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# The Impact of Bioactive Molecules to Stimulate Tooth Repair and Regeneration as Part of Restorative Dentistry

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For years, dental surgeons have used a limited number of capping agents to keep teeth alive. The most efficient was calcium hydroxide. Lessons from developmental biology have provided a better understanding of the genes involved in normal and pathologic processes. Added to an arsenal of transcription factors, growth factors and a series of extracellular matrix (ECM) molecules pave the road for controlled tissue repair and regeneration. These bioactive molecules constitute a large family that will provide

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the tools to modify everyday practice in dentistry substantially in the near future. The investigations discussed in this article are aimed at promoting the healing or regeneration of dental pulp which, in this context, either forms a mineralized barrier of limited size (the so-called ''reparative dentinal bridge'') or induces a more extensive mineralization area, with the prospect of filling the crown and root pulp partially or totally. The effects of a few ECM molecules have been investigated with these aims. The authors have used them in vivo, in an animal model developed in their laboratory (Fig. 1A), and in vitro, on clonal cell lines of odontoblast precursors obtained from mouse embryo transgenic for an adenovirus-SV40 recombinant plasmid. The effects induced by bioactive molecules on the pulp and on cells are under investigation, and this report summarizes the effects of direct implantation of these cells in the pulp. This review summarizes the experimental approaches performed by the authors' group, with greatly appreciated contributions from a network of collaborators.

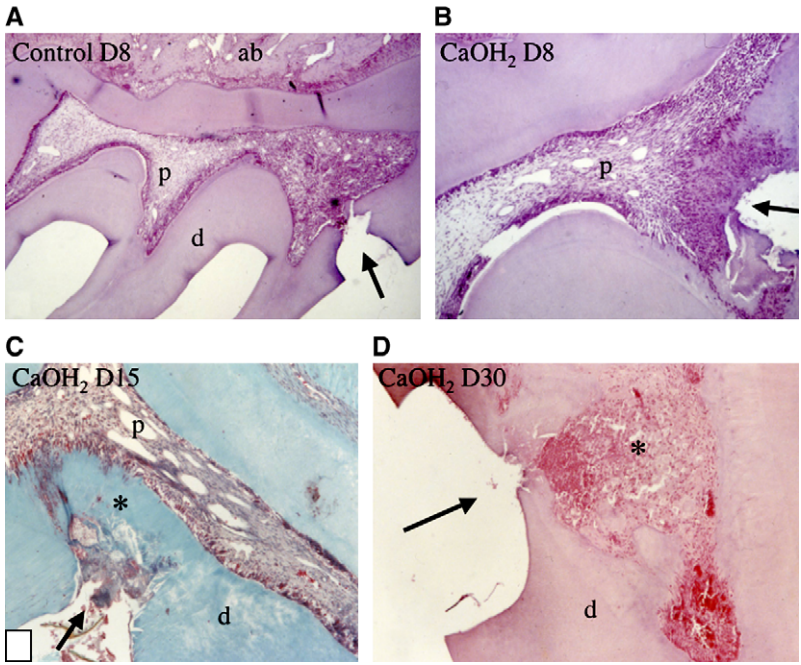


Fig. 1. (*A*) Eight days after the preparation of the cavity and pulp exposure (D8), an inflammatory process is seen in the mesial part of the pulp chamber. (*B*) At day 8 (D8), pulp capping with calcium hydroxide induces a limited reaction; (*C*) 2 weeks (D15) after capping, a reparative dentin bridge starts to be formed (*); and (*D*) after 1 month (D30), a thick heterogenous dentin bridge fills the mesial part of the pulp (*). The arrow indicates the cavity that has been prepared and location of the pulp exposure. ab, alveolar bone; d, dentin; p, pulp.

## A brief historical survey

### *Calcium hydroxide: cellular mechanisms leading to the formation of a dentinal bridge*

For more than 60 years, dental surgeons have used calcium hydroxide as a direct capping agent to induce the formation of a reparative dentinal bridge. In doing so, this bioactive material contributes to the repair of a pulp exposure [1]. Indirect capping with calcium hydroxide used as cavity liner contributes to the formation of reactionary dentin. The reaction is the result of the biologic properties of $Ca_2OH_2$. As a pulp-capping agent, the high alkaline pH of the preparation induces a burn of limited amplitude at the surface of the pulp exposure. Below the scar, within a few days and when the inflammatory process starts to be resolved, reparative cells are recruited in the central part of the pulp (see Fig. 1B). A first cell division then occurs in the central part of the pulp. The two daughter cells migrate toward the wounded area, where a second cell division occurs [2]. Fully differentiated odontoblasts, which are postmitotic cells, do not participate in these events, and there is evidence that adult resident stem cells that have properties in common with bone-marrow stem cells initiate this process [3–5]. Another possibility is that cell phenotype plasticity allows some cells, already differentiated, to dedifferentiate and then redifferentiate into odontoblast-like or osteoblast-like cells. Therefore, the so-called ''pulp stem cells'' are more likely multipotent or intermediary undifferentiated cells. This does not exclude the possibility that endothelial cells, pericytes, and pulp fibroblasts also may be candidates for contributing to the population of cells that are recruited, and this constitutes the first step in a series of events that are not understood fully. Cell proliferation is the second event. When a sufficient number of cells is obtained to cover the whole surface that has to be repaired, the cells start their final differentiation.

The reparative process long has been considered as resulting from odontoblast-like cell activity. These cells also are termed neo-odontoblasts or third-generation odontoblasts. There is evidence that at the beginning of the reparative bridge construction, newly differentiated osteoblasts contribute to osteodentin formation (see Fig. 1C, D). At a later stage, and after a terminal phase of polarization, osteoblast/odontoblast precursors become odontoblast-like cells, expressing molecules that are shared by bone and dentin. Actual identification of the terminal phenotype of cells involved in reparative dentin formation is difficult, because the cell types cannot be distinguished easily and they differ only by the level of expression of some ECM molecules. For example, the dentin sialophosphoprotein (DSPP) has been considered a molecule expressed exclusively by odontoblasts and, therefore, a phenotypic marker. Now, DSPP, which is secreted and split immediately into dentin sialoprotein (DSP) and dentin phosphoprotein (DPP), is known to be expressed not exclusively by odontoblasts but also by

osteoblasts in a 1:400 ratio [6]. This lack of specific markers renders the precise identification of the reparative cells difficult.

