Prion Protein (PrP) in Human Teeth: An Unprecedented Pointer to PrP's Function

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Abstract

Although prion protein's (PrP) involvement in transmission of degenerative neurological diseases has been subjected to considerable scrutiny, its physiological role is still obscure. The distribution of PrP in dental tissues was investigated using three different methods: immunohistochemistry, cell culture, and scanning electron microscopy. PrP knockout mice were found to have marked anomalies in dentin structure. In human teeth, cementoblasts and odontoblasts showed prominent staining for PrP at levels comparable to those of nerve fibers. Epithelial rests of Malassez, which are remnants of a cell type formerly forming enamel, were also positive. Thus, all PrP-positive cells in human dentition are in some way involved in calcified tissue formation. This suggests a previously undetected function of prion protein in healthy vertebrates as evidenced by an obvious phenotype in PrP knockout mice. Periodontal and pulpal tissue exposed by disease or trauma might represent a clinically relevant entry point for prions incorporated orally and thus a possible mode of infection. *(J Endod 2007;33:110–113)*

Key Words

Prion protein (PrP), teeth, immunohistochemistry, SEM

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Prion protein (PrP) is a developmentally regulated (1) glycoprotein found in all mammals. Sequences related to PrP have been reported in other vertebrates including fish (2, 3). The process of refolding from the native (PrP^{C}) to an alternative conformation (PrP^{Sc}) is believed to be a key event in the pathogenesis of disorders collectively referred to as "transmissible spongiform encephalopathies" (TSEs/"prion diseases"), the prototype of which is scrapie in sheep.

According to structures found in PrP's gene promoter suggestive of housekeeping genes (4), PrP^{C} is ubiquitously detectable. Bendheim et al. (5) found it in nearly all hamster tissues. Although the highest levels of PrP are found in the CNS, it can also be detected in the peripheral and enteric nervous system and non-neural tissues (6). Horiuchi et al. (6) detected it in the parotid gland in sheep by immunoprecipitation. Ironside et al. (7) reported negative PrP immunocytochemistry in salivary glands of victims of the new variant of Creutzfeldt–Jakob disease (vCJD), but found it in the trigeminal ganglion.

Since the advent of variant Creutzfeldt–Jakob disease (8), which preferentially affects young people, different theories regarding the mode of transmission have been put forward. One of these theories propagates the idea that adolescents could have been infected through wounds arising from teething and tooth loss (9).

We investigated the distribution of PrP in dental tissues by: (1) immunostaining of human teeth (antibody 6H4); (2) reverse transcriptase–polymerase chain reaction (RT-PCR)/Western blot (antibody 6H4) of cultured human dental pulp, periodontal ligament, and ectomesenchyme cells; and (3) scanning electron microscopy (SEM) of molars from knockout ($Prnp^{0/0}$, Zrch I), wild-type, and PrP-overexpressing rescue mice.

Materials and Methods

Human teeth and tooth germs were collected from healthy patients undergoing surgical and/or orthodontic treatment. The study protocol was approved by the Ethics Committee of the University of Düsseldorf and informed consent was granted by each patient.

Immunohistochemistry

After extraction, teeth were immediately fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) and decalcified in 4 M formic acid for 2 weeks. Tissues were cryoprotected in a 30% sucrose solution in 0.1 M TB for 48 hours, embedded in Jung Tissue Freezing Medium (Leica, Bensheim, Germany), snap frozen in liquid N_2, and stored at −80°C. Sections of 40 μm were cut on a cryostat.

At room temperature, under constant shaking, sections were incubated in 0.3% H_2O_2 in 0.05 M TBS for 20 minutes to inhibit endogenous peroxidases. Non-specific antibody binding was blocked with 2% bovine serum albumine (PAA, Pasching, Austria) and 10% normal horse serum (Vector Laboratories, Burlingame, CA, USA) in 0.05 M TBS for 30 minutes. Sections were incubated with anti-PrP antibody 6H4 (1:3000) (Prionics, Schlieren, Switzerland) for 48 hours at 4°C, 1 hour in biotinylated horse anti-mouse antibody (1:500; Vector), Vectastain ABC kit (Vector), and developed in 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) containing 0.0013% $NiSO_4$ and 0.004% H_2O_2.

