Proliferation of dental pulp fibroblasts in response to thrombin involves mitogen-activated protein kinase signalling

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Abstract

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Aim To examine the involvement of mitogen-activated protein kinases (MAPK) signalling on thrombin-stimulated human dental pulp fibroblasts (DPF).

Methodology Dental pulp fibroblasts were isolated from dental pulp connective tissue of third molars and expanded *in vitro*. Expression of thrombin receptors was analysed by RT-PCR, and cell proliferation was measured by ^{3}[H]-thymidine incorporation assay. Phosphorylation levels of MAPK were determined by Western blot analysis, and alkaline phosphatase activity was measured to serve as a marker for odontogenic differentiation. Statistical analysis was performed by Student's *t*-test.

Results Dental pulp fibroblasts express the thrombin receptors protease-activated receptor-1 (PAR-1), PAR-3 and PAR-4. Measurement of ^{3}[H]-thymidine incorporation revealed a dose-dependent increase of DNA synthesis in response to thrombin treatment. The thrombin-induced mitogenic activity was decreased by the extracellular signal-regulated protein kinase (ERK) signalling inhibitor PD98059 ($P < 0.05$), and by SB203580 ($P < 0.05$), a p38 MAPK inhibitor. Western blot analysis demonstrated increased phosphorylation of ERK in DPF following stimulation with thrombin, while p38 MAPK and c-Jun NH_2-terminal kinase (JNK) were not activated. Alkaline phosphatase activity of DPF remained unchanged upon incubation with thrombin.

Conclusions These results suggest that signalling via MAPK mediates the mitogenic activity of thrombin on DPF and may thus play a role during the early stages of pulp repair.

Keywords: dental pulp fibroblasts, dental pulp stem cells, ERK, p38 MAPK, protease activated receptor, thrombin.

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Introduction

Dentine has the potential of self-repair as mesenchymal progenitor cells within the pulp tissue can proliferate and differentiate into dentine-producing odontoblasts (Gronthos *et al.* 2000, Tziafas *et al.* 2000, Smith & Lesot 2001, Gronthos *et al.* 2002). This multistep cascade of cellular events is coordinated by local growth factors that are produced in response to injury. Isolated dental pulp fibroblasts (DPF) can serve as an *in vitro* model to determine the effects of growth factors possibly involved in pulp repair (Ohbayashi *et al.* 1999, Matsushita *et al.* 2000).

Thrombin is a serine protease that is activated upon tissue damage during the ongoing coagulation cascade (for review, see Dery *et al.* 1998, Coughlin 2000, Macfarlane *et al.* 2001). Thrombin exerts cellular effects via G-protein-coupled protease-activated receptors (PARs), in addition to its role in the formation of a blood clot. PARs are activated by proteolytic cleavage of a specific site in the extracellular domain, which, in turn, acts as a ligand. Among the four known PAR receptors, thrombin activates PAR-1, PAR-3 and PAR-4, but not PAR-2, which is cleaved by trypsin, tryptase and the coagulation factors VIIa and Xa (Dery *et al.* 1998, Coughlin

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2000, Macfarlane *et al.* 2001). Thrombin is mitogenic for a large number of cell types that are involved in tissue repair, e.g. smooth muscle cells (Madamanchi *et al.* 2001), endothelial cells (Lafay *et al.* 1998), osteoblasts (Abraham & Mackie 1999) and DPF, which express PAR-1 (Sundqvist *et al.* 1995, Chang *et al.* 1998). The question of whether or not DPF express PAR-3 and PAR-4 remains unanswered. Moreover, no information exists on whether thrombin promotes the differentiation of DPF into the odontogenic lineage.

