Osteoblasts Engulf Apoptotic Bodies During Alveolar Bone Formation in the Rat Maxilla

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ABSTRACT

During bone formation, as in other tissues and organs, intense cellular proliferation and differentiation are usually observed. It has been described that programmed cell death, i.e., apoptosis, takes place in the control of the cellular population by removing of the excessive and damaged cells. Although it is generally accepted that apoptotic bodies are engulfed by professional phagocytes, the neighboring cells can also take part in the removal of apoptotic bodies. In the present study, regions of initial alveolar bone formation of rat molars were examined with the aim to verify whether osteoblasts are capable of engulfing apoptotic bodies, such as professional phagocytes. Rats aged 11–19 days were sacrificed and the maxillary fragments containing the first molar were removed and immersed in the fixative solution. The specimens fixed in glutaraldehyde-formaldehyde were processed for light microscopy and transmission electron microscopy. For the detection of apoptosis, the specimens were fixed in formaldehyde, embedded in paraffin, and submitted to the TUNEL method. The results revealed round/ovoid structures containing dense bodies on the bone surface in close contact to osteoblasts and in conspicuous osteoblast vacuoles. These round/ovoid structures showed also positivity to the TUNEL method, indicating that bone cells on the bone surface are undergoing apoptosis. Ultrathin sections showed images of apoptotic bodies being engulfed by osteoblasts. Occasionally, the osteoblasts exhibited large vacuoles containing blocks of condensed chromatin and remnants of organelles. Thus, these images suggest that osteoblasts are able to engulf and degrade apoptotic bodies.

Key words: osteoblasts; apoptosis; alveolar bone; bone cells; bone formation

Osteoblasts are responsible for the production and mineralization of preosseus matrix. The active osteoblasts are generally plump, cuboidal, and polarized cells that exhibit well-developed rough endoplasmic reticulum and conspicuous Golgi sacules in their large cytoplasm (Sodek and McKee, 2000; Mackie, 2003). These cells establish a continuous layer on newly forming bone surfaces. It has also been shown that osteoblasts are involved in initiating the mineralization process by releasing matrix vesicles (Anderson, 1995; Arana-Chavez et al., 1995; Weismann et al., 2005). Moreover, the osteoblasts contain high concentration of alkaline phosphatase that appears to play a role in the bone mineralization (Manolagas, 2000).

During bone formation, as in other tissues and organs, intense cellular proliferation and differentiation are usually observed. These cellular events are controlled by growth factors and cytokines produced in the bone micro-

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environment; moreover, several systemic hormones and mechanical signals can act on bone cells (Raisz and Rodan, 1998; Manolagas, 2000; Sodek and McKee, 2000). It has been described that programmed cell death, i.e., apoptosis, takes place in the control of the cellular population by removing of the excessive and damaged cells (Raff, 1998; Zhang and Xu, 2002; Abraham and Shaham, 2004). Apoptosis is a complex process of cell death that involves a series of molecular events, including DNA fragmentation, which can be detected in situ by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) method (Gavrieli et al., 1992; Zhang and Xu, 2002; Lockshin and Zakeri, 2004). In addition, programmed cell death can be characterized by typical morphological changes (Wyllie et al., 1980; Lincz, 1998; Lockshin and Zakeri, 2004). Apoptotic cells frequently breaks into round/ovoid membrane-bounded bodies originating the apoptotic bodies, which are quickly internalized and degraded by phagocytic cells (Wyllie et al., 1980; Raff, 1998; Lockshin and Zakeri, 2004).

In skeletal tissues, apoptosis plays an essential role in the maintenance of bone mass since this process of cell death controls the lifespan of bone cells (Manolagas, 2000). In addition, it has been suggested that apoptosis of osteoblasts/osteocytes could be considered a mechanism to attract osteoclasts to specific bone regions and thereby contributes to the control of bone remodeling (Noble et al., 1997; Tomkinson et al., 1998; Boyce et al., 1999; Verborgt et al., 2000). Although it is generally accepted that apoptotic bodies are engulfed and digested by professional phagocytes, the neighboring cells can also take part in the removal of apoptotic bodies (Wyllie et al., 1980; Fadok et al., 1992; Hetts, 1998; Raff, 1998). Thus, it seems reasonable to consider that osteoblasts could play a role in the removal of the apoptotic bodies during bone formation. In the present study, sections stained with hematoxylin and eosin (H&E), TUNEL method and transmission electron microscopy were used with the aim to detect images of apoptotic cells being engulfed by osteoblasts during initial alveolar bone formation of rat molars.