Controls included (1) omission of primary or secondary antibody (negative control), (2) a second prion protein specific monoclonal antibody (8H4, Alicon AG, Zürich, Switzerland) (positive control), and (3) Western Blot of protein isolated from brain of $Prnp^{0/0}$ and wild-type mice to demonstrate the specificity of the 6H4 antibody.

Cell Culture

Permanent human molar teeth and tooth germs were used for cell culture. Immediately after extraction, teeth were repeatedly rinsed with PBS and the crowns were separated from the roots using a sterile diamond bur (Meissinger, Düsseldorf, Germany). Pulpal tissue from the crown and root canals was sampled for cell culture (pulpal cells). The adherent periodontal ligament (periodontal ligament cells) was scraped off using a sterile curette (Hu-Friedy, Leimen, Germany). Ectomesenchymal tissue was harvested from tooth buds (ectomesenchymal cells). All tissue samples were minced ($\leq$1 mm^3), sequentially digested with a mixture of 1 mg/mL collagenase (type IV, Sigma, Deisenhofen, Germany) and 4.5 U/mL elastase (type IV, Sigma) in serum-free medium (2 hours at 37°C). The released cells were washed with serum-containing medium to inhibit collagenase and transferred to 10-cm^2 cell-culture flasks (Greiner GmbH, Frickenhausen, Germany). Cells were grown in a medium consisting of DMEM F-12, 11% FBS, 5 μg/mL insulin-transferrin-sodium selenite, penicillin-streptomycin, and 2 mM glutamine (all from Sigma). The cultures were incubated in humidified atmosphere (5% CO_2, 37°C). Primary cells were passaged at the time of confluency (3–4 weeks) and the first subculture cells were used for RNA isolation.

RNA Isolation and PCR

For harvesting, cells were trypsinized, centrifuged, resuspended in Qiazol (Qiagen, Hilden, Germany), and snapfrozen in liquid N_2. After storage at −80°C, cells were thawed and passed through disposable syringe needles of 0.9 mm (20 gauge) and 0.6 mm (23 gauge) diameter, respectively. RNA was precipitated by the phenol-chloroform procedure. Genomic DNA was digested with DNase I (Qiagen). RNA was purified with RNeasy MinElute columns (Qiagen) and reverse transcribed with Revert-Aid (Fermentas, Vilnius, Lithuania) using random hexamer primers, according to the manufacturer's instructions, followed by amplification with PCR using Taq polymerase (Qiagen). Sequences for custom made primers were determined using the program Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The primer sequences were 5′-CCG AGT AAG CCA AAA ACC AA-3′ (forward primer) and 5′-TCC CTC AAG CTG GAA AAA GA-3′ (reverse primer) (Operon, Cologne, Germany). The forward primer thus corresponded to the amino acid sequence Pro_{102}–Ser_{103}–Lys_{104}–Pro_{105}–Lys_{106}–Thr_{107}–Asn_{108} of the human prion protein. The reverse primer bound 511 bp downstream on the complementary strand of the nucleotide sequence (corresponding to roughly 170 amino acids, which is beyond the coding region of the mature protein).

Scanning Electron Microscopy

Prion protein knockout mice of the Zürich I type ($Prnp^{0/0}$) (10), heterozygotes ($Prnp^{+/0}$), tga20 mice (overexpressing the prion protein 10-fold) (11), and C57BL wild-type mice were sacrificed, after which jaws were dissected out and immediately fixed in 2% glutaraldehyde at 4°C for at least 24 hours. The jaw halves were mounted on Palavit G (Heraeus Kulzer, Hanau, Germany) blocks. A transverse section of the molars was ground using a cylindrical bur under visual control until the pulp chamber was cut open. To remove residual pulp tissue, the blocks were treated with 1.5% sodium hypochlorite (NaOCl) with an ultrasonic bath for 10 minutes followed by 1.5% NaOCl without an ultrasonic bath for 1 hour, and 0.1% trypsin without an ultrasonic bath for 24 hours.

