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Inflammatory and immunological aspects of dental pulp repair

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Abstract

The repair of dental pulp by direct capping with calcium hydroxide or by implantation of bioactive extracellular matrix (ECM) molecules implies a cascade of four steps: a moderate inflammation, the commitment of adult reserve stem cells, their proliferation and terminal differentiation. The link between the initial inflammation and cell commitment is not yet well established but appears as a potential key factor in the reparative process. Either the release of cytokines due to inflammatory events activates resident stem (progenitor) cells, or inflammatory cells or pulp fibroblasts undergo a phenotypic conversion into osteoblast/odontoblast-like progenitors implicated in reparative dentin formation. Activation of antigen-presenting dendritic cells by mild inflammatory processes may also promote osteoblast/odontoblast-like differentiation and expression of ECM molecules implicated in mineralization. Recognition of bacteria by specific odontoblast and fibroblast membrane receptors triggers an inflammatory and immune response within the pulp tissue that would also modulate the repair process.

Keywords

Dental pulp; Tissue repair; Extracellular matrix molecules; Inflammation; Immune cells

1. Introduction

For decades, the treatment of choice for dental pulp exposure has been calcium hydroxide [Ca(OH)₂] capping [1]. Ca(OH)₂ is highly alkaline and consequently induces the formation of a scar at the surface of the exposed pulp. The capping procedure leads to a local inflammatory

process, and afterwards, pulp cells are recruited. They proliferate and differentiate into odontoblast/osteoblast-like cells that produce an extracellular matrix that turns out to be a scaffold for a mineralized dentinal reparative bridge [2] (Fig. 1a–d). However, this structure is not homogeneous, the newly formed osteodentin includes tunnels, equivalent to osteocyte lacuna and pulp remnants [3] (Fig. 1d). Unfortunately this inhomogeneous reparative barrier is permeable and cannot resist re-colonization by bacteria. Clinical failures in capping therapy have led to the search for new therapeutic agents. The potential of bioactive agents such as dentin extracellular matrix (ECM) molecules or stem (progenitor) cells is currently under study. Analysis of the cellular and molecular mechanisms underlining the regenerative processes, emphasize the role of ECM molecules, progenitor cells, and the importance of the carrier.

Preparation of the cavity, the pulp exposure and the implantation into the pulp of carrier loaded with cells or bioactive molecules into the pulp all induce inflammation. All studies report that the healing sequence includes an initial inflammation process. The currently accepted concept is that the reparative process follows after inflammation is resolved. We now have evidence that inflammation is a prerequisite for tissue repair. We present evidence here supporting the concept that inflammation is a prerequisite for tissue repair, which would not occur if this essential step was omitted [4].

2. Pulp cells

In the healthy adult tooth, odontoblasts and cells of the sub-odontoblastic layer, the so-called Höehl cell layer [5], form a thin border located between the inner margin of the dentin and the outer limit of the pulp. These post-mitotic polarized cells are responsible for the production of physiological *primary* and *secondary dentins*, depending on whether the dentin formation takes place before tooth eruption for the former or after eruption when the tooth becomes functional for the later. Although decreasing in number and activities, odontoblasts produce dentin ECM molecules continuously as long the tooth is alive and a gradual thickening of dentin takes place over time.

By contrast, in the dental pulp, fibroblasts or pulpoblasts [6] are renewed constantly [7,8]. In the fully mature and even in senescent teeth, the pulp remains a soft tissue. However, aging influences pulp mineralization, and in many cases pulp stones or areas of diffuse mineralization develop gradually. The reduced mineralization potential of dental pulp fibroblasts underlines that they are distinctly different entities from odontoblast/ sub-odontoblastic Höehl's cells, even if they share a common embryological origin.

In vitro, cells that grow from pulp explants seem to be mostly fibroblast-like cells bearing biological specificities [9] (Figs. 2 and 3). In addition to the different expressions of ECM molecules, it is now well documented that odontoblasts and pulp fibroblasts differ in their response to oral pathogens, another argument in favor of pulpoblast specificities [6,9,17]. Their relationship of structural fibroblast-like cells with odontoblast/osteoblast progenitors is not clearly elucidated. However, in some experimental conditions, pulpoblasts may contribute to the formation of mineralized nodules [9].

It is now well established that the normal dental pulp contains heterogeneous cell populations including a majority of fibroblast-like cells, but also inflammatory and immune cells (dendritic cells (DCs), histiocytes/macrophages, T-lymphocytes) (Figs. 2-4 and 7), and latent or dormant pulp stem cells (progenitors), mostly involved in self-renewal. After damage of the tooth, the progenitors may contribute to pulp repair and mineralization. Nerves (axons and Schwann's cells), vascular and perivascular cells (endothelial cells of the intima, vascular smooth muscle cells of the media and Rouget's pericytes) are also present within the pulp [10].

