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Effects of platelet-derived growth factor isoforms on plasminogen activation by periodontal ligament and gingival fibroblasts

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Background and Objective: Platelet-derived growth factor isoforms and components of the plasminogen activator system are expressed at higher levels during periodontal regeneration. Recombinant platelet-derived growth factor-BB is approved for the treatment of periodontal defects. In the present study we investigated the effect of platelet-derived growth factor isoforms on the plasminogen activator system in periodontal fibroblasts.

Material and Methods: Human periodontal ligament fibroblasts and gingival fibroblasts were exposed to platelet-derived growth factor isoforms. Changes in urokinase-type plasminogen activator, tissue-type plasminogen activator, plasminogen activator inhibitor-1 and plasminogen activator inhibitor-2 transcript levels by platelet-derived growth factor-BB were monitored with a quantitative reverse transcription-polymerase chain reaction. Urokinase-type plasminogen activator and plasminogen activator inhibitor-1 protein levels were assessed by immunoassays. The effects of platelet-derived growth factor-BB on mitogen-activated protein kinase and phosphoinositol-3 kinase/Akt signaling were investigated by western blot and inhibitor studies. Casein zymography and kinetic assays revealed the size and activity, respectively, of the plasminogen activators.

Results: We found that incubation of periodontal ligament fibroblasts and gingival fibroblasts with platelet-derived growth factor-BB resulted in enhanced levels of urokinase-type plasminogen activator and plasminogen activator inhibitor-1 transcripts, but not of tissue-type plasminogen activator and plasminogen activator inhibitor-2. Platelet-derived growth factor-BB also increased urokinase-type plasminogen activator and plasminogen activator inhibitor-1 release into the culture medium. Phosphorylation of extracellular signal-regulated kinase, p38, c-Jun N-terminal kinase and Akt was observed in fibroblasts of both origin. Inhibition of phosphoinositol-3 kinase signaling abrogated the platelet-derived growth factor-BB effect on plasminogen activator inhibitor-1 production. Casein zymography revealed enzymatic activity of the urokinase-type plasminogen activator in cell-conditioned media and lysates of periodontal ligament fibroblasts and gingival fibroblasts. Exposure of gingival fibroblasts, but not of periodontal ligament fibroblasts, to platelet-derived growth factor isoforms moderately increased total plasminogen activation in the medium.

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Conclusion: These findings suggest that periodontal ligament fibroblasts attempt to maintain an equilibrium of the plasminogen activator system in the presence of platelet-derived growth factor isoforms.

Periodontal ligament connects the tooth root to the alveolar bone and the connective tissue of the gingiva. The intrinsic potential for remodeling and repair depends on specialized cells, such as fibroblasts, residing in this multifunctional tissue (1–3). Periodontal ligament fibroblasts and gingival fibroblasts not only produce extracellular matrix (3,4), they also contribute to its degradation under physiologic conditions (3) and at sites of chronic inflammation (5). Matrix degradation can be regulated by controlling the conversion of plasminogen into plasmin. Plasmin is a serine protease that cleaves fibrin and other matrix components, for example fibronectin, laminin and proteoglycans (6). Plasmin also activates proforms of enzymes and growth factors such as matrix metalloproteinase-9 and transforming growth factor-β, respectively (7,8).

Plasminogen conversion into plasmin is determined by the ratio of plasminogen activators and plasminogen activator inhibitors. The serine proteases urokinase-type plasminogen activator and tissue-type plasminogen activator are antagonized by plasminogen activator inhibitor-1 and plasminogen activator inhibitor-2, which together with the urokinase-type plasminogen activator receptor, represent the plasminogen activator system. Plasminogen activators and their inhibitors are expressed at different levels in healthy and diseased periodontal tissue, as concluded from analyses of crevicular fluid (5,9,10) and histological sections (11). *In vitro*, periodontal ligament fibroblasts and gingival fibroblasts are among the cell types that can control plasminogen conversion into plasmin (12,13). However, knowledge about the regulation of the plasminogen activator system by signaling molecules expressed within the periodontal microenvironment is limited to epidermal growth factor, interleukin-1 and transforming growth factor-β (12–14).

