

The MAP Kinase Pathway Is Involved in Odontoblast Stimulation via p38 Phosphorylation

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Abstract

Introduction: We have previously shown that the p38 gene is highly expressed in odontoblasts during active primary dentinogenesis, but is drastically down-regulated as cells become quiescent in secondary dentinogenesis. Based on these observations, we hypothesized that p38 expression might be upregulated, and the protein activated by phosphorylation, when odontoblasts are stimulated such as during tertiary reactionary dentinogenesis. **Methods:** We stimulated immortalized, odontoblast-like MDPC-23 cells, alone or in combination, with heat-inactivated *Streptococcus mutans*, EDTA-extracted dentine matrix proteins (DMPs), or growth factors, including transforming growth factor (TGF)- β 1, tumor necrosis factor- α (TNF- α), and adrenomedullin (ADM). We used ELISA to measure the resulting phosphorylation of the p38 protein, as well as its degree of nuclear translocation. **Results:** Our results suggest that the p38-MAP-Kinase pathway is activated during odontoblast stimulation in tertiary dentinogenesis by both p38 phosphorylation and enhanced nuclear translocation. **Conclusions:** Data indicate that odontoblast behaviour therefore potentially recapitulates that during active primary dentinogenesis. (*J Endod* 2010;36:256–259)

Key Words

Dental pulp, dentinogenesis, MAP kinase, odontoblast

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Dentinogenesis is the process of dentine secretion that continues throughout the life of a tooth. Primary and secondary dentinogenesis, occurring before and after eruption, respectively, are physiologic processes, whereas tertiary dentinogenesis, which can be either reactionary or reparative, occurs in response to injury (1). Primary and secondary dentins are histologically similar and are deposited at 4 and 0.4 $\mu\text{m}/\text{d}$, respectively.

Reactionary dentinogenesis is the secretion of a tertiary dentine matrix by surviving odontoblasts in response to an appropriate stimulus (2). The dentin matrix is permeable by virtue of its tubular structure, and, therefore, after injury to the tooth and/or subsequent restorative procedures, this may allow molecules to diffuse and contact the pulp. Such substances may include bacteria, toxins, and/or dentin matrix proteins (DMPs). Because the pulp is enclosed by a rigid, mineralized tissue shell, dentin matrix degradation by acid bacterial products begins before the disease process reaches the pulp. Notably, growth factors derived from the dentin have been shown to reach and stimulate the odontoblast layer, inducing new dentine secretion in those areas of the dentin-pulp complex that are in direct tubular connection with the traumatic agent (3).

It has been suggested that, during tooth repair and tertiary reactionary dentinogenesis, the healing process recapitulates developmental events (4). Transforming growth factor- β 1 (TGF- β 1) has been shown to play an important role in tooth development (5, 6), particularly in odontoblast differentiation (7, 8). Transforming Growth Factor β 1 (TGF- β 1) is also sequestered within the dentin matrix (9) and may be released during carious disease or by restorative agents commonly used in dentistry that dissolve mineralized components, such as EDTA, calcium hydroxide (10), or mineral trioxide aggregate (11). Notably, TGF- β 1 has also been shown *in vivo* and *in vitro* to stimulate odontoblast behavior (12–14).

It would be clinically valuable to be able to activate quiescent odontoblasts for dentine repair, and, thus, it is important to understand the regulatory control of cellular and molecular signaling in these cells. However, only limited information currently exists on these molecular events that occur during reactionary dentinogenesis. In a previous study, we provided evidence that changes in the secretory activity of odontoblasts reflect differential transcriptional control and that, therefore, the transcriptome of the odontoblast evolves as the cell matures (15, 16). Among the dataset of genes whose expression changed during odontoblast maturation, we identified the *p38* transcript to be abundantly expressed in odontoblasts during primary dentinogenesis while being significantly down-regulated in secondary dentinogenesis. Based on these observations, we hypothesized that p38 signaling might be involved in regulating odontoblast activity, and the present study aimed to determine whether the p38 protein is up-regulated and phosphorylated upon odontoblast stimulation.

