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Relationship of cellular proliferation to expression of osteopontin and bone sialoprotein in regenerating rat periodontium

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Abstract. Cellular repopulation was studied in a model in which adjacent mineralising and soft connective tissue matrices are regenerated. Window wounds were created through alveolar bone, with either preservation or removal of periodontal ligament, in 30 male Wistar rats. Three animals per time period were killed on days 1, 3, 7, 14, and 28 after surgery for each wound type. Cellular proliferation in alveolar bone and periodontal ligament was assessed by ^{3}H-thymidine labelling 1 h before death, followed by radioautographic analysis. Cellular differentiation was determined by the temporal expression of the bone-related markers osteopontin and bone sialoprotein, using immunohistochemical methods. In regenerating periodontium, osteopontin was expressed earlier than bone sialoprotein, which was restricted to alveolar bone. After wounding, transient expression of osteopontin was detected in the periodontal ligament at days 1 and 3. In general, wounding induced fivefold higher proliferation and clonal growth of periodontal ligament cells compared to the unwounded (control) side. Combined immunostaining and radioautography demonstrated colocalisation of osteopontin in sites with high numbers of labelled cells in both nascent periodontal ligament and regenerating alveolar bone at days 3 and 7. In contrast, bone sialoprotein, which appeared in regenerating alveolar bone on days 14–28 after wounding, was expressed much later than the peak of cellular proliferation. We conclude that (1) the intact periodontal ligament influences cell proliferation and osteopontin expression; (2) osteopontin is an early marker of periodontal tissue regeneration that is temporally and spatially associated with intensive cell proliferation and migration in osteogenic and periodontal ligament cell populations; and (3) bone sialoprotein is expressed after proliferation at sites of mineralising bone formation.

Key words: Periodontium – Osteopontin – Bone sialoprotein – Cell proliferation – Rat (Wistar)

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Introduction

Identifying mechanisms that regulate the migration, proliferation, and differentiation of cell populations is essential for an understanding of basic processes determining connective tissue homeostasis and regeneration. The periodontium is a useful model for studies of connective tissue homeostasis as it contains cell populations that synthesise and remodel soft and mineralising connective tissues. Notably, the periodontal ligament (PL) is a dense, fibrous, and highly vascular connective tissue interposed between two mineralised tissues, the cementum of the tooth root and alveolar bone. The PL contains populations of osteoblasts, fibroblasts, and cementoblasts which are confined within the narrow dimensions of this tissue and which are capable of synthesising bone, fibrous connective tissue, and cementum, respectively. These tissues are critical for tooth anchorage, support, proprioception, and absorption of physical stresses associated with mastication.

Cell kinetic experiments in rodent molar teeth have shown that PL cells comprise a renewal system in steady state (McCulloch and Melcher 1983a; McCulloch et al. 1989). The fibroblast progenitor population undergoes extensive turnover in the maintenance of the cellular steady state (McCulloch and Melcher 1983b) and is located in the middle of the PL adjacent to blood vessels (Gould et al. 1977; McCulloch and Melcher 1983a). Studies using homograft recombinations of enamel organs and papillary, pulpal, and follicular mesenchyme (Palmer and Lumsden 1987), as well as cell kinetic experiments (Roberts and Chase 1981; McCulloch and Melcher 1983b), have revealed that the tooth-related PL contains cementoblast precursors and the bone-related PL contains osteoblast precursors. Although a significant proportion of PL cells is likely osteogenic (Roberts and Chase 1981; Cho et al. 1992), bone formation by these cells is normally inhibited since the width of the PL is preserved over the lifetime of a mammal (McCulloch and Melcher 1983a). Paracrine inhibition of osteogenesis by PL fibroblasts has been suggested as a possi-

ble regulatory mechanism that preserves PL width (Line et al. 1974; Ogiso et al. 1991, 1992) and that helps maintain the domains of the osteoblastic, fibroblastic, and cementoblastic lineages.

In mineralised tissue formation, several non-collagenous matrix proteins, including osteopontin (OPN), osteocalcin, alkaline phosphatase, SPARC (osteonectin), and bone sialoprotein (BSP), are secreted by differentiating osteogenic cells. Analysis of the expression of these proteins has been used to identify discrete stages in the formation of bone in vivo (Yoon et al. 1987; Chen et al. 1992) and in vitro (Lian and Stein 1992; Yao et al. 1994). However, application of this approach to the study of PL cells in steady-state conditions has limitations due to an overlap in the temporal expression of OPN and BSP, the heterogeneity of PL cell populations (McCulloch and Bordin 1991), and the existence of cells at different stages of differentiation (Aubin et al. 1992). If PL cells could be stimulated to proliferate synchronously, then the temporal expression of matrix proteins could be more accurately related to the developmental stage of nascent cell populations in repopulating wound sites. In this context the periodontal window-wound model developed by Melcher (1970) facilitates study of PL cell populations as a synchronous cohort of proliferating cells that is generated shortly after wounding (Gould et al. 1980). Further, it is possible to delete selectively portions of the PL so that the regulation of osteogenic cells by adjacent soft connective tissue cells can be studied. In this report we examine the relationship between the proliferation and differentiation of cells that repopulate the PL in the periodontal window-wound model. We have labelled proliferating cells with ^{3}H-thymidine and used specific antibodies to OPN and BSP to identify secreted proteins associated with differentiating cells.