In response to a slow carious decay, pathological abrasion, or to superficial tooth preparation by a dental surgeon, the odontoblasts that are still alive have the capacity to produce a *reactionary dentin*, more or less similar to the physiological dentin (tubular orthodentin) [11] (Fig. 5). In case of an invasive carious lesion, the odontoblasts are destroyed by bacteria toxins or altered by noxious molecules released by the restorative material, by necrotic cells or by enzymes released by the degradation of the extracellular matrix. The cells located in the Höehl (sub-odontoblastic) layer may in that case ultimately differentiate and replace the wounded cells in producing a layer of reactionary dentin beneath a calcio-traumatic line (Fig. 5).

If the pulp is exposed, odontoblasts and Höehl cells can no longer perform repair of the lesion and another process takes place. Stem cells or progenitors located within the pulp become recruited. They proliferate and differentiate into osteoblast-like or odontoblast-like cells [12, 13] and start to produce an ECM, which will ultimately undergo mineralization. This cascade of events leads to the elaboration of a *reparative dentin* [10,11], in the form of a thin dentinal bridge occluding the exposure site, or a bone-like structure (osteo-dentin) filling partially or totally the pulp [12] (Fig. 6).

Radioautographic data obtained on monkey using tritiated thymidine after pulp capping with calcium hydroxide have shown that a first replication is observed in the central part of the pulp, and latter a second cell division occurs in the outer part of the pulp, near the pulp exposure [14]. The distribution of labeled cells indicates that cells involved in the reparative process divide at least twice, and that progenitors initially located in the central pulp migrate toward the exposure site. This further suggests that a continuous influx of differentiating cells that contribute to the formation of a dentinal bridge, excluding odontoblasts and Höehl's cells in the formation of reparative dentin. Since these are post-mitotic cells, they would have lost the potential for self-renewal.

It seems clear that these reparative cells originate within the pulp. Further, only a subpopulation of the pulp cells seems susceptible to be committed and to be involved in the repair process. The identification of stem cells within the dental pulp [15,16] has led to the hypothesis that the reparative cell progenitors are stem cells, but this has not been yet clearly demonstrated.

Progenitors or adult stem cells exist as dormant or latent cells, and are scarce in the sound pulp [15,16]. During the inflammatory process there is a general increase in the number of pulp cells, but it is not known if this increase is related to fibroblasts proliferation, or represent a massive migration of inflammatory cells, or to proliferation of stem cells.

With respect to immune/inflammatory phenomena, some pulp cells express class II antigens. They are implicated in the immune reaction (Table 1, and references [17-35]). Immune cells are present both in the normal and pathologic pulp [22-25]. In the normal pulp, they regulate cell population density, and are crucial in the control of cell proliferation and apoptosis [7,8, 25]. They may contribute in the exposed pulp to resolve inflammatory processes. They are mainly DC and macrophages that activate T-lymphocytes [22-25]. These cells are motile. They migrate independently, in contrast with pulp fibroblasts that are closely associated through junctional complexes, and therefore presumably translocate as a whole from the central to the outer part of the pulp, as a syncytial structure.

In the intact pulp, two distinct DC populations have been identified. CD11c⁺ are present at the pulp–dentin border, beneath occlusal fissures, whereas F4/80⁺ DCs are almost concentrated in the perivascular region of the inner pulp and in the sub-odontoblastic layer. CD11c⁺ dendritic cells express Toll-like receptors 2 and 4 and are CD205 positive. F4/80⁺ migrate from the inner pulp, increase in size and display CD86 expression [36]. They disappear entirely from the pulp, 24 h after experimental stimulation. Therefore within the pulp, two distinct types of dendritic cells are identified, the superficial CD11c⁺ acting as sentinels and the migrating interstitial

F4/80+. Each displays distinct territories and plays specific role(s) in response to external stimuli via the dentinal tubules [36] (Fig. 7).

A link between inflammatory molecules and the regulation of pulp cell population has been suggested earlier by our studies on the effects of essential fatty acids deficiency (EFAD) [7]. When rats were fed with EFAD diet, the cell density was increased twofold to threefold within the central and outer pulp, respectively, a time-depending effect related to the period of EFAD-diet administration (Table 2).

In these studies, we did not observe mitotic cells within the pulp, and therefore we suggested that the increased pulp cell population might result from a decrease or arrest of the apoptotic events that normally occur [7,25]. By contrast, this diet did not modify either the number or shape of the odontoblasts. As the essential fatty acids are transformed into prostaglandins and leukotrienes known mediators of the inflammation processes, it may be that these mediators may play role in the balance regulating cell death and cell renewal, as it is the case for other cells and tissues [37,38].

