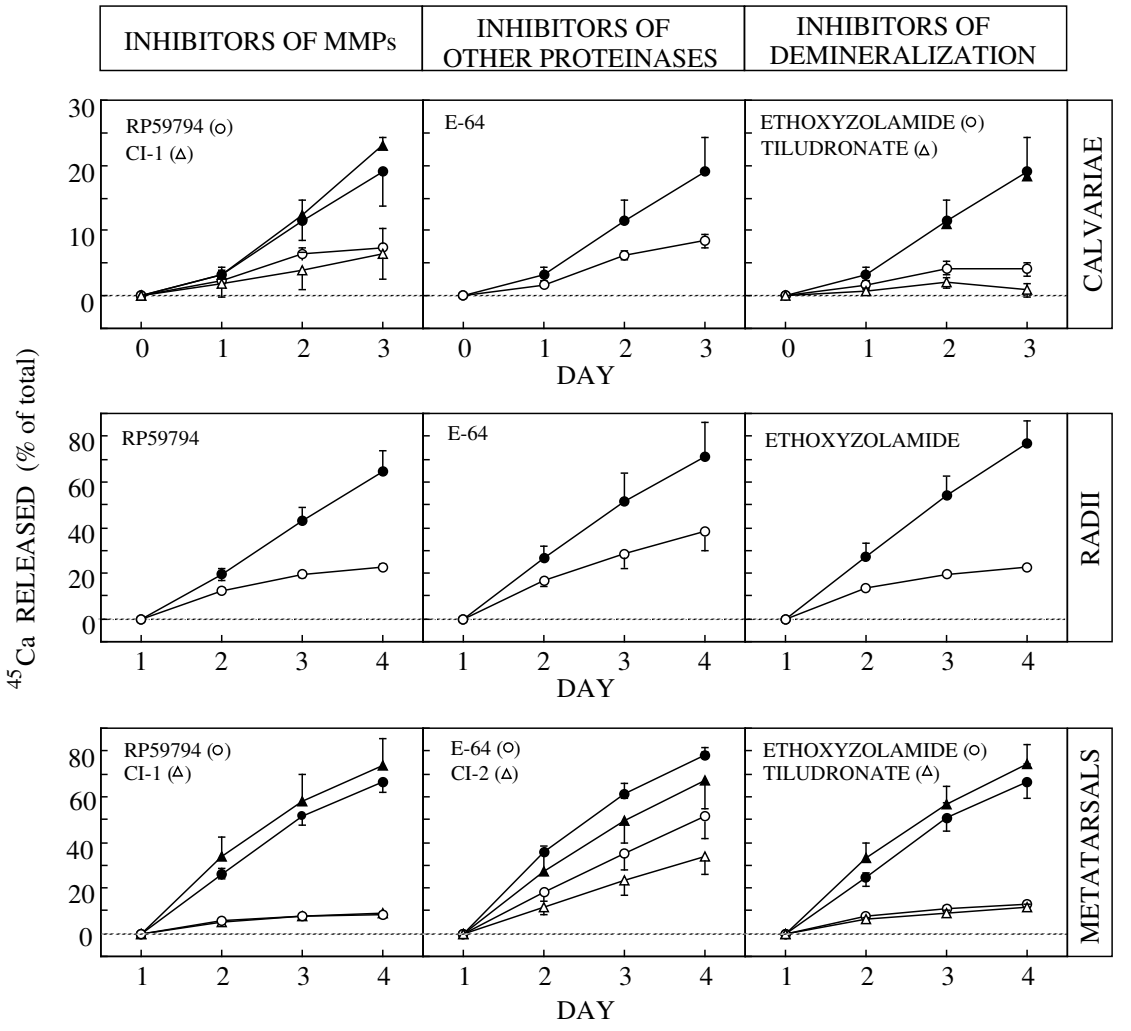

Fig. 2. Comparative effect of drugs on the resorption of different types of bone explants. Calvariae (A), radii (B) or metatarsals (C) were cultured either in the presence of 25 μM RP59794, 50 μM CI-1, 42 μM E-64, 50 μM CI-2, 10 μM ethoxyzolamide, 30 μM tiludronate (open symbols as indicated, ○,△), or in their absence (corresponding closed symbols, ●,▲). Each point is the mean ± s.d. of the ^{45}Ca release determined in 4 independent cultures. All the drugs inhibit significantly ^{45}Ca release (P<0.05).

tively after 1, 2 and 3 days. Note also that the average number of nuclei is 1 in the po, 1.9±0.2 in the bc and 2.8±0.5 in the cc after 1 day culture (means ± s.d.). The latter value remains constant during the whole culture. This shows that the fusion of TRAP+ cells occurs progressively during their migration, and that the osteoclasts reach complete maturity once they reach the cc.