Materials and methods

Wound model

Thirty male Wistar rats weighing 110–130 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:12 h light:dark cycle. Surgery was performed between 10:00–12:00 hours on the 3rd day after the arrival of animals. Periodontal window wounds (Gould et al. 1977; Lekic et al. 1996) were created by removal or preservation of PL under general anaesthesia (Halothane, N_2O). The rationale for preservation or removal of the PL was to provide a simple method by which to assess the relative importance of the PL in the repopulation response in healing periodontal wounds. Animals were observed closely and warmed for 1 h after the surgery. Three animals per time period were killed for each type of wound by N_2O asphyxiation on days 1, 3, 7, 14, and 28 after surgery. An hour prior to sacrifice, rats were injected IP with 1 μCi/g body weight ^{3}H-thymidine (specific activity=20 Ci/mmol; NEN, Oakville, Ont., Canada) diluted with PBS (pH 7.4) to 2.0 ml.

Tissue preparation

Immediately after killing, the lower right mandibles were removed and separated between the mid-incisor and third molar areas to facilitate fixation and decalcification. Tissues were fixed in periodate-lysine-paraformaldehyde (McLean and Nakane 1974) at pH 7.4 for 24 h at 4°C, demineralised for 24 h in 0.2 N HCl, and washed in PBS for 20 h. Mandibles were trimmed to include first and second molar regions and dehydrated in graded ethanol, cleared in toluene, and embedded in paraffin. Frontal sections (5 μm in thickness) were attached to glass slides previously etched with 50% (v/v) nitric acid. Every tenth section was used for histology to assess the site of the window wound.

Immunohistochemistry

Sections were prepared for immunostaining using monoclonal antibodies to rat OPN and BSP (Developmental Studies Hybridoma Bank, University of Baltimore, School of Medicine, Baltimore, Md., USA) as described previously (Lekic et al. 1996). The intensity of OPN and BSP staining was classified by visual inspection. The localisation of proteins was studied in the alveolar bone, in the periodontal ligament, and in a zone of periodontal ligament adjacent to alveolar bone, as described in detail for the labelling indices. Tabular depiction of these data was obtained from assessment of the modal staining intensity from at least nine sections from three different animals for each time and site.

Radioautography

Sections attached to glass 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. After exposure the slides were developed in Kodak D-19 developer and stained with haematoxylin and eosin through the emulsion. For some specimens, sections that had been stained previously for immunohistochemistry were prepared for radioautography as described above.

^{3}H-thymidine labelling and assessment of clonal growth

The middle section in each ten-section ribbon was examined with a light microscope at ×250. All measurements were made with an intraocular grid system (250 μm×250 μm) containing 100 squares of 625 μm^2 each. Using methods described earlier (McCulloch and Melcher 1983b), a cell was considered to be labelled if more than five silver grains overlaid its nucleus (P<0.001). Haematopoietic and endothelial cells were excluded from total cell counts. Endothelial cells were distinguished by their squamous shapes, their locations adjacent to the lumina of blood vessels, and the bulging of their nuclei into the lumina.

Counts of labelled and unlabelled cells in seven sites (62 500 μm^2) from each section were obtained for each slide to assess proliferation in each zone. The areas for analysis were the PL at the margin of wounded and intact PL (site 1); a location just peripheral to the margin of wounded alveolar bone (site 2); the PL adjacent to the tooth (site 3); the middle of the wound bounded by alveolar bone (site 4); the PL 250 μm superior to the wound edge (site 5); the PL on the unwounded side of the periodontium (site 6); and the alveolar bone on the unwounded side (site 7). The labelling index for each zone was calculated: number of labelled cells/number of total cells×100. To assess clonal growth of PL progenitors, labelled nuclei located within 25 μm of one another were considered to have arisen from a common precursor and were designated as clustered (McCulloch and Melcher 1983b). The percentage of clustering for each zone was calculated: number of clustered cells/number of labelled cells×100.

Morphometric assessment of new tissues

Three sections for each animal of immunostained and radiolabelled periodontal wounds with either removed PL or preserved PL from the first and last experimental days were analysed

morphometrically. Slides were coded so that the time of sacrifice and the type of wound were unknown at the time of measurement. Image analysis was used to firstly assess the area of debris in the preserved PL after the removal of alveolar bone (day 1). Secondly, the area of the drilled bone (day 28) was measured by digitising the reversal line in the bone at the cut margin. This measure provided an estimate of the original wound outline for each animal at each sacrifice time. These two areas were then expressed as a mean±SE or as the percentage of the maximum area for each time point (mean±SE of the percentage).

Statistical methods

The raw labelling index and clustering index data were kept separate, and the means were calculated for each animal, type of surgery, sacrifice day, and examined site. The mean from each animal was considered as an independent sample, and these data were then assessed by analysis of variance. Differences of labelling index and clustering index between zones were examined by ANOVA and were considered significant at $P<0.05$. Data were expressed as mean±SE.

Results

Histological examination demonstrated that window wounds with either removal or preservation of the PL healed spontaneously by day 28 after surgery and exhibited architecture that was similar to unwounded tissue (Fig. 1A–E). Image analysis showed that the percentage area of the debris after wounding with preservation of PL was 11.8±0.3% and that the percentage of cells in the PL with normal histological appearance (i.e., not pycnotic) was 91.3±0.5% (Fig. 1C). Notably, by day 3, there was no evidence of debris in wounds with preserved PL. Periodontal window wounds exhibited nearly complete restoration of the alveolar bone, the PL, and the cementum on the wounded site. Newly formed alveolar bone in wounds with removal of PL exhibited somewhat larger trabecular spaces than wounds with preservation of PL (Fig. 1D,E). Moreover, image analyses revealed that in wounds with removal of PL the alveolar bone was thinner in a bucco-lingual dimension and occupied less area than in wounds with preservation of PL [121 015±3202 μm^2 (removed) and 146 275±4106 μm^2 (preserved); $P<0.01$]. Despite these differences, the organisation of the PL was similar to unwounded control sites (Fig. 1D,E).

