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Temporal and spatial mRNA expression of bone sialoprotein and type I collagen during rodent tooth movement

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SUMMARY To investigate the mechanism of bone formation during tooth movement, *in situ* hybridization was performed with digoxigenin-labelled RNA probes to detect bone sialoprotein (BSP) and type I collagen mRNAs in the dentoalveolar tissue of 72 Sprague–Dawley rats. An elastic band was inserted between the first and second right maxillary molars, and the teeth experimentally moved for 1, 3, and 7 days. The left first maxillary molar was used as the control. For the untreated molars, osteoblasts and osteocytes near the distal surface of the interradicular septum (IRS) expressed a high level of both BSP and type I collagen mRNAs, while cells on the mesial side of the IRS showed a low level of these mRNAs. For the first molars subjected to experimental tooth movement, a high level of type I collagen mRNA expression was found in the osteoblasts on the tension side of the IRS after 1 day of experimental tooth movement. A high level of BSP mRNA was detected after 3 days of experimental tooth movement. However, a negligible amount of both mRNAs was found in cells on the compression side.

These results support the hypothesis that BSP may be involved in mineralization during physiological bone remodelling. On application of orthodontic force, osteoblasts were activated and induced to express BSP mRNA, which is involved in bone remodelling due to orthodontic force. In addition, response to the orthodontic force was observed in osteocytes.

Introduction

Orthodontic tooth movement induces bone formation along the tension area of the periodontal ligament (PDL). Bone formation in orthodontic tooth movement has mainly been studied by histological and biochemical methods (Oppenheim, 1911; Schwarz, 1932; Macapanpan *et al*., 1954; Reitan, 1967). Recently, molecular biology techniques such as *in situ* hybridization have been applied to determine the localization of specific mRNA in dental tissues (Karimbux *et al*., 1992; Lukinmaa *et al*., 1992; Nakagawa *et al*., 1994; Takano-Yamamoto *et al*., 1994; Karimbux and Nishimura, 1995). However, the mechanism of bone formation has not been fully elucidated.

Osteoblasts play a major role in bone formation and produce type I collagen, which is the major organic component of the mineralized bone matrix. Besides collagens, osteoblasts also express many kinds of non-collagenous proteins. In the past two decades the major non-collagenous proteins of bone have been isolated and characterized. These include two sialoproteins (osteopontin and bone sialoprotein), osteonectin, and osteocalcin. All of these are acidic proteins that have a strong affinity for hydroxyapatite and most of these proteins bind calcium ions. Osteonectin and osteopontin are not specific to mineralizing tissues and have been identified in a number of non-mineralizing tissues (Stenner *et al*., 1986; Butler, 1989). While osteocalcin has been shown to be tissue specific, this protein

does not appear to be necessary for tissue mineralization (Price *et al*., 1982). However, osteocalcin appears to be important in bone resorption as a chemoattractant of osteoclast precursors, because its expression is strongly enhanced by 1,25-dihydroxyvitamin D_3 (Glowacki *et al*., 1989). In contrast, bone sialoprotein (BSP) is specifically expressed in cells that are directly involved in the formation of mineralizing connective tissue matrices (Oldberg *et al*., 1988; Fisher *et al*., 1990; Chen *et al*., 1991a,b, 1992, 1994; Sodek *et al*., 1992). Additionally, BSP has the ability to bind hydroxyapatite through its 2–3poly glutamic acid sequences (Kasugai *et al*., 1991; Nagata *et al*., 1991), and the ability to mediate cell attachment through an RGD sequence that is recognized by the vitronectin α_2 receptor (Oldberg *et al*., 1988; Miyauchi *et al*., 1991). BSP seems to play a specific role in mineralization of bone, and this protein may be a useful marker of bone formation.

To date, no study has examined the expression of BSP mRNA in response to tooth movement. Therefore, the mRNA expression of BSP and type I collagen was investigated in dentoalveolar tissue to further elucidate the mechanism of bone formation during orthodontic treatment.

