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REGULAR ARTICLE

A.L.J.J. Bronckers · W. Goei · W.L. van Heerde
E.A.W.J. Dumont · C.P.M. Reutelingsperger
S.M. van den Eijnde

Phagocytosis of dying chondrocytes by osteoclasts in the mouse growth plate as demonstrated by annexin-V labelling

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Abstract Endochondral ossification in the epiphyseal growth plate of long bones is associated with programmed cell death (PCD) of a major portion of the chondrocytes. Here we tested the hypothesis that at the ossification front of the epiphyseal growth plate osteoclasts preferentially phagocytose chondrocytes that are undergoing PCD. We injected biotin-labelled annexin-V (anx-V-biotin, an early marker of PCD) intravenously in young adult mice. After 30 min of labelling, long bones were recovered and the tissue distribution examined of anx-V-biotin-labelled cells in the growth plate using ABC-peroxidase histochemistry. Positive staining for anx-V-biotin was detected in hypertrophic chondrocytes still present in closed lacunae at some distance from the ossification front. At the ossification front, chondrocyte lacunae were opened and close contacts were seen between tartrate-resistant acid phosphatase-positive osteoclasts and hypertrophic cartilage cells. Osteoclasts were significantly more frequently in contact with anx-V-biotin-labelled chondrocytes than with unlabelled chondrocytes. Osteoclasts also contained labelled and unlabelled phagocytic fragments within their cytoplasm. We conclude that in the growth plate osteoclasts preferentially phagocytose hypertrophic chondrocytes that are dying, suggesting these dying cells may signal osteoclasts for their removal.

Key words Endochondral ossification · Apoptosis · Hypertrophic chondrocytes · Osteoclasts · Mouse (FVB)

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A.L.J.J. Bronckers (✉) · W. Goei
Department of Oral Cell Biology, ACTA, Vrije Universiteit,
van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands
e-mail: ALJJ.Bronckers.ocb.acta@med.vu.nl
Fax: +31–20–4448683

W.L. van Heerde · E.A.W.J. Dumont · C.P.M. Reutelingsperger
Department of Biochemistry and Cardiology,
Cardiovascular Research Institute Maastricht (CARIM),
University of Maastricht, Postbox 616, 6200 MD Maastricht,
The Netherlands

S.M. van den Eijnde
MGG Department of Clinical Genetics,
and Institute of Plastic Surgery,
Erasmus University Medical School, 3000 DR Rotterdam,
The Netherlands

Present address:
S.M. van den Eijnde, Department of Molecular Cell Biology
and Genetics, CARIM, University of Maastricht, Postbox 616,
6200 MD Maastricht, The Netherlands

Introduction

During endochondral bone formation, chondrocytes embedded in a cartilage-specific matrix will proliferate, differentiate and form a hypertrophic zone of cartilage that calcifies. Subsequently the calcified cartilage is replaced by bone at the ossification front. A considerable portion of the chondrocytes in the zone of hypertrophy undergoes programmed cell death (PCD), which includes various cell morphologies (Clarke 1990; Gibson et al. 1995; Hatori et al. 1995; Bronckers et al. 1996a; Roach 1997; Horton et al. 1998; Roach and Clarke 1999); whereas at particular locations other hypertrophic chondrocytes transdifferentiate into cells with osteoblast-like characteristics (Bianco et al. 1998; Harada and Ishizeki 1998; Roach 1997). Previously we have examined PCD in developing alveolar jaw bones of neonatal rodents by transferase-mediated end labelling (Bronckers et al. 1996a), which labels DNA fragments that are generated relatively late in the PCD pathway (Collins et al. 1997). We noticed that osteocytes with nuclear fragmentation were more frequently phagocytosed than osteocytes with intact nuclei. Based on that observation, we and others proposed that conceivably dying osteocytes transmit signals through the canalicular network that subsequently attract and/or activate osteoclasts to locally start bone resorption and thus initiate bone remodelling (Noble et al. 1997; Burger and Klein Nulend 1999). Recent experimental data agree with such a concept (Verborgt et al. 2000).