Effect of wounding on proliferation and clonal growth of periodontal ligament cells

Assessment of the percentage of ^{3}H-thymidine-labelled cells (labelling index) and the proportion of these labelled cells that were clonally distributed (clustering in-

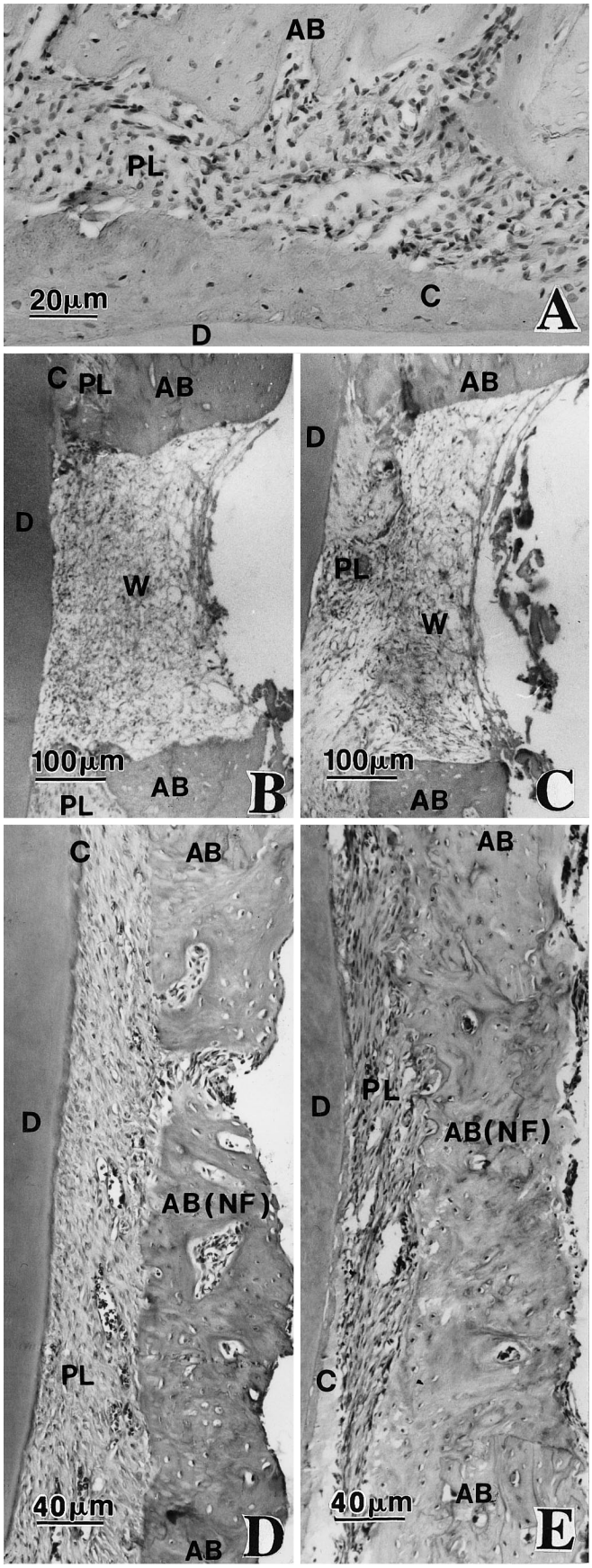

Fig. 1. Micrographs of control side (**A**) and window wounds at day 1 (**B, C**) and day 28 (**D, E**) after surgery with removed (**B, D**) or preserved (**C, E**) periodontal ligament. *AB*, Alveolar bone; *AB(NF)*, newly formed alveolar bone; *C*, cementum; *D*, dentin; *PL*, periodontal ligament; *W*, wound. Haematoxylin and eosin. ×500 (**A**), ×100 (**B, C**), ×250 (**D, E**)