For years, inflammation in the tooth has been considered mostly as a negative factor leading to pulp destruction by necrosis or apoptosis. This seems to be an oversimplification. Some data on the reaction to carious lesions and/or implantation of biomolecules suggest that the inflammatory reaction might be a prerequisite for the burst of progenitors implicated in the pulp repair [10].

3. Regulation of dental pulp repair by immune cells in carious teeth

Recent data have shown the role of odontoblasts and pulp fibroblasts in the regulation of the dental pulp immune and inflammatory responses to cariogenic bacteria and suggest that interactions between immune/inflammatory cells and odontoblasts and/or their precursors influence the pulp repair process [10,17,18,20,^{24,26},27,29,33,34]. In this section we will review the relationship between inflammatory processes, immune cells, and odontoblasts and pulp repair in carious teeth.

The clinical reality of pulp capping is that a carious lesion precedes the therapy. This implies that progression of bacteria from the outermost enamel to the pulp–dentin interface triggers inflammatory and immune events in the underlying dental pulp through the diffusion of bacteria pathogen by-products into dentin tubules. These events may be prevented when reactionary/reparative dentin is formed by odontoblasts at the pulp–dentin interface, eliminating the bacteria insult and blocking the route of infection. In the absence of odontoblast reaction, or in case of odontoblast death, bacterial invasion leads to irreversible pulpitis, pulp necrosis, infection of the root canal system and periapical disease [39,40].

In the dental pulp, when dentin is being destroyed by caries, immature antigen-presenting dendritic cells rapidly migrate to the odontoblast layer facing the lesion in a strategic location near the foreign antigens [22,41]. Then, a progressive and sequential accumulation of T-lymphocytes, macrophages, neutrophils and B-lymphocytes occurs in the pulp, concomitantly with the deepening of the dentin caries lesion, the increase of the bacterial insult and the development of the pulp inflammatory process [24,42]. It is thus reasonable to assume that close relationships exist between immune cells, especially DCs that are involved in the initial steps of the pulp immune response, and odontoblasts in order to regulate both DC migration and odontoblast dentinogenic activities [45] (Fig. 7c).

The rapid accumulation of DCs into the odontoblast layer during the early phase of pulp repair has suggested that odontoblast-derived chemotactic molecules might be responsible for the recruitment of DCs that ensure immunosurveillance in the pulp tissue or patrolling in pulp

blood vessels [45]. This hypothesis was recently confirmed by *in vitro* data that demonstrated the role of odontoblasts in triggering immune/inflammatory events in response to specific bacterial components [17,21,43,46]. In particular, it was shown that odontoblasts grown *in vitro*, express specific cell membrane receptors of the Toll-like family (TLR1-6 and 9) that enable them to sense various types of bacterial and viral pathogens and to initiate innate immune responses [21]. Among these receptors, TLR2, 3, 5 and 9 are up-regulated in odontoblasts by lipoteichoic acid (LTA), a cell wall component of Gram-positive bacteria [46]. TLR2 is also up-regulated by lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria [43].

LTA sensing by odontoblasts induces synthesis of small chemotactic cytokines, called chemokines, involved in the recruitment of immune cells to the infectious and inflammatory sites. These include CCL2 and CXCL10 that are involved in dendritic cell and T-lymphocyte migration, respectively [44]. CCL2 is considered a key element in the recruitment of circulating blood dendritic cells and their migration through the endothelial barrier. Chemokines more specifically involved in the trafficking through the pulp parenchyma to the site of pathogen invasion remain to be identified. Differences exist in odontoblast and pulp fibroblast innate immune responses to LTA and LPS, with differential up-regulation of the Toll-like receptors TLR2 and TLR4, and the chemokines CXCL2, CXCL10, CCL7, CCL26 and CXCL11. Odontoblasts were also found to be more potent DC attractants than pulpoblasts, whatever the TLR agonist used [17].

Odontoblasts challenged with LTA also down-regulate the major dentin matrix components type I collagen and dentin sialophosphoprotein (DSPP) [21]. A similar DSPP down-regulation was observed in cells from a dental odontoblast-like cell line stimulated with high doses of lipopolysaccharide [46]. Odontoblasts thus repress their specialized functions of dentin matrix synthesis and mineralization when stimulated with microgram range doses of bacterial by-products, a condition that mimics the rapid progression of bacteria to the pulp–dentin interface in actively developing dentin caries lesions. It is not known whether odontoblasts respond differently to low or very low doses of bacterial components, a condition encountered in slowly developing caries lesions and possibly more favourable to the deposition of reactionary dentin by odontoblasts.