An early event in all types of PCD is exposure of phosphatidylserine (which normally resides only at the inner leaflet of plasma membranes) at the outer cell surface, while the plasma membrane is still intact and DNA fragmentation not yet started (Vermes et al. 1995; van den Eijnde et al. 1997a, 1997b, 1999; van Engeland et al. 1998). Annexin-V (anx-V) has a high affinity for phosphatidylserine (van Heerde et al. 1995) and is a powerful tool with which to visualise exposure of this phospholipid at outer cell surfaces (Koopman et al. 1994; Vermes et al. 1995; for review, van Engeland et al. 1998). The advantage of this procedure is in the early detection of PCD compared with other techniques (van Heerde et al. 1995). Recently, anx-V-labelling was used to study PCD in early development in a number of tissues (van den Eijnde et al. 1997a, 1997b, 1999), including suture formation in calvarial bones (Bourez et al. 1997) but not in endochondral ossification.

In the present study, we tested the hypothesis that PCD of hypertrophic chondrocytes in the growth plate leads to osteoclast recruitment and phagocytosis by osteoclasts. Such a concept might link PCD of hypertrophic chondrocytes to removal of the calcified cartilage as part of the process of ossification. We therefore examined: (1) at which time point dying chondrocytes become apparent in the hypertrophic cartilage zone (i.e. before any contacts with osteoclasts), and (2) the number of contacts between osteoclasts and vital or dying chondrocytes at the ossification front. To detect early stages of PCD in the hypertrophic zone, we labelled chondrocytes with anx-V-biotin in situ.

Materials and methods

Animals

Adult FVB mice, 8 weeks of age, 30–36 g body weight, were injected intravenously with 1 mg (three mice) or 0.5 mg (two mice) anx-V-biotin in the tail vein. Anx-V-biotin (Apoptests-Biotin, product B500) was obtained from NeXins Research (Hoeven, The Netherlands).

Anx-V-biotin staining

Thirty minutes after injection, the mice were killed and the long bones of the lower extremities removed and fixed by immersion in 4% formalin in HEPES buffer at 4°C (Bourez et al. 1997). They were decalcified in 4% EDTA in phosphate-buffered saline (PBS) for 4–6 weeks and embedded in paraffin (Bourez et al. 1997). Six-micrometre-thick longitudinal sections were cut, mounted on poly-L-lysine-coated glass slides and incubated overnight at 37°C to improve adherence. Sections were dewaxed in xylene and rehydrated, incubated with 3% H_2O_2 in PBS for 5 min to block endogenous peroxidase, rinsed and blocked with 30% normal goat serum in PBS (Vector Labs, Burlingame, Calif., USA). Next, sections were incubated at 4°C overnight with ABC-peroxidase complex (Elite kit, Vector Labs), rinsed 3 times for 10 min in PBS and developed in freshly prepared DAB substrate solution (Zymed, Calif.) for 15–20 min (dark-brown colour). Sections were subsequently stained for tartrate-resistant acid phosphatase (TRAP).

TRAP staining

To locate osteoclasts and osteoclast precursor cells, TRAP staining was carried out on anx-V-biotin-stained sections. Sections were preincubated with 5 mM potassium tartrate for 15 min, covered with tartrate containing TRAP substrate solution and incubated at 37°C for periods of 15–30 min (Bronckers et al. 1996a). Sections were counterstained with hematoxylin (blue nuclei), mounted in 50% glycerol, 50% PBS and covered with a glass coverslip.

