Cell Tissue Res (2000) 302:353–363
DOI 10.1007/s004419900165

REGULAR ARTICLE

P. D'Aoust · C.A.G. McCulloch · H.C. Tenenbaum
P.C. Lekic

Etidronate (HEBP) promotes osteoblast differentiation and wound closure in rat calvaria

Received: 16 September 1999 / Accepted: 23 November 1999 / Published online: 3 November 2000

Abstract The mechanisms that regulate the migration, proliferation and differentiation of osteogenic cell populations in vivo are poorly understood. Elucidation of these mechanisms is essential for an understanding of the basic processes that determine mineralized connective tissue homeostasis and regeneration. Bisphosphonates are known to regulate bone metabolism, in part through effects on osteoclastic resorption. Given previous data from other in vitro and in vivo investigations, we considered that they could also affect the proliferation and differentiation of osteoblasts in vivo. We tested this hypothesis using a bisphosphonate (ethane-1-hydroxy-1,1-bisphosphonate, HEBP, etidronate) and a calvarial wound model in which osteogenic differentiation and bone formation are coordinately induced by the wounding stimulus. Wounds through the calvarial bone were created in 20 male Wistar rats. After surgery, animals were treated every day for 1 or 2 weeks with HEBP or saline (controls) and five rats in each group were killed at 1 or 2 weeks following surgery. Cellular proliferation and clonal growth were assessed by ^{3}H-thymidine labeling 1 h before death followed by radioautography. Cellular differentiation of osteogenic cell populations was determined by immunohistochemical staining for osteopontin and bone sialoprotein. Von Kossa and toluidine blue staining were used for the assessment of mineralization and osteoid formation, and for morphometric analysis of wound closure. At 1 and 2 weeks after surgery HEBP promoted wound closure (>twofold greater than controls, $P<0.001$) and mineralized/osteoid tissue formation in the bony compartment of the wound (>50% higher than saline controls, $P<0.05$). In HEBP-treated animals there was a >50% increase in intracellular staining for osteopontin in the endosteum-lined spaces adjacent to the wound ($P<0.05$) and increased staining for osteopontin in the nascent bone at the wound margin (>50% greater than controls, $P<0.05$). However, there were reduced cell counts and labeling indices at stromal precursor sites (65% reduction compared to controls; $P<0.01$). As HEBP increased osteopontin expression and osteoid/mineralized tissue formation but reduced the proliferation of precursor cells, we conclude that in addition to blockade of bone resorption and mineralization, this drug, at doses which also reversibly inhibit mineralization, may promote osteoblast differentiation as well.

Keywords Osteogenesis · Wound healing · Bisphosphonates · Osteopontin · Bone sialoprotein · Rat calvaria · Radioautography · Rat (Wistar)

Introduction

The mechanisms that regulate the migration, proliferation and differentiation of osteogenic cell populations are crucial processes that determine mineralized connective tissue homeostasis and regeneration. To study homeostatic mechanisms at sites where contiguous soft and mineralized tissue domains interact, it is important to use model systems that comprise cells from spatially and functionally discrete origins. Calvarial tissue provides a useful model for such studies since its cell populations synthesize and remodel soft (dura mater) and mineralizing connective tissue (i.e., calvarial bone). While in close apposition, the soft and mineralizing cell populations retain their spatially discrete domain structure (Marden et al. 1993). Preservation of these cellular domains appears to be important for physiological growth and remodeling, and for the healing of calvarial wounds (Marden et al. 1994).

C.M. and H.T. are supported by a Medical Research Council (MRC) of Canada Group Grant. P.L. is supported by an MRC of Canada Operating Grant

P. D'Aoust · C.A.G. McCulloch · H.C. Tenenbaum
Medical Research Council Group in Periodontal Physiology, Faculty of Dentistry, University of Toronto, Toronto, Canada

P.C. Lekic (✉)
Department of Dental Diagnostics and Surgical Sciences, Faculty of Dentistry, University of Manitoba, 780 Bannatyne Ave., Winnipeg, Manitoba, Canada, R3E OW2
Tel.: +1 204 789 3507, Fax: +1 204 789 3913

The synthesis and turnover of cells and extracellular matrix molecules in calvarial bone are mediated by a complex mixture of cell populations including the progenitors for osteoblasts and fibroblasts. These progenitor cells proliferate and migrate to produce more differentiated cells that can synthesize bone and the extracellular matrix of the dura mater respectively (Kenley et al. 1994). Various experimental and clinical perturbations have been used to facilitate studies of mineralized connective tissue homeostasis in vivo including wounding (Levy et al. 1994; Lekic et al. 1997). Some of these wounding protocols rely in part on the transient depletion of osteogenic cell populations. For example, selective disruption of the cellular domains that are required to preserve mineralized connective tissue homeostasis was described in the calvarial wound model developed by Turnbull and Freeman (1974). Defined portions of the calvarial bone were excised to generate a sequence of differentiation steps that lead to complete regeneration of calvarial bone (Marden et al. 1993). However, the mechanisms that regulate these differentiation steps in vivo are poorly understood.

A wide variety of pharmacological agents have been used that either inhibit or stimulate osteogenic cell differentiation in vivo including reconstituted collagen (Todescan et al. 1994) and osteogenic growth factors (Malpe et al. 1997; Hsieh and Graves 1998) including the bone morphogenetic proteins (Kenley et al. 1994; Rutherford et al. 1992) and bisphosphonates (King et al. 1971). Bisphosphonates are known regulators of bone metabolism that could be used potentially to probe osteogenic cell differentiation in healing calvarial wounds. While these agents are used therapeutically to regulate the relatively slow remodeling that occurs in the mature unwounded skeleton, we considered that their use might provide fresh insights into osteogenic differentiation in a wound model where large numbers of synchronous cohorts of cells are induced to differentiate by the wounding stimulus.

The bisphosphonates of therapeutic importance are synthetic analogues of inorganic pyrophosphate that inhibit bone resorption and, at higher doses, mineralization in vivo (Fleisch 1991), but also affect bone formation in vitro as well as other measures of osteogenic cell differentiation (Goziotis et al. 1995). We have shown that moderately high doses of bisphosphonates (particularly HEBP) affect cell proliferation and increase the production of type I collagen (as osteoid) and expression of alkaline phosphatase (Goziotis et al. 1995), osteopontin (OPN) and bone sialoprotein (BSP; Lekic et al. 1997) in osteoblastic cells. These investigations have shown that the excess osteoid formed in the presence of HEBP can mineralize once HEBP treatment is stopped. In addition, although mineralization is inhibited by HEBP, such inhibition is not complete, meaning that some mineralization does still occur in the drug's presence, albeit at a much reduced rate. Finally in this regard, the mineral that does form following cessation of HEBP treatment is significantly more dense than the control (Torontali et al. 1994). Cognizant of these effects of bisphosphonates in vitro, we studied the effects of the bisphosphonate HEBP on the proliferation and differentiation of osteogenic cells in a wounded mineralized connective tissue that regenerates within a relatively short period of time. We assessed mineralized bone and osteoid with von Kossa and toluidine blue staining respectively, labeled proliferating cells with ^{3}H-thymidine and osteoid synthesis rate using ^{3}H-proline, and used immunohistochemistry to identify the production of OPN and BSP in differentiating cells. Our hypothesis was that HEBP promotes osteoblastic differentiation and bone formation in calvarial wounds. The data show an increase in bone formation that may be due to HEBP-induced differentiation of osteoblastic cells at endosteal sites enriched with precursors, and to an increase in the rate of bone formation by already differentiated osteoblastic cells.