After being mounted onto aluminum stubs (Plano, Wetzlar, Germany), the jaws were sputtered with gold in an Edwards Sputter Coater S150B for 90 seconds. The specimens were observed under a DSM950 scanning electron microscope (Zeiss, Oberkochen, Germany) at 20-kV accelerating voltage. Aspects of the specimens that showed the dentin surface as perpendicular to the line of vision as possible were selected.

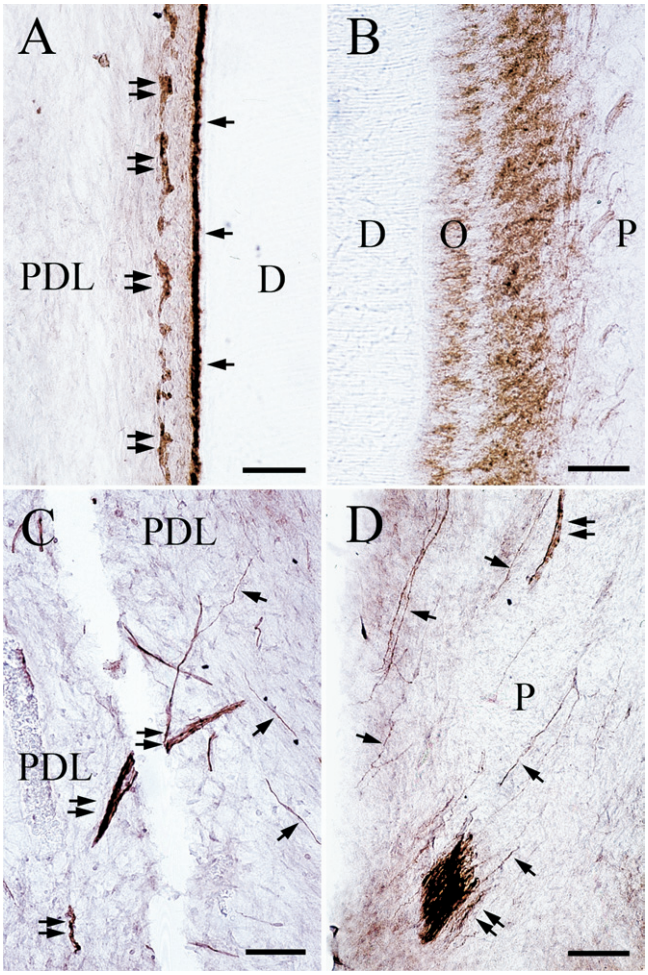

Figure 1. PrP-immunohistochemistry of dental tissues. PrP in cells of the periodontal ligament and of the dentin–pulp complex from human tooth germs before completion of apical foramen growth. Cells engaged in biomineralization (cementoblasts, *A*; odontoblasts, *B*) were primarily found to be immunoreactive to PrP antibody 6H4. Immunohistochemical reactivity for 6H4 in the ERM (double arrows, *A*) was also detected. Although odontoblast cell bodies were immunopositive, their processes did not react with 6H4 (*B*). In the periodontal ligament (*C*) and dental pulp (*D*), 6H4 was identified in thick nerve fiber bundles (double arrows) and in numerous thin varicose nerve fibers (arrows), which were often distributed around blood vessels. PDL, periodontal ligament; D, dentin; O, odontoblasts; P, pulp. Bars: 100 μm.

Photographs of dentin samples were analyzed morphometrically using ImageJ software (V1.35p, National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij). The mean size and number of dentin tubules were evaluated in 25 SEM photographs of each group (constituting an area of 10^4 μm^2). Results were statistically analyzed using one-way ANOVA and post hoc test (Scheffé procedure, SPSS V8.0, SPSS Inc., Chicago, IL, USA). We assumed a statistically significant level of $p < 0.01$.

