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Sources of Dentin-Pulp Regeneration Signals and Their Modulation by the Local Microenvironment

Fanny Chmilewsky, PhD,* Charlotte Jeanneau, PhD,* Jacques Dejou, PhD,*[†] and Imad About, PhD*

Abstract

Many aspects of dentin pulp tissue regeneration have been investigated, and it has been shown that dentin pulp has a high regeneration capacity. This seems to be because of the presence of progenitor cells and inductive regeneration signals from different origins. These signals can be liberated after the acidic dissolution of carious dentin as well as from pulp fibroblasts and endothelial cells in cases of traumatic injury. Thus, both carious lesions and pulp cells provide the required mediators for complete dentin-pulp regeneration including reparative dentin secretion, angiogenesis, and innervation. Additionally, all dentin pulp insults including carious "infection," traumatic injuries, application of restorative materials on the injured dentin pulp, and subsequent apoptosis are known activators of the complement system. This activation leads to the production of several biologically active fragments responsible for the vascular modifications and the attraction of immune cells to the inflammatory/injury site. Among these, C5a is involved in the recruitment of pulp progenitor cells, which express the C5a receptor. Thus, in addition to dentin and pulp cells, plasma should be considered as an additional source of regeneration signals. (*J Endod* 2014;40:S19–S25)

Key Words

Complement, odontoblast, progenitor cells, pulp-dentin regeneration

Tissue regeneration strategies require 3 major elements: stem cells, scaffolds, and activation signals. Although many studies were performed to develop a "suitable" scaffold for each particular tissue, numerous investigations were devoted to better understand the stem cells' properties and potential in tissue regeneration both *in vitro* and *in vivo* (1, 2). However, the signals that orchestrate the regeneration processes were not given much attention. Indeed, stem cells are resting cells residing beside differentiated cells within an intact and functional tissue. With few exceptions, the regenerative function of these cells is induced only after tissue injury in cases of trauma or under pathological conditions. Stem cell induction appears as the result of activation signals originating from the modified microenvironment. This pinpoints the fact that understanding what happens in case of pathology/injury is essential in understanding the role of activation signals in the regeneration processes (3).

This is of prime importance in cases of dentin-pulp regeneration, which occurs under carious or traumatic injuries. These events lead to an inflammatory reaction, which is believed to be the initial step of tissue regeneration. Within the tooth, dentin-pulp regeneration can take multiple forms depending on the causative agent and the severity of the tissue alteration. This varies from a "simple" up-regulation of the underlying odontoblast synthetic activity, which leads to regenerating a protective reactionary dentin, to more severe dentin-pulp injuries. These usually imply complete pulp dentin regeneration including not only reparative dentin synthesis but also angiogenesis and innervation. This regeneration seems to be dependent not only on the interaction of the dentin with the pulp but also on the interaction between different pulp cell types (4) and between the progenitor cells and other components of the immune/inflammatory response such as the complement biologically active C5a fragment (5). Thus, dentin-pulp regeneration in case of pulp injury requires the activation and proliferation of progenitor cells as well as their migration and differentiation at the injury site. All these processes are orchestrated by signaling molecules. In this review, we discuss the origin of the regeneration signals under normal and pathological conditions and their modulation by inflammation and restorative procedures.

Pulp Progenitor Cell Differentiation *In Vitro* Is Dependent on the Culture Conditions

It has long been established that dental pulp contains progenitor cells capable of dentin regeneration (6). Although other localizations and niches cannot be excluded, 3 independent investigations using 3 different approaches have clearly identified a specific origin of these cells in the perivascular area (1, 7, 8). When isolated with specific surface markers such as CD34/vascular endothelial growth factor receptor 2 (VEGFR2), these cells can undergo adipogenic, chondrogenic, neuronal, and odontoblastic differentiation depending on the culture conditions (1, 6). Additionally, these progenitor cells are heterogenous and comprise a subpopulation of CD31⁻/CD146⁻, which can undergo endothelial differentiation when cultured with a medium containing basic fibroblast growth factor 2 (FGF-2) and vascular endothelial growth factor (VEGF). This has been shown by the formation of tubular structures on Matrigel (San Jose, CA) and the expression of many endothelial cell markers (1). Taken together, these data clearly indicate that modifying the culture conditions/local environment affects the cell fate/differentiation potential.