PARs can signal via mitogen-activated protein kinases (MAPK), a family of serine/threonine kinases that link receptor activation in the cell membrane with gene expression in the nucleus ((Coughlin 2000); for review on MAPK, see Chang & Karin 2001, Pearson *et al.* 2001, Johnson & Lapadat 2002)). MAPK family members are extracellular signal-regulated protein kinases (ERK), c-Jun NH_2-terminal kinase (JNK) and p38 MAPK. MAPK are activated by phosphorylation of an upstream kinase, e.g. Raf-mitogen-activated protein kinase (MEK), which phosphorylates ERK1 and ERK2. Activated ERK1 and ERK2, in turn, phosphorylate transcription factors that induce the expression of target genes. ERK signalling predominantly mediates the effects of mitogens, whereas JNK and p38 MAPK are activated by inflammatory cytokines. MEK activity is inhibited by the pharmacological compound PD98059, whereas SB203580 blocks p38 MAPK (Pearson *et al.* 2001). Thrombin activates MAPK signalling, e.g. in astrocytes (Wang *et al.* 2002), smooth muscle cells (Tokunou *et al.* 2001) and T-cells (Maulon *et al.* 2001), but no information is available whether DPF behave similarly.

The purpose of this study was to determine the expression pattern of thrombin receptors in DPF and to investigate whether the effects of thrombin on proliferation and odontogenic differentiation involve MAPK signalling.

Materials and methods

Isolation and cultivation of dental pulp fibroblasts

Third molars were collected from three adults (age: 23, 27 and 45 years) who had given their written consent. Dental pulp connective tissue was separated from the root and digested in 3 mg mL^{-1} collagenase type I and 4 mg mL^{-1} dispase (Roche, Basel, Switzerland) for 3 h at 37 °C as described recently by Gronthos *et al.* (2000). The released cells were seeded into T-75 culture flasks at a density of 5×10^3 cm^{-2}. DPF were cultured in alpha modification of Eagle's medium (αMEM; Gibco/Life Technologies, Grand Island, NY, USA) supplemented with 10% foetal calf serum (FCS; Gibco), antibiotics and antimycotics (Gibco). Cells were grown in a humidified atmosphere at 37 °C in 5% CO_2. Experiments were performed with DPF not exceeding 10 passages.

RT-PCR analysis of PAR-1, PAR-3 and PAR-4

Total RNA was extracted from 1×10^6 DPF with TRIzol reagent (Gibco). Aliquots of 1 μg total RNA were primed by random hexamers and converted into cDNA using a kit following the instructions of the manufacturer (MBI Fermentas, St Leon, Rot, Germany). The following primers were used for RT-PCR analysis as described recently by Hamilton *et al.* (2001): PAR-1 (forward) 5′-TGTGAACTGATCATGTTTATG-3′, PAR-1 (reverse) 5′-TTCGTAAGATAAGAGATATGT-3′; PAR-3 (forward) 5′-GAAAGCCCTCATCTTTGCAG-3′, PAR-3 (reverse) 5′-AGGTGAAAGGATGGACGATG-3′; PAR-4 (forward) 5′-GGCAACCTCTATGGTGCCTA-3′, PAR-4 (reverse) 5′-TTCGACCCAGTACAGCCTTC-3′. Reactions were performed in a Perkin Elmer GeneAmp PCR System 2400 with an initial denaturation at 94 °C for 2 min, followed by 36 cycles of 94 °C/30 s, 55 °C/30 s, 72 °C/60 s and an additional 7-min polymerization step at 72 °C. Amplified PCR products were subjected to 1.5% agarose gels and photographed with a digital scanning system (Bio-Rad Laboratories, Hercules, CA, USA).

^{3}[H]-thymidine incorporation

Dental pulp fibroblasts were seeded at 5×10^4 cells cm^{-2} in 96-well plates (Packard, Meriden, CT, USA) in αMEM containing 10% FCS and antibiotics. The following day, DPF were stimulated with 0.1, 0.3, 1, 3 and 10 U thrombin mL^{-1} serum-free medium, i.e. αMEM supplemented with 2.5 μg mL^{-1} insulin–transferrin–selenium (Boehringer Mannheim, Mannheim, Germany) and antibiotics. To investigate the role of ERK and p38 MAPK in thrombin signalling, PD98059 at 30 μM and SB203580 at 10 μM were added to thrombin at 3 U mL^{-1} serum-free medium. Cells were pulse-labelled with ^{3}[H]-thymidine (0.5 μCi $well^{-1}$; Amersham Pharmacia Biotech, Little Chalfont, UK) for the last 6 h of culture and subjected to liquid scintillation counting (Packard).