The MMP inhibitors CI-1 and RP59794 completely prevent the invasion of cc by TRAP+ cells, even after a 3-day culture (Figs 1F, 4). Concomitantly, these drugs cause an accumulation of nuclei of TRAP+ cells in the po+bc, thus showing that they do not stop the formation of new TRAP+ cells. The formation of TRAP+ cells is, however, somewhat smaller in the treated metatarsals than in the controls. The noninvaded cc of treated metatarsals becomes invaded (Fig. 4) and undergoes resorption (not shown) as soon as the inhibitors are removed from the culture fluids. Thus the inhibition of invasion by CI-1 and RP59794 is reversible. Because TRAP+ cells of CI-1 treated metatarsals seemed to get closer to the mineralization front of the bc than those of RP59794 treated ones, a more detailed analysis of the po+bc zone was performed in plastic sections. It showed indeed that in the RP59794 treated explants, only 22% of TRAP+ cells reach the mineral layer after a 1-day culture, whereas in CI-1 treated explants as many as 44% of the TRAP+ cells are in contact with mineral. Thus

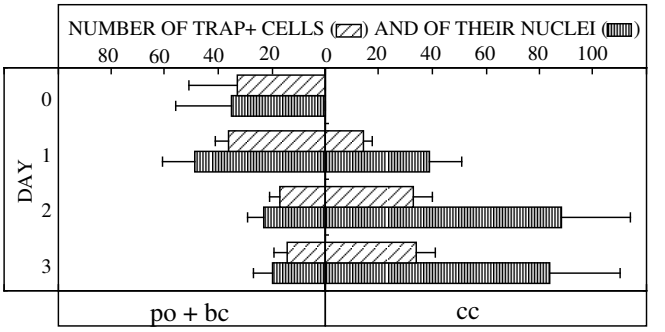

Fig. 3. Effect of a culture on the number of TRAP+ cells and of their nuclei, counted in and outside the cc. Metatarsals were cultured in control conditions for the indicated times. The number of TRAP+ cells (▨) and of their nuclei (▥) localized inside and outside the cc of the diaphysis of each metatarsal were counted. Counts inside the cc are shown to the right of the '0' axis, those outside the cc (i.e. in po + bc) to the left. Each bar (left + right part) expresses thus the total numbers in one metatarsal. Counts at day 0, 1, 2 and 3 are the means ± s.d. of, respectively, 14, 14, 16 and 12 experiments, each involving at least 9 metatarsals.

RP59794 inhibits more strongly the migration of the TRAP+ cells than does CI-1. Noteworthy also, the mean number of nuclei per cell in the bc is not affected by RP59794 and is