MATERIALS AND METHODS

Holtzman rats aged 11–19 days, fed ad libitum, were sacrificed with chloral hydrate (600 mg/kg). The maxillary fragments containing the first molar with surrounding periodontal tissues were removed and immediately immersed in the fixative solution. This study was performed in accordance with the principles of laboratory animal care (NIH publication 85-23, 1985) and national laws on animal use were observed.

Light Microscopy

Glycol methacrylate embedding. The specimens containing alveolar bone were fixed in a mixture of 4% glutaraldehyde and 4% formaldehyde (derived from paraformaldehyde) buffered at pH 7.2 with 0.1 M sodium phosphate for 16 hr at room temperature. After decalcification for 7 days in a 7% solution of EDTA (ethylenediaminetetraacetic acid) containing 0.5% of formaldehyde, buffered at pH 7.2 with 0.1 M sodium phosphate (under constant agitation), the specimens were dehydrated in graded concentrations of ethanol and embedded in glycol methacrylate (Historesin-JUNG, Germany). Sections 2 μm thick were stained with Gill's hematoxylin and eosin (Cerri and Sasso-Cerri, 2003).

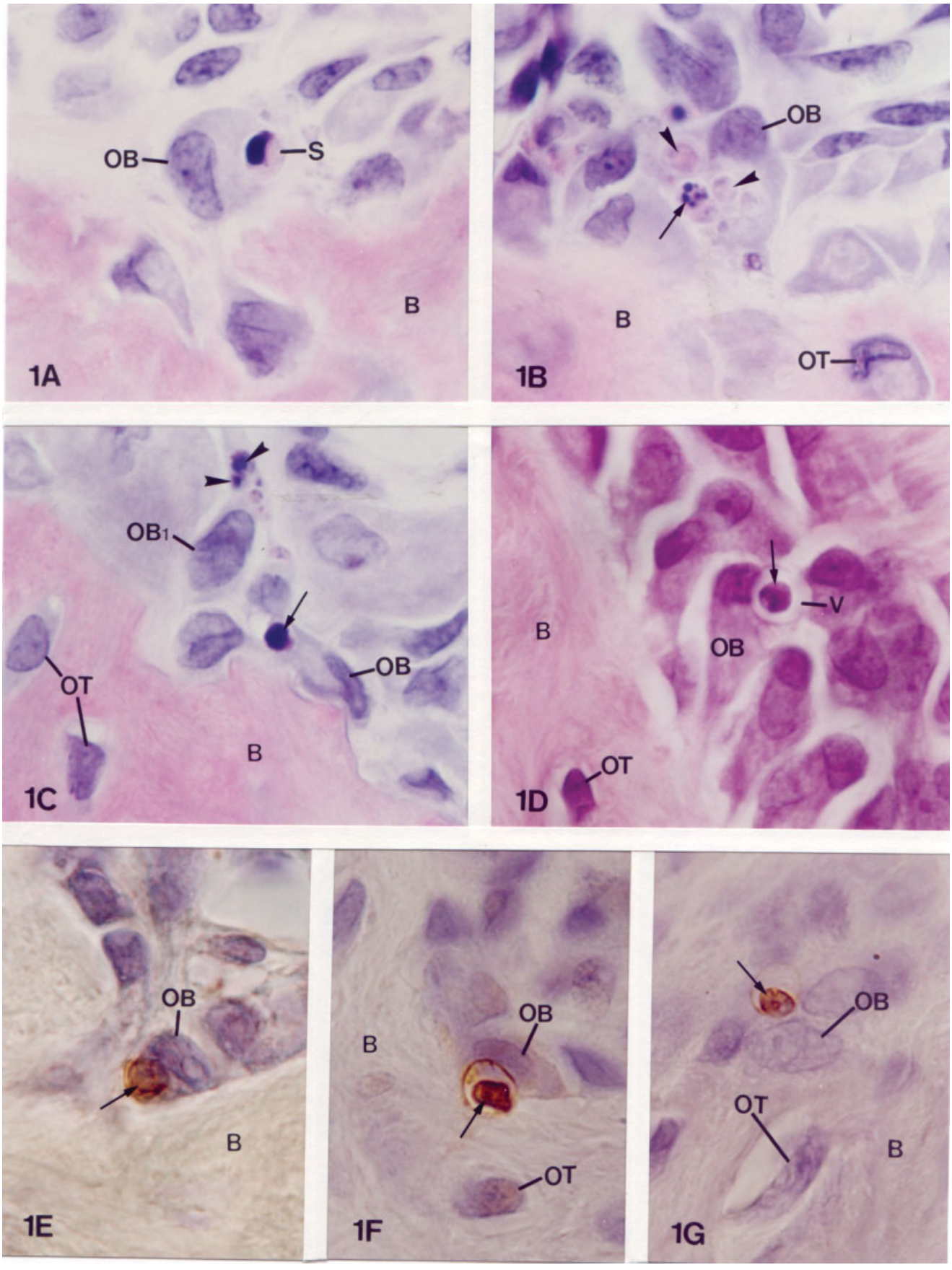
Paraffin embedding. The specimens were fixed in 4% formaldehyde (prepared from paraformaldehyde) buffered at pH 7.2 with 0.1 M sodium phosphate for 48 hr at room temperature. Subsequently, the specimens were decalcified for 7 days in a 7% solution of EDTA, prepared as described above, dehydrated, and embedded in paraffin. Sections 6 μm thick were collected onto silanized slides and stained with H&E; some sections were submitted to the TUNEL method.

