Recruitment and Characterization of the Cells Involved in Reparative Dentin Formation in the Exposed Rat Molar Pulp after Implantation of Amelogenin Gene Splice Products A+4 and A−4

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Purpose: To understand the mechanisms of action of amelogenin splice products A+4 and A−4 and their potential as therapeutic agents in dentin repair.

Materials and methods: Two amelogenin peptides, specific gene splice products A+4 and A−4, were adsorbed onto agarose beads and implanted via dentin perforations into the pulps of the first maxillary molars of rats for 8, 15, 30 and 90 days. Beads soaked in buffer only were used as controls.

Results: Inflammatory processes were seen shortly after implantation, but the pulps recovered gradually. At 8 days, cells recruited by both A+4 and A−4 soaked beads formed rings around the beads, and the proliferating cell nuclear antigen (PCNA) method revealed that a proliferation centre formed in the central part of the pulp and near the perforation. Cell proliferation occurred also in the root along a sub-odontoblastic border. The cells present around the beads were stained positively by anti-osteopontin. In contrast anti-dentin sialoprotein labelled cells near the perforation, but not the cells in closest association with the bead surface. These observations suggest that post-mitotic cells differentiated into osteoblast-like, rather than odontoblast-like cells. A+4 produced a thick dentinal bridge at the perforation, whereas A−4 contributed both to the formation of reparative dentin in the coronal pulp and to the total closure of the root canal. Mineralization was restricted, even after 90 days, to the pulp and did not extend to the periodontal tissues.

Conclusions: These results suggest that the amelogenin peptides may be used to stimulate reparative dentin formation, and have the potential to be a substitute for endodontic treatment.

Key words: pulp, reparative dentin, amelogenin, cell differentiation

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Recently, *in vivo* experiments have shown that bioactive molecules such as Bone Morphogenetic Proteins (BMP)-2, 4 and 7 (Nakashima, 1990, 1994a,b; Rutherford et al, 1993; Six et al, 2002a,b), bone sialoprotein (BSP) (Decup et al, 2000), and a few other extracellular matrix (ECM) molecules (Goldberg et al, 2001, 2003; Six et al, 2002b) may be used in pulp-capping situations. The bioactive molecules stimulate the recruitment of stem cells, if any persist, in the adult pulp as suggested by Gronthos et al (2000, 2002), or stimulate the dedifferentiation or redifferentiation of resident adult cells into odontoblast-like or osteoblast-like cells. Furthermore, once recruited or stimulated, the cells divide and after at least two divisions in the coronal pulp the resultant osteoblast-like and odontoblast-like cells secrete an extracellular matrix, which subsequently mineralizes and forms reparative dentin. This can be in the form of a dentinal bridge or a diffuse pulp mineralization (Goldberg and Smith, 2004). The mechanisms involved in such processes are not fully understood.

Additional information is necessary to determine the cascade of events that regulate such processes. However, even with such limited understanding such investigations demonstrate the prospect of new treatments in vital pulp therapy (Tziafas et al, 2000). Evidently many molecules of the ECM may be potential agents for stimulating physiological reparative dentin formation (Lesot et al, 1993).

We became interested in the possibility of inducing reparative dentin formation using molecules extracted from rat and bovine dentin that had been shown to display a chondrogenic-inducing activity on fibroblasts in culture (Amar et al, 1991; Nebgen et al, 1999). Although extracted from dentin, these molecules were shown to be intact-specific amelogenin gene splice products, not products of the degradation of the larger amelogenin molecule (Veis et al, 2000). The two splice product peptides were comprised from the expression of amelogenin gene exons 2,3,4,5,6d,7 ([A+4], 8.1 kDa), and 2,3,5,6d,7 ([A−4], 6.9 kDa). Although differing only by the 14 amino acid sequence encoded by exon 4, the two peptides had strikingly different effects in the fibroblast culture system. [A+4] induced the rapid expression of transcription factor Sox 9, whereas on a different time scale, [A−4] elevated the expression of transcription factor Cbfa1 (Veis et al, 2000). Both factors are necessary to drive cartilage and bone formation. In an initial pilot study we showed that both molecules may induce controlled mineralization in the dental pulp (Six et al, 2002c).

In the present study, we examined the short-term and long-term effects of the implantation of the two molecules into experimentally created dentin defects in rat molars, as compared with control implants and control defects without any implant. Two particular points were considered. First, in view of the ultimate question of the use of these peptides in human tooth repair: was there any potential risk of diffuse mineralization, and could the mineralization reaction be turned off? Thus, implantations up to 3 months were examined. Second, from a more basic view: what was the mechanism of repair, and which cells were involved in the response? In this study, PCNA was used as an immunocytochemical marker for the visualization of cells undergoing cell division. PCNA appears in the nucleus during the late G1 phase, increases during the S phases, and declines during the G2 and M phases of the cell cycle (Celis and Celis, 1985; Robbins et al, 1987; Hall et al, 1990). Finally, specific phenotype markers were used to determine the nature of the cells involved in the reparative process: where they osteoblast-like or odontoblast-like?

