Dentin Regeneration in vitro: the Pivotal Role of supportive cells

I. About

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ABSTRACT

The elaboration of dentin-pulp engineering strategies requires the investigation of not only progenitor cell potentials but also their interactions with other non-progenitor "supportive" cells. Under severe caries lesions, progenitor cells may be activated by growth factors released after the acidic dissolution of carious dentin. However, dentin regeneration has also been observed after traumatic injuries without any significant dentin dissolution. This raises questions about the origin of signals involved in progenitor cell activation, migration, and differentiation. Study models such as the entire tooth culture and co-cultures of pulp and endothelial cells highlighted the role of interactions between the different pulp cell types and the pivotal role they play in dentin regeneration. Injured pulp fibroblasts secrete growth factors involved in progenitor cell activation and differentiation as well as neoangiogenesis which may pave the pathways for progenitor cell migration. This appears to be the first paper to focus on this very important field in dental pulp biology.

The dental pulp is rather complex and contains a heterogeneous population of fibroblasts (Moule *et al*., 1995) expressing growth factors such as Bone Morphogenetic Proteins (BMPs) and receptor types IA and II (Gu *et al*., 1996). They also express members of the TGF-ß superfamily, receptor types I and II for TGF-ßs. These cells also express the vascular endothelial growth factor (VEGF) in healthy human pulp and in cases of irreversible pulpitis (Artese *et al*., 2002). The expression of this factor in healthy tissues is relevant to physiological angiogenesis, while its expression in pulp cells from irreversible pulpitis is suggestive of a possible role in angiogenesis under pathological conditions. The expression and secretion of VEGF, FGF-2, and PDGF by pulp fibroblasts has also been recently reported (Tran-Hung *et al*., 2008). Their increase after physical injuries is indicative of a possible role in traumatic injuries.

Moreover, pulp cells from both rats and humans express mRNAs and release the corresponding neurotrophic proteins. While the production of neurotrophic factors by dental pulp cells plays an important role in tooth innervation during development, continued production by mature pulp cells seems to be involved in other functions, such as the control of neuronal survival, guidance of nerve processes, and regulation of innervation density (Nosrat *et al*., 2004). It has been shown, for example, that explants of young rat trigeminal ganglia (TG) extend neurites when co-cultivated with pulpal tissue explants, suggesting that pulpal cells stimulate growth of TG axons with soluble molecules (Lillesaar *et al*., 1999). Thus, the pulp-supportive cells (all non-progenitor cells) have to be regarded as acting cells in the sequence of events regulating dentinogenesis, vasculature, and innervation of the dental pulp.

This paper will focus on the pulp progenitors' capacity to regenerate dentin and shed light on the pivotal role of supportive cells in angiogenesis and dentin regeneration.

DENTIN REGENERATION *IN VITRO*

Evidence on the presence of pulp progenitor cells in the pulp able to regenerate dentin came from *in vitro* investigations based mainly on cultures of enzyme-dissociated pulp cells or explants from third molars. The obtained cells showed many characteristics of odontoblasts: When cultured in appropriate conditions, they exhibited morphological polarization and synthesized mineralization nodules. They express molecular markers of odontoblasts, such as dentin sialoprotein (DSP) and nestin. The cells at the appearance site of these nodules express a high level of alkaline phosphatase, indicating a high mineralization potential, and several molecules present in the dentin are expressed in these nodules: collagen I, osteonectin, and DSP (About *et al*., 2000a, 2002a). The appearance of the nodules is accompanied by the expression of functional molecules involved in the mineralization process, such as DSP and connexin 43 (About *et al*., 2002b). Fourier transform infrared microspectroscopic analysis of the mineralized nodules revealed that the spectra obtained from the mineralized matrix were very similar to those of dentin, particularly at the mineralization front from teeth of the same age (About *et al*., 2000a). Pulp cells are responsive to growth factors affecting the odontoblasts. When pulp explants

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are incubated with beads soaked in BMP-4, an up-regulation of nestin can be observed. This is indicative of odontoblastic differentiation, since nestin is considered a specific marker of the human secretory odontoblast (About *et al*., 2000b).

