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Apoptosis is coordinately regulated with osteoblast formation during bone healing

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Abstract. The ultimate fate of the expanded pool of osteoblasts formed following a typical bone injury is unclear. Since necrosis has not been described in the latter stages of bone healing, there must be some other mechanism by which obsolete osteoblasts are cleared from an injury site. We therefore evaluated the possibility that their removal is pre-programmed, by investigating the occurrence of apoptosis in rats that received a standardized bone injury. Histological evidence identical to that found in tissues known to exhibit apoptosis was obtained, thereby showing that programmed cell death was a normal concomitant of fracture healing. The concentration of apoptotic bodies reached its maximum after the differentiative response had peaked, suggesting that the two processes were coordinated. The same result was found in a second group of rats that received the same bone injury plus a simultaneous standardized soft-tissue injury. The combined injuries resulted in more osteoblasts and more apoptotic bodies, but an identical temporal relationship between the peak responses in the two parameters. The results suggested that osteoblasts were removed from the injury site via apoptosis, and that the process was coordinately regulated with differentiation. Since the number of apoptotic bodies per osteoblast varied during healing, it is likely that apoptosis was associated with healing and not merely with osteoblast concentration.

Keywords: Apoptosis, osteoblasts, bone healing, cell regulation, differentiation, osteoprogenitor cells

Introduction

Following fracture, the stem cells of bone proliferate and differentiate into the osteoblasts that form the reparative matrix. Although the role of osteoblasts in fracture repair is established, the ultimate fate of the expanded population of cells is less clear. Some become osteocytes, some become bone-lining cells (Parfitt, 1987; Miller et al., 1989) and some probably die. Since phagocytic cells and focal necrosis are not normally prominent at a bone injury site beyond the first few post-injury days (Fawcett, 1986; Weiss, 1988), the mechanism responsible for eliminating osteoblasts is unclear. Manipulation of the rate at which osteoblasts are cleared from bone could present a new alternative in the treatment of pertinent bone pathology.

Apoptosis is a process by which cells are deleted from a population via triggering of an inherent program that ultimately leads to cell death in the absence of an inflammatory response (Kerr et al., 1972). Cells undergoing apoptosis shrink and separate from their neighbors, possibly as a consequence of the loss of water, and chromatin becomes condensed beneath the nuclear envelope. The cell corpse then breaks up into membrane-bound fragments (apoptotic bodies) containing varying ratios of cytoplasm and chromatin that become engulfed by nearby cells. The dead cell is removed without disturbing local tissue architecture or function, and without initiating inflammation.

Increased rates of apoptosis occur almost exclusively in proliferating or proliferated tissues. As examples,

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apoptosis has been observed during embryological development (Vaux et al., 1992), in association with cancer, (Savill, 1994) after clonal expansion in the immune system (Squier et al., 1995) and following tissue hyperplasia (Bursch et al., 1985; Walker et al., 1989). The purpose of this study was to use histological methods to ascertain whether apoptosis might account for a decrease in osteoblasts during the course of fracture healing.

Materials and methods

Animals

Male Fischer rats (Harlan Sprague—Dawley, Indianapolis, IN, USA) were used. In one group, a bone defect 1.1 mm in diameter and 0.5 mm deep was created in the anteromedial region of the tibia using a hand-held burr. To eliminate possible endosteal influences on the healing response, the defect did not penetrate the medullary cavity. Although the model does not mimic fracture as seen in the clinic, it proved more reproducible than previously described models and was suitable for testing the hypotheses of interest. In a second group, to explore the role of soft-tissue injury (a necessary concomitant of bone injury), a model soft-tissue injury was created by removing 1000 (by weight) from the most distal aspect of the anterior tibialis muscle. The bone and muscle injuries were both administered in the same limb during one operation. Further details regarding the surgical procedures are given elsewhere (Landry et al., 1996).

The rats were sacrificed 1, 2, 3, 5, 7 and 14 days following operation (five rats in each group at each time interval), except that no rats were recovered at 14 days in the group that received both injuries. Non-injured animals (10 animals, 20 tibias) served as non-operative controls. All operations and sacrifices (carbon-dioxide suffocation) were carried out between 10 :00 and 14:00 to minimize potential effects due to circadian rhythms. All animal procedures were approved by the Institutional Animal Care and Use Committee.