The Transplantation Site Affects the Pulp Progenitor's Fate

Similar results indicate that the microenvironment also controls the fate of progenitor cells *in vivo*. This effect is nicely shown through transplantation experiments. When STRO-1–positive pulp progenitor cells were mixed with hydroxyapatite/tricalcium phosphate ceramic powder followed by transplantation into immunocompromised mice, dentin-pulp–like tissue was generated (9). Similarly, when they were transplanted on dentin slices, pulp cells generated tubular reparative dentin on the slice surface (10). Surprisingly, when progenitor cells from exfoliated teeth were transplanted with hydroxyapatite *in vivo*, bone regeneration was obtained (11). This was also best shown in autotransplantation experiments in which progenitor cell transplantation with β -tricalcium phosphate into swine mandibular critical bone defects led to bone regeneration (2). Interestingly, when pulp progenitor cells that express CD34 and VEGFR2 but not CD31 or CD146 were injected into an ischemic site of a mouse hind limb, they led to re-establishment of blood flow and vascularization (1). On the other hand, when the same cells were labeled with fluorescent Dil-acetylated-LDL (Biomedical Technologies Inc, Schiltigheim, France) dye and autotransplanted into amputated pulp of dog teeth in a scaffold of collagen type I and type III, the Dil-labeled cells were observed in the amputated area closely related to newly formed capillaries and expressed several proangiogenic factors implying trophic action on endothelial cells (12). Similarly, when stem cells from exfoliated deciduous teeth were seeded onto tooth slice/scaffolds and implanted subcutaneously into immunodeficient mice, they differentiated into functional odontoblasts and endothelial cells only after the addition of VEGF (13).

These data show clearly that pulp progenitors differentiate into odontoblastic cells when transplanted into the pulp and into other cell types *in vitro* or when transplanted into other sites *in vivo*. This indicates that the regeneration signals affecting progenitor cell differentiation originate from the local environment.

Injuries and Restorative Materials Modify the Dentin-Pulp Microenvironment

Dentin pulp is subject to injuries with various degrees of severity. These can be caused by carious or traumatic injuries and the subsequent application of a restorative material. All these injuries are responsible for dentin-pulp modifications. Indeed, dentin bioactive molecules such as growth factors can be solubilized and liberated from the dentin by acids that can be produced during the carious injury progression because of bacterial metabolism. These factors can also be released from the dentin during the total-etching restorative procedures in which dentin etching does not only remove the smear layer and smear plugs but also results in a superficial demineralization of the underlying dentin (14). Thus, during dentin demineralization that occurs after carious irritation, these signaling molecules can be released from the dentin matrix and diffuse to reach the pulp cells. The secretory activity of the odontoblasts is then stimulated, leading to the production of a reactionary dentin (15–17). This effect has also been shown by the fact that both calcium hydroxide and mineral trioxide aggregate can solubilize transforming growth factor beta 1 (TGF- β 1) from the dentin (18, 19). Similarly, both materials together with Biodentine (Septodont, Saint-Maur-des-Fossés, France) have been shown to induce the secretion of significant quantities of TGF- β 1 from cultured human pulp fibroblasts (20). Given the well-established role of this growth factor in progenitor cell recruitment and odontoblast differentiation, this explains, at least in part, the therapeutic success of these materials in direct pulp capping or in deep cavities. Opposed to these bioactive materials, a decrease in TGF- β 1 secretion was observed after applying the Xeno III self-etching adhe-

sive (Dentsply DeTrey GmbH, Konstanz, Germany). Additionally, it has been reported that resinous monomers reduce the secretion of other growth factors from pulp cells such as FGF-2 and appear to be responsible for the intracellular accumulation of dentin sialoprotein and osteonectin within the endoplasmic reticulum (21). These proteins are involved in extracellular matrix mineralization and need to be secreted to accomplish their function. This accumulation explains why after pulp cells were incubated even with nontoxic concentrations of resinous monomers the mineralization was inhibited (22).

