

Review

Bioactive Molecules Stimulate Tooth Repair and Regeneration

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(Accepted for publication, June 20, 2006)

Abstract:Dentin extracellular matrix proteins display multifunctional properties. Firstly, they participate to the mineralization processes either as promoters or as inhibitors of crystal nucleation or crystal growth. Secondly, they act as signaling molecules implicated in the differentiation of odontoblast progenitors. These molecules may be used to promote the recruitment of odontoblast progenitors, the proliferation and the final differentiation into functional odontoblast-like or osteoblast-like cells implicated in pulp repair. This has been evaluated through a series of experiments carried out *in vivo* on the rat first maxillary molar and *in vitro* on onto/osteo progenitors. Along this line, BMP7 (OP1) induced in the crown a fibrous osteodentin-like structure where unmineralized pulp remnants were seen. In addition, the mesial root canal was totally filled with a homogeneous dentin-like structure. The bone sialoprotein (BSP) stimulated within one month the formation of a reparative dentinal bridge and the complete closure of the coronal pulp with an atubular homogeneous reparative dentin. Dentonin, a peptide from MEPE, implanted into the exposed pulp produced more rapidly than the two previous molecules reparative mineralization in the coronal pulp and also occlusion of the lumen of the root canal. Implanted in the exposed pulp, A+4 and A-4, two spliced amelogenin gene products, induce either the formation of a reparative dentinal bridge (A+4) or a more diffuse mineralization (A-4). The mechanisms of proliferation and differentiation were studied in parallel in an *in vivo* situation after implantation in the first maxillary molar of the rat, and *in vitro* on odontoblast progenitor cell lines. These molecules may contribute to pulp repair and promote new strategies in dental therapies.

Keywords:Pulp repair, Dentin extracellular matrix molecules, BMP7, Bone sialoprotein, MEPE, Amelogenin, Odontoblast progenitor cell lines.

Introduction

For years, dental surgeons have used a limited number of capping agents in order to keep teeth alive. The most efficient was calcium hydroxide. Lessons from developmental biology have provided a better understanding of the genes and molecules that are involved in normal and pathological 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. The series of investigations presented here aimed at promoting the healing or regeneration of the dental pulp, and in this context to form a mineralized barrier either of limited size (the so-called reparative dentinal bridge) or

to induce more extensive mineralization area, with the prospect of filling partially or totally the crown and root pulp. This review is aimed at summarizing the experimental approaches that have been carried out by our group, with the greatly appreciated contributions from a network of collaborators.

A rapid 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 exposure¹. Indirect capping with CaOH₂ used as cavity liner, contributes to the formation of reactionary dentin. The reaction is due to the biological properties of Ca₂OH₂. As a pulp-capping agent, the high alkaline pH of the

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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 (Plate 1 b). A first cell division then occurs in the central part of the pulp. The two daughter cells migrate towards the wounded area, where a second cell division occurs²⁾. Fully differentiated odontoblasts, that are post-mitotic cells, do not participate in these events, and there is some evidence that adult resident stem cells that share properties in common with bone-marrow stem cells initiate this process³⁻⁵⁾. Another possibility is that cell phenotype plasticity allows some cells, already differentiated, to de-differentiate and then re-differentiate 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 may also be candidates for contributing to the population of cells that are recruited, and this constitutes the first step of a series of events that are not fully understood. Cell proliferation is the second event. When a sufficient number of cells have been obtained in order to cover the whole surface that has to be repaired, the cells start their final differentiation.

The reparative process has long been considered as resulting from odontoblast-like cells activity. These cells were also termed neo-odontoblasts or third generation odontoblasts. Some evidence has now been obtained that at the beginning of the reparative bridge construction, newly differentiated osteoblasts contribute to osteodentin formation (Plate 1 c, 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, as the cell types can not be easily being distinguished, differing only by the level of expression of some extracellular matrix molecules. For example, the dentin sialo phospho protein (DSPP) has long been considered as a molecule expressed exclusively by odontoblasts, and therefore as a phenotypic marker. Now, we know that DSPP, which is secreted and immediately split into dentin sialoprotein (DSP) and dentin phosphoprotein (DPP), is not exclusively expressed by odontoblast but also by osteoblasts in a 1/400 ratio⁶⁾. This lack of specific markers renders the precise identification of the reparative cells difficult.

