

## RESEARCH REPORTS

Biological

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J Dent Res 80(7): 1665-1671, 2001

# Expression of Bone Matrix Protein mRNAs by Primary and Cloned Cultures of the Regenerative Phenotype of Human Periodontal Fibroblasts

## ABSTRACT

The successful regeneration of periodontal tissues is dependent, in part, on the ability of cells to reconstitute the mineralized tissues of cementum and bone. The aim of the present study was to characterize regeneration-associated cells in terms of their ability to express mineralized tissue macromolecules. Following guided tissue regeneration, cell cultures were established from regenerating tissue, periodontal ligament, and gingiva. Additionally, these cells were transfected, and single-cell-derived clones were established. Following treatment with platelet-derived growth factor-BB and insulin-derived growth factor-1, the presence of mRNA for alkaline phosphatase, osteocalcin, bone sialoprotein, osteopontin, and bone morphogenetic proteins-2 and -4 was assessed. The three cell types expressed similar mRNA levels for alkaline phosphatase, bone morphogenetic protein-2, and bone morphogenetic protein-4, whereas the expression of osteopontin, osteocalcin, and bone sialoprotein was greater in the periodontal ligament and regenerating tissue fibroblasts compared with the gingival fibroblasts. The two growth factors did not affect the expression of any of the genes. This study has identified markers that correlate with the known ability of periodontal ligament and regenerating tissue-derived fibroblasts to facilitate regeneration of the mineralized tissues of the periodontium.

**KEY WORDS:** osteopontin, osteocalcin, bone sialoprotein, periodontal regeneration, fibroblast heterogeneity.

## INTRODUCTION

A primary goal of periodontal therapy is regeneration of the tissues that have been destroyed by periodontitis. Guided tissue regeneration is a surgical technique that utilizes barrier membranes to exclude undesirable cells, such as epithelial and gingival connective tissue cells, from the periodontal defect, thus allowing for repopulation by cells capable of regenerating the desired structures (*i.e.*, cementum, bone, periodontal ligament). However, because predictable regeneration is not always achieved by this and other regenerative techniques (Laurell *et al.*, 1998), the cellular and molecular events occurring during this type of wound healing require ongoing investigations. The heterogenous nature of the fibroblasts that populate the periodontium, together with the complex interactions which occur between hard and soft tissues during periodontal wound healing, is a poorly understood concept which is of paramount importance in periodontal regeneration.

The aims of the present study were to establish and characterize primary fibroblast cultures, and single-cell-derived clones, obtained from the regenerating tissues and to compare them with gingival and periodontal ligament fibroblast cultures obtained from adjacent tissues in the same patient. Since restoration of cementum and bone lost to periodontitis is an essential requirement of periodontal regeneration, we have hypothesized that cells of a regenerating phenotype should express mRNA for proteins associated with hard-tissue formation. Hence, we assessed the expression of mRNA for osteopontin, osteocalcin, bone sialoprotein, alkaline phosphatase, and bone morphogenetic proteins-2 and -4 (BMP-2 and BMP-4), because these proteins have unique distributions within the periodontium, are secreted at different stages of osteogenic cell differentiation, and/or play important roles in cementogenesis and osteogenesis (Bronckers *et al.*, 1994; MacNeil *et al.*, 1995; Boskey, 1996; Thesleff and Nieminen, 1996; Lee, 1997). The properties exhibited by these proteins make them potentially useful as markers of osteoprogenitor/cementoprogenitor cells residing within the periodontium.

## MATERIALS & METHODS

### Surgical Procedure

Following attainment of institutional ethics approval and informed consent from three subjects, buccal flaps were raised around periodontal defects associated with non-carious teeth destined for extraction in preparation for immediate denture construction. Granulation tissue was removed from the defect, and the tooth surface was thoroughly debrided prior to placement of an expanded polytetrafluoroethylene membrane (W.L. Gore & Associates, Flagstaff, AZ, USA). Following a six-week healing period, the membrane was removed, the regenerated tissue was excised, a biopsy of the buccal gingiva was obtained, and the tooth was extracted.

Received May 15, 2000; Last revision March 8, 2001;  
Accepted March 26, 2001

A supplemental appendix to this article is published electronically only at <http://www.dentalresearch.org>.