Platelet-derived growth factor isoforms are signaling molecules that can form five different dimers (AA, BB, AB, CC and DD) (15). In periodontal tissue, platelet-derived growth factor-A, platelet-derived growth factor-B and their corresponding receptors are expressed during regeneration (16,17). Platelet-derived growth factor-BB is the sole isoform that activates all combinations of platelet-derived growth factor receptors αα, αβ and ββ (15). Platelet-derived growth factor-BB shows a positive effect on adhesion of fibroblasts to periodontal diseased root surfaces (18) and causes a strong mitogenic and chemotactic response (19,20). Recently, recombinant platelet-derived growth factor-BB has been approved for treatment of periodontal defects (21). Autocrine effects of platelet-derived growth factor-BB in periodontal ligament fibroblasts may not be possible (22); however, the cells express platelet-derived growth factor-C and -D (22). Platelet-derived growth factor-CC and -DD are produced as latent proforms that have to be prototypically activated involving the plasminogen activator system (23,24).

Platelet-derived growth factor signaling in periodontal ligament fibroblasts can be mediated by the activation of extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal protein kinase, which belong to the family of mitogen-associated protein kinases (MAPK) (25). Platelet-derived growth factor isoforms also activate phosphoinositol-3 kinase/Akt signaling in skin fibroblasts, which has not previously been shown for periodontal fibroblasts (26). Platelet-derived growth factor isoforms can modulate the plasminogen activator system, involving ERK signaling, in vascular smooth muscle cells (27). The phosphoinositol-3 kinase/Akt pathway also acts as a negative regulator of plasminogen activator inhibitor-1 expression in endothelial cells (28). Whether or not platelet-derived growth factor isoforms can modulate the plasminogen activator system in periodontal ligament fibroblasts and gingival fibroblasts involving MAPK and phosphoinositol-3 kinase/Akt signaling remains to be determined.

Here we tested the hypothesis that active platelet-derived growth factor isoforms can control matrix remodeling of periodontal tissue by altering the capacity of periodontal ligament fibroblasts and gingival fibroblasts to convert plasminogen into plasmin.

Material and methods

Cell culture and basic experimental settings

Primary human periodontal ligament fibroblasts and gingival fibroblasts were prepared, following an established protocol, from the extracted retromolars of six donors after informed consent was obtained, and the fibroblasts were stored in liquid nitrogen until required for analysis. The donors were selected on the basis of an absence of previous history of inflammation in the retromolar tissue. Periodontal ligament fibroblasts and gingival fibroblasts were prepared by outgrowth from explant cultures. Cells of not more than 10 passages were plated into culture dishes at 5×10^4 cells/cm^2 in α-minimal essential medium (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% fetal calf serum, 100 U/mL of penicillin G and 100 μg/mL of streptomycin, in a humidified atmosphere at 37°C. The following day, growth medium was changed to serum-free medium, with or without the following recombinant human platelet-derived growth factor isoforms: platelet-derived growth factor-AA, platelet-derived growth factor-AB, platelet-derived growth factor-BB and platelet-derived growth factor-CC (all R & D systems, Minneapolis, MD,

USA), at 100 ng/mL. After 24 h, conditioned medium was harvested and cell lysates were prepared by sonification in a buffer containing 50 mM Tris, pH 8.8, 38 mM NaCl and 0.1% Triton X-100. Debris was eliminated by centrifugation. Cell lysates and cell-conditioned medium were stored frozen at −20°C.

