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Plasmin is involved in inflammation via protease-activated receptor-1 activation in human dental pulp

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ABSTRACT

Plasmin is a proteolytic enzyme produced from plasminogen by plasminogen activators. We investigated the function of plasmin in human dental pulp fibroblast-like cells. Plasmin induced an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in a concentration-dependent manner. Expression of mRNA for protease-activated receptor-1 (PAR-1) was detected, and the PAR-1 activating peptide SFLLRN induced an increase in $[Ca^{2+}]_i$ in the cells. The plasmin-induced increase in $[Ca^{2+}]_i$ was inhibited in the presence of the PAR-1 antagonist SCH79797. Plasmin stimulated the expression of interleukin-8 (IL-8) mRNA and prostaglandin E_2 release, which are involved in inflammation. These effects of plasmin on expression of IL-8 mRNA and prostaglandin E_2 release were inhibited in the presence of the PAR-1 antagonist SCH79797. These results suggest that plasmin activates PAR-1 and is involved in inflammation in human dental pulp.

1. Introduction

Plasmin is a proteolytic enzyme produced from plasminogen by plasminogen activator (PA). It is well known that plasmin digests fibrin threads and other substances in blood, such as fibrinogen, Factor V and prothrombin, causing blood hypocoagulability. Moreover, plasmin is considered to be involved in wound healing, tissue remodeling, angiogenesis and cell proliferation, because plasmin is capable of activating latent matrix metalloproteases for matrix degradation [1,2]. Furthermore, plasmin has been demonstrated to activate cell signaling and functions. In platelets, plasmin activates phospholipase C and protein kinase C, thus stimulating Ca^{2+} mobilization and protein phosphorylation [3]. In peripheral monocytes, plasmin has been shown to be a potent and specific chemoattractant acting via a cyclic GMP-dependent pathway [4]. In endothelial cells, plasmin stimulates arachidonate release [5].

Protease-activated receptors (PARs) are a family of G-protein-coupled seven-transmembrane-domain receptors that include four members: PAR-1, PAR-2, PAR-3 and PAR-4 [6,7]. PAR-1, PAR-3 and PAR-4 are activated by thrombin, whereas PAR-2 is known to be activated by trypsin, tryptase and coagulation factors VIIa and Xa, but not by thrombin. These peptidases activate PAR via cleavage of the extracellular N-terminal domain, which then enables the new N-terminus to interact distally within the same molecule to activate G-protein-coupled signal transduction pathways [6,7]. PARs are widely distributed, especially throughout the alimentary system, and play various physiological roles such as modulation of salivary, gastric or pancreatic glandular exocrine

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secretion, gastrointestinal smooth muscle motility, gastric mucosal cytoprotection, and suppression of visceral pain [8–11]. In addition, PAR activation has been suggested to induce inflammatory responses. In human respiratory epithelial cells, activation of PAR-1, PAR-2 and PAR-4 stimulates release of interleukin (IL)-6, IL-8 and prostaglandin E_2, a proinflammatory cytokine, a proinflammatory chemokine and an encosanoid involved in inflammation, respectively [12]. In human gingival fibroblasts, PAR-1 stimulates IL-6 production [13].

Dental pulp is a tissue of mesenchymal origin and consists of an odontoblast layer adjacent to the dentin and immunocompetent connective tissue with nerves and vascular elements. Mechanical, chemical and microbial irritation of the dental pulp can provoke pain and an inflammatory response. Pulpal disease is associated with tissue degradation. Elastinolytic and collagenolytic activities have been reported to be increased in diseased human dental pulp with suppurative pulpitis and necrosis [14]. Cytokines have been demonstrated to stimulate the production of elevated levels of matrix metalloproteinase [15]. These observations indicate that inflammation processes in dental pulp resemble those in other tissues.

As is the case for inflammation in other tissues [1,2], it has been considered that the PA/plasmin system plays an important role in pulpitis, inducing further pulpal disease, because proinflammatory cytokines such as TNF-α and IL-1α stimulate tissue-type PA (tPA) synthesis and release in human dental pulp cells [16,17]. Recently, we have also reported that IL-1β stimulates the expression of mRNA for urokinase-type PA (uPA) and release of uPA in human dental pulp cells [18]. However, the function of plasmin is not well understood. In the present study, we demonstrated that plasmin activates PAR-1 and consequently stimulates an inflammatory response in human dental pulp cells.

