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Isolation of murine cementoblasts: unique cells or uniquely-positioned osteoblasts?

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While cementoblasts express a number of mineral-related proteins, including bone sialoprotein (BSP), osteopontin (OPN) and osteocalcin (OC), these proteins do not appear to be expressed by cells of the intermediate dental follicle/periodontal ligament (PDL). This information was utilized in an experimental strategy to isolate presumptive cementoblasts from the root surface of day 24 murine mandibular first molars. Using microscopic dissection techniques, molars were carefully extracted from their alveolar crypts and subjected to trypsin-collagenase digestion to remove adherent cells. Primary cultures were established and assayed for expression of proteins known to be expressed by cementoblasts at this timepoint *in vivo* (i.e. BSP, OPN, OC) and also an odontoblast-specific protein (i.e. DSP) to rule out contamination by pulpal cells. A subgroup of cells were found to express Type I collagen (89% of cells), BSP (46%), OPN (23%) and OC (30%); DSP was not detected within these cultures. We propose that cells within this heterogeneous population, which express this profile of osteogenic proteins, represent cementoblasts. The availability of a cementoblast cell line will make possible rigorous and controlled *in vitro* analysis of these cells and allow for determination of the unique characteristics of these cells not shared with other cells, particularly osteoblasts.

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Since the earliest description of root cementum by the noted physiologist Jan Purkinje in 1835 (1), considerable interest and debate has surrounded this dental tissue. Cementum is unique in histologic terms yet it shares many properties with other mineralized tissues, particularly bone (2, 3). There is a need to determine the cell(s) and products responsible for formation of cementum and, subsequently, to establish those properties which define cementum and cementoblasts as unique versus bone and osteoblasts, respectively.

Light and electron microscope criteria, based on the presence (cellular) or absence (acellular) of cells and the source of collagen fibers (extrinsic vs. intrinsic), have been used to classify cementum into five recognized subtypes (3, 4). It is currently unknown if the histologic differences observed between these subtypes are due to differences in the cells synthesizing these subtype matrices (e.g. different types/phenotypes of cementoblasts) or environmental influences in different regions of the developing/mature root surface (e.g. rate of cementum matrix synthesis/mineralization; differences in mechanical loading between cementum subtypes; etc.). All cementum subtypes differ from bone in being avascular, non-innervated, and possessing low remodeling potential. However, examination of other characteristics and properties of cementum, especially the cellular cementum subtypes, indicates that this tissue is bone-like. The organic/inorganic distribution of matrix elements in cementum (96% mineral; 27% organic; 12% water) closely mimics bone (27% mineral; 30% organic; 25% water) as does the overall distribution

of amino acid and glycosaminoglycan constituents (3, 5–8). The organic matrix of cementum, like bone, is mainly composed of Type I collagen, lesser amounts of Type III collagen (2) and a myriad of non-collagenous proteins including bone sialoprotein (BSP), osteopontin (OPN), and osteocalcin (OC). And, importantly, diseases/syndromes which affect bone also markedly alter the cementum matrix; for example, hypercementosis accompanies Paget's disease, cementum fails to form in hypophosphatasia, decreased cementum formation occurs during hypopituitarism, and defective cementum is a dental characteristic of cleidocranial dysplasia.

These observations suggest that the matrices of cementum and bone are closely related and also that cementoblasts (here also termed root lining cells) and osteoblasts are phenotypically similar. Whether cementoblasts are phenotypically unique or rather "osteoblast variants" is an important question that must be addressed in order to understand developmental mechanisms and to devise therapeutic methods (e.g. substrate modification, use of matrix/growth factors, etc.) to enhance the formation and regeneration of cementum in post-disease situations. This paper describes the initial step in a strategy to isolate and culture (murine) root lining cells and to initiate controlled experiments at the cell, protein and gene level to characterize these cells and to compare their behavior to that of osteoblasts.