Whatever the biologic agent that stimulates reparative dentin formation, an ECM molecule or a growth factor, and whatever the type of cells implicated in the process, the three successive steps (described previously)—recruitment, proliferation, and differentiation—all are required. Long after the introduction of calcium hydroxide in dental therapies, the effects of other bioactive molecules were investigated. In the early 1990s, Rutherford and coworkers [7,8] demonstrated that indirect capping with bone morphogenetic protein (BMP)-7, also named osteogenic protein (OP-1), was able to stimulate the formation of either reactionary dentin or, as direct capping agent, reparative osteodentin in the root canal of monkeys. These pioneering studies have opened new avenues for regenerative biologic therapies.

## *A few definitions*

To avoid misunderstandings, a consensus has to be reached on definitions. Reactionary dentin results from the stimulation of odontoblasts after carious decay or after the preparation of a cavity. The subodontoblast cell layer, the so-called ''Höhl's layer,'' also may produce a similar beneficial reaction. Beneath a calciotraumatic line, a layer of tubular dentin is formed, more or less in continuity with the dentin formed previously in nonpathologic conditions. Although the staining agent ''stains all'' that is used to visualize phosphorylated molecules, it interacts only weakly with reactionary dentin, suggesting reduced phosphorylation of the dentin matrix; the structure of this dentin apparently is normal [9].

Reparative dentin formation is under the control of events described previously. Some pulp cells produce it exclusively. If a wound is superficial, such as in a small accidental exposure during the preparation of a cavity, again the subodontoblastic layer, Höhl's layer, may be involved in the repair process. If a dentin carious process has reached the pulp or if the pulp exposure is large, the odontoblasts and the subodontoblastic layer are destroyed. In this case, reparative pulp cells are recruited, proliferate, and, after differentiation, are implicated in the formation of an atubular or osteodentin structure. The terminology used to describe the different types of dentin reflects the similarities between osteodentin and bone where osteocytes are located within lacunae. In contrast, in orthodentin, highly differentiated and polarized odontoblasts display cell bodies that are not embedded in the mineralized tissue but always are located beneath the predentin and at the periphery of the pulp. Protracted odontoblast processes alone are anchored inside the lumen of the tubules in dentin and may extend up to the dentin-enamel junction. The biologic differences between reactionary and reparative dentin are established clearly [10].

*New molecules, new approaches, and concepts*

The biologic properties, a putative role of BMPs or transforming growth factor-beta (TGF-β), and their putative role in dentin repair led to several studies to determine the effects of these molecules on dentin repair [11–15]. These studies conclude that BMPs or TGF-β may induce reparative dentin formation. Gene expression was found in human and animal dental pulps for BMPs and for their receptors [16–19]. They both probably contribute to the beneficial reaction. The intimate mechanisms mediating this action remain obscure, however.

Two major views arise from the available literature. First, there is converging evidence that reparative processes recapitulate early developmental events that lead to dental tissue formation [20]. In support of this mechanism, c-jun and jun-B proto-oncogenes [21] and the Delta-Notch signaling normally expressed at early stages of tooth development [22] are re-expressed during pulp healing. This suggests that transcription factors, growth factors, or ECM molecules may contribute to promote reparative dentin formation or, moreover, promote partial or total pulp mineralization. Pioneering investigations already are undertaken that support this mechanism [23–25].

Second, it seems important to mention a few points regarding the potential multifunctional nature of ECM molecules. This multifunctionality is a well-accepted concept that has to be taken into account in the interpretation of many studies. For a long time, the paradigm was that one molecule plays one single role. According to this way of thinking, a molecule recognized as a member of the ECM is involved in the tissue structure alone. The concept recently has been revisited or modified, and it is now well recognized that most molecules display more than one function.

For example, native collagen subunit association is regulated by the cleavage of the C-terminal nonhelicoidal extensions by a C-procollagen peptidase involved in the processing of a structural protein. The C-proteinase also was identified as a BMP-1, a molecule that is a growth factor [26]. In this specific case, the enzyme has two different functions.

As another example of multifunctionality, for years amelogenin was considered a molecule implicated only in enamel formation. Most studies were devoted to the structural aspect of the molecule. With the discovery of the spliced forms of small molecular weight amelogenins arose the perception that such amelogenin gene splice products have potential signaling properties [27]. Enamelysin (matrix metalloproteinase-20 [MMP-20]) also is expressed in cells that are not ameloblasts and, therefore, not implicated in enamel formation. These two examples suggest either that isoforms of a family of molecules obtained by alternative splicing may play different biologic roles or that after partial degradation, residual peptides may be converted in an activated form and display functional properties hidden in the intact molecule. This sheds light on the complex effects of ECM implanted in a given tissue to promote healing or regeneration.

Consequently, molecules involved in embryonic development may be used as reparative or regenerating agents. This is a result not only of the intrinsic properties of the molecules to rejuvenate a tissue but also of the multifunctionality of such bioactive molecules.

In the course of these investigations, two different approaches have been used. Various ECM molecules were implanted in exposed pulp tissue, anticipating that some cells would be specifically recruited, would proliferate, and finally would differentiate into cells involved in the formation of reparative dentin. Preliminary results have been obtained with a second approach involving clonal pulp cells that produce, after direct implantation in a target zone, a specific ECM capable in some cases of mineralizing further.

## Dentin matrix proteins composition, functions, and bioactive potentials

Dentin is a complex tissue, produced mostly by odontoblasts. Many ECM molecules already are identified. Some are associated with the mineralization process or initiation of the crystal formation or crystal growth, whereas others are acting as inhibitors (Table 1).