Long after the introduction of CaOH₂ in dental therapies, the effects of other bioactive molecules were investigated. In the early 1990's, Rutherford and coworkers⁷⁻⁸⁾ demonstrated that indirect capping with Bone Morphogenetic Protein-7 (BMP-7, also named Osteogenic Protein- OPI) was able to either stimulate the formation of reactionary dentin or, as direct capping agent, reparative osteodentin in the root canal of monkeys. These pioneering studies have open new avenues for regenerative biological therapies.

A few definitions

The biological differences between reactionary and reparative dentin are clearly established¹⁰⁾.

Reactionary dentin results from the stimulation of odontoblasts after carious decay or after the preparation of a cavity. The sub-odontoblast cell layer, the so-called Höhl's layer, may also produce similar beneficial reaction. Beneath a calcio-traumatic line, a layer of tubular dentin is formed, more or less in continuity with the dentin previously formed in non-pathological conditions. Although the staining agent "stains all" that is used to visualize phosphorylated molecules, interacts only weakly with reactionary dentin, suggesting reduced phosphorylation of the dentin matrix, the structure of this dentin is apparently normal⁹⁾.

Reparative dentin formation is under the control of events that have been summarized above. Exclusively some pulp cells produce it. If the wound is superficial such as in a small accidental exposure during the preparation of a cavity, again the subodontoblastic layer, the so-called Höhl 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 sub-odontoblastic layer are destroyed. In this case, reparative pulp cells are recruited, proliferate and after differentiation, are implicated in the formation of an atubular or a 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 located beneath the predentin and at the periphery of the pulp.

New molecules, new approaches and concepts

The biological properties, a putative role of BMPs or TGFβ and their putative role in dentin repair lead to several studies to determine the effects of these molecules on dentin repair¹¹⁻¹⁵⁾. These studies concluded that BMPs or TGFβ may induce reparative dentin formation. Gene expression was found in human and animal dental pulps for BMPs and for their receptors as well¹⁶⁻²⁵⁾. They both probably contribute to the beneficial reaction. However, the intimate mechanisms mediating this action still remain obscure.

It seems important to mention a few points regarding the potential multifunctional nature of ECM molecules. This multifunctionality is nowadays a well-accepted concept that has to be taken into account in the interpretation of many studies. For example native collagen subunit association is regulated by the cleavage of the C-terminal non-helicoïdal extensions by a C-procollagen peptidase involved in the processing of a structural protein. The C-proteinase was also identified as a Bone Morphogenetic Protein 1, a molecule that is a growth factor²⁶⁾. In this specific case, the enzyme has two different functions.

As another example of multifunctionality, for years amelogenin was considered to be a molecule only implicated in enamel

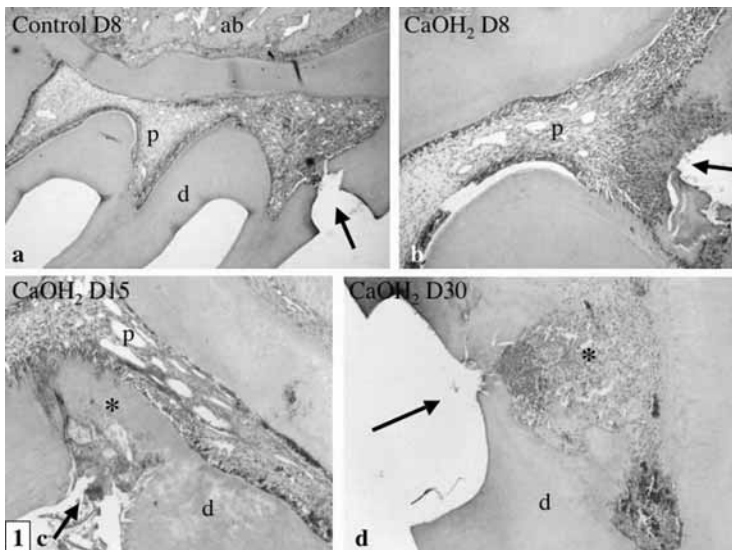


Plate 1 a: Eight days after the preparation of the cavity and pulp exposure (D8), an inflammatory process is seen in the mesial part of the pulp chamber. b: At day 8 (D8) pulp capping with calcium hydroxide induces a limited reaction, c: two weeks (D15) after capping a reparative dentin bridge starts to be formed (asterisk), and d : after one month(D30) a thick heterogenous dentin bridge fills the mesial part of the pulp (asterisk).