Dentin Acts as a Reservoir of Regeneration Signals

Many growth factors are assumed to play a role in pulp-dentin regeneration (23, 24). Among these factors, VEGF, platelet-derived growth factors, FGF-2, and TGF- β 1 are secreted and sequestered in the dentin (25, 26). These factors can be liberated by the acidic environment at the carious site and diffuse via the dentin tubules to stimulate the underlying odontoblasts to secrete a reactionary dentin locally. Among these factors, TGF- β 1, TGF- β 3, and bone morphogenetic protein 7 have been shown to up-regulate odontoblast secretory activity (25, 27).

On the other hand, the odontoblasts that border the dental pulp act as sensors of external stimuli. These cells have cytoplasmic processes throughout the dentin and lie in close proximity to sensory unmyelinated nerve fibers, which extend from the dental pulp to the inner half of the dentin (28). Emerging evidence supports a role for odontoblasts as sensory cells that may sense external stimuli and transduce the signal to nearby neural cells, which is supported by the close apposition of the odontoblasts to the dentinal nerve terminals (29). Recent data have shown that these cells express transient receptor potential (TRP) ion channels (30). These channels are known for their capacity to directly mediate nociceptive functions because of thermal and chemical stimuli. Odontoblasts express transient receptor potential vanilloide 1 that responds to noxious heat and transient receptor potential cation channel melastin 8 and ankyrin A1 that mediate cool and noxious cold sensations, respectively. In addition, these receptors are sensitive to chemical stimulation (30). Thus, although the dentin pulp is the main target of bacteria/toxins and restorative materials, odontoblasts deposit a protective reactionary dentin because they act as sensor cells that are responsive to dentin-solubilized regeneration signals.

Pulp Cells Secrete Regeneration Signals after Pulp Injury

Severe caries lesions or deep cavity preparations may lead to odontoblast destruction. However, a new generation of odontoblast-like cells is responsible for reparative dentin apposition within the pulp chamber (31). This results in tubular dentin secretion discontinuous from the physiological dentin in terms of tubular structure. The reparative dentin secretion is a complex process requiring the presence of responsive progenitor cells as well as the appropriate signals for their activation and differentiation. Again, growth factors liberated under caries lesions play a role in reparative dentin secretion; TGF- β 1, FGF-2, and BMP-2 and -4 seem to be involved in the proliferation and differentiation of pulp cells (32, 33).

However, after surgical pulp amputation, healing can occur with hard tissue formation in germ-free animals independent of growth factor release from the dentin (34, 35). This is suggestive of the autoreparative pulp potential after traumatic injuries.

Indeed, investigating pulp fibroblasts' and endothelial cells' secretory activity revealed some interesting aspects related to their functions both under normal and pathological conditions. It has been shown that

pulp fibroblasts isolated from human third molars secrete FGF-2, VEGF, and platelet-derived growth factors *in vitro*. When these cells were stimulated by a traumatic injury, their secretion of growth factors significantly increased, and this increase was observed 6 hours after the injury (36). The same results were obtained with endothelial cells, which secrete both VEGF and FGF without any stimulation (4). However, after a physical injury to the endothelial cell layer, a significant increase of the growth factors was observed within hours of injury. Interestingly, this reveals that the injury itself induced a modification of the microenvironment via the secretion of growth factors. In an attempt to understand the consequences of this secretion on pulp regeneration processes, a coculture system allowed the investigation of the consequences of a physical injury on neoangiogenesis *in vitro*. After performing physical injuries to pulp fibroblasts, the culture medium containing the soluble factors was collected and was applied onto endothelial cell cultures. Surprisingly, endothelial cells started to organize into closed “tubular” structures corresponding to neoangiogenesis *in vitro*

(37). In addition to FGF-2 effects on angiogenesis, this factor may act as a mitogen for pulp progenitor cells. It stimulates the proliferation of rat immature dental pulp cells and has an inductive activity on mineralized calcium production in 3-dimensional cultures of dental pulp cells in collagen type I gel. Exogenous TGF-βs, FGFs, and bone morphogenetic proteins have shown their potential in signaling reparative dentinogenesis. Their involvement was investigated after direct application onto the pulp by evaluating pulp-dentin regeneration (24, 25, 38, 39). Moreover, pulp cells from both rats and humans express messenger RNAs and release the corresponding neurotrophic proteins. Although the production of neurotrophic factors by dental pulp cells plays an important role in tooth innervation during development, continued production by mature pulp cells is involved in nerve growth and guidance (40). This has been shown by the fact that explants of young rat trigeminal ganglia extend neurites when cocultivated with pulpal tissue explants, suggesting that pulpal cells stimulate the growth of trigeminal ganglia axons with soluble molecules