Recent studies demonstrate the complex biologic effects of some ECM components. They are mineralization promoters or inhibitors, but they also play a role in cell differentiation. For example, such dual functions are reported for DPP, also named phosphophoryn, a molecule that triggers dentin mineralization and regulates the gene expression and differentiation of a mouse osteoblastic cell line, a mouse fibroblastic cell line, and human mesenchymal stem cells via the integrin/mitogen-activated protein kinase (MAPK) signaling pathway [28]. Therefore, in addition to the well-documented role in mineral nucleation, DPP plays a role in cell differentiation and other novel signaling functions. Along the same lines, overexpression of dentin matrix protein (DMP)-1 induces the differentiation of embryonic mesenchymal cells to odontoblast-like cells [29]. DMP-1 is regulated by c-fos and c-jun transcription factors and plays a role in early osteoblast differentiation [30]. Signaling effects also are well documented with the amelogenin gene splice products, A+4 and A−4 [27]. The complexity is enhanced by the fact that matrix molecules are substrates for MMPs or for metalloproteinases, and fragments of the processed proteins may be biologically active. For example, DMP-1 is processed physiologically by BMP-1/tolloid-like proteinases [31]. DSP and amelogenin are substrates for MMP-2 [32]. Dentin mineralization and enamel formation are impaired by inhibitors of MMPs [32,33]

Although the concept of a pulp-dentin complex has been developed, the composition of the dental pulp differs from dentin. Table 2 summarizes the pulp ECM composition, which differs substantially from that of dentin. In addition, most of the molecules associated with the mineralization process are absent or present in minute amounts. Therefore, implantation of such molecules into the dental pulp involves introducing an exogenous protein.

Table 1
Composition and properties of the extracellular matrix molecules located in dentin and, therefore, involved in dentinogenesis

| Molecules synthesized by odontoblasts | | |
|---|---|---|
| Collagens, 90%<br>type I (90%),<br>including type I trimer (11%),<br>type III (1%–3%),<br>type V (1%–3%) | Constitute a scaffold (may be used as a scaffold or a carrier for bioactive molecules) | 3-D—network indirectly implicated in dentin or pulp mineralization |
| Noncollagenous proteins, 10%<br>Phosphorylated molecules<br>SIBLINGs | DSPP 72-kd mice, 90–95–kd rat, 155-kd bovine, cleaved into DSP (N-terminal) (53 kd) and DPP (C-terminal):large amount of aspatic acid, phosphoserine, initiator and modulator of dentin apatite crystal formation | Chromosome 4, locus q20–21, *Dspp* gene defect is associated with dentinogenesis imperfecta<br>DSP: 6 Pi/mol<br>DPP: ~209 Pi/mol |
| | DMP-1 (61 kd), activation of OC | Chromosome 4, q20–21 473 aa, proteolytically processed into 37-kd (N-terminal) and 57-kd (C-terminal) fragments |
| | #Bone acidic glycoprotein (BAG75), 75 kd | |
| | BSP (75 kd) | Chromosome 4, q20–21 303 aa, 5.85 Pi/mol |
| | Nucleator of initial apaptite crystals, inhibitor of the growth of crystals, attachment molecule (RGD), collagen fibrillation | Chromosome 4, q20–21 301 aa, 13 Pi/mol<br>Chromosome 4, locus 4q21 |
| | OPN (44 kd), RGD sequence, polyaspatic acid motifs | Mineralization inhibitor |
| | Inhibitor of HaP formation | A peptide fragment containing the RGD and SGDG sequence stimulate bone formation |
| | MEPE | |
| | 56.6 kd = osteocyte factor (OF45) | |

(*continued on next page*)

Table 1 (*continued*)

| Molecules synthesized by odontoblasts | | |
|---|---|---|
| Other phosphorylated matrix proteins | Amelogenins (spliced forms) A−4 (6.9 kd) and A+4 (8.1 kd), phosphorylated in Ser16 of the RAP domain (N-terminal) | Chromosomes x and y<br>Signaling molecules |
| | Small leucine-rich proteoglycans (SLRP) CS/DS | Decorin and biglycan may be phosphorylated |
| | Decorin = biglycan: 40 kd | Contribute to dentin mineralization |
| Nonphosphorylated proteins | KS SLRP: fibromodulin (42 kd), lumican (38 kd), osteoadherin (42 kd) | Repressor mineralization?<br>Expressed by odontoblasts: adhesion, mineralization inhibitor? |
| | OC (5.7– 6.8 kd), a member of the γ-carboxyglutamic acid containing (gla-) protein family (10–12.7 kd) | Mineralization inhibitor? |
| | Osteonectin (= SPARC protein = BM40) (43 kd) | |
| Enzymes | | |
| Metalloproteinases | MMPs: MMP-1, MMP-2, MMP-9, MMP-3, MMP-20 | Collagenase, gelatinases, stromelysin 1, enamelysin |
| | MT1-MMP | |
| | TIMPs 1–3 | |
| Others enzymes | Serine proteases, acid phosphatase, alkaline phosphatase | |
| Growth factors | TGF-β1, insulin-like growth factors I and II | |
| Proteins taking origin from the serum, not synthesized by odontoblasts | | |
| | α2HS glycoprotein (50 kd) | Present exclusively in mineralized tissues |
| | Albumin | Lipid carrier |
| Lipids | | |
| Membrane lipids | 66% | Related to dentin mineralization (crystal ghosts) |
| Lipids associated with the mineralized phase | 33% acidic phospholipids | |

*Abbreviations:* #, very similar to; CS/DS, chondroitin sulfate/dermatan sulfate; HS, heparan sulfate; KS, keratan sulfate; TGF-β1, transforming growth factor-beta 1; TIMP, tissue inhibitor of metalloproteinase.