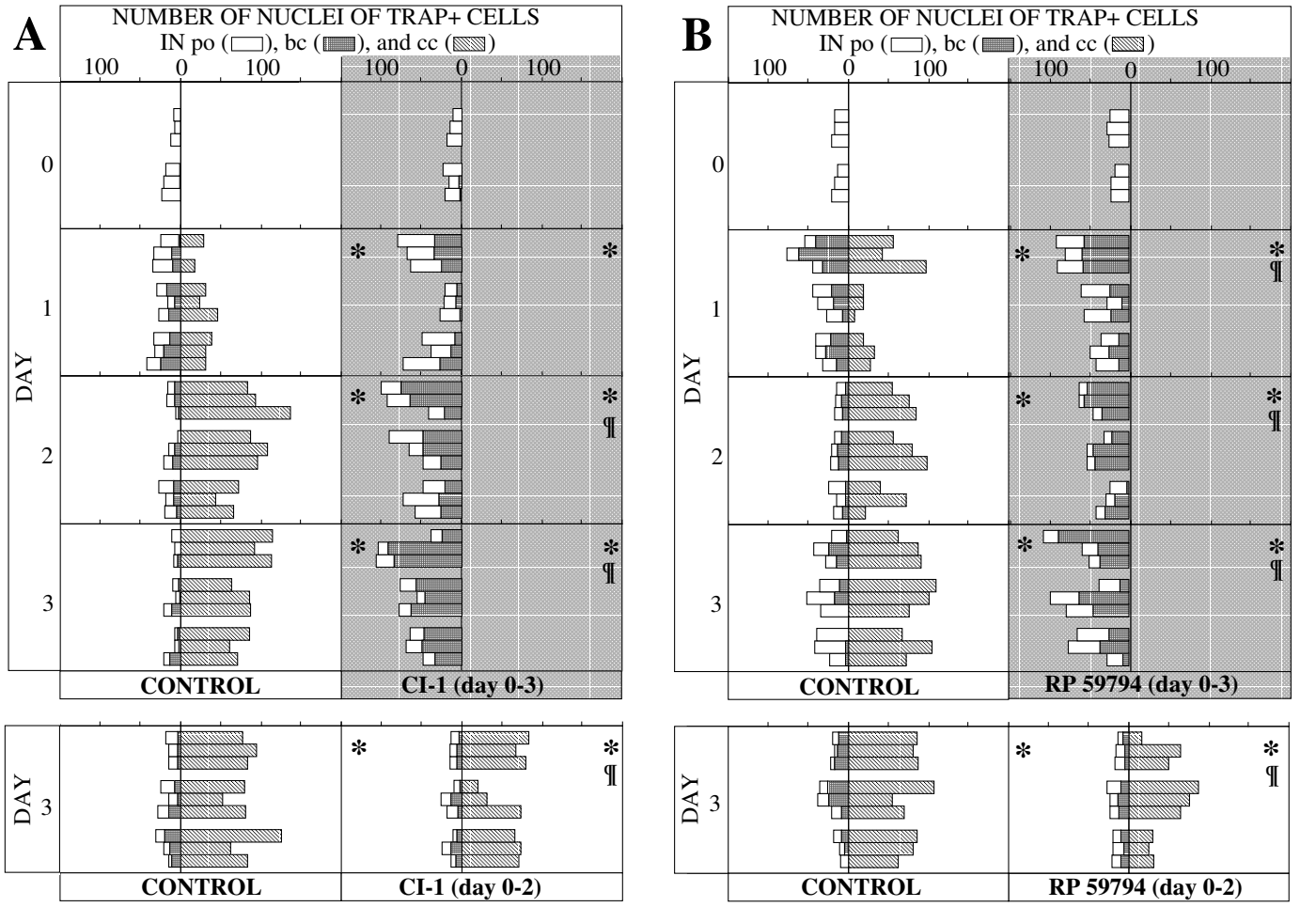

Fig. 4. Effect of MMP inhibitors on the migration of TRAP+ cells to the cc. Metatarsals were obtained from 2 litters of 17-day-old fetal mice. One litter was used for testing CI-1 (A), the other for testing RP59794 (B). The metatarsal triads of the left limbs were cultured in control conditions and those of the corresponding right limbs were cultured in the presence of 50 μM CI-1 (A) or 25 μM RP59794 (B) for the indicated days. The nuclei of the TRAP+ cells were counted in the po (block □), in the bc (block ▒), and in the cc (block ▨) of each metatarsal. Each horizontal bar obtained by juxtaposing these 3 blocks (po + bc + cc) represents the total number of nuclei in one metatarsal. Each bar is positioned so as to show the counts in po+bc to the left of the 'o' axis, and those in the cc to the right. The counts in each pair of triads of metatarsals (i.e. controls and tests) are represented facing each other. The lower graph in A and B shows the situation at day 3, when CI-1 or RP59794 is added only during the first 2 days. * at the left or at the right of the '0' axis: significant effect of the inhibitor on the number of nuclei of TRAP+ cells respectively in po+bc or in cc ($P<0.05$); ¶: significant effect of the inhibitor on the number of nuclei of TRAP+ cells in the whole metatarsal (i.e. po+bc+cc) ($P<0.05$).

enhanced in the presence of CI-1 (2.4±0.7 nuclei/cell in the bc of CI-1 treated metatarsals, as compared to 1.6±0.4 in the bc of controls). The addition of CI-1 at day 1 (instead of day 0), to metatarsals whose cc is already invaded and is undergoing resorption, results in a lowering of the cell and nuclei counts in the cc by the next day, thus indicating that MMP dependent migration occurs throughout the whole culture (not shown).