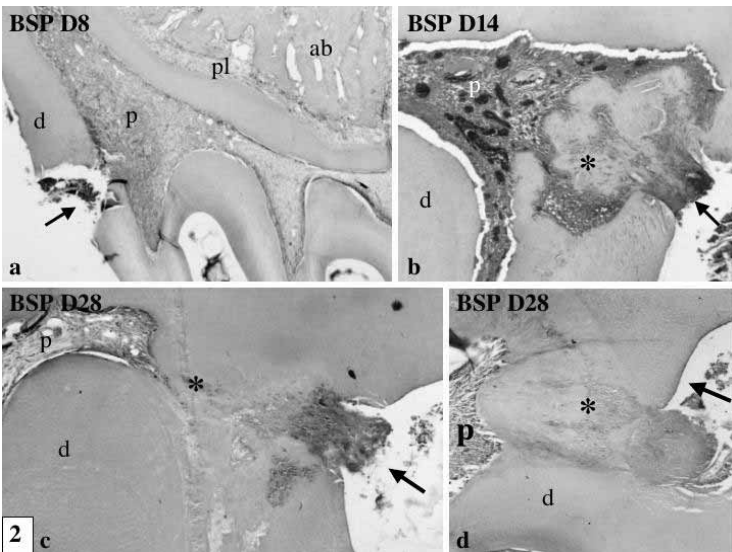


Plate 2 a: implantation of BSP leads to an initial moderate inflammatory pulp reaction after 8 days (D8), b: the reaction is not resolved after 2 weeks (D14). The formation of reparative dentin starts around dentin debris that has been pushed into the pulp during pulp exposure. c and d: after one month (D28), a dense and homogeneous reparative dentin formation (asterisks) fills totally the mesial part of the pulp.

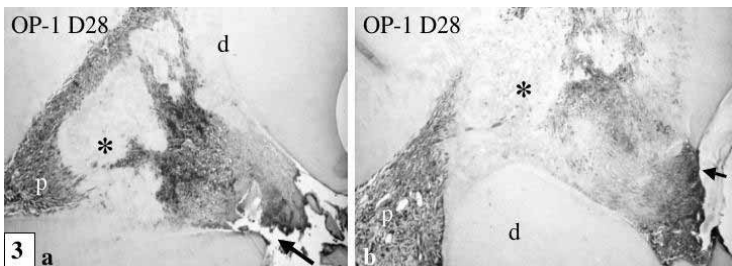


Plate 3 a and b: One month (D28) after implantation of OP-1 a heterogeneous formation of the osteodentin type fills the mesial part of the pulp chamber (asterisks). Pulp remnants contribute to the heterogeneity of the reparative structure.

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 properties²⁷. It has also been shown that enamelysin (MMP-20) is expressed in cells that are not ameloblasts, and therefore are not implicated in enamel formation. These two examples suggest either that isoforms of a family of molecules obtained by alternative splicing may play different biological roles, and/or that after partial degradation, residual peptides may be converted

in an activated form and display functional properties hidden in the intact molecule. This shed lights on the complex effects of ECM implanted in a given tissue in order to promote healing or regeneration.

In the course of our 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 differentiate into cells involved in the formation of reparative dentin. Preliminary results have been obtained with a second approach