Extracellular matrix factors associated with root lining cells

Table 1 summarizes factors reported to be expressed, localized or affiliated with root lining cells. Initial investigations in this area concentrated on extraction and identification of factors archived within the matrix of mature cementum, while more recent studies have used techniques directed at synthesizing cells *in vivo*. Studies on the biochemical properties and composition of mature cementum demonstrate that protein extracts of cementum stimulate migration (9), attachment (10–12), proliferation (13) and protein synthesis (14) of gingival fibroblasts and periodontal ligament fibroblasts. Cementum also contains Arg-Gly-Asp (RGD) associated adhesion proteins including BSP (12, 15, 16), OPN (11, 12, 16–18), cementum attachment protein (CAP) (19, 20) and fibronectin (21). McKee & Nanci (17), using immunogold labeling, and MacNeil *et al.* (22–24), employing immunocytochemistry and *in situ* hybridization methods, demonstrated that OPN and BSP are major components of cementum and are synthesized by cementoblasts. Similar techniques have been used to identify other noncollagenous proteins in

Table 1

Molecular factors associated with cementum. Included in this table are both established factors as well as factors suggested to be important for cementum formation/maintenance but not yet established. (Modified and updated from ref. 18)

Proposed activity	Developing cementum	Mature cementum
1. Adhesion	OPN (11, 25, 26)	OPN (16, 17)
	BSP (22, 24, 35–37)	BSP (12, 15, 16, 22–24)
	FN (38)	FN (15, 21)
	LM (38)	55 kDa protein (15)
	Type I collagen (2, 24)	Tenasin (21)
		CAP (10, 19, 20)
2. Chemoattraction	FN (38)	Protein extracts (9)
3. Differentiation/mineralization	BSP (22, 24)	BSP (15, 16)
	OPN (11, 25, 26)	OPN (16, 17)
	ON/sparc (40, 41)	ON/sparc (26)
	OC (45)	"Gla" proteins (27)
	HERS-secreted factors	OC (25, 26)
	ameloblastin (32, 33)	Proteoglycans (7)
	enamel proteins (42, 43)	Protein extracts (46)
	TGFβ (2, 44)	ALP (47)
	IGF-1 (2)	
4. Mitogens	TGFβ (2, 44)	CGF (13, 28, 29)
	IGF-1 (2)	
5. Matrix biosynthesis	Type I, III, V, VI XIV collagens (48)	Protein extracts (14)
		Type I collagen (24, 45)

Abbreviations: BSP – bone sialoprotein. CAP – cementum adhesion protein. CGF – cementum growth factor. HERS – Hertwig's epithelial root sheath. IGF-1 – insulin-like growth factor 1. OC – osteocalcin. ON – osteonectin. OPN – osteopontin. FN – fibronectin. LM – laminin. TGFβ – transforming growth factor β. EGF – epidermal growth factor.

cementum, including osteocalcin (OC) (25, 26), γ-carboxyglutamic acid (27), osteonectin (26), proteoglycans (7) and cementum-derived growth factor (CGF) (28, 29). While CAP and CGF have been proposed to be specific for cementum, these proteins have yet to be fully characterized and appear to have some homology to known bone proteins (20, 28). A report by TENORIO & CRUCHLEY (2) further supports an association between cementoblasts and osteoblasts; using immunohistochemistry in rat, an antibody (anti-E11) thought specific to differentiated osteoblasts and newly-formed osteocytes was found localized to cementoblasts of cellular cementum.

Epithelial proteins, including laminin (30, 31) and ameloblastin (32, 33), are also expressed by cells in the vicinity of the cementum surface of the forming root. It is suspected that these proteins are synthesized by the adjacent epithelial root sheath but the role of these proteins in cementoblast differentiation remains largely unknown and subject to considerable debate (34).

Experimental strategy

Our current knowledge of osteoblast function has been largely derived from studies using primary osteoblast cultures and immortalized or transformed cell lines (50–53). It is evident that a similar strategy must be followed to characterize cementoblasts if we are to advance our understanding of this cell population beyond the indirect evidence currently available. Employing what are now considered classical techniques for osteoblast isolation (54), we describe here an experimental protocol to isolate and initially characterize murine cementoblasts (45).

