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Dentin-specific proteins in MDPC-23 cell line

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Only four established odontoblast-like cell lines have been reported in the literature (1–6). Of the four, only two synthesize dentin-specific proteins. These studies report that the cell line MO6-G3 synthesizes phosphophoryn (DPP), dentin sialoprotein (DSP) and dentin matrix protein-1 (DMP-1), while MDPC-23 synthesizes DSP, but not DMP-1. The objective of the present study was to determine whether polyclonal antibodies to rat DSP and DPP would label odontoblasts on microscopic sections of day-19 fetal mouse incisor odontoblasts as well as cultured cells of the MDPC-23 cell line. The spontaneously immortalized MDPC-23 cell line was derived from fetal mouse molar papillae, made continuous by the 3T6 method and cloned by dilution. These cultures have been passaged 77 times after cloning, form multilayered nodules, and have high alkaline phosphatase activity. The data show positive reactivity in odontoblasts in 19-d mouse fetal incisors as well as in cultures of MDPC-23 cells by fluorescence and confocal microscopy. In addition, these cultures were characterized by phase microscopy and scanning and transmission electron microscopy. These findings suggest that MDPC-23 cells are of the odontoblast lineage.

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Established cell lines, which are of the odontoblast lineage or which mimic odontoblasts, may have utility for a variety of purposes to the dental scientist. These include (a) synthesis of large amounts of specific molecules including protein, mRNA and DNA for study of the process of dentinogenesis, (b) surveying a variety of molecules such as hormones, growth factors and cytokines for possible effects on cell function, and (c) initial tests for effects of extrinsic molecules such as dental materials and environmental molecules for possible effects on odontoblast function and potential for altering dentinogenesis. A number of primary cultures of pulpal cells from a variety of species have been isolated and reported in the literature. There have been reports of only four established cell lines from dental pulp. Kasugai *et al.* (1) and Kawase *et al.* (2) both reported development of cell lines from rat dental pulp by the 3T3 method. Although both cell lines synthesize osteopontin (OPN), it appears that neither express phosphophoryn (DPP) or dentin sialoprotein (DSP). MacDougall *et al.* (3–5) reported a cell line (MO6-G3) immortalized from fetal mouse molar dental papillae mesenchyme by transfection with a retrovirus carrying a temperature-sensitive mutant of SV-40 large T antigen, followed by cloning by dilution. MO6-G3 was shown to be positive for DPP and DSP at both the transcriptional and translational levels, and for DMP-1 at the transcriptional level. In addition, it was shown to express type I collagen, alkaline phosphatase (ALP) and calbindin D 28K. The MDPC-23 cell line makes transcription products for DSP, type I collagen, ALP, OPN and osteocalcin (OCN), but is negative for DMP-1 (6).

The MDPC-23 cell line was developed as a spontaneously immortalized cell line derived from fetal mouse first molar papillae cells and cloned specifically to have high ALP activity, the ability to form multilayered nodules, and a cell doubling time of less than 24 hours. The objective of the present study was to determine if, by immunohistochemistry or confocal microscopy, DSP and DPP could be localized by means of fluorescein-labeled secondary antibodies to rat DSP and a moderately phosphorylated rat DPP (DPP-MP), respectively, in cultured MDPC-23 cells as

well as odontoblasts of 19-d fetal mouse incisor teeth.

Material and methods

The cell line was established by removing the dental papillae from 18–19-d fetal mice, dispersing these mesenchymal cells with 0.25% trypsin and growing them in complete medium (α-MEM with 10% FCS, and supplemented with penicillin, streptomycin and glutamine) at 37°C in a humidified incubator with 5% CO_2/95% air. These cultures were allowed to proliferate and were subcultured at half density through 7 d, and then were placed on a 3T6 regimen (subcultured every 3 d at 600 000 cells/50 μm dish) for spontaneous immortalization of the cell line (7). After 28 passages (approximately 3 months), the cells were cloned by dilution, with the first cloned cell designated as "passage 0" of this mouse dental papilla cell-23 (MDPC-23) cell line.