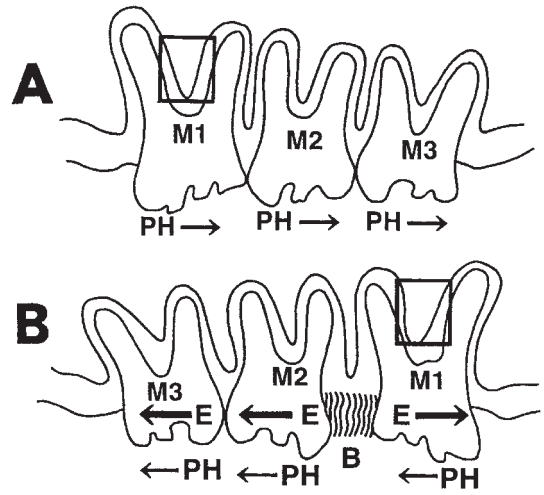

Figure 1 Schematic representation of the rat maxillary molar block. The boxed area was selected for light microscopic observation. (A) The untreated left side was used as the control. (B) The right side was subjected to 7 days of experimental tooth movement. PH (thin arrow), direction of physiological tooth movement; E (thick arrow), direction of experimental tooth movement; B, elastic band; M1, M2, and M3 indicate the first, second, and third maxillary molars, respectively.

Materials and methods

Histological preparations

A total of 72 seven-week-old, male Sprague–Dawley rats weighing 210–250 g were used. According to the method of Waldo (1953), a segment of orthodontic elastic band (1/4-inch 2oz; Unitek/3M, Tokyo, Japan) was inserted in the interproximal space between the maxillary right first and second molars (Figure 1) for 1, 3, or 7 days. The rats were fed a standard laboratory diet (CE-2, Nihon Clea, Tokyo, Japan) and water *ad libitum* throughout the experiment. The left side of the maxilla was used as the control. On the indicated day, the animals were fixed by perfusion of 4 per cent freshly prepared paraformaldehyde in phosphate-buffered saline (PBS, pH 7.2) for 15 minutes under nembutal anaesthesia. The maxillary bone was dissected immediately, immersed in the same fixative overnight at 4ºC, and then demineralized in Morse's solution (22.5 per cent formic acid, 10 per cent sodium citrate) at 4ºC for 6 days. The tissue was dehydrated in an ascending series of ethanol, rinsed in chloroform and embedded in paraffin. Each sample was sliced into 6-μm sections in a sagittal direction, and the sections were mounted on glass slides that had been pre-coated with 3-aminopropyl triethoxysilane. After drying, the mounted sections were stored at 4ºC until use.

Preparation of probe

Rat BSP cDNA (912-base pair Eco RI fragment; Chen *et al*., 1991b), and the cDNA encoding rat type I collagen α_2(I) chain (900-base pair Pst I fragment; Genovese *et al*., 1984) were used. Each cDNA was subcloned into a pBLUESCRIPT vector (Stratagene, La Jolla, CA, USA). The BSP cDNA-containing vector was cleaved by BamHI to produce the antisense probe and this vector was cleaved by EcoRI to produce the sense probe. The type I collagen cDNA-containing

vector was cleaved by BamHI to produce the antisense probe, and this vector was cleaved by HindIII to produce the sense probe. Digoxigenin (DIG)-labelled, single-stranded antisense and sense RNA probes were prepared using a DIG RNA labelling kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. These probes were diluted with Hybridization Solution I (Maxim Biotech, San Francisco, CA, USA) to a final concentration of 700 ng/ml.