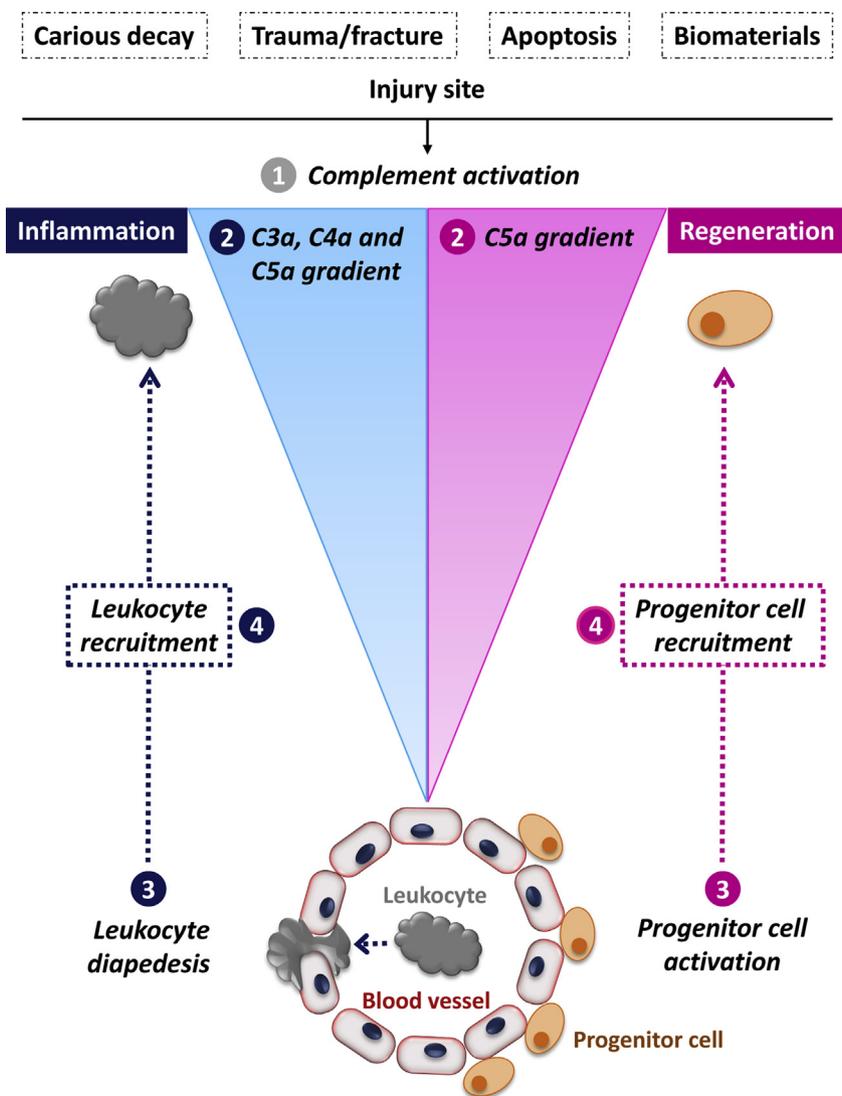


Figure 1. Complement activation initiates inflammation and regeneration simultaneously in dentin pulp. Carious decay, traumatic injuries, apoptosis, and biomaterial application lead to complement activation at the tissue injury site. The soluble fragments C3a, C4a, and C5a produced after complement activation modify the vascular permeability indirectly by inducing basophiles/mastocytes degranulation. This leads to a massive liberation of histamine and serotonin, which increases vascular permeability. They also act on endothelial cell permeability by inducing fiber stress formation, which facilitates leukocyte diapedesis upon contraction. The created gradient of these fragments guides leukocyte migration toward the infected/inflamed tissue. In parallel, C5a activates pulp progenitor cells, and the produced C5a gradient guides their migration toward the injured tissue to initiate the dentin-pulp regeneration process.