Materials and methods

Wound model and HEBP treatment

Twenty Wistar male rats weighing 130–150 g on the day of surgery were caged in pairs, fed water and food ad libitum and kept in a room with a 12 h light/dark cycle. Surgery was performed between 10:00 and 12:00 hours on the 3rd day after the arrival of the animals. Two calvarial wounds (~0.8 mm in diameter) were created on the left and right side of the parietal bone while the animal was under general anesthesia (halothane, N_2O) as previously described (Turnbull and Freeman 1974). Following surgery, groups of animals (N=5/group) were treated daily with HEBP subcutaneously at a dose of 15 mg/kg body weight beginning immediately after surgery, or were given saline only (control). Group H1 was treated for 1 week and group H2 for 2 weeks with HEBP and killed by N_2O asphyxiation. Rats in the two control groups were treated with saline for 1 week (C1) or for 2 weeks (C2) following wounding. In addition, since it was possible that wound closure might be accelerated because of HEBP's ability to inhibit osteoclastic cell function, another group was added that received an even more potent inhibitor of bone resorption, an aminobisphosphonate (APD). The rationale for using this drug was that if inhibition of bone resorption was playing a stimulatory role with respect to bone wound healing, this would be likely to occur in rats treated with APD. Thus, animals in one group received APD (1 mg/kg) for a period of 1 week (A1) followed by a recovery period at 2 weeks (A2) following a similar regime to that for HEBP-treated animals. Finally, inasmuch as stimulation of excess osteoid formation would not in and of itself confer any great advantage on bone wound healing, it was thought that it was important to demonstrate that newly formed osteoid could in fact mineralize following cessation of HEBP administration. To this end another group, H3, that was treated with HEBP over the initial week of healing was then permitted to recover from HEBP treatment for an extra week. One hour before death all rats were injected intraperitoneally with 2 ml phosphate-buffered saline (PBS, pH 7.4) containing ^{3}H-thymidine (specific activity = 20 Ci/mmol; NEN, Oakville, ON, Canada) at 1 µCi/g body weight. The calvariae were removed immediately after sacrifice and one-half were fixed for preparation of undemineralized sections and the other half for preparation of demineralized sections. For the purposes of this investigation, morphometric measurements were made for all groups but a detailed assessment of cellular kinetics and immunostaining was restricted to the groups actively receiving HEBP as these showed the most significant increases in healing as will be outlined below. For similar reasons only, morphometry was carried out in H3 samples, as in this case the prime outcome measure was related to whether or not the newly formed osteoid was capable of mineralization.

Tissue preparation

The left calvarial halves were fixed in 10% neutral buffered formalin, washed, dehydrated in ethanol, and embedded in Spurr resin, and 5-µm sections were cut in the transverse plane. Specimens were sectioned until the total periphery of the wound was observed in cross section, at which point every third section was chosen for histomorphometry. The sections were stained with von Kossa and toluidine blue for the assessment of mineralized and non-mineralized bone respectively and for morphometric analysis of wound closure on coded slides (to maintain blindness for assessment of data).

The right calvarial halves were fixed in periodate lysine paraformaldehyde (McLean and Nakane 1974) at pH 7.4 for 24 h at 4°C, demineralized for 24 h in 0.2 N HCl, and washed in PBS for 20 h. The specimens were then bisected with a sharp scalpel in the center of the wound to facilitate embedding and tissue orientation so that the sectioning could start from the middle of the wound. The calvarial specimens were dehydrated in graded ethanol, cleared in toluene, and embedded in paraffin. Sections in the coronal plane (5 µm in thickness) were attached to coded glass slides for immunohistochemical and radioautographic analyses.

Immunohistochemistry

Sections were stained with a mouse monoclonal anti-rat OPN antibody (Hybridoma Bank; Iowa City, IA) or a rabbit polyclonal anti-rat BSP antibody (kindly provided by Dr. J. Sodek) as described previously (Langille and Solursh 1990; Frank et al. 1993; Rajshankar et al. 1998). The specificity of the OPN and BSP antibodies has been confirmed by immunoprecipitation of the respective proteins from radiolabeled rat bone cell cultures (Kasugai et al. 1991). Control slides were treated with an irrelevant antibody (anti-human CD4 lymphocyte antigen; Coulter Electronics, Burlington, ON, Canada). The intensity of OPN and BSP staining was classified by visual inspection as intense (3), moderate (2), weak (1) or negative (0) in relation to the expression of these proteins in the surrounding intact bone. A detailed account of this analytical procedure has been described previously (Lekic et al. 1996a). The area of OPN and BSP expression was examined under a light microscope (Laborlux K, Leica, Wetzlar, Germany) with an intraocular grid (250×250 µm) containing 100 squares of 625 µm each. The staining for each protein was estimated by: staining = staining intensity in each grid × area stained in the whole sampling grid. The localization of proteins was studied in the intact, unwounded bone adjacent to the wound site (site 1), in the bone compartment of the wound at the wound margin (site 2), as well as in the central compartment of the defect at a distance of >400 µm from the cut margins (site 3, Fig. 1). As no tissue regeneration occurred in the surrounding bone and endosteum (site 1), instead of measuring the tissue staining, we measured the percentages of cells containing intracellular OPN and BSP at this site (Fig. 1). Staining data were obtained from assessments of at least nine sections from three different animals for each time (1 and 2 weeks) and each of the three sites (Fig. 1) for the C1, C2, H1 and H2 groups.

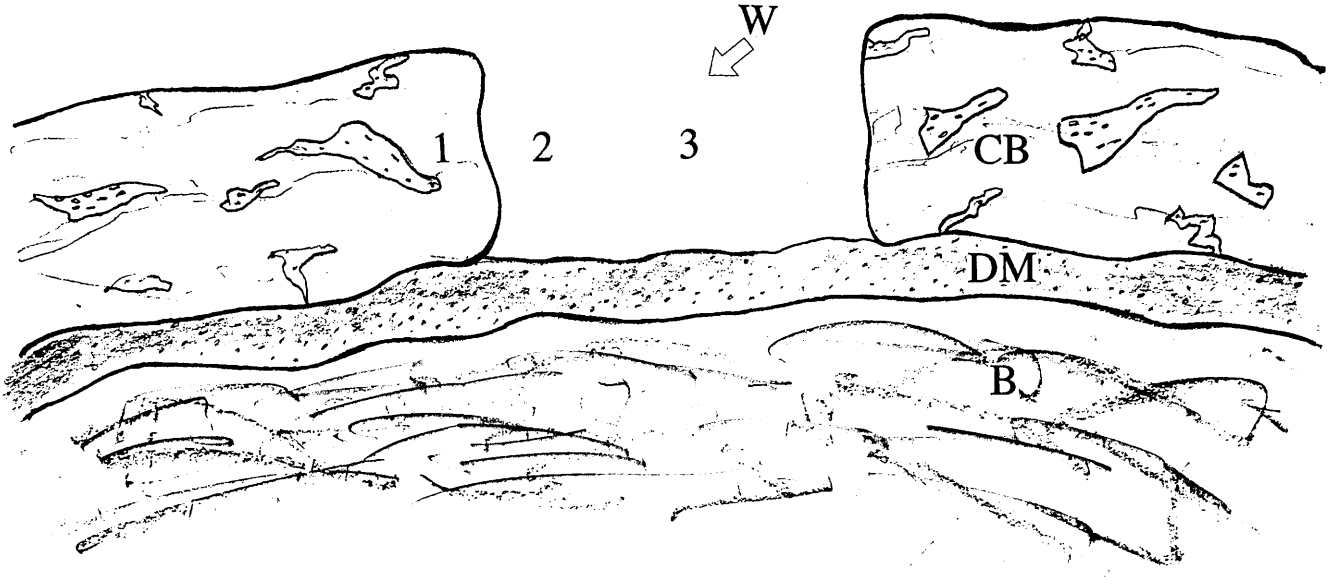
Fig. 1 Key for all sites for morphometric analyses and photomicrography. Diagram of sagittal cut through the rat cranium showing calvarial bone (*CB*), dura mater (*DM*), wound (*W* and *large arrow*) and brain (*B*). The sites analyzed include: the surrounding bone adjacent to the wound (site 1), the bone compartment of the wound within 200 µm of the bone margin (site 2), and the central compartment of the wound, >400 µm from the wound margin (site 3)

Cellular proliferation

Immunostained slides were dipped in full-strength Kodak NTB-2 emulsion, randomly distributed to light-tight, dry boxes, and exposed for 2 weeks at 4°C as described previously (Lekic et al. 1996b). After exposure, the slides were developed in Kodak D-19 developer and stained through the emulsion with hematoxylin and eosin.

The middle section in each ten-section ribbon was examined with a light microscope (Laborlux K, Leitz, Wetzlar, Germany) at a magnification of ×250. All measurements were made with an intraocular grid system (see above) and a cell was considered to be labeled if more than five silver grains overlaid its nucleus ($P<0.001$). Background counts obtained from sections of control (unlabeled) animals and application of the Poisson distribution were used to derive this figure (McCulloch et al. 1989).