Hybridization procedure

The hybridization procedure was a slight modification of that described by Nomura *et al*. (1988) and Okamura *et al*. (1993). Prior to hybridization, the tissue sections were deparaffinized in xylene and rehydrated through a series of graded ethanol and PBS. The slides were fixed by placing them in 4 per cent paraformaldehyde in PBS for 20 minutes. They were then placed in a solution of 1–3 μg/ml proteinase K in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for 10–15 minutes at 37ºC, and then post-fixed by placing them in 4 per cent paraformaldehyde in PBS for 10 minutes. They were then immersed in a solution of 0.25 per cent acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 minutes, and rinsed in an ascending series of ethanol. After air-drying, the tissue sections were hybridized with the probe in a humidified chamber at 42ºC for 16 hours. Following hybridization, the sections were washed in 2 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate) at 42ºC for 30 minutes, and then treated with 10 μg/ml RNase in TNE (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA) at 37ºC for 30 minutes. Additional washes (once in TNE at 37ºC for 10 minutes, once in 2 × SSC at 42ºC for 20 minutes, and twice in 0.5 × SSC at 42ºC for 20 minutes each) were performed.

Detection of hybridized probes

The slides were rinsed with DIG buffer 1 (100 mM Tris-HCl, pH 7.5, 150 mM NaCl), and pre-incubated in the same buffer with 1.5 per cent blocking reagent and 0.1 per cent bovine serum albumin for 1 hour. They were then incubated with alkaline phosphatase-labelled anti-digoxigenin antibody diluted 1:500 with buffer 1 at room temperature (RT) for 45 minutes, and then washed twice in buffer 1 at RT for 15 minutes each. The slides were rinsed with DIG buffer 3 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$), and colour development was achieved with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium salt (NBT), which had been placed in DIG buffer 3. Levamisole (0.24 mg/ml) was added to the reaction mixture to inactivate intrinsic alkaline phosphatase. The colour reaction was allowed to proceed for 6–12 hours and was stopped with TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). Some sections were counter-stained with methyl green. The area of the interradicular septum (IRS) of the first molar was selected for light microscopic observation.

Results

First molar on the untreated control side

Rat molars drift distally under physiological conditions. An intense positive signal of BSP and type I collagen mRNAs was observed in osteoblasts and osteocytes that were adjacent to the distal surface of the IRS, where bone was formed physiologically (Figure 2). Osteocytes that were located at the distal part of the IRS contained a much higher signal intensity of BSP and type I collagen mRNAs than osteocytes that were located at the mesial part of the IRS. The level of expression of the two proteins in osteocytes located between the distal and mesial parts was lower than that in osteocytes at the distal part, and decreased proportionally with the distance from the distal part of the IRS. The rate of decrease of BSP mRNA was greater than that of type I collagen mRNA. Many fibroblast-like cells in the PDL contained type I collagen mRNA and some of these cells contained a small amount of BSP mRNA. Microscopic examination of the mesial surface of the IRS showed a typical image of resorbing bone together with osteoclasts and bone-lining cells. Some of the