Some cultures were allowed to grow to confluence in complete medium plus ascorbic acid (AA; 50 μg/ml) with or without β-glycerophosphate (β-GP; 2 mM) in order to encourage formation and mineralization of multilayered nodules. AA was used to promote collagen and extracellular matrix formation (8). β-GP was necessary for mineralization of the multilayered nodules (9). Three passages of this cloned cell line (7, 37 and 77) have been maintained for study. This paper presents microscopic and immunohistochemical data on passage 7 cells. Phase contrast microscopy was used to follow the cells during plating, culture maintenance, and multilayered nodule development.

These antibodies to rat DPP-MP (10, 11) and rat DSP (12) were developed in the laboratory of one of the investigators (W.T.B.). Fluorescence microscopy, immunohistochemistry, and confocal microscopy were used to determine whether DSP or DPP was being expressed. Specifically, the cultures were fixed with 100% methanol at −10°C for 15 min and allowed to air dry. They were then incubated with 1% goat serum in PBS for 20 min to block non-specific binding, followed by washing twice with PBS. Next, they were incubated with diluted affinity column-purified antibodies to rat dentin-specific proteins (DSP or DPP-MP; 1:1000 dilution) overnight, and washed twice with PBS. Control cultures were treated with PBS rather than primary antibody. Next, the slides were incubated (1:50 dilution) with secondary antibody (goat anti-rabbit affinity purifed IgG) conjugated with FITC (Vector Laboratories, Burlingame, CA, USA) for 60 min, followed by washing twice with PBS. The cultures were cover-slipped with a Vectashield mounting media (Vector) on glass slides. Fetal CD-1 mouse heads (19 d), which served as positive *in vivo* controls, were sagitally sectioned and fixed in Bouin's solution for 6 h. They were then washed in running water overnight, processed through an ethanol series for dehydration, embedded in paraffin, and sectioned at between 5 and 10 μm. The sections from the fetal heads were treated with antibodies in a manner similar to the cultured cells. Finally, the same sections which were viewed by fluorescence microscopy were also viewed by a krypton-argon laser of a Bio-Rad MRC-600 confocal setup utilizing a Nikon Diaphot inverted microscope. The excitation wavelength for FITC label was 490 nm and emission wavelength was 525 nm. Confocal images were digitized, captured by PhotoShop 3.0 (Adobe Systems, San Jose, CA, USA), and printed on a Kodak XLS 8600 dye-sublimation printer.

Cells observed with scanning electron microscopy (SEM) were plated on clean plastic coverslips or on hydrated rat tail tendon type I collagen gels on glass coverslips in the bottom of 24-well dishes. These cultures were maintained for up to 28 d, in the presence of AA +/− β-GP. Cultures were fixed with 2.5% buffered glutaraldehyde, dehydrated through an ethanol series, and placed in 100% hexamethyldidilizane for three changes before drying overnight. The cultures were then sputter-coated with gold and examined with an AMRAY scanning electron microscope with electron dispersive spectroscopic (EDS) analysis.

Cultures observed by transmission electron microscopy (TEM) were fixed with 2.5% buffered glutaraldehyde, post-fixed in buffered OsO_4, and dehydrated through an ethanol series. Cultures on polystyrene were treated with 2-hydroxypropyl-methacrylate and then polymerized in Epon® resin (Ladd Research Industries, Burlington, VT, USA) Cultures on collagen gels were lifted out of the well and processed through propylene oxide as the intermediate between ethanol and Epon.