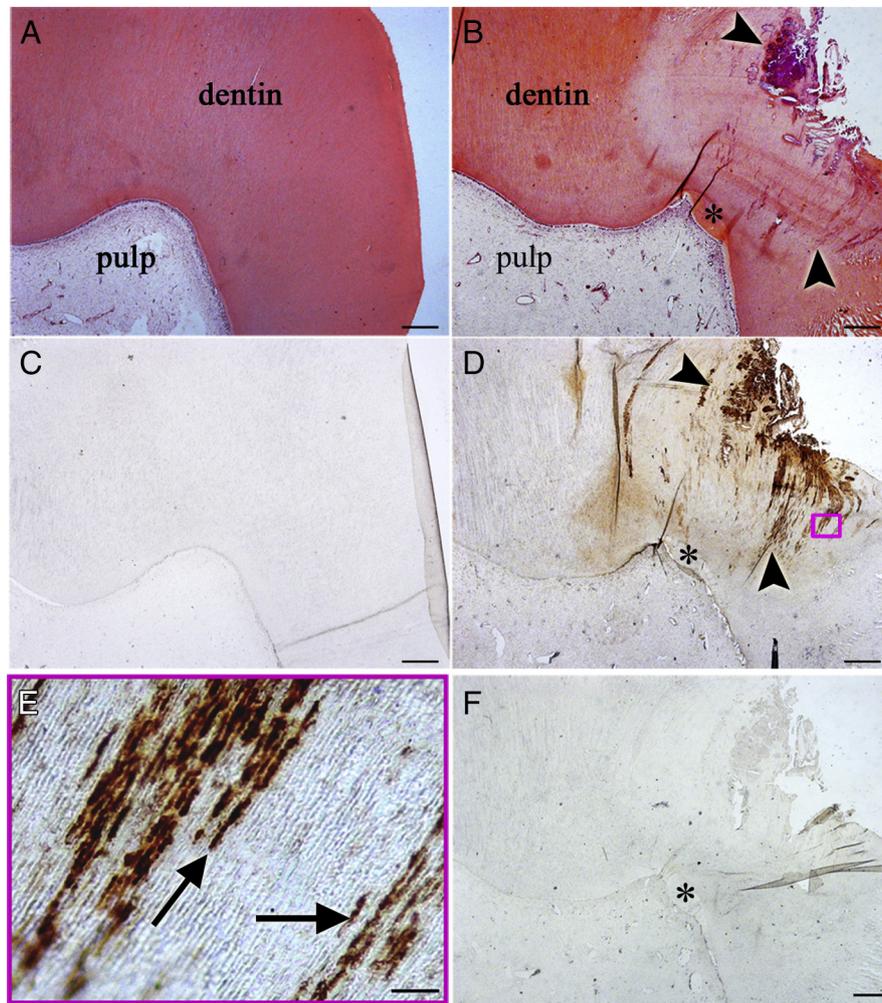


Figure 2. Complement activation *in vivo*. Immunohistochemistry was used to investigate complement activation through the formation of the cytolytic membrane attack complex (C5b–9) in (A and C) intact and (B and D–F) carious teeth. (C) Although C5b–9 complex was not detected in noncarious dentin of the intact tooth, (D) its labeling was intense in the carious tooth, and (E) the labeling correlated to the bacterial location within the dentin tubules. (F) No labeling was observed in the controls. (A and B) Hematoxylin-eosin stain. Arrowheads indicate caries lesions, arrows indicate C5b–9 binding of bacteria in dentin tubules, and the asterisk indicates reparative dentin. Scale bars: A–D and F = 500 μm , E = 20 μm .

(41). This clearly shows that any local tissue modification has direct consequences on dentin-pulp regeneration.

Complement Activation Produces Essential Molecules Initiating Inflammation

The coagulation and complement systems are efficient plasma cascades that play essential roles in inflammation and immune defense mechanisms. The complement system is a major component of the immune system, consisting of a proteolytic cascade of more than 30 plasma and cellular proteins (42). It is activated via 3 principle pathways:

1. The classic pathway is mainly activated by the formation of antigen-antibody complexes but can also be activated by biomaterials (43), apoptotic/necrotic cells, and pathogen-associated molecular patterns.
2. The lectin pathway is known to be activated directly by pathogen surface and especially by carbohydrates and other pathogen-associated molecular patterns.
3. The alternative pathway is constitutively activated by spontaneous hydrolysis but may be triggered directly by foreign surfaces like microorganisms or man-made biomaterials (44).