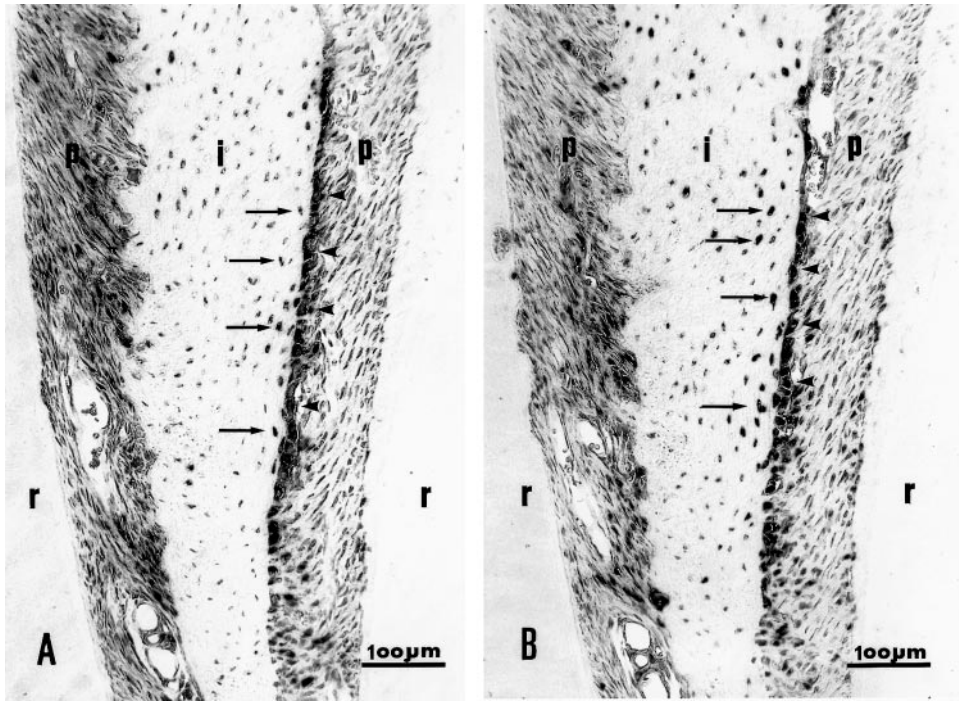

Figure 2 Results of hybridization of BSP and type I collagen RNA antisense probes to sections of the first molar on the untreated control side. A highly positive signal of the mRNAs encoding BSP (A) and type I collagen (B) was observed in osteoblasts (arrowheads) and osteocytes (arrows) that were close to the distal surface of the interradicular septum. r, tooth root; p, periodontal ligament; i, interradicular septum (magnification ×37.5).

bone-lining cells at the mesial surface contained both mRNAs. There was uneven staining among cementoblasts, some of which contained a small amount of both mRNAs.

First molar on the experimental side

After one day of experimental tooth movement, the first molar had moved mesially. Across the IRS, the PDL was stretched on the mesial side and compressed on the distal side. A faint signal of BSP mRNA was detected in a few osteoblasts in the tension area of the PDL (Figure 3A). However, these osteoblasts and the fibroblast-like cells in the same area expressed a higher level of type I collagen mRNA than the respective cells on the control side (Figure 3B). BSP and collagen type I mRNAs were not detected in the compressed area.

After 3 days of experimental tooth movement, an intense signal of BSP mRNA was detected in osteoblasts in the tension area of the PDL, and a moderate signal of BSP mRNA was found in some fibroblast-like cells in this area (Figure 4A). A strong hybridization signal of type I collagen mRNA was present not only in osteoblasts and osteocytes in the tension area, but also in several layers of cells adjacent to the osteoblasts in the tension area (Figure 4B). Many fibroblast-like cells in the PDL also showed a strong positive signal of type I collagen mRNA. BSP mRNA and collagen type I mRNA could not be detected in the compressed area.

After 7 days of experimental tooth movement, there was uneven staining of both mRNAs in the osteoblasts in the tension area of the PDL (Figure 5A,B). Some of the osteoblasts showed a relatively weak signal of both mRNAs. An intense signal of BSP and collagen type I mRNAs was found in newly-embedded osteocytes, while no signal was detected in other osteocytes (Figure 5C,D). Many fibroblast-like cells showed