The above-mentioned arguments clearly indicate that pulp cells differentiate *in vitro* into odontoblast-like cells. However, the dentin produced is atubular in nature, and a question has been raised about the possibility of pulp cells producing tubular reparative dentin. Recent works showed that mixing pulp cells with hydroxyapatite/tricalcium phosphate ceramic powder and transplanting them into immunocompromised mice generated a dentin-pulp-like tissue (Gronthos *et al*., 2002). Moreover, after transplantation onto dentin slices, pulp cells generated reparative tubular dentin on the slice surface (Batouli *et al*., 2003).

The fact that isolated cells secrete atubular reparative dentin, while the presence of either a dentin slice or hydroxyapatite/tricalcium phosphate powder leads to the generation of tubular reparative dentin, indicates that the signals required for the production of tubular dentin originate *in vivo* and/or from direct contact with the hydroxyapatite. The origin of the precursor cells giving rise to new odontoblasts and the signaling pathways involved in their differentiation have not been clearly identified and are still a matter of debate.

Progenitor cell population sources within the pulp include cells of the layer of Höhl, fibroblasts, and undifferentiated mesenchymal cells (reviewed in Smith *et al*., 2001; Goldberg and Smith, 2004). However, using progenitor surface markers such as STRO-1 in human (Shi and Gronthos, 2003) or CD34+/VEGFR2+ in porcine dental pulps (Iohara *et al*., 2008), three recent and independent investigations have clearly identified a specific origin of these cells in the perivascular area. Additionally, the activation of these cells at the perivascular area was obtained in a human entire tooth culture after pulpal injury (Téclès *et al*., 2005). Although this perivascular origin seems to be well-established, other localizations and derivations cannot be excluded. Moreover, the recent investigations also add to our knowledge that these progenitor pulp cells are heterogeneous with regard to their differentiation potential hierarchy into different cell types (Iohara *et al*., 2008). Indeed, $CD34^{+}$-VEGFR2/FLK^{+} cells seem to be heterogeneous with regard to surface markers. The $CD31^{+}$-$CD146^{-}$ subpopulation exhibits a multilineage differentiation potential, including chondrogenic, adipogenic, neuronal, and odontoblastic potentials, while the subpopulation $CD31^{-}$-$CD146^{-}$ can additionally give rise to endothelial cells. This was demonstrated *in vitro* by the formation of tubular structures on Matrigel together with expression of endothelial cell markers and functional capacity. It was also shown *in vivo* by injection of these cells into an ischemic site of a mouse hind limb and the re-establishment of blood flow and vascularization after 7 days (Iohara *et al*., 2008).

Analysis of these data has provided valuable information regarding the dentinogenic capacity of pulp progenitor cells. However, we must remember that the maintenance and regulation of normally quiescent stem cells are tightly controlled by the local microenvironment. These cells can be activated by appropriate signals under carious and traumatic injuries. Carious dentin has been suggested as the source of progenitor-activating signals under carious injuries. But no information is so far available on the role of supportive cell signals in reparative dentin secretion under traumatic injuries.

