Journal of Orthopaedic Trauma
Vol. 13, No. 5, pp. 356–362

Regulation of Osteoblast Levels During Bone Healing

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Objective: To confirm the occurrence of programmed cell death of osteoblasts during bone healing and to evaluate the role of interleukin-1β (IL-1β) in regulating osteoblast concentration.
Study Design: Electron microscopic study of the response of rats to a controlled bone injury, and a randomized controlled study of the effect of IL-1β administered continuously for three days.
Methods: A standardized defect (1.1 millimeter in diameter, 0.5 millimeter deep) was created unilaterally on the anteromedial surface of the tibia. In some animals, the injury site was recovered five days after operation and processed for ultrastructural evaluation of osteoblasts in the callus. In another group, IL-1β was delivered to the bone defect using micro-osmotic pumps (0.5 nanograms/hour); control rats received vehicle only. The bones were recovered one to fourteen days after injury, and concentrations of proliferating cells, osteoblasts, and apoptotic bodies were determined. The amount of callus that formed in the defect was measured.
Results: Osteoblasts in the callus exhibited ultrastructural changes characteristic of cells undergoing apoptosis, including condensation of chromatin, membrane blebbing, formation of apoptotic bodies, and phagocytosis by nearby osteoblasts. Addition of IL-1β significantly increased the number of osteoblasts at the injury site and significantly decreased the number of apoptotic bodies in relation to the number of osteoblasts. The amount of callus in the bone defect was not affected by IL-1β treatment.
Conclusion: The role of programmed cell death of osteoblasts as a normal concomitant of bone healing was confirmed. Evidence was found suggesting that IL-1β mediated the appearance and disappearance of osteoblasts, possibly by affecting the rates of differentiation and apoptosis, respectively. Understanding these mechanisms conceivably could lead to the ability to control osteoblast levels at an injury site.
Key Words: Interleukin-1β, bone healing, osteoblasts, apoptosis.

A salient feature of the response of bone to trauma is the dynamic change in osteoblast levels that occurs during healing. The inflammatory milieu at a bone injury site contains many substances that might mediate local cellular changes, but the mechanisms that control the appearance and disappearance of osteoblasts have not been established.

Interleukin-1β (IL-1β), a polyfunctional polypeptide cytokine produced by many cell types including monocytes and macrophages, is present at bone injury sites (5,6,27). In model systems, IL-1 stimulated bone cell proliferation (3,25), protein synthesis (3), and osteoclastic resorption (2,10,29).

IL-1β is synthesized as a thirty-one-kilodalton protein that is converted into its active form by cleavage catalyzed by IL-1β converting enzyme (ICE) (28). ICE is one of the major effector agents of programmed cell death (apoptosis) (19,33), a process by which cells are deleted from a population by triggering of an inherent program that ultimately leads to cell death in the absence of an inflammatory response (14). IL-1β can stimulate its own production (4), indicating that interaction of IL-1β with its receptor may be coupled to the expression or activity of ICE.

Increased rates of apoptosis occur almost exclusively in cases involving proliferating or proliferated tissues, including embryological development (30), cancer (24), clonal expansion in the immune system (26), and hyperplasia (31). Thus, apoptosis appears to be a general strategy for removing cells that are no longer needed. Withdrawal of cytokines can be a trigger for induction of apoptosis in many cell types (17,20,22,23), including osteoblasts (12). Interactions with the extracellular matrix may also play a role in regulating the survival of osteoblasts (9).

We previously described an animal model for studying traumatic bone healing (15) and showed that apoptosis occurred and was coordinately regulated with osteoblast formation (16), suggesting that the apoptotic cells were osteoblasts. The first goal of this study was to confirm, at the ultrastructural level, the light-microscope implications

Accepted February 12, 1999.
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This work was supported by grants to M.L.O. from the Orthopaedic Research and Education Foundation and the Mid-America Orthopaedic Association, and to K.K.S. from the Orthopaedic Trauma Association. The authors have received nothing else of value.
This manuscript does not contain information about medical devices.

of the earlier work that osteoblasts underwent programmed cell death. The second goal was to test the hypothesis that the mechanisms controlling the appearance and disappearance of osteoblasts were coordinated in part by IL-1β.