The complement system activation by either pathway leads to the production of 3 kinds of effectors that allow the complement system to fulfill 3 essential roles regarding immune defense mechanisms. First, C3b and C5b opsonins coat the pathogen surface to enhance its clearance by the phagocytic system. Second, the membrane attack complex (C5b–9) fixation into the lipid membrane of cells and pathogens leads to transmembrane pore formation and cell lysis. Finally, the anaphylatoxins (C3a, C4a, and especially C5a) are important mediators of inflammation inducing the vascular permeability by stimulating histamine and serotonin release from mastocytes and basophils and fiber stress formation in endothelial cells. Moreover, these anaphylatoxins are powerful chemotactic factors of leukocyte recruitment to the complement activation site (Fig. 1).

Complement Activation Is Also Involved in the Regeneration Process

Tissue regeneration and inflammation are tightly linked processes, and tissue regeneration is completely dependent on the inflammatory reaction. This has been clearly shown in the context of myocardial infarction in which the corticoids used to decrease inflammation significantly reduced the tissue regeneration process (45). The complement

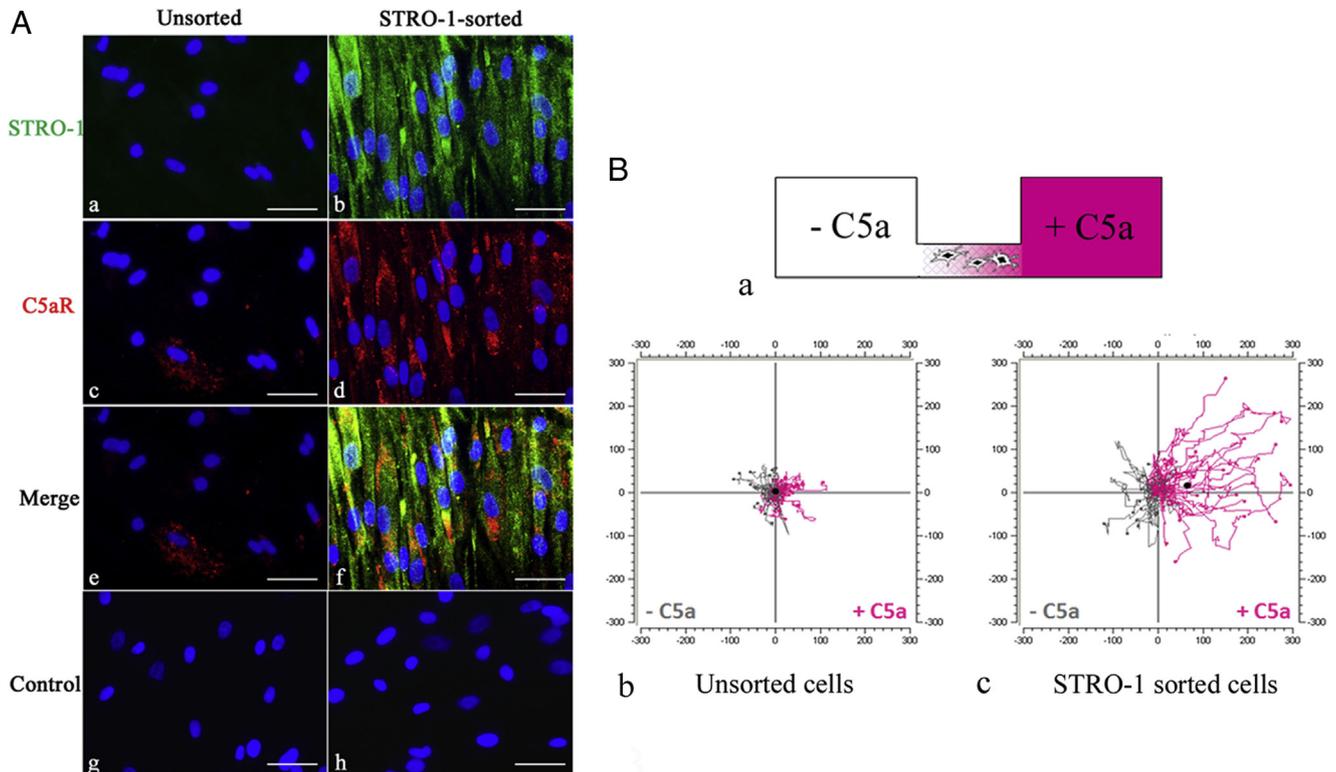


Figure 3. (A) The expression of C5aR by pulp progenitor STRO-1–sorted cells. (a) Although STRO-1 was not expressed by unsorted cells, (b) this surface marker was expressed by all STRO-1–sorted cells. Similarly, (c) although only a few unsorted cells showed the expression of C5aR, (d) all STRO-1–sorted cells appeared to express C5aR. (e) Merged images of STRO-1 and C5aR revealed that some unsorted cells expressed C5aR, (f) whereas STRO-1–sorted cells coexpressed both STRO-1 and C5aR. No immunostaining was observed with the (g) negative unsorted or (h) on STRO-1–sorted cells. Scale bars = 50 μm . (B) C5a induces selective pulp progenitor STRO-1–sorted cell migration. (a) A schematic representation of the migration system used (μ -slide chemotaxis 3-dimensional chamber cross-section). Pulp cells were seeded into a C5a gradient and followed for 48 hours. (b and c) Representative plots of cell migration over a 48-hour period: (b) unsorted cells' trajectory plots and (c) STRO-1–sorted cells' trajectory plots. (b) No directed migration can be measured for unsorted cells, (c) whereas STRO-1–sorted cells migrate toward the C5a gradient.