ACID DISSOLUTION OF DENTIN IN CARIOUS TEETH LIBERATES GROWTH FACTORS FOR PROGENITOR CELL ACTIVATION AND DIFFERENTIATION

Mild or moderate caries lesions stimulate the odontoblasts beneath the lesion to secrete reactionary dentin. This dentin is tubular, and the tubules are continuous with those in the physiological dentin (Tziafas *et al*., 2000). The secretion of reactionary dentin is believed to be due to growth factors initially secreted by the odontoblasts and sequestered in the dentin (Finkelman *et al*., 1990; Cassidy *et al*., 1997; Roberts-Clark and Smith, 2000). These factors can be liberated by the acidic environment at the carious site and diffuse to stimulate the underlying odontoblasts to secrete reactionary dentin locally. Among these factors, transforming growth factors TGF-β1 and TGF-β3 and bone morphogenetic protein BMP-7 have been shown to be able to up-regulate odontoblast secretory activity (Sloan and Smith, 1999; Sloan *et al*., 2000). Severe caries lesions or deep-cavity preparations may lead to odontoblast destruction. The damaged dentin is replaced by a reparative dentin secreted by a new generation of odontoblast-like cells (Fitzgerald *et al*., 1990). 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 the induction of their activation and differentiation.

Again, growth factors liberated under caries lesions play a role in reparative dentin secretion: TGFβ1, FGF-2, and BMP-2 and -4 seem to be involved in the proliferation and differentiation of pulp cells, thus providing the chemotactic signals to recruit progenitor pulp cells at the injury site (Shiba *et al*., 1995; Martin, 1997).

TRAUMATIC INJURIES PROVIDE VALUABLE INFORMATION ON THE PIVOTAL ROLE OF SUPPORTIVE CELLS IN DENTIN REGENERATION AND ALTERNATIVELY EXPLAIN PROGENITOR CELL RECRUITMENT AT THE INJURY SITE IN THE ABSENCE OF AN ACIDIC ENVIRONMENT

It has been demonstrated that, after surgical pulp amputation, healing can occur with hard-tissue formation in germ-free animals independent of growth factor release from the dentin (Tsuji *et al*., 1987; Inoue and Shimono, 1992). This is suggestive of the autoreparative pulp potential after traumatic injuries. Similarly, the preparation of pulpal cavities in cultures of entire human immature third molars revealed perivascular activation after 1 day which exhibited a gradient directly related to the injury site. The BrdU labeling was strong around the blood vessels surrounding

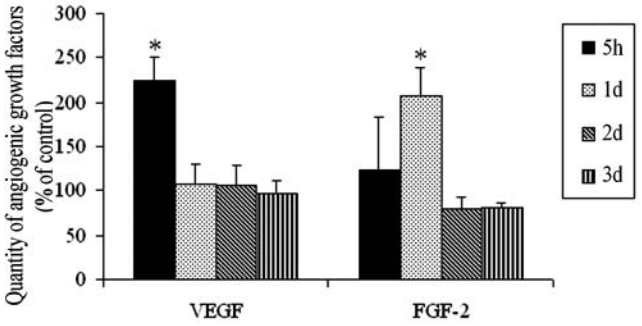

Figure 1. Growth factor secretion increases after endothelial cell injury. Confluent cultures of human umbilical vein endothelial cells (HUVEC) were cultured in EGM medium and used to investigate growth factor secretion. Ten straight lines of 3 cm length *per* dish were created with sterile scalpels to disrupt the cell monolayer. The media obtained after a contact period of 5 hrs, 1, 2, or 3 days with injured or intact cells were used for the quantification of angiogenic factors. Results are expressed as percentage of control (growth factor secretion by intact cells). Statistics were analyzed by the Mann-Whitney test. *$p < 0.05$.

the pulpal cavity and decreased in those away from the cavity. After culture for 2 wks, the labeled cells migrated to the injury site and secreted reparative dentin without any significant acidic dissolution of growth factors such as under caries lesions (Téclès *et al*., 2005, 2008). This raises questions about the source of progenitor activating, migrating, and differentiating signals. The data noted above are highly suggestive of a potent role of pulp-supportive cells in dentin regeneration and complete pulp healing which has not so far been given much consideration.