Material and methods

Timed pregnant CD-1 mice were obtained from Charles River Labs, Cambridge, MA, USA. Mice at day 41 of development (i.e. 24 d post-natal) were sacrificed by decapitation. Day 41 animals were selected based on results from our previous studies demonstrating high levels of expression for BSP, OC and OPN mRNA by cells along the root surface of molars at this time point (22–24), suggesting that these cells are actively involved in formation of cementum.

A schematic description of the experimental approach used to isolate cementoblasts is provided in Fig. 1. First, mandibles were dissected from surrounding tissues, washed in Hank's balanced salt solution (HBSS) and then hemisected into halves by incision through the midline symphysis. Using a dissecting microscope, first molars were carefully removed by bisecting the periodontal ligament and removing the molar devoid of surrounding bone or bone cells. The reliability of the dissection technique was confirmed by histological examination of random samples of a) intact, undissected mandibles/molars (for reference), b) the alveolar crypt following removal of the first molars, and c) isolated molars. The standard techniques used for tissue processing and H&E staining have been previously described (22–24).

Molars were rinsed with HBSS, pooled ($n=150$) in HBSS, and then placed in a 15 ml centrifuge tube containing Dulbecco's Modified Eagle medium (DMEM) with 2 mg/ml collagenase and 0.25% trypsin for 2 h at 37°C (53). To confirm cell removal, a representative number of digested molars were examined histologically as described above. The cell suspension (approximately 10^6 cells) was removed and pelleted in a microcentrifuge at 2000 *g* for 10 min at 4°C. The supernatant was carefully aspirated and cells washed twice with DMEM containing 20% fetal calf serum (FCS) and 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were resuspended in 2 ml of DMEM/20% FCS and transferred to tissue culture treated glass chamber slides (Nunc, Naperville, IL, USA) and grown in a humidified atmosphere of 5% CO_2 at 37°C.

Once cultures reached 80% confluency (at approximately 27 d), cells were fixed in 2% paraformaldehyde and processed for *in situ* hybridization as described in detail in a previous publication (24). Probes used were: BSP: PM-BSP: mouse cDNA in PCR II vector containing a 1 Kb PCR product of mouse BSP inserted by TA Cloning (55) (a gift from Dr. M. Young, NIH/NIDR). OPN: 2ar mouse: (JB6 epidermal cell library), pGEM3 plasmid containing a portion of the coding region of mouse OPN (56) (a gift from Dr. D. Denhardt, Rutgers University). Osteocalcin: mouse OC cDNA cloned into pSP65 cloning vector (57). Type I collagen: mouse α2(I) procollagen cDNA (58). DSP: 230 bp fragment of mouse DSP cDNA cloned into pGEM7 (59) (a gift from Drs. H. Ritchie and W. T. Butler, University of Texas, Houston). Hybridization signal was visualized using dark and light field microscopy. Where appropriate, the number of cells expressing specific markers was determined by computer image analysis using LPLab Spectrum software (Signal Analytics, Vienna, VA, USA). Four fields were counted for each probe and data expressed as percent of cells expressing the marker compared with total cells, averaged for four fields.

Results

Table 2 summarizes findings derived from histologic examination of representative tissues. Following