Results

From the time of plating, the cells of these clonal cultures were epithelioid, rather than fibroblastic (i.e. spindle-shaped or stellate). In addition, every cell, whether individual or in clusters or nodules, was covered with multiple small processes of the cytoplasmic membrane (Fig. 1). When cells were plated at low density (1000–5000 cells/cm^2) in complete medium plus AA and grown to confluency and beyond, the cells grew into clusters and then into multilayered nodules (Fig. 2). If plated at higher density (30 000–50 000 cells/cm^2), distinct multilayered nodules were less apparent before the cultures became confluent. Cells plated on tissue

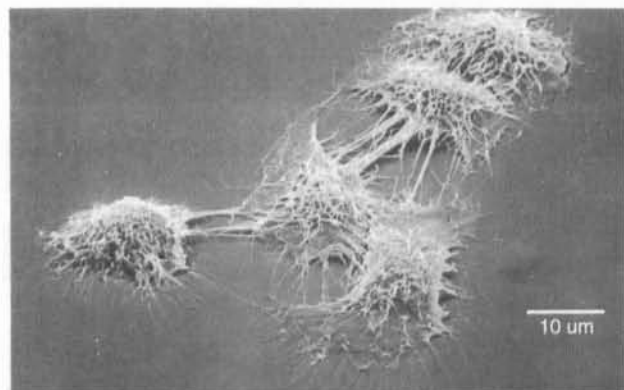

Fig. 1. Phase contrast micrograph of MDPC-23 cells (passage 7) 1 d after plating at 1000 cells/cm² on tissue culture plastic. Cells are epithelioid and tend not to migrate far from where they were plated. Cells have many fine cellular processes on their cytoplasmic membranes (× 1900).

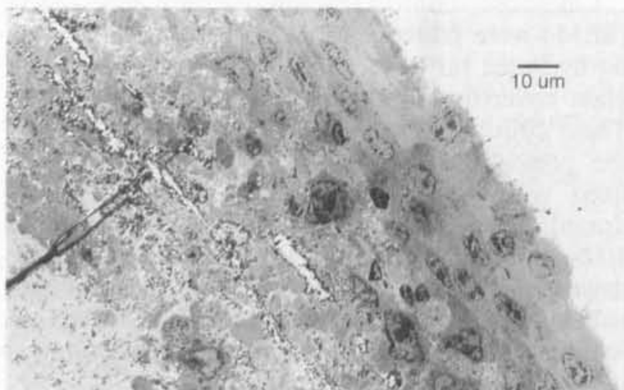

Fig. 2. TEM of section through multilayered nodule 8–9 cell layers thick. Viable cells are underlaid by necrotic cells on collagen gels. These cells are epithelioid rather than fibroblastic. The top cells are cuboidal and display multiple cell processes of the cytoplasmic membrane (× 1000).

culture plastic tended to have a lower profile than those on hydrated collagen gels. The cell processes stretched out and attached to substrate as well as to other cells whether they were plated on plastic or on collagen gels. Finally, when grown on collagen gels, random multilayered nodules reached 6–10 vital cell layers in thickness within 10 days (Fig. 2). Below the vital cells, necrotic cells could also be observed, immediately adjacent to the collagen gel. Between cells in the multilayered nodules, new collagen was expressed. When 2–5 mM β-GP was added to the cultures, spicules of mineral could be observed surrounding newly synthesized collagen fibers (data not shown).

Pure cultures of MDPC-23 cells were plated at high density and grown for 1 wk before treating them with either rabbit anti-rat DSP or anti-rat DPP-MP, followed by FITC-labeled goat anti-rabbit IgG. These cultures gave a positive reaction for both proteins, with the anti-DSP reaction being