TUNEL method. For detection of DNA breaks, the Kit Apop-Tag Plus (Oncor, Gaithersburg, MD) was used in the paraffin sections (Gavrieli et al., 1992). The sections were deparaffinized and treated with proteinase K solution (20 μg/ml in PBS) for 15 min at room temperature. Subsequently, the sections were washed in distilled water and immersed in 3% hydrogen peroxide for 15 min. After several washings in PBS (50 mM sodium phosphate and 200 mM NaCl at pH 7.4), the sections were immersed in equilibration buffer at room temperature for 20 min. The sections were then incubated in TdT enzyme (terminal deoxynucleotidyl transferase) at 37°C for 1 hr in a humidified chamber and the reaction was stopped by immersion in a stop/wash buffer. After several washings, the sections were incubated in antidigoxigenin-peroxidase for 30 min at room temperature. The reaction was revealed with 0.06% 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical, St. Louis, MO) in PBS for 3–6 min and the sections were counterstained with hematoxylin. Involuting mammary gland sections, provided by the manufacturer of the Kit, were used as positive controls for the TUNEL method. For negative controls, the sections were incubated in medium lacking TdT enzyme. The H&E-stained glycol methacrylate and paraffin sections and the sections submitted to the TUNEL method were examined and photographed in a light microscope Olympus BX-50.

Transmission Electron Microscopy

The fragments containing alveolar bone were fixed in a mixture of 4% glutaraldehyde and 4% formaldehyde (freshly prepared from paraformaldehyde) buffered at pH 7.2 with 0.1 M sodium cacodylate at room temperature for 16–20 hr. Then the specimens were decalcified for 5 days in a 7% solution of EDTA containing 0.5% formaldehyde buffered at pH 7.2 in 0.1 M sodium cacodylate. After washing in 0.1 M sodium cacodylate at pH 7.2, the speci-