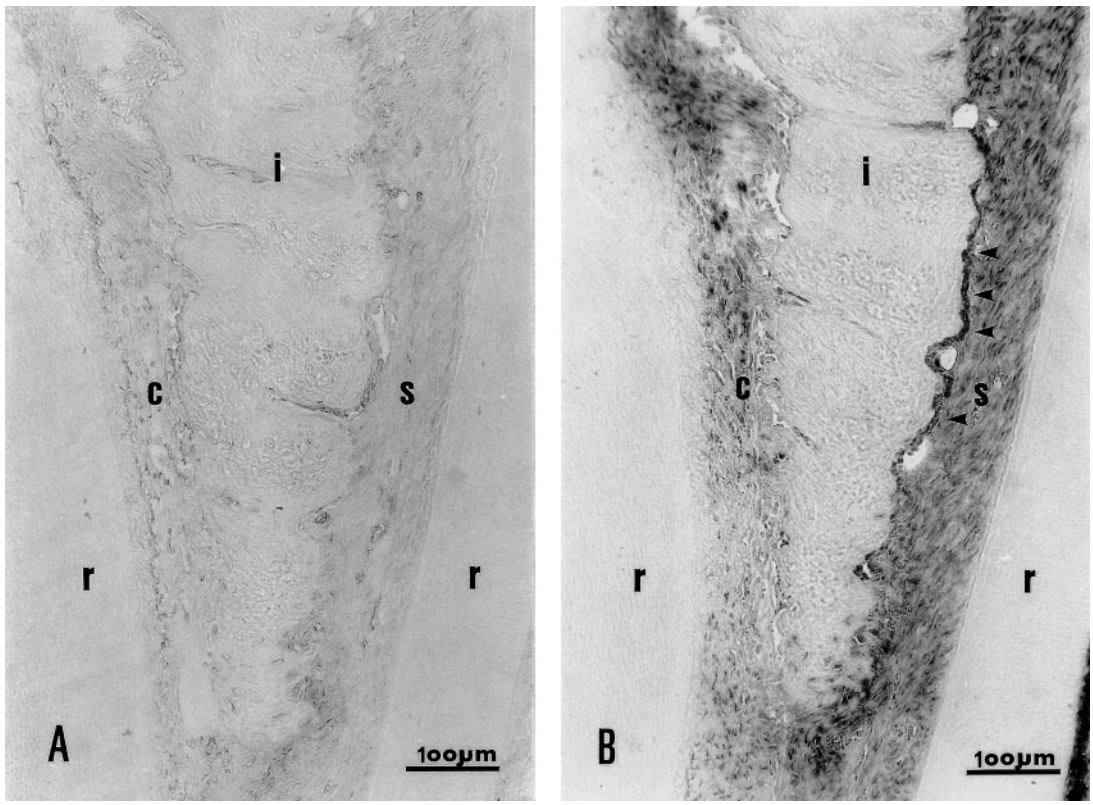

Figure 3 Results of hybridization of BSP and type I collagen RNA antisense probes to sections of the first molar that had been subject to experimental tooth movement for one day. Only a faint signal of BSP mRNA is observed (A), while an intense signal of type I collagen mRNA is observed in osteoblasts (arrowheads) and fibroblast-like cells in the stretched periodontal ligament (B). r, tooth root; c, compressed periodontal ligament; s, stretched periodontal ligament; i, interradicular septum (magnification ×37.5).

positive reaction of type I collagen mRNA and some fibroblast-like cells also showed relatively weak, but positive reaction of BSP mRNA. Cells in the compressed area of the PDL contained a trace amount of both mRNAs. No signal was detected in any of the sections that had been hybridized with the sense RNA probes (Figure 5E).

Discussion

Expression of BSP and type I collagen mRNAs in physiological tooth movement

Physiologically rat molars move distally (Sicher and Weinmann, 1944). This phenomenon involves bone resorption at the mesial and bone formation at the distal surface of the IRS. Consistent with these facts, intense signals of BSP and type I collagen mRNAs were found in a cell layer of osteoblasts on the distal surface of the untreated side. BSP is considered to be expressed by mature osteoblasts and to be involved in the formation of hydroxyapatite crystals (Chen *et al.*, 1991a,b, 1992, 1994). The results of this study support this hypothesis, as a highly intense positive signal of BSP and type I collagen mRNAs was observed in osteoblasts adjacent to the mineralization front. On the other hand, along the mesial surface of the IRS, where bone was physiologically resorbed, only a few scattered cells expressed both BSP and collagen type I mRNA. This may represent the bone remodelling phenomenon, in which bone