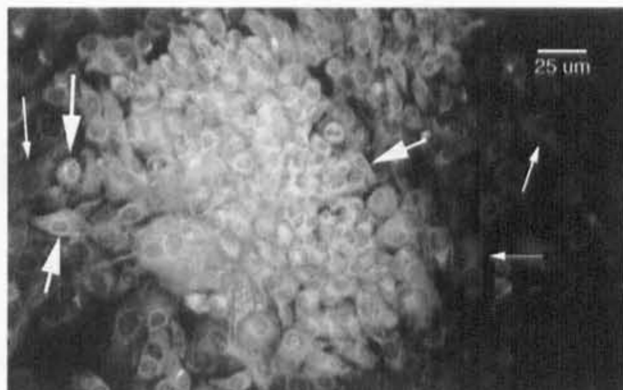

Fig. 3. FITC-labeled antibody for rat DPP-MP in mixed cultures of MDPC-23 cells (passage 7) and mouse gingival fibroblasts, each plated at 30 000 cells/cm² and grown 1 wk on glass coverslips in complete medium plus AA. The MDPC-23 cells, whether they occur in dense multilayered nodules or as individual cells, have a bright yellowish green cast in this micrograph (large arrows). They are smaller cells with smaller nuclei as compared to the mouse gingival fibroblasts. The FITC label tends to camouflage the nucleus in some of the MDPC-23 cells, which are prominent in the nodules. The mouse gingival fibroblasts are larger, stellate cells with larger nuclei than MDPC-23 cells (small arrows). They occur individually around the nodules, are not clustered, and have a deeper green cast than do the MDPC-23 cells (× 480).

slightly stronger than the anti-DPP-MP reaction. In order to confirm the specificity of the antibodies for these cells, mixed cultures of MDPC-23 cells and mouse gingival fibroblasts were plated, each at high density, in the same medium for 1 wk and again reacted with these antibodies. In Fig. 3, the MDPC-23 cells formed many multilayered nodules in which the FITC-labeling for DPP was visualized as a bright yellowish green label principally in multilayered nodules, but also in some surrounding clusters and individual cells. The mouse gingival fibroblasts were larger, stellate cells with distinctly larger nuclei, around the periphery of the nodules and were unlabeled. Likewise in Fig. 4, multilayered nodules of MDPC-23 cells were brightly labeled for DSP with FITC, while surrounding single, stellate gingival fibroblasts were unlabeled. The background of the original photographs was a dull, drab-green and this was brightened enough by photographic processing to visualize the gingival fibroblasts. Control slides in which PBS had been substituted for the primary antibody gave a dull, olive-drab staining of the entire slide without definition of the MDPC-23 cells as bright yellowish-green (data not shown).

As controls for immunoreactivity of these antibodies with odontoblasts, 19-d fetal mouse jaws were chosen. This was the approximate age of the animals from which the cultures were derived. Also, incisor teeth were observed because odontoblast differentiation varied from precursor cells to young

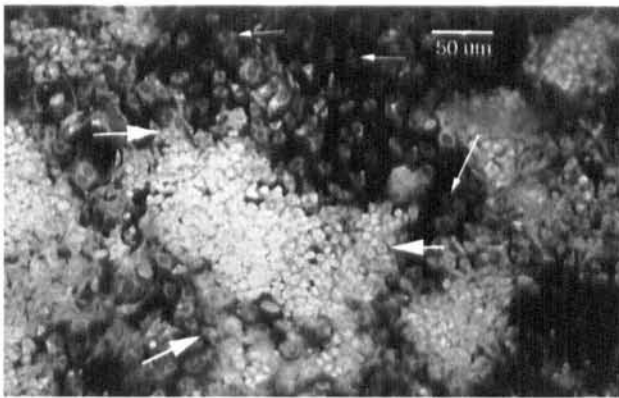

Fig. 4. FITC-labeled antibody for rat DSP in mixed cultures of MDPC-23 cells (passage 7) and mouse gingival fibroblasts, each plated at 30 000 cells/cm², grown 1 wk on glass coverslips in complete medium plus AA. Like FITC-staining for DPP-MP, the antibody is more intense in the multilayered nodules and smaller clusters of cells (large arrows) than in surrounding gingival fibroblasts (small arrows), which grow as individual cells (×280).