## Cell Isolation and Culture

Periodontal fibroblasts were isolated and cultured as reported previously (Somerman *et al.*, 1988). Briefly, the periodontal ligament, gingival, and regenerating tissues were cut into small pieces and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS), 50 units/mL penicillin, and 50  $\mu$ g/mL streptomycin. Cells between the 4th and 5th transfers in culture were used.

## Transfection and Establishment of Clones

The fibroblast cultures were transfected by use of the putative transforming genes E6 and E7 from the human papilloma virus type 16 prototype, which were contained in the retrovirus vector, pLXSN (Shiga *et al.*, 1997). The amphotropic virus LXSN16E6E7 was produced by the NIH 3T3-derived mouse fibroblast packaging line PA317 and used to infect the three primary fibroblast cell lines, which were subsequently selected in standard culture medium containing G418 (1 mg/mL). We obtained clones of the transfected cells by seeding them into 24-well plates using a limiting dilution of 0.3-0.4 cells/well. Clone growth was microscopically assessed on a daily basis to ensure that each clone had originated from a single cell. Following the establishment of the clones, 9 clonal populations (representing 3 from each cell type) derived from one patient were randomly selected for analysis.

## Growth Factor Stimulation

Upon reaching 80% confluence, the cells were exposed to culture media containing various growth factors, including: 0.2% FCS, 10% FCS, 10 ng/mL PDGF-BB in 0.2% FCS, 100 ng/mL IGF-1 in 0.2% FCS, and a combination of 10 ng/mL PDGF-BB and 100 ng/mL IGF-1 in 0.2% FCS. These conditions were based on previous studies which have shown them to stimulate a variety of periodontal cells with regard to cell proliferation and matrix protein synthesis (Haase *et al.*, 1998).

## RT-PCR Analysis

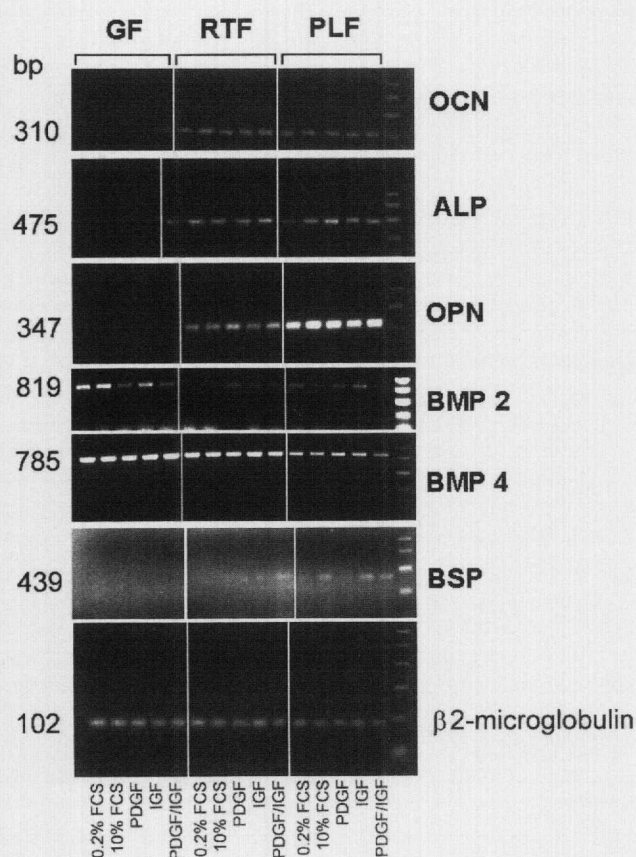
Total cellular RNA was isolated from the three populations by means of a Rneasy Mini Kit (QIAGEN Pty Ltd, Victoria, Australia) as described previously (Haase *et al.*, 1998). cDNA was synthesized from 1  $\mu$ g of total RNA which was primed with Oligo dT. The final 20- $\mu$ L reaction mixture, containing 1 x reverse transcriptase buffer (Promega, Madison, WI, USA), dCTP, dGTP, dATP, and dTTP each at 500  $\mu$ M, 20 U of RNase inhibitor, and 100 U of M-MLV enzyme, was incubated at 37°C for 1 hr followed by 99°C for 5 min.