MATERIALS AND METHODS

Animals

Forty-four male Sprague Dawley rats, 6-7 weeks old (~150 g), were used in the implant experiments. All experiments were performed under an institutionally approved protocol for the use of animals in research. Anaesthesia in each case was with a single intraperitoneal injection of Chloral (400 mg/kg body weight).

Amelogenin Peptides

Rat incisors' amelogenin corresponding to the secreted form of the product of exons 2, 3, 5, 6D, 7 (LRAP, A−4), and exons 2, 3, 4, 5, 5, 6D, 7 (A+4) were prepared as glutathione S-transferase (GST) fusion proteins (Veis et al, 2000).

Cavity Preparation and Implantation of the Bioactive Molecules

Cavities were prepared in the mesial aspect of the first maxillary molars following gingival electrosurgery as previously described (Decup et al, 2000). Pulp perforation was carried out with the tip of a steel probe. During that step, small dentin fragments were pushed into the pulp. Affi-gel agarose beads (70-150 μm in diameter) (Biorad, Hercules, CA) were equilibrated with PBS for use as controls. Beads were incubated with 50 μg of the A+/−4 peptides in 75 μL PBS at 37 °C for 1 h. It was estimated that 0.2 μg of A+4 or A−4 was absorbed on each bead. Three to 7 beads were inserted into each cavity and pulp perforation and left for defined periods according to the schedule in Table 1. All cavities were filled with an encapsulated GIC (GC Fuji IX GP Fast) kindly provided by the GC Corporation (GC Eur N.V. Leuven, Belgium). Premeasured material was mixed with an amalgamator according to the manufacturer's recommendation and applied directly into the cavities (1 capsule/cavity). A total of 88 molars were examined.

Block sections including the three maxillary molars and the surrounding periodontal tissues were fixed in 4% paraformaldehyde buffered with 0.1 M sodium cacodylate at pH 7.2-7.4 for 4 h. They were rinsed in

Table 1 Schedule of implants, treatments, recovery time and number of teeth

Implant period	8 days	15 days	30 days	3 months
Agarose beads, Control	4	4	13	9
A+4	6	6	7	9
A−4	6	6	8	10

the cacodylate solution and then demineralized in 4.13% EDTA or in sodium formiate. The tissue was dehydrated in graded ethanols and embedded in Paraplast (Oxford Labware, St Louis, MO, USA). Five μm thick sections were cut then dewaxed and stained with Masson's trichrome, or haematoxylin-eosin.

A pilot study was carried out with an electron microprobe prior to the experiment reported here. That study established that the material induced by pulp capping inside the pulp is located in the mesial pulp chamber, expanding with time along the root, homogeneously mineralized and mostly formed with Calcium and Phosphate in a ratio (1.66) that allows identification as a hydroxyapatite. Therefore, we are confident that the material observed after demineralization was originally mineralized and represent true reparative dentin or pulp mineralization.

Immunohistochemistry

Sections, prepared for immunohistochemistry were treated with methanol+0.4% hydrogen peroxide to inhibit endogenous peroxidases, rinsed in double-distilled water, and blocked with PBS-1% BSA at 4 °C overnight. The sections were treated with primary antibody (see Table 2), then developed with the appropriate peroxidase-conjugated secondary anti-body (DAKO, Glostrup, DK) followed by 3-3′ diaminobenzidine (Sigma) and H_2O_2 for 20 min. They were mounted in Aquamont (Gurr).

RESULTS

Controls (Agarose Beads and Dentin Debris)

Eight days after implantation of dentin debris inserted into the pulp during pulp perforation and implantation of control agarose beads, a burst of inflammatory cells was seen in the mesial part of the coronal pulp, in the area where the trauma occurred (Plate 1, a). After 15 days, reparative dentin formation had begun, separated from the dentin formed before pulp implantation by a continuous calciotraumatic line. Residual inflammation was still present in the pulp (Plate 1, b). After 90 days, the inflammatory process had totally disappeared. A continuous thin layer of reparative dentin was formed, embedding the agarose beads (Plate 1, c).