The specificity of the effect of RP59794 and CI-1 on migration was assessed by comparing their effect to that of CI-2 and of other inhibitors of osteoclast resorbing activity, i.e. E-64, ethoxyzolamide and tiludronate. Clearly, none of these agents stops the invasion of cc (Figs 5 and 6). One day culture in their presence lowers, however, the number of nuclei in the cc and enhances concomitantly those in the po+bc as compared to controls (except for tiludronate). These opposite effects in the cc and in the po+bc indicate a slower migration of TRAP+ cells in the presence of CI-2, E-64 and ethoxyzolamide. One more day culture in the presence of these agents results in a further migration into the cc. In contrast with the other inhibitors, tiludronate lowers the number of nuclei in the cc without concomitant enhancement of their numbers in the po+bc (Fig. 6). The cells were also smaller. These effects could be ascribed to a decreased formation of TRAP+ cells (Löwik et al., 1988), or to a toxic effect on the mature osteoclasts (Sato et al., 1991). Whatever the explanation, our results show that TRAP+ cells are able to migrate in the presence of an irreversible inhibitor of bone resorption. Noteworthy, all these inhibitors allow invasion up to the middle of the cc (not shown), even when they inhibit strongly the release of ^{45}Ca (Fig. 2C). This is in accordance with the incomplete mineralization of the lacunae of the hypertrophic chondrocytes as shown by von Kossa staining (Fig. 1A). Some of the inhibitors affect transiently the number of nuclei per cell (not shown); the number of nuclei per cell tends to increase as compared to

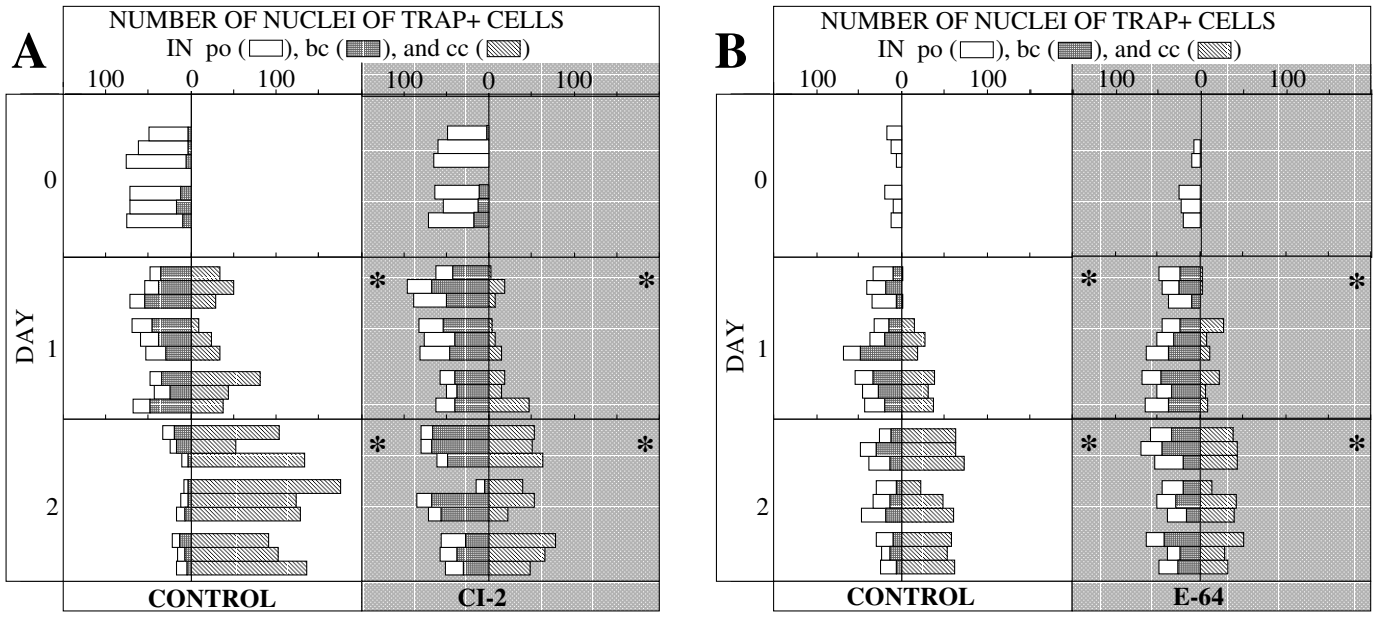

Fig. 5. Effect of the protease inhibitors CI-2 and E-64 on the migration of TRAP+ cells to the cc. Experimental procedure and presentation of the data are as in Fig. 4. The concentrations of CI-2 (A) and E-64 (B) were, respectively, 50 and 42 μM.