Casein zymography

Cell-conditioned medium and cell lysates were size fractionated by 10% nonreducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis, together with controls of purified urokinase-type plasminogen activator and recombinant tissue-type plasminogen activator (both Technoclone GmbH, Vienna, Austria). Gels were washed twice, for 30 min each wash, in 2.5% Triton X-100, placed onto substrate gels containing 2% (w/v) nonfat dry milk, 0.25 mM Tris-HCl, pH 7.6, 1% agarose, ×0.25 phosphate-buffered saline and 5 μg/mL of plasminogen, and then incubated in a humidified chamber at 37°C overnight. Plasminogen activator activity was visualized as clear zones in the substrate gel. Gels were also supplemented with aprotinin at 5 kallikrein-units/mL (Sigma, St Louis, MO, USA) and were used without the addition of plasminogen as a control.

Caseinolysis kinetic assay

Fifty-microlitre samples of conditioned medium, and cell lysates and the same volume of a substrate solution containing 50 mM Tris, pH 8.8, 38 mM NaCl, 4% casein and 20 μg/mL of plasminogen (Technoclone GmbH), were incubated in microtiter plates at 37°C overnight. Measurement of kinetic turbidity at 405 nm and 37°C was performed using a microplate reader. Optical density was assessed every 20 min for 16 h. Plots were obtained to calculate the time required for the half-maximal lysis of casein (13).

Immunoassays for urokinase-type plasminogen activator and plasminogen activator inhibitor-1

Total urokinase-type plasminogen activator and total plasminogen activator inhibitor-1 were measured in conditioned medium prepared from periodontal ligament fibroblasts and gingival fibroblasts following incubation with or without platelet-derived growth factor-BB at 100 ng/mL and the inhibitors for ERK (U0126; Cell Signaling Technology, Beverly, MA, USA), p38 (SB203580; Sigma), c-Jun N-terminal protein kinase (SP600125; Calbiochem, San Diego, CA, USA) and phosphoinositol-3 kinase (LY294002; Sigma), all at 10 μM, by a specific enzyme-linked immunosorbent assay (Technoclone GmbH).

One-step quantitative reverse transcription-polymerase chain reaction analysis of gene expression

Periodontal ligament fibroblasts and gingival fibroblasts in six-well plates were incubated with or without 100 ng/mL of platelet-derived growth factor for 24 h.

Cellular RNA was isolated using an RNeasy mini kit (Qiagen, Hilden Germany) and treated with DNAse I (Invitrogen Corporation) following the manufacturer's instructions. Reverse transcription (RT) and polymerase chain reaction (PCR) were performed in a one-step methodology (SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR Kit; Invitrogen Corporation), according to the instructions of the manufacturer, on a 7000 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The following primers were used (29,30): htPA forward, GGCCTTGTCTCCTTTCTATTCG; htPA reverse, GCGGCTGGATGGGTACAGT; huPA forward, AAGGACAAGCCAGGCGTCTA; huPA reverse, AAAATGACAACCAGCAAGAAAGC; huPAR forward, GGTGACGCCTTCAGCATGA; huPAR reverse, CCCACTGCGGTACTGGACAT; hPAI-1 forward, CACAAATCAGACGGCAGCACT; hPAI-1 reverse, CATCGGGCGTGGTGAACTC; hPAI-2 forward, TGGTCCTGGTGAATGCTGTCTACT; hPAI-2 reverse, TGAGCCGAGTTTACACGGAAAGGA; hβactin forward, GCATCCCCCAAAGTTCACAA; hβactin reverse, AGGACTGGGCCATTCTCCTT. Amplification was performed at one cycle of 50°C for 3 min followed by one cycle of 95°C for 5 min; 40 cycles of 95°C for 15 s, and 60°C for 30 s, followed by a final cycle of 40°C for 1 min. The ΔΔCt (where Ct = threshold cycle) method was used to calculate the fold up-regulation. The expression level of the target gene was quantified by the concentration-dependent PCR circle number at which the amplification is distinguishable over background. Each Ct sample of the target gene was normalized by subtracting the Ct of the endogenous reference hβactin (ΔCt = Ct target gene − Ct hβactin). The ΔCt of the stimulated cells (ΔCts) was subtracted from the ΔCt of the untreated cells (ΔCtu) (ΔΔCt = ΔCt − ΔCtu). The target gene expression level of the stimulated cells compared with the level of the untreated cells was calculated as follows: x-fold of unstimulated control = $2^{(-\Delta\Delta Ct)}$. PCR amplification was performed in triplicate and repeated with cells from three independent donors.