Implantation of Agarose Beads Loaded with A+4

Eight days after implantation of agarose beads soaked in A+4, the inflammatory process was severe (Plate 2 a, b). Inflammation was still present after 15 days (Plate 2 c, d) and even at 30 days (Plate, 2 e). No evidence of inflammation was detectable at 90 days. In contrast to the controls, a reparative dentinal bridge started to form at 15 days, and became thicker by day 30. A thick dentinal bridge formed by non-tubular dentin occluded the perforation. The lumen of the root canal was not totally filled in most of the implanted molars (Plate 2, f) but the closure was total due to dentin formation in a few implanted molars (Plate 2, g).

Implantation of Agarose Beads Loaded with A−4

After 8 days, the inflammatory reaction was less intense than with A+4 (Plate 3 a, c). A dense ring of cells was seen around implanted beads (Plate 3, b). After 15 days, reparative dentin formation began in the crown part, appearing as a thick dentinal bridge, filling or not filling the perforation (Plate 3, d). A re-

Table 2 Antibodies used to investigate cell proliferation and the phenotype of the cells recruited in the reparative process

Antibody	Aims and origin	Concentration, dilution	Secondary antibody
Proliferation Cell Nuclear Antigen (PCNA) Monoclonal mouse antibody	Marker of proliferation PCNA (Ab-1) Ref NA03 Oncogen Research Product USA	1/75	Polyclonal goat antibody 1/100 (Dako, Glostrup, DK)
Dentin sialoprotein (DSP) Polyclonal anti-rabbit	Phenotypic marker of odontoblasts. Source: Generous gift from Dr. W.T. Butler, UTHS, Houston, Texas, USA	1/200	Pig anti- rabbit IgG 1/100
Osteopontin (OPN) Polyclonal anti-rabbit	Phenotypic marker of both osteoblasts and odontoblasts. LF 123 – source: generous gift from Dr. L. Fisher, NIH, NIDCR, Bethesda, ML, USA	1/100	Pig anti- rabbit IgG 1/100

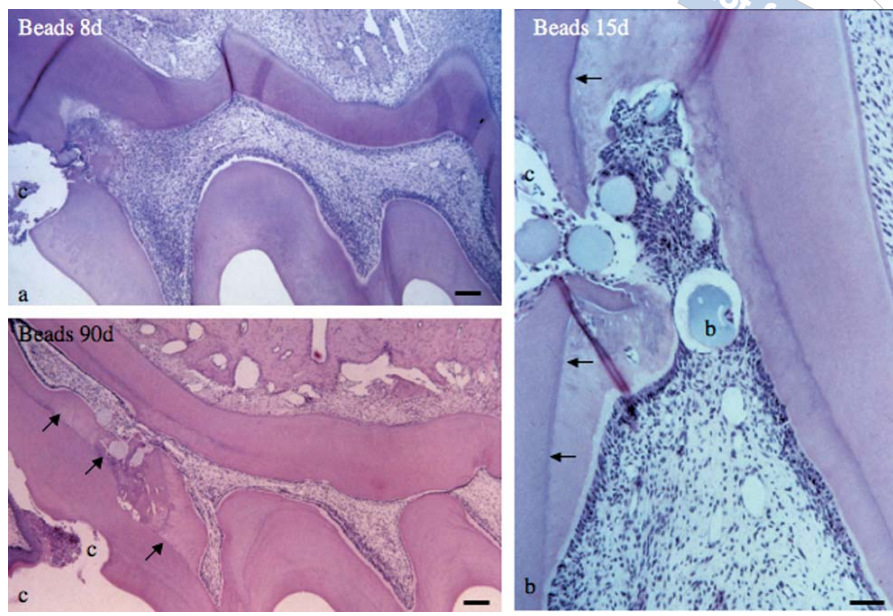

Plate 1 Effects of implanted agarose beads in the pulp of rats' maxillary molars. **a**. After 8 d inflammatory cells are seen in the mesial part of the pulp, in a pulp area close to the perforation. **b**. After 15 d, the inflammatory process is firmly reduced. A thin reparative dentin layer formed, beneath a calciotraumatic line (arrows), however the perforation is not totally filled by a dentinal bridge. During the preparation of the tooth, agarose beads (b) were pushed into the pulp. **c**. After 90 d, agarose beads implanted into the pulp stimulate the formation of an irregular layer of reparative dentin (arrows). c: cavity, b: agarose bead. Bar= 100 μm.