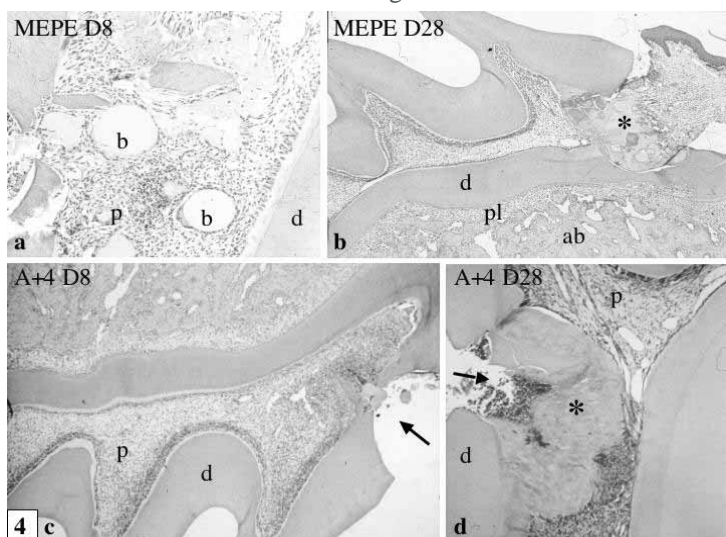


Plate 4 a: Eight days after implantation of agarose beads (b) loaded with a small fragment of MEPE, an inflammatory process is seen. Cells are grouped around the beads. b: One month after MEPE implantation (D28) a thick reparative dentinal bridge is formed, occluding the pulp exposure. Agarose beads and dentin debris are embedded in the reparative dentin (asterisk). 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 thick arrow indicates the cavity which has been prepared and location of the pulp exposure. All figures: p=pulp, d=dentin, ab= alveolar bone, pl: periodontal ligament.

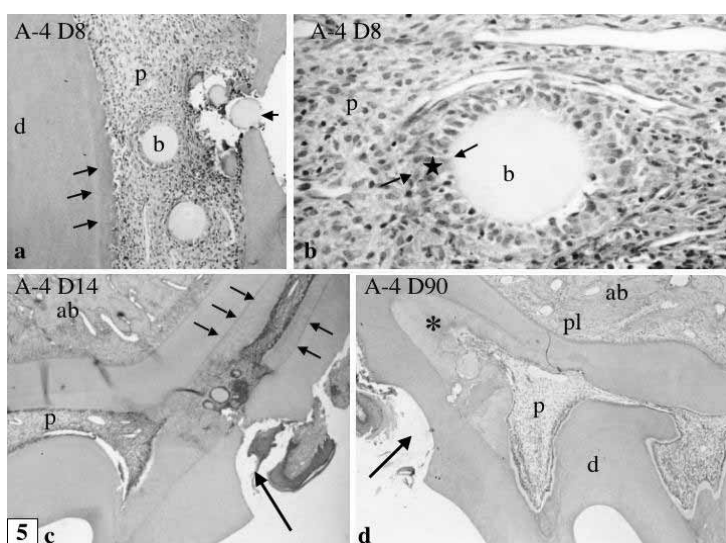


Plate 5 a: Eight days after implantation of agarose beads loaded with A-4, inflammation is moderate. b: Densely packed around an agarose bead used as A-4 carrier, a ring of cells that underwent terminal differentiation is seen at Day 8. c: After 14 days (D14), reparative dentin is forming actively in the mesial aprt of the pulp. The diameter of the root canal is reduced by the formation of dentin limited by the calciotraumatic lines (arrows). d: After three months (D90), the mesial pulp chamber and the mesial root canal are totally filled by reparative dentin. The periodontal ligament (PL) is maintained in shape and width. Alveolar bone (ab) is apparently unchanged.

involving clonal pulp cells that produce, after direct implantation in a target zone, a specific extracellular matrix capable in some cases to further mineralize.

Dentin matrix proteins composition, functions and bioactive potentials

Dentin is a complex tissue, mostly produced by odontoblasts. Many ECM molecules have been already identified. Some are associated with the mineralization process, initiation of the crystal formation and/or crystal growth, while others are acting as inhibitors. They also play a role in cell differentiation. For example, such dual functions have been reported for Dentin PhosphoProtein (DPP) also named phosphophoryn, a molecule that triggers dentin mineralization but also 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/MAPK signaling pathway²⁸. Along the same line, over-expression of DMP-1 induces the differentiation of embryonic mesenchymal cells to odontoblast-like cells²⁹. DMP-1 is regulated by c-Fos and c-Jun transcription factors and plays role in osteoblast early differ-

entiation³⁰. Signaling effects are also well documented with the amelogenin gene spliced products A+4 and A-4²⁷. The complexity is even enhanced by the fact that matrix molecules are substrates for MMPs or for metalloproteinases, and fragments of the processed proteins may be also biologically active. For example, DMP-1 is physiologically processed by BMP-1/Tolloid-like proteinases³¹. DSP and amelogenin are substrates for MMP-2³². Dentin mineralization and enamel formation are impaired by inhibitors of MMPs^{32,33}.