Table 2
Extracellular matrix components of the dental pulp

| | | |
|---|---|---|
| Collagens, 34% of the whole | Type I, 56%<br>Type III, 41%<br>Type V, 1%–3%<br>Type IV<br>Type VI and fibrillin | |
| Noncollagenous proteins | Large proteoglycans | CS/DS, KS, HS: versican |
| | Small leucine-rich proteoglycans | Decorin, biglycan |
| | Fibronectin | Two forms: cellular and fibronectin taking origin from the blood serum |
| | Elastin | |
| | MMPs | MMP-1, MMP-2, MMP-9, MMP-3, MMP-20 |
| Inflammatory molecules | Interleukin-1, prostaglandines, vasoactive intestinal polypeptide-like | |
| Growth factors | TGF-β1, BMP-2, BMP-4, BMP-6 | |
| Growth factor receptors | BMPR-I | Activin receptor-like kinase-1 (ALK-1), ALK-2, BMPR-1A, ALK-4, ALK-5, ALK-6, or BMP 1B |
| | BMPR-II | Type II receptor of BMP |
| Cellular and extracellular matrix lipids | | Phospholipids |
| Molecules that are not present or are present in minute amounts | No OC, no osteonectine, no DPP, no DSP, no DMP-1, no BSP (traces of sialoprotein) | |

The properties of a few of these molecules suggest their potential as inducers of mineralized tissues, taking into account the known composition of the dental tissues and the data available on in vivo distribution and in vitro effects in cell free systems or in cell cultures. To this end, the authors established a reliable animal model to study the stimulation of pulp repair, then explored the effects of implantation of bone sialoprotein (BSP), OP-1, Dentonin (a peptide of matrix extracellular phosphoglycoprotein [MEPE]), and two spliced forms of dentin amelogenins (A+4 and A−4) in the exposed pulp of rat molar. In a second strategy, the authors performed in vitro studies on odontoblast progenitor cell lines corresponding to different steps of differentiation. This strategy allowed characterizing by reverse transcription–polymerase chain reaction (RT-PCR) the short-term effects of bioactive molecules on the level of expression of some genes and transcription factors—such as Runx2, Pax9, Msx1, and Msx2—and ECM molecules—such as type II collagen, osteocalcin (OC), and DSP. These odontoblast precursors also were implanted in the pulp.

## Mechanisms involved in the in vivo reparative processes

### *The in vivo model of exposed rat molar pulp*

In 1990, Ohshima [34] described the changes in odontoblasts and pulp capillaries after cavity preparation in the rat maxillary molar. The cavity was prepared in the mesial aspect of the first maxillary molar. The authors modified this experimental protocol by using electrosurgery to remove the gingival papilla before any cavity preparation. This allows a more consistent preparation of half moon cavities in the cervical area of the rat molar. This location avoids interference with the pulp horns, which biologically are different in the enamel free area from the rest of the pulp chamber. Secondly, this cervical location allows a better mechanical resistance to occlusal pressures and, consequently, fewer restorative fillings with glass ionomer cements (GIC) are lost. The cavity is drilled with carbide burs in fewer than 2 seconds. The preparation of the cavity and its filling with a GIC induce only a slight inflammatory reaction [35]. The residual dentin then is pushed with a steel probe, which allows obtaining a pulp exposure of limited size. This process avoids stretching of the pulp tissue around the bur and uncontrolled pulp damages. The projection of dentin debris inside the pulp may release ECM molecules, however, contributing to spontaneous pulp healing with the resultant formation of reparative dentin [23–25]. In spite of this complication, this system provides an excellent animal model for study of the effects of bioactive molecules on the dental pulp [36].

## Experimental results: in vivo and in vitro approaches

### *Bone sialoprotein/collagen implantation*

Among the dentin ECM that seems to display intrinsic bioactive properties for promoting the formation of a reparative tissue, BSP is a good candidate. Implantation of the molecule in calvaria leads to rapid repair of a critical defect greater than or equal to 8 mm, with stimulation of the recruitment of bone forming cells, which differentiate and subsequently form a small amount of cartilage, replaced by bone [37–39]. In addition, BSP is a phosphorylated protein with tyrosine sulphatation and an arginine-glycine-aspartate (RGD) attachment sequence. Several stretches of polyglutamic acid are involved in binding to hydroxyapatite [40]. BSP enhances the fibrillogenesis of collagen [41]. BSP messenger RNA is expressed by odontoblasts of the incisor during dentinogenesis, and a polyclonal antibody directed against BSP reacts positively with epitopes located in odontoblast cell bodies and processes and with the peritubular dentin [42,43]. In addition, pulp cells do not express BSP.

Eight days after the implantation of BSP with gelatin as a carrier, an inflammatory process was seen (Fig. 2A). Some loci of mineralization appeared around the dentin fragments that were pushed into the pulp

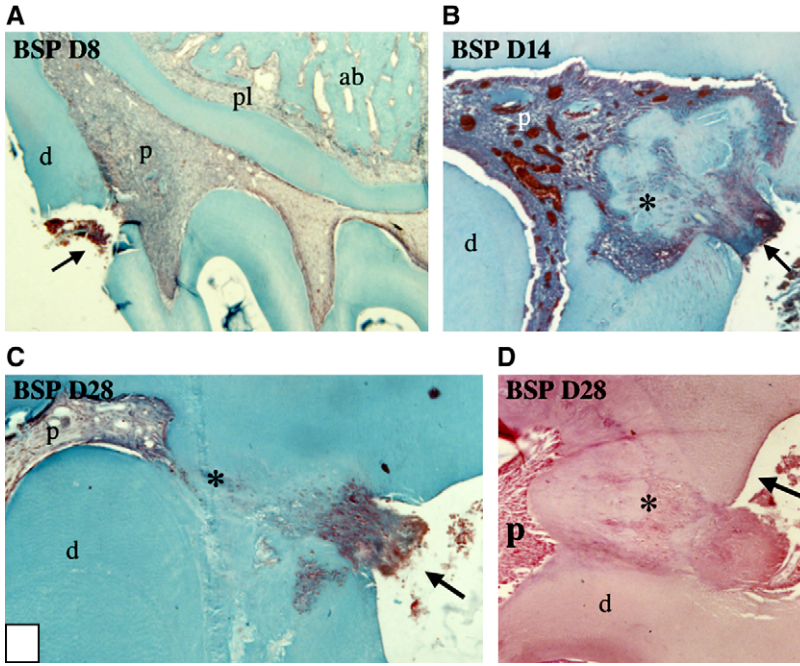


Fig. 2. (*A*) Implantation of BSP leads to an initial moderate inflammatory pulp reaction after 8 days (D8); (*B*) the reaction is not resolved after 2 weeks (D14). The formation of reparative dentin starts around dentin debris that has been pushed into the pulp during pulp exposure. (*C*, *D*) After 1 month (D28), a dense and homogeneous reparative dentin formation (*) fills the mesial part of the pulp totally. The arrow indicates the cavity that has been prepared and location of the pulp exposure. ab, alveolar bone; d, dentin; p, pulp; pl, periodontal ligament.