Counts of labeled and unlabeled cells in four sampling grids (62,500 µm² each) from one section of each slide were obtained to assess proliferation. The sites for analysis were: the intact calvarial bone surrounding the wound (site 1), the bone compartment of the wound (site 2, within 200 µm of the bone margin) and the central portion of the wound (site 3, >400 µm from the cut bone margin; Fig. 1). The labeling index for each area was calculated as follows: LI = number of labeled cells/number of total cells × 100. To assess clonal growth of progenitor cells, labeled nucleus located within 25 µm of another labeled nucleus were considered to have arisen from a common precursor and were designated as clustered (McCulloch and Melcher 1983). The percentage of clus-



tered cells at each site was calculated as follows: CI = number of clustered cells/number of labeled cells × 100.

Morphometric assessment

Three sections from each animal in the HEBP-treated and APD-treated groups were analyzed morphometrically and slides were coded so that the sacrifice day and the type of treatment were unknown at the time of measurement. Wound closure and quantity of mineralized tissue within the wound were analyzed by light microscopy (Metallux 3, Leitz) with the aid of a bone morphometry program (Bioquant, R&M Biometrics, Nashville, TN). Osteoid tissue as well as wound closure were expressed as percentages (mean±SEM%) of the original size of the wound which was estimated from cut-lines in the bone. The area of the regenerating calvarial bone at the drilled site was measured after digitizing the area of the OPN or BSP-stained tissue in the bone compartment of the wound.

In a previous study where increased bone-osteoid volume was demonstrated following treatment with HEBP, there was still some question as to whether this arose secondary to inhibition of resorption or stimulation of matrix formation. Therefore, in addition to the experiments outlined above, it was necessary to demonstrate more directly that increased bone volume seen in animals treated with HEBP was due to an increase in *matrix synthesis* as seen in vitro. This was accomplished by labeling newly synthesized bone-osteoid with tritiated proline and identifying labeled bone matrix using autoradiography. Although assessment of matrix formation within the calvarial wounds would have been ideal, pilot studies indicated that bone formation occurs in many planes within a wound. As such, the identification of tritiated proline label fronts was not possible within wounds. Therefore, bone-osteoid formation rates were assessed on bone appositional surfaces that were not wounded, such as on the periosteal surface of the femur, as follows. To measure the distance between the double label we injected rats intraperitoneally with ^{3}H-proline (2 μCi/g, specific activity 21 Ci/mmol) 24 h apart on days 3 and 4. Following the sacrifice of animals and the tissue preparation (see above), quantification of the distance between the bands or thickness of band widths was accomplished with light microscopy (Metallux 3, Leitz) and with the aid of the bone morphometry program from the Bioquant morphometric system (R&M Biometrics, Nashville).

Statistical methods

Raw data were kept separate and the means for each animal, type of treatment, sacrifice day, and examined site were calculated. The mean from each animal was considered as an independent sample and these data were then assessed by analysis of variance (ANOVA) to evaluate the differences between the five treatment groups with respect to labeling indices, clustering indices, and morphometric and immunostaining assessments at the different sites. Differences between groups were examined by ANOVA and were considered significant at $P<0.05$. Data were expressed as means ± SEM. Post hoc comparisons were made using Duncan's multiple-range test.

Results

Histomorphometry

We measured the closure of calvarial wounds to determine whether HEBP was pharmacologically active in regenerating mineralized tissue. Independent of the type of treatment, calvarial wounds healed progressively over the duration of the experiment. At 1 week after wounding, the HEBP-treated group demonstrated a twofold increase in closure compared to the saline group ($P<0.05$; Fig. 2) as determined by von Kossa/toluidine blue staining. Notably, HEBP-treated animals also exhibited a twofold increase in wound closure when compared with the APD treatment ($P<0.001$). However, animals treated for 2 weeks with HEBP exhibited the greatest closure of calvarial wounds (mostly osteoid) based on the relative volume of new bone matrix and mineralized matrix in the defect (>100% more than saline controls at 2 weeks; $P<0.05$; Fig. 2). The difference in wound closure between saline- and HEBP-treated rats can be appreciated in the histological photographs of the specimens stained with von Kossa and toluidine blue (Fig. 3a,b). Collectively, these findings indicated that systemic treatment with HEBP induces a significant increase in initial bone closure in healing calvarial wounds, which was maintained after 2 weeks as well.

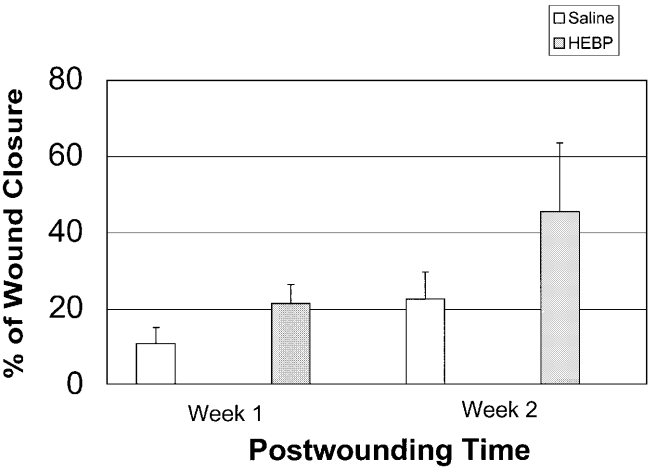


Fig. 2 Morphometric assessment of the percentage of wound closure formed within the wound site. The percentage of wound closure that has closed the drill site was estimated as a function of the original volume of the drilled site and has been expressed as mean percentage of wound closure ± SEM. Note that for the same post-wounding time HEBP-treated animals showed increased wound closure. Animals were treated continuously with either saline or HEBP for 1 or 2 weeks

Osteoid matrix production

As shown in Fig. 3b, when animals in H3 were permitted to "recover" from HEBP treatment, the newly formed osteoid was capable of mineralization. Interestingly, APD did not induce significant increases in wound closure relative to saline control (1.3-fold, $P>0.05$, and Fig. 3a,c). There was still visible non-mineralized osteoid in H3 (Fig. 3b), while most of the newly formed osteoid was not mineralized in animals treated with HEBP for two full weeks (Fig. 3d). To assess this more meaningfully, we calculated the ratio of osteoid to mineralized bone in the tissues growing into the total wound area. This showed that whereas there was about 19% osteoid in animals treated with saline only, there was greater than 60% osteoid in animals treated with HEBP