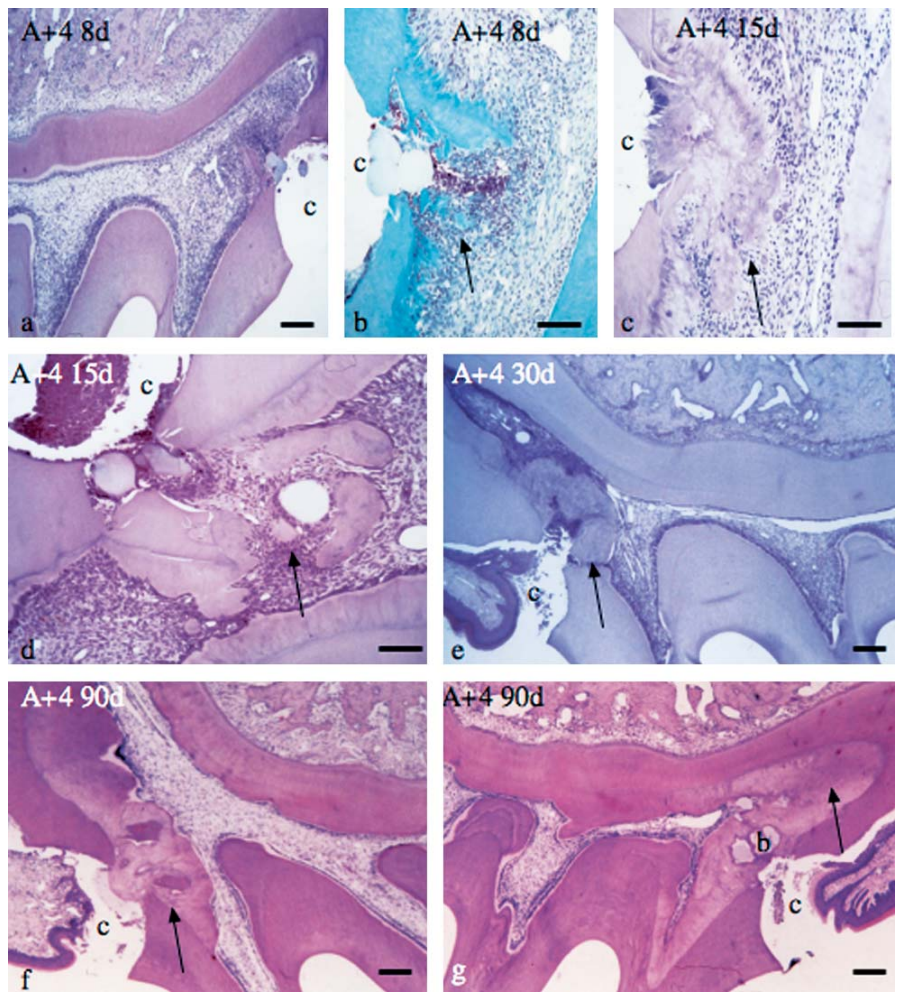

Plate 2 Effects of the implantation of A+4 into the dental pulp of the first maxillary molar. **a**. and **b**. After 8 d, inflammatory cells are seen in the pulp near the perforation (arrow). **c**. and **d**. After 15 d a reparative dentinal bridge starts to be formed (**c** arrow), filling the perforation. Thicker reparative formations may be also observed in some pulps, which have not yet merged (**d** arrow). **e**. Thirty days after implantation a thick reparative dentin bridge is formed. Inflammation is not totally resolved, as it appears in the mesial root canal. **f**. and **g**. Ninety d after implantation of agarose beads as carrier for A+4, a thick reparative dentin bridge is formed both in the coronal part (**f** arrow) and filling a large part of the root canal (**g** arrow). c: cavity, b: agarose bead. Bar=100 μm.