during the preparation of the teeth at 14 days (see Fig. 2B). After 1 month, the mesial part of the pulp chamber was filled with a homogeneous atubular dentin (see Fig. 2C, D). Inflammatory processes were resolved [36]. In the controls, after calcium hydroxide pulp capping, poorly filled interruptions, appearing as channels, porosities, or large osteodentin areas, were seen, which induce discontinuities in the dentinal bridge. Therefore, protection against possible bacterial recontamination was questionable. The major defect of calcium hydroxide is that long-term evaluations are unable to protect the pulps efficiently. This should not be the case after BSP implantation, because the reparative material is more homogeneous and should lead to a more efficient protection of the pulp.

### *Bone morphogenetic protein-7 (osteogenic protein-1)/ collagen implantation*

BMP-7 (OP-1) is used as a lining agent indirectly promoting the formation of reactionary dentin and is implanted directly in exposed pulp to

induce reparative dentin by many groups [7,8,11,12,19,44]. The authors determined the effects of OP-1 in their first rat maxillary molar model for dentin repair [15]. After implantation of OP-1 (1, 3, or 10 μg of recombinant BMP-7/implant) mixed with small collagen pellets, an inflammatory process was observed that was not totally resolved after 1 month. After 30 days, the mesial portion of the coronal pulp was filled with a heterogeneous osteodentin porous material. Globular structures did not merge and interglobular spaces were filled with pulp remnants. Reparative dentin formation was defective in the crown of the teeth (Fig. 3A, B). In contrast, the mesial roots were filled with a homogeneous dense material located beneath a calciotraumatic line. No appearance of lumen of root canal was detectable on serially cut sections.

The striking difference between the coronal and radicular pulp in rats treated with OP-1 was an unexpected result. The differences between the crown and root dentin are documented and understood poorly. During crown formation, the enamel organ that expresses large amounts of amelogenin surrounds the embryonic pulp. The formation of the root is driven by Hertwig's sheath, which does not express amelogenin, although most of other enamel proteins are expressed. In some species, dentin is lacking phosphorylated proteins. Differences also appear with respect to the vascular network and nerve development.

In the crown, radioautographic investigation [2] and proliferation cell nuclear antigen (PCNA) immunodetection provide evidence that reparative cells are recruited in the central part of the coronal pulp and later move toward the lateral area where the reparative process takes place. In the root, an initial localization in the central part is not apparent, but PCNA-labeled cells are seen already in the lateral subodontoblastic area. This provides some explanation of the fact that reparative dentin is deposited along the root canal walls, with a gradual reduction in diameter of the root pulp, leading to complete obliteration of the lumen.

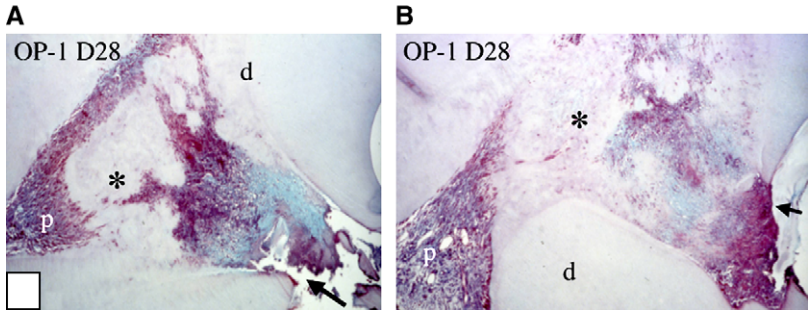


Fig. 3. (*A*, *B*) One month (D28) after implantation of OP-1, a heterogeneous formation of the osteodentin type fills the mesial part of the pulp chamber (*). Pulp remnants contribute to the heterogeneity of the reparative structure. The arrow indicates the cavity that has been prepared and location of the pulp exposure. d, dentin; p, pulp; pl, periodontal ligament.

*Dentonin: a peptide derived of the matrix extracellular phosphorylated glycoprotein*

MEPE is a member of the SIBLING (small integrin-binding ligand, N-linked glycoprotein) family [45]. Similar to DMP-1, MEPE has high serine content and contains a hydrophobic leader sequence and RGD and SGDG motifs. It is described as a mineralization inhibitor [46–48]. Dentonin (Acologix, Emeryville, California) (also known as AC-100) is a 23-amino-acid fragment of MEPE that stimulates the proliferation of dental pulp stem cells and their differentiation in vitro [49].

In experiments to determine the effect of MEPE on pulp repair, the authors used another carrier more appropriate for experiments using molecules that are soluble in aqueous culture media, the Affigel (Biorad, Hercules, California) agarose beads (75–150 μm in diameter).

Agarose is a sulphated galactan, which may induce a biologic effect per se. Therefore, a control group was included with the exposed pulp implanted with agarose beads alone. After agarose bead implantation, an inflammatory reaction could be seen at 8 days, but it resolved at 90 days. Some reparative dentin was formed after 15 days. Beneath a reparative dentinal bridge, however, after 90 days coronal pulp still was present and the lumen of the root canal was reduced but pulp tissue remained. Hence, the contribution of agarose beads was not negligible but was restricted compared with the major effects of the bioactive molecules.