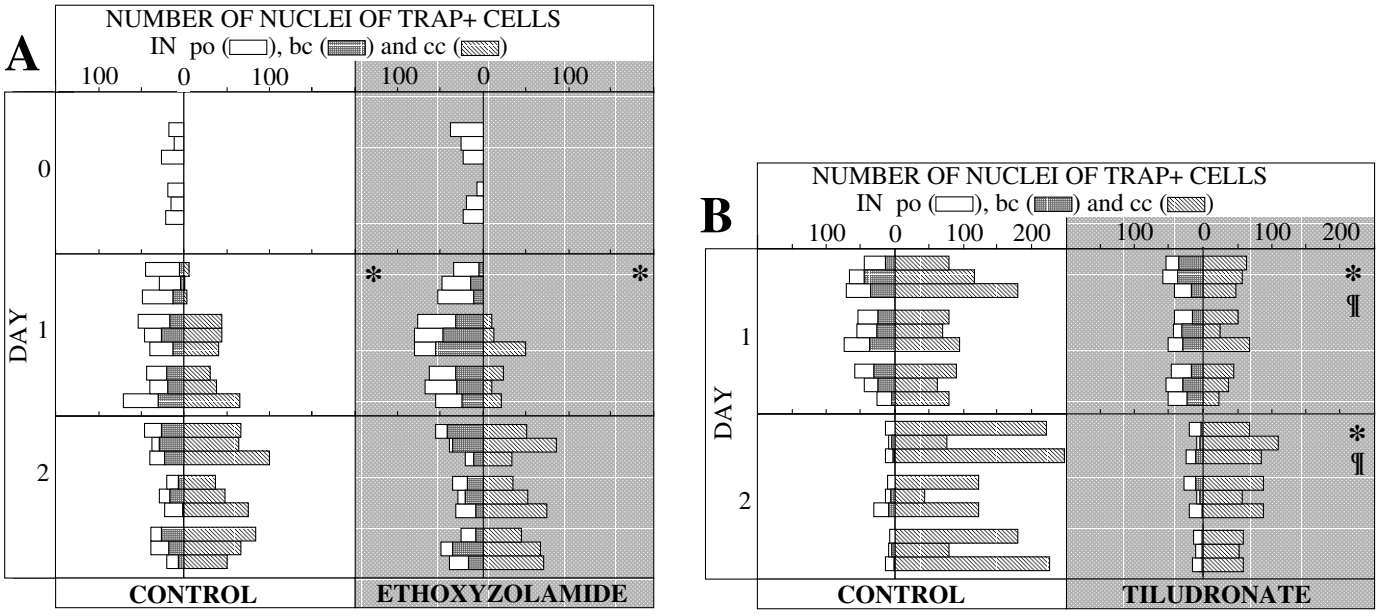

Fig. 6. Effect of inhibitors of demineralization on the migration of TRAP+ cells to the cc. Experimental procedure and presentation of the data are as in Fig. 4. The concentrations of ethoxyzolamide (A) and tiludronate (B) were, respectively, 10 and 30 μM.

controls when more nuclei are counted in the bc, and to decrease when the concentration of nuclei in a given zone is smaller. None of the inhibitors appear to affect the intrinsic ability of cells to fuse.

In situ hybridization

To specify the role of MMPs in the migration of (pre)osteoclasts, in situ hybridizations were performed with RNA probes for interstitial collagenase and gelatinase B, 2 MMPs that appear important for bone resorption (Delaissé et al., 1993; Okamura et al., 1993; Grigoriadis et al., 1994; Hill et al., 1994; Reponen et al., 1994; Tezuka et al., 1994; Wucherpfenning et al., 1994; Okada et al., 1995).