Table 1 summarizes the immunohistochemical staining found at day 1 (D1) and day 3 (D3) after beads implantation with a marker of proliferation (PCNA), of the osteoblastic recruitment (RP59), osteopontin (OPN) and dentin sialoprotein (DSP). Note that at that period of time, DSP is not present, suggesting that cells bearing an odontoblast phenotype are not yet differentiated.

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

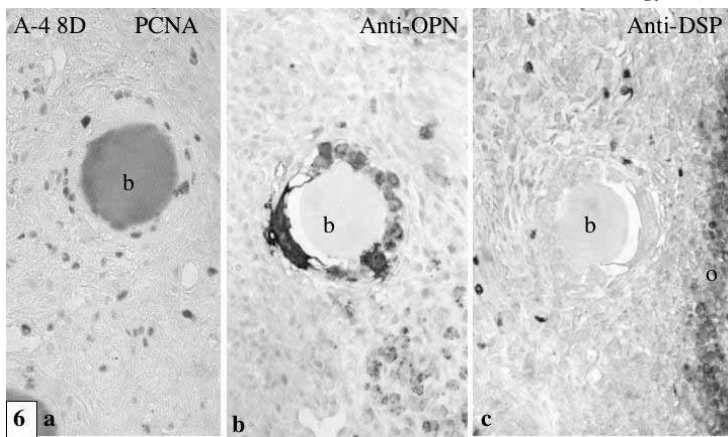


Plate 6 Eight days (D8) after A-4 implantation in an exposed pulp, cell proliferation is visualized by PCNA staining (a). The dividing cells are located some distance away from the bead (b). Anti- OPN staining reveals that the cells located around the beads are positively labeled (b), whereas a few cells 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 odontoblast cell bodies (o).

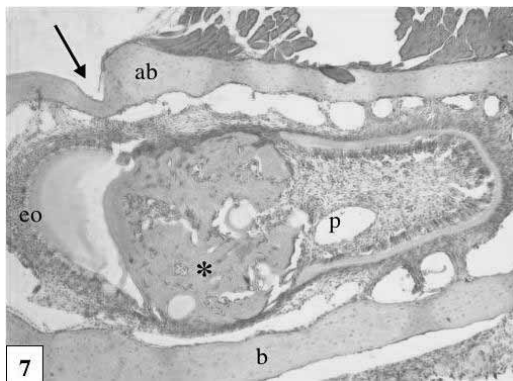


Plate 7 Eleven days after the injection of odontoblast progenitor cells in the pulp of mouse incisor, together with agarose beads used in this case only to visualize the injection site, reparative dentin is formed massively (asterisk) in the upper part of the pulp, beneath the enamel organ (eo). The arrow indicates the site where the injection of cells and beads was made

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

We have first established a reliable animal model for to study the stimulation of pulp repair, have then explored the effects of implantation of OP-1, BSP, Dentonin, a peptide of MEPE, and two spliced forms of dentin amelogenins (A+/-4) in the exposed pulp of rat molar. In a second strategy, we carried out *in vitro* studies on odontoblast progenitor cell lines corresponding to different steps of differentiation. This strategy allowed us to first to characterize by RT PCR the short term effects of bioactive molecules on the level of expression of some genes and transcription factors such as Runx2, Pax 9, Msx1 and 2, and ECM molecules such as type II collagen, osteocalcin, DSP. These odontoblast precursors were also implanted in the pulp.

Mechanisms involved in the *in vivo* reparative processes
The *in vivo* model of exposed rat molar pulp

In 1990, Ohshima³⁴ described the changes in odontoblasts and pulp capillaries following cavity preparation in the rat maxillary molar. The cavity was prepared in the mesial aspect of the first maxillary molar. We modified this experimental protocol by using electrosurgery to remove the gingival papilla prior to 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 are biologically 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 less than 2 seconds. The preparation of the cavity and its filling with a GIC were shown to only induce a slight inflammatory reaction³⁵. The residual dentin is then pushed with a steel probe, which allows to obtain a pulp exposure of limited size. This process also avoids stretching of the pulp tissue around the bur and uncontrolled pulp damages. However, the projection of dentin debris inside the pulp may release ECM molecules, contributing to spontaneous pulp healing with the resultant formation of reparative dentin²³⁻²⁵. In spite of this complication, this system provided an excellent animal model for study of the effects of bioactive molecules on the dental pulp³⁶.