INTERACTIONS BETWEEN ENDOTHELIAL CELLS AND PULP FIBROBLASTS PLAY A MAJOR ROLE IN BOTH NEOANGIOGENESIS AND PROGENITOR CELL ACTIVATION AND MIGRATION TO THE INJURY SITE

It has been well-established that injured endothelial cells release signaling molecules that initiate the inflammatory reaction and the healing process (Martin, 1997). When physical injuries were performed on these cells in an insert culture system, progenitor pulp cell migration to the injury site was observed. At early time periods, the migrating cells were randomly localized on the endothelial cells. However, at 14 days, most of the migrating cells were strikingly reorganized and aligned along the injury site (Mathieu *et al*., 2005). These new data add to our knowledge that pulp progenitor cells can migrate in response to endothelial cell injury. This may help us to understand the successful tubular dentin production after transplantation of progenitor cells in highly vascularized tissues (Gronthos *et al*., 2000; Braut *et al*., 2003). This may be due to the release of signaling molecules from injured endothelial cells for the recruitment of transplanted cells, together with the fact that pulp cells express growth factors necessary for their own differentiation (Tziafas *et al*., 2000; Tran-Hung *et al*., 2008). Indeed, endothelial cells secrete FGF-2 and VEGF, and this secretion significantly increases shortly after the injury (Fig. 1). While FGF-2 enhances cell proliferation, VEGF exerts a strong chemotactic effect on human pulp cells in a dose-dependent manner (Matsushita *et al*., 2000). Similarly, co-cultures of pulp fibroblasts with endothelial cells on Matrigel extracellular matrix showed that pulp fibroblasts induce the organization of endothelial cells into closed tubular structures corresponding to angiogenesis *in vivo*. This starts very early (24 hrs), and completely closed structures were obtained after 6 days. The process is mediated *via* soluble factors: FGF-2, VEGF, and PDGF (Tran-Hung *et al*., 2006). The secretion of these factors significantly increased after injury to pulp fibroblasts. This is very important for pulp biology function in cases of traumatic injury, since it indicates that these factors can affect progenitor cells at a distance from the injury site. The significance of these results was clearly demonstrated when these growth factors were investigated by simulation of the clinical application of dental materials such as incubation of the pulp fibroblasts with resinous monomers. Incubating the injured fibroblasts with 2-hydroxyethylmethacrylate significantly affected the secretion of these factors. It decreased FGF-2 secretion in a dose-dependent manner and completely suppressed mineralization nodule formation *in vitro,* even when applied at a non-toxic concentration (10 μM) (About *et al*., 2002a; Tran-Hung *et al*., 2008). This shows that they are involved in both neoangiogenesis and reparative dentin formation. Indeed, neoangiogenesis is required not only for bringing nutrients and oxygen during the healing process, but also for paving the pathways for progenitor cell migration to the injury site.

INTERACTIONS BETWEEN PULP-SUPPORTIVE AND PROGENITOR CELLS ARE ESSENTIAL FOR DENTIN REGENERATION

In addition to the effects of secreted growth factors on neoangiogenesis, analysis of recent data demonstrated that the FGF-2 and VEGF secreted from endothelial cells and/or pulp fibroblasts directly affect progenitor cell differentiation. They are involved in the differentiation of the side-population $CD34^{+}$-VEGFR2/FLK^{+} and $CD31^{-}$-$CD146^{-}$ into both odontoblasts and endothelial cells. Similarly, when stem cells from exfoliated deciduous teeth (SHED) 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 (Sakai *et al*., 2010).

Taken together, these results clearly demonstrate that injured pulp-supportive cells such as endothelial cells and pulp fibroblasts liberate growth factors which can directly influence the progenitor cell differentiation into odontoblastic or endothelial cells. The migration of progenitors to the injury site can be speculated to occur *via* newly formed blood vessels resulting from pulp fibroblast-endothelial cell interactions. Thus, the supportive cells play a major role in the dentin-pulp physiology in normal and pathological conditions through the expression of growth factors. Consequently, fibroblasts, endothelial cells, perivascular non-progenitor cells, and all other pulp cells should be regarded not as inert supportive cells, but rather as pivotal supportive cells providing essential signals, with a very significant role not only in dentin regeneration but also in all processes involved in complete pulp healing, including angiogenesis and innervation.