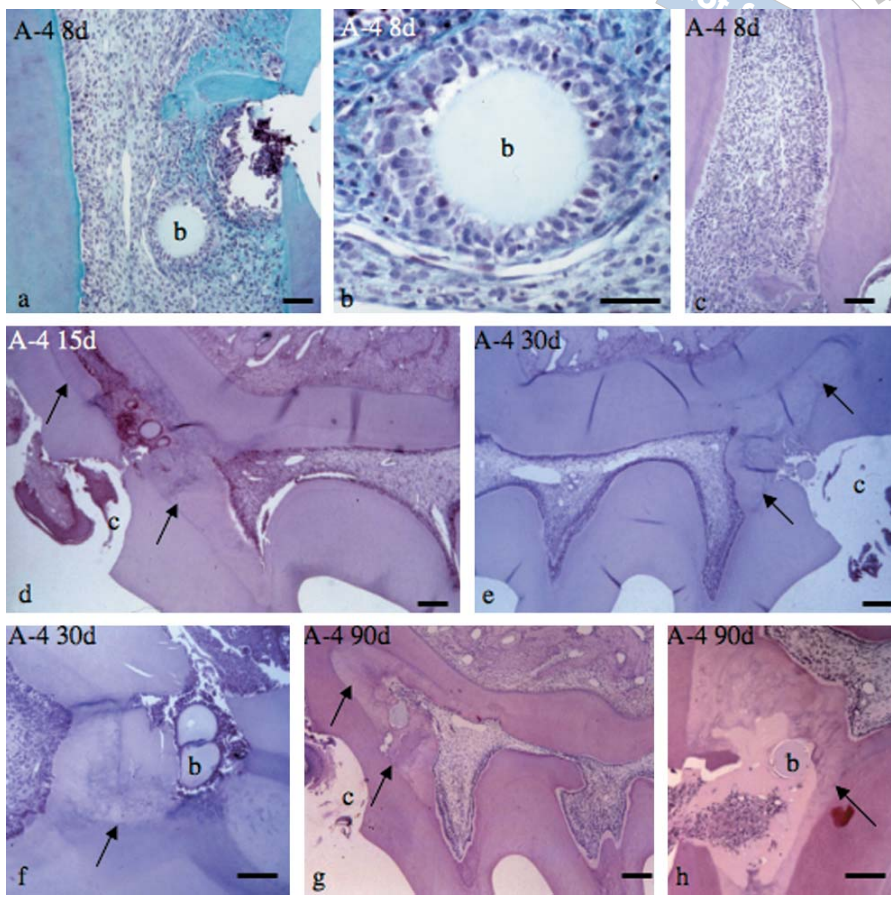

Plate 3 Effects of the implantation of A−4 into the dental pulp of the first maxillary molar. **a. b**. and **c**. Eight d after implantation, a moderate inflammatory process is seen. Cells that are involved in the reparative processes are recruited and grouped around the beads. **d**. After 15 d, a large part of the mesial coronal pulp is filled with reparative dentin. In the root part of the tooth, the lumen is reduced by the formation of a reparative dentin, limited by a calciotraumatic line (arrows). **e**. and **f**. After 30 d, the coronal pulp is largely filled with reparative dentin, and the lumen of the root canal is totally closed (arrows). **g**. and **h**. The formation of reparative dentin is stable after 90 d and did not interfere with alveolar ligament, cement or bone structures. c: cavity, b: agarose bead. Bar= 100 μm.

sidual lumen was seen in the root canal, although formation of thick reparative dentin displayed a tendency to obstruct the lumen. The newly formed non-tubular reparative dentin was separated from the dentin formed before any implantation by a calciotraumatic line. Thirty days after implantation, there was no further evidence of any inflammatory process, and the mesial part of the coronal pulp was filled with reparative dentin. The lumen of the pulp in the mesial root of the molar was totally filled by a mineralized structure, up to the apex of the root (Plate 3 e, f). After 90 days, the mineralization process was limited to the tooth and did not expand to the surrounding tissues. The ligament and apical zone were anatomically normal, without changes in thickness, and the cementum formation was not excessive (results not shown). Both the mesial coronal pulp and the root canal were filled in most of the teeth. Remnants of dentin debris and residual agarose beads formed inclusions into the reparative dentin (Plate 3 g, h).

Proliferation Cell Nuclear Antigen (PCNA) Immunolabelling

Eight days after implantation, PCNA revealed two distinct localizations. Initially, labelled cells were seen in the coronal mesial pulp, in an area located near the perforation. Cells were grouped in a central part, forming a proliferation centre in a central area where beads had been implanted, and the cells were dispersed at the periphery of the pulp, in an area subjacent to the perforation. Secondly, labelled cells were seen only in the root, at the periphery of the canal and not in a central part, forming a dotted line in the sub-odontoblastic border of the pulp (Plate 4, a). After 15 days, no labelled cells were detected in the root pulp. In the crown part, labelling was decreased, compared with the sections examined 8 days after implantation. Labelled cells were found either in a pulp area near the perforation, or around agarose beads. In this case, cells were not in direct contact with the agarose bead, but at the periphery of an unlabelled