Materials and Methods

Isolation of Dentin Matrix Proteins

Dentin matrix proteins were isolated as previously described (10). Briefly, dentin matrix components were extracted from powdered dentin using 10% EDTA (pH = 7.2) supplemented with the protease inhibitors n-ethylmaleimide (10 mmol/L) (Sigma, Dorset, UK) and phenyl-methyl-sulphonyl fluoride (5 mmol/L) (Sigma). Extractions were performed with agitation over 14 days at 4°C and with the extraction solution changed daily. Extracts were transferred to dialysis tubing (Scientific Laboratory Supplies, Nottingham, UK) and dialyzed exhaustively for 10 days against repeated

changes of distilled water. Dialyzed extracts were lyophilized by using a Modulyo freeze dryer (Edwards, UK). The EDTA dentin matrix extracts were stored at -20°C before use.

Cell Culture

Mouse odontoblast-like cells (MDPC-23) (17) were seeded and cultured in 96-well plates (Appleton-Woods, Birmingham, UK) at a density of 25,000 cells per well in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin (Sigma), and 200 mmol/L glutamine (Sigma). Cells were cultured in a humidified incubator in a 5% carbon dioxide atmosphere at 37°C for 24 hours before stimulation.

Cell Stimulation

Cells were stimulated for 15 minutes, 1 hour, 3 hours, and 24 hours with tumor necrosis factor α (TNF- α) (100 ng/mL; Cellomics, Berks, UK), rh-TGF β 1 carrier-free (0.2 ng/mL; R&D Systems, UK), EDTA-extracted DMPs (1 $\mu\text{g}/\text{mL}$), or adrenomedullin (10^{-11} mol/L, Sigma). As controls, cells were stimulated for 30 minutes before TGF- β 1 stimulation with anti-TGF- β 1 antibody (10 $\mu\text{g}/\text{mL}$, R&D Systems); anisomycin (40 ng/mL, Sigma), which induces p38 phosphorylation; the p38 inhibitor SB203580 (0.6 $\mu\text{g}/\text{mL}$, Sigma); or *Streptococcus mutans* (SM) (10^9 bacteria/mL based on chains of 10 cocci). Alternatively, control cells were prestimulated with combinations of TGF- β 1 + TNF- α , adrenomedullin (ADM) + SM, TGF- β 1 + SM, or DMPs + SM (all at concentrations previously described).

P38 Phosphorylation Enzyme-linked Immunosorbent Assay

Phosphorylated p38 expression was measured by using a cell-based, p38-mitogen-protein activated kinase (MAPK) enzyme-linked immunosorbent assay kit (Raybiotech, Norcross, GA). After stimulation, cells were fixed for 20 minutes in 10% formalin at room temperature (RT) with shaking and then treated with quenching buffer for 20 minutes at RT. After incubation with blocking solution, cells were incubated with the primary antiphospho-p38 antibody (Thr180/Tyr182) for 2 hours at

RT and then with horseradish peroxidase (HRP)-conjugated antimouse immunoglobulin G antibody for 1 hour. TMP-One-Step reagent (Raybiotech, Norcross, GA) was then added to each well, and the plates were incubated for 30 minutes at RT. After stopping the reaction, the absorbance of each well was read immediately at 450 nm with a spectrophotometer (Universal Plate Reader ELx800; Bio-Tek Instruments Inc, East Sussex, UK). All experiments were performed in triplicate.

p38 Phosphorylation and Translocation Analysis

To determine whether the various treatments activated p38, we examined both the phosphorylation and nuclear translocation of this protein using high-content analysis. After stimulation, cells were fixed in 1% formaldehyde in phosphate-buffered saline for 1 hour. The fixed plates were processed at Imagen Biotech (Manchester, UK) by permeabilization and stained with antiphospho-p38 antibody (Cat# K0100041; Thermo Fisher). Plates were analyzed on an Arrayscan (Cellomics, Thermofisher, Leicestershire, UK) using the compartmental analysis algorithm (Fig. 1).

Results

p38 Phosphorylation Enzyme-linked Immunosorbent Assay

Anisomycin stimulates the MAPK pathway by activating p38 (18). In this study, phosphorylated protein was expressed at a basal level in untreated MDPC-23 cells and was further activated in cells after anisomycin stimulation, with a peak level reached at 15 minutes (Fig. 2). The expression levels then progressively decreased, returning to basal levels by 24 hours. Exposure of cells to the p38 inhibitor SB203580 caused no detectable reduction in p38 protein expression.

Similar expression profiles were observed after cells were stimulated with TGF- β 1, ADM, or TNF- α . The incubation of cells with the anti-TGF- β 1 receptor alone reduced protein expression levels compared with the control. The preincubation of cells with antibody before incubation with rh-TGF- β 1 led to no increase in protein activation, confirming previous findings that the activation of the p38-MAPK pathway depends on TGF- β 1 receptor binding.