Alkaline phosphatase activity and histochemistry

Dental pulp fibroblasts were plated at 1×10^4 cells cm^{-2} in 24-well plates (Corning) in αMEM containing 10%

FCS and antibiotics. The next day, the medium was supplemented with 3 U thrombin mL^{-1}. Cells were grown for 6 days with a medium change on day 3. Alkaline phosphatase activity was determined in cell lysates containing 0.2% Triton X-100 (Sigma, St Louis, MO, USA). Aliquots of each sample were incubated with alkaline phosphatase substrate (20 mM diethanolamine, 150 mM NaCl, 2 mM $MgCl_2$ and 5 μM *p*-nitrophenylphosphate) for 5–30 min at room temperature. Total cellular protein was determined by BCA-kit as recommended by the manufacturer (Pierce Chemical Co., Rockford, IL, USA). Alkaline phosphatase activity, which is defined as the release of 1 nmol *p*-nitrophenol per minute per microgram of total cellular protein, was measured. For histochemical staining of alkaline phosphatase, cells were fixed with 10% neutral buffered formalin for 15 min at room temperature and incubated with a substrate solution containing 4 mg of naphthol AS-TR phosphate in 0.15 mL of *N,N′*-dimethylformamide and 12 mg of fast blue BB salt (all Sigma) in 15 mL of 100 mM Tris–HCl (pH 9.6). After rinsing with distilled water, the cultures were photographed.

Western blot analysis

Subconfluent DPF were grown in serum-free medium for 24 h followed by stimulation with 0.1, 0.3, 1 and 3 U thrombin mL^{-1} for 5 min (dose response), or 5, 15, 45 and 135 min with 3 U thrombin mL^{-1} (time response). Cells were lysed with radioimmuno precipitation assay (RIPA) buffer (50 mmol Tris–HCl (pH 7.5), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) containing phosphatase and protease inhibitors (1 mM dithiothreitol, 50 mM β-glycerophosphate, 20 μg mL^{-1} aprotinin, 1 μg mL^{-1} leupeptin, 1 mM sodium orthovanadate and 400 μM phenylmethylsulfonylfluoride). Cell debris was eliminated by a 10-min centrifugation step at 10 000 ***g***. The protein concentration of the cell lysates was determined using the BCA-kit (Pierce). Equal amounts of cell extracts were separated by 10% SDS–PAGE and transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech). Membranes were blocked in 5% nonfat, dried milk in TBS-T (50 mM Tris (pH 7.5), 0.15 M NaCl, 0.1% Tween 20) and incubated with antiphospho ERK1/2 (clone E-4, 1 : 1000), anti-ERK1 (clone K-23, 1 : 1000), antiphospho p38 MAPK (clone D-8, 1 : 1000) and anti-p38 MAPK (clone C-20, 1 : 1000), antiphospho, JNK (clone G-7, 1 : 1000) and anti JNK antibodies (clone C-17, 1 : 1000; all antibodies Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. The first antibody was detected with the appropriate secondary antibody (Dako, Glostrup, Denmark) using the ECL method (Amersham Pharmacia Biotech). Radiographs were photographed with a digital scanning system.

Statistical analysis

Statistical analysis was performed by Student's *t*-test at a significance level of $P < 0.05$.