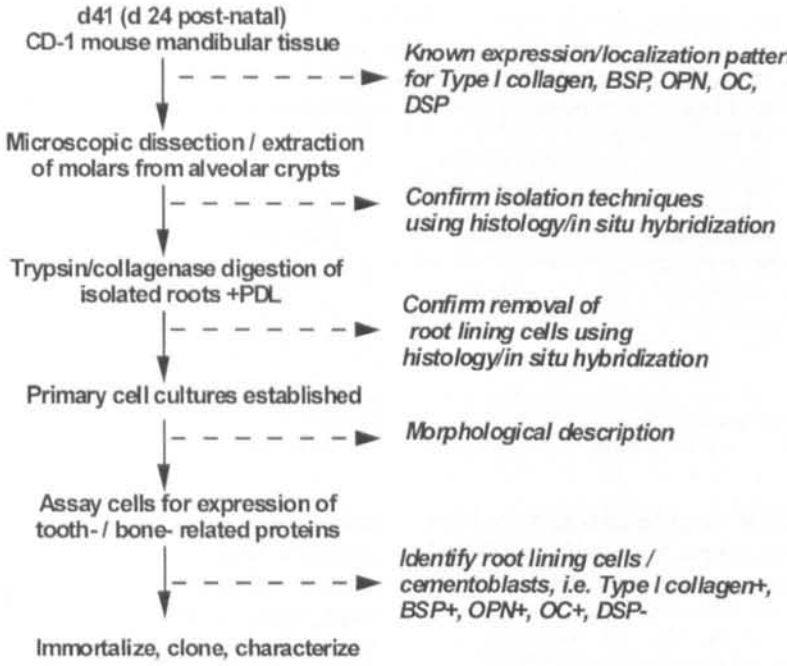

Fig. 1. Experimental strategy to isolate root lining cells/cement blasts from murine molar roots.

Table 2

Outline of findings at sequential aspects of cell isolation protocol, i.e. tissues in vivo, *residual alveolar crypts, extracted molar roots, root surfaces following enzymatic digestion and isolated cells. Standard histology was used to verify absence/presence of cells while* in situ *expression of osteocalcin was used as a marker in the isolation of root lining cells/cementoblasts*

	1. *In vivo* pre-extraction	2. Residual alveolar bone crypt	3. Extracted root prior to digestion	4. Extracted root following digestion	5. Isolated cells
Cells					
Cementoblasts/RLC	+	−	+	−	+
PDL fibroblasts	+	+	+	−	+
Osteoblasts/ABLC	+	+	−	−	−
Odontoblasts	+	−	+	+	−
OC Expression					
Cementoblasts/RLC	+	NA	+	NA	+
PDL fibroblasts	−	−	−	NA	−
Osteoblasts/ABLC	+	+	NA	NA	NA
Odontoblasts	+	NA	+	−	NA

OC = Osteocalcin. RLC = Root lining cells. ABLC = Alveolar bone lining cells. NA = Not applicable, i.e. not present.

tooth removal, lining osteoblasts and some periodontal ligament tissue could be clearly detected along the PDL aspect of the alveolar bone crypt. To further confirm that isolated molars did not contain alveolar bone cells, *in situ* hybridization using the OC probe was performed on 10 extracted molar specimens. OC expression was noted in odontoblasts and in cementoblasts adherent to the root surface but was not seen throughout the neighboring PDL, suggesting absence of alveolar bone osteoblasts. Following enzymatic digestion, all adherent cells/tissues seen prior to digestion were absent from the root surface, suggesting successful PDL/cementoblast cell removal. Primary cultures displayed a heterogeneous cellular morphology including spindle-shaped and cuboidal cell types, suggesting the presence of both PDL and cementoblast cell types.

Table 3 summarizes *in situ* hybridization results for primary cell cultures. The percentages of cells within the total population, *in vitro*, expressing Type I collagen, BSP, OPN, OC and DSP were calculated by counting four fields for each of the five probes and averaging over total cells counted. Almost all cells, i.e. 89%, expressed Type I collagen. The number of cells expressing BSP, OPN and OC was 46%, 30% and 23%, respectively.

Expression for all proteins, especially BSP,

Table 3

Comparison of gene expression (mRNA) for Type I collagen, BSP, OPN, OC, and DSP as determined by in situ *hybridization in tissues* in vivo *and primary cultures* in vitro. In vivo *results are given for mRNA expression* in situ *at day 41; similarly,* in vitro *data for mRNA expression in primary cultures reflect cells obtained from day 41 mouse molars*

	In vivo (native tissues)			*In vitro* (primary cultures)	
mRNA	Osteoblasts	PDL	Cementoblasts	Cells	% total cells
Type I collagen	+++	+++	+++	+++	89%
BSP	++	–	+++	+++	46%
OPN	+	–	++	++	30%
OC	+++	–	++	++	23%
DSP	–	–	–	–	0%

Intensity of mRNA signal: – = absence/none; + = low level, ++ = moderate level, +++ = high level.

ranged from undetectable in some cells to very high levels of expression in others. No cells expressed DSP. Sense probe controls were negative.