Close spatial relationships between odontoblasts and DCs in the peripheral pulp under caries lesions suggest that DCs might play a role in odontoblast differentiation and/or modulation of their synthesis activity [47]. DCs fail to produce bone morphogenetic proteins (BMPs) in culture [48] but express transforming growth factor-beta (TGF- β 1) [49]. The production of these factors by DCs in inflamed dental pulp tissue *in vivo* remains to be determined. A direct effect of DCs on odontoblasts and/or their precursors might occur through CCL20, a chemokine produced, among other cells, by activated DCs [50-52]. Indeed, *in vitro* stimulation of human dental pulp cells and mesenchymal stem cells with CCL20 was shown to augment DSPP gene expression [53,54].

Clinically, pulp repair taking place after calcium hydroxide capping, occurs when bacterial contamination is limited and inflammation is mild. Repair is never achieved in the presence of a long lasting chronic infection, which invariably results in pulp necrosis. Therefore there is an acceptable threshold that clinicians should access, but the limits between mild and acute infections are difficult to be determined. In many cases the success or the failure of the capping provides the actual answer.

4. Experimental pulp capping of healthy exposed pulp with bioactive ECM molecules: an experimental approach

As it is difficult to determine the virulence of carious bacteria, the characteristics of the lesion, of the oral environment and the intrinsic resistance of the patient, there is a need for experimental models with well-controlled parameters. One model of $\text{Ca}(\text{OH})_2$ capping of a sound tooth in the rat, although not being fully relevant from a clinical point of view, provides a way to study the cellular and molecular mechanisms occurring during induced pulp repair.

During the last several years, we have developed this animal model allowing a controlled *in vivo* approach to studying pulp reaction to extracellular molecules. A half-moon cavity prepared on the mesial aspect of the rat first maxillary molar, and the pulp is then exposed by pressure with the tip of a steel probe [55]. Bioactive molecules can then be implanted into the pulp using collagen pellets or agarose beads as a carrier for these molecules. The first recordable event with all the molecules that were investigated is an inflammatory process appearing within the first 3–7 days after implantation of the molecules and resolving within 2 weeks. This observation leads to reconsider the importance of the inflammatory process in the initial step of the healing cascade.

We will limit this review to the direct capping effects of calcium hydroxide and ECM molecules. Though calcium hydroxide is still nowadays a gold standard in dentistry, capping with ECM molecules is extremely promising, producing a reparative mineralized tissue that has better structural properties than that produced in the presence of calcium hydroxide.

Studies on the interactions of ECM molecules with dental pulp show that the properties of ECM molecules are more complex and multifunctional than was initially believed. ECM molecules interact with the network of collagen fibrils to participate in the tri-dimensional structure of the complex scaffold of connective tissues. As matricellular proteins, they serve as biological modulator with functions including interactions with integrins and other cell surface receptors [56]. They capture and release soluble extracellular factors such as growth factors (cytokines), proteases, calcium, hormones, etc. [57]. Finally, ECM molecules can direct intracellular signaling, resulting in the activation of transcription factors and other signaling cascades [57]. These multifunctional ECM molecules include amelogenin gene splice products $A + 4$ [9,12,13,58] and also dentonin, a peptide from MEPE [59,60]. A combination of these various factors might be required in pulp repair.

We previously reviewed the various ECM dentin molecules used as capping agents [10]. They include some of the small integrin binding ligand, N-linked glycoprotein (SIBLING) family [61], such as bone sialoprotein (BSP) [55] (Fig. 8a–d), matrix extracellular phosphoglycoprotein (MEPE) [59], dentin matrix protein-1 (DMP-1). Also present in bone and dentin matrices, growth factors such as BMPs, $\text{TGF}\beta$ have led to dentin repair. However these growth factors are not adequately therapeutic as they produce a porous osteodentin with tunnel defects that contain pulp debris [3,62]. Implantation of two spliced forms of amelogenin ($A + 4$ and $A - 4$) [9,11,12] induced the formation of homogeneous dentinal bridge or massive pulp mineralization (Figs. 9 and 10). These amelogenin isoforms may thus provide a material adaptable to clinical needs, and therefore we have concentrated our studies on the effects of these molecules.

$A + 4$ and $A - 4$ are low molecular weight (6–10 kDa) polypeptides isolated from the rat incisor dentin matrix. *In vitro*, they were found to stimulate embryonic muscle fibroblasts to express sulfated proteoglycans and type II collagen, characteristic of cartilage. For this reason the molecule was originally designated as being a chondrogenic inducing agent (CIA) [63]. The CIA was further identified as a low molecular mass amelogenin polypeptide [64]. Veis et al.