Results

DPF express PAR-1, PAR-3 and PAR-4

To determine the expression pattern of thrombin receptors, RT-PCR analysis of 1 μg aliquots of total RNA extracted from 1×10^6 DPF was performed. Amplification signals for PAR-1, PAR-3 and PAR-4 were observed when cDNA from DPF was subjected to PCR amplification (Fig. 1). For control, PCR analysis using total RNA without prior reverse transcription was performed and showed no specific bands (data not shown).

Effects of thrombin on ^{3}[H]-thymidine incorporation and alkaline phosphatase activity

Incubation of 5×10^4 DPF cm^{-2} from all three donors with thrombin resulted in a dose-dependent increase of ^{3}[H]-thymidine incorporation (Fig. 2a). Thrombin, at a concentration of 10 U mL^{-1}, was most potent in this regard, and increased the rate of DNA synthesis by 8–24-fold when compared to unstimulated controls. In the presence of thrombin at 3 U mL^{-1}, inhibition of

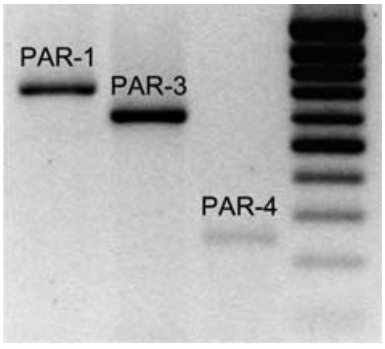

Figure 1 Expression profile of thrombin receptors PAR-1, PAR-3 and PAR-4 by DPF. RT-PCR analysis of PAR expression profile in DPF. RNA was extracted, reverse-transcribed and amplified with specific primers for PARs. Amplification products were separated on 1.5% agarose gels, stained with ethidium bromide, and were photographed. Lane 1, PAR-1; Lane 2, PAR-3; Lane 3, PAR-4; Lane 4, 100-bp ladder, respectively.

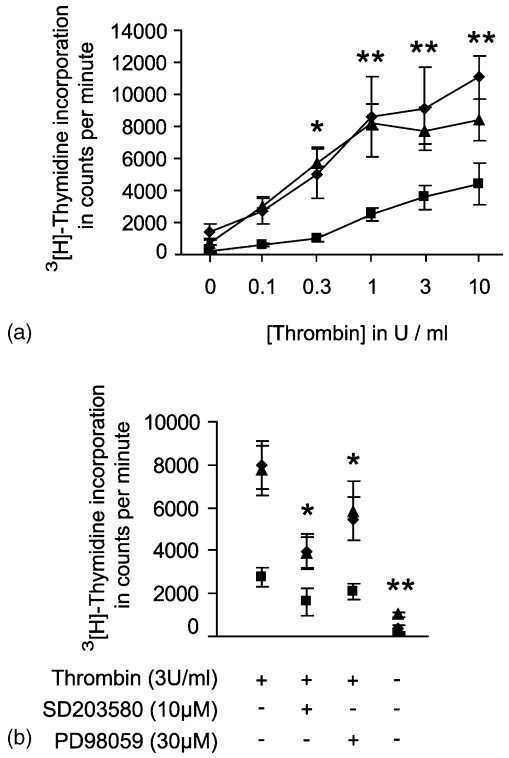

Figure 2 Effects of thrombin on ^{3}[H]-thymidine incorporation of DPF. (a) Dose-response curve of the indicated concentrations of thrombin on ^{3}[H]-thymidine incorporation by three individual preparations of DPF expressed as triangle, squares and rhombus. $^*P < 0.05$ and $^{**}P < 0.01$ versus unstimulated cells. (b) Effects of inhibitors of MAPK signalling SB203580 at 10 μM and PD98059 at 30 μM ^{3}[H]-thymidine incorporation by DPF. DPF were stimulated for 24 h as described in the 'Materials and methods' section and pulse-labelled with ^{3}[H]-thymidine for the last 6 h of culture. ^{3}[H]-thymidine incorporation was determined by liquid scintillation counting. Values are given as counts per minute per well. Results are shown as mean ± SD. $^*P < 0.05$ and $^{**}P < 0.01$ versus cells stimulated with thrombin at 3 U mL^{-1} without inhibitors.