Western blot analysis

Periodontal ligament fibroblasts and gingival fibroblasts in six-well plates were serum-starved overnight and exposed for 15 min to platelet-derived growth factor-BB at 100 ng/mL before being lysed in sodium dodecyl sulfate buffer containing phosphatase and protease inhibitors. Cell debris was eliminated by centrifugation. Equal amounts of cell extracts were size-fractionated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were transferred onto nitrocellulose membranes (GE Healthcare UK Ltd, Bucks., UK). Membranes were blocked with 5% w/v nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 and then incubated with a 1 : 1000 dilution of antibodies against phospho-ERK1/2 (clone E-4; Santa Cruz Biotechnology, Santa Cruz, CA, USA), ERK1 (clone K-23; Santa Cruz Biotechnology), phospho-p38 MAPK (clone #9211; Cell Signaling Technologies, Danvers, MA, USA), p38 MAPK (clone C-20, Santa Cruz Biotechnology), phospho-c-Jun N-terminal protein kinase (clone #9251; Cell Signaling Technologies),

c-Jun N-terminal protein kinase (clone C-17; Santa Cruz Biotechnology), phospho-Akt (Ser473; clone #9271; Cell Signaling Technologies),or phospho-Akt (Thr308; clone #9275; Cell Signaling Technologies). The first antibody was detected with the appropriate secondary antibody (polyclonal goat rabbit immunoglobulin horseradish peroxidase, #P0448, and polyclonal rabbit mouse immunoglobulin horseradish peroxidase, #P0260; Dako; Glostrup, Denmark) using a chemiluminescence substrate (GE Healthcare).

Statistical analysis

Data were compared with the paired Student's *t*-test. To increase statistical power, we pooled the immunoassay data of periodontal ligament fibroblasts and gingival fibroblasts. A significant difference was assigned at the $p < 0.05$ level.

Results

Effect of platelet-derived growth factor-BB signaling on transcription and translation of the plasminogen activation system

Recombinant platelet-derived growth factor-BB was tested for the ability to modulate the plasminogen activation system in periodontal ligament fibroblasts and gingival fibroblasts. The system included the plasminogen activators urokinase-type plasminogen activator and tissue-type plasminogen activator as well as the corresponding antagonists plasminogen activator inhibitor-1 and plasminogen activator inhibitor-2. Quantitative RT-PCR analysis showed that platelet-derived growth factor-BB incubation increased urokinase-type plasminogen activator transcript levels in periodontal ligament fibroblasts to 10.5 ± 6.6-fold ($p = 0.03$). Urokinase-type plasminogen activator in gingival fibroblasts and urokinase-type plasminogen activator receptor, tissue-type plasminogen activator, plasminogen activator inhibitor-1 and plasminogen activator inhibitor-2 were expressed in both cell types but did not reach the level of significance (Table 1). However, immunoassays revealed a 2.5-fold increase of total urokinase-type plasminogen activator ($p = 0.04$) and a 2.3-fold increase of total plasminogen activator inhibitor-1 ($p = 0.02$) in the cell-conditioned medium when periodontal ligament fibroblasts and gingival fibroblasts were exposed to platelet-derived growth factor-BB (Table 2).

Effects of platelet-derived growth factor-BB on the activation of MAPK and phosphoinositol-3 kinase/Akt signaling pathways