Tissue processing

The anteromedial region of the tibia was recovered, demineralized, processed and sectioned completely, at 4 pm in the longitudinal plane. For each animal, sections were selected from the middle of the defect, from halfway between the middle of the defect and its medial edge, and from halfway between the middle and lateral edge. Sections were stained with methyl green and thionin, or with toluidine blue and basic fuchsin. In the rats that did not receive the bone defect, sections were selected as if the defect were present.

The criteria for identification of pertinent cell types, based on morphological and histochemical properties, was described previously (Landry et al., 1996). Briefly, the resting cambium consists of 2—5 layers of alkaline phosphatase-positive cells, termed bone cells, that have a spindle-shaped darkly-staining nucleus, typically 10 x 5 pm, with scanty resolvable cytoplasm at x 400. They are morphologically identical to one another but are distinguishable on the basis of location and response to various stimuli. The bone-lining cell is located next to bone and can be activated following injury to become an osteoblast, after which it either returns to the resting state, or becomes sequestered in bone as an osteocyte, in which state it is no longer AP-positive. The osteoprogenitor cell is a cambial bone cell found above the bone surface. It is also activatable, from which state it either divides or differentiates to become an osteoblast. When activated, the osteoprogenitor cell exhibits a relatively light staining nucleus, contains 1—2 nucleoli, and is found in the upper region of the cambium. An osteoblast is a cell that is actively expressing and secreting collagen, osteonectin, osteocalcin, osteopontin and other matrix proteins. The osteoblast is a cell approximately 15 pm in its longest dimension, sometimes cuboidal in shape, that contains a nucleus with two nucleoli, extensive basophilic cytoplasm and a prominent Golgi apparatus.

Measurements

The qualitative description of apoptosis was determined in the sections stained with toluidine blue and basic fuchsin. Quantitative determinations of apoptotic bodies were made in the same sections by counting the number of apoptotic bodies (ABs) in the defect, and those in the cambium within ±3 mm from the center of the defect. In the tibias that lacked the defect, the 6-mm length was chosen from the corresponding anatomical location. ABs were identified as membrane-bound structures containing various ratios of pyknotic chromatin and condensed cytoplasm (Kerr et al., 1972, 1987; Ferguson & Anderson, 1981). They ranged in size from 1 to 7 pm, and were distinguished from lymphocytes and mitotic figures by their size and by the presence of a cytoplasmic halo, respectively (Montironi et al., 1994). All intra- and intercellular structures that met these criteria were counted as ABs, and the counts were normalized by the length of the corresponding bone surfaces and expressed as the number of ABs per millimeter of bone.

Differentiation was assessed in the sections stained with methyl green and thionin by counting, again separately, the number of osteoblasts in the cambium and within the defect. Both counts were also normalized by the corresponding lengths and expressed as the number of osteoblasts per millimeter of bone. A cell was recognized as an osteoblast if it displayed basophilic cytoplasm, a lightly-stained nucleus with nucleoli, and a pale Golgi.

For each animal, the results for both ABs and osteoblasts were averaged over the three sections and the

mean was used in all statistical evaluations. The data was evaluated using the unpaired f-test and AN OVA at a significance level of *0.05*. All listed means are ± SE.

Results

Occurrence of apoptosis in bone cells following injury was established by comparing histological features observed at the bone injury site (Figs 1, 2) with those reported in other tissues and cells undergoing apoptosis (Hopwood & Levison, 1975; Ferguson & Anderson, 1981; Bursch et al., 1985; Kerr et al., 1987).

1. In cambial and callus cells following bone injury, loss of contact with neighboring tissue occurred that resulted in the formation of an empty space (halo) around the cell corpse (Fig. 1, top left). Rounding-up and halo formation evidenced apoptosis in rat liver during regression of chemically-induced hyperplasia (Bursch et al., 1985).
2. Blebbing of the plasma membrane occurred at the bone injury site (Fig. 1, top right), similar to that seen in mammary epithelial cells 2 days post-weaning (Walker et al., 1989).
3. Membrane-bound structures formed from a cell corpse (Fig. 1, bottom) that were identical to structures described in many reports as apoptotic bodies (Kerr et al., 1972, 1987; Savill, 1994).