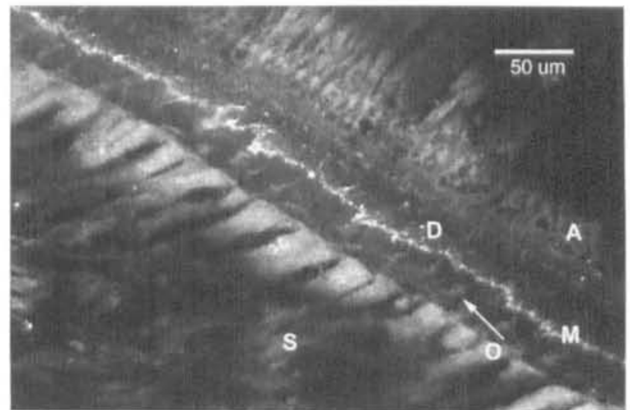

Fig. 6. DSP localized in the anterior one-third of mandibular incisor of 19-d fetal CD-1 mouse. Odontoblasts (O), odontoblastic processes (arrow), and mineralization front (M) have a strong granular labeling reaction with FITC-conjugated secondary antibody to anti-rat DSP. New dentin apical to the mineralization front also has granular labeling.There is a lower level of labeling in the subodontoblastic cells as well as in the ameloblastic (A) layer (×400).

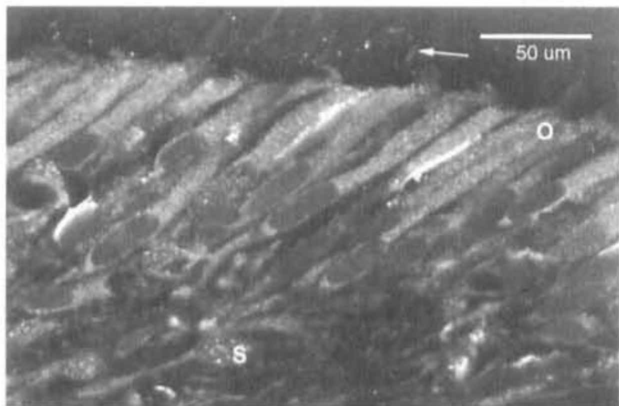

Fig. 5. DPP-MP localized in anterior one-third of mandibular incisor of 19-d fetal CD-1 mouse. Confocal microscopy of the odontoblasts (O) shows a fine granular structure which represents FITC-conjugated secondary antibody to anti-rat DPP-MP located both apically and basally to the nucleus, and over some odontoblastic processes. In addition, there is some labeling of subodontoblastic cells (S). The bright lines are thought to be artifactual, perhaps caused by the fixation technique (×550).

odontoblasts in these teeth, and because the odontoblasts of the first molars at this age were making very little dentin matrix. The site of the confocal micrograph in Fig. 5 is in the anterior one-third of the mandibular incisor where the odontoblasts have begun to differentiate. This micrograph shows the labeled material (FITC-labeled secondary antibody for rabbit anti-DPP-MP) as a granular deposition both apically and basally to the non-labeled nucleus of each odontoblast. In addition, there was some labeling of subodontoblastic cells, as well as a low-level labeling of odontoblastic processes. In Fig. 6, a similar zone in an adjacent section through the same incisor was labeled with FITC-conjugated secondary antibody to rabbit anti-rat DSP. At the same dilutions of primary antibody as with DPP-MP, the label for DSP was more intense in the portion of the odontoblasts apical to the nuclei, in the the odontoblastic processes, at the mineralization front, and in the mineralizing dentin matrix. A lower level of labeling was observed in subodontoblastic cells as well as in the layer of young ameloblasts.