Fig. 1. Light micrographs of portions of alveolar bone stained by H&E (**A**–**D**) and submitted to the TUNEL method (**E**–**G**). In A, a round structure (S) exhibiting block of condensed chromatin (strongly stained by hematoxylin) is partially surrounded by a large osteoblast (OB) apposed to the bone surface (B). In B, an OB appears to be engulfing round structures (arrowheads), which are next to the bone surface (B). One of them (arrow) contains small and basophilic dense spots. In C, a polarized OB carpeting the bone surface exhibits a round body strongly stained by hematoxylin (arrow) inside a vacuole. Small basophilic structures (arrowheads) are present next to other osteoblast (OB1). In D, a typical OB on the bone surface (B) shows a round dense structure (arrow) inside a large clear vacuole (V). In E–G, the TUNEL-positive structures exhibit yellow-brown color. In E, a round TUNEL-positive structure (arrow) on the bone surface (B) is in close juxtaposition to an OB, which exhibits TUNEL-negative nucleus. In F and G, osteoblasts (OB) apposed to the bone surface (B) exhibit large vacuoles containing TUNEL-positive irregular structures (arrows). OT, osteocyte. Magnification, 1,390×.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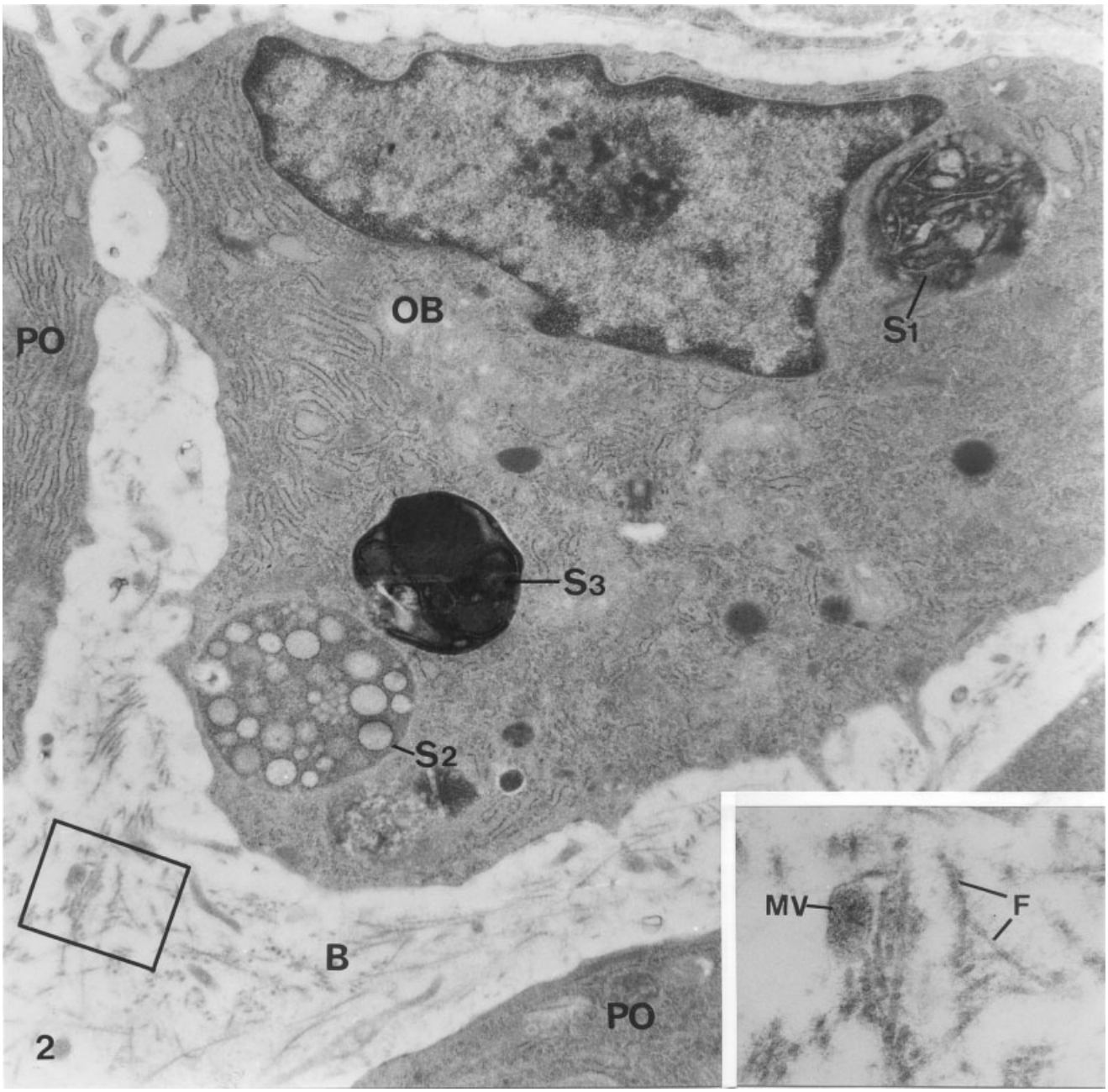