Aliquots of 2  $\mu$ L (for  $\beta$ -2-microglobulin, alkaline phosphatase, bone morphogenetic protein-2, and bone morphogenetic protein-4) and 4  $\mu$ L (for osteopontin, osteocalcin, and bone sialoprotein) of the total cDNA were amplified with 10 pmol of 5' and 3' primer, 1x PCR buffer (Promega), dCTP, dGTP, dATP, and dTTP each at 0.4 mM, 1.5 mM MgCl<sub>2</sub>, and 0.5 U of Taq polymerase. A PCR product for bone sialoprotein was obtained only by increasing the primer and MgCl<sub>2</sub> concentrations to 50 pmol and 3 mM, respectively. A minimum of 3 PCR reactions was carried out for each cDNA sample. Amplifications were performed in a thermal cycler (MJ Research, Watertown, MA, USA). Twenty-five cycles were used for  $\beta$ 2-microglobulin and alkaline phosphatase, 30 cycles for BMP-2 and BMP-4, and 35 for osteopontin, osteocalcin, and bone sialoprotein. The primer sequence for bone sialoprotein (F:GCCTGTGCTTTCTCAATG and R:TTCCTTCCTCTCCTCCTC) was designed by means of the GCG program "Prime" (Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, Madison, WI, USA), whereas previously published sequences were used for osteopontin, osteocalcin, alkaline phosphatase (Rickard *et al.*, 1996), BMP-2, and BMP-4 (Li *et al.*, 1998). Reaction products were analyzed and visualized by electrophoresis of 10- $\mu$ L samples in 1.5-2% agarose gels containing 0.5  $\mu$ g/mL ethidium bromide. The identity of the PCR products was confirmed by sequencing. We quantified the DNA bands by measuring their density using image analysis (NIH Image Analysis version 1.57, Bethesda, MD, USA). Statistical analysis of the results was carried out by one-way ANOVA.

## RESULTS

### Primary Cultures

A representative agarose gel from one patient is shown in Fig. 1. The RT-PCR analyses (Fig. 2) showed that there were significantly higher levels of osteocalcin and osteopontin mRNA expression in the regenerating tissue fibroblasts and periodontal ligament fibroblasts compared with the gingival fibroblasts ( $p < 0.01$ ). Furthermore, bone sialoprotein mRNA expression was noted only in the periodontal ligament fibroblasts and regenerating tissue fibroblasts. There were no significant differences noted between periodontal ligament fibroblasts and regenerating tissue fibroblasts with respect to the mRNA expression of any of the proteins. Furthermore, the



**Figure 1.** Representative agarose gel showing RT-PCR products from one patient. The expression of osteopontin, osteocalcin, and bone sialoprotein in periodontal ligament (PLF) and regenerating tissue fibroblasts (RTF) is up-regulated compared with gingival fibroblasts (GF). OCN = osteocalcin; ALP = alkaline phosphatase; OPN = osteopontin; BSP = bone sialoprotein; BMP-2 = bone morphogenetic protein-2; BMP-4 = bone morphogenetic protein-4.

**Table.** RT-PCR Product Band Intensities (relative to  $\beta$ -2 microglobulin) of Mineralized-tissue-associated mRNAs Derived from Primary Cultures and Clones at the Baseline (0.02% FCS) level

	OPN	OCN	BSP	ALP	BMP-2	BMP-4
Primary Cultures <sup>a</sup>						
Mean (Range)						
GF	0.09 (0-0.26)	0.08 (0-0.16)	0	0.31 (0-0.67)	0.35 (0.15-0.45)	0.51 (0.3-0.68)
RTF	0.55 (0.25-0.84)	0.29 (0.1-0.47)	0.08 (0-0.2)	0.49 (0.26-0.88)	0.27 (0.15-0.36)	0.56 (0.45-0.68)
PLF	0.94 (0.14-1.7)	0.52 (0.43-0.58)	0.15 (0.04-0.28)	0.64 (0.1-1.6)	0.39 (0.3-0.45)	0.42 (0.38-0.48)
Clones <sup>b</sup>						
Mean (SD)						
G1	0	0	0	0.18 (0.03)	0.46 (0.12)	1.23 (0.38)
G2	0.32 (0.08)	0	0	0.11 (0.02)	0.81 (0.24)	0.83 (0.16)
G3	0.15 (0.04)	0	0	0	0.43 (0.14)	0
R1	0.35 (0.07)	0.16 (0.03)	0	0.48 (0.07)	0.81 (0.28)	1.28 (0.32)
R2	0.8 (0.16)	0	0	0.22 (0.03)	0.78 (0.22)	1.76 (0.43)
R3	0.55 (0.12)	0.17 (0.07)	0.18 (0.04)	0.63 (0.11)	0.52 (0.16)	0.52 (0.15)
P1	0.26 (0.11)	0.32 (0.12)	0	0	0.82 (0.24)	0.93 (0.21)
P2	0.74 (0.18)	1.24 (0.36)	0.15 (0.07)	0.75 (0.09)	0.22 (0.12)	1.21 (0.24)
P3	0.21 (0.09)	0.73 (0.22)	0.24 (0.07)	0.41 (0.05)	0.58 (0.23)	0.43 (0.11)