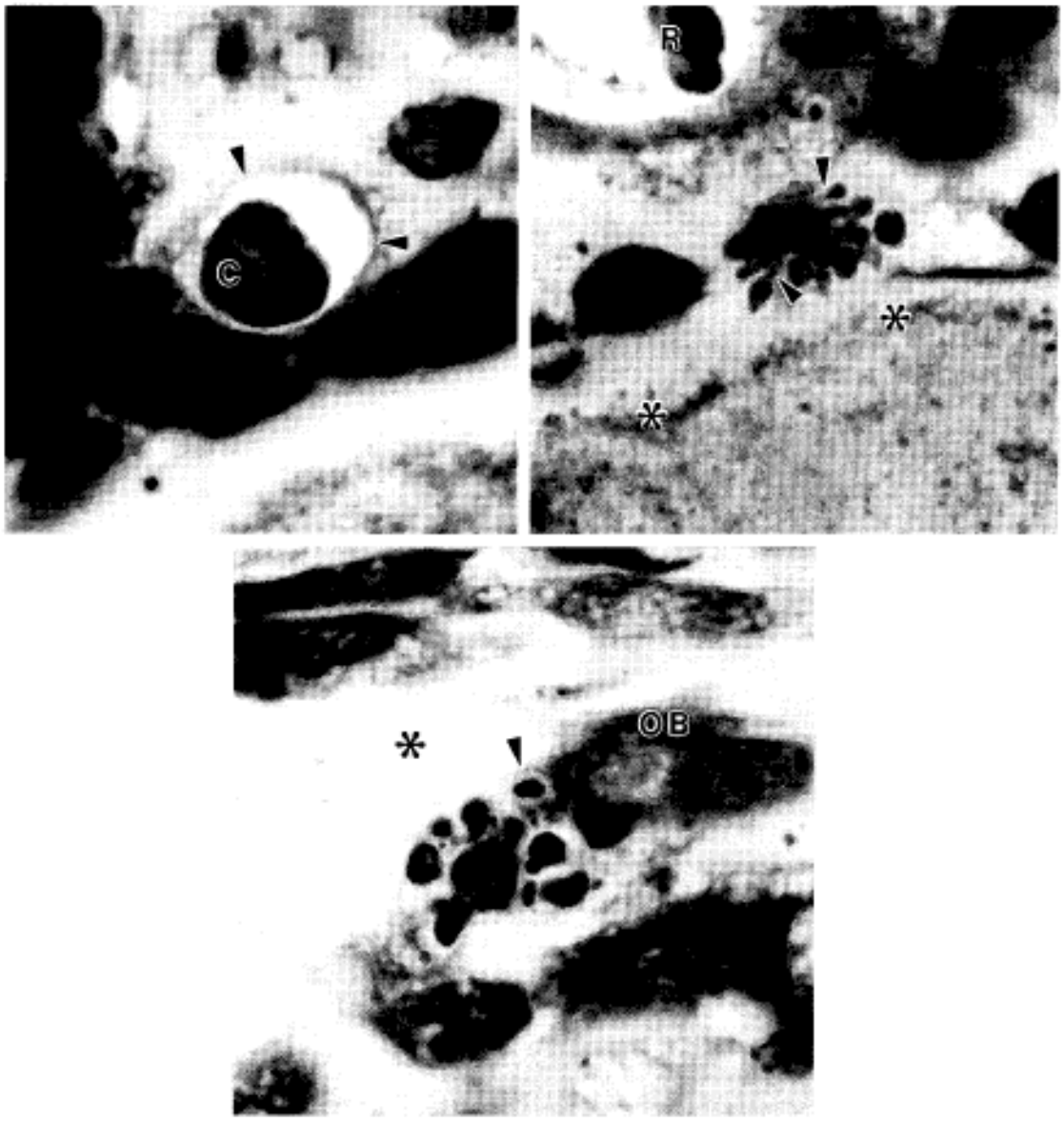

Fig. 1 Apoptosis within the callus following bone injury, lop left: loss of cell contact with the extracellular matrix (arrowheads) due to shrinkage of the apoptotic cell (C). lop right: membrane protuberances (arrowheads) formed on the surface of a cell lining an osteoid edge (asterisks). R, red blood cells. Bottom: Apoptotic bodies formed by sealing of the membrane at the trailing edges of the protuberances. Approximately ten apoptotic bodies were within the cell space bordered by osteoid (asterisk), some with a distinct halo (arrowhead). OB, osteoblast. x 2000.