Control tissues

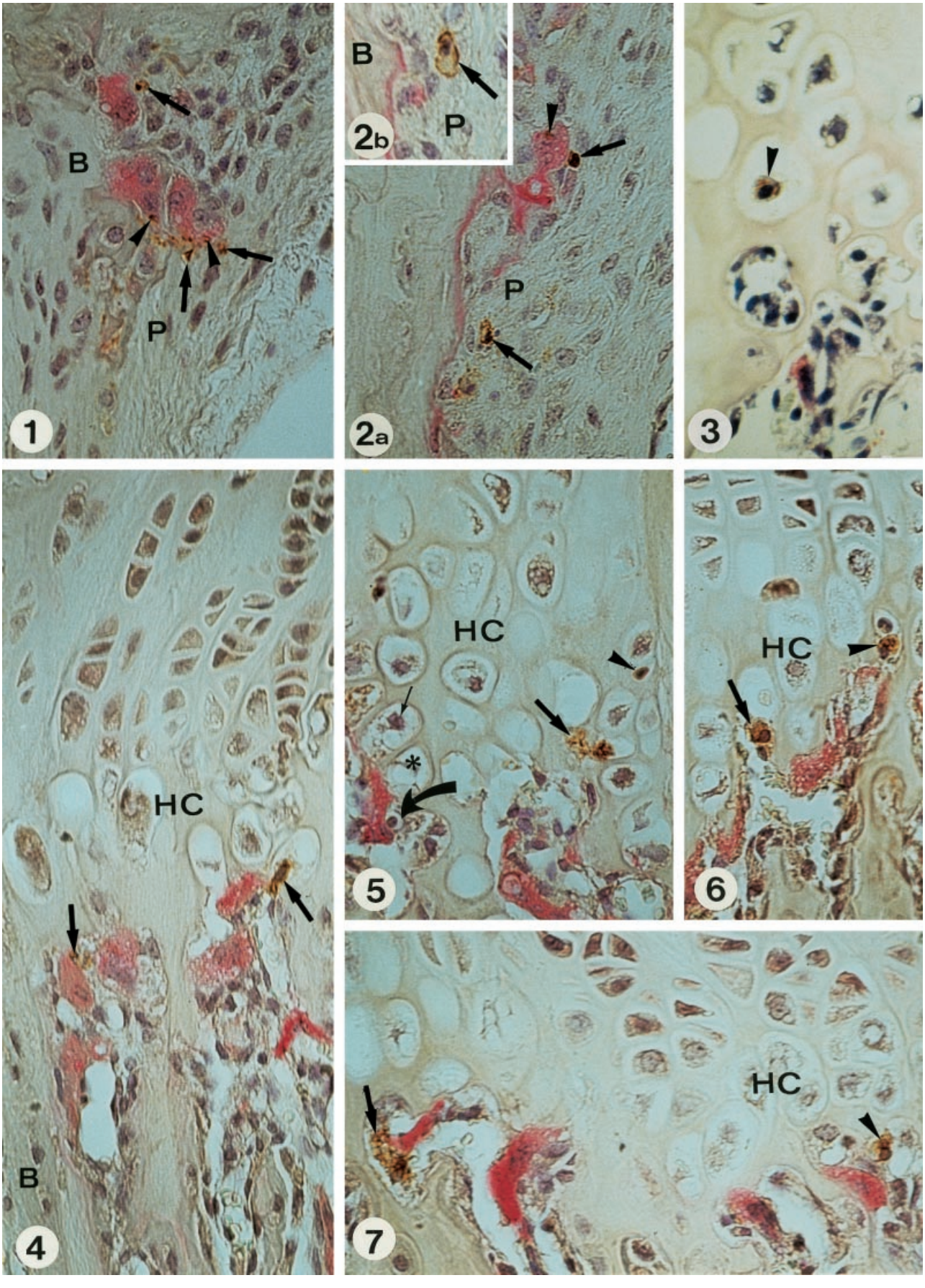
As a negative control, staining of the following tissues was carried out: (1) tissues from mice injected with HEPES buffer instead of anx-V-biotin; (2) tissues from anx-V-biotin-injected animals in which the ABC-peroxidase step was omitted; (3) tissues from mice injected with heat-inactivated anx-V-biotin (10 min at 56°C; Bourez et al. 1997; van den Eijnde et al. 1997a, 1997b); and (4) tissues from mice injected with a mutant biotinylated anx-V, which does not bind to phosphatidylserine (Mira et al. 1997). The following cells were used as a positive control: follicle cells of corpora lutea in ovaria (van Engeland et al. 2000); transitional ameloblasts in developing incisors (Bronckers et al. 1996b); enterocytes at the tip of villi of intestine (Gavrieli et al. 1992); and developing interdigital tissues of developing limb buds of embryonic mice; all established sites of PCD.