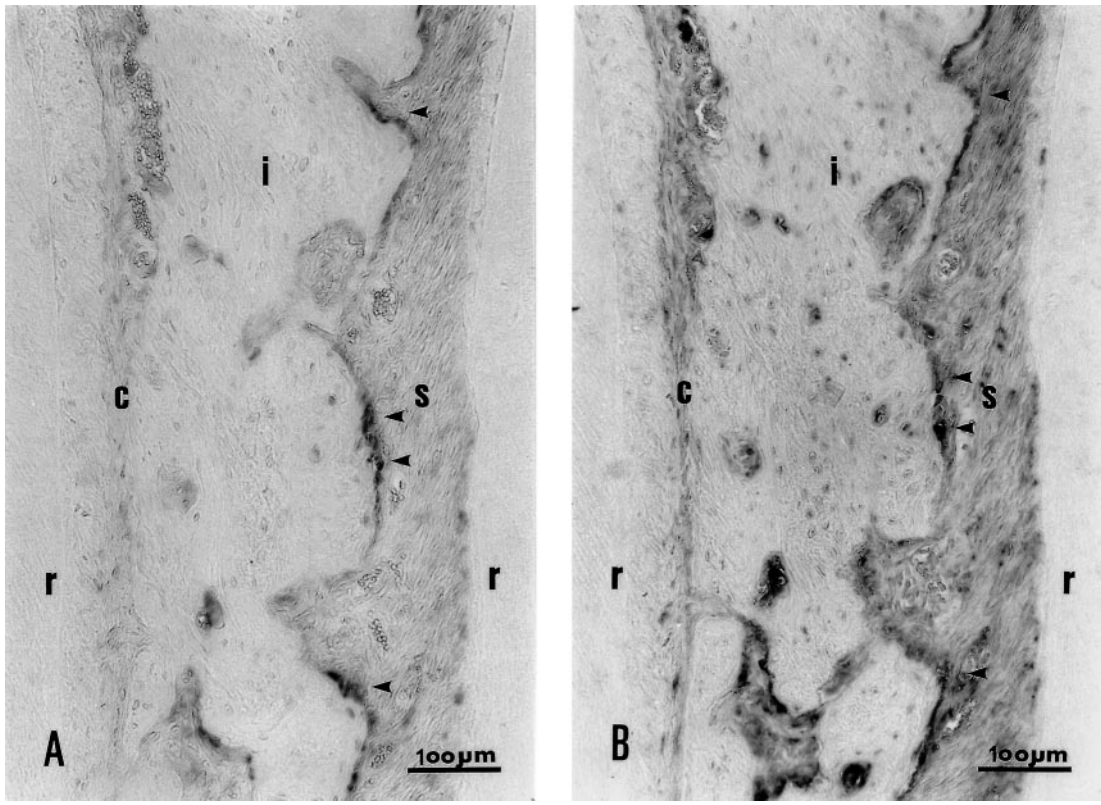

Figure 4 Results of hybridization of BSP and type I collagen RNA antisense probes to sections of the first molar that had been subjected to experimental tooth movement for three days. (A) An intense signal for BSP mRNA is observed in osteoblasts (arrowheads) along the mesial surface of the interradicular septum. (B) An intense signal for type I collagen mRNA is observed in osteoblasts (arrowheads) and fibroblast-like cells in the stretched periodontal ligament. r, tooth root; c, compressed periodontal ligament; s, stretched periodontal ligament; i, interradicular septum (magnification ×37.5).

formation and resorption occur simultaneously at the same site, and on the mesial surface of teeth under physiological movement, resorption dominates formation.