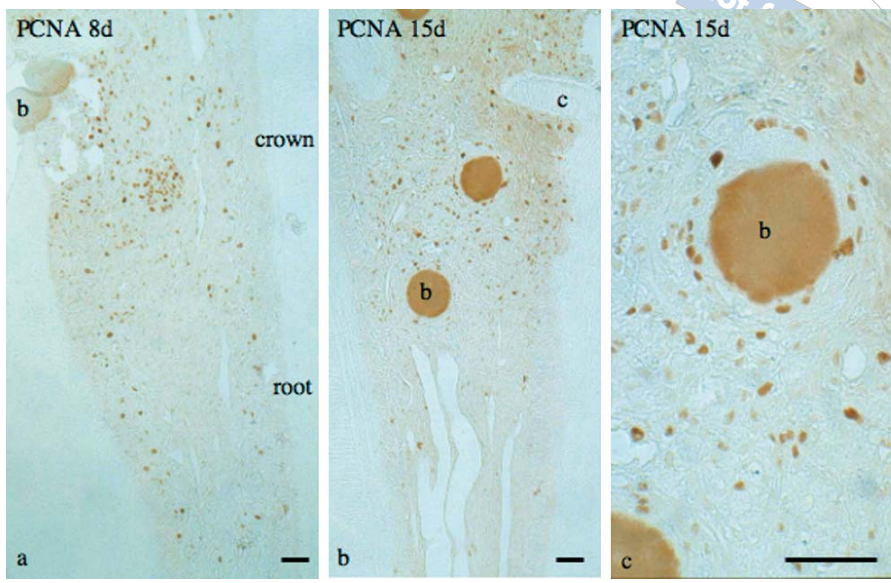

Plate 4 Cell proliferation in the implanted pulp visualized with PCNA. **a**. After 8 d, in the crown part of the pulp, labelled cells are seen in the central part of the pulp, may be near agarose beads and in the pulp area near the perforation. In the root part, labelled cells are present in a sub-odontoblastic border, but not in the central part. **b**. and **c**. After 15 d no labelled cells are detectable in the root pulp. In contrast, PCNA-positive cells are present in the crown part, some distance away from agarose beads. c: cavity, b: agarose bead. Bar= 100 μm.

ring of cells wrapping the beads (Plate 4 b, c). At later stages, no staining became detectable in the zone of interest, due to reparative dentin formation.

Characterization of the Phenotype of Reparative Cells

For the two bioactive molecules, immunolabelling with anti-DSP was only positive in the sound part of the tooth for the odontoblasts present at the periphery of the pulp. The cells located at the surface of the pulp near the perforation were intensely positive, but the cells located most closely around the carrier beads were unstained with the anti-DSP antibody (Plate 5 a, b). In contrast, using anti-OPN, some cells were positively stained within the pulp, near the implanted beads. The most intense staining was seen in the cells forming a ring around the beads at 8 and 15 days.

DISCUSSION

The present results establish that implantation of amelogenin gene splice products in the pulp of rats' molars induces and accelerates the formation of reparative dentin with A+4 and A−4 as compared to control. In addition to a thicker area of mineralization filling the mesial coronal pulp, the lumen of the root canal was occluded with a homogeneous mineralized structure by the action of A−4, while dentin bridge formation was more extensive with A+4.

Comparison with Controls and other Bioactive Molecules

The introduction of dentin debris into the pulp allows the release of growth factors and matrix molecules, which induce reparative dentin formation (Tziafas et al, 1992). The biological effects of dentin debris alone have been investigated previously (Decup et al, 2000). At day 15, reparative globular structures were seen in the mesial pulp, but no tendency to fill the perforation was detected. Finally, 30 days after implantation, a large area of osteodentin was formed in the mesial pulp, including large round mineralized structures separated by empty spaces containing pulp remnants. Implantation of agarose beads contributes to additional effects, and there is a significant possibility that beads, formed from sulphated galactan, also have a potential for inducing some pulp healing. However, in no case did the lumen of the root canal display any sign of closure when agarose beads were implanted alone. The insertion of 3-7 beads into the pulps means that the peptide concentration may influence interpretation of the results. However, despite slight variations, we observed a general tendency in the 6-10 teeth treated per group, apparently unrelated to a possible dose response. Difference in the thickness of the reparative dentin between the controls and after A+4 and A−4 implantation argues in favour of a higher therapeutic potential of the two amelogenins in dentin repair. In this context, it should be noted that A−4 induces pulp mineralization both in the crown and root, compared with A+4, which solely induces the formation of a dentinal bridge in