2. Materials and methods

2.1. Materials

Fetal calf serum (FCS), α-minimal essential medium (α-MEM), fungizone and trypsin were obtained from GIBCO BRL Life Technologies (Tokyo, Japan). Penicillin G and kanamycin were from Meiji Seika (Tokyo, Japan). Fura-2/AM was purchased from Dojindo Labs (Tokyo, Japan). Human plasmin (recombinant, >95% pure) was purchased from Calbiochem (Darmstadt, Germany). The PAR-agonist peptides SFLLRN, SLIGKV, TFRGAP, GYPGQV were obtained from Bachem AG (Bubendorf, Switzerland). N3-Cyclopropyl-7-{[4-(1-methylethyl)phenyl]methyl}- 7H-pyrrolo[3,2-f]qinazoline-1,3-diamine (SCH79797) was purchased from Tocris Bioscience (Ellisville, MI). Human α-thrombin was obtained from Haematologic Technologies Inc. (Essex Junction, VT). A prostaglandin E_2 enzyme immunoassay (EIA) kit was from Cayman Chemical Co. (Ann Arbor, MI).

2.2. Cell culture

Human dental pulp (fibroblast-like) cells were obtained from the extracted third molars under aseptic conditions from patients aged 20 years during orthodontic treatment. The patients gave informed consent before providing the samples. After the dental pulp had been extracted, the tissue was minced, placed on a 35-mm tissue culture dish, and covered with a sterilized glass coverslip. α-MEM supplemented with 10% FCS and antibiotics (20 U/ml penicillin G, 100 μg/ml kanamycin, 250 ng/ml fungizone) was used for culture. Once cell growth from the explants had reached confluence, the cells were detached with 0.05% trypsin in phosphate-buffered saline and subcultured in culture flasks. For the experiments, the cells from passages 6–9 were plated at 2×10^5 cells/ml medium. This study was approved by the ethics committee of Nihon University School of Dentistry at Matsudo (No. EC02-078).

2.3. Measurement of intracellular Ca^{2+} concentration

Human dental pulp cells (1×10^5) were cultured on thin, circular, 13.5-mm glass plates in α-MEM containing 10% FCS. Confluent cells were preincubated with 2 μM fura-2/AM in α-MEM for 30 min at 37 °C. After loading with fura-2/AM, the glass plates with the attached cells were washed twice and put into quartz cuvettes containing Krebs–Ringer–Hepes solution [120 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.96 mM NaH_2PO_4, 0.2% glucose, 0.1% bovine serum albumin, 1 mM $CaCl_2$, and 20 mM Hepes (pH 7.4)] for determination of intracellular Ca^{2+}. The fluorescence of the fura-2-loaded cells was measured with a CAF-110 spectrofluorometer (Nihon Bunkou, Japan) with excitation at 340 nm and 380 nm and emission at 500 nm. $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence intensities [19].

2.4. RT-PCR

Human dental pulp cells (1×10^6) were cultured in tissue culture dishes (10 cm) in α-MEM containing 10% FCS. When the cells were confluent, they were incubated in α-MEM containing 1% FCS for 24 h. When the effect of plasmin on IL-8 mRNA expression was examined, the cells were further incubated in 1%FCS α-MEM for 1 h with or without plasmin. Total cellular RNA was extracted from the cells using an RNeasy mini kit (QIAGEN®). The procedure for isolation of RNA was in accordance with the protocol provided with the RNeasy mini kit. cDNA synthesis and amplification by RT-PCR were conducted using a One Step RT-PCR kit. For the PCR mixture, RNA (100 ng) and oligonucleotide primers (500 nM) were used. The PCR primers for PAR-1, PAR-2, PAR-3, PAR-4, IL-8 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed with reference to the reported sequences, as shown in Table 1 [20,21]. The GeneAmp PCR system 9600 (PerkinElmer) was programmed for cDNA synthesis, and the procedure involved pre-denaturation for 30 min at 95 °C, followed by 22 thermal cycles of denaturation for 30 s at 94 °C, primer annealing for 30 s at 55 °C, chain elongation for 30 s at 72 °C, and final extension for 10 min at 72 °C. The PCR fragments were electrophoresed on 2.0% agarose gels and subsequently stained with ethidium bromide.