Results

According to our expectations, PrP was present in nerve fibers. Furthermore, prominent PrP-immunostaining was found in odontoblasts (cells forming and calcifying dentin matrix) and cementoblasts (cells forming and calcifying cementum matrix). Epithelial rests of

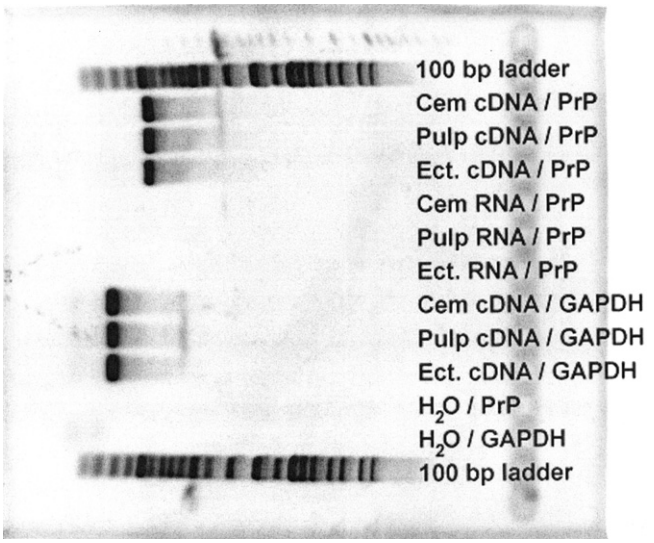

Figure 2. RT-PCR of cultured human cells. Lanes 3–5: cementoblast, pulp, and ectomesenchyme cDNA (primer: PrP). Controls: Lanes 6–8: cementoblast, pulp, and ectomesenchyme RNA (primer: PrP); Lanes 9–11: cementoblast, pulp, and ectomesenchyme cDNA (primer: GAPDH); Lane 12: H_2O (primer: PrP); Lane 13: H_2O (primer: GAPDH); lanes 2 + 14: 100-bp ladder (500 bp is darker).

Malassez (ERM, remnants of a cell type formerly forming enamel) also stained positive for PrP. No other cells, for example, periodontal or pulpal fibroblasts, were found to contain PrP (Fig. 1).

In the cultured cells (cementoblasts, pulp cells, and ectomesenchyme cells), PrP expression was evident at the mRNA level as demonstrated by PCR. Identity of PCR products was verified by sequencing (Fig. 2). The Western Blot revealed the presence of PrP protein in all cultured cell lines (data not shown).

Comparisons by SEM between molars of wild-type, $Prnp^{0/0}$ knockout, $Prnp^{+/0}$, and tga20 mice revealed a functional influence of PrP on dentin structure (Fig. 3). The genotypes (except heterozygotes) demonstrated statistically significant differences in mean area and number of tubules.

Mean area was lowest for tga20 and highest for $Prnp^{0/0}$ mice: tga20 (5.64 μm^2) < $Prnp^{+/+}$ (7.03 μm^2) < $Prnp^{0/0}$ (8.93 μm^2) ($p < 0.01$, Fig. 4*a*). A reverse order was found for the number of dentin tubules: $Prnp^{0/0}$ (381.71/10^4 μm^2) < $Prnp^{+/+}$ (481.63/10^4 μm^2) < tga20 (543.46/10^4 μm^2) ($p < 0.01$, Fig. 4*b*).

Discussion

Only those human dental cells engaged in biomineralization contain PrP at levels comparable to those of nerve fibers. Both cementoblasts and odontoblasts showed prominent staining for PrP. Positive staining was also found in the ERM. Thus, all PrP-positive cells in the human dentition are in some way involved in calcified tissue formation and their origin is—as it is for neural cells—ectodermal.

The role of PrP in cells forming calcified dental tissues was verified by SEM analysis. The analysis revealed significant structural anomalies in the dentin of molars in PrP knockout, as well as in PrP overexpressing mice. These anomalies (variability of diameter and number of tubules) indicate an influence of PrP on odontoblast function. To our knowledge, this study is the first report about a phenotype in PrP knockout mice. Consequently, we suggest a functional influence of PrP on odontoblasts demonstrating a physiological role for PrP during dentinogenesis. Whether a lack of PrP influences other calcified tissues remains to be demonstrated.