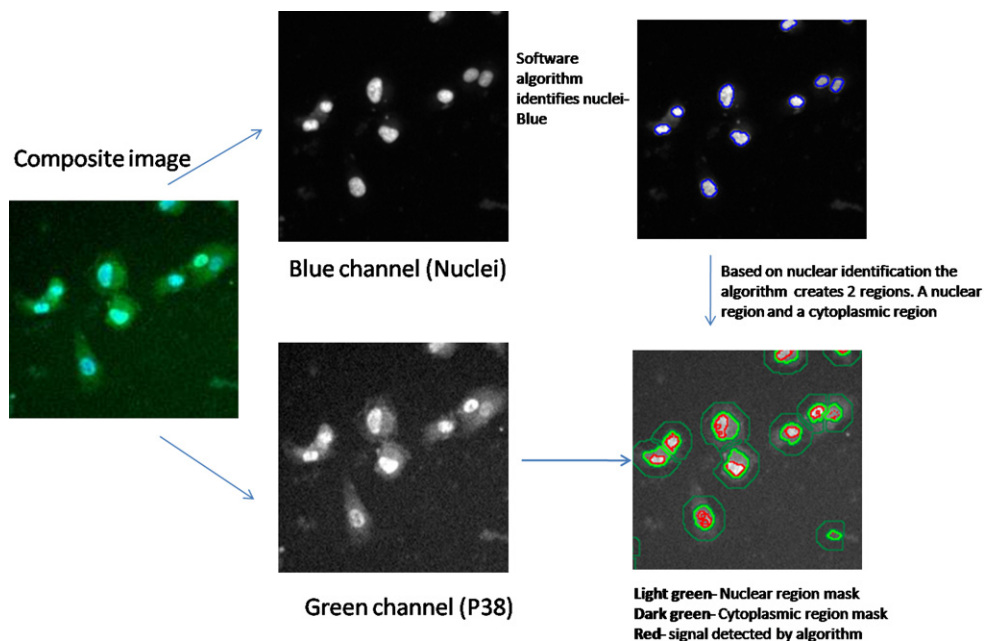


Figure 1. Principles of the cell analysis performed using an Arrayscan (Cellomics, Thermofisher, USA) and the compartmental analysis algorithm.