2.5. Determination of prostaglandin E_2

Cells (5×10^4) were plated in 24-well plates in α-MEM containing 10% FCS. The confluent-stage cells were incubated in α-MEM containing 1% FCS for 24 h, and were stimulated

Table 1 – Primers for RT-PCR

Gene name	Primer	Product size (bp)
PAR-1	Forward 5′-CTAGGTTGGTAGAGTTTAGCCC-3′	514
	Reverse 5′-TGATCAGTTCACAGCCAATCCC-3′	
PAR-2	Forward 5′-AATGCTGCGATCTTCTGCCATG-3′	430
	Reverse 5′-CCTACTGTGCAATTCCCATCTG-3′	
PAR-3	Forward 5′-CGTCCATCCTTTCACCTACCCG-3′	580
	Reverse 5′-TCTCTGTGATGGCTGTCCTTGT-3′	
PAR-4	Forward 5′-TCTATGGTGCCTACGTGCCCA-3′	381
	Reverse 5′-TTATGAGGACACCACCCACACTG-3′	
IL-8	Forward 5′-ATGACTTCCAAGCTGGCCGTGGCT-3′	294
	Reverse 5′-TCTCAGCCCTCTTCAAAAACTTCTCGAG-3′	
GAPDH	Forward 5′-ATCACCATCTTCCAGGAG -3′	318
	Reverse 5′-ATGGACTGTGGTCATGAG-3′	

with plasmin or SFLLRN for 10 min. The prostaglandin E_2 concentration in the culture medium was measured using an EIA kit according to the manufacturer's instructions.

2.6. Statistical analysis

Results are presented as means ± S.E. for the number of experiments indicated. Tukey test was performed for statistical analysis.

3. Results

3.1. Plasmin-induced Ca^{2+} mobilization

Plasmin has been reported to induce Ca^{2+} mobilization in platelets [3,22] and brain capillary endothelial cells [23]. Therefore, we first examined whether plasmin induces Ca^{2+} mobilization in human dental pulp cells. As Fig. 1A shows, plasmin (200 nM) induced an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in fura-2-loaded human dental pulp cells. $[Ca^{2+}]_i$ started to increase at 30 s after stimulation with plasmin, gradually reached a peak level at 90 s, and then decreased to a steady level. Fig. 1B summarizes the peak levels in $[Ca^{2+}]_i$ induced by various concentrations of plasmin. The increase in $[Ca^{2+}]_i$ induced by plasmin (10–200 nM) in human dental pulp cells was concentration dependent.

3.2. Effect of PAR-activating peptides on Ca^{2+} mobilization in human dental pulp cells

It has been reported that the plasmin-induced increase in $[Ca^{2+}]_i$ is caused by activation of PARs [22,23], and that human dental pulp cells express PARs [24,25]. Therefore, we next examined the effects of PAR-activating peptides on Ca^{2+} mobilization in human dental pulp cells. It is known that 4 subtypes of PAR exist: PAR-1, PAR-2, PAR-3 and PAR-4 [6,7]. When the fura-2-loaded cells were stimulated with PAR-activating peptides, SFLLRN (100 μM), which activates PAR-1, clearly induced an increase in $[Ca^{2+}]_i$, whereas SLIGKV (100 μM), TFRGAP (100 μM) and GYPGQV (100 μM), which activate PAR-2, PAR-3 and PAR-4, respectively, had no or a weaker effect on $[Ca^{2+}]_i$, as shown in Fig. 2A. Fig. 2B summarizes the concentration-response curves of the peak $[Ca^{2+}]i$ induced by the PAR-activating peptides. SFLLRN (1–100 μM) caused a concentration-dependent increase in $[Ca^{2+}]_i$, and the maximum response was obtained at 100 μM, whereas the peptides activating PAR-2, PAR-3 and PAR-4 failed to induce a clear increase in $[Ca^{2+}]_i$. These results suggest that human dental pulp cells express PAR-1.

3.3. RT-PCR analysis of PAR subtypes in human dental pulp cells

To confirm the expression of mRNA for PARs in human dental pulp cells, RT-PCR analysis was performed using the primers