is known to play a major role in initiating and amplifying the inflammatory reaction. This role is mainly mediated by the C3a and C5a anaphylatoxins. Although the importance of C3a still raises some debates (46), that of C5a is well established. Indeed, C5a has a powerful chemotactic effect on C5aR-expressing cells. Basically, this receptor is mainly expressed by immune cells such as neutrophils, eosinophils, basophils/mastocytes, monocytes/macrophages, dendritic cells, and B and T lymphocytes (47, 48). However, in addition to various immune cells, several nonimmune cells express C5aR including endothelial cells; astrocytes, cells from skin, intestine, and heart; and human mesenchymal stem cells (49). This receptor is also expressed by cells involved in the mineralization process, such as osteoblasts (50). In addition, it has been shown that some stem/progenitor cells express this receptor (51).

C5a and Tissue Regeneration

Several investigations showed the involvement of C5a in tissue regeneration. This has been studied in the liver, which has a very high regeneration capacity (51). Indeed, when C5-deficient mice were subjected to a toxic damage after CCL4 chemokine treatment, their liver could not regenerate. However, liver regeneration was observed after injecting C5 or C5a to the treated animals (52). This role has also been shown in cardiac tissue regeneration in which C5a not only induced the migration of progenitor cells but also their differentiation (53). After human bone fracture, a significant increase of C5aR expres-

sion was observed in mesenchymal progenitors during osteogenic differentiation. This study also showed that C5a induces the migration of mesenchymal progenitor cells as well as osteoblasts showing C5a involvement in fractured bone regeneration (54).

Dental Pulp Is Subject to Various Insults Leading to Complement Activation

Dental pulp is subject to various types of injuries, which lead to an inflammatory reaction. These include carious decay, traumatic injuries/fractures, and the therapeutic application of a restorative material. It has been shown that biomaterials that contain free OH, NH₃, or carboxylic acid groups are involved in the complement activation (55, 56). It has also been reported that Ni(2+) and Co(2+) induce complement activation in plasma samples as shown by the cleavage of complement factor C3 to C3b (55). Additionally, during the management of pulp lesions, odontoblast and pulp cells may undergo apoptosis (56). All these events are well-established complement activation mechanisms (Fig. 1).