<sup>a</sup> The primary culture data are presented as the mean of the three patients studied, and the range is included to show the level of inter-patient heterogeneity that was encountered.

<sup>b</sup> The data derived from the cloned cultures are presented as the mean and standard deviation (SD) of triplicate samples.

three cell populations did not express any significant differences in the BMP-2, BMP-4, and alkaline phosphatase mRNA expression. PDGF and IGF-I did not have a significant effect on the mRNA expression of any of the molecules studied.

There was considerable heterogeneity within each set of three regenerating tissue, periodontal ligament, and gingival fibroblast populations with regard to the mRNA expression of all of the molecules (Table). For example, only one of the three gingival fibroblast populations expressed osteocalcin mRNA, whereas two periodontal ligament fibroblast populations expressed high levels of alkaline phosphatase mRNA, while the expression level in the remaining population was low.

### Clones

Single-cell-derived clones were successfully established from the transfected primary cultures. Initial assessment indicated that the general growth rates and protein synthesis were not different from those of the parent cultures (results not shown). The baseline (0.2% FCS-treated) mineralized tissue marker mRNA expression by the clones, as measured by the RT-PCR product band intensities (relative to  $\beta$ -2-microglobulin), is presented in the Table. The mRNA expression in the 9 clonal populations was consistent with the findings in the primary cell populations. There was considerable heterogeneity with respect to the mRNA expression of the majority of the proteins studied. Generally, there were greater incidence and intensity of bone sialoprotein, osteocalcin, osteopontin, and alkaline