We next examined the effect of platelet-derived growth factor-BB on the phosphorylation of MAPK and Akt in periodontal ligament fibroblasts and gingival fibroblasts. Western blot analysis showed that incubation of periodontal ligament fibroblasts and gingival fibroblasts with platelet-derived growth factor-BB resulted in an increased phosphorylation of ERK, p38, c-Jun N-terminal protein kinase and Akt (Fig. 1). To determine which of these signaling pathways affected the expression of urokinase-type plasminogen activator and plasminogen activator inhibitor-1, periodontal ligament fibroblasts and gingival fibroblasts were exposed to platelet-derived growth factor-BB in the presence of pharmacologic inhibitors that block ERK, p38, c-Jun N-terminal protein kinase and phosphoinositol-3 kinase signaling. Immunoassay of conditioned medium showed that only phosphoinositol-3 kinase signaling was required to mediate the effect of platelet-derived growth factor-BB on plasminogen activator inhibitor-1 production. Plasminogen activator inhibitor-1 increase was independent of ERK, p38 and c-Jun N-terminal protein kinase signaling in periodontal fibroblasts (Table 2). Inhibition of ERK, p38, c-Jun N-terminal protein kinase and Akt did not modulate the effects of platelet-derived growth factor-BB on urokinase-type plasminogen activator production (Table 2).

Effect of platelet-derived growth factor isoforms on total plasminogen activation in periodontal ligament fibroblasts and gingival fibroblasts

Based on the ability of platelet-derived growth factor-BB to enhance urokinase-type plasminogen activator and plasminogen activator inhibitor-1 production in periodontal ligament fibroblasts and gingival fibroblasts, as determined by immunoassay, it can be suggested that periodontal fibroblasts attempt to maintain an equilibrium of the plasminogen activator system. To confirm this assumption, we included a casein zymography and a kinetic assay, both of which integrate the activity of plasminogen activators and the corresponding plasminogen inhibitors.

Table 1. Quantitative reverse transcription-polymerase chain reaction analysis of periodontal ligament fibroblasts and gingival fibroblasts exposed to platelet-derived growth factor-BB

	PDLF	GF
tPA	1.5 ± 1.0	0.8 ± 1.1
uPA	10.5 ± 6.6*	6.4 ± 7.3
uPAR	1.1 ± 0.9	2.4 ± 3.7
PAI-1	4.4 ± 1.3	1.6 ± 1.9
PAI-2	1.4 ± 0.7	1.0 ± 0.2

Periodontal ligament fibroblasts from three donors and gingival fibroblasts from five donors were exposed to 100 ng/mL of platelet-derived growth factor-BB for 24 h and the transcripts were amplified by one-step quantitative reverse transcription-polymerase chain reaction analysis. Expression levels were normalized to β-actin and given as x-fold of unstimulated controls. The results are expressed as mean ± SD, *$p < 0.05$.

GF, gingival fibroblasts; PAI-1, plasminogen activator inhibitor-1; PAI-2, plasminogen activator inhibitor-2; PDGF, platelet-derived growth factor; PDLF, periodontal ligament fibroblasts; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen receptor.

Table 2. Immunoassay of periodontal ligament fibroblasts and gingival fibroblasts exposed to platelet-derived growth factor-BB

	w/o	U0126	SB203580	SP600125	LY294002
Total uPA (ng/mL)					
PDLF					
Control	12 ± 9	14 ± 61	5 ± 8	10 ± 7	7 ± 7
PDGF-BB	21 ± 11	27 ± 8	28 ± 17	22 ± 14	12 ± 4
Increase	1.8	2.0	1.9	2.3	1.7
GF					
Control	6 ± 2	4 ± 1	8 ± 4	5 ± 2	4 ± 2
PDGF-BB	13 ± 12	23 ± 9	23 ± 19	14 ± 8	15 ± 9
Increase	2.4	6.1	2.8	2.9	3.8
PDLF and GF					
Control	9 ± 7	9 ± 7	11 ± 7	7 ± 5	5 ± 5
PDGF-BB	17 ± 12	25 ± 8	26 ± 17	18 ± 11	13 ± 6
Increase	2.5*	3.7**	3.8*	3.4**	2.5*
Total PAI-1 (ng/mL)					
PDLF					
Control	45 ± 38	41 ± 34	37 ± 15	29 ± 14	53 ± 57
PDGF-BB	101 ± 40	55 ± 20	83 ± 13	39 ± 12	38 ± 21
Increase	2.3	1.3	2.3	1.3	0.7
GF					
Control	56 ± 28	55 ± 40	41 ± 13	50 ± 32	69 ± 66
PDGF-BB	132 ± 82	79 ± 33	105 ± 120	72 ± 37	69 ± 31
Increase	2.3	1.4	2.6	1.4	1.0
PDLF and GF					
Control	51 ± 31	48 ± 35	39 ± 14	40 ± 25	61 ± 58
PDGF-BB	117 ± 62	67 ± 28	94 ± 17	56 ± 31	53 ± 30
Increase	2.3*	1.4*	2.4**	1.4**	0.9