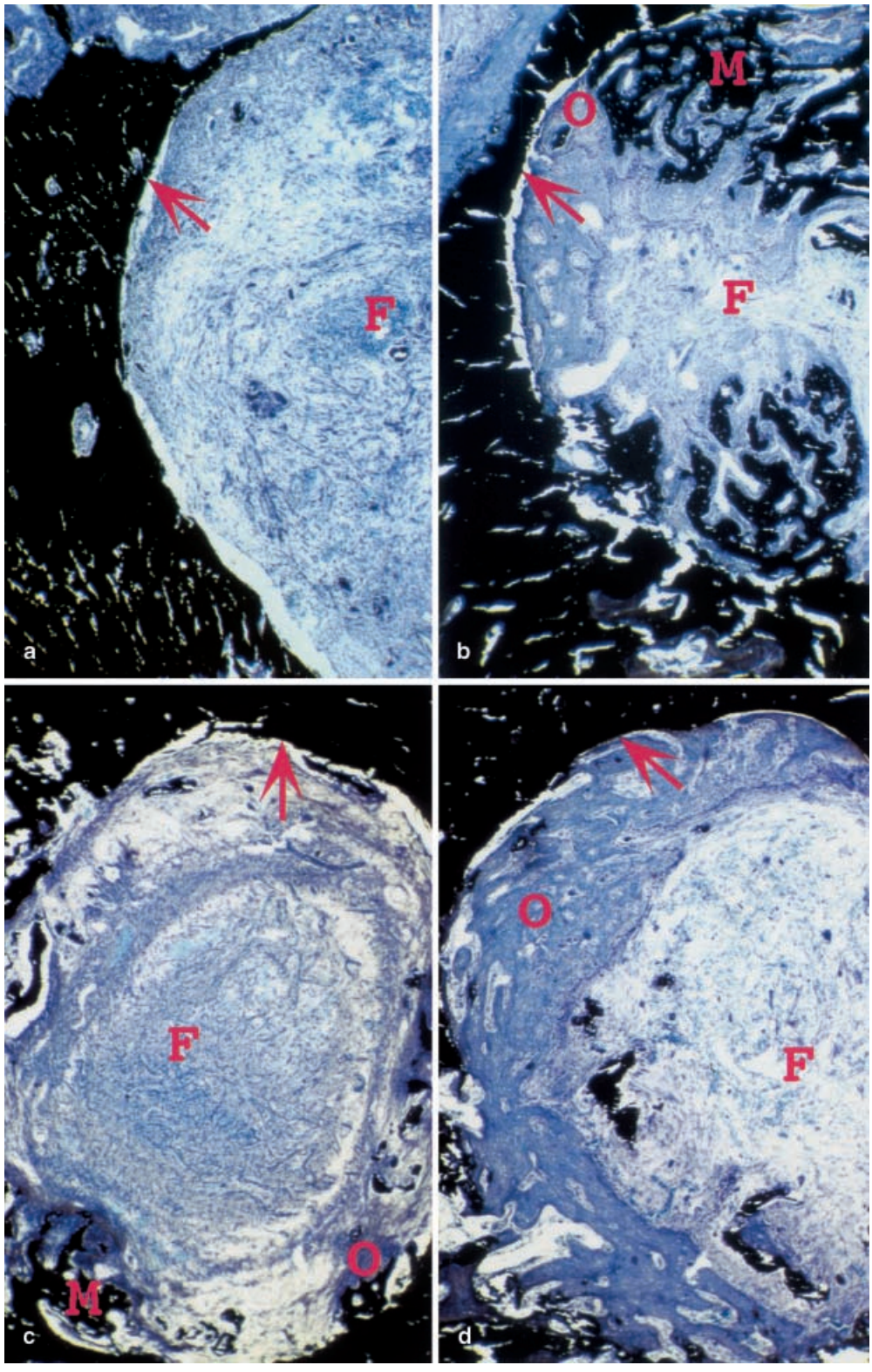


Fig. 3 **a** A 2-week wound closure in rats treated with saline is demonstrated. There was little wound closure in this specimen, with the wound edge (*arrow*) clearly seen and the central portions of the wound being filled mainly with fibrous tissue (*F*). In contrast, animals treated with HEBP for 1 week and then saline **(b)** showed the presence of both mineralized bone (*M*) and residual osteoid (*O*) that appears to be radiating inwards from the wound edge (*arrow*). Animals treated with APD **(c)** followed by saline had much smaller amounts of mineralized bone (*M*) and osteoid (*O*) at the wound edge (*arrow*). In animals treated for 2 full weeks with HEBP **(d)**, there was little mineralized bone while wound closure from the wound edge (*arrow*) was accomplished almost completely with unmineralized bone or osteoid (*O*). All wounds still had central zones of fibrous tissue (*F*) at 2 weeks. ×105 **(a–d)**

($P<0.05$). The percentage osteoid returned to baseline levels of approximately 18% 1 week following cessation of HEBP treatment in H3 and was indistinguishable from saline control levels. This trend was also suggested when relative mineralization (Fig. 4) was examined *in different sites within the wound* (i.e., sites 2 and 3) at 1 week but also showed some interesting variations. In relation to this, the relative volume of mineralized matrix in the bone compartment of the defect (site 2, Fig. 4) was >50% higher than that in saline controls at 1 week

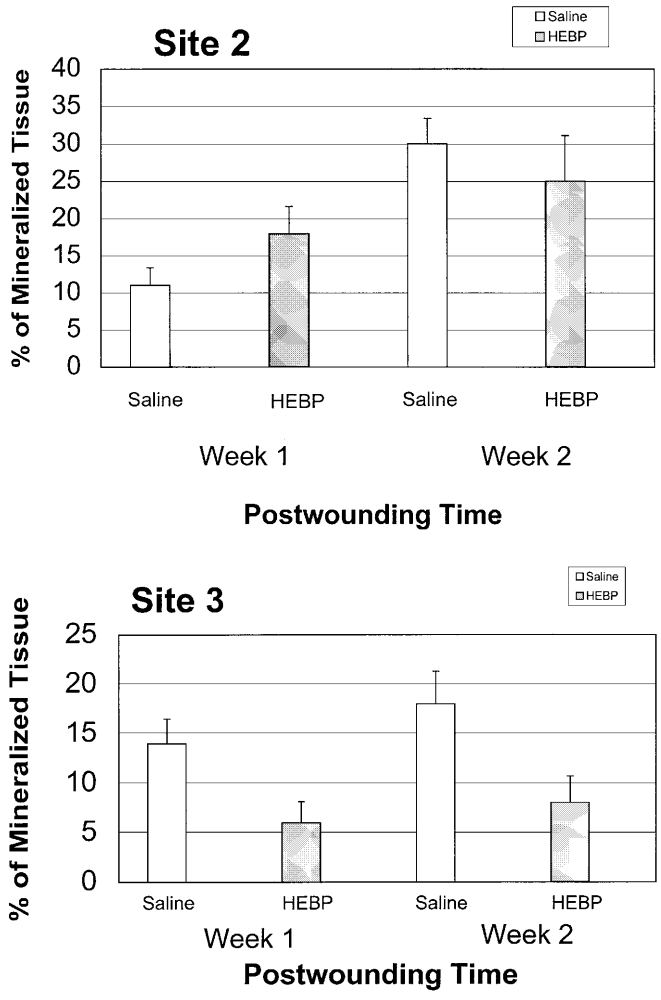


Fig. 4a,b Histomorphometric assessment of the percentage of mineralized tissue formation within the defect at the indicated wound sites. The percentage of mineralized and osteoid tissue in the wound is a function of the volume of the entire wound and has been expressed as mean percentage of mineralized and osteoid tissue ± SEM. Note that both HEBP- and saline-treated animals showed a sustained increase in mineralized tissue formation for the examined healing times. As in Fig. 2, animals were treated continuously with either saline or HEBP for 1 or 2 weeks

(*P*=0.2). However, for the same postwounding time (1 week) the relative volume of mineralized tissue in the central compartment (site 3, Fig. 4) was >250% greater in controls (saline) than in the HEBP-treated animals (*P*<0.07). In animals treated for 2 weeks with HEBP, the volume of mineralized tissue in the bone compartment was not significantly different than controls (*P*>0.2; site 2, Fig. 4) but there was still greatly reduced mineralized tissue in the central compartment (<50%; *P*<0.05; site 3, Fig. 4) compared with controls. Therefore HEBP treatment promoted appositional osteoid and mineralized tissue formation in regenerating bony defects at sites of existing bone where there are abundant osteogenic cells available but inhibited mineralization at sites where osteogenic cells must differentiate only after extensive proliferation of precursors (site 3).

In addition to increases in the relative levels of osteoid matrix caused by HEBP's inhibitory effects on mineralization, it was possible to demonstrate that there was also a clear increase in osteoid matrix synthesis too, even in non-wounded sites. The findings (Fig. 5a,b) demonstrated that, in saline-treated rats, it was not possible to distinguish between the two ^{3}H-proline labels as they were too close together (Fig. 5b). However, the two labels in non-wounded femur could be more readily distinguished in HEBP-treated animals (Fig. 5a), which is in itself evidence for stimulation of matrix production by HEBP. Given the fact that two separate lines could not be distinguished at all in the control groups and not at all times in the HEBP-treated group, we elected to measure the total thickness of the lines within the labeled tissues and also to measure the distance from the outermost label to the appositional border of subperiosteal bone. In addition to corroborating the label width measurements, the latter measurement would also permit a postlabeling assessment of matrix production to determine whether the labeling itself had any effect on our findings. There was almost a twofold (*P*=0.003) increase in the total width of label in the HEBP-treated animals compared with saline-injected controls. This difference was maintained when measuring the distance of the outermost margin of the label to the edge of bone (twofold, *P*=0.03).