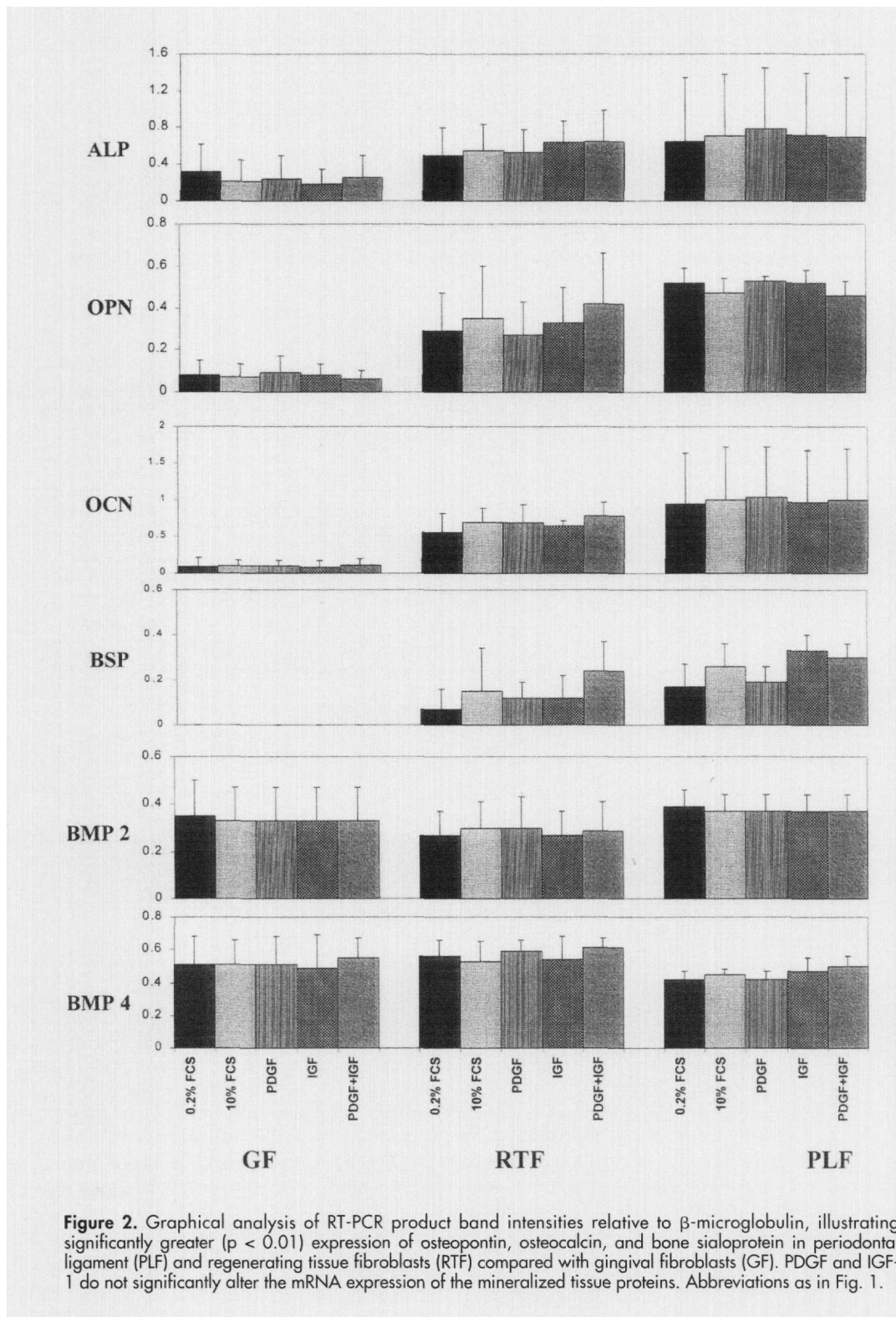
phosphatase mRNA expression in the regenerating tissue fibroblasts and periodontal ligament fibroblasts compared with those of the gingival fibroblasts. Of particular interest was the ubiquitous and strong expression of mRNA for all the hard-tissue-associated proteins that was observed in several clones, namely, R3, P2, and P3.

The PDGF and IGF effect on mRNA expression by two representative clones from each cell population is illustrated in Fig. 3. The PDGF and IGF effect on mineralized tissue-associated genes was more pronounced in certain clonal populations. For example, osteopontin, osteocalcin, alkaline phosphatase, and BMP-2 mRNAs were all up-regulated by PDGF and/or IGF in the R1 clones. There was no correlation between mineralized tissue marker expression and cellular proliferation, as measured by a <sup>3</sup>H-thymidine incorporation assay, in response to growth factor exposure. Indeed, PDGF and IGF-I had a mitogenic effect on the clonal populations (see Fig. in electronic Appendix), similar to that observed in primary cultures of periodontal cells (Haase *et al.*, 1998).

### DISCUSSION

This study has attempted to characterize cells isolated from regenerating periodontal defects. The findings, which show that regenerating tissue fibroblasts and periodontal ligament fibroblasts express higher levels of mRNA for mineralized-tissue-associated proteins compared with gingival fibroblasts, complement earlier immunohistochemical studies showing that these proteins accumulate in regenerating periodontal defects (Amar *et al.*, 1997; Ivanovski *et al.*, 2000).





**Figure 2.** Graphical analysis of RT-PCR product band intensities relative to  $\beta$ -microglobulin, illustrating significantly greater ( $p < 0.01$ ) expression of osteopontin, osteocalcin, and bone sialoprotein in periodontal ligament (PLF) and regenerating tissue fibroblasts (RTF) compared with gingival fibroblasts (GF). PDGF and IGF-1 do not significantly alter the mRNA expression of the mineralized tissue proteins. Abbreviations as in Fig. 1.