Discussion

The MDPC-23 mouse odontoblast-like cell line is unique in that the cells are smaller than fibroblasts, are epithelioid in shape, and have multiple small cell membrane processes from the time they are plated until they form multilayered nodules. They tend to proliferate and form clusters of cells at the point of attachment to the substrate, rather than migrating after cell division. The cell line is also unique in that it is the only spontaneously transformed mouse cell line described to date which synthesizes the proteins, DSP and DPP-MP, which are thought to be dentin-specific and synthesized mainly by odontoblasts. They also synthesize other proteins common to other mineralized tissues such as ALP, type I collagen, OPN, and OCN (6).

The antibodies used for the DSP immunostaining were affinity-purified polyclonal antibodies raised in rabbit against rat incisor dentin proteins extracted and purified by column chromatography (12). D'Souza *et al.* (15), utilizing this antibody preparation, found that DSP was first expressed in young odontoblasts and their processes in 5–11 d postnatal rat first molars, as the predentinal matrix was being secreted and before onset of mineraliz-

ation. These authors also reported staining of early pre-dentin. BRONCKERS *et al.* (16), also using this antibody preparation, localized DSP to newly differentiated odontoblasts, predentin and dentin of 1- and 3-d neonatal rat first and second molars. In addition, they reported labeling of pulpal cells and preameloblasts in these teeth. BUTLER & RITCHIE (17) and RITCHIE *et al.* (18) reported from *in situ* hybridization studies that DSP mRNA was present in polarized odontoblasts of 2-d-old rat molars. Preodontoblasts, non-polarized cells of the dental papilla, and osteoblasts surrounding the young tooth organ did not hybridize with the DSP riboprobe. Studies using antibodies to DSP in mouse tissues have not been reported previously.

The antibody used for DPP immunolocalization was a rabbit polyclonal antibody to one of several chromatographic fractions of rat dentin phosphophoryn (10, 11). It is thought that "moderately phosphorylated" fraction (DPP-MP) may have the same protein backbone as the lowly phosphorylated fraction (LP) and one of two highly phosphorylated fractions (HP2). Other immunolabeling studies with this particular antibody have not been reported to date. However, there is abundant evidence that odontoblasts actively synthesize and secrete phosphophoryns. For example, MUNKSGAARD *et al.* (19) showed that phosphate- and serine-containing proteinaceous material extracted from odontoblasts which were attached to the dentin walls of rat incisors, after having been treated with ^{3}H-serine and ^{33}P-phosphate, co-chromatographed with carrier phosphoprotein. Further, NAKAMURA *et al.* (20) reported immunostaining of odontoblasts by labeled monoclonal antibody for fetal calf phosphophoryn in decalcified fetal calf molars. The immunolabel was present in the body of odontoblasts and odontoblastic processes in the predentin. In nondecalcified teeth, DPP label was also found in circumpulpal dentin, but not in mantle dentin. MACDOUGALL *et al.* (21), using rabbit polyclonal antibodies to 2-d Swiss-Webster mouse dentin DPP (72 kDa), reported localization of antibody in the monocellular layer of odontoblasts, the odontoblastic processes which traversed the predentinal matrix (but not in the predentinal matrix), and in mineralized dentin matrix of sections of 1-d postnatal mouse molar tooth organs. MACDOUGALL *et al.* (22) also demonstrated immunofluorescent localization of similar anti-DPP antibodies in organ cultures of Swiss-Webster molar tooth germs removed at 16 d of gestation and cultured another 10 d. Again, DPP was localized to odontoblasts, odontoblastic processes and mineralizing dentin matrix when tooth germs were maintained in serum-supplemented medium. Later, MACDOUGALL *et al.* (3) demonstrated by immunohistochemistry that DPP also labeled cultured cells of the virally-transformed mouse odontoblast-like cell line, MO6-G3.