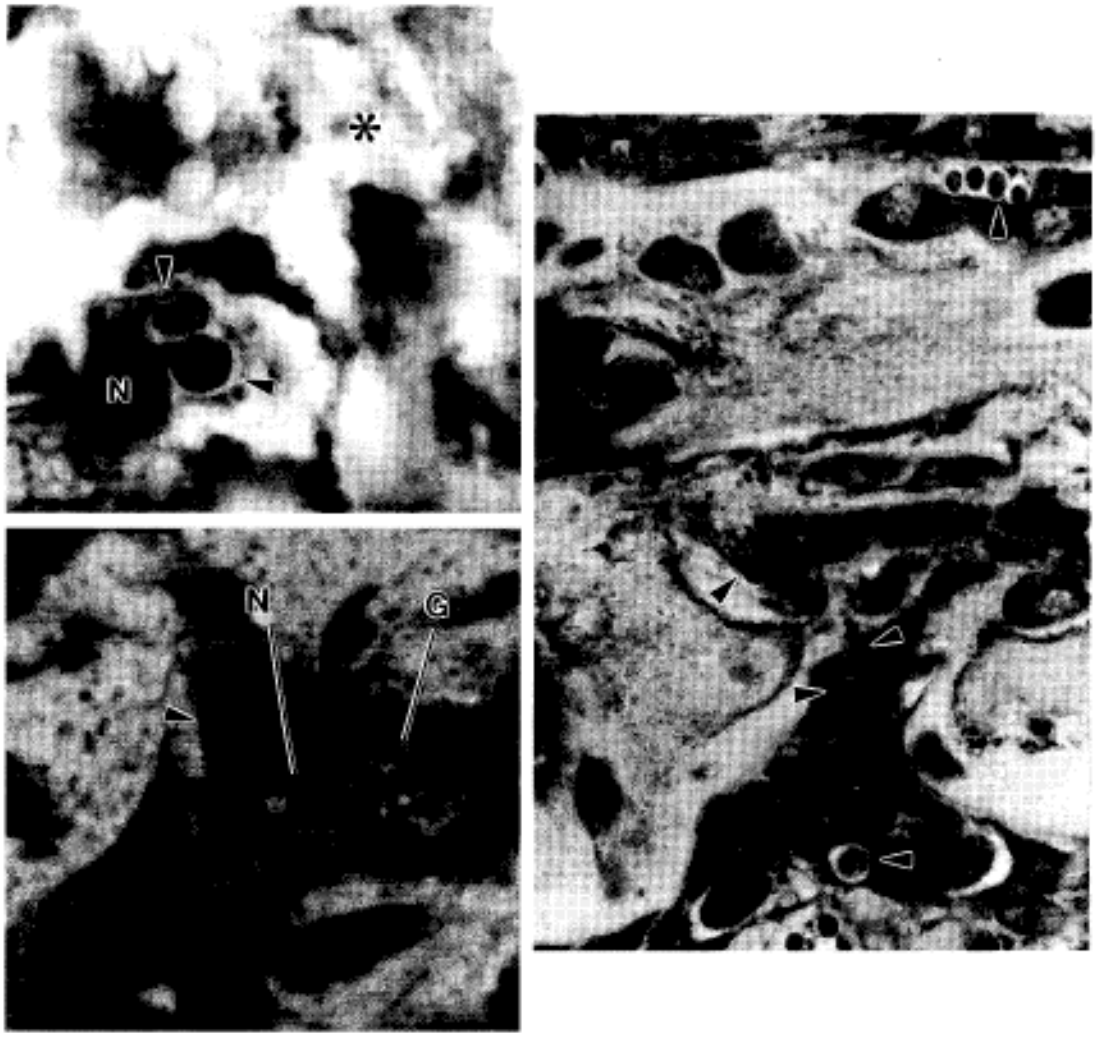

Fig. 2 Apoptosis within the callus following bone injury, lop left: two apoptotic bodies (arrows) within the callus (asterisk) displaying distinct haloes and containing cytoplasm and densely stained chromatin remnants; N, nucleus of a nearby cell. Bottom left: an apoptotic body (arrowhead) engulfed by an osteoblast identified by its Golgi apparatus (G) and nucleus (N) with prominent nucleolus. Right: apoptotic bodies (arrowheads) observed singly and in groups distributed throughout the callus. The fibrous periosteum was located immediately above, and the original cortical surface immediately below, the callus segment shown. All x 2000 except bottom (x 750).