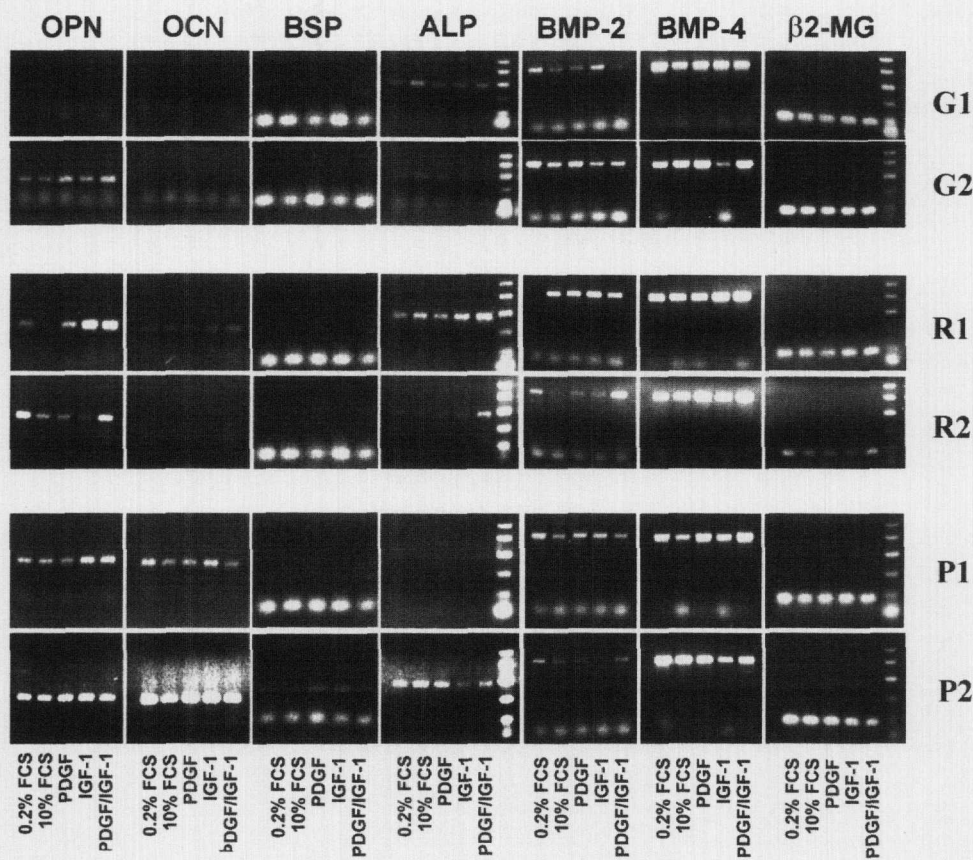
The increased osteopontin, osteocalcin, and bone sialoprotein mRNA expression by the regenerating tissue fibroblasts and periodontal ligament fibroblasts is significant, because these proteins are secreted at different stages during osteogenic cell differentiation (Boskey, 1996) and have unique distributions within the periodontium (Bronckers *et*

*al.*, 1994; MacNeil *et al.*, 1995; Lekic *et al.*, 1996). Furthermore, the mRNAs for these proteins are expressed in a specific temporal and spatial manner during cementogenesis (MacNeil *et al.*, 1995; D'Errico *et al.*, 1997). Therefore, the strong mRNA expression noted for osteopontin, osteocalcin, and bone sialoprotein indicates the potential for hard-tissue formation by a subpopulation of cells residing within the periodontal ligament and regenerating defect. The expression of these genes may be used to identify cells of the osteoblastic/cementoblastic lineage within these populations.

Bone sialoprotein, in particular, is an excellent marker for mineralized-tissue-specific cells because of its role as initiator of biomineralization (Hunter and Goldberg, 1993). Furthermore, bone sialoprotein is localized in cementum and bone but not within the periodontal ligament (MacNeil *et al.*, 1995; D'Errico *et al.*, 1997). Since some undifferentiated fibroblastic pre-osteoblasts have been shown to express bone sialoprotein mRNA (Liu *et al.*, 1994), it may be speculated that the source of bone sialoprotein mRNA noted within the periodontal ligament fibroblast populations could be cementoblast and/or osteoblast precursors.

Although earlier studies have reported increased activity of alkaline phosphatase in periodontal ligament fibroblast cultures

(Somerman *et al.*, 1988), our findings of no significant difference in the level of alkaline phosphatase mRNA expression among the regenerating tissue, periodontal ligament, and gingival fibroblasts are consistent with recent reports of alkaline phosphatase activity in gingival fibroblasts (Carnes *et al.*, 1997).