MATERIALS AND METHODS

A bone defect was created unilaterally in the tibia of male Fisher rats (200 to 250 grams) as described previously (15). Briefly, 1.1-millimeter-diameter, 0.5-millimeter-deep bone defects were made that did not communicate with the medullary canal, thus eliminating the influences of the intramedullary repair system. The defect site was irrigated with sterile water to remove bone particles that could initiate bone induction, and micro-osmotic pumps (model 1003D, Alzet, Palo Alto, CA, U.S.A.) loaded with either human recombinant IL-1β (Promega, Madison, WI, U.S.A.) dissolved in phosphate-buffered saline (vehicle), or vehicle only, were implanted subcutaneously in the abdomen. The pump output tubing (polyvinyl chloride, 0.64 millimeters outside diameter, Bolab, Lake Havasu City, AZ, U.S.A.) was sutured to the soft tissue (outside the region of interest) so that the discharge port was lying directly over the bone defect. The skin was closed with 4.0 nylon suture, and the rats were caged individually and fed and watered ad libitum.

The pump delivered 0.5 nanograms of IL-1β/microliter/hour for seventy-two hours or, in the controls, an equivalent volume of vehicle. The cytokine was delivered for seventy-two hours following injury because IL-1 is maximally expressed in fracture callus during that period (6). The concentration was chosen on the basis of in vitro reports suggesting that 0.1 to 10 nanograms/milliliter was associated with increased protein and DNA synthesis in bone cells (3,10,25). The total dose administered (180 nanograms/kilogram) was significantly less than both therapeutic dose levels in human studies (one microgram/kilogram) (4) and the dose shown to cause bone resorption in animals (375 micrograms/kilogram) (2).

The bones were recovered one, two, three, five, seven, ten, and fourteen days postinjury ($n = 5$ for each treatment condition at each time point). These recovery times were chosen on the basis of earlier work that showed these recovery times were necessary and sufficient to permit a reliable determination of the time-dependent pattern of cellular changes that occurred after injury. The animals were pulse-labeled with tritiated thymidine (intraperitoneal injection, one microcurie/gram of body weight, ICN Biomedicals, Costa Mesa, CA, U.S.A.) one hour before sacrifice (carbon dioxide suffocation).

The specimens were fixed in 10 percent neutral buffered formalin and decalcified using 0.12 percent disodium ethylenediaminetetraacetate dihydrate (EDTA) and 5.5 percent hydrochloric acid (Cal-EX, Fisher Scientific, Pittsburgh, PA, U.S.A.). A ten-millimeter segment of bone centered around the bone defect was embedded in epoxy (JB-4, Polysciences, Warrington, PA, U.S.A.) and sectioned in the longitudinal plane, then three trios of sections were selected from the lateral, central, and medial aspects of the defect. One section from each trio was dipped in photographic emulsion (Ilford K5, Polysciences), processed for autoradiography, and counterstained with van Gieson's stain; a second set of sections from each trio was stained with methyl green/thionin, and the third set was stained with toluidine blue/basic fuchsin.

An additional group of ten rats that received no treatment were killed five days after receiving the bone injury and used for ultrastructure studies of the periosteal callus. The specimens were fixed with modified Karnovsky's solution, pH 7.4, two millimolar magnesium chloride, and demineralized in 14 percent EDTA in cacodylate buffer, embedded with Spurr's resin, sectioned with a diamond knife, stained with uranyl acetate and lead citrate, and examined in an electron microscope (Hitachi H-300, San Francisco, CA, U.S.A.).

Proliferation was assessed in the autoradiographs by counting the number of cells with more than five grains per nucleus. Osteoblasts were counted using the methyl green/thionin sections. Due to a technical error that occurred during histological processing, no data regarding cell counts were obtained from the control group recovered ten days after operation.

Apoptotic bodies (ABs) were counted in the stained toluidine blue/basic fuchsin sections. ABs were identified as membrane-bound structures surrounded by a halo that were one to seven micrometers in diameter and contained various ratios of pyknotic chromatin and condensed cytoplasm (7,13,14).