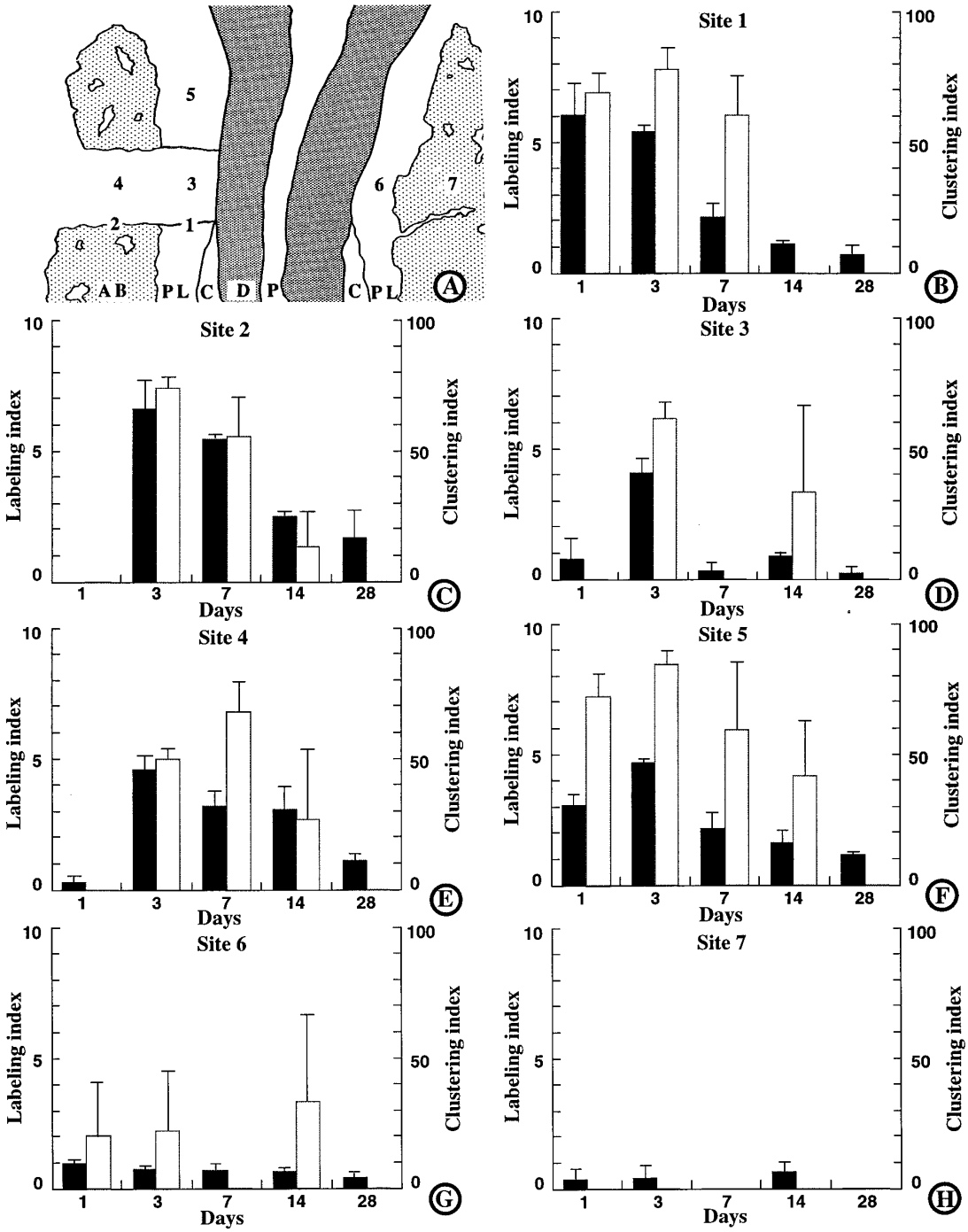

Fig. 2A–H. ^{3}H-labelling (*solid bars* labelling index) and clustering (*open bars* clustering index) of cells at wound sites (*1–5*) and control sites (*6, 7*) with removal of periodontal ligament (days 1–28 after surgery). *AB*, Alveolar bone; *C*, cementum; *D*, dentin; *P*, pulp; *PL*, periodontal ligament

dex) provided a quantitative approach to assess the proliferative behaviour of progenitor cells and their spatial distribution in the repopulation response of wounded PL. Regardless of wound type, labelling and clustering indices of the contralateral (unwounded) side [Figs. 2, 3 (sites 6, 7)] revealed similar, low values throughout the experimental period. The most intense proliferative response in the PL to wounding was noted at days 1 and 3 at the cut PL margin [Fig. 2 (site 1)]. High labelling and clustering indices were also found on day 1 at sites 250 μm away from the wound margin [Fig. 2 (site 5)] and reached maximal levels at day 3.

At days 3 and 7, both types of wounds revealed significantly higher (P<0.001) labelling and clustering indices in the PL [Figs. 2, 3 (sites 1, 3, 5)] than the control side [Figs. 2, 3 (site 6)]. Adjacent to the tooth root (site 3), after initially intense proliferation at day 3, the labelling indices sharply decreased (by tenfold) at day 7 and thereafter. This large reduction of labelling index in the middle of the repopulating PL was seen in both types of wounds.