Fig. 2. Electron micrograph of a portion of alveolar bone during early formation. A large osteoblast (OB) apposed on the preosseus matrix (B) exhibits three round structures (S1, S2, and S3) containing material with variable appearance. The structures S1 and S2 appear to contain profiles of organelles; the S3 contains material exhibiting strong electron opacity. The inset shows a higher magnification view of a portion of the bone matrix exhibiting collagen fibrils (F) and matrix vesicle (MV). PO, portions of osteoblasts. Magnification, 14,500×; inset, 35,000×.

mens were postfixed in 0.1 M cacodylate-buffered 1% osmium tetroxide for 1 hr at room temperature and subsequently treated with 2% uranyl acetate for 2 hr. After dehydration in graded concentrations of ethanol, the specimens were treated with propylene oxide and embedded in Araldite (Electron Microscopy Sciences, Fort Washington).

Semithin sections stained with 1% toluidine blue were examined in a light microscope and suitable regions containing alveolar bone were selected for trimming of the blocks. Ultrathin sections were collected on grids, stained with 1% uranyl acetate and lead citrate, and examined in a Philips CM 200 transmission electron microscope.

RESULTS

In the initial stages of formation of the alveolar bone, numerous large and polarized osteoblasts were observed forming a continuous layer on the bone surfaces. After