Experimental results: *in vivo* and *in vitro* approaches
BMP-7 (OP-1) /collagen implantation

Bone morphogenetic protein (BMP-7) also named osteogenic protein-1 (OP-1) has been used both as lining agent indirectly promoting the formation of reactionary dentin or implanted directly in exposed pulp to induce reparative dentin by many groups^{7, 8, 11, 12, 19, all reviewed in 44.} We determined the effects of OP-1 in our first rat maxillary molar model for dentin repair¹⁵. After implantation of OP-1 mixed with small collagen pellets, we observed an inflammatory process that was not totally resolved after one 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 (Plate 3 a, b). In contrast, the mesial roots were filled with a homogeneous dense material located beneath a calcio-traumatic line. No appearance of lumen of root canal was detectable on serially cut sections.

The striking difference between the coronal and radicular pulp in rat treated with OP-1 was an unexpected result. The differences between the crown and root dentin are poorly documented and understood. It is clear that during crown formation, the enamel organ, which expresses large amounts of amelogenin, surrounds the embryonic pulp. Whereas the formation of the root is driven by the Hertwig's sheath, which does not express amelogenin, though 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 investigation²⁾ as well as the proliferation cell nuclear antigen (PCNA) immunodetection have provided evidences that reparative cells are recruited in the central part of the coronal pulp, and later move towards 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 on 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.

Bone sialo protein (BSP)/collagen implantation

Among the dentin ECM that seems to display intrinsic bioactive properties for promoting the formation of a reparative tissue, BSP was a good candidate. Implantation of the molecule in calvaria leads to rapid repair of a critical defect 8mm, with stimulation of the recruitment of bone forming cells, which differentiate and subsequently formed a small amount of cartilage, replaced by bone³⁷⁻³⁹⁾. In addition, it is known that BSP is a phosphorylated protein with tyrosine sulphatation and a RGD attachment sequence. Several stretches of polyglutamic acid are involved in binding to hydroxyapatite⁴⁰⁾. BSP enhances the fibrillogenesis of collagen⁴¹⁾. BSP mRNA 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 (Plate 2a). Some loci of mineralization appeared around the dentin fragments that were pushed into the pulp during the preparation of the teeth at 14 days (Plate 2 b). After one month, the mesial part of the pulp chamber was filled with a homogeneous atubular dentin (Plate 2 c, d). Inflammatory processes were resolved³⁶⁾. In the controls, after CaOH₂ pulp capping, poorly filled interruptions, appearing as channels,

porosities and/or large osteodentin areas were seen which induce discontinuities in the dentinal bridge. Therefore, protection against possible in bacterial recontamination was questionable. This is certainly the major defect of calcium hydroxide that long-term evaluations have shown to be unable to efficiently protect the pulps. This should not be the case after BSP implantation, since the reparative material is more homogeneous and should lead to a much more efficient protection of the pulp.

Dentonin, a peptide derived of the Matrix Extracellular Phosphorylated glycoprotein (MEPE).

MEPE is a member of the SIBLING family. Similar to DMP-1, MEPE has high serine content, contains a hydrophobic leader sequence and RGD and SGDG motifs. It is described as a mineralization inhibitor⁴⁶⁻⁴⁸⁾. Dentonin (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 the experiments to determine the effect of MEPE on pulp repair, we used another carrier, more appropriate for an experiment using molecules that are soluble in aqueous culture media, the Affi-gel agarose beads (75-150mm in diameter).