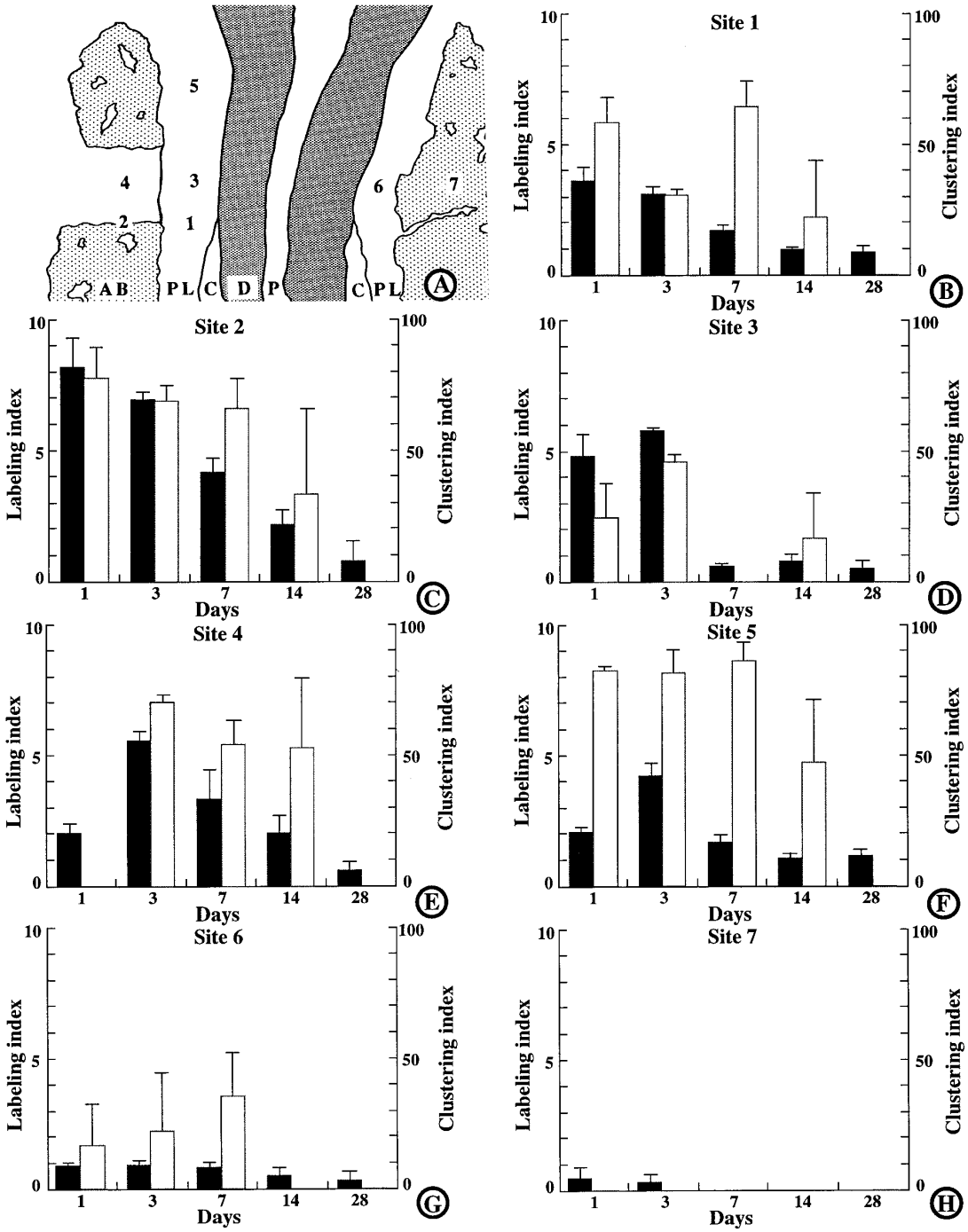

Fig. 3A–H. ^{3}H-labelling (*solid bars* labelling index) and clustering (*open bars* clustering index) of cells at wound sites (*1–5*) and control sites (*6, 7*) with preservation of periodontal ligament (days 1–28 after the surgery). *AB*, Alveolar bone; *C*, cementum; *D*, dentin; *P*, pulp; *PL*, periodontal ligament

Periodontal ligament modulation of cell proliferation

Shortly after wounding, we detected large and significant differences in labelling and clustering indices that were dependent on the type of wound. At the cut PL margin, in wounds involving the removal of PL, there was a twofold higher labelling index [$P<0.01$ (days 1 and 3)] and clustering index (day 3) compared to wounds with preserved PL [Figs. 2, 3 (site 1)]. At more advanced stages of healing (after day 7), there were no significant differences in labelling and clustering indices between the two types of wounds ($P>0.05$). Similarly, when the PL was preserved, the labelling index at day 1 in site 5, which was 200 μm away from the wound margin, was significantly ($P<0.05$) lower than wounds with removed PL at the same site [Fig. 3 (site 5)]. However, from day 3 onwards, labelling indices were essentially the same at this site regardless of the type of wound [Figs. 2, 3 (site 5)]. In contrast to wounds with preserved PL, wounds with removed PL did not exhibit significant