All cell counts were performed within ± three millimeters of the defect center (the central six millimeters of the ten-millimeter processed bone segment) and expressed as the total number of cells or ABs divided by the corresponding length along the cortical bone surface. For each animal, the results were averaged over the three representative sections. Interobserver variance was not assessed, but all counts were made in a blinded fashion and were reproducible to better than 10 percent. Histomorphometric measurements of callus in the defect were made using a computer-based measuring system (R&M Biometrics, Nashville, TN, U.S.A.) and the toluidine blue/basic fuchsin sections.

Mann–Whitney U tests were used to analyze the data (presented as mean ± standard error); $p < 0.05$ was regarded as significant. All animal procedures were approved by the Animal Care and Use Committee of the LSU Medical Center.

RESULTS

Osteoblasts in the callus exhibited the range of ultrastructural features definitive of cells undergoing apoptosis (Fig. 1). These include formation of apoptotic bodies (Fig. 1A, E, and F), degeneration of cytoplasmic organelles and enlargement of endoplasmic reticulum cisternae (Fig. 1B and C), margination and condensation of chromatin (Fig. 1C), cytoplasmic and nuclear blebbing (Fig. 1D), and phagocytosis by nearby normal osteoblasts (Fig. 1A and E). Osteoblasts were capable of completely engulfing apoptotic cells (Fig. 1A and F). Pale cells containing apoptotic bodies were seen near the vasculature (Fig. 1B). Their lack of