4. The ABs in the callus contained variable amounts of cytoplasm and chromatin; the latter sometimes lined a portion of the membrane (Fig. 2, top left). The distinct crescent shape of condensed chromatin against the plasma membrane in ABs was displayed in epithelial cells in post-castration rat prostate (Kerr et al., 1987).
5. Osteoblasts were seen containing ABs (Fig. 2, top right), indicating that osteoblasts were capable of phagocytosis. Frequently, ABs are engulfed by normal neighboring cells of the same lineage even though the cell type was not ordinarily ascribed a phagocytic role. For example, similar observations were relied upon to confirm occurrence of apoptosis in human endometrium (Hopwood & Levison, 1975).

ABs were observed among the cells of the cambium and throughout the callus along the length of the bone segment that was studied and within the defect, not only in specific locations. Apoptosis occurred wherever osteoid formation was present; apoptotic events were typically seen in cells that lined the osteoid surface (Figs 1, 2). It was determined in a previous study of the time-dependent histological changes following bone injury that the cells lining the osteoid were alkaline-phosphatase positive, and were osteoblasts (Landry et al., 1996).

Regulation of apoptosis was evaluated by examining the time-dependent changes in concentration of ABs in relation to changes in osteoblastic concentration. In the cambium, osteoblastic concentration peaked around day 5, but ABs were not observed above the baseline level prior to day 3 post injury, after which their number increased significantly (Fig. 3A).

In the defect, differentiation lagged behind that in the

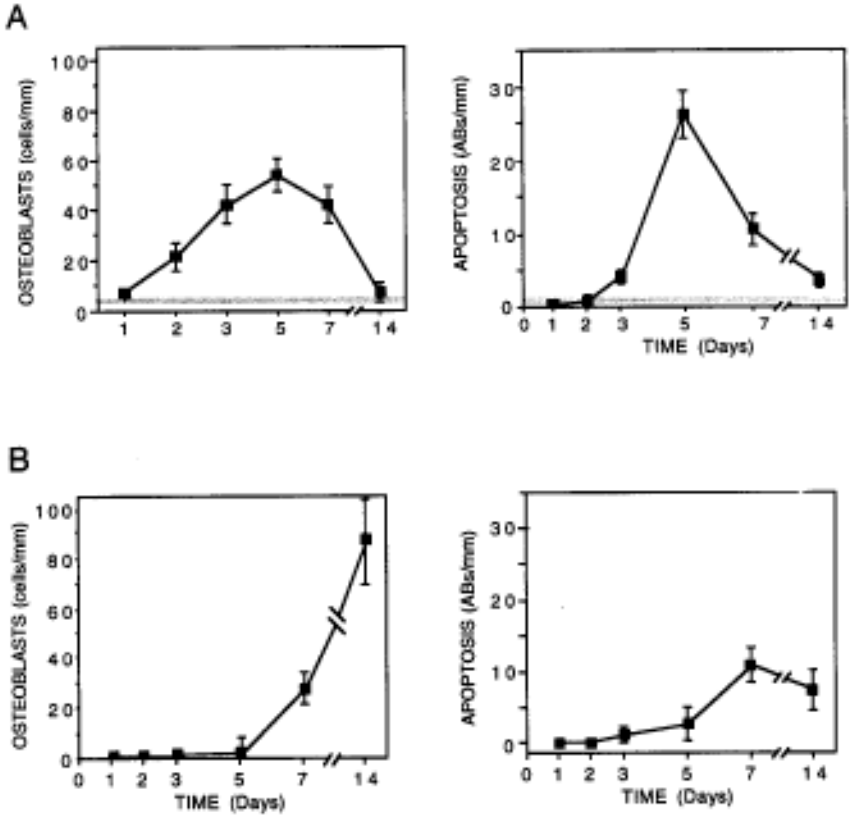

Fig. 3 Osteoblastic response and apoptosis in rats that received the bone injury. A, in the cambium immediately adjacent to the bone defect; B, in the defect. Shaded bars indicate the means ±SE in the resting (uninjured) bone: osteoblasts, 4.7 ± 0.6 cells/mm; apoptosis, 0.8 = 0.2 ABs/mm (n =20).

cambium because 1-2 days were needed for the injured fibrous periosteum to re-seal to the cortical surface (in the model, a necessary event for bone formation in the defect (Landry et al., 1996)). A substantial increase in osteoblasts occurred beginning around day 7 (Fig. 3B), which corresponded with an increase in ABs.