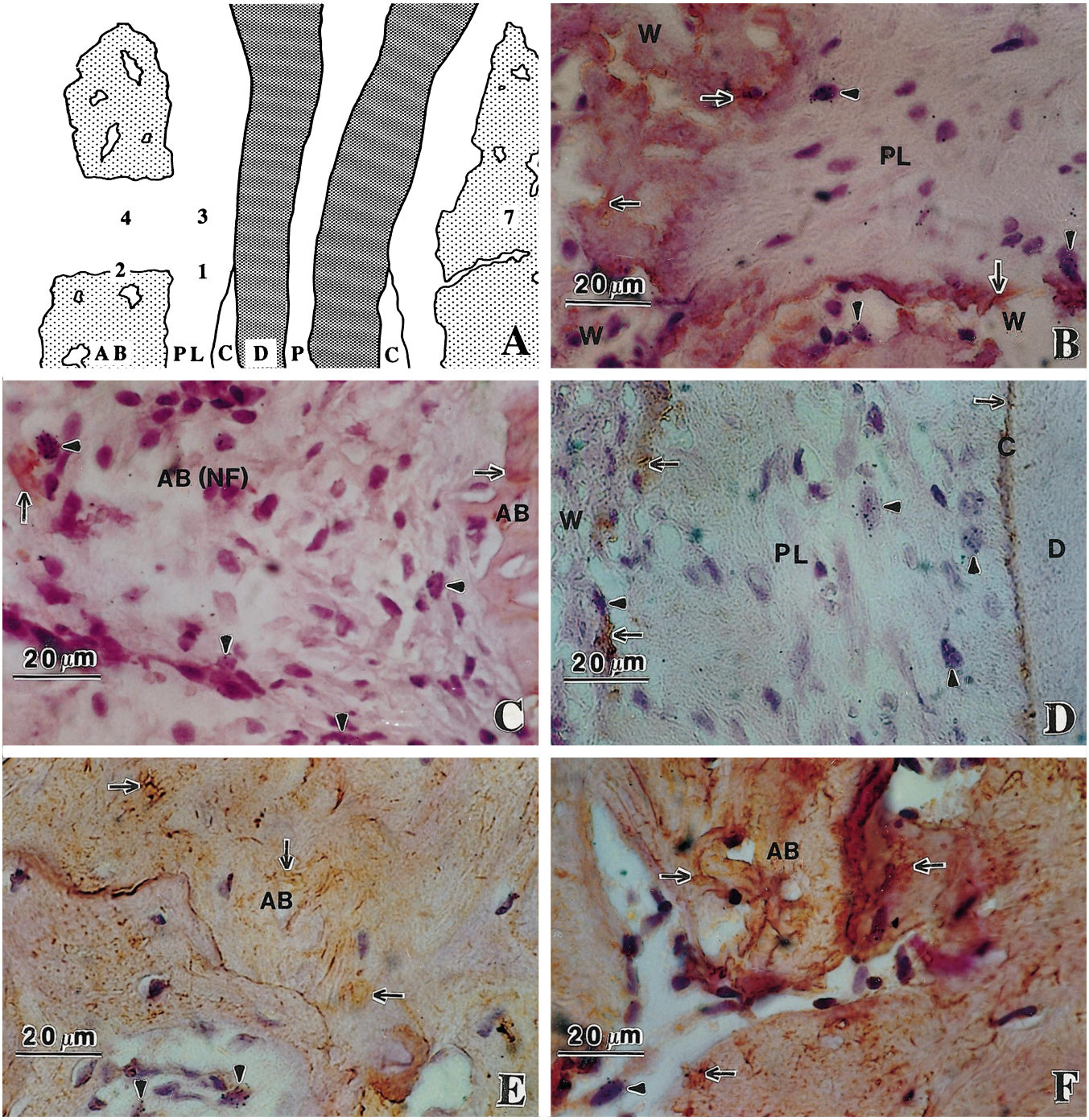

Fig. 4A–F. Combined radioautography for detection of ^{3}H-thymidine (*arrowheads*) and immunohistochemistry for localisation of either osteopontin or bone sialoprotein in regenerating periodontal ligament and alveolar bone with removal or preservation of periodontal ligament. **A** Sampling sites (*1–4*, *7* sites 1–4, 7). **B** Osteopontin expression (*arrows*) at the cut margins (site 1) of surrounding periodontal ligament at day 1 after wounding with removal of periodontal ligament. Note ^{3}H-thymidine labelling of cells in periodontal ligament adjacent to the wound area. **C** Osteopontin expression (*arrows*) in early stages of alveolar bone formation (site 2) at day 3 after the surgery with removal of periodontal ligament. **D** At day 3 after wounding with preservation of periodontal ligament, osteopontin expression (*arrows*) was present on margins of the ligament (site 3) and cementum. **E** Strong bone-sialoprotein expression (*arrows*) in newly formed alveolar bone (site 4) at day 28 after wounding with preservation of periodontal ligament. **F** Intact alveolar bone at the control site (site 7) shows intense bone-sialoprotein expression (*arrows*). *AB*, Alveolar bone; *AB(NF)*, newly formed alveolar bone; *C*, cementum; *D*, dentin; *P*, pulp; *PL*, periodontal ligament; *W* wound. ×1000

Table 1. Qualitative estimates for expression of osteopontin (*OPN*) and bone sialoprotein (*BSP*)

Time after wounding	Site 1		Site 2		Site 3		Site 4		Site 7	
	OPN	BSP	OPN	BSP	OPN	BSP	OPN	BSP	OPN	BSP
Removal of periodontal ligament										
Day 1	+++	–	–	–	–	–	–	–	++	+++
Day 3	++	–	++	–	++	–	++	–	++	+++
Day 7	–	–	++	–	–	–	++	–	++	+++
Day 14	–	–	++	+++	–	–	++	+++	++	+++
Day 28	–	–	++	+++	–	–	++	+++	++	+++
Preservation of periodontal ligament										
Day 1	+++	–	–	–	++	–	–	–	++	+++
Day 3	+++	–	++	–	+++	–	++	–	++	+++
Day 7	–	–	++	+	–	–	++	–	++	+++
Day 14	–	–	++	+++	–	–	++	+++	++	+++
Day 28	–	–	++	+++	–	–	++	+++	++	+++

– Negative, + weak, ++ moderate, +++ intense

numbers of labelled cells at day 1 adjacent to the cut bone surface (site 2) or at the exposed root surface (site 3). By day 3, there was no difference.

Bone-cell proliferation

Following removal of PL there was little detectable proliferative activity in cells adjacent to the wounded alveolar bone or in the middle of the wound hole at day 1 after surgery [Fig. 2 (sites 2, 4)]. With preserved PL, day 1 after wounding at sites adjacent to the surrounding unwounded bone margin there were very few repopulating cells. However, a high percentage of these cells were proliferating since the labelling index was found to be very high [Fig. 3 (site 2)]. From day 3 onwards, there was a gradual decrease in proliferation of the cells repopulating alveolar bone regardless of the type of wound [Figs. 2, 3 (sites 2, 4)]. Nonetheless, there was a sustained higher level of cellular proliferation within the newly formed alveolar bone in both types of experimental wounds, and at day 28, the labelling index was still significantly higher than that on the control side [Figs. 2, 3 (sites 2, 4) and Figs. 2, 3 (site 7); $P<0.001$].

Relationship of periodontal ligament preservation to osteopontin and bone-sialoprotein expression

Within wound sites involving removed or preserved PL, transient OPN staining (days 1 and 3 only) was observed on the cut PL margins (Fig. 4A,B; Table 1) but this staining was not associated with any recognisable tissue debris. Transient and weak OPN (but not BSP) expression was noted in the PL 200 μm away from the wound margin (days 1 and 3). In the middle of the wound adjacent to the root surface (Fig. 4A,D; Table 1), specimens with removed PL exhibited OPN staining at days 1 and 3 while wounds with removed PL showed only transient OPN expression at day 3. OPN staining was observed in early stages of alveolar bone formation (day 1) right on the cut bone surfaces, but adjacent to this bone surface there was no detectable OPN staining until day 3 (Fig. 4C; Table 1). There was no BSP staining in the PL wound site regardless of preservation or removal of PL except in discrete areas where dentin or cementum were damaged by drilling. Wounds involving removal or preservation of PL exhibited OPN staining prior to BSP staining in regenerating alveolar bone. During alveolar bone regeneration (days 14 and 28), BSP staining appeared in new bone (Fig. 4A,E; Table 1) and was similar in intensity to the unwounded alveolar bone (Fig. 4A,F). Transient and occasional expression of OPN in the PL of unwounded tissue was noted only at high magnification (×2500) and in the proximity of proliferating PL cells.