The mRNA for collagenase is detected (Fig. 7B,C,D) only at the level of the cc of the metatarsals, an area where only hypertrophic chondrocytes are present at the beginning of the culture. During culture, there is a decrease of the signal, while the marrow cavity is developing and hypertrophic chondrocytes are disappearing. The parallel between collagenase expression and the fate of hypertrophic chondrocytes suggests

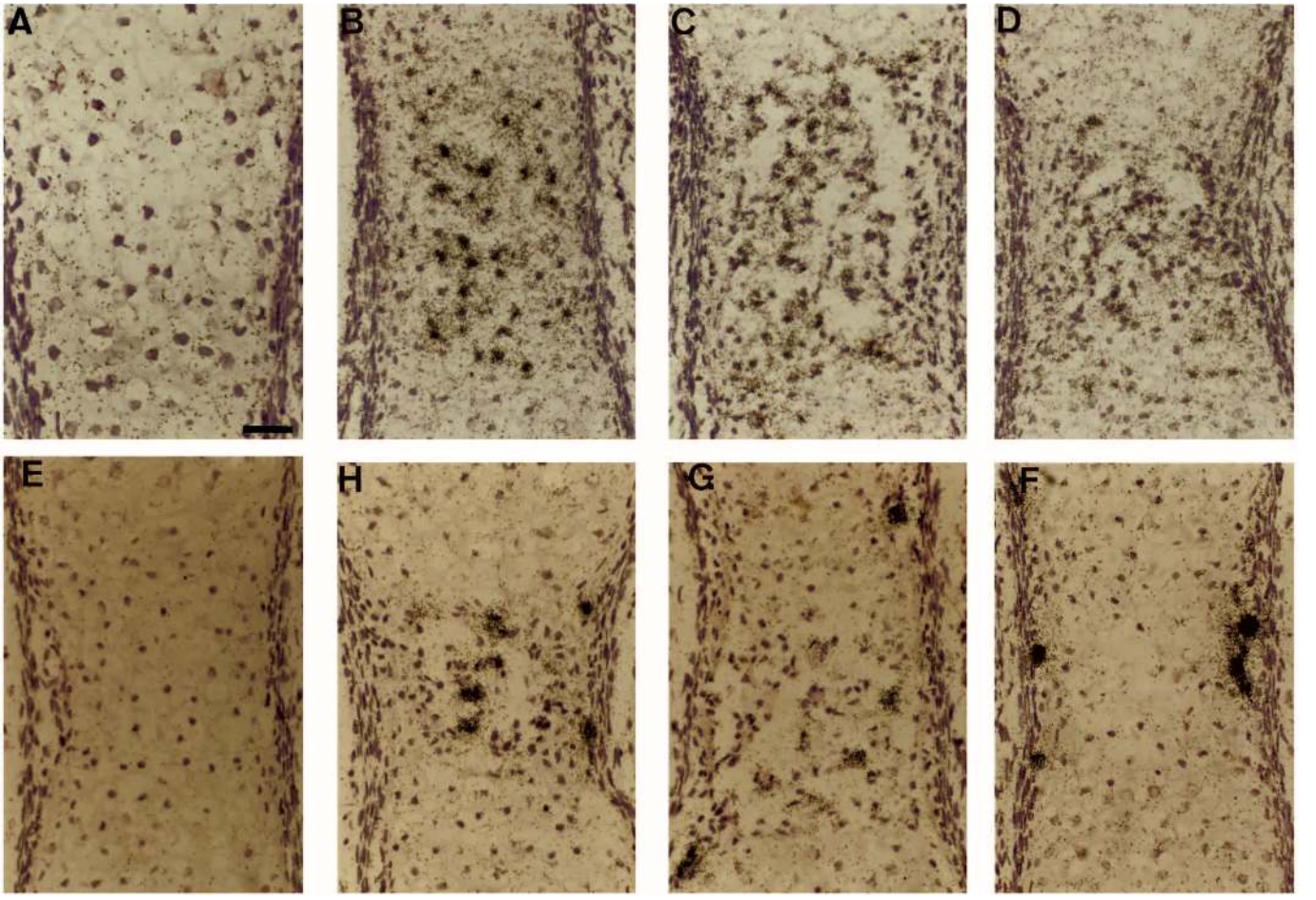

Fig. 7. Time course of the expression of interstitial collagenase and gelatinase B in the diaphysis of cultured metatarsals. Expression of collagenase (B,C,D) is detected only at the level of the cc. It appears as well delimited spots after 1 day culture (B) and more diffusely after 2 (C) and 3 (D) days when the marrow cavity is developing. Gelatinase B is detected only at the periphery of the cc after 1 day culture (F), but also in the cc after 2 (G) and 3 (H) days culture. Hybridizations with sense probes were performed after 1, 2 or 3 days culture and were negative (shown here after 1 day culture for collagenase (A) and gelatinase B (E)). Bar, 40 μm.

that collagenase is synthesized mainly by these cells, as reported previously (e.g. Brown et al., 1989). The analysis of adjacent sections stained with methylene blue did not allow us to determine whether collagenase is also expressed in the osteoclasts resorbing the cc.