Nakagawa *et al.* (1994) examined the expression of type I collagen mRNA by *in situ* hybridization with an oligodeoxynucleotide probe, and demonstrated that type I collagen mRNA is expressed uniformly in rat periodontal tissue. In contrast, site-specific expression of type I collagen mRNA using an RNA probe, on the bone side of the PDL was found in the present study. These conflicting observations are presumably due to a difference in the type of probes used. An RNA probe is much more specific than an oligodeoxynucleotide probe. The results of this investigation are consistent with the suggestion that a higher level of remodelling activity of the PDL occurs in the area near the alveolar bone (Anderson, 1967; Melcher and Correia, 1971).

Expression of bone sialoprotein and type I collagen mRNAs during experimental tooth movement

A histological study showed that upon insertion of an elastic band, osteoblasts appear on the bone surface of the tension side of the PDL on approximately the first day, and that new bone formation begins within 2 days (Azuma, 1970). The results of the present investigation are consistent with these findings. On the untreated control side, an inactive type of bone-lining cell

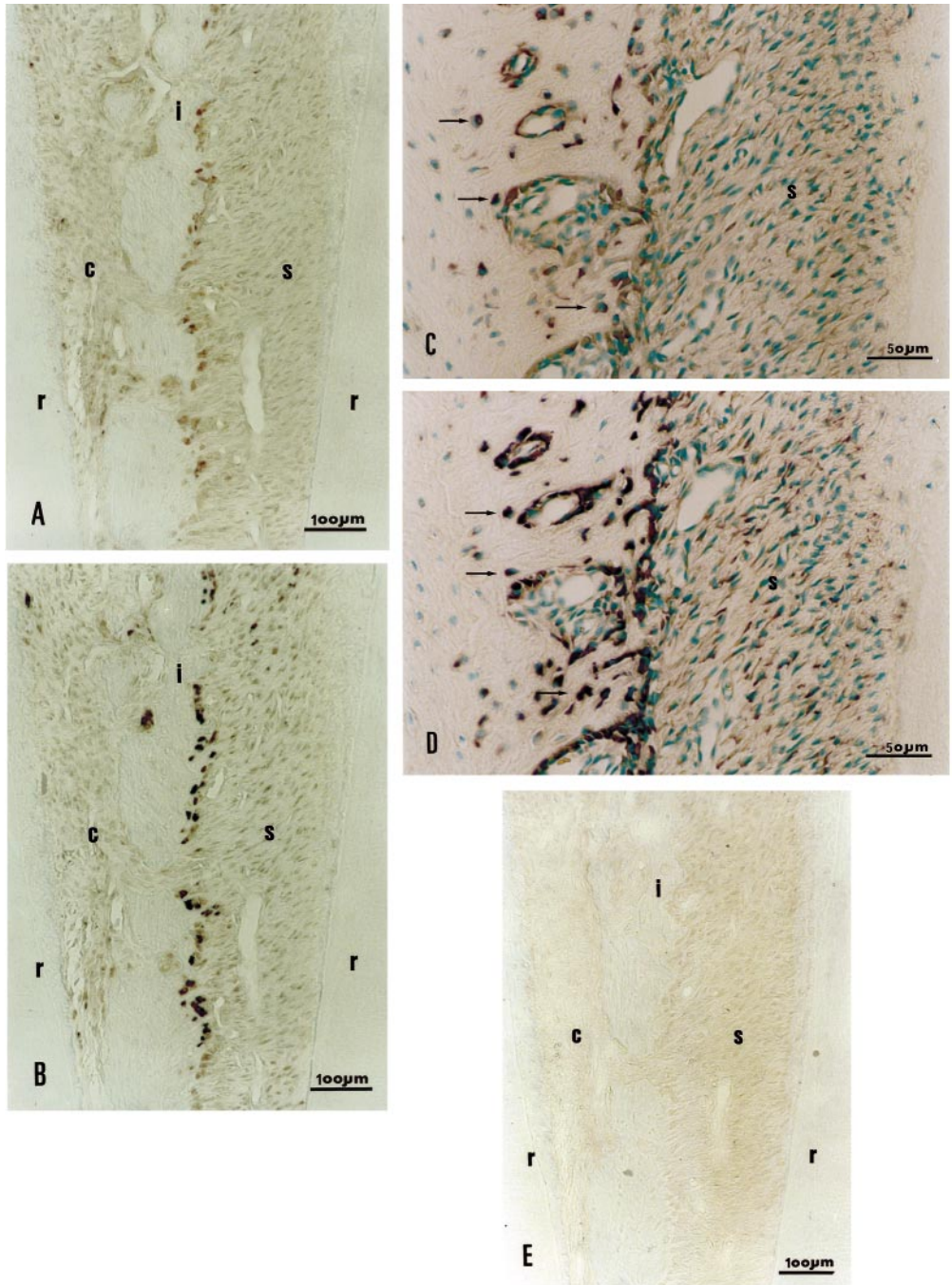

Figure 5 Results of hybridization of BSP and type I collagen RNA antisense probes to sections of the first molar that had been subjected to experimental tooth movement for 7 days. In the sections hybridized with BSP probe (A), and those hybridized with type I collagen probe (B), there is uneven staining in osteoblasts along the tension area of the periodontal ligament. Some of these cells show weak signals for both mRNAs. Intense signals of the mRNA encoding BSP (C) and that encoding type I collagen (D) are observed in newly-embedded osteocytes (arrows). (E) Sense probe shows negative results. r, tooth root; c, compressed periodontal ligament; s, stretched periodontal ligament; i, interradicular septum (magnifications: A, B, and E, ×37.5; C and D, ×75).

was scattered along the mesial surface of the IRS, where bone was being resorbed physiologically. After initiation of experimental tooth movement, the mesial surface of the IRS became the tension side of the PDL. Consequently, active expression of type I collagen mRNA was observed in osteoblasts on the mesial surface of the IRS on day 1, while only a faint signal of BSP mRNA was detected. However, by day 3 of the experimental tooth movement, a positive