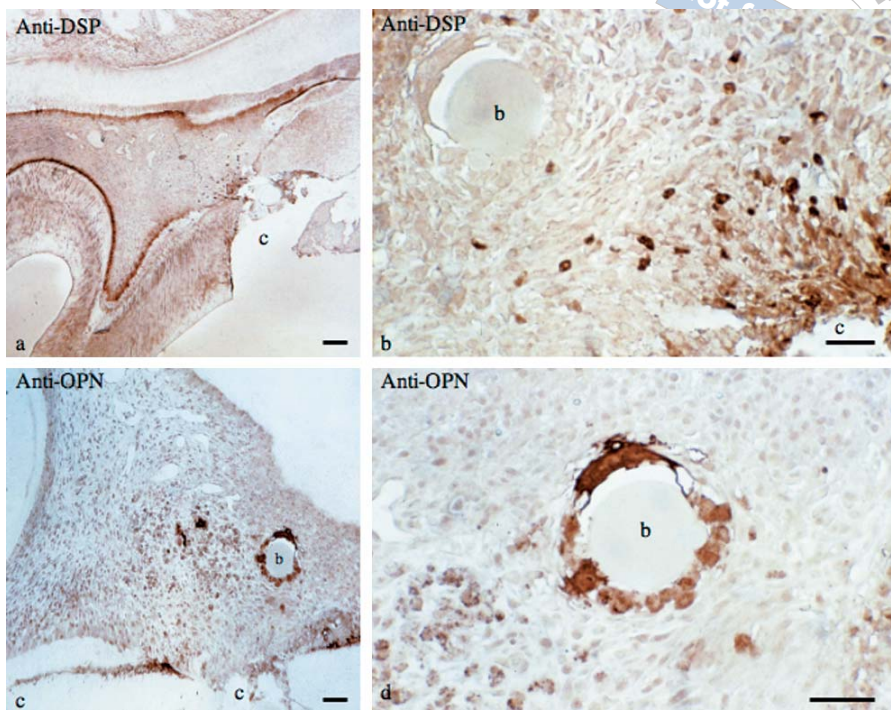

Plate 5 Identification of proteins expressed by reparative cells. **a**. and **b**. DSP-immunolabelling is positive in odontoblasts' cell bodies and processes, as could be expected from an odontoblast specific protein. Bone was unlabeled. The pulp is negative, except near the perforation where a few cells are positively stained. The staining of the cells around the beads is totally negative. **c**. and **d**. Osteopontin antibody positively stains a few pulp cells but not odontoblasts. A group of pulp cells is positively stained in the central crown area. The staining reaches a maximum for the cells located at the surface of the beads, whereas only intracellular dots are stained in the cells located some distance away. c: cavity, b: agarose bead. Bar=100 μm.

the crown area. Thus the two peptides might have different uses when considering them as potential dentin repair agents. A−4 obviously has a superior potential for pulp occlusion whereas A+4 has better limited activity as a dentin repair agent.

Defects in the formation of dentinal bridges induced by $Ca(OH)_2$ hamper reparative dentin function as a true permeability barrier. In case of bacterial reinfection, fissures and channels in the bridge do not allow pulp to resist. To some extent the formation of reparative dentin in the crown with amelogenin gene splice products was similar to what was formed in response to BSP implantation; i.e. an atubular dense and homogeneous dentinal bridge (Decup et al, 2000). In contrast, with BMP-7 (OP-1) implantation, where a porous osteodentin was obtained, the imperfect dentin bridges were unable to resist a second attack (Six et al, 2002a). Clearly, the structures obtained after amelogenin implantation provide more efficient permeability barriers.

From a clinical point of view, these bioactive molecules offer three interesting possibilities. The first comprises an increased thickness of reactionary dentin, thereby providing good protection against the harmful effects of the non-polymerized monomers released by the composites or by resin reinforced glass ionomers. Secondly, if there is no infection reparative dentin provides a tool that allows the pulp to remain alive, Thirdly, the total mineralization of root canals opens new possibilities, and a potential use in endodontic therapy. Apparently A+4 is a good candidate in situations covered by the first two points, whereas A−4 fulfils the requirements for the third point, and opens up innovative prospects.

Cell Proliferation and Differentiation

The experimental approach used has provided information on the series of events during the first 15 days after implantation. In longer term implants mineralization masks the biological events that are still occurring. Importantly, inflammatory processes occur during the first days after the preparation of the cavity. Inflammatory cells were still present even 30 days after implantation with A+4, and probably for a little less time with A−4. Hitherto, no precise identification of these cells has been made, but some of these cells may be precursors involved in the cascade of events leading to the formation of reparative dentin. It is still not known if, in the inflammatory cell population, a few cells may shift their phenotype and act as stem cells that may differentiate into reparative cells. There is also a possibility that in this burst of cells, inflammatory cells are co-located or interact with stem cells, which may be totally independent from the former

but will further proliferate and differentiate into odonto/osteoblasts involved in the reparative processes. At that stage the existence of common roots shared by different cell populations should be considered. An alternative possibility may be that distinct lineages, not yet identified, co-exist in this amalgam of cells.

The PCNA immunostaining that was carried out showed where cell proliferation was occurring. At 8 days after agarose bead implantation, two distinct areas were labelled. Cells were labelled in the coronal pulp in a large zone near the perforation, and more distantly in the central part of the pulp in a proliferating centre – near but not directly related to the agarose beads. This corresponds more or less with the zone of inflammation; however, among the large population of cells present in the area, only a few of the inflammatory cells were PCNA stained. This suggests either that proliferation was restricted to a small number of cells, or to some specific lineage. It is interesting to note that proliferation was never seen in close association with the carrier beads, but always at a short distance from them. For example, the first row of cells around the beads was never labelled. Fitzgerald et al (1990) reported that after $Ca(OH)_2$ capping and tritiated thymidine labelling a first cell division occurred in the central pulp, and that later the cells migrated near the perforation, where they underwent a second cell division. The interpretation provided by theses authors fits in well with the concept of a central proliferation centre and the burst of labelled cells migrating toward the wounded area observed at 8 days with the PCNA technique. The reduction of labelling observed at 15 days, suggests that a large part of the cells involved in repair had already been recruited, and once initiated, proliferation gradually decreased. No substantial difference was detected between A+4 and A−4 in this respect.