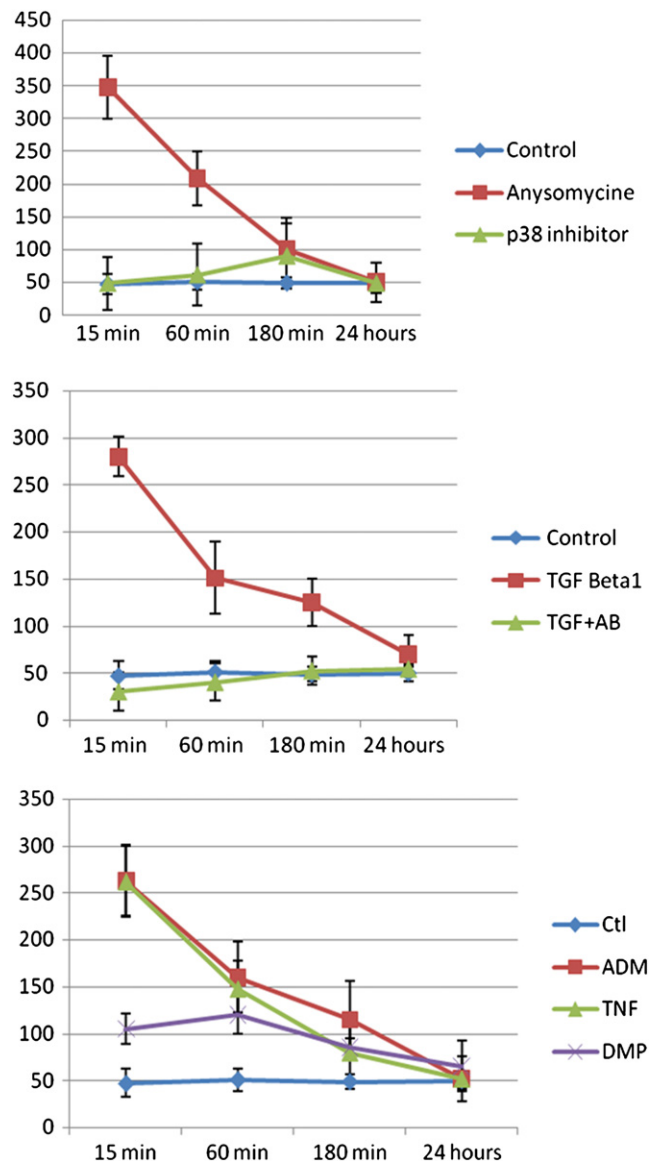


Figure 2. Phosphorylated p38 protein expression at a basal level in untreated MDPC-23 cells and after cell treatment with anisomycin, SB203580 (p38 phosphorylation inhibitor), TGFβ1, TGFβ1+antiTGFβ1 antibody, ADM, TNFα, Dentine Matrix Proteins for 15, 60, 180 mins and 24 hours. Bar = Standard deviation. *: statistically significant difference from control.

Significantly, DMP stimulation also induced p38 phosphorylation, albeit with a different temporal profile. At 15 minutes, protein was expressed at levels only two-fold above control, whereas expression was increased almost five-fold with ADM or TNF-α, and nearly 6.5-fold with TGF-β1 exposure. However, peak expression levels, which approached those achieved with other molecules, were reached only after 1 hour. At 3 hours, the protein expression level after DMP stimulation was higher than with other treatments, and at 24 hours phospho-p38 remained 25% overexpressed compared to that of the control, at which time expression levels in cells treated with other molecules had returned to baseline.

Segmental Analysis of Cytoplasmic and Nuclear Expression of P-p38

All molecules induced an activation and translocation of the protein but at different levels. With TNF-α, phospho-p38 appeared nearly completely translocated; however, after stimulation with SM,

only half the activated protein was present in the nucleus. Notably, TGF-β1 was most effective for both p38 activation and protein translocation. DMPs and ADM exhibited similar effects, inducing activation of the protein and resulting in 75% of the protein being translocated after 1 hour of exposure.

When the stimulatory molecules were applied in combination, to potentially better mimic the *in vivo* situation, protein activation was increased. TNF-α together with TGF-β1 increased P-p38 expression by 55% compared with TNF-α exposure alone and by 15% compared with TGF-β1 exposure alone. Growth factors applied in combination with SM also led to a synergistic effect compared with SM or growth factors stimulation alone (Fig. 3).

Discussion

The MAPK/ERK pathway is a signal transduction pathway that couples intracellular responses to the binding of growth factors to cell surface receptors and has been implicated in odontoblast differentiation (19). The role of TGF-β1 in odontoblast differentiation during primary development and in tertiary dentinogenesis is well established (4, 20–22), with several recent publications indicating that TGF-β1 regulates the MAPK pathway (23, 24). Because similarities have been described between primary development and the healing process (4, 25), we wished to investigate the role of the MAPK pathway in these processes and to determine whether odontoblasts might be stimulated in a model of reactivation of dentine secretion during reactionary dentinogenesis.

The stimulation of odontoblast-like cells with several growth factors, such as an *in vitro* model of tertiary dentinogenesis, or with SM, such as an *in vitro* model of caries, caused an increase in p38 phosphorylation and its nuclear translocation. This enhancement of translocation is significant because apart from the HSP27 pathway most components of the p-38 MAPK pathway reside in the nucleus, where p38 acts as a transcription factor.

TNF-α, ADM, and TGF-β1 showed similar patterns of p38 activation, whereas DMPs appeared to induce a different pattern of p38 activation. The cocktail of growth factors in DMPs appeared to have a weaker but more long-lasting effect on cells than did the growth factors or bacterial stimulants alone. The effect of DMPs on p38 protein persisted past the point where the effect of growth factors alone had culminated. It is likely that this response profile better mirrors the events occurring in caries disease in which DMP components are released in combination. In view of the fact that tissue regeneration is a balance between inflammation and repair, the comparatively weaker effect of DMPs may serve the biological role of protecting the dental pulp by preventing irreversible inflammation.