Discussion

This study attempted to use existing information regarding the expression pattern of cementoblasts, periodontal ligament fibroblasts, and osteoblasts in vivo to isolate and culture cells from the murine root surface characteristic of cementoblasts. A major technical dilemma in such an endeavor is avoidance of contamination of isolated cementoblasts by neighboring cells with similar but distinct cellular characteristics, namely periodontal ligament fibroblasts, osteoblasts, and odontoblasts. This would normally require the use of a marker protein unique to cementoblasts, but unfortunately one does not currently exist, at least with universal acceptance by the scientific community. Therefore, our strategy was to first devise a dissection technique that could reliably isolate cells lining the tooth root (cementoblasts/PDL fibroblasts) from cells lining the alveolar crypt (osteoblasts) and then, having avoided osteoblast contamination, use molecular techniques to differentiate between the remaining cell types, i.e. cementoblasts, PDL fibroblasts and odontoblasts. Histologic examination of isolated molars and the residual alveolar crypt strongly suggested that we were successful in the initial task. While lining osteoblasts remained visibly attached to the alveolar bone surface, osteoblasts, as determined by *in situ* hybridization for osetocalcin, could not be detected in tissues adherent to extracted root surfaces.

The timed and spatial expression pattern for osteocalcin as well as the other proteins used here (i.e. Type I collagen, BSP, OPN and DSP) had already been carefully defined during sequential periods of root/PDL development in mouse (22–24). These initial studies allowed us to define day 41 of tooth development as an optimal time to conduct cell isolation experiments. At this time point, OPN, BSP and OC are each expressed by root lining cells and are not expressed by cells within the neighboring PDL (23, 24); this finding facilitated use of a molecular method (i.e. *in situ* hybridization) to distinguish between PDL- and cementoblastic-type cells digested from tissues adherent to the root surface, *in vitro*. Morphological analysis of isolated cultured cells indicated that a heterogeneous population of cells was present in primary cultures. Significantly, a subgroup of cells could be defined which expressed high mRNA levels of genes characteristic of root lining cells/cementoblasts *in vivo*, i.e. BSP, OPN, and OC. We propose that cells *in vitro* expressing BSP, OPN, and OC are derived from the root surface and are cementoblasts. To our knowledge, only one other study has focused on isolating and characterizing "cementoblast-like" cells, *in vitro*; in that study, ARZATE *et al.* (60), described cells isolated from a cementoma tumor that produced BSP and collagen Type I and V.

As pulpal tissue was not removed or isolated from molar roots prior to enzymatic digestion, it was also important to rule out contamination with odontoblasts. Importantly, odontoblasts can express BSP, OPN and OC to varying levels during dentinogenesis; thus, the availability of a marker protein for odontoblasts become critical. DSP is a sialoprotein expressed specifically by odontoblasts and pre-secretory ameloblasts during rat and mouse dentinogenesis (59, 61). As none of the cells isolated here expressed DSP, it is proposed that odontoblasts have been successfully excluded by the technique employed.

As cells were derived from the total available root surface, it is unlikely that these cells are exclusively associated with one cementum subtype. At day 41 of murine root development, acellular cementum is the predominant form, although small

amounts of cellular cementum can be detected in the most apical regions of the root. Hence, while the majority of cultured cells are likely related to acellular cementum, other origins cannot be ruled out.

Our short- and long-range goals are to immortalize these primary cultures to establish clonal cell populations. These cell populations may provide an excellent model to study cementoblasts at the molecular level *in vitro*, including their response to osteotropic factors and their expression of perhaps specific extracellular matrix proteins, transcription factors, etc. Results from these experiments may help clarify the relationship between cementoblasts and other mineralizing cell types.

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