Agarose is a sulphated galactan, and which may induce a biological effect *per se*. Therefore, a control group was included with the exposed pulp implanted with agarose beads alone. Indeed, after agarose bead implantation, an inflammatory reaction could be seen at 8 days, but it is resolved at 90 days. Some reparative dentin was formed after 15 days. However, beneath a reparative dentinal bridge, after 90 days coronal pulp was still present and the lumen of the root canal was reduced but still pulp tissue remained. Hence, the contribution of agarose beads was not negligible, but was restricted compared to the major effects of the bioactive molecules. The initial recruitment of reparative pulp cells by Dentonin peptides is faster that with the two previous molecules reported above. At day 8 after implantation, a ring of differentiating cells is located at the periphery of beads. Around the dentin debris that has been pushed during the pulp exposure, precocious mineralization is detectable (Plate 4, a). After two weeks, loci of initial mineralization, confluence of mineralization nodules (calcospherites) and massive formation of reparative dentin is observed. The transformation of the mesial coronal pulp in]to a homogeneous mineralized tissue is slower thereafter. Dentin debris, remnants of the agarose beads, and other material were embedded into a thick area of reparative dentin occluding largely the coronal pulp (Plate 4 b). 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 A+4 and A-4 in the exposed pulp and in an ectopic non-mineralizing gingival tissue - In vitro effects of the amelogenin gene splice products on odonto/osteoblast precursors.

Implantation in the exposed pulp

A small molecular weight molecule that initially appeared as a chondrogenic-inducing agent was isolated from calf and rat dentin⁵⁰. This molecule was shown to be an amelogenin⁵¹. Soon after the existence of two small molecular weight amelogenin splice products (A+4 and A-4) were identified in an odontoblast library. A+4 is derived from all exons from 1 to 7, but lacking the 5' sequence of exon 6, to synthesize an 8.1kDa alternatively spliced amelogenin. A-4 was similar to A+4, but did not include exon 4, to produce a 6.9kDa alternatively spliced amelogenin⁵². A+4 was shown to induce the rapid expression of the transcription factor Sox9, whereas A-4 elevated the transcription of Cbfa1⁵². 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, due to the gradual obstruction of the lumen by reparative dentin. The initial inflammatory process (Plate 4c) was decreasing at 15 days, but still some residual inflammatory process was present even 30 days after implantation (Plate 4d). No inflammation was detectable after 90 days. At that time, one observed, beneath a thick dentinal bridge, a pulp reduced in size both 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 (Plate 5a). A dense ring of cells was seen around the beads (Plate 5b). At day 14, reparative dentin formation was seen both in the crown where diffuse mineralization of the pulp was rapidly developing but did not fill totally the mesial part of the pulp chamber (Plate 5c). This pulp area was occluded at day 30 with a diffuse mineralized tissue, as shown by studies carried out 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, due to the extensive formation of a homogeneous reparative dentin layer. At day 30, the root canal lumen was not visible, and was totally occluded by 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 (Plate 5d).

Using the PCNA immunostaining, it was seen that cell proliferation occurred in the central part of the crown pulp, some distance away from the beads (Plate 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 positively immunolabeled for osteopontin (OP) and BSP, but negative for DSP (Plate 6 b, c). Therefore this suggests that they are cells differentiating into osteoblast lineage cells. Surprisingly, a few cells in the central pulp and beneath the exposure area were found to be positive for DSP, while those located around the beads never

display any significant labeling (Plate 6c). DSP is mostly a dentin protein, although found to be expressed at very low level in bone.

After either A+ or A-4 implantation, two groups of cells differentiated simultaneously, providing odontoblasts near the pulp exposure and osteoblasts in a more centrally located area, which may be implicated in osteodentin formation. Alternatively, it is possible that a single group of cells of the osteo/odontoblastic lineage is recruited and proliferate. 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 where they are implicated in reparative dentin formation². Doing so, they apparently shift from an osteoblast phenotype to an odontoblast phenotype. In order to get a better understanding of the process, we investigate the reaction at early time points after implantation (1 and 3 days)⁵³. Immunohistochemical data shows 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+ (Table 1 and 54, 55).