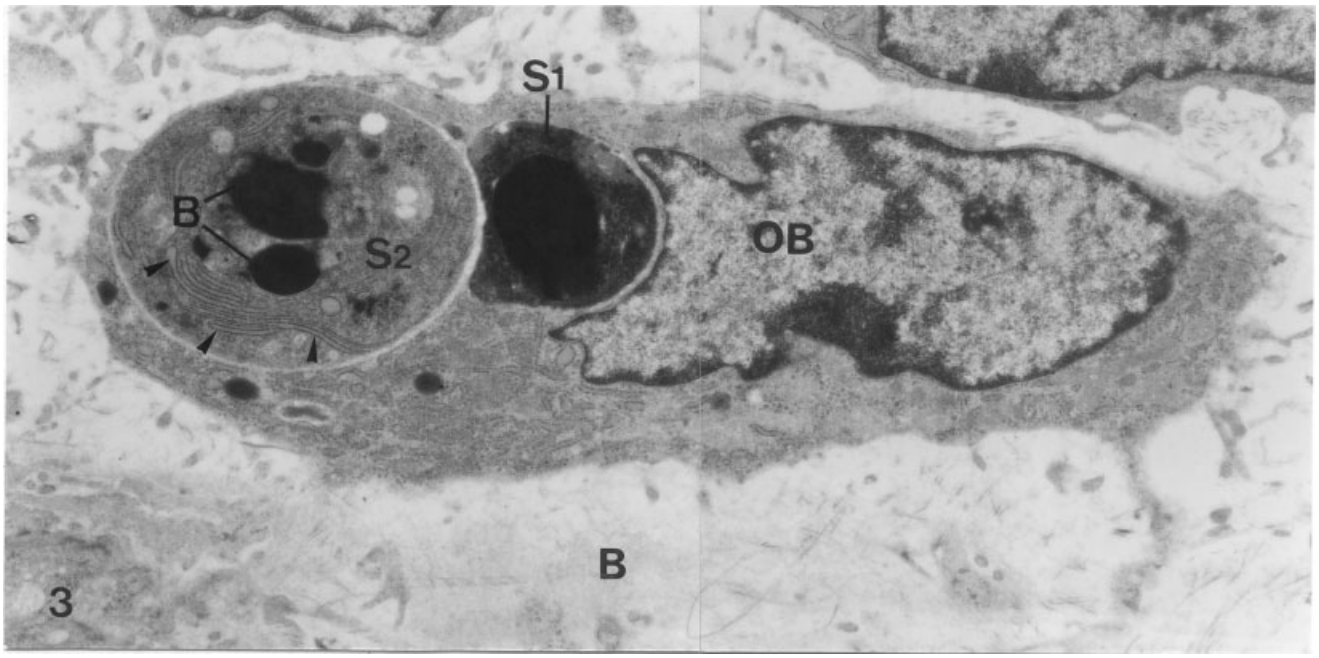

Fig. 3. Electron micrograph of a portion of alveolar bone during early formation. A typical secretory osteoblast (OB) adjacent to a layer of unmineralized bone matrix (B) appears to be engulfing round/ovoid structures (S1 and S2). The smaller structure S1 exhibits compact and electron-opaque material; the structure S2 contains small electron-opaque bodies (B), typical of condensed chromatin, and apparently intact rough endoplasmic reticulum cisternae (arrowheads). Magnification, 9,000×.

extensive examination of the H&E-stained preparations, some round/ovoid structures containing bodies strongly stained by hematoxylin were observed on the bone surfaces. Occasionally, these dense structures were observed in close juxtaposition to typical osteoblasts. Sometimes the large and polarized osteoblasts appeared to surround partially these round/ovoid structures containing basophilic masses of condensed chromatin (Fig. 1A). In the regions of active bone formation, several round/ovoid structures adjacent to bone surface were observed almost entirely surrounded by cytoplasmic processes of osteoblasts (Fig. 1B). Some osteoblasts, apposed on the bone surfaces, showed dense round/ovoid bodies strongly stained by hematoxylin within their cytoplasm. Frequently, these dense bodies were surrounded by clear halo typical of vacuolar structure (Fig. 1C and D).

The TUNEL method revealed brown-yellow structures typical of TUNEL positivity on the bone surfaces. TUNEL-positive round/ovoid structures were found on bone surfaces, often in close association with osteoblasts that exhibited TUNEL-negative nuclei (Fig. 1E). Occasionally, the osteoblasts exhibited conspicuous vacuoles containing brown-yellow round/ovoid structures (Fig. 1F and G). Brown-yellow structures were observed in the sections of involuting mammary gland used as positive controls for the TUNEL method. The sections incubated in the absence of TdT enzyme were negative (data not shown).

In ultrathin sections, regions of the alveolar bone in the initial stages of formation were identified by the presence of preosseus matrix exhibiting matrix vesicles intermingled with collagen fibrils irregularly distributed. A continuous layer of osteoblasts was juxtaposed to newly forming bone matrix (Fig. 2). After extensive examination of several sections, some typical osteoblasts juxtaposed to the bone matrix exhibited round/ovoid structures inside their cytoplasm. Some of these structures exhibited compact electron-opaque bodies while other structures contained remnants of organelles (Fig. 2). Small round/ovoid structures exhibiting blocks of condensed chromatin were also observed on the bone surface in proximity to intact osteoblasts. Sometimes osteoblasts extended cytoplasmic processes that surrounded partially these round/ovoid structures (Fig. 3). Occasionally, osteoblasts exhibited large vacuoles containing granular material and irregular electron-opaque bodies, similar to condensed chromatin (Fig. 4).

DISCUSSION

The present study showed round/ovoid structures containing dense bodies, strongly stained by hematoxylin, on bone surfaces in close contact to osteoblasts and in conspicuous osteoblast vacuoles. Moreover, the TUNEL-positive structures, suggestive of apoptosis, were also observed adjacent to osteoblasts as well as inside osteoblasts. These results reinforce the interpretation that the images of dense round/ovoid bodies observed in H&E preparations are indicative of apoptosis. The TUNEL method reveals DNA fragmentation that is part of the cascade of events observed during cell death (Gavrieli et al., 1992; Zhang and Xu, 2002; Lockshin and Zakeri, 2004). The apoptotic bodies present in osteoblasts were often surrounded by a clear halo, suggesting therefore a vacuolar structure, which was confirmed by the ultrastructural results. These images suggest that apoptotic bodies, probably derived from dying bone cells, can be internalized by osteoblasts. These findings are supported