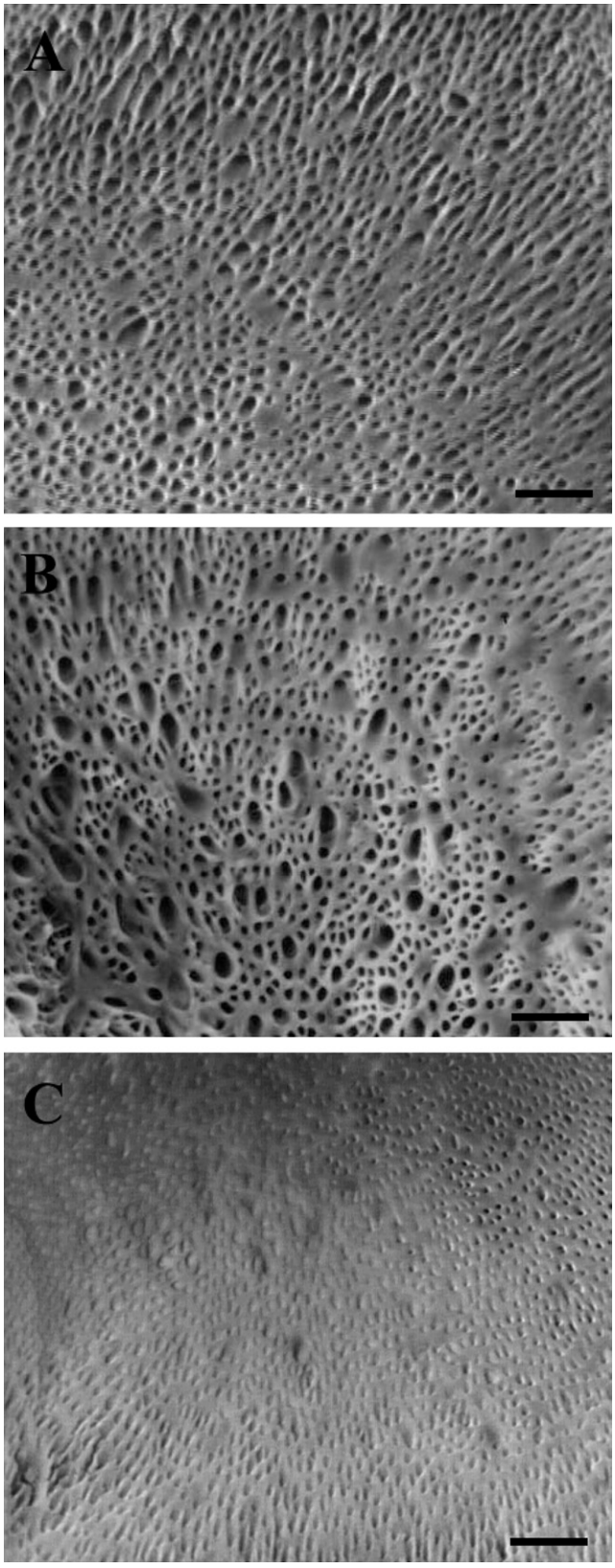

Figure 3. Pulpal aspects of murine dentin (×500). Wild-type dentin shows a homogeneous structure. Orifices of dentin tubules show little variability in diameter (*A*). Tubuli of $PrP^{0/0}$ dentin are bigger and show great variability in diameter (*B*). Tubuli of tga20 dentin are smaller and show little variability in diameter (*C*). Bar: 20 μm.