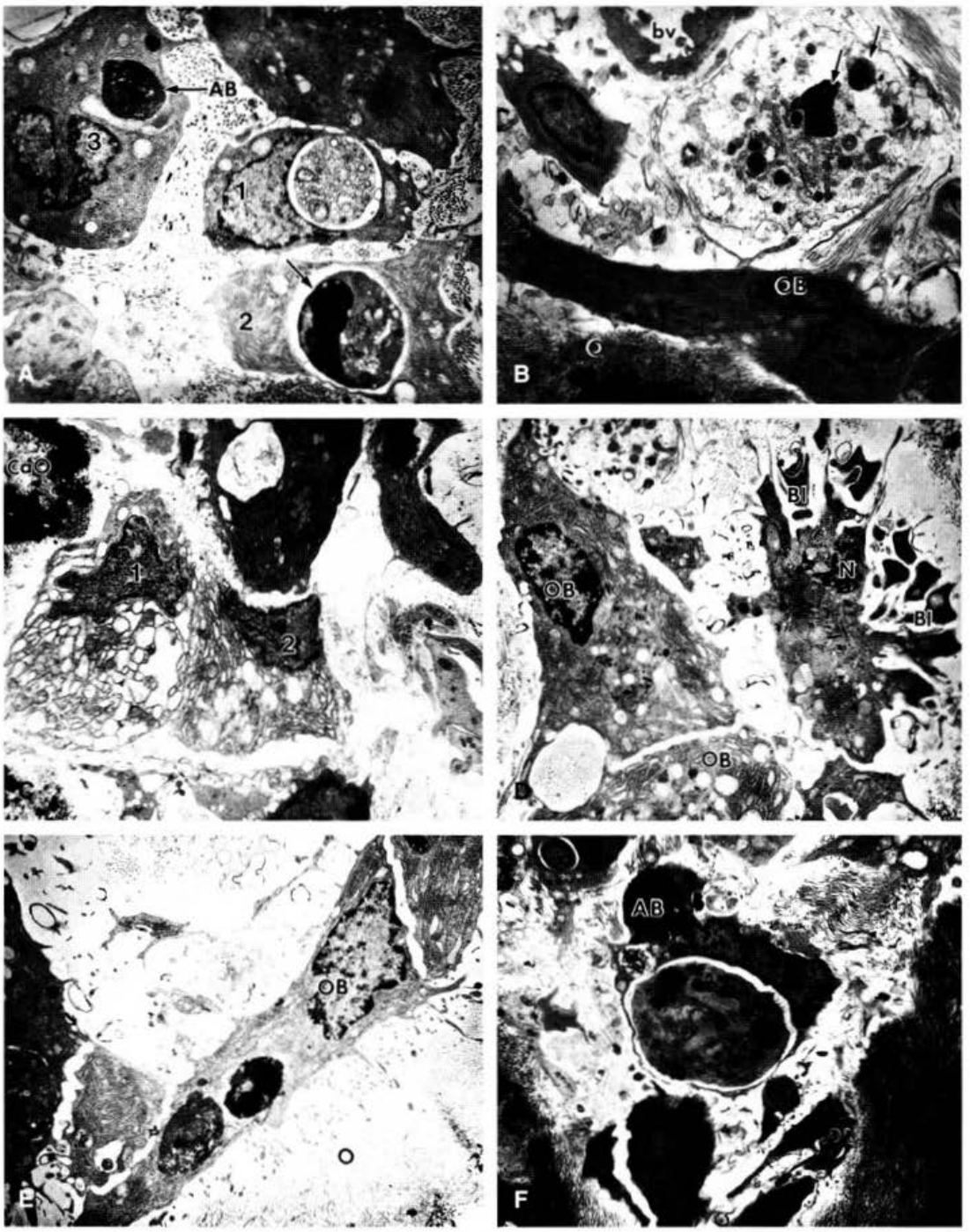

FIG. 1. Osteoblasts five days after bone injury. The range of ultrastructural features characteristic of apoptosis can be seen. **A:** Osteoblasts concentrically arranged around osteoid (out of view on the periphery). Two osteoblasts (1, 2) contain large phagosomes surrounded by a clear space. Osteoblast 2 contains the remnants of a nucleus (arrow), rough endoplasmic reticulum, and membranous material. Osteoblast 3 contains a large, dense apoptotic body (AB). The elongate granules of the typical osteoblast are evident in the top right osteoblast. 5,000×. **B:** An elongate osteoblast (OB) lying along osteoid (O). A pale cell (either an apoptotic cell or a macrophage) with dense apoptotic bodies (arrows) of various size and several Golgi stacks, otherwise devoid of granules, is above the OB near a vessel (bv). 5,000×. **C:** Two apoptotic cells (1, 2) lying along calcified osteoid (CaO). These cells have extensive, dilated, empty rough endoplasmic reticulum and swollen mitochondria. Nuclei show irregular chromatin condensation. 4,000×. **D:** A cell near the osteoblast (OB) line of cells displays several cytoplasmic blebs (Bl) as well as nuclear (N) blebbing. The cell is separated from osteoid by bone-lining osteoblasts (OB). 5,000×. **E:** An osteoblast (OB) extends cell processes through the osteoid (O). Two large, dense apoptotic bodies are apparent in the cytoplasm. 5,000×. **F:** An osteoblast–phagocytic cell surrounds an apoptotic cell with thin cytoplasmic extensions. This osteoblast–phagocytic cell contains several dense apoptotic bodies (AB) in addition to the larger phagosome with nuclear remnants and membranous whorls. 4,000×. Cells 2 and 3 in Panel A and the cell in Panel F were determined to be osteoblasts because of their cytoplasmic resemblance to nearby osteoblasts and their positions near or on osteoid.