[65] identified two specific amelogenin cDNAs in the rat incisor tooth odontoblast/pulp library, corresponding to the low molecular weight CIA. The first resulted from expression of amelogenin gene exons 2, 3, 4, 5, 6d, and 7 [A + 4, 8.1 kDa] and the second from exons 2, 3, 5, 6d and 7, the expression of exon 4 being omitted [A - 4, 6.9 kDa]. When A + 4 was added to the culture medium, Sox9 expression was stimulated in cultured pulp cells, whereas A - 4 stimulated Runx2/Cbfa1 mRNA expression in these same cell populations.

In the mouse enamel organ, the larger full length (M194) and near full length (M180) amelogenin isoforms are the principal amelogenin splice products to direct the formation of the mineralized enamel. The M194 mRNA includes both the full exons 6 and 4 sequences, while M180, the major mature amelogenin mRNA, still excludes exon 4. The corresponding short forms, M73 (A + 4) and M59 (A - 4, LRAP) include, or exclude the 14 amino acid sequence encoded by exon 4. M194, M180, the two initially secreted full length forms of amelogenin [66] and M59 proteins are all produced, at different levels, within the mouse enamel organ, with M73 being present at much smaller levels. By contrast, M73 and M59 are expressed by odontoblasts at the perinatal period of development [67,68].

Amelogenin isoforms have been shown to promote differentiation of odontoblasts, acting as signal molecules [65,69]. Through how this signal is transmitted is not yet fully elucidated. A direct link apparently exists between the presence of an amelogenin membrane receptor and the internalization of the molecule. This is likely a non-specific process since internalization happens also with cells that are not odontoblasts or pulp cells. The lysosome-associated membrane protein-1 (Lamp-1) has been suggested as one possible receptor for amelogenin, at least for the spliced isoforms A-4 or LRAP [70-72], from the following observations:

- ^3H [A-4] binds to the cell surface of myoblasts, and biotin-labeled [A-4] binds to polarizing ameloblasts and odontoblasts [70].
- Exogenously added amelogenin moves into the cell as Lamp-1-positive vesicles containing internalized amelogenin localize in the perinuclear region [71].
- Lamp-1 is up-regulated in the presence of recombinant human LRAP [72].

Altogether the three reports suggest that Lamp-1 plays a role as membrane receptor, although the mechanism for how the amelogenin peptides are internalized and relay signals leading to the cell commitment to an odontoblast phenotype, remains to be understood. However, few studies have addressed this question and it is possible that the recognition of Lamp-1 as receptor does exclude the possibility that other molecules involved in the membrane to nucleus traffic are also required. Jacob and Veis [73] have shown recently that Lamp-3 is also an [A - 4] amelogenin isoform receptor. After internalization of the molecule and its receptor into an ameloblast cell-line, the nitric oxide (NO) signaling pathway is activated. The reaction is modulated by nitric oxide synthetases (NOS), known enzymes expressed by inflammatory cells. As tissue inflammation and pulp repair seem to be tightly linked, the novel relation discovered between A - 4, Lamp-1 and the NO/NOS reaction cascade needs to be further explored.

These data led to a number of questions.

First, is the entire amelogenin peptide internalized or is it smaller peptides or domains of amelogenin resulting from enzyme degradation that penetrate into the cells? MMPs or other catalytic molecules that are released during the inflammatory processes contribute to ECM molecule fragmentation. The small peptides or molecular domains that are released or are exposed after enzyme hydrolysis, may be involved in the bioactivity of the molecule. However the question remains open.

Secondly, what is the role of inflammatory processes in the cascade of events leading to pulp repair? Is it a simple consequence of the preparation of the teeth, including pulp exposure and a reaction to the implantation of agarose beads used as bioactive molecule carrier? Or, are inflammatory molecules and other cytokines involved in the commitment of specific progenitors? Some of the fibroblast-like pulp cells, which are present mostly as feeder cells, may de-differentiate and transdifferentiate, and subsequently become involved in the reparative process, as this has been suggested for adult reserve stem cells [35]. Alternatively, another possibility that should be explored is that cell plasticity would play role in the phenotypic inter-conversion of inflammatory cells toward the osteo/odonto progenitor lineage [74].

5. In vivo pulp implantation of ECM molecules: short-term reaction

Because inflammation is an early event, we studied what happens at 1, 3 and 7 days after *in vivo* implantation of amelogenin gene spliced product in the first maxillary molar [8].