Complement Activation and Dentin-Pulp Regeneration

It has been shown that carious injuries, like any bacterial infection, activate complement as revealed by the cytolytic membrane attack complex (C5b–9) formation in human third molars (5). This complex binds onto the bacterial membrane as shown in the

TABLE 1. Sources of Dentin-Pulp Regeneration Signals

Sources	Insults	Mediators	Effects
Dentin/odontoblast (14–18, 23, 24, 37–39)	Cariou	FGF-2, VEGF, PDGF, TGF- β 1...	Progenitor and pulp cell proliferation, angiogenesis, directing progenitor cell migration, odontoblastic differentiation
Pulp fibroblasts (27, 31, 32)	Cariou, trauma/biomaterial	FGF-2, VEGF, PDGF, TGF- β 1	Pulp cell proliferation, angiogenesis
Endothelial cells (4)	Cariou, trauma	FGF-2, VEGF,	Directing progenitor cell migration
Plasma (5)	Cariou, trauma	C5a	

FGF-2, basic fibroblast growth factor 2; PDGF, platelet-derived growth factor; TGF- β 1, transforming growth factor beta 1; VEGF, vascular endothelial growth factor.

tubules of decayed dentin and pulp (Fig. 2A–F). This activation implies the generation of biologically active fragments including a C5a gradient production. In an attempt to understand the potential role of this fragment in dentin-pulp regeneration, a cell migration design was used to study the interactions between pulp progenitor cells and the C5a fragment (5).

Using the magnetic cell sorting approach of human pulp progenitor cells with the mesenchymal STRO-1 stem cell marker, it has been shown that the isolated pulp progenitors also express C5aR as shown by immunofluorescence double staining (Fig. 3A). This has also been shown in human teeth in which both STRO-1 and C5aR were coexpressed by the same cells in the perivascular area, which corresponds to the perivascular niche of pulp progenitor cells (5). When STRO-1–sorted progenitor cells expressing C5aR were subjected to a C5a gradient in a migration chamber, they migrated toward a C5a gradient, whereas the nonsorted cells exhibited random movements (Fig. 3B). The interaction specificity between C5a and STRO-1–sorted progenitor cells has been shown by adding the C5aR antagonist W54011, which significantly inhibited the binding (5).

Thus, after complement activation, a C5a gradient is produced, and this gradient guides the pulp progenitor cells to the complement activation site/inflammatory site (Fig. 1). This clearly shows the involvement of C5a in 1 of the early and essential steps of dentin-pulp regeneration. Indeed, after odontoblast disintegration in deep/severe carious/traumatic injuries, a migration of progenitor cells is required to replace the missing odontoblasts and to secrete a protective reparative dentin. Although the C5a fragment is produced within minutes of complement activation and provides a very quick signal for initiating the inflammatory reaction, it is also involved in the regeneration process via the recruitment of pulp progenitor cells.

These findings do not exclude the involvement of other mechanisms during this critical step. Indeed, dentin and pulp matrix components (57), liposaccharides (58), and stromal cell–derived factor 1 (SDF-1) (59) have been shown to contribute to pulp cell migration. Interestingly, among the cells expressing SDF-1 receptor, a high percentage was STRO-1 positive and had progenitor cell properties such as colony formation, high proliferation rate, a multilineage differentiation potential, and expressed chemokine receptor 4 (CXCR4) (60, 61). Also, it is well established that CXCR4-expressing cells are responsive to SDF-1, which can attract CXCR4-positive cells from their niches in response to stimulations related to tissue damage or infection (60). In line with this, it has been shown that SDF-1 and CXCR4 messenger RNA expression increases in inflamed dental pulps (59).

These data contribute to a better understanding of the mechanisms involved in progenitor cell migration to the infectious/injury site in order to regenerate the damaged dentin. Thus, in addition to the signals produced from the dentin, pulp fibroblasts, and endothelial cells, complement activation and its consequences to pulp regeneration clearly show that the plasma should be considered as an important additional source providing C5a as a new signal involved in dentin-pulp regeneration.

Conclusions

Regeneration signals of the dentin pulp have different sources (Table 1). The soluble extracellular matrix molecules of dentin, pulp fibroblasts, and endothelial cells are involved in complete dentin-pulp regeneration. This review adds to our understanding that complement activation is involved in the regeneration process by inducing progenitor cell recruitment. This regeneration process is rather complex implying many signals and starts very quickly during the inflammatory reaction after any dentin-pulp damage.

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