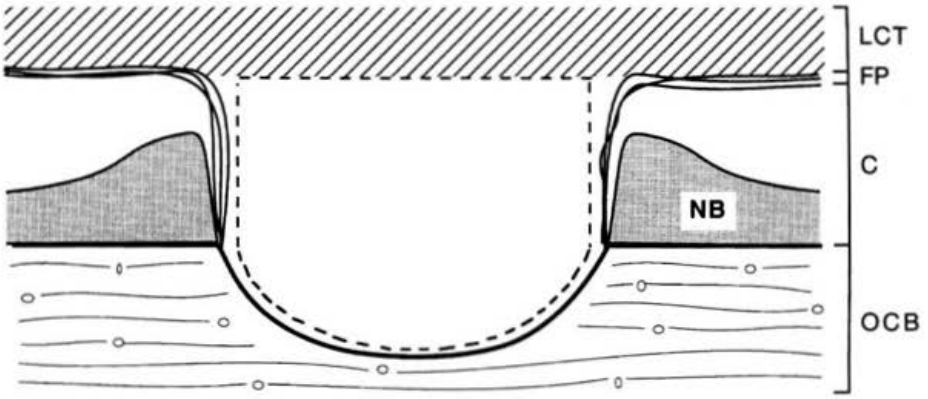

FIG. 2. Tissue compartments within the region of interest five days after making the bone defect. OCB, original cortical bone; C, cambium; FP, fibrous periosteum; LCT, loose connective tissue. The shaded region in the cambium (NB) is the new bone deposited on the original cortical surface. The injury site (dashed line) is the cylindrical region centered on the bone defect and bounded inferiorly by the OCB and superiorly by the LCT.

ribosomes and rough endoplasmic reticulum suggested that they were apoptotic cells. However, sections containing the nucleus revealed no evidence of apoptotic features, possibly indicating that the pale cells were macrophages. The presence of apoptotic bodies in osteoclasts was not observed.

An adequate characterization of the time-dependent changes that occurred after injury required separate determinations of changes in the cambium adjacent to the defect and in the injury site (15). The tissue compartments are depicted in Figure 2 as they appeared five days after injury.

Osteoblast concentrations in the cambium increased in both the IL-1β and control groups after injury, but the increases were consistently greater in the IL-1β-treated group; the differences were statistically significant at Day 5 and Day 14 (Fig. 3A). At the injury site, the osteoblast level was significantly lower in the IL-1β group on Day 7 postinjury but was significantly higher on Day 14 (Fig. 3B).

In both groups, peak proliferation in the cambium occurred two to three days after injury at more than 500 times above baseline. The cambial proliferating cells were previously shown to be almost entirely osteoprogenitor cells, as determined by the presence of alkaline phosphatase (15). The amount of proliferation was apparently not altered by treatment with IL-1β (Fig. 4A). At the injury site, the proliferating populations consisted of both positive and negative cells (osteoprogenitors and fibroblasts, respectively) (15), and again IL-1β had no demonstrable effect on the pattern of proliferation (Fig. 4B).

In the cambium, ABs were not seen above baseline levels until after Day 3 postinjury (Fig. 5A), at which time the osteoblast levels were near their maximum values (Fig. 3A). In the defect, osteoblast levels lagged behind those in the cambium (Fig. 3) because one to two days were needed for the injured fibrous periosteum to reseal to the cortical surface, a necessary event for the appearance of osteoblasts in the defect (15). Consequently, the appearance of ABs in the defect was delayed relative to the cambium (Fig. 5B). IL-1β treatment had no effect on the absolute numbers of ABs (with the exception of Day 7, Fig. 5B). However, the number of ABs depends on the number of osteoblasts that are available to undergo apoptosis. Consequently, the number of ABs per osteoblast is the pertinent parameter for examination of the effect of IL-1β on apoptosis. When the AB levels were evaluated in relation to osteoblast levels, a significant effect of IL-1β administration on apoptosis was seen at Day 14 in the cambium and at the injury site (Fig. 6).