ERK signalling by PD98059 at 30 μM and p38 MAPK signalling by SB203580 at 10 μM decreased the [^{3}H]-thymidine incorporation rate by 27 ± 9% ($P < 0.05$) and 47 ± 11% ($P < 0.05$), respectively (Fig. 2b). Alkaline phosphatase activity was not affected when DPF were cultured in the presence of thrombin at a concentration of 3 U mL^{-1} for 6 days (Fig. 3).

Effects of thrombin on MAPK phosphorylation

Dental pulp fibroblasts, which were grown for 24 h in serum-free medium, were treated with thrombin at a concentration of 3 U mL^{-1} for time periods ranging from 5 to 135 min. At all time points, phosphorylation signal intensity of the two isoforms, ERK1 and ERK2, was increased. Dose-response experiments were performed at the 5-min time-point, where strong phosphorylation of ERK1 and ERK2 was detectable. Western blot analysis of DPF revealed increased phosphorylation of ERK in response to thrombin at 0.1–3 U mL^{-1} when compared to unstimulated controls. Phosphorylation of the other two investigated MAPK, p38 MAPK and JNK, was detectable in DPF, but there was no further increase in the thrombin group at any of the time points investigated (Fig. 4).

Discussion

The present study demonstrates that DPF express the whole panel of currently known PARs that are activated by the protease thrombin. In this *in vitro* model, thrombin was mitogenic for DPF, but did not increase alkaline phosphatase activity, a marker for odontogenic differentiation. Treatment of DPF with thrombin increased phosphorylation of ERK, and the mitogenic effect of thrombin was reduced by inhibitors of ERK and p38 MAPK signalling. These data suggest that thrombin is mitogenic for DPF and exerts its effects involving ERK and p38 MAPK signalling.

Injury of the dental pulp can result in the formation of thrombin. The protease is required for the formation of a blood clot, which provides a provisorial extracellular matrix for the ongoing repair processes, and it can directly activate cells via cleavage of an extracellular domain of the receptor. Evidence has been provided that DPF express PAR-1, PAR-3 and PAR-4, suggesting that these cells are potential targets to become activated by thrombin. This observation is in agreement with studies where the expression of PAR-1 by DPF was reported (Chang *et al.* 1998). Among the three investigated thrombin receptors, PAR-4 showed a low expression level in cultured DPF. High expression levels of PAR-4 by DPF cultured under *in vitro* conditions were, however, not required to mediate the mitogenic effects of thrombin. The observation of this study and those of others suggest that thrombin can activate the mitogenic expansion of repair cells after pulp injury (Sundqvist *et al.* 1995, Chang *et al.* 1999).

This study investigated whether the mitogenic effects of thrombin involve MAPK signalling. PD98059 was used to inhibit ERK signalling. PD98059 is a specific inhibitor of MEK that does not inhibit p38 MAPK and JNK activity. The inhibition of ERK signalling decreased

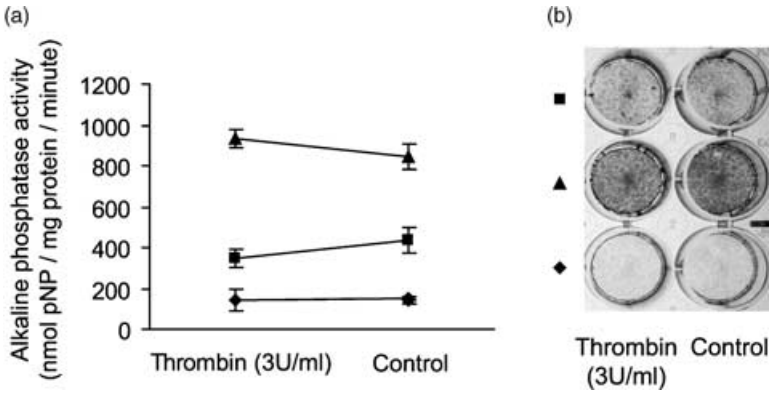

Figure 3 Effects of thrombin on alkaline phosphatase activity of DPF. (a) Alkaline phosphatase activity of DPF in response to thrombin. DPF were stimulated for 6 days with thrombin at 3 U mL^{-1}. Enzymatic activity was determined in cell lysates and normalized to total cellular protein. Results are shown as mean ± SD. (b) Histological staining of alkaline phosphatase activity in DPF.