Using agarose beads as carriers for bioactive molecules in our A ± 4 experiments, we have always noticed an initial moderate pulp inflammation at the exposure site. This inflammation was partially resolved after 8 days and totally at day 15. In a very few cases, some inflammation persisted at day 30, which was never observed at day 90. Examination of our control teeth (sham or implanted with beads alone), showed that both the surgical procedure and agarose bead implantation induce some self-repair. There is a tendency to enhanced pulp healing [12, 13], though the repair process is either arrested or very slow, when compared to the effects of A ± 4 loaded beads. This observation is likely explained by the intrinsic bioactive properties of agarose beads, and the fact that rat teeth appear more resistant to necrosis than human teeth. Indeed, agarose is a linear sulphated galactan that displays anti-viral and anticoagulant properties, and therefore may contribute to pulp healing. In any case, the degree of inflammatory reaction appears dependent on the skillfulness of the operator, and the bioactive molecule under investigation (A + 4 vs. A - 4).

These observations suggest that inflammation alone initially contributes to the repair process of healing, though after initiation of this process bioactive molecules are essential in the formation of reparative dentin. Indeed, cell proliferation reaches a maximum 7 days after implantation of control beads (not loaded with amelogenin), but the cells do not differentiate toward an osteoblast/odontoblast phenotype [13].

In short-term experiments, 1, 3 or 7 days after A + 4 or A - 4 implantation, osteopontin (OPN), which is both a matrix structural molecule and an inflammatory marker [75,76] was gradually increased in the A + 4 implanted pulps. At 7 days, expression began to decrease. In contrast, after A - 4 implantation, OPN labeling was maximal at 3 days [13]. At later periods of time OPN was used exclusively as a bone cell marker because no inflammatory reaction was detected [12]. This labeling was roughly parallel with what was observed using a RP59 antibody, a marker of bone marrow cells, primitive mesenchymal cells, erythroid cells, megacaryocytes, hematopoietic precursor cells and osteo/odontoblast progenitors [77-79]. Therefore, after an initial inflammatory burst, the committed cells underwent differentiation toward an osteoblast-like phenotype.

When agarose beads either with or without A + 4 or A - 4 were ectopically implanted in the non-mineralizing mucosa of the cheeks in rats and mice [80], the presence of CD45 positive cells, which are inflammatory cells issued from a leukocyte lineage, were visible as a large and dense infiltrate in the lamina propria at day 3. The number of these cells was reduced at day 8 and they were barely detectable at day 30. Only a few of these CD45 positive cells expressed the I-A^k molecule, a marker of macrophage and dendritic cells, were positively stained. At day 8, RP59 positive cells started to appear, suggesting an initial differentiation toward an osteoblastic lineage. Labeling was observed for Sox9, a chondrogenic/osteogenic marker.

Expression of BSP and OPN was observed at day 30, but DSP was never detectable. However, von Kossa staining failed to visualize any mineralized nodules. Because the cells are PCNA negative, we therefore assume that the labeled cells are recruited from the vascular compartment or migrate from another compartment and are not due to local cell proliferation.

Under appropriate conditions, it is possible for CD45 positive monocyte-derived progenitors to be oriented toward osteogenic or chondrogenic lineages [74]. This plasticity is also recognized from circulating skeletal stem cells, mesenchymal stem cells and fibroblast-like cell populations [81-83]. The results of this *in vivo* study suggest that the CD45⁺ positive cells that persist around the beads at day 30 are leukocyte-derived mesenchymal progenitors that express osteo-chondrogenic markers under the influence of A ± 4 molecules, at least in cheeks mucosa [79]. Whether comparable events also take place in the pulp still remain to be explored.

Similarly, inflammatory events may contribute to the very early phases of pulp repair, especially if there is a direct link between the release of cytokines and the commitment of some pulp cells towards the odont/osteoblast progenitor phenotype. After *in situ* implantation inside the pulp, the cells proliferate, though this is obviously not the case when implantation occurs ectopically in the cheek mucosa. The similar commitment of some cells to an odont/osteoblast progenitor phenotype in these two dissimilar niches, suggests that there are a few cells that are specifically committed and differentiate toward a chondro/osteogenic lineage.

These cells likely require a specific environment to produce mineralized nodules or bone-like structures. When implanted into the pulp, the bioactive molecules are stimulating the formation of mineralized reparative area of the osteodentin type. We propose that as seen from the control teeth implanted with beads without A ± 4, the inflammatory reaction may initiate steps of the intracellular cascade, but the reaction is arrested and does not result into the final differentiation of osteoblast/odontoblast-like cells. This indicates that bioactive molecules function by participating in the recruitment, proliferation and differentiation of progenitor cells.