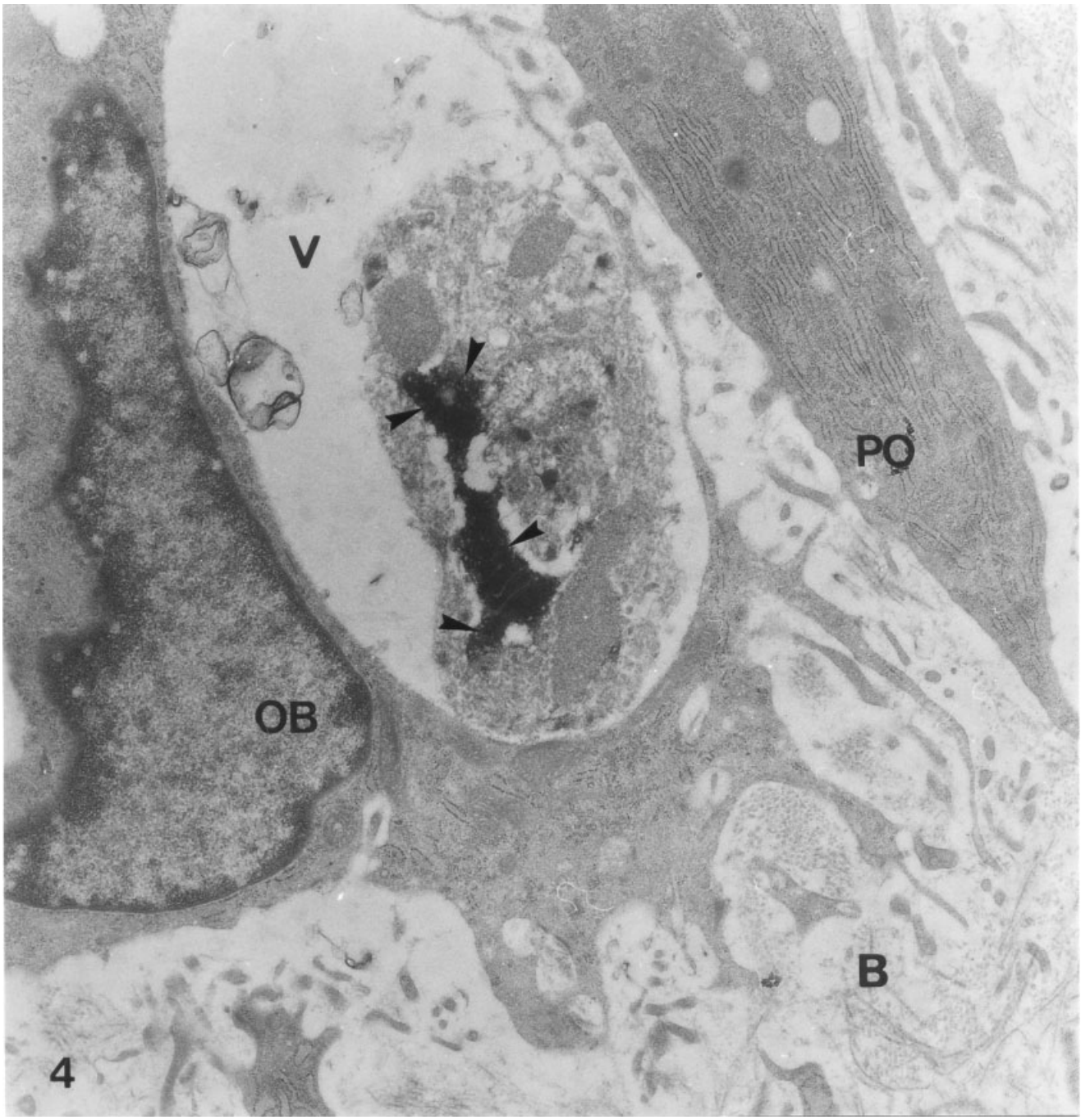

Fig. 4. Electron micrograph of a portion of alveolar bone during early formation. An irregularly shaped osteoblast (OB) adjacent to bone matrix (B) shows a large vacuole (V) containing granular material and remnants of organelles. Note the presence of an irregular material exhibiting electron opacity similar to condensed chromatin (arrowheads). PO, portion of an osteoblast. Magnification, 13,700×.