Periodontal ligament fibroblasts and gingival fibroblasts from four donors were exposed to 100 ng/mL of platelet-derived growth factor-BB at for 24 h and conditioned media were analyzed for urokinase-type plasminogen activator and plasminogen activator inhibitor-1. Experiments were performed with and without the mitogen-activated protein kinase (MAPK) inhibitors U0126 (extracellular signal-regulated kinase), SB203580 (p38), and SP600125 (c-Jun N-terminal protein kinase) or the phosphoinositol-3 kinase inhibitor LY294002, at 10 μg/mL each. The results are expressed as mean ± standard deviation,*$p < 0.05$, **$p < 0.01$.

GF, gingival fibroblasts; PDGF, platelet-derived growth factor; PAI-1, plasminogen activator inhibitor-1; PDLF, periodontal ligament fibroblasts; uPA, urokinase-type plasminogen activator; w/o, without.

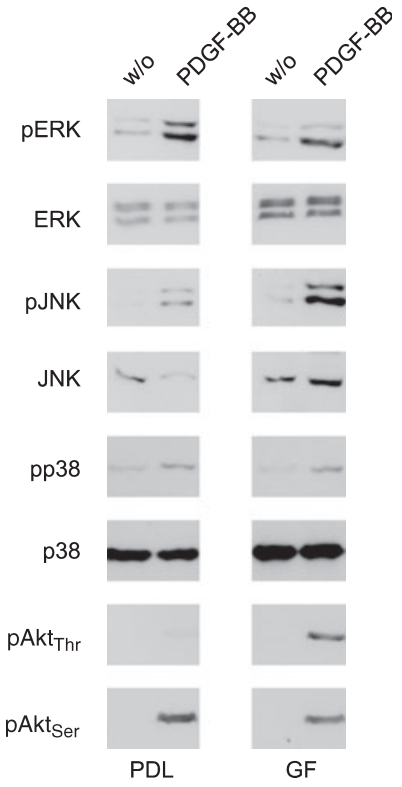

Fig. 1. Characterization of signaling pathways of periodontal ligament fibroblasts and gingival fibroblasts activated by platelet-derived growth factor-BB. Periodontal ligament fibroblasts and gingival fibroblasts in six-well plates were serum-starved overnight and then exposed for 15 min to 100 ng/mL of platelet-derived growth factor-BB. Cell lysates were size fractionated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. Phosphorylated (pERK, pp38, pJNK, pAkt) and unphosphorylated (ERK, p38, c-Jun N-terminal protein kinase) proteins were detected by western blot analysis. ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal protein kinase; PDGF-BB, platelet-derived growth factor-BB; Ser, serine; Thr, threonine; w/o, without.

Conditioned medium and cell lysates from periodontal ligament fibroblasts and gingival fibroblasts revealed a proteolytic activity at the level of urokinase-type plasminogen activator in the casein zymography (Fig. 2A). This pattern was independent of incubation of periodontal ligament fibroblasts and gingival fibroblasts with the platelet-derived growth factor isoforms (Fig. 2B). In the kinetic assay, activation of platelet-derived growth factor signaling increased the plasminogen-activation capacity of gingival fibroblast-conditioned medium, but not of the corresponding cell lysates (Fig. 3). None of the platelet-derived growth factor isoforms changed the plasminogen activation capacity of the conditioned medium and cell lysates of periodontal ligament fibroblasts (Fig. 3).