As a concluding remark it worth noting that we induced the formation of reparative dentin with most of the ECM molecules when they were used as pulp capping agents. However, we noted that BSP and A – 4 implantation leads to large pulp mineralization, very similar morphologically to an atubular orthodentin, located in the crown and in the root as well. Therefore these molecules appear to somewhat differentially act at all stages of the cascade of reactions leading to dentin formation, including cell, proliferation, and differentiation and finally the formation of reparative tissue (see for review [10]). In contrast, dentonin, peptides of MEPE acts mostly on the initial steps and to a lesser extend on the final events [84].

6. Conclusion

In conclusion, experimental data obtained on pulp repair of healthy and carious teeth, highlight the potential importance of the initial inflammatory reaction. In experimental capping of exposed sound pulps or after implantation of bioactive molecules in ectopic sites, this initial step seems to be associated with the commitment of latent or dormant progenitors (stem cells). In a non-pathologic situation, inflammatory mediators such as prostaglandins and leukotrienes regulate cell proliferation and cell survival. This function is directed to all pulp cells but does not discriminate between structural fibroblasts, inflammatory and immune cells or stem cells. In case of experimental capping or in caries diseases, inflammation seems also to favour a cell phenotype reorientation and to promote the transdifferentiation of some inflammatory cells into odontoblast-like or osteoblast-like progenitors. The same might occur for pulpoblasts, which are present as structural, or feeder cells, but these may still retain potential for transdifferentiation. Other data obtained on carious teeth suggest that odontoblasts are affected

by stimulation of the expression of Toll-like receptors and the beneficial release of cytokines within the pulp [85].

Therefore, for many years the importance of inflammation in pulp healing has been underestimated, considered only to be an undesirable effect, leading in most case to pulp necrosis. In view of recent results the inflammatory process should be re-examined to understand the potentially beneficial effect of this process. These studies will pave the way for a better understanding of the initial molecular and cellular events leading to pulp repair, and the development of ideal materials to promote pulp healing.

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Fig. 1. Pulp capping with Ca(OH)_2 at day 2 (a), day 8 (b), day 15 (c) and day 30 (d). The cavity was prepared on the mesial aspect of the first maxillary molar. During pulp exposure, some dentin fragments were pushed into the pulp. The slight inflammation is resolved at day 15. A reparative dentin bridge starts to be formed at day 8 (b), and becomes thicker at days 15 and 30 (c and d). Pulp remnants and tunnels are seen even at day 30.

Fig. 2. Rat incisor pulp observed with light microscopy. The cell population is dense, heterogeneous, mostly constituted by fibroblast-like cells (pulpoblasts). Some cells are clear (arrow), whereas most others are darker.

Fig. 3.
Pulp cells observed with the transmission electron microscope. Most cells display a fibroblast-like appearance. Here a cell becomes apoptotic (arrow).

Fig. 4. Macrophage (m) in a healthy pulp. Tubulo-vesicular structures are seen, forming phagolysosomes. In the center of the cell lysosomes (ly) are implicated in the degradation and destruction of apoptotic debris and residual extracellular matrix molecules.

Reactionary dentin

Fig. 5.

Reactionary dentin (Rd) separated from normal dentin (d) by a calico-traumatic line (star). This dentin is formed by the odontoblasts (O), secreting collagen in the predentin (Pd). Pulp cells (p) are not implicated in the formation of this physio-pathological dentin.

Fig. 6. Reparative dentin (star) formed 15 days (a) and 30 days (b) after implantation of A-4 by differentiated pulp cells.

Fig. 7.

(a) Immunoperoxidase staining of dendritic cells (brown) in the healthy human dental pulp with an anti-HLA-DR antibody. Sections were slightly counterstained with toluidine blue. V, blood vessel; P, pulp core. (b and c) Immunoperoxidase staining of T-lymphocytes (brown) in healthy and inflamed human dental pulps with an anti-CD3 antibody. In healthy tissue (b), rare T-lymphocytes are scattered within the pulp. In inflamed pulp (c), T-lymphocyte density is highly increased. Sections were slightly counterstained with toluidine blue. P, pulp core; V, blood vessel; Od, odontoblast layer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.” has been incorporated in the caption to Fig. 7)

Fig. 8. Pulp capping with a bone sialoprotein (BSP). Preparation of the cavity and pulp exposure at day 0 (a). Moderate inflammation is seen in the pulp at day 8 after implantation of collagen pellets loaded with BSP (b). The formation of reparative dentin starts around day 15 (c). At day 30, a homogeneous atubular reparative dentin layer (star) closes totally the pulp exposure (d).

Fig. 9.