Gelatinase B expression is detected (Fig. 7F,G,H) predominantly in the po+bc at the beginning of the cultures, but also in the cc later on. This shift is reminiscent of the migration of (pre)osteoclasts from the po to the cc. The presence of mRNA for gelatinase B in osteoclasts was confirmed in our study by analysing adjacent sections stained with methylene blue (not shown), and is in line with recent reports (Grigoriadis et al., 1994; Reponen et al., 1994; Tezuka et al., 1994; Wucherpfenning et al., 1994; Okada et al., 1995).

DISCUSSION

The growth and the maintenance of the skeleton require a strict control of the access of (pre)osteoclasts to specific sites of the bone. Metatarsals of 17-day-old mouse embryos, are at an interesting developmental stage for investigating this process. The core of their diaphysis consists of still intact cc that must be replaced by a marrow cavity; they have only preosteoclasts, all located in the po and separated from the cc by a seam of 15-25 μm thick osteoid and by a very thin and discontinuous layer of mineral. Upon culture, these metatarsals develop as they do in vivo. The maturing preosteoclasts move across the osteoid and invade the cc, transforming it into a 'marrow' cavity, while the osteoid further develops into a bone collar. The present work shows that the activity of MMPs is obligatory for the migration of (pre)osteoclasts across the mineralizing osteoid.

The main evidence for the requirement of MMPs for (pre)osteoclast migration is that MMP inhibitors inhibit this process. Several data indicate that these inhibitors act truly through an effect on MMP activity. The inhibitions are achieved by 2 drugs (CI-1 and RP59794) differing by their reactive groups and reported to be selective for MMPs (Delaissé et al., 1985; Lelièvre et al., 1990). Their relative inhibitory activity on migration is in accordance with their relative inhibitory effect on MMPs; CI-1 is a weaker inhibitor of MMP than RP59794 and allows a larger displacement of the cells in the bc than does RP59794, while CI-2 is a weaker inhibitor than CI-1 (Delaissé et al., 1985) and allows the invasion of the cc, although at a slower rate than in control bones.

Furthermore, these drugs appear to inhibit migration without any significant side effect. Indeed inhibitions of migration are fully reversible and are thus not due to toxicity. An accumulation of TRAP+ cells in the po+bc accompanies the blockage of migration, thus indicating that the generation of TRAP+ cells is not prevented. The formation of TRAP+ cells in treated metatarsals is, however, somewhat smaller than in control bones. The tendency to such a reduction is also seen upon treatment with drugs that inhibit the resorption of the cc without stopping migration, and could thus simply be due to the modulating effect that resorption might exert on the recruitment of new osteoclasts (Van de Wijngaert et al., 1988). Anyway, the absence of invasion of cc cannot be ascribed to an antiproliferative effect of CI-1 or RP59794, such as could result from the MMP inhibitor impaired processing of TNF-α (e.g. Gearing et al., 1994), a cytokine that stimulates the proliferation of osteoclast precursors in metatarsals (Van der Pluijm et al., 1991). Indeed, the cells responsible for resorption have already reached a post-proliferative stage at the onset of the cultures, as shown by irradiation (Scheven et al., 1986) or by an antimototic agent (present work). Note also that the MMP inhibitors do not prevent the fusion of the TRAP+ cells into multinucleated osteoclasts. CI-1, but not RP59794, even enhances the number of nuclei per TRAP+ cell as compared to controls; this could be related to the higher density of TRAP+ cells in contact with the mineral in CI-1 treated bones, since the contact of TRAP+ cells with mineral is believed to favor their fusion (Baron et al., 1986).