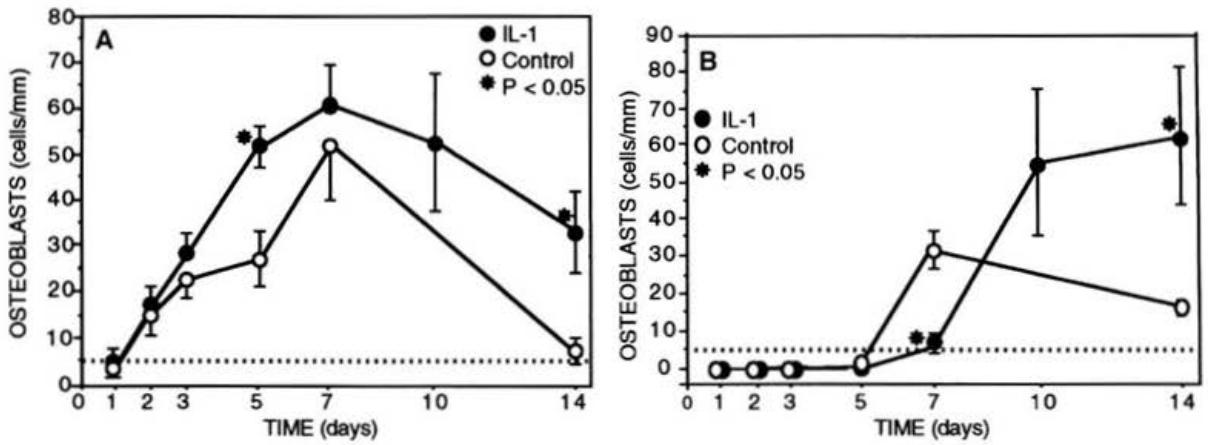

FIG. 3. Effect of interleukin-1β on osteoblast concentration in the cambium (**A**) and in the defect (**B**) after injury (on day zero). The dotted line (4.7 ± 0.6 cells/millimeter) corresponds to the value in uninjured bone (11).

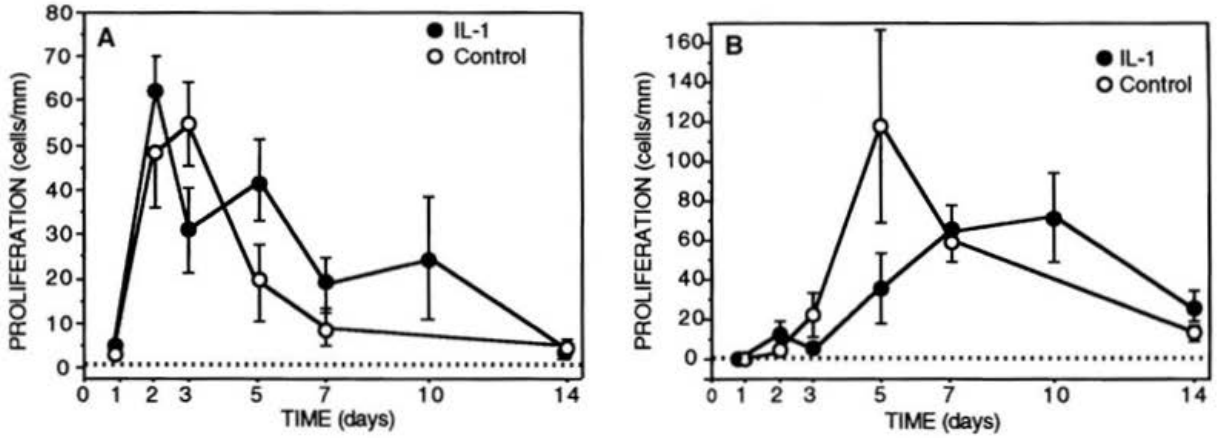

FIG. 4. Effect of interleukin-1β on proliferation of osteoprogenitor cells in the cambium (**A**) and at the injury site (**B**) after injury (on Day 0). The dotted line (0.1 ± 0.1 cells/millimeter) corresponds to the value in uninjured bone (11).