by ultrastructural observations that revealed round/ovoid structures exhibiting condensed chromatin surrounded partially by cytoplasmic processes of osteoblasts. Furthermore, dense round/ovoid bodies, similar to condensed chromatin, and remnants of cellular structures with variable appearance were also observed inside osteoblasts. These structures are characteristic of apoptotic bodies as described by others (Wyllie et al., 1980; Baratella et al., 1999; Cerri et al., 2000; Boabaid et al., 2001; Palumbo et al., 2003). Although the TUNEL method alone is not considered a specific maker for apoptosis (Grasl-Kraupp et al., 1995), the morphological characteristic is a conclusive hallmark of apoptosis (Danial and Korsmeyer, 2004). Thus, taken together, it seems that apoptotic cells/bodies

are engulfed and digested by osteoblasts on the bone surfaces. However, the images of apoptotic bodies on the bone surfaces being engulfed by osteoblasts were scarce and therefore rarely found. It could at least in part be because the apoptotic bodies are quickly removed from the tissues (Wyllie et al., 1980; Raff, 1998; Abraham and Shaham, 2004).

Although bone cell death has been investigated mostly at sites of active bone remodeling (Noble et al., 1997; Boabaid et al., 2001; Cerri et al., 2003), the present study concerns cell death at early developing bone. At this stage, the uncalcified bone matrix is characterized by the presence of membrane-bound bodies, i.e., matrix vesicles between the collagen fibrils (Anderson, 1995; Arana-Chavez et al., 1995; Weismann et al., 2005). Moreover, this newly forming matrix is surrounded by a single layer of juxtaposed osteoblasts (Arana-Chavez et al., 1995). During bone formation, as in other tissues and organs, cellular proliferation and differentiation are accompanied by programmed cell death, i.e., apoptosis (Boyce et al., 1999; Rice et al., 1999; Palumbo et al., 2003), which eliminates excessive and eventually abnormal cells (Wyllie et al., 1980; Raff, 1998; Zhang and Xu, 2002). Thus, it is reasonable to suggest that the apoptotic bodies observed within osteoblasts may be at least in part derived from osteoblasts and/or preosteoblasts in differentiation. It has been proposed that apoptosis of osteogenic lineage cells may regulate the rate of bone formation (Jilka et al., 1998; Rice et al., 1999; Palumbo et al., 2003). Zimmermann (1992) has suggested that dying osteoblasts could be associated with the bone mineralization process. Therefore, it seems that apoptosis may perhaps play an important role in the development and growth during early bone formation.

Although it has been shown that osteoblasts undergo apoptosis during developing bone (Taniwaki and Katchburian, 1998; Boabaid et al., 2001; Palumbo et al., 2003), the images of apoptotic bodies being engulfed by osteoblasts have not been demonstrated. During bone remodeling, apoptotic osteoblasts/osteocytes generate signals that attract and activate osteoclasts to bone-specific sites (Noble et al., 1997; Tomkinson et al., 1998; Boyce et al., 1999; Verborgt et al., 2000). In addition, the images of dying bone cells internalized by osteoclasts have been observed, indicating that apoptotic cells may be recognized by osteoclasts (Bronckers et al., 1996; Jilka et al., 1998; Taniwaki and Katchburian, 1998; Boabaid et al., 2001; Cerri et al., 2003). It is known that the cells undergoing apoptosis expose the phosphatidylserine on the outer surface of the plasma membrane. Thus, the macrophages and neighboring cells immediately recognize apoptotic cells/bodies, which are quickly engulfed and digested (Fadok et al., 1992; Hetts, 1998; Lincz, 1998; Platt et al., 1998; Abraham and Shaham, 2004). In the present study, our findings suggest that osteoblasts may recognize and engulf dying bone cells on the bone surfaces. Previous studies support the idea that several cells, in addition to macrophages, remove apoptotic bodies by a mechanism similar to that of phagocytosis. Thus, fibroblasts appear internalize apoptotic bodies in the periodontal ligament (Cerri et al., 2000), and epithelial cells of the stellate reticulum are also able to engulf apoptotic bodies during involuting enamel organ (Baratella et al., 1999). Furthermore, acid phosphatase activity was observed in cementoblasts (cells similar to osteoblasts), suggesting that these cells could present a phagocytotic activity (Yajima et al., 1989). Thus, although the osteoblasts synthesize and secrete bone matrix, it is reasonable to suggest that these cells can engulf and degrade apoptotic bodies during the initial stages of alveolar bone formation around rat molars.

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