Pulp cell proliferation is seen at day 8 (a) by PCNA staining in the coronal pulp near the agarose beads (b) used as carrier for A+/-4. In the root, labeling is seen in a sub-odontoblastic border at day 8. At day 15, labeling is seen near the beads, but not at direct contact (see b and d). No labeling is detectable in the root. Cells are grouped around agarose beads loaded with amelogenin isoforms resulting from spliced genes. Masson's trichrome staining in (c), immunostaining for PCNA in (d), showing that proliferating cells are located near the beads. The cells differentiate toward a osteoblast/odontoblast phenotype, as shown by osteopontin (OPN) immunostaining (e); bar = 100 μ m.

Fig. 10.

Effects of pulp capping with A+4 (a) and A-4 (b-d). Whereas A+4 induces mostly the formation of reparative dentin (star), A - 4 firstly stimulate the formation of a reparative dentin bridge (b and d), and afterward stimulate the closure of the lumen of the root canal (c). b = beads; bar = 100 μ m.

Table 1
Intracellular and ECM molecules of the normal and inflammatory pulp and dentin

Normal pulp	Carious teeth/ inflammatory process
Odontoblasts and Hhl's sub-odontoblastic layer Post-mitotic cells - no proliferation - MIB-1 negative negative; nestin, a few bcl2 positive cells (apoptosis); integrins: mature odontoblasts, αvβ5 and β1, new differentiated odontoblasts and TGFβ receptors and BMPR; FGF2, ILGF I and II; CCL2 not expressed by healthy tissues (odonto and pulp) [18]	Enhanced Toll-like receptor (TLR) [TLR1-6 and 9] gene expression (but not TLR7, 8 and 10); TLR2 initiates innate immune response - chemokines - recruits immature dendritic cells; CXCL2 and CXCL10 are increased; re-expression of N-cadherin [19,20,17]
Immunocompetent pulp cells Low proliferation, confined to radicular pulp—a few MIB-1 positive cells; peripheral T-lymphocytes; helper/inducer T cells (CD4+); cytotoxic/suppressor T cells (CD8+); monocytes/macrophages; B-lymphocytes; class II antigen expressing cells (dendritic cells); non-detectable levels of CD44; integrins: αv, β1, β5 and β8, but not β6 [22-25]	PMN-cell infiltration is enhanced by LPS; preferential activation of CD8+ T cells; CCL7, CCL26 and CXCL11 increased by LPS in pulp fibroblasts; high level of CD44 positive cells [17,26-29]
Pulp cells E-selectin, P-selectin, ICAM-1, VCAM-1 and PCAM-1; connexin 43 (intercellular junctions); ALP positive cells; stem cells; STRO-1 positive cells [30]	Alteration of markers in prepared teeth; increased nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) and macrophage NOS [31]
Pulp cytokines IL-2; TGFβ1; BMP-2, -4, -6; BMP receptors: BMPR-I activin receptor-like kinase-1 (ALK-1), ALK-2, BMPR-1A, AKLK-4, ALK-6 (or BMPR-1B); BMPR-II [9]	Activation by LPS and TNF of the nuclear factor kappa B (NF-κB), immunologic transcription factor of MHC such as: IFN-γ, IL-2, IL-4, IL-6 and -10; IL-1α, IL-1β and TNF-α; S100A8, A9 and A13 are enhanced; HGF production stimulated by IL-1α, TNF-α and PGE ₂ ; LPS-induced enhanced VEGF expression; vasoactive intestinal polypeptide-like [32-34]
Dentin and pulp extracellular matrix Dentin: collagen I; siblings (DSPP, DSP;	Caries dentin: gradual degradation of
	Caries pulp: degradation of

Normal pulp	Carious teeth/ inflammatory process
DGP; DPP; DMP-1; osteopontin; BSP; MEPE); non-phosphorylated proteins (NCP); osteocalcin, osteonectin, SLRPs, albumin, $\alpha 2$ HS glycoprotein, growth factors; phospholipids (cells and matrix) [9]	(56%), coll III (41%), coll V, VI, IV, collagen type XVIII α 1; NCP: CS/DS, KS, HSPGs, versican; fibronectin; elastin, osteocalcin; IGF-2, discordin domain tyrosine kinase-2, cyclin-dependent kinase 6; MMP13 (collagenase-3); MMP 2 and MMP3 [9,35]
	collagen and massive rapid degradation of non-collagenous proteins (NCP) by bacteria proteases and MMPs
	collagens and NCP; MMP9

Table 2

Cell density per unit area ($4500 \mu\text{m}^2$) in the central part and sub-odontoblastic outer part of the pulp (mean $\pm$ S.D.) in normal and essential fatty acid deficient (EFAD) rats

Diet	Central pulp	Outer sub-odontoblastic area
Normal/normal	10.80 ± 3.80	11.00 ± 1.40
Deficient/normal	21.35 ± 3.50	18.00 ± 3.05
Normal/deficient	34.70 ± 3.10	22.80 ± 2.30