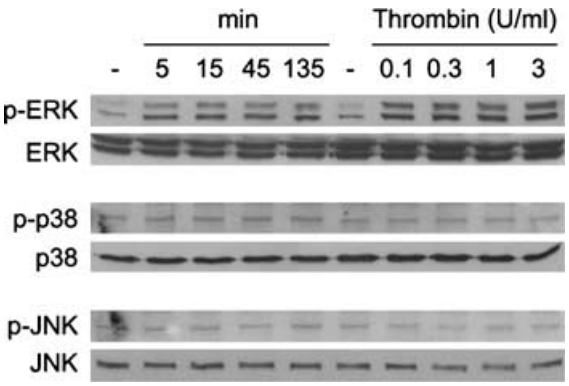

Figure 4 Effects of thrombin on MAPK phosphorylation in DPF. Serum-starved DPF were exposed to thrombin at 3 U mL^{-1} for 5, 15, 45 and 135 min. In parallel cultures, DPF were exposed to thrombin at 3, 1, 0.3 and 0.1 U mL^{-1} for 5 min. Cell lysates were separated on a 10% SDS–PAGE and blotted onto a nitrocellulose membrane. Phosphorylated and unphosphorylated MAPK were detected by Western blot analysis.

thrombin-induced proliferation of DPF. PD98059 did not completely suppress thrombin-induced DNA synthesis, suggesting that there might be other pathways leading to cell proliferation. To determine whether the mitogenic effects of thrombin require p38 MAPK signalling, SB203580, a selective p38 MAPK inhibitor, was added to DPF during the stimulation period. The presence of SB203580 decreased the mitogenic activity of DPF in response to thrombin. These observations indicate that, besides ERK, p38 MAPK activity is also required to mediate the mitogenic activity of thrombin on DPF.

To further investigate the mechanism of thrombin-induced, MAPK-dependent cell proliferation, a Western blot analysis of the phosphorylated proteins was performed. Phosphorylation of ERK was increased, whereas no increase was observed in p38 MAPK and JNK. Western blot analysis, however, showed that p38 MAPK and JNK are phosphoprylated and are thus active in DPF. Although thrombin does not increase p38 MAPK phosphorylation, it requires p38 MAPK activity to achieve a maximum mitogenic response in DPF. No conclusions can be drawn about the involvement of JNK in thrombin signalling as no specific kinase inhibitors are currently available.

Besides proliferation, pulp repair requires the differentiation of DPF towards the odontogenic lineage (Smith & Lesot 2001). The potential of DPF to form dentine-like tissue was confirmed in an *in vivo* model (Gronthos *et al.* 2000, Gronthos *et al.* 2002). In the current study, alkaline phosphatase activity, a commonly used odontogenic differentiation marker, was not increased in DPF that were cultured in the presence of thrombin. These data suggest that thrombin is not a key regulator of odontogenic differentiation, at least in this *in vitro* setting. In agreement with these findings, alkaline phosphatase activity was not increased in osteoblastic cells in a similar cell culture model (Abraham & Mackie 1999). These findings do exclude the possibility that thrombin can enhance the effects of other growth factors that stimulate the odontogenic differentiation of DPF.

Conclusions

The present study showed that thrombin is a mitogenic factor for dental pulp fibroblasts and exerts its function via ERK and p38 MAPK signalling. These results suggest a role of MAPK signalling during the early stages of pulp repair.

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