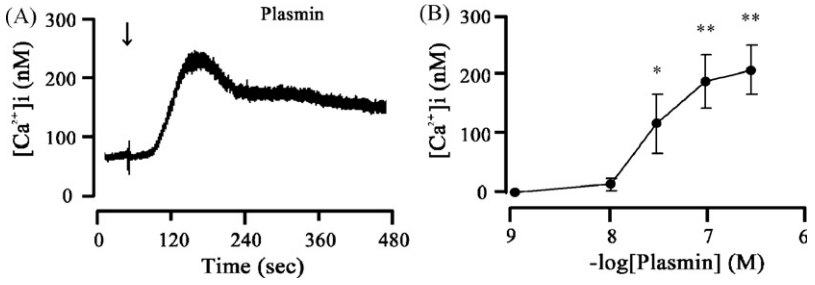

Fig. 1 – Plasmin-induced increase in $[Ca^{2+}]_i$ in human dental pulp cells. (A) Ca^{2+} mobilization in fura-2-loaded human dental pulp cells stimulated with 200 nM plasmin. Plasmin was added to the medium at the time indicated by the arrow. This result is representative of 4 independent experiments. (B) Concentration-dependent effect of plasmin. Fura-2-loaded human dental pulp cells were stimulated with various concentrations of plasmin. The control levels were subtracted from the peak levels induced by plasmin. Values are means ± S.E.M. for 3 independent experiments. *P < 0.05; **P < 0.01.

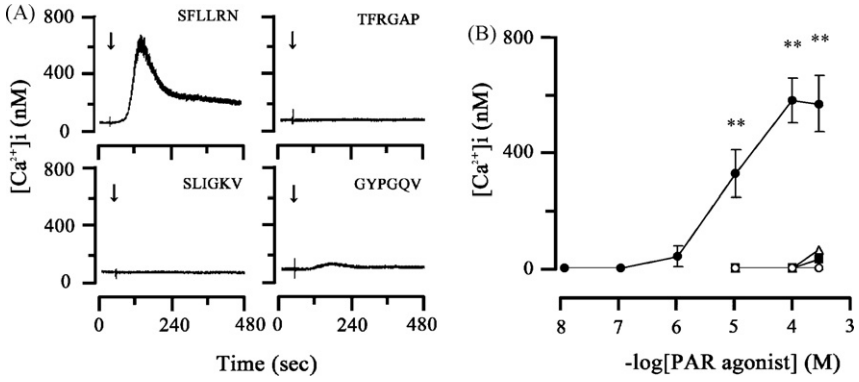

Fig. 2 – Effects of the PAR-activating peptides on $[Ca^{2+}]_i$. (A) Ca^{2+} mobilization induced by PAR-activating peptides in fura-2-loaded human dental pulp cells. SFLLRN (100 μM), SLIGKV (100 μM), TFRGAP (100 μM) or GYPGQV (100 μM), PAR-1, PAR-2, PAR-3 and PAR-4 activating peptides, respectively, were added to the medium at the time indicated by the arrow. These results are representative of 3–6 independent experiments. (B) Concentration-dependent effects of PAR-activating peptides. Fura-2-loaded human dental pulp cells were stimulated with various concentrations of PAR-activating peptides, SFLLRN (closed circle), SLIGKV (closed squire), TFRGAP (open circle) and GYPGQV (open triangle). The control levels were subtracted from the peak levels induced by the peptides. Values are means ± S.E.M. for 3 independent experiments. $^{**}P < 0.01$.

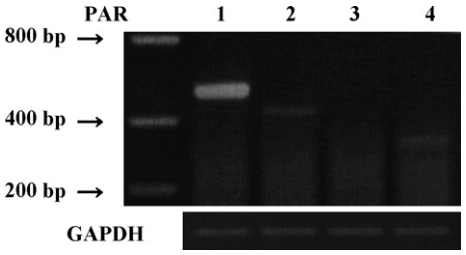

Fig. 3 – Expression of PAR mRNAs in human dental pulp cells. RT-PCR was performed using the primers specific to each type of PAR, as described in Section 2. The PCR products were subjected to electrophoresis through a 2% agarose gel and subsequently stained with ethidium bromide. The lower panel shows the levels of GAPDH mRNA as a control. These results are representative of 3 independent experiments.

for each subtype of PAR. As shown in Fig. 3, when the specific primers for PAR-1 mRNA were used for amplification, a single band with the predicted size (514 bp) was detected at high strength. Bands of the PCR product were also detected using the primers for PAR-2 and PAR-4 mRNAs, but these were much weaker than the band of PAR-1 mRNA. No PCR product was found for PAR-3 mRNA. These results suggest that PAR-1 is the dominant PAR subtype functioning in human dental pulp cells.