In an attempt to elicit more osteoblasts than would otherwise have occurred in response to the standardized bone defect, a second group of rats received a significant muscle injury along with the bone injury. And as hypothesized, more osteoblasts were observed during days 2-5 ($P<0.05$) (Fig. 4A compared with Fig. 3A). In both the cambium and the defect, increased concentrations of ABs occurred in phase with corresponding increases in osteoblastic concentration. However, the increase in ABs on day 5 following muscle/bone injury was not statistically significant when compared with the bone-injured group (36.9+7.2 compared with 26.3±3.2, in Figs 4A and 3A, respectively).

Discussion

Following a surgically-induced defect in the tibia, cells in the callus were observed to become round, bleb, and fragment into membrane-bound chromatin-containing structures, 1—7 gm in diameter, that become engulfed by other osteoblasts (Figs 1, 2). The observed changes were histologically identical to those that occurred in tissues known to be exhibiting apoptosis (Hopwood & Levison, 1975; Ferguson & Anderson, 1981; Bursch et al., 1985; Walker et al., 1989; Montironi et al., 1994), thereby showing that programmed cell death is a normal concomitant of fracture healing in our model. Apoptosis in osteoclast precursors has been reported, and its occurrence in bone cells in vitro and during calvarial development has been suggested (Furtwiingler et al., 1985; Greenfield et al., 1992; McCabe et al., 1995). However, occurrence of apoptosis at a bone injury repair site has not previously been documented and quantified.

We did not address the question of the relative contributions of the various cell types to the process of formation of apoptotic bodies. However, in the cambium it seems likely that the ABs were derived mostly from osteoblasts because: (1) osteoblasts and ABs were temporally coordinated (Figs. 3, 4); (2) the apoptotic events were almost always observed in cells on the surface of osteoid, and it was demonstrated previously that cells at that location are almost exclusively osteoblasts (Landry et al., 1996); (3) the engulfing cells exhibited the normal phenotypic appearance of osteoblasts and it

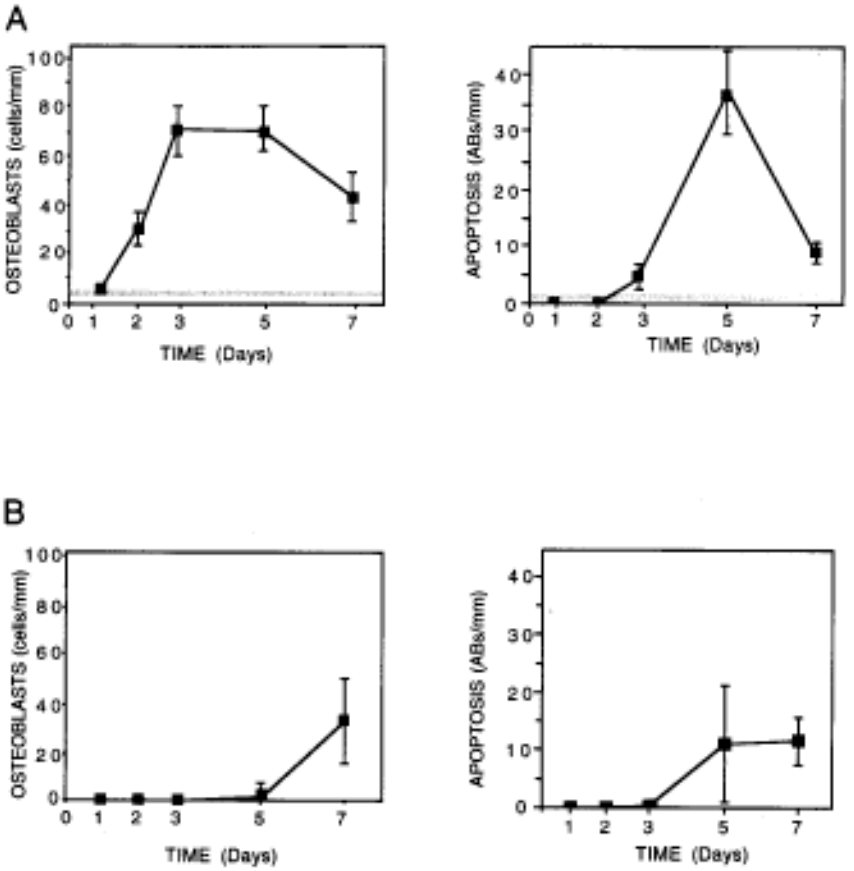

Fig. 4 Osteoblastic response and apoptosis in rats that received simultaneous muscle and bone injuries. A, in the cambium immediately adjacent to the bone defect; B, in the defect. Shaded bars indicate the means ± SE in the resting (uninjured) bone: osteoblasts 47±06 cells/mm; apoptosis, 0.8 =0.2 ABs mm (n = 20).