Cellular proliferation

At sites 2 and 3, the percentage of ^{3}H-thymidine-labeled and clonally distributed cells was the same, independent of the type of treatment (site 2: the bony compartment, 1 or 2 weeks after wounding; HEBP group; Labeling Indices = 3.1%, 3.0%; Clustering Indices = 55.6%, 58.3%; saline controls: Labeling Indices = 2.9%, 2.7%; Clustering Indices = 56.6%, 50.0%). Notably, proliferation of cells in the intact bone adjacent to the wound (site 1) was reduced >50% at 1 week in HEBP-treated animals compared with controls (*P*=0.06). Accordingly, after 1 week of HEBP treatment the total cell count derived from sampling grids in the bony compartment of the wound and in the middle of the wound was <65% compared with controls (1 week saline; *P*<0.001; sites 2, 3). However, 1 week after the HEBP treatment proliferation of cells in the middle of the wound (site 3) was increased >80% compared with controls (*P*<0.05), indicating the presence of more primitive proliferating cells. At the end of the 2-week experimental period, animals treated with HEBP exhibited a total cell count that was not significantly different from controls in the periphery (site 2, Fig. 6; *P*>0.2) but was significantly different when compared with controls in the central portion of the wound (site 3, Fig. 6; *P*<0.05). These findings are consistent with the lack of difference of labeling index at the 2-week period and could indicate that HEBP initially suppressed the proliferation of precursor cells from the surrounding bone and thereby reduced the total cell count. Subsequently this difference was dissipated, presumably due to cell migration within the wound.

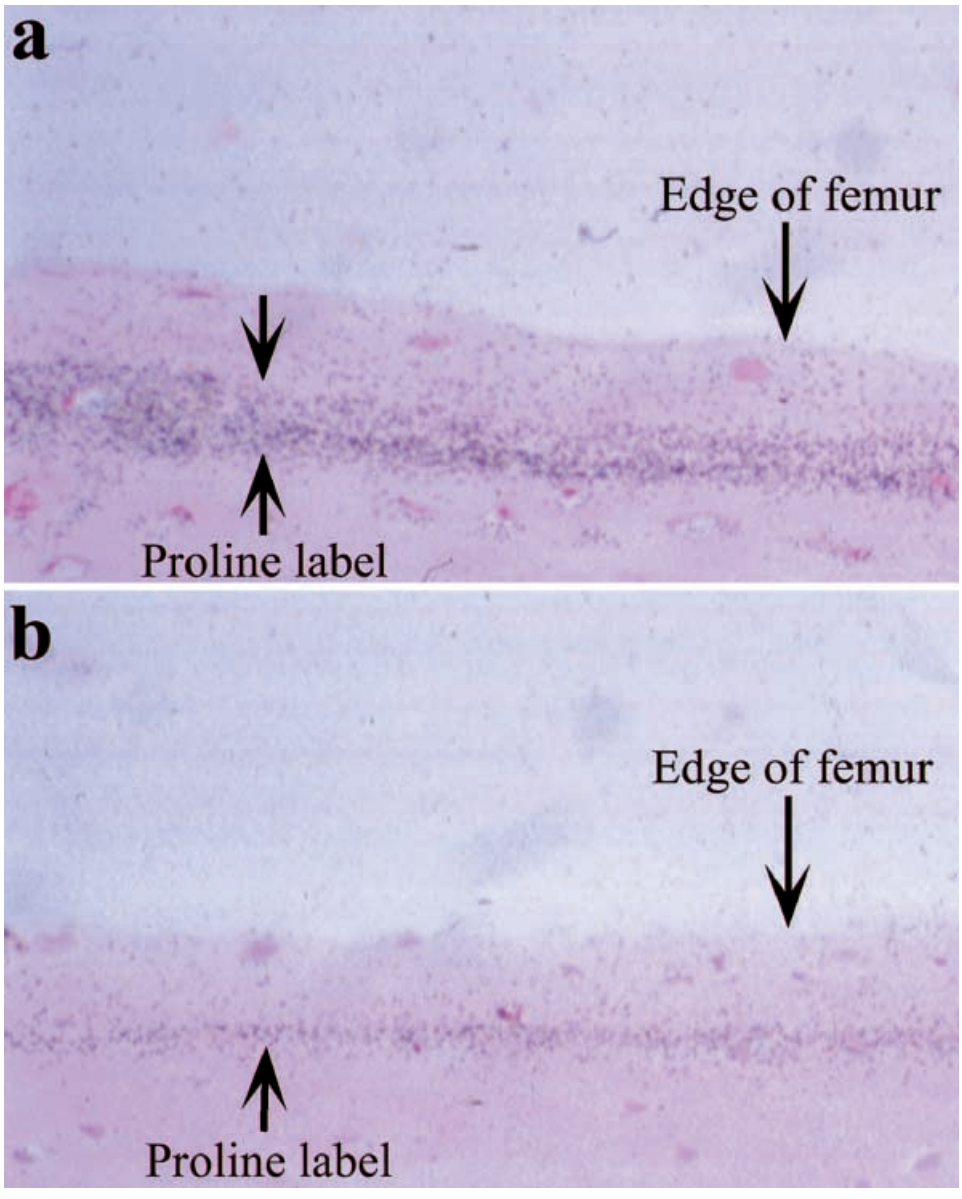


Fig. 5a,b Proline label lines are shown in these autoradiographs (*arrows*). The animals were treated with HEBP **(a)** or saline **(b)**. In animals treated with saline **(b)** only one thin single line can be discerned. However, in animals treated with HEBP **(a)**, the label lines are not only thicker but in some areas two single lines can be discerned a little more clearly (e.g., *on the right side*). The distance from the label front to the edge of the femur is also greater in HEBP-treated animals. ×312 **(a,b)**

Osteogenic differentiation

We used OPN and BSP as differentiation markers of mineralizing connective tissue cell populations (Fig. 7a–d). OPN staining that ranged from very low to intense was observed at all examined sites independent of the treatment or the experimental period (Fig. 8). In animals treated with HEBP (1 or 2 weeks) the OPN staining in the bony compartment of the wound (site 2, Fig. 8) was >80% higher than in controls ($P<0.05$). In the central portion of the defects at 1 week after wounding, OPN staining was >100% higher in controls than HEBP-treated animals (site 3, Fig. 8, $P=0.3$). However, at 2 weeks after wounding OPN staining in the central portion of the defect (site 3, Fig. 8) was >50% higher in the HEBP-treated animals than in controls ($P<0.005$).

In contrast to OPN staining, BSP was not seen in the central portion of the wound during the initial healing phase (1 week) in HEBP-treated animals (site 3, Fig. 8). Staining for BSP in the bony compartment of the wound (site 2, Fig. 8) was similar at 1 and 2 weeks after surgery for both treatment groups ($P>0.2$; Figs. 3e, 8). However, in the central portion of the wound the BSP expression was >eightfold higher in controls compared with HEBP-treated animals (site 3, Fig. 8, $P<0.001$). Collectively, these data showed that HEBP alters the spatial and temporal staining patterns for OPN and BSP and point to an important effect on switching of osteogenic differentiation markers in healing bony defects.

We assessed only intracellular OPN and BSP immunostaining of cells at site 1 (Fig. 8). In the HEBP-treated animals there were >50% higher percentages of OPN+ve cells at 1 week after wounding but only a small difference at 2 weeks. For BSP staining, there were 50% higher percentages at 1 week and >twofold higher percentages of BSP+ve cells at 2 weeks after wounding ($P<0.001$).

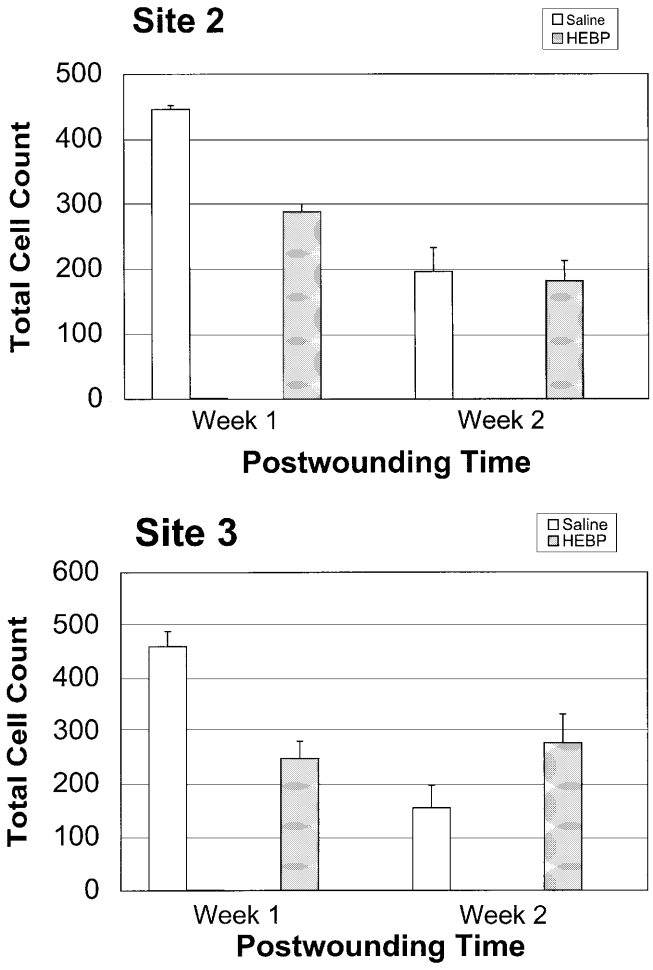


Fig. 6a,b Total cell counts are depicted in this figure (±SEM) in the bony compartment (*site 2*) and in the central compartment (*site 3*) of the wound. In site 2 there is a decrease in total cell count from week 1 to week 2 in control animals. However, total cell counts also appear to be reduced in HEBP-treated animals as compared with the control at 1 week while the total cell counts are similar by 2 weeks. A similar control pattern is seen in site 3; however, the total cell count is now higher by week 2 in HEBP-treated animals as compared with the control

These findings on intracellular staining are consistent with the tissue staining for BSP and OPN in the bony defect of the wound and illustrate a significant inductive effect of HEBP on osteoblast differentiation.