3.4. *Inhibition of plasmin-induced Ca^{2+} mobilization by PAR-1 antagonist*

We next examined the effect of a PAR-1 antagonist on Ca^{2+} mobilization in human dental pulp cells. SCH79797 is a potent and selective PAR1 antagonist, which antagonizes the thrombin-generated tethered ligand [26]. In the cells pretreated with SCH79797 (20 μM) for 10 min, the increase in $[Ca^{2+}]_i$ induced by the PAR-1 activating peptide SFLLRN (100 μM) or the PAR agonist α-thrombin (20 nM) was clearly inhibited (Table 2). In the SCH79797-pretreated cells, plasmin (100 nM) also failed to induce an increase in $[Ca^{2+}]_i$, as shown in Table 2. SCH79797 had no effect on the basal $[Ca^{2+}]_i$ (data not shown). These observations strongly suggest that the effect of plasmin is coupled to PAR-1 activation.

3.5. *Plasmin-induced IL-8 mRNA expression and prostaglandin E_2 release coupled to PAR-1 activation*

We next examined whether plasmin is involved in inflammation in human dental pulp. Fig. 4 shows the effects of plasmin and SFLLRN on expression of mRNA for the proinflammatory chemokine IL-8 in human dental pulp cells. When the cells were stimulated with plasmin (100 nM) and SFLLRN (100 μM) for 1 h, the expression of IL-8 mRNA was markedly enhanced.

Table 2 – Inhibitory effect of PAR-1 antagonist on plasmin-, SFLLRN- and α-thrombin-induced increase in $[Ca^{2+}]_i$

	SCH79797	
	2 μM	20 μM
	Activation (% of control)	
Plasmin	71.38 ± 15.12	10.41 ± 7.86**
SFLLRN	81.81 ± 6.24	14.01 ± 2.14**
α-Thrombin	61.94 ± 10.04	9.46 ± 2.31**

After pretreatment with the PAR-1 antagonist SCH79797 (2 or 20 μM) for 10 min, the cells were stimulated with plasmin (100 nM), the PAR-1-activating peptide SFLLRN (100 μM) or the PAR agonist α-thrombin (20 nM). Inhibitory ratio was expressed as a percentage of the control (plasmin-, SFLLRN- or α-thrombin-induced increase in $[Ca^{2+}]_i$ in the absence of the antagonist). Values are means ± S.E.M. for 3 independent experiments.
** $P < 0.01$.

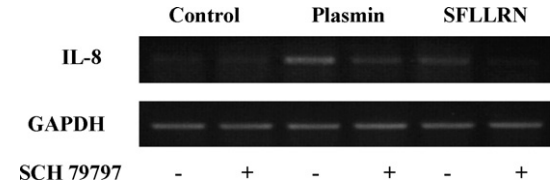

Fig. 4 – Plasmin- and SFLLRN-induced IL-8 mRNA expression in the absence or presence of PAR-1 antagonist. After treatment without or with the PAR-1 antagonist SCH79797 (2 μM) for 30 min, the human dental pulp cells were stimulated with 100 nM plasmin or 100 μM SFLLRN for 1 h, and then their total RNAs were subjected to RT-PCR. The PCR products were subjected to electrophoresis through a 2% agarose gel and subsequently stained with ethidium bromide. The lower panel shows the levels of GAPDH mRNA as a control. These results are representative of 6 independent experiments.

Table 3 – Plasmin- and SFLLRN-induced prostaglandin E_2 release in the absence or presence of PAR-1 antagonist

	SCH79797	
	(−)	(+)
	Prostaglandin E_2 (pg/10^5 cells)	
Control	233.0 ± 29.0	290.8 ± 25.7
Plasmin	2962.8 ± 6.2**	571.4 ± 7.1
SFLLRN	1406.1 ± 75.0**	563.9 ± 24.1

After treatment without or with the PAR-1 antagonist SCH79797 (2 μM) for 10 min, the human dental pulp cells were stimulated with 100 μM plasmin or 100 μM SFLLRN for 10 min. Prostaglandin E_2 released into the conditioned medium was determined using an EIA kit. The results are representative of 4 independent experiments.

** $P < 0.01$.

However, in the presence of the PAR-1 antagonist SCH79797 (2 μM), plasmin- or SFLLRN-induced IL-8 mRNA expression was reduced. These observations suggest that plasmin-induced IL-8 mRNA expression is coupled to PAR-1 activation in human dental pulp cells.

Next we investigated the effect of PAR-1 activation on release of prostaglandin E_2, an eicosanoid involved in inflammation, in human dental pulp cells. As shown in Table 3, when the cells were stimulated with plasmin (100 nM) or SFLLRN (100 μM) for 10 min, prostaglandin E_2 release was clearly enhanced. However, such plasmin- or SFLLRN-induced prostaglandin E_2 release was markedly reduced in the presence of SCH79797 (2 μM). These results strongly suggest that plasmin functions as an inflammatory factor via PAR-1 activation in human dental pulp.