A previously unsuspected second location of PCNA positive cells was seen in the root, far from the pulp perforation and implantation zone. A few cells were aligned along a sub-odontoblastic border. None of the odontoblasts and the cells in the Höhl layer was labelled. This is quite logical as odontoblasts and Höhl's cells are post-mitotic cells. This distribution was seen in the root at day 8 but not at day 15, whereas in the crown a large part of the staining persisted even longer. One possibility is that cells proliferate in the root and contribute to the healing process after migration toward the wounded area. This implies the presence of a cell reservoir in the root. Alternatively, this second group of PCNA positive cells is only devoted to the closure of the lumen. Again, as suggested in our BMP-7 article (Six et al, 2002a), substantial differences appear between the crown and root pulp, recognized not only in the context of pulp healing, but also in relation with some important biological functions.

Immunostaining demonstrated that cells located near the surface of the wounded pulp were DSP positive, whereas OPN positive cells were grouped around, and in close association with the carrier beads. Again, this was seen both for A+4 and A−4. For a long time, it was believed that DSPP and the two daughter molecules DSP and DPP (the N- and C-portions obtained after cleavage of the original molecule) were dentin specific. Recently, Qin et al (2002) showed that DSPP was also found in bone, although in a very low, but not negligible, 1/400 ratio. Hence, it cannot be assumed that the presence of DSP is related to the odontoblast phenotype alone, although it is highly probable that this is actually the case. OPN is mostly found in bone cells, but is also detected in odontoblasts (Chen et al, 1993). The two phosphorylated molecules are members of the SIBLING family, and expressed by the co-localized genes on human chromosome 4q20-21. They are all presumably implicated in dentin mineralization, but also in cell signalling. The two antibodies were used because we were looking for the characterization of reparative cells, of the odontoblast-like or osteoblast-like types. Currently, there is a lack of specific markers that avoid any possible overlap between the two types of cells (Goldberg and Smith, 2004). Despite these limitations, the immunohistochemical staining suggests that DSP is strongly expressed in the cells directly implicated in the formation of reparative dentin at the surface of the wounded pulp. Thus, these have the characteristics of odontoblast-like cells. In contrast, the cells positively immunostained with anti-OPN near and around the carrier beads are osteoblast-like cells. The difference of phenotype may be related to the position of the cells within the pulp and to the place where the repair process occurs, influencing the acquired phenotype. It is also possible that two distinct types of cells are recruited and play different roles in the repair process.

In conclusion, the present investigation allowed us to answer a few questions. Cell divisions do occur in the pulp cells once they are recruited into the coronal part in the central proliferating centre and near the perforation. Perhaps this is related to two successive steps. In the root, another group of cells aligned along the sub-odontoblastic border may con-

tribute to the burst of new cells migrating in the coronal direction, or the cells may stay locally, and be implicated in the gradual closure of the root canal lumen. Comparing the data from Plates 4 and 5, it is clear that the proliferating cells grouped immediately around the carrier beads express OPN, whereas DSP is expressed in cells some distance away, near the wound perforation. Thus, two groups of labelled cells coexist, in adjacent, but geographically distinct areas. This therefore suggests that the cells implicated in pulp mineralization are more of the osteoblastic-type rather than odontoblast-like cells, which are favoured where dentinal bridge formation is required. It is well documented that A+4 stimulates the expression of *Sox 9* transcription factor, whereas A−4 stimulates the expression of the transcription factor *Cbfa1*, involved in the differentiation of chondrocytes and osteoblasts (Veis et al, 2000). As there are neither Type II collagens nor chondrocytes in dentin and pulp, the question of the initial mechanisms of recruitment of the cells involved in reparative dentin formation is still open. How are the specialized cells selected from among a heterogeneous population? Are there real stem cells in the pulp? Or can differentiated resident cells present in the mature tissue dedifferentiate and redifferentiate into reparative odontoblasts? In any case, at the moment we cannot determine: which cells are recruited at the early stage immediately after trauma; which genes activate the process of selection/differentiation; and which transcription factors and/or matrix molecules are involved in the cascade of activation of cells which have been recruited? We appear to be near the limits of such *in vivo* investigations, despite their interesting bridge to potential clinical approaches to dentin and pulp repair. It is highly probable that answers to the mechanistic questions now have to be obtained by *in vitro* approaches.

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