is typically the case that ABs are engulfed by neighboring cells of the same kind (Hopwood & Levison, 1975). However, it is possible that apoptosis also occurred among other cell types such as osteoprogenitors.

In animals that received the bone injury alone, the peak in ABs in the cambium coincided with the peak in osteoblastic concentration (Fig. 3A). This observation supports the idea that apoptosis served as a mechanism for eliminating excess osteoblasts because it would be expected that the mechanism responsible for removing osteoblasts would not become operative prior to termination of the differentiative response. Too few observations were made in the defect after day 7 to permit a similar analysis regarding apoptosis at that location.

In our view, occurrence of more ABs would be expected, but in the same temporal relationship to osteoblastic formation in the case of an injury that resulted in a higher osteoblastic concentration than that produced by the model bone injury. The evidence reasonably supported this prediction (Fig. 4); addition of soft-tissue injury resulted in significantly more osteoblasts and an associated apparent increase in concentration of ABs at day 5 (26.3 $\pm$ 3.2 compared with 36.9 $\pm$ 7.2 in Figs. 3A and 4A, respectively). This difference was not statistically significant, possibly because

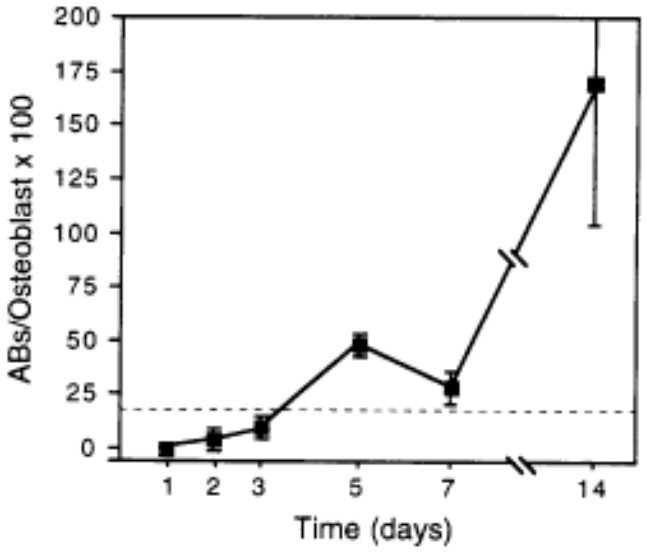

Fig. 5 Apoptosis in the cambium of rats that received the bone injury normalized by the corresponding osteoblast concentration. Data from Figure 3. The dotted line indicates the value in uninjured bone.

too few animals were studied (n = 5 in each group). Thus, in both groups studied, the temporal and quantitative pattern of changes in osteoblasts and ABs plausibly indicated that apoptosis served to remove cells, probably osteoblasts, from the injury site.

The observed association between apoptosis and increased osteoblast concentrations raised the further question of whether the increase in ABs was associated with fracture healing or merely with the increased levels of osteoblasts. We reasoned that if the association were between apoptosis and osteoblasts then the AB levels normalized by the osteoblast levels should be relatively time-independent. On the other hand, a consistent time-dependent pattern would suggest that the fracture healing process itself (irrespective of the osteoblast concentration) affected the likelihood that osteoblasts would become apoptotic. The normalization was performed on the cambial data from the rats that received only the bone injury (because only that group contained non-zero data at a sufficient number of the points). The resulting pattern was clearly time-dependent (Fig. 5), indicating that osteoblast levels were not the sole determining factor of apoptosis.

In summary, apoptosis was a concomitant of the bone-healing response in the model studied. Since healing in rodent bone is commonly accepted as an appropriate model for bone healing generally, apoptosis may be the basic mechanism by which osteoblasts are deleted following callus formation. The evidence suggests that the occurrence of apoptosis was coordinated with the appearance of the osteoblasts elicited by the injury, and that regulation of apoptosis was associated with the healing process.

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