Bacterial contamination of the pulp is usually described as the most important factor in pulp disease. In our experiments, when cells were stimulated with SM, only half of the activated protein was translocated. Based on this observation, we hypothesize that bacteria might stimulate the cytoplasmic pathway better, whereas growth factors might directly induce transcription. It should be noted that bacteria alone are not able to induce odontoblast activation but that they exhibit a synergistic effect when combined with growth factors. It is thus critical, when evaluating the healing and toxic effects of various agents, that they be tested both alone and in combination. Further study is needed to determine the effect of a range of concentrations of DMPs and other agents to allow the development of a more accurate and reliable *in vitro* model of the carious disease process.

Notably, our results are in agreement with the differential phospho-p38 expression that has been observed during tertiary dentinogenesis. As has been suggested by several authors, this finding may indicate that the behavior of odontoblasts during tertiary dentinogenesis

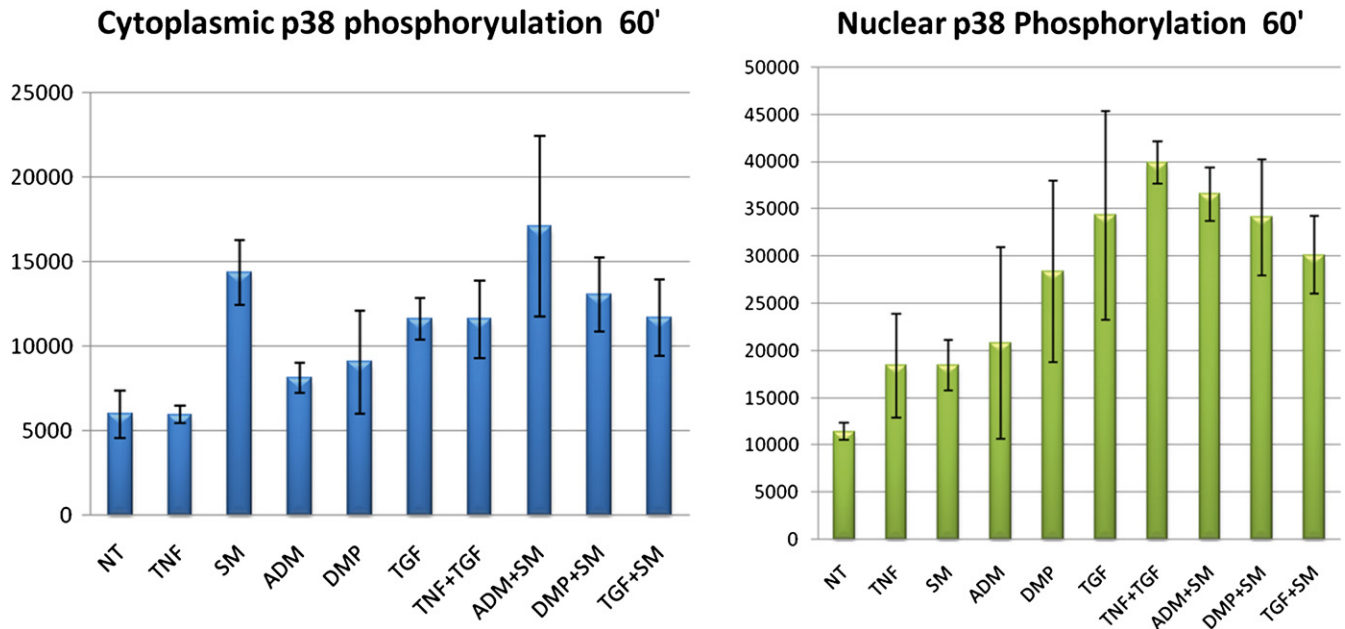


Figure 3. Phosphorylated p38 protein expression in cytoplasmic and nuclear localizations of MDPC-23 cells, after stimulation with TNF- α , *Streptococcus mutans* (SM), ADM, DMPs, TGF- β 1, TNF α +TGF- β 1, ADM+SM, DMP+SM, TGF- β 1+SM for 60 minutes. (DMP, dentine matrix protein preparation; SM, *Streptococcus mutans*). Bar = standard deviation.

recapitulates the development of the primary cells (4, 25). Understanding cellular behavior in response to caries disease may allow the development of new approaches in regenerative dentistry that use biological and pharmacologic control of cell behavior, much as p38 phosphorylation is currently exploited as a target of anti-inflammatory drugs. Better understanding of the regulation of the p38 pathway during tertiary dentinogenesis might also allow the development of new therapies, in particular for novel pharmaceuticals that facilitate bonding systems to better control the healing process and enhance the secretory activity of odontoblasts.

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