Discussion

The central finding of this study is that HEBP accelerates mineralized/osteoid tissue formation and wound closure. We suggest that these effects are due in part to the HEBP-induced upregulation of osteogenic cell activity. The major emphasis for research on bisphosphonates in the past relates to their ability to inhibit bone resorption (Fleisch 1989), a property that should actually inhibit wound closure. Our data provide support for the notion that HEBP may also regulate osteogenic cell differentiation, further contributing to acceleration of wound closure.

In spite of past therapeutic successes (Averbach 1993; Blomqvist and Eloma 1996; Delmas and Meunier 1997), the principal mechanism for bisphosphonate-induced increases in bone mass remains unknown. In particular, the possible effects of these agents on the proliferation and differentiation of osteogenic cell populations is poorly understood. In addition to the expected increase in wound closure observed in earlier reports of periodontal wound healing (Lekic et al. 1997), it is evident that HEBP promoted mineralized/osteoid tissue formation and the expression of differentiation markers as seen previously in vitro (Goziotis et al. 1995; Igarashi et al. 1997). Histomorphometry demonstrated that in HEBP-treated animals there was a significant increase in early wound closure and an increase in the percentage area of osteoid plus mineralized tissue in the healing defects. We have found previously that HEBP increases bone volume (Lekic et al. 1997) and, in this study, increases in matrix production as measured by ^{3}H-proline uptake. These HEBP-mediated changes in bone mass could be regulated through changes in bone resorption or bone formation or both (Goziotis et al. 1995). However, the fact that labeled proline data show increased bone matrix synthesis in vivo as well as in vitro (Goziotis et al. 1995) coupled with the findings showing that APD, a powerful inhibitor of resorption, did not stimulate wound closure suggests that the predominant effect of HEBP is on bone formation.

In support of the above, previous studies indicate that ^{3}H-HEBP labeled 3–15 times more osteoblast than osteoclast surfaces (Masarachia et al. 1996). This increased affinity of HEBP for osteoblasts and the previously demonstrated effect on promotion of osteoblast differentiation (Lekic et al. 1997; Klein et al. 1998) could in part explain the enhanced osteoid/mineralized tissue production in the bony compartment of the wound. On the other hand, the increased affinity of HEBP for osteoblasts may help to inhibit bone resorption since bisphosphonates can abrogate IL-6 production in human osteoblastic cells (Giuliani et al. 1998). Although not shown here, there were few if any osteoclasts (on the basis of thrombospondin-related anonymous protein, TRAP, staining) observed in any of the treatment groups including the control. Thus, HEBP- or APD-mediated inhibition of osteoclast activity would not be expected to contribute very much to wound healing in any case. Conceivably, the newly formed, thicker osteoid tissue in the HEBP-treated animals mineralizes (King et al. 1971; Tenenbaum et al. 1992), thereby leading to increased bone volume.

Effect of HEBP on proliferation of osteogenic precursor cells

The relatively high percentages of ^{3}H-thymidine-labeled cells in the endosteal spaces of the surrounding bone ad-

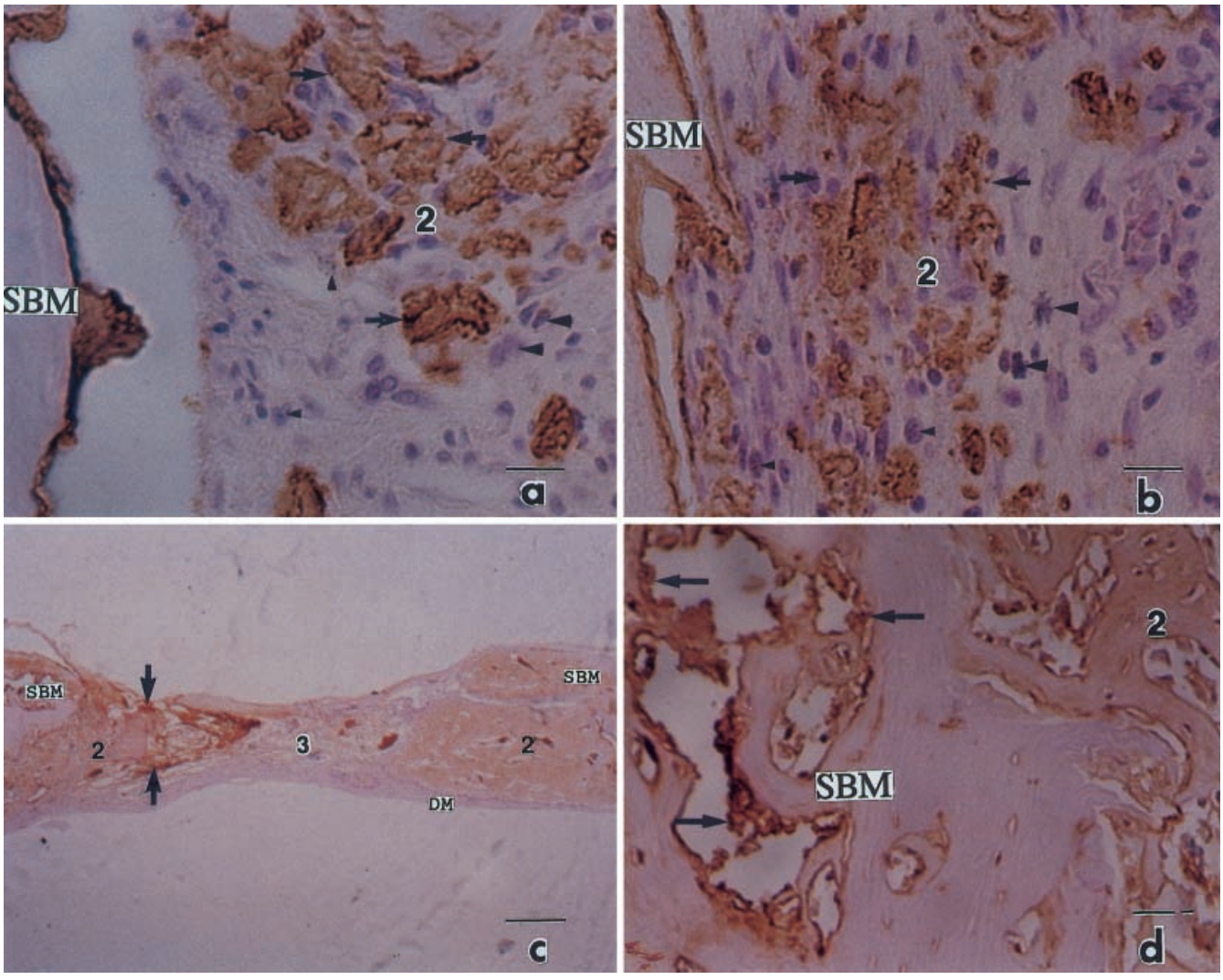


Fig. 7 **a** HEBP-treated, 1-week postwounding specimen, showing ^{3}H-thymidine-labeled cells in the bony compartment of the wound (*larger arrowheads*) and clustering of labeled cells (*smaller arrowheads*) in the proximity of OPN-stained cells (*arrows*). **b** Saline-treated control, 1 week postwounding specimen showing similar labeling (*larger arrowheads*) in the HEBP-treated specimen (Fig. 3c) and clustering (*smaller arrowheads*) of labeled cells in the proximity of positive OPN immunostaining (*arrows*) (*2* bony compartment of the wound). **c** Photomicrograph of an HEBP-treated, 2 weeks postwounding specimen showing intense BSP staining (*arrows*) in the bony compartment of the wound (*2*) (*DM* dura mater, *2* bony compartment of the wound, *3* central portion of the wound). **d** Photomicrograph of an HEBP-treated, 2 weeks postwounding specimen showing a high percentage of BSP+ve cells (*arrows*) at the endosteal spaces of the surrounding bone margin (*SBM*). Bony compartment of the wound (*2*). *Bars* 20 μm **(a,b,d)**