Morphometric analysis

An ocular grid (175×260 µm containing 10-µm squares) was positioned over the epiphysial growth plate, with the long axis parallel to the ossification front in a total of eight sections. At ×400 magnification, the following cells were counted with a light microscope: (1) all chondrocytes in the hypertrophic zone; generally and ideally this zone consisted of a layer about three cells wide, though some variation was seen locally; (2) TRAP-positive osteoclasts present at the ossification front; (3) the hypertrophic chondrocytes that were either anx-V-biotin-positive (brown) or unlabelled and in physical contact with osteoclasts; also when an osteoclast had opened a lacuna but was not yet in physical contact with the (often shrunken) chondrocyte-cell body, it was considered to be in contact; in case more TRAP-negative mononuclear cells had entered the lacunae and the chondrocyte could not be distinguished, contacts were scored as negative; (4) hypertrophic anx-V-biotin-positive or unlabelled chondrocytes lying in an opened lacuna in the absence of osteoclasts; (5) anx-V-biotin-positive hypertrophic chondrocytes in closed lacunae. For each mouse, two (one mouse) or three sections (two mice) were counted and the numbers averaged per mouse. These mean countings were then averaged over the three mice, giving the mean values per section. To examine the relationship between anx-V-biotin-labelled cells and osteoclasts, the cumulative countings from all eight sections were used. Not all hypertrophic chondrocytes will undergo cell death, but they might do so within a certain zone near the ossification front. Therefore we calculated the number of unlabelled chondrocytes that potentially could undergo PCD by multiplying the total number of hypertrophic chondrocytes by two-thirds, assuming that throughout the growth plate the hypertrophic zone at this developmental stage was approximately three cells wide and that only the two layers most near the ossification front undergo PCD – as based on location of the anx-V-stained hypertrophic chondrocytes in this area. To test statistical significance, the potential relation between various groups was tested by the χ^2-test (2-tailed).

Results

An anx-V-biotin-positive reaction was observed in areas where many TRAP-positive osteoclasts were present,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such as in the periosteum of the metaphysis either between osteoclasts and bone or between osteoclasts and overlying soft connective tissue of the periosteum (Figs. 1, 2, 3). Also intense staining was observed at the cartilaginous trabecular ends at the transition between the metaphysis and diaphysis and at the ossification front where blood vessels and osteoclasts invade the growth plate (Figs. 4, 5, 6, 7). The cytoplasm of some of the actively resorbing osteoclasts contained areas with intracellular anx-V-biotin-positive cell debris of either a fine

Table 1 Anx-V-biotin-positive and negative hypertrophic chondrocytes in epiphyseal growth plate

State of lacuna in hypertrophic zone	Hypertrophic chondrocytes in the epiphyseal growth plate/section (*n*)[1]	
	Anx-V-biotin-negative	Anx-V-biotin-positive
Opened lacuna, osteoclast contact[2]	8±5	2±1
Opened lacuna, no osteoclast contact	3±1	<1
Closed lacuna	108±39	2±1
Total in closed and opened lacunae	120±45	4±1

[1] Mean ± SD for three mice (2–3 sections/mouse). All chondrocytes throughout the hypertrophic zone up to the ossification front were counted
[2] The mean (± SD) number of 25±12 osteoclasts/section was counted. Cumulative counts in the three mice were 216 osteoclasts

Table 2 Anx-V-biotin-labelled hypertrophic chondrocytes in epiphyseal growth plate that contact osteoclasts at the ossification front

State of lacunae in "PCD zone" of hypertrophic cartilage	Hypertrophic chondrocytes in the epiphyseal growth plate (*n*)[1]	
	Anx-V-biotin-negative	Anx-V-biotin-positive
Opened lacuna, osteoclast contact	76	16*
Opened lacuna, no osteoclast contact	31	3
Closed lacuna in PCD zone[2]	607	14
Total in closed and opened lacunae in PCD zone	714	33