Spatial relationship of osteopontin and bone-sialoprotein expression to cell proliferation

Light-microscopic radioautographs with combined OPN immunostaining demonstrated large numbers of labelled cells at the cut wound margin of the PL beginning at day 1 after the surgery [Fig. 2 (site 1)] compared to the corresponding control side of the PL and in the alveolar bone [Fig. 2 (site 7)]. Frequently, we noted focal and intense cell proliferation in the vicinity of OPN staining. In wounds with removal of PL, there was intense OPN expression at days 1 and 3 in the PL at the cut wound margin (Fig. 4A,B; Table 1), the same site in which a sixfold increase in labelling index compared to the control side was observed (Fig. 2 [site 1]). At day 3, high labelling indices [Fig. 2 (site 3)] and intense OPN expression were also detected transiently in the middle of the repopulated PL (Table 1), but both labelling index and OPN expression decreased sharply thereafter. Within the newly formed bone, OPN expression (Table 1) and high labelling indices [Fig. 2 (site 4)] were observed at days 3 and 7. The expression of BSP appeared later than the initial OPN expression (Table 1), and the appearance of BSP staining was contemporaneous with decreased labelling indices [Table 1 (days 14 and 28)].

In wounds with preserved PL, there was intense expression of OPN at days 1 and 3 (Table 1) and high labelling indices [Fig. 3 (site 3)]. In the newly formed alveolar bone, OPN appeared at day 3 and thereafter (Table 1) at the site adjacent to very high labelling indices [Fig. 3 (site 4)]. However, in site 2, although there was OPN staining on the cut bone margin, there was no OPN detectable adjacent to the bone margin, and in this site, labelling indices were high. As in wounds with the removal of PL, BSP appeared after the expression of OPN (Table 1) and was associated temporally with decreased labelling indices [Fig. 3 (site 4)].

Discussion

Osteopontin and bone-sialoprotein expression

We have studied the appearance and localisation of two non-collagenous proteins whose expression has been associated previously with discrete stages of mineralised tissue formation (Chen et al. 1992; Yao et al. 1994). Here we have shown the expression of these proteins in the regeneration of rat periodontium. The detection of OPN in both mineralised and soft regenerating periodontal tissues at early stages of wounding healing is consistent, in part, with expression of this protein by differentiating mineralised tissue-forming cells. OPN is part of a cement-like layer on the surface of damaged bone (McKee and Nanci 1995) and is first expressed in rat tissues by pre-osteoblastic cells (Mark et al. 1987; Chen et al. 1992) as a 55-kDa protein (Kubota et al. 1989) prior to mineralised bone formation. In our studies a discrete line of OPN staining was observed, not only on the cut bone surfaces, but also at the severed surface of the PL where it served to define the border of the nascent PL and the wound site. Thus the expression of OPN by PL cells may support the concept that the resident cell populations have osteogenic potential (Roberts and Chase 1981; Cho et al. 1992), a concept that is also supported by the high levels of alkaline phosphatase expressed by many of these cells (Groenveld et al. 1993).

Since OPN is also expressed by non-osteogenic cells (Denhardt and Guo 1993), it is possible that OPN expression in the wounded periodontium is unrelated to osteogenesis. While the restricted temporal and spatial localisation of OPN staining argues against a macrophage/lymphocyte origin (Patarca et al. 1989), expression of OPN by proliferating PL fibroblasts in vitro has been detected with the same antibody used in this study (R. Zohar, C.A.G. McCulloch, and J. Sodek, unpublished data). Thus it is conceivable that the immunostaining could reflect expression by proliferating/migrating PL fibroblasts in vivo. As such, the expression of OPN may be related to the repair of the periodontal ligament by specialised, non-osteogenic cells. This view is consistent with the known heterogeneous nature of PL fibroblast populations (McCulloch and Bordin 1991).

It is also conceivable that some OPN staining could be due to the incomplete removal of bone fragments. In the morphometric analysis, tissue debris occupied a significant portion of wounds with preserved PL. However this debris was not seen at day 3 or thereafter, indicating that the material was rapidly phagocytosed. Further, since no immunostaining for OPN and BSP was associated with the debris, it is unlikely that the transient expression of OPN in wounds was due to bone fragments, which are therefore unlikely to affect local osteogenesis. The debris more likely represents dead or dying blood cells which had migrated into the wound after the removal of the remaining portion of the alveolar bone with the dental probe.

BSP expression occurred after OPN expression and was restricted to the alveolar bone compartment. This finding is consistent with the temporal expression of BSP in bone formation and with the proposed role of BSP as a nucleator of mineralisation (Kasugai et al. 1992; Hunter and Goldberg 1993; Yao et al. 1994). The lack of BSP expression by PL cells also indicates that these cells do not normally express the full repertoire of differentiation-associated proteins in osteogenesis and may explain, in part, the lack of mineralisation of the PL after wounding. Compared to the biphasic expression of OPN, BSP is synthesised only by fully differentiated osteoblasts in association with de novo bone formation and may represent an earlier stage phenotypic marker of osteoblasts in comparison to the 44-kDa OPN and osteocalcin (Chen et al. 1992; Kasugai et al. 1992; Yao et al. 1994).

Repopulation of wound

Analysis of cell proliferation by comparison of labelling and clustering indices demonstrated a threefold-to-fivefold higher percentage of labelled and clustered cells in wounded sites compared to unwounded sites at 24 h after wounding, and these higher levels persisted up to day 7 after surgery. The sustained proliferation and the relatively high proportion of progenitor cells that were clonally distributed suggests a prolonged repopulation response after wounding. Proliferating PL progenitor cells have a paravascular location (Gould et al. 1980; McCulloch 1985) and, at least in wound sites, are derived from adjacent, unwounded periodontal ligament (Gould et al. 1980). Thus our finding of high labelling indices in the unwounded PL adjacent to the wound sites indicates that these proliferating cells likely contribute to the repopulation of the denuded wound site. Our data also showed a transient increase in labelling and clustering indices in the PL only at day 3 in PL-removed wounds, indicating that the increase in the proportion of proliferating cells was likely a result of migration from adjacent unwounded PL or bony sites.