jacent to the calvarial wound indicate that these proliferating precursor cells contribute to the cell repopulation of the extirpated wound as has been shown previously in alveolar bone (Gould et al. 1980). Cells repopulating calvarial wounds include fibroblast and osteoblast precursor cells (Marden et al. 1993), both of which may be regulated by bisphosphonates (Goziotis et al. 1995; Lekic et al. 1997). Consistent with these data we found that animals treated with HEBP exhibited reduced proliferation of osteoblast precursor cells in the endosteal spaces of the surrounding intact bone. Conceivably, and as indicated in previous studies (Goziotis et al. 1995; Lekic et al. 1997), it is possible that HEBP selectively stimulates the differentiation but reduces the proliferation of osteogenic cells as has been shown previously in an in vitro study of cultured rat calvarial cells (Sodek et al. 1995).

HEBP promotes osteoblast differentiation and closure of calvarial wounds

We found that animals treated with HEBP exhibited changes in the staining intensity and localization of OPN and BSP compared with controls. The expression of these proteins has been associated with discrete stages of mineralized tissue formation (Chen et al. 1992; Yao et al. 1994). For OPN, previous studies have shown that this protein is expressed by osteogenic cells and also by non-osteogenic cells (Denhardt and Guo 1993), whereas staining for BSP is more exclusively associated with differentiated mineral-

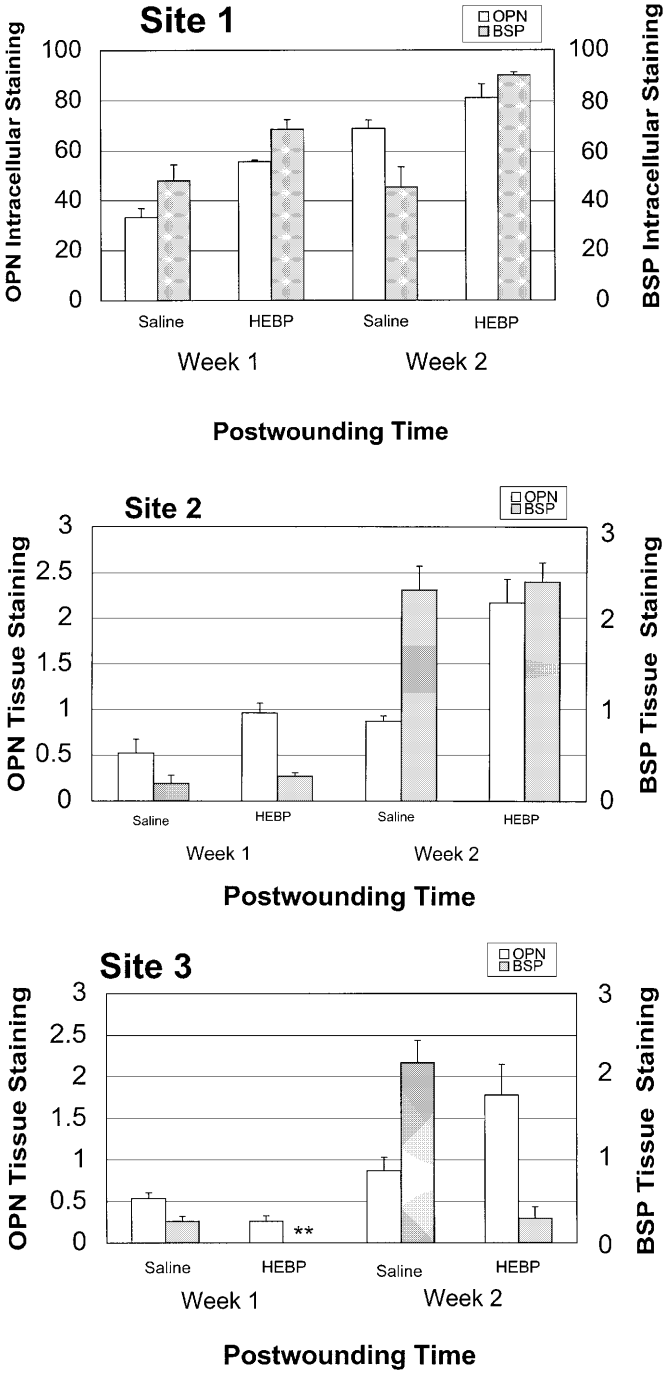


Fig. 8a–c Semiquantitative assessment of OPN (mean staining ± SEM) and BSP (mean staining ± SEM) in sites indicated. No detectable staining is denoted by **. Note increased OPN and BSP staining at sites 1 and 2 in the HEBP-treated animals. For site 1, data represent the percentage of cells that contain intracellular staining for either OPN or BSP (mean ± SEM)

ized tissue-forming cells (Bianco et al. 1991; Chen et al. 1991, 1992). Notably, HEBP-treated animals exhibited increased expression for OPN at sites adjacent to the wound margin, demonstrating that HEBP may promote osteoblast differentiation. In animals treated with HEBP for 1 week there were greater numbers of OPN-stained cells which were confined to the bone compartment of the wound. These cells presumably originated from the surrounding bone but did not undergo extensive proliferation as suggested by the ^{3}H-thymidine data shown here and earlier (Lekic et al. 1996b). In the central compartment of the wound, OPN and BSP expression were significantly reduced in animals treated with HEBP for 1 week, suggesting that either the migratory capacity and/or the proliferation of precursor cells was reduced. Expression of BSP, a known nucleator of mineralization (Kasugai et al. 1991), was completely absent in the central portion of the wound for the 1st week. This is consistent with the findings of an initial inhibitory effect of HEBP on mineralization (Klein et al. 1998; Leyhausen et al. 1998), whereas mineralization of osteoid will eventually occur either with prolonged HEBP treatment or with cessation of the same in vivo (King et al. 1971) or in vitro (Tenenbaum et al. 1992) as we observed at 2 weeks in the present report. It is also interesting to note that full inhibition of mineralization with HEBP did not occur and that mineralization patterns were not always completely consistent with BSP-staining patterns. This points to the probability that there are multiple mineralization pathways that may not be dependent on BSP. Indeed, in a BSP-null mouse (unpublished observations by Dr. Jane Aubin), there were no obvious changes in bone mineralization. However, we intend to determine how wounds heal in these animals with and without HEBP in future studies.

We conclude that HEBP promotes osteoblast differentiation and matrix-producing activity, but reduces the proliferation and possibly the migration of more primitive osteogenic precursor cells. These alterations in cell metabolism lead to enhanced osteoid and, ultimately, mineralized tissue formation at the periphery of calvarial wounds. Given the stimulatory effects on bone matrix synthesis and the fact that mineralization will still occur in the presence of HEBP (albeit more slowly) and once HEBP treatment is stopped as shown by others (King et al. 1971; Wesselink and Beertsen 1994), our results could have significant implications for therapeutic interventions in which bisphosphonates may be used potentially to promote integration of endosseous implants or closure of osseous defects following injuries. However, it must also be emphasized that the biomechanical properties of the bone formed in the non-weight-bearing calvaria may be such that it would not confer a biomechanical advantage over control. Thus, further studies focusing on the biomechanical characteristics of bone formed with HEBP treatment need to be done before this can be used clinically.

Acknowledgements We thank Monique Berard for the figure preparations.