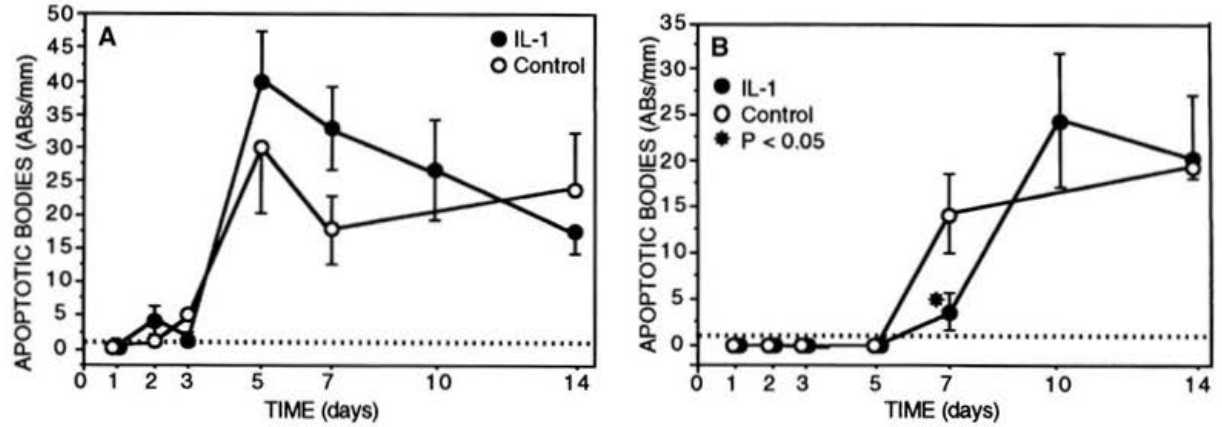

FIG. 5. Effect of interleukin-1β on apoptosis in the cambium (**A**) and at the injury site (**B**) after injury (on day zero). The dotted line (0.8 ± 0.2 apoptotic bodies/millimeter) corresponds to the value in uninjured bone (12).

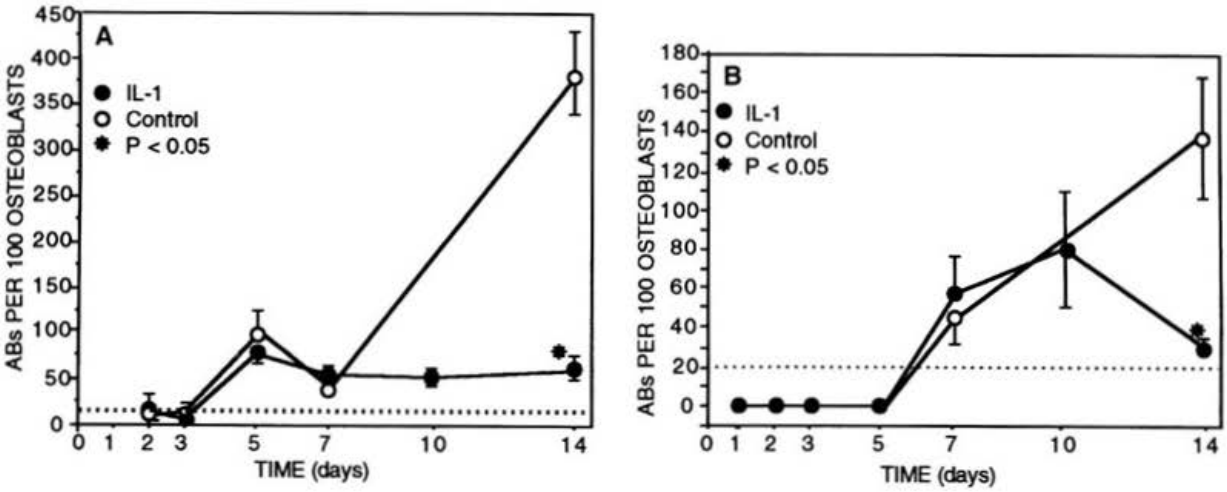

FIG. 6. Effect of interleukin-1β on apoptosis in the cambium (**A**) and in the defect (**B**) after injury (on day zero) normalized by the corresponding osteoblast concentration. Data in Panels A and B derived from Figures 1A and 3A and Figures 1B and 3B, respectively. The dotted line corresponds to the value in uninjured bone.

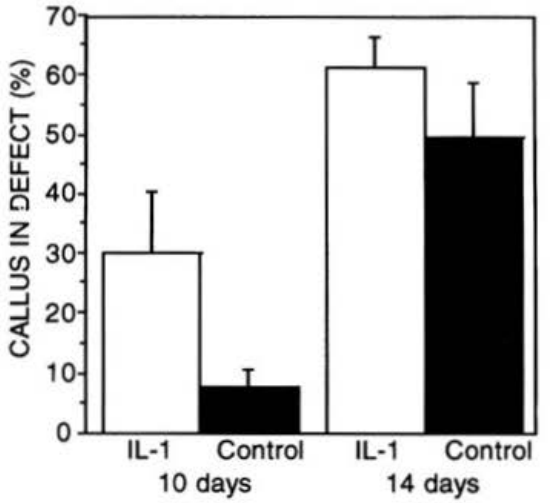

FIG. 7. Effect of interleukin-1β on percent callus in the defect ten and fourteen days after injury.