4. Discussion

In the present study, we demonstrated that plasmin functions in human dental pulp cells. Plasmin is produced from plasminogen by the action of PA. Two types of PA, tPA and uPA, are expressed by numerous tissues. Although both PAs activate plasminogen, tPA is primary implicated in fibrinolysis because of its high affinity for fibrin and the extracellular matrix, whereas uPA promotes inflammatory cell chemotaxis and proliferation via interaction with high-affinity cell surface receptors (uPAR) [27,28]. In human dental pulp cells, it has been reported that TNF-α and IL-1α induce tPA synthesis and secretion [16,17] and that IL-1β induces uPA synthesis and secretion [18]. Therefore, it is likely that plasmin is produced by the PAs secreted from cells stimulated with these inflammatory cytokines.

In human dental pulp cells, plasmin stimulated the expression of IL-8 mRNA and prostaglandin E_2 release. IL-8 is a member of the chemokine family and is produced by a wide range of cell types such as monocytes, macrophages and fibroblasts [29]. This chemokine primarily mediates the activation and migration of neutrophils from peripheral blood into tissue and is involved in the initiation and amplification of inflammatory processes in response to various kinds of pathogens [29]. Therefore, it is conceivable that IL-8 plays an important role in inflammation in human dental pulp. On the other hand, prostaglandin E_2 is a member of the eicosanoid family and is a principal mediator of inflammation in various diseases [30]. Inflamed human dental pulps have been reported to contain higher levels of prostaglandins, including prostaglandin E_2, than asymptomatic pulp [31]. The chemical mediator bradykinin and the inflammatory cytokines TNF-α and IL-1β stimulate prostaglandin E_2 production in human dental pulp cells [32,33]. TNF-α and IL-1β also induce gene expression of cyclooxygenase, the enzyme responsible for producing prostaglandins, in dental pulp cells [34]. These observations imply that prostaglandin E_2 is important in the process of pulp inflammation. Therefore, the effects of plasmin we demonstrated in this study suggest that plasmin is involved in inflammatory processes in dental pulp.

We also demonstrated that the effects of plasmin on IL-8 mRNA expression and prostaglandin E_2 release were coupled to PAR-1 activation. So far, 4 types of PAR, PAR-1, PAR-2, PAR-3 and PAR-4, have been discovered and cloned [6,7]. Previously, it has been reported that PAR-1 and PAR-3 mRNAs were detected predominantly in human pulp fibroblasts-like cells [24], whereas another study found that these cells expressed only PAR-2 mRNA [25]. In the present study, PAR-1 mRNA was found to be expressed predominantly. Furthermore, among the PAR-activating peptides, SFLLRN, which activates PAR-1, clearly induced a dose-dependent increase in $[Ca^{2+}]_i$. Moreover, the SFLLRN-induced increase in $[Ca^{2+}]_i$ was inhibited by the PAR-1 antagonist SCH79797. These observations strongly suggest that PAR-1 is expressed and functions in human dental pulp fibroblast-like cells, although the reason for the difference in expression of PARs is not clear. Although dental pulp cells used in this experiment are fibroblast-rich, the cultured cells probably contain other kinds of cells such as odontoblast progenitor cells, because isolated dental pulp cells has been reported to be induced to differentiate into odontoblast-like cells and generate dentin-like mineral structure in vitro [35,36]. Therefore, the difference of expression of PARs appears to be caused by the presence of different kinds of cells.

PAR-1 has been known to be activated by cleavage of arginine-41/serine-42 peptide bond located within the exodo-

main by thrombin [37]. Plasmin has been demonstrated to directly cleave and activate the receptor similar to thrombin [38,39]. It has also been demonstrated that plasmin induces gene expression and mitogenesis in fibroblasts [40,41], elastase secretion in macrophages [42], and migration of Chinese hamster ovary cells expressing recombinant integrin [43] via PAR-1 activation. Similarly, since plasmin-induced IL-8 mRNA expression and prostaglandin E_2 release was inhibited by a PAR-1 inhibitor, it is suggested that PAR-1 activation is essential for the effects of plasmin in human dental pulp cells. Taken together with previous data, the present results suggest that plasmin is involved in inflammatory processes via PAR-1 activation in human dental pulp.

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