References

Asahina I, Sampath KT, Nishimura I, Hauschka PV (1993) Human osteogenic protein-1 induces both chondroblastic and osteoblastic differentiation of osteoprogenitor cells derived from newborn rat calvaria. J Cell Biol 123:921–933

Averbuch SD (1993) New bisphosphonates in the treatment of bone metastasis. Cancer 72:3443–3452
Bianco P, Fisher LW, Young MF, Termine JD, Robey PG (1991) Expression of bone sialoprotein (BSP) in developing human tissues. Calcif Tissue Int 49:421–426
Blomqvist C, Eloma (1996) Bisphosphonate therapy in metastatic breast cancer. Acta Oncol 35:81–83
Chen J, Shapiro HS, Wrana JL, Reimers S, Heersche JNM, Sodek J (1991) Localization of bone sialoprotein (BSP) expression to the site of mineralized tissue formation in fetal rat tissues by in situ hybridization. Matrix 11:133–143
Chen J, Shapiro HS, Sodek J (1992) Developmental expression of bone sialoprotein mRNA in rat mineralized connective tissues. J Bone Miner Res 8:987–997
Delmas PD, Meunier PJ (1997) The management of Paget's disease of bone. N Engl J Med 335:1016–1021
Denhardt DT, Guo X (1993) Osteopontin: a protein with diverse functions. FASEB J 7:1475–1482
Fleisch H (1989) Bisphonates: a new class of drugs in diseases of bone and calcium metabolism. In: Brunner KW, Fleisch H, Senn HJ (eds) Bisphosphonates and tumor osteolysis. Springer, Berlin Heidelberg New York, pp 1–29
Fleisch H (1991) Bisphosphonates: pharmacology and use in the treatment of tumour-induced hypercalcaemic and metastatic bone disease. Drugs 42:919–944
Frank JD, Balena R, Masarachia P, Sedor JG, Cartwright ME (1993) The effects of three different demineralization agents on osteopontin localization in adult rat bone using immunohistochemistry. Histochemistry 99:295–301
Giuliani N, Pedrazzoni M, Passeri G, Girasole G (1998) Bisphosphonates inhibit IL-6 production by human osteoblast-like cells. Scand J Rheumatol 27:38–41
Gould TRL, Melcher AH, Brunette DM (1980) Migration and division of progenitor cell populations in periodontal ligament after wounding. J Periodontal Res 15:20–42
Goziotis A, Sukhu B, Torontali M, Dowhaniuk M, Tenenbaum HC (1995) Effects of bisphosphonates APD and HEBP on bone metabolism in vitro. Bone 16:317–327
Hsieh SC, Graves DT (1998) Pulse application of platelet-derived growth factor enhances formation of a mineralizing matrix while continuous application is inhibitory. J Cell Biochem 69:169–180
Igarashi K, Hirafuji M, Adachi H, Shinoda H, Mitani H (1997) Effects of bisphosphonates on alkaline phosphatase activity, mineralization and prostaglandin E_2 synthesis in the clonal osteoblast-like cell line MC3T3-E1. Prostaglandins Leukot Essent Fatty Acids 56:121–125
Kasugai S, Todescan R, Nagata T, Yao KL, Butler WT, Sodek J (1991) Expression of bone matrix proteins associated with mineralized tissue formation by adult rat bone marrow cells in vitro: inductive effects of dexamethasone on the osteoblastic phenotype. J Cell Physiol 147:111–120
Kenley R, Marden L, Turek T, Jin L, Ron E, Hollinger JO (1994) Osseous regeneration in the rat calvarium using novel delivery systems for recombinant human bone morphogenic protein-2 (rhBMP-2). J Biomed Mater Res 28:1139–1147
King WR, Francis MD, Michael WR (1971) Effect of disodium ethane-1-hydroxy-1,1-diphosphonate on bone formation. Clin Orthopaed Rel Res 78:251–270
Klein BY, Ben-Bassat H, Breuer E, Solomon V, Golomb G (1998) Structurally different bisphosphonates exert opposing effects on alkaline phosphatase and mineralization in marrow osteoprogenitors. J Cell Biochem 68:186–194
Langille RM, Solursh M (1990) Formation of chondrous and osseous tissues in micromass cultures of rat frontonasal and mandibular ectomesenchyme. Differentiation 44:197–206
Lekic P, Sodek J, McCulloch CAG (1996a) Osteopontin and bone sialoprotein expression in regenerating rat periodontal ligament and alveolar bone. Anat Rec 244:50–58
Lekic P, Sodek J, McCulloch CAG (1996b) Relationship of cellular proliferation to expression of osteopontin and bone sialoprotein in regenerating rat periodontium. Cell Tissue Res 285:491–500
Lekic P, Rubbino I, Krasnoshtein F, Cheifetz S, McCulloch CAG, Tenenbaum H (1997) Bisphosphonate modulates proliferation and differentiation of rat periodontal ligament cells during wound healing. Anat Rec 247:329–340
Levy FE, Hollinger JO, Szachowicz EH (1994) Effect of a bioresorbable film on regeneration of cranial bone. Plast Reconstr Surg 93:307–311
Leyhausen G, Lorenz B, Zhu H, Geirtsen W, Bohnensack R, Muller WEG, Schroder HC (1998) Inorganic polyphosphate in human osteoblast-like cells. J Bone Miner Res 13:803–812
Malpe R, Baylink DJ, Linkhart TA, Wergedal JE, Mohan S (1997) Insulin-like growth factor (IGF)-I, -II, IGF binding proteins (IGFBP)-3, -4, and –5 levels in the conditioned media of normal human bone cells are skeletal site-dependent. J Bone Miner Res 12:423–430
Marden LJ, Quigley NC, Reddi AH, Hollinger JO (1993) Temporal changes during bone regeneration in the calvarium induced by osteogenin in critical size calvarial defects. Calcif Tissue Int 53:262–268
Marden LJ, Hollinger JO, Chaudhari A, Turek T, Schaub RG, Rone E (1994) Recombinant human morphogenic protein-2 is superior to demineralized bone matrix in repairing craniotomy defects in rats. J Biomed Mater Res 28:1127–1138
Masarachia P, Weinreb M, Balena R, Rodan GA (1996) Comparison of the distribution of ^{3}H-alendronate and ^{3}H-etidronate in rat and mouse bones. Bone 19:281–290
McCulloch CAG, Melcher AH (1983) Cell migration in the periodontal ligament of mice. J Periodontal Res 18:339–352
McCulloch CAG, Barghava U, Melcher AH (1989) Cell death and the regulation of populations of cells in the periodontal ligament. Cell Tissue Res 255:129–138
McLean IW, Nakane PK (1974) Periodate-lysine-paraformaldehyde fixative: a new fixative for immunoelectron microscopy. J Histochem Cytochem 22:1077–1083
Rajshankar D, McCulloch CAG, Tenenbaum HC, Lekic PC (1998) Osteogenic inhibition by rat periodontal ligament cells: modulation of bone morphogenetic protein-7 activity in vivo. Cell Tissue Res 294:475–483
Rutherford RB, Sampath TK, Rueger DC, Taylor TD (1992) Use of bovine osteogenic protein to promote rapid osseointegration of endosseous implants. Int J Oral Maxillofac Implants 7: 297–301
Sodek J, Chen J, Nagata T, Kasugai S, Todescan R, Li IWS, Kim RH (1995) Regulation of osteopontin expression in osteoblasts. Ann N Y Acad Sci 760:223–239
Tenenbaum HC, Torontali M, Sukhu B (1992) Effects of bisphosphonates and inorganic pyrophosphate on osteogenesis in vitro. Bone 13:249–255
Todescan R, Sodek J, Davies JE (1994) Reconstituted collagen produces different healing reactions in bony and soft tissue peri-implant compartments. Int J Oral Maxillofac Implants 9:298–304
Torontali M, Koo I, Holmyard DP, Tomlinson G, Grynpas MD, Tenenbaum HC (1994) Backscattered electron image assessment of mineral density in bone formed in vitro. Scanning Microsc Intl 4:125–134
Turnbull RS, Freeman E (1974) Use of wounds in parietal bone of the rat for evaluating bone marrow for grafting into periodontal defects. J Periodontal Res 9:39–43
Van-Beek E, Hoekstra M, Van de Ruit M, Lowik CWGM, Papapoulos SE (1994) Structural requirements for bisphosphonate actions in vitro. J Bone Miner Res 9:1875–1882
Wesselink PR, Beertsen W (1994) Ankylosis of the mouse molar after systemic administration of 1-hydroxyethylindene-1,1-bisphosphonate (HEBP). J Clin Periodont 21:465–471
Yao KL, Todescan R, Sodek J (1994) Temporal changes in matrix protein synthesis and mRNA expression during mineralized tissue formation by adult rat bone marrow cells in culture. J Bone Miner Res 9:231–240