Callus formation within the defect was noteworthy beginning about Day 10. No differences attributable to IL-1β were seen at Day 10 or Day 14 (Fig. 7).

DISCUSSION

Callus that formed in response to the standard injury was remodeled by osteoclasts beginning about Day 7 (15). Before substantial remodeling occurred, however, many of the osteoblasts that secreted the callus matrix were eliminated. We found previously that the number of ABs increased relative to the number of osteoblasts during healing, suggesting that programmed cell death of osteoblasts might account for their elimination (16). Confirming evidence that osteoblasts in the callus became apoptotic was found in the present study. We observed cells with the organellar composition and ultrastructure of osteoblasts that exhibited the range of ultrastructural changes typically seen during apoptosis (Fig. 1). Observation of these changes in a particular histological cell type is definitive evidence of apoptosis in that cell type (7,14). These results do not prove that all ABs were derived from osteoblasts, but they do show that apoptosis of osteoblasts is a normal concomitant of bone healing in the model. This conclusion is consistent with the reported role of apoptosis in removal of chondrocytes during endochondral fracture healing (8,18) and closure of the growth plate (1) and in the removal of osteocytes during remodeling (21).

The hypothesis that IL-1β coordinated the mechanisms that regulate the appearance and disappearance of osteoblasts was tested by adding exogenous IL-1β to the injury site for three days. Addition of IL-1β resulted in more osteoblasts in the cambium and in the defect (Fig. 3) and fewer ABs relative to the number of osteoblasts (Fig. 6). Taken together, these results suggest that IL-1β was somehow involved in both the appearance and disappearance of injury-induced osteoblasts. This connection might be mediated by ICE. It is possible that different ICE isoforms are involved in apoptosis and production of mature IL-1β (32). Consequently, the role of ICE as an effector mechanism remains unclear.

A possible explanation for the role of IL-1β in regulating the changing levels of osteoblasts is as follows. IL-1β was produced locally early in the postinjury period and mediated the expansion of the osteoblast population. The osteoblasts secreted bone matrix while in the presence of IL-1β or for a fixed period after it was no longer present, and then underwent programmed cell death. Exogenous IL-1β resulted in increased osteoblast levels, probably by means of increased differentiation because no evidence of increased proliferation was found (Fig. 3). The added IL-1β delayed the normal decrease of IL-1β to baseline. Consequently, triggering of apoptosis was also delayed, resulting in a marked reduction in the number of apoptotic bodies per osteoblast (Fig. 6). The significance of this process with respect to callus formation in the defect cannot be assessed on the basis of the present data (Fig. 7).

Delivery of IL-1β had no demonstrable effect on the injury-induced proliferative response (Fig. 4). In contrast, in vitro studies suggested that IL-1β increased proliferation (3,11,23). The difference in implication could be explained by statistical imprecision in the animal data or by noncomparability in IL-1β concentration. Other possibilities include an inadequate (for proliferation) dose of IL-1β, the absence of a necessary cofactor in vivo that was present in vitro, or the presence of an inhibitor in vivo. These considerations underscore the desirability of studying bone regulatory phenomena in vivo, despite the variability inherent in even well-controlled animal models.

The model used was a cortical defect that did not extend into the medullary canal (Fig. 2). The rationale for this model was that it facilitated study of the essential cellular changes of bone in response to trauma. Fracture healing, in contrast, typically involves additional biomechanical and physiological conditions including motion at the injury site, interactions with marrow cells, and the inflammatory consequences of soft-tissue injuries. Any of these factors could, in principle, modulate the regulatory processes that would otherwise occur.

In summary, apoptosis is a normal concomitant of healing in our animal model. Evidence was found suggesting that IL-1β mediated the appearance and disappearance of osteoblasts, possibly by affecting the rates of differentiation and apoptosis, respectively. Understanding these mechanisms conceivably could lead to the ability to control osteoblast levels at an injury site.

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