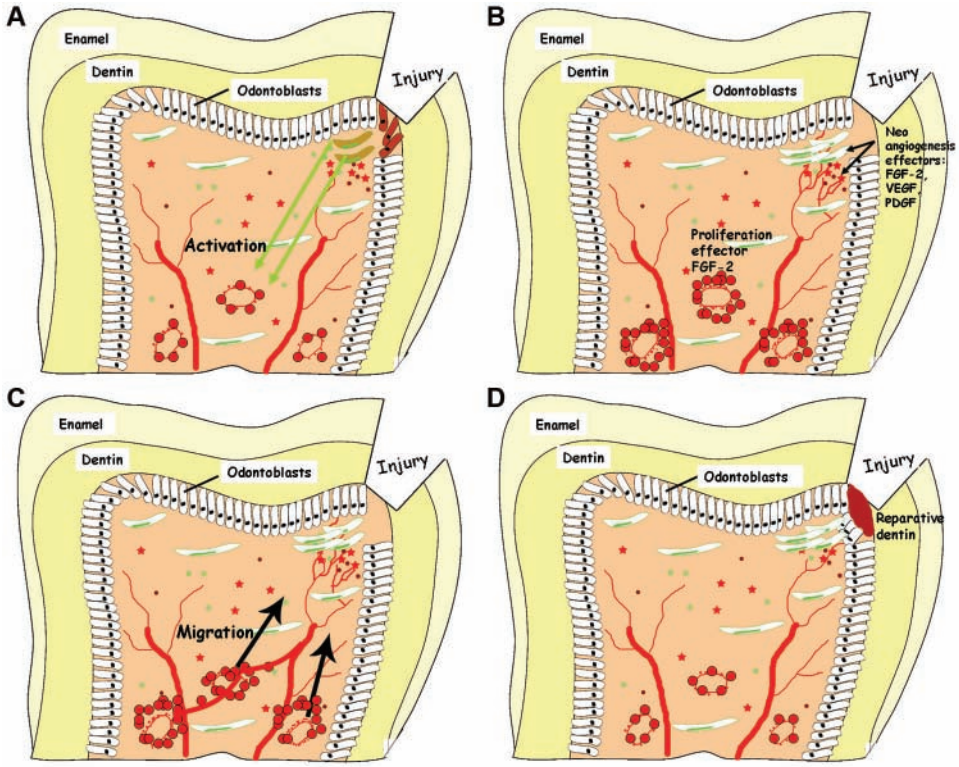

Figure 2. Sequence of events after traumatic injury and possible role of supportive cells. **(A)** Injured pulp fibroblasts secrete FGF-2, VEGF, and PDGF very early (5 hrs). This secretion activates pulp progenitor cells at the perivascular area. **(B)** FGF-2 induces proliferation of progenitor cells one day after its secretion, while neoangiogenesis starts at 24 hrs, and the process is completed after 6 days due to the secretion of FGF-2, VEGF, and PDGF by injured pulp cells. **(C)** Progenitor cell migration to the injury site after 1 to 2 wks (*via* newly formed vessels). **(D)** Regression of newly formed blood vessels and odontoblast differentiation and reparative dentin secretion at the injury site after 2 wks due to FGF-2 and VEGF secretion.

CONCLUSIONS

Based on injury models *in vitro*, this paper highlighted the pivotal role of pulp-supportive cells and clearly showed that these cells are not in the pulp just to "fill a space," but rather play a major role in producing the activation signals of pulp progenitor cells, directing their migration and differentiation at the injury site to elaborate the reparative dentin (Fig. 2). Additionally, the interactions between these cell types merit more in-depth attention, since they play a major role in neoangiogenesis, which is a prerequisite for progenitor cell migration. Analysis of these data will set the basis of future tissue engineering studies, and, to the best of my knowledge, this is the first paper devoted to this very important topic in dental pulp biology.

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