The role of MMPs in the migration of (pre)osteoclasts is clearly distinct from the previously reported roles of MMPs in the bone resorption processes. It is not related to the removal of mineralized matrix in the subosteoclastic resorption compartment (Everts et al., 1992; Delaissé et al., 1993; Okada et al., 1995). Indeed, the inhibition of migration occurs already before the cells reach the mineralization line. Anyway, this mineralization line is thin and discontinuous and should not be an important barrier for the migration of the (pre)osteoclasts, some of which even pass between mineral clusters. Furthermore, inhibitors of key processes of the resorption compartment of mature osteoclasts (Delaissé and Vaes, 1992) are unable to stop the invasion of the cc, i.e. inhibitors of demineralization of two different types and an inhibitor of cysteine proteinases, that all inhibit bone resorption as efficiently and as rapidly as do the MMP inhibitors (checked in the present work). The migrations are, however, somewhat slower in the presence of these inhibitors. This may be interpreted in different ways; cysteine proteinases may be involved in the degradation of extracellular matrix constituents (Murphy and Reynolds, 1993) or in the activation of MMPs (Delaissé and Vaes, 1992); or demineralization might help some cells to get through the layer of mineral of the bc.

The role of MMPs in migration of (pre)osteoclasts is also distinct from the role that osteoblastic MMPs might play in the removal of osteoid, thereby exposing the mineral to the osteoclasts (Chambers et al., 1985). Indeed, it is remarkable that the passage of the maturing osteoclasts through the nascent bc proceeds concomitantly to its further development and to its mineralization. Thus the present role of MMPs involves only focal lysis and specifically concerns migration. It is of interest in this respect, that we show that the migrating (pre)osteoclasts express gelatinase B (MMP-9), a protease that appears involved in a series of processes where cells move through or invade connective tissues, such as migration of keratinocytes in wound healing, invasion of trophoblasts in embryo implantation, or tumor invasion (e.g. Salo et al., 1994; Librach et al., 1991; Watanabe et al., 1993). The role of gelatinase B in (pre)osteoclast migration might be similar to the one it plays in the latter processes (where its mode of action has, however, not yet been elucidated at the molecular level). Thus our observations may give clues to understand the physiological meaning of the expression of gelatinase B in early precursors of the osteoclast lineage (Grigoriadis et al., 1994), as well as in mature osteoclasts (Wucherpfennig et al., 1994; Tezuka et al., 1994; Reponen et al., 1994; Okada et al., 1995). In addition, we show the expression of interstitial collagenase in hypertrophic chondrocytes in accordance with other reports (e.g. Brown et al., 1989). Thus it may also be speculated that this collagenase activity releases collagen fragments from the cc (Scheven et al., 1988; Alini et al., 1992), which might diffuse to the bc and act as chemoattractant for the (pre)osteoclasts (Malone et al., 1982).

It should be stressed that the role of MMPs in (pre)osteoclast migration is obligatory, since the blockage of migration persists after a 3-day culture in the presence of MMP inhibitors. Furthermore, the implication of other proteinases in migration was questioned, and no indication was found for the participation of plasminogen activator generated plasmin activity (Hoekman et al., 1992; Leloup et al., 1994), or for an obligatory role of cysteine proteinases (present work), although there is good evidence for the participation of these enzymes in several other situations where cells migrate (Mignatti and Rifkin, 1993). The strict dependence of migration on MMP activity suggests that regulators of MMP activity may be key agents in the mechanism responsible for targeting the (pre)osteoclasts specifically to the future marrow cavity and restricting their resorptive activity at that level. These regulators may be any agent able to modulate MMP expression or release, or able to activate or inhibit MMP enzymatic activity. Interestingly, chemoattractants were shown to induce release of gelatinase B from neutrophils and monocytes (Opdenakker and VanDamme, 1992), a cell type close to osteoclasts. Noteworthy also, high levels of endogenous tissue inhibitor of MMPs (TIMP) have been detected in the bc of developing bones (e.g. Nomura et al., 1989). Thus TIMP might restrict the lysis of the bc to focal points during the migration of (pre)osteoclasts. It is also of interest that some modulators of the MMP/TIMP balance, such as TNF-α, TGF-β and LIF (Murphy and Reynolds, 1993; Richards et al., 1993) are able to interfere with the migration of (pre)osteoclasts into the cc of metatarsals (Van der Pluijm et al., 1991; Dieudonné et al., 1991; Van Beek et al., 1993).

In conclusion, this work demonstrates that MMPs are indispensable for the migration of (pre)osteoclasts. This role is reminiscent of the MMP dependent migration of other cell types, and is distinct from the previously reported roles of MMPs in the bone resorption processes. We propose that regulation of MMP activity is an important component of the mechanism that controls where and when bone resorption will be initiated.

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