[1] Cumulative values of eight sections from three mice
[2] The actual number of chondrocytes in closed lacunae in the *total* epiphyseal growth plate was 910. This number was multiplied by two-thirds to give the population of 607 hypertrophic chondrocytes in a *"PCD zone"*(i.e. the two layers most near the ossification front)
PCD programmed cell death
*The proportion of anx-V-biotin-positive hypertrophic cells in contact with osteoclasts (16 of a total of 33 positive cells) at the ossification front was significantly higher (χ^2-test: $P<0.00001$, 2-tailed) than the proportion of anx-V-biotin-negative hypertrophic chondrocytes in contact with osteoclasts in the zone potentially suitable for PCD to occur (76 of a total of 714 negative cells)

◀ **Figs. 1, 2** Annexin-V (anx-V)-positive material at sites of bone resorption in the periosteum of the metaphysis. Periosteum (*p*) of mouse long bone (*b*) near the growth plate.

Fig. 1 *Arrowheads* point to anx-V-positive (*brown*) material within the osteoclasts (*red*); the *arrows* indicate anx-V-positive cellular material in extracellular space contact with osteoclasts. ×440

Fig. 2 a The *arrowheads* point to anx-V-positive (*brown*) material within the osteoclasts (*red*); the *arrows* indicate anx-V-positive cellular material in extracellular space contact with osteoclasts. **b** The membrane of a periosteal cell is labelled with anx-V (*arrow*). ×440

Figs. 3–7 Anx-V-positive cells in the hypertrophic zone of the growth plate.

Fig. 3 A hypertrophic chondrocyte (*arrowhead*) with condensed nucleus and positive (*brown*) cytoplasm trapped in a closed lacunae near the ossification front. ×440

Fig. 4 At the right-hand side, an osteoclast (*red*) that is opening a lacuna and is in contact with a positive (*brown*) chondrocyte (*arrow*). On the left-hand side are two osteoclasts, one of which is in contact with positive extracellular granular material, presumably remnants of a chondrocyte (*arrow*). ×440

Fig. 5 A positive chondrocyte within a closed lacuna, with some positive material nearby in the matrix (*straight large arrow*). The *arrowhead* points to a small but positive chondrocyte with condensed nucleus. The osteoclast (*bent large arrow, left-hand side, bottom*) lies near an empty lacuna (*asterisk*) and contains in its cytoplasm a clear vacuole with a condensed nucleus characteristic for an (unstained) ingested cell in the process of degradation (Bronckers et al. 1996a). Note that the adjacent lacuna contains a hypertrophic chondrocyte (*small straight arrow*) without a sign of anx-V staining or nuclear changes. ×440

Fig. 6 A positive (*brown*) chondrocyte (*arrowhead*) in a closed lacuna at the ossification front and another (positive) chondrocyte (*arrow*) in an openend lacuna not in contact with an osteoclasts. ×440

Fig. 7 A contact between a positive chondrocyte (*arrow*) and an osteoclast (*red*) invading the lacuna. The *arrowhead* on the right-hand side indicates a positive chondrocyte in a closed lacuna, with an osteoclast very nearby. Most hypertrophic cells are not stained with anx-V. Some flattened and early chondrocytes and osteoblasts (Figs. 4, 6, 7) stain dark purple-brown for haematoxylin which is different from the bright brown anx-V staining. ×440

granular nature or a coarse and discrete nature (Figs. 1, 2). Occasionally also unlabelled phagocytic material was found (i.e. condensed phagocytosed nuclei in various stages of degradation within a clear vacuole; see Bronckers et al. 1996a).