signal for BSP mRNA was evident in these cells. These results suggest that the osteoblasts that appear on day 1 are not fully differentiated; however, they become mature enough to express the BSP mRNA by day 3. On day 7, a fewer number of cells showed an intense signal of both mRNAs. It seems that the activities of bone formation and PDL remodelling are decreasing at this point. The fibroblast-like cells of the PDL also expressed a lower level of both mRNAs. The fibroblasts in the PDL are more similar to osteoblasts than to fibroblasts in other tissues (Kawase *et al*., 1988). These similarities between fibroblasts and osteoblasts also exist in the PDL.

Function of osteocytes

During physiological tooth movement, osteocytes that are adjacent to the distal surface of the IRS expressed a high level of BSP and collagen type I mRNA. These newly-embedded osteocytes in bone matrix are considered to be very active. This is consistent with the suggestion that osteocytes have the ability for bone formation (Owen, 1963; Owen and MacPherson, 1963). The intensity of the signal for BSP and type I collagen mRNAs gradually decreased in the osteocytes along the mesial direction of the IRS. These results indicate that osteocytes localized in the mesial part of the IRS are less active than those in the distal part.

Interestingly, on experimental tooth movement, osteoblasts and active osteocytes adjacent to the distal surface, stopped expressing mRNAs to BSP and collagen type I, because the physiological bone-formation side became the resorption side. In this respect, the organization of the cellular network in bone, where osteocytes are connected to each other via cell processes and gap junctions (Doty, 1981; Palumbo *et al*., 1990a,b), assumes significance. This cellular network is considered to be important for detecting mechanical stress, such as distortion of bone matrix. It is possible that the network detected the stress of experimental tooth movement and induced osteocytes to stop expressing BSP mRNA and type I collagen mRNA. As observed after 7 days of experimental tooth movement, osteocytes that were newly embedded after treatment was started exhibited a positive signal for both mRNAs, while the existing osteocytes that had been embedded prior to treatment showed negligible signalling of both mRNAs. It seems that the cellular network of new osteocytes is not connected to that of old osteocytes.

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