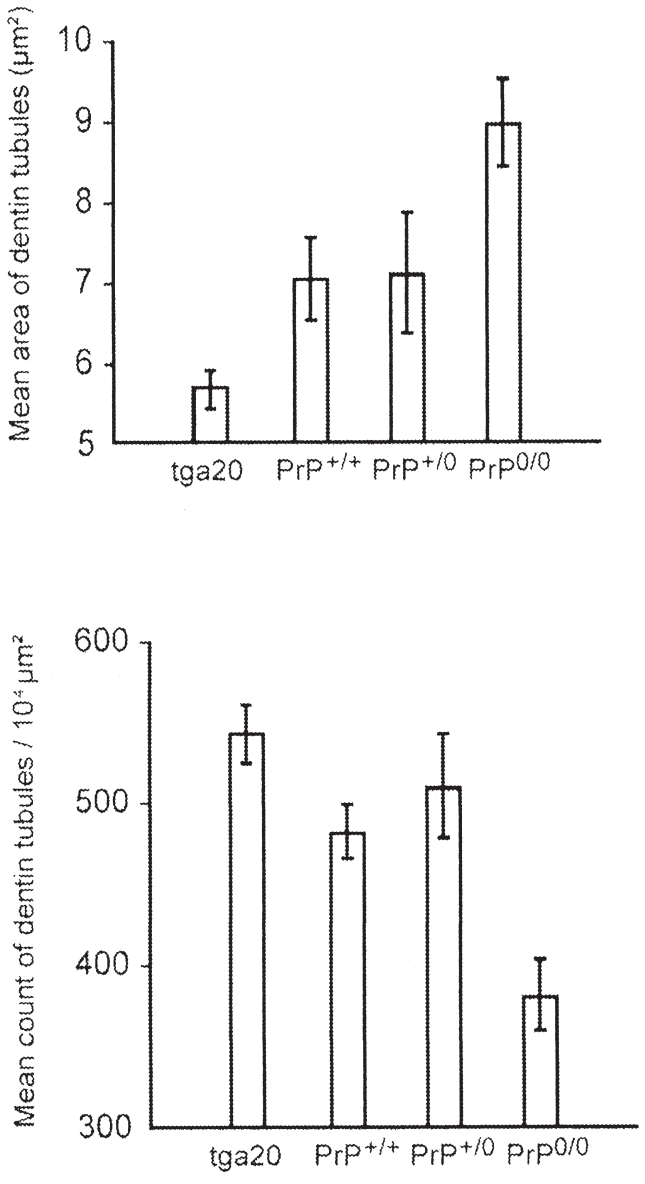

Figure 4. (*a*) Mean area of dentin tubules (mean ± 2 SE). (*b*) Mean count of dentin tubules (mean ± 2 SE).

Using in situ hybridization, Salès et al. (12) detected PrP mRNA in the tooth bud of hamsters at embryonal day E14.5. Manson et al. (13), using the same method with mouse embryos, found hybridization in the dental lamina at E13.5 and in tooth buds at E16.5. The hybridization signal at this last stage was reported to be higher than that in any other tissue of the embryo (13). Thus, an important role for PrP^C in the oral cavity was to be expected. Our present study demonstrates a remarkable correlation between the cells engaged in biomineralization of hard tissue in the oral cavity and their expression of PrP^C.

Clinical relevance might be expected when considering transmission of TSEs, which was previously demonstrated by lesions in oral mucous membranes in mice (14), by the dental pulp in hamsters (15), and the tongue in hamsters (16, 17). Pulpal tissue can be exposed by carious lesions, cavity preparation, or trauma. PrP-containing cells might also be exposed by periodontal diseases, which are widespread among the population worldwide. Additionally, therapeutic intervention could be attributed to the risk of infection by contaminated dental instruments (such as endodontic files, burs, or curettes) (18). Thus, in case of exposure, human pulpal and periodontal cells might be able to transport and propagate prions, thereby opening up an interface between prions incorporated orally and the CNS by the trigeminal ganglion.

Finally, as functional dentition is a prerequisite for most vertebrates to attain the reproductive age, and thus to propagate their genes, PrP's involvement in odontogenesis could be another reason for the evolutionary conservation of PrP (19) in the lineage of vertebrates.

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