There were very few labelled cells at the cut edge of the alveolar bone or at the denuded root surface in wounds with removed PL at day 1, while in contrast there were high labelling indices in wounds with preserved PL. We found that, although labelling indices were high at this site, the actual numbers of repopulating cells were very low. Thus the repopulating cells were relatively rare but were enriched with progenitors. The

marked difference between PL-preserved and PL-removed wounds almost certainly reflects the expected delay in repopulation of the latter by proliferating fibroblasts. Cell division occurs in adjacent, unwounded tissue and these cells migrate into the wound. Several growth factors, including platelet-derived growth factor and epidermal growth factor, have been shown to have chemotactic activity for PL cells and enhance their proliferation (Terranova and Wikesjo 1988; Wang et al. 1994). We suggest that the increases of labelling indices in the nascent alveolar bone (site 2) at day 1 after wounding with preservation of PL could be explained by the initial proliferation and migration of cells from the PL. Cells from adjacent endosteum-lined spaces could also contribute to the repopulation of nascent alveolar bone (McCulloch et al. 1987). Notably, there was a large reduction in the labelling index of cells in the middle of the PL wound site at day 7 and thereafter, indicating that the migrating cells in the nascent PL stopped proliferating after entry into the wound site. In contrast, cells in the other wound compartments exhibited a more sustained proliferation, suggesting that factors associated with the denuded root surface inhibited cell proliferation. This conjecture is supported by previous findings of very low labelling indices of PL cells in sites adjacent to the root surface and extending outwards for about 30 μm (McCulloch and Melcher 1983a; McCulloch et al. 1989). The nature of this factor is unknown but may include mineral-bound cytokines, such a transforming growth factor-β, which can inhibit fibroblast proliferation.

Relationship of cell proliferation to osteopontin and bone-sialoprotein expression

The initial appearance of OPN on the border of the nascent PL at days 1 and 3 after surgery may reflect the regulatory role of PL cells in defining the location and the temporal sequence of PL and alveolar bone regeneration. Indeed a high percentage of labelled and clustered cells in the nascent PL were temporally and spatially associated with OPN expression. The contemporaneous appearance of OPN at sites of increased labelling and clustering indices suggests that PL cell subpopulations have a relatively high proliferative capacity and express OPN, independent of their osteogenic potential. We think it is unlikely that the labelled cells in the PL are activated macrophages or lymphocytes since they do not proliferate in periodontal window wounds (Gould et al. 1980). As BSP staining was not detected in the PL, we conclude that, in healing wounds, PL cells are incapable of contributing to osteogenesis. Indeed, if they were, the PL would mineralise across its width (Ogiso et al. 1991, 1992). Conceivably, the movements of the tooth roots during masticatory function may inhibit the ingrowth of bone cells into the PL space during the wound-healing process. Analogous phenomena have been observed in healing bone around orthopaedic implants in which small amplitude movements result in colonization of the implant by fibroblasts instead of osteogenic cells (Pilliar et al. 1981) and the formation of a fibrous ligament instead of bone. The actual mechanism of osteogenic inhibition is suggested by earlier work showing that the secretion of prostaglandin E2 by PL fibroblasts inhibits osteogenesis (Ogiso et al. 1991, 1992).

Wounds with preserved PL exhibited expression of OPN in the PL adjacent to the tooth root at day 1 after the surgery while in wounds with removed PL this expression was delayed (day 3), following the appearance of high numbers of proliferating cells. Thus the presence of intact PL influenced both local cell proliferation and the expression of non-collagenous matrix proteins that are associated with osteogenic cells. However, the coupling of OPN expression to proliferation was notably absent in the PL adjacent to the cut edge of the alveolar bone when wounds involved preservation of the PL (day 1). In these sites the few cells that were present were frequently labelled with ^{3}H-thymidine but did not express OPN at day 1 after surgery. By day 3, cells in this site continued to proliferate and expressed OPN. This finding indicates that the PL contains low numbers of proliferating, but OPN-negative, cell populations which are involved in the initial repopulation response at the cut bone surface. Later, during the presumptive differentiation of osteogenic cells and OPN-positive PL cells in the wound site, there was increased deposition of both OPN and BSP into the nascent bone matrix and a decrease in the proportion of labelled cells (days 7–28 after the surgery). These findings show that, in a more complex connective tissue like the PL, there is considerable heterogeneity of OPN expression by PL cells.

The relationship between non-collagenous protein expression and cell proliferation was more clear-cut with respect to BSP, which was expressed only by osteogenic cells. Notably, BSP staining of the newly formed alveolar bone was evident only after there was a significant decrease in the percentage of labelled cells and clustered cells (after day 14), at a time when cell differentiation was more advanced and new bone formation had been initiated. By the end of the experimental period (day 28), BSP expression was still intense, and the labelling and clustering indices were as low as those on the control side, reflecting the final stage of osteoblast differentiation and bone formation and remodelling of the PL.

Collectively these data indicate that the presence of an intact PL regulates the kinetics of cell proliferation and the appearance of OPN in healing wounds. The expression of OPN is not restricted to cells that participate in early stages of bone formation but is also associated with the proliferation status of discrete PL subpopulations. In contrast, BSP (a marker of differentiated osteoblasts) is expressed after OPN, is restricted to osteogenic tissues, and is not associated with cell proliferation.

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