The initial recruitment of reparative pulp cells by Dentonin peptides is faster than that with the two previous molecules described previously. At day 8 after implantation, a ring of differentiating cells was located at the periphery of beads. Around the dentin debris pushed during the pulp exposure, precocious mineralization was detectable (Fig. 4A). After 2 weeks, loci of initial mineralization, confluence of mineralization nodules (calcospherites), and massive formation of reparative dentin were observed. The transformation of the mesial coronal pulp into a homogeneous mineralized tissue was slower thereafter. Dentin debris, remnants of the agarose beads, and other materials were embedded into a thick area of reparative dentin occluding largely the coronal pulp (see Fig. 4B). Such events are not detected in the root. Hence, Dentonin seems to be a good inducer of pulp mineralization in vivo.

*In vivo implantation of AM+4 and A−4 in the exposed pulp and in an ectopic nonmineralizing gingival tissue: in vitro effects of the amelogenin gene splice products on odontoblast and osteoblast precursors*

*Implantation in the exposed pulp*

A small molecular weight molecule that appeared initially as a chondrogenic-inducing agent was isolated from calf and rat dentin [50]. This molecule was an amelogenin [51]. Soon after, the existence of two small molecular weight amelogenin splice products (A+4 and A−4) was identified

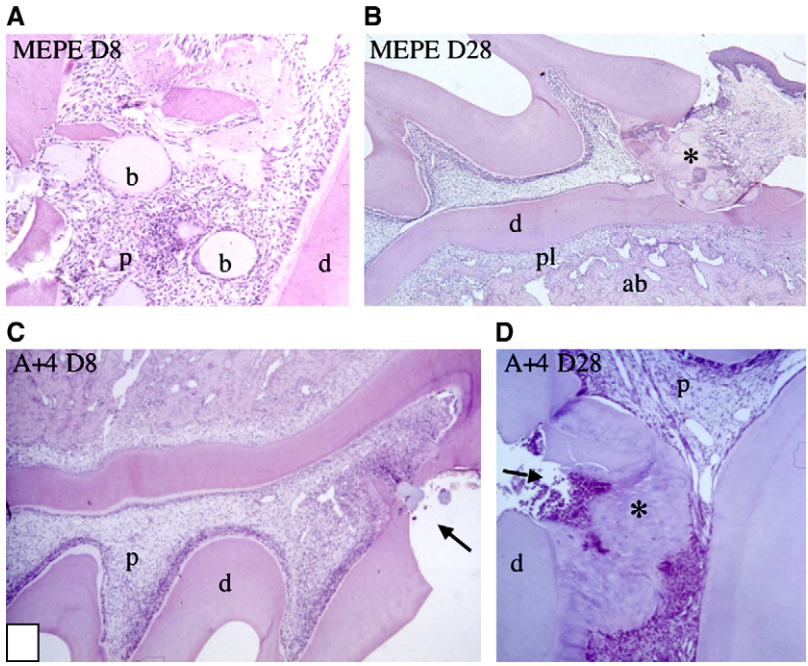


Fig. 4. (*A*) Eight days after implantation of agarose beads (b) loaded with a small fragment of MEPE, an inflammatory process is seen. Cells are grouped around the beads. (*B*) One month after MEPE implantation (D28), a thick reparative dentinal bridge is formed, occluding the pulp exposure. Agarose beads and dentin debris are embedded in the reparative dentin (*). (*C*) Day 8 after implantation in the pulp of agarose beads loaded with A+4. (*D*) One month after A+4 implantation (D28), a thick homogeneous reparative dentinal bridge occludes the pulp exposure. An inflammatory process is not yet resolved in the root canal pulp. The arrow indicates the cavity that has been prepared and location of the pulp exposure. ab, alveolar bone; d, dentin; p, pulp; pl, periodontal ligament.

in an odontoblast library. A+4 is derived from all exons from 1 to 7, but lacks the 5′ sequence of exon 6 to synthesize an 8.1-kd alternatively spliced amelogenin. A−4 was similar to A+4 but did not include exon 4 to produce a 6.9-kd alternatively spliced amelogenin [52]. A+4 induced the rapid expression of the transcription factor Sox9, whereas A−4 elevated the transcription of Cbfa1 [52]. These two spliced forms soaked on agarose beads were implanted in the exposed pulp.

Implantation of A+4 leads to the formation of a thick and homogeneous dentinal bridge. The pulp in the root canal was reduced in diameter as a result of the gradual obstruction of the lumen by reparative dentin. The initial inflammatory process (see Fig. 4C) was decreasing at 15 days, but residual inflammatory process was present 30 days after implantation (see Fig. 4D). No inflammation was detectable after 90 days. At that time, observed beneath a thick dentinal bridge was a pulp reduced in size in the

crown and root of the teeth. In some cases, there was no pulp tissue remaining in the mesial root.

Eight days after the implantation of agarose beads loaded with A−4, inflammation was moderate in contrast with the control and the other experimental groups (Fig. 5A). A dense ring of cells was seen around the beads (see Fig. 5B). At day 14, reparative dentin formation was seen in the crown where diffuse mineralization of the pulp was developing rapidly but did not fill the mesial part of the pulp chamber totally (see Fig. 5C). This pulp area was occluded at day 30 with a diffuse mineralized tissue, as shown by studies performed on undemineralized section of molars examined with an electron microprobe combined with a scanning electron microscope. In parallel, at day 14, the lumen of the root canal displayed a reduced diameter as a result of the extensive formation of a homogeneous reparative dentin layer. At day 30, the root canal lumen was not visible and was occluded totally by