Discussion

The plasminogen activation system can play a role in periodontal tissue regeneration and mediates the catabolic activity of chronic inflammation (1,2,10). To understand the pathophysiologic mechanisms of periodontal disease and the function of recombinant platelet-derived growth factor-BB in therapy, deeper insights into the regulation of the plasminogen activation system are required. At present there is no direct evidence that platelet-derived growth factor signaling regulates the plasminogen activator system in periodontal tissue. The aim of the present study was to determine changes in the plasminogen activation system in periodontal ligament fibroblasts and gingival fibroblasts when treated with platelet-derived growth factor isoforms. We also investigated the signaling pathways that are activated in response

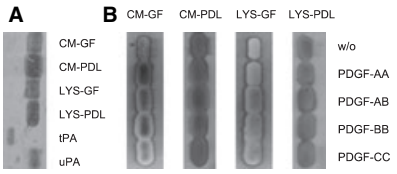

Fig. 2. Casein zymography of periodontal ligament fibroblasts and gingival fibroblasts exposed to platelet-derived growth factor isoforms. (A) Conditioned medium and cell lysates of untreated periodontal ligament fibroblasts and gingival fibroblasts were subjected to casein zymography. Purified urokinase-type plasminogen activator and recombinant tissue-type plasminogen activator served as positive controls. (B) Periodontal ligament fibroblasts and gingival fibroblasts were exposed to 100 ng/mL of platelet-derived growth factor isoforms for 24 h, and conditioned medium and cell lysates were subjected to casein zymography. Plasminogen activator activity was visualized as clear zones in the substrate gel and were photographed. CM, conditioned medium; GF, gingival fibroblasts; LYS, cell lysates; PDGF-AA, -AB, -BB, -CC; platelet-derived growth factor-AA, -AB, -BB, -CC; PDL, periodontal ligament fibroblasts; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; w/o, without.

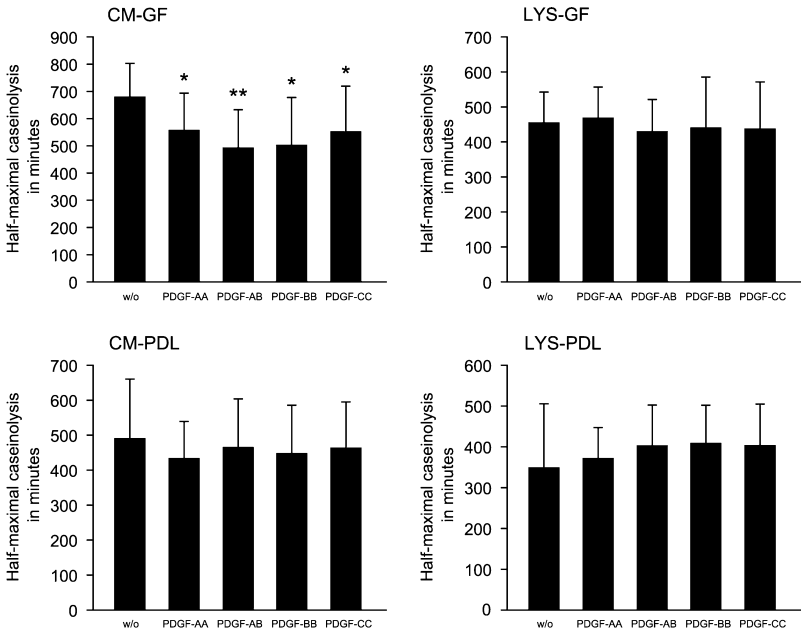

Fig. 3. Caseinolysis kinetic assay of periodontal ligament fibroblasts and gingival fibroblasts exposed to platelet-derived growth factor isoforms. Periodontal ligament fibroblasts and gingival fibroblasts from six donors were exposed to 100 ng/mL of platelet-derived growth factor isoforms at for 24 h. Cell-conditioned medium and cell lysates were subjected to casein substrate solution containing plasminogen. The kinetics of the changing turbidity was determined using a microplate reader. Data points represent the