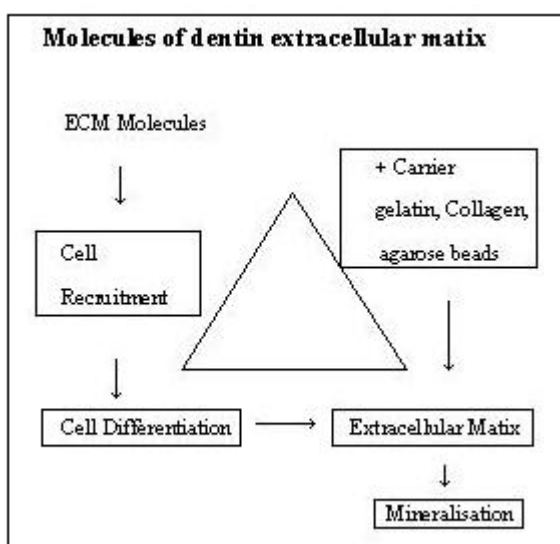
In vitro effects of A+/-4 on odontoblast progenitors

As the early events leading to the reparative process are poorly understood, we have started to investigate by RT PCR the nature of the stimulation with A+/-4 on two clones of odontoblast precursors previously described⁵⁶. The two cell lines used both reacted to A+ or A-4, and Sox9 expression was stimulated. Lhx6, a member of the LIM-homeobox containing genes encoding transcriptional regulators, was expressed transiently at 24h, whereas Lhx7 was expressed constantly at 6h, 24h and 48h but by one clone alone. Osteocalcin and DSP were also expressed at 48h by another odontoblastic clone.

Early effects (6-24h) 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 (48h). Differences were noted between the type of cells, their stage of differentiation between the respective effects of A+4 and A-4, and in relation with the time course⁵⁷. This cascade of events implies the presence of receptors, specific or not, and intracellular signaling pathways now under focus.

In vivo implantation in ectopic site

Because an inflammatory reaction was seen in most of our pulp implantations, we wondered whether some inflammatory cells could contribute to the process of dentin formation. It has been reported that some stem cells take origin from bone marrow⁵⁸ while others may derive from circulating CD14⁺ monocytes, which may differentiate into osteoblast or chondroblast progenitors⁵⁹. In order to elucidate if the cells we were dealing with were resident cells or circulating cells we decided to implant agarose beads



loaded with A+ or A-4 in the gingiva of mice, a non-mineralizing tissue where osteo/odontoblast progenitors is not expected.

Three days after implantation, a strong inflammatory process was observed in the lamina propria. The inflammatory cells appeared of the leukocyte lineage as suggested by their CD45+ and I-A^s staining. This inflammatory process was decreased at day 8 and barely detectable at day 30. When beads were loaded with A+ or A-4, some cells were RP59 positive. These cells also expressed Sox9. PCNA staining was negative, therefore suggesting that the RP59 positive cells are not derived from the proliferation of resident stem cells, but rather from the migration of circulating cells with progenitor properties. Surprisingly these cells displayed a positive labeling for BSP and OPN, but remain negative for DSP. Despite the fact that these cells expressed some molecules considered to be specific markers for 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 seems to be under the control of the tissue, 4- as already reported *in vitro* and *in vivo* in other contexts^{27,52} the two amelogenin peptides A+ and A-4, implanted in the dental pulp have a differential effect.

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

Prospective area of research: cell recruitment by ECM or mineralized ECM produced by osteo/odontoblast precursors.

Two different strategies have been used at the moment. Either

one implants bioactive molecules and we may expect that an appropriate group of cells will be recruited, will proliferate and differentiate into cells that produce an ECM with mineralizing potential, or one implants odontoblast/osteoblast precursors that would promote pulp mineralization. Along this line, the injection of some of our clones of immortalized odontoblast precursors in the pulp provides interesting preliminary results. In fact, the injection of a population of cloned cells⁵⁶ in the mouse mandibular incisor induced the formation of a huge area of osteodentin within 11 days (Plate 7). Further experiments using these cells are currently in progress.

In conclusion,

we have investigated the possible contribution of a series of extracellular matrix molecules to the formation of reparative dentin. We 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, or 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 better contribute to clarifying the biological cascade of events reported here. Shortcuts may be found by direct implantation of some specific cells in the pulp, as apparently 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.

Acknowledgements for funding support

This work was supported by an INSERM/CNRS grant (Ingénierie tissulaire program). Support was also provided by grants from the French Institut for Dental Research (IFRO). This work was also partially supported by grants in aid for scientific researches from the Ministry of Education, Culture, Sports, Science and Technology (#15209060,17406027, 17591910, 17591911).

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