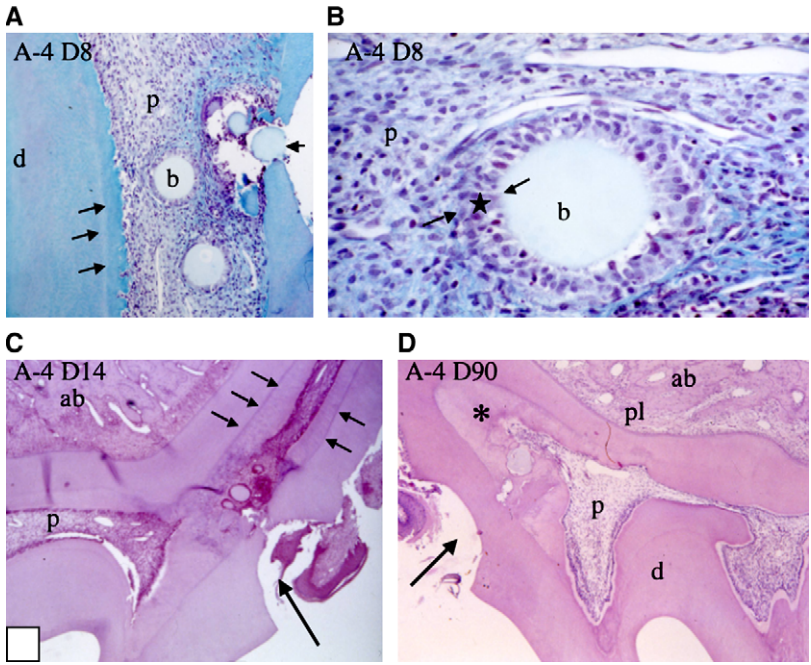


Fig. 5. (*A*) Eight days after implantation of agarose beads loaded with A−4, inflammation is moderate. (*B*) Densely packed around an agarose bead used as A−4 carrier, a ring of cells that underwent terminal differentiation is seen at day 8. (*C*) After 14 days (D14), reparative dentin is forming actively in the mesial part of the pulp. The diameter of the root canal is reduced by the formation of dentin limited by the calciotraumatic lines (*arrows*). (*D*) After 3 months (D90), the mesial pulp chamber and the mesial root canal are totally filled by reparative dentin. The periodontal ligament is maintained in shape and width. Alveolar bone apparently is unchanged. The arrow indicates the cavity that has been prepared and location of the pulp exposure. ab, alveolar bone; d, dentin; p, pulp; pl, periodontal ligament.

a homogeneous mineralized tissue. At day 90, it was seen that the effect of the molecule was limited to the pulp without any alteration of the periodontal ligament (see Fig. 5D).

Using PCNA immunostaining, it was observed that cell proliferation occurred in the central part of the crown pulp, some distance away from the beads (Fig. 6A). In the root, proliferation occurred only in the subodontoblastic area and never in the central part. The ring of cells located around the beads was immunolabeled positively for osteopontin (OPN) and BSP but negatively for DSP (see Fig. 6B, C). This suggests that they are cells differentiating into osteoblast lineage cells. A few cells in the central pulp and beneath the exposure area were found positive for DSP, whereas those located around the beads never displayed any significant labeling (see Fig. 6C). DSP mostly is a dentin protein although expressed at very low level in bone.

After either A+4 or A−4 implantation, two groups of cells differentiated simultaneously, providing odontoblasts near the pulp exposure and osteoblasts in an area located more centrally, which may be implicated in osteodentin formation. Alternatively, it is possible that a single group of cells of the osteoblastic and odontoblastic lineage is recruited and proliferates. Initially located in the central part of the pulp, around the beads, they may migrate from the central part of the pulp toward the peripheral wounded area

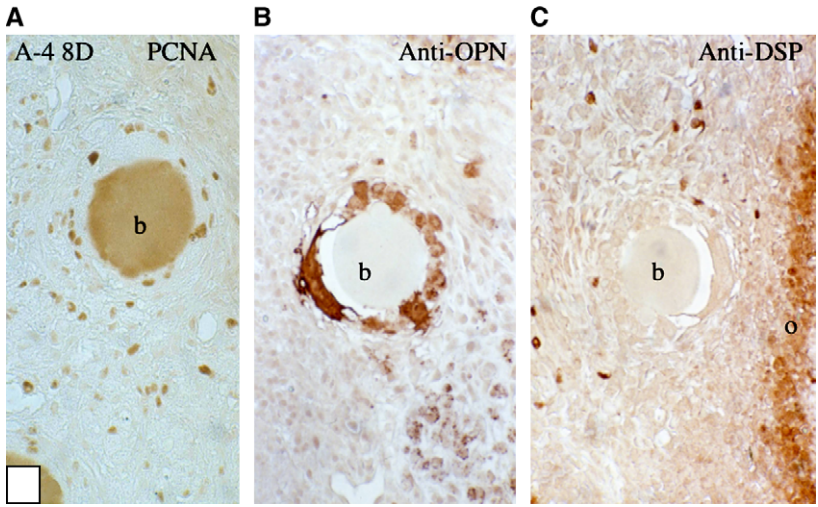


Fig. 6. Eight days (D8) after A−4 implantation in an exposed pulp, cell proliferation is visualized by PCNA staining (*A*). The dividing cells are located some distance away from the bead (*B*). Anti-OPN staining reveals that the cells located around the beads are positively labeled (b), whereas a few cells distributed throughout the central part of the pulp are positively stained with anti-DSP (*C*). They are located some distance away from the bead. DSP antibody stains positively odontoblasts cell bodies (o). The arrow indicates the cavity that has been prepared and location of the pulp exposure.

where they are implicated in reparative dentin formation [2]. Doing so, they apparently shift from an osteoblast phenotype to an odontoblast phenotype. To get a better understanding of the process, the authors investigated the reaction at early time points after implantation (1 and 3 days) [53]. Immunohistochemical data show that cells proliferate (PCNA) at both time points. RP59 is a marker of osteoblast recruitment also detected in primitive mesenchymal cells, erythroid cells, and megacaryocytes. A few pulp cells are RP59 positive at day 3, mostly after stimulation with A−4. The reaction is weaker but still positive with A+4 (Table 3) [54,55].