**Figure 3.** Agarose gel showing mRNA expression of mineralized tissue proteins in clonal populations and their responsiveness to growth factors PDGF and IGF-1. The clones appear to be more responsive to the growth factors than the primary cultures. Abbreviations as in Fig. 1, plus G = gingival fibroblast clones, R = regenerating tissue fibroblast clones, and P = periodontal ligament fibroblast clones.

BMP-2 and BMP-4 play important roles in tooth morphogenesis (Thesleff and Nieminen, 1996). During development, the translation of these proteins is highly regulated in both a spatial and temporal sense (Lee *et al.*, 1997). Hence, although there were no differences in mRNA expression for BMP-2 and BMP-4 among the regenerating tissue, periodontal ligament, and gingival fibroblasts, this may not correlate with the protein expression by these cells.

The extensive heterogeneous expression of mineralized tissue protein mRNA by the three cell populations is not surprising, since the cells inhabiting the periodontium are noted for their considerable heterogeneity (Hassell and Stanek, 1983; Giannopoulou and Ciamasoni, 1996; Kuru *et al.*, 1998). Osteoblast precursors and mature osteoblasts are also considerably heterogeneous in terms of mRNA expression for alkaline phosphatase, osteocalcin, osteopontin, and bone sialoprotein (Liu *et al.*, 1997).

The limitations of quantifiable data obtained by the use of the highly sensitive RT-PCR method must be noted. Indeed, Northern blotting would have provided more directly quantifiable results. However, as has been the case in previous studies (Nohutcu *et al.*, 1997; Parkar *et al.*, 1999), we were unable to use Northern blotting successfully to detect osteoblast-associated genes in human periodontal cells. Although this limits the quantitative conclusions drawn from our results, the scarcity of

information on the expression of osteoblast-associated genes by cultured human periodontal cells necessitates the pursuit of this method of analysis.

In periodontal cells, PDGF and/or IGF-1 have been shown to affect extracellular matrix synthesis *via* the up-regulation of collagen (Matsuda *et al.*, 1992) and proteoglycan (Haase *et al.*, 1998) synthesis, as well as BMP-2 and BMP-4 mRNA (Li *et al.*, 1998). In this study, PDGF and IGF-1 did not significantly affect the mRNA expression of any of the proteins studied. The findings of this study are supported by the reported lack of PDGF and/or IGF-1 effect on osteocalcin, osteopontin, and/or alkaline phosphatase mRNA expression in osteoblast and their precursors (Canalis and Lian, 1988; Tanaka and Liang, 1995; Thomas *et al.*, 1999). However, IGF-1 and PDGF have been reported both to stimulate and to inhibit various mineralized tissue molecules in a variety of *in vitro* models (Tanaka and Liang, 1995; Langdahl *et al.*, 1998; Strayhorn *et al.*, 1999). Hence, it appears that the effects of PDGF and IGF-1 on osteoblast-associated genes are dependent on a variety of factors, including growth factor concentrations and duration of application, source and phenotype of the cells under investigation, as well as the stage of cell differentiation.

The successful transfection and subsequent cloning of human periodontal cells are of particular interest, as is the apparent ability of the clones to retain the characteristics of the parent cultures. The establishment of cloned human cells from regenerating periodontal tissues now provides us with a stable *in vitro* system for the study of various phenotypes (*e.g.*, fibroblast, cementoblast, and osteoblast precursors) involved in regeneration. The study of clonal populations may be used to overcome the difficulties associated with the extensive heterogeneity of primary cultures of periodontal cells. Indeed, the influence of PDGF and IGF-1 on osteoblast-associated gene expression was more pronounced in certain clones compared with the primary cultures, and this finding can be attributed to the use of isolated phenotypes.

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In conclusion, this study has shown that human regenerating tissue fibroblasts have phenotypes consistent with an ability to produce mineralized tissues. The increased level of mineralized tissue mRNA in periodontal ligament

fibroblasts and regenerating tissue fibroblasts compared with gingival fibroblasts supports the concept that cells originating from within the periodontal ligament (including those from cementum and bone) have the ability to regenerate lost periodontal attachment. Furthermore, these findings reinforce the necessity for investigating means to facilitate periodontal regeneration through the promotion of selective repopulation of the defect by cells with a regenerative phenotype.

## ACKNOWLEDGMENTS

The transfection of the three primary cell lines was carried out at the Lions Human Immunology Laboratories, Brisbane, Queensland, Australia. This study was supported by the Australian National Health and Medical Research Council.

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