In the present study, FITC-labeled secondary polyclonal antibodies to rabbit anti-rat DSP or DPP-MP were used to visualize these two proteins in the MDPC-23 cell line. Controls for the specificity of the primary antibodies were (a) PBS substitution for primary antibodies and (b) odontoblasts of mandibular incisor teeth from 19-d fetal CD-1 mice. Use of PBS in place of the primary antibodies in the control slides of mixed cell cultures resulted in no difference in staining between MDPC-23 cells and mouse gingival fibroblasts. The MDPC-23 cells were not stained with FITC. In sections of mouse heads, labeling for these two proteins was in the anterior one-third of the incisor teeth. When viewed by confocal microscopy, the antibody for DSP (Fig. 6) gave a slightly stronger labeling than did the antibody for DPP-MP (Fig. 5). This may have been because of different titers of antibodies in the preparations. Within the odontoblasts, DSP label was most intense apical to the nucleus and could also be seen in the odontoblastic processes as they crossed the predentin (Fig. 6). The label was also intense at the mineralization front. The dentin matrix, which was beginning to mineralize as judged by adjacent H&E sections, was labeled at a lower level than the mineralization front. Finally, there was some labeling of the apical portions of some young, polarized ameloblasts of these 19-d fetal mouse incisors. The FITC label for DPP-MP (Fig. 5) was also granular, was localized throughout the cytoplasm and cytoplasmic processes, but was not observed at the mineralization front or in dentin. This label was apparent throughout the cytoplasm, but not in the nuclei. Finally, low-level labeling for both DPP-MP and DSP were observed within the cytoplasm of cells in the sub-odontoblastic layer (Figs. 5, 6).

Recently, there have been two papers reporting that DSP and DPP are sequentially related, that they may be derived either from a bicistronic gene, or may be segments of one large open reading frame of DNA . RITCHIE *et al.* (23) described an 801 bp sequence immediately downstream from the 3′ end of odontoblast-specific cDNA identified as rat DSP. According to these authors, the N-terminal amino acid sequence corresponds well to that of one form of phosphophoryn, HP2 (10). MACDOUGALL *et al.* (5) described a larger 4420 bp cDNA sequence protein (DDSP) which includes both DSP and DPP sequences, derived from screening a mouse molar tooth library. The corresponding protein sequence for DSPP contains 940 amino acids derived from a single open reading frame. At the amino terminus, the peptide (DSP) is 75%

homologous with rat DSP. Downstream, within a sequence identified as DPP, there is a RGD sequence followed by repeats of aspartic acid and serine. The latter sequence is consistent with that of rat dentin phosphoprotein (phosphophoryn; DPP). Both HP-1 and HP-2 sequences can be found in the DSPP molecule. In addition, MacDougall suggests that DPP-MP may be a part of the linker region between the DSP and DPP coding regions. Thus, it is expected that the odontoblasts in the fetal mouse incisors as well as the MDPC-23 cells should stain positively with antibodies to both DSP and DPP-MP.

It has been suggested by several investigators that both DSP and DPP (24–26) may be secreted through the odontoblastic processes directly into the mineralization front at the predentin-dentin junction. However, in the present study only antibodies to rat DSP were found at the mineralization front and in mineralizing dentin. Antibodies to rat DPP-MP were maintained within the cytoplasm of the odontoblasts. One possible explanation is that the DSP antibodies were bound to an epitope of a secreted protein, perhaps the DSPP molecule, while the DPP-MP antibodies were bound to a protein ("linker protein") which remained in the cytoplasm of odontoblasts and was not secreted. Finally, a few cells immediately beneath the odontoblastic layer appeared to be faintly labeled with the antibodies of both DSP and DPP. These products appear to be entirely cytoplasmic.

In conclusion, we have presented evidence that our spontaneously immortalized and cloned odontoblastic-like cell line derived from 19-d fetal CD-1 mouse first molar papillae (MDPC-23) are positive for expression of both DSP and DPP-MP molecules, are epithelioid in shape from the time of plating, and make multilayered nodules when maintained beyond three days without passaging. The presence of DSP and DPP-MP in odontoblasts in the same region of fetal mouse incisor teeth as well as in the MDPC-23 cloned cell line suggests that MDPC-23 is a part of the odontoblastic lineage.

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