*In vitro effects of A+4 and A−4 on odontoblast progenitors*

Because the early events leading to the reparative process are understood poorly, the authors are using RT-PCR to investigate the nature of stimulation with A+4 and A−4 on two clones of odontoblast precursors (described previously) [56]. The two cell lines used reacted to A+4 or A−4, and Sox9 expression was stimulated. Lhx6, a member of the LIM-homeobox–containing genes encoding transcriptional regulators, was expressed transiently at 24 hours, whereas Lhx7 was expressed constantly at 6 hours, 24 hours, and 48 hours but by one clone alone. OC and DSP also were expressed at 48 hours by another odontoblastic clone.

Early effects (6–24 h) were detected on the expression of diverse transcription factors involved in bone cartilage and odontoblast differentiation; then, expression of ECM molecules, such as OC and DSP, was activated (48 h). Differences were noted between the type of cells in their stage of differentiation between the respective effects of A+4 and A−4 and in relation with the time course [57]. This cascade of events suggests the presence of receptors, specific or not, and intracellular signaling pathways now in focus.

*In vivo implantation in ectopic site*

Because an inflammatory reaction was seen in most of the pulp implantations, the authors wondered whether or not some inflammatory cells could contribute to the process of dentin formation. It is reported that some stem cells take origin from bone marrow [58], whereas others may derive from

Table 3
Immunohistochemical staining found at day 1 (D1) and day 3 (D3) after bead implantation with a marker of proliferation (PCNA), of the osteoblastic recruitment (RP59), OPN, and DSP

| | Beads D1 | Beads D3 | A+4 D1 | A+4 D3 | A−4 D1 | A−4 D3 |
|---|---|---|---|---|---|---|
| PCNA | + | − | ± | ++ | ± | +++ |
| RP59 | + | − | + | ± | − | ++ |
| OPN | ± | + | − | + | ± | ++ |
| DSP | − | − | − | − | − | − |

Note that at the time, DSP is not present, suggesting that cells bearing an odontoblast phenotype are not yet differentiated.

circulating $CD14^+$ monocytes, which may differentiate into osteoblast or chondroblast progenitors [59]. To elucidate if the cells were resident cells or circulating cells, the authors decided to implant agarose beads loaded with A+4 or A−4 in the gingiva of mice, a nonmineralizing tissue where osteoblast and odontoblast progenitors is not expected.

Three days after implantation, a strong inflammatory process was observed in the lamina propria. The inflammatory cells seemed of the leukocyte lineage, as suggested by their $CD45^+$ and $I\text{-}A^k$ staining. This inflammatory process was decreased at day 8 and barely detectable at day 30. When beads were loaded with A+4 or A−4, some cells were RP59 positive. These cells also expressed Sox9. PCNA staining was negative, suggesting that the RP59 positive cells are not derived from the proliferation of resident stem cells, rather from the migration of circulating cells with progenitor properties. These cells displayed a positive labeling for BSP and OPN but remained negative for DSP. Despite the fact that these cells expressed molecules considered specific markers of a bone-like mineralized tissue, no mineralization was detected, in contrast with the observations after implantation of the amelogenin peptides in the pulp. This suggests that (1) differentiation of osteogenic precursors is not necessarily dependant on local resident stem cells; (2) the presence of precursors results either from the migration of cells from the bone marrow or circulating monocytes mixed within the inflammatory cell population; (3) the last events leading to a mineralization phenomenon are more specific and seem to be under the control of the tissue; and (4) as reported elsewhere, in in vitro and in vivo [27,52], the two amelogenin peptides, A+4 and A−4, implanted in the dental pulp, have a differential effect.

This set of experiments shows that the amelogenin peptides provide a useful tool for investigating the mechanisms involved in reparative dentin formation and in pulp reaction. Further studies are needed to determine which part of the molecule is active biologically and how this action is mediated.

## Prospective area of research: cell recruitment by extracellular matrix or mineralized extracellular matrix produced by osteoblast and odontoblast precursors

Two different strategies have been used. One implants bioactive molecules, and we may expect an appropriate group of cells to be recruited and to proliferate and differentiate into cells that produce an ECM with mineralizing potential. The other one implants odontoblast/osteoblast precursors that promote pulp mineralization. The injection of some of the clones of immortalized odontoblast precursors in the pulp provides interesting preliminary results. The injection of a population of cloned cells [56] in the mouse mandibular incisor induces the formation of a huge area of osteodentin within 11 days (Fig. 7). Further experiments using these cells are currently in progress.

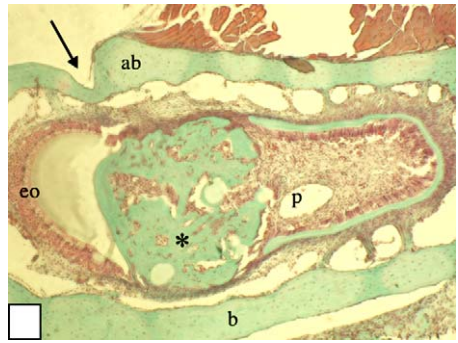


Fig. 7. Eleven days after the injection of odontoblast progenitors cells in the pulp of mouse incisor, together with agarose beads used in this case only to visualize the injection site, reparative dentin is formed massively (*) in the upper part of the pulp, beneath the enamel organ. The arrow indicates the site where the injection of cells and beads was made. The arrow indicates the cavity that has been prepared and location of the pulp exposure. ab, alveolar bone; eo, enamel organ; p, pulp.

In conclusion, the authors have investigated the possible contribution of a series of ECM molecules to the formation of reparative dentin and conclude that BSP (a fragment of MEPE) and some amelogenin gene splice products (A+4 and A−4) stimulate either the formation of a reparative dentinal bridge, the closure of the mesial coronal pulp chamber, or the total closure of the root canal. This choice of bioactive molecules is not exhaustive and others may be used in this context, such as DSP, DPP, or DMP-1. Combined in vivo and in vitro approaches to study the function of amelogenin may contribute to clarifying the biologic cascade of events. Shortcuts may be found by direct implantation of specific cells in the pulp, as this approach also works. These two tissue-engineering strategies may contribute to substantial changes in the concept of promoting healing and regeneration of altered dental tissues.

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