In the epiphyseal growth plates, a small number of chondrocytes in closed lacunae was anx-V-biotin-positive (Fig. 6). Sometimes the nucleus of these positive hypertrophic cells had not yet condensed. Other positive chondrocytes were found either in opened chondrocyte lacunae (Fig. 6) and in close contact with TRAP-positive osteoclasts (Figs. 4, 7) or in a closed lacuna at some distance from the chondro-osseous junction. The anx-V-biotin-positive hypertrophic chondrocytes were located roughly in the two layers most closely to the ossification front. Tables 1 and 2 represent the numbers per section and cumulative numbers for all sections, respectively. Table 2 indicates that about 3% of all hypertrophic chondrocytes (33 of 1050) was positive for anx-V-biotin. Of these positive chondrocytes, 48% (16 of 33) were in physical contact with osteoclasts, whereas 42% (14 of 33) were still lying in closed lacunae (Table 2). Of the unlabelled hypertrophic cells in the calculated zone where potentially PCD can occur, only 10% (76 of 714) were in contact with osteoclasts. Statistical analysis showed that anx-V-biotin-positive hypertrophic chondrocytes were significantly more frequently in contact with osteoclasts than unlabelled hypertrophic chondrocytes. Positive controls showed staining as expected, and negative controls did not show any staining.

Discussion

The present results support the hypothesis that osteoclasts preferentially phagocytose dying hypertrophic chondrocytes. This was based on the following observations: (1) the preferential contacts between osteoclasts and labelled chondrocytes in opened lacunae at the ossification front; (2) the presence of phagocytic labelled (and unlabelled) cell debris in osteoclasts, suggesting dying cells are internalized, in agreement with published reports that osteoclasts are capable of ingesting osteocytes and hypertrophic chondrocytes (Elmardi et al. 1990; Bronckers et al. 1996a; Harada and Ishizeki 1998). Furthermore, we detected a positive labelling of chondrocytes in closed lacunae, showing that PCD in hypertrophic chondrocytes starts at some distance from the ossification front. This suggests that PCD of the chondrocytes starts independently from the presence of osteoclasts, in agreement with other reports (Gibson et al. 1995; Hatori et al. 1995; Bronckers et al. 1996a; Roach 1997; Roach and Clarke 1999). Collectively, these data are in favour of the hypothesis that osteoclasts preferentially contact and phagocytose dying chondrocytes.

In non-skeletal tissues, during early stages of PCD phosphatidylserine exposure to the extracellular space is one of the signals to phagocytic recognition leading to ingestion and digestion by neighbouring cells or by specialized macrophages (Fadok et al. 1992). Our data indicate that in analogy to macrophages also osteoclasts may recognize phosphatidylserine complexes present at the outer surface of dying chondrocytes. Hence, it is tempting to speculate that exposed phosphatidylserine groups or other factors involved in the PCD pathway act as local signals from the dying late chondrocyte to the osteoclasts to facilitate its ingestion and degradation.

In view of our results, it remains to be elucidated whether osteoclasts at the ossification front are also attracted to the more remote, dying early chondrocytes, which would require a signalling mechanism for phagocytosing cells over a longer distance. Roach (1997) found for chicken femurs that up to 10 cell diameters around the tips of “marrow” tunnel cells with condensed nuclei could be located and that the incidence of PCD (detected by TUNEL staining) was increased near sites of resorption. A potential chemoattractant for osteoclasts is endothelial monocyte-activating polypeptide II (EMAP II), a proinflammatory cytokine activated by caspase-like activity involved in PCD but not in necrosis. EMAP-II attracts macrophages and is highly expressed at apoptotic sites during ongoing ossification (Knies et al. 1998). Other candidate chemotactic factors may be produced by secretory phospholipase A2 (PLA2), which is present in chondrocytes as well as in cartilage matrix (Nevalainen and Haapanen 1993) and may be active at plasma membranes exposing phosphatidylserine (Hack et al. 1997). If hypertrophic chondrocytes produce soluble, PCD-associated factors involved in osteoclast recruitment over longer distances, these factors probably will have access to the ossification front, since, vice versa, systemically applied anx-V-biotin is capable of labelling chondrocytes early in the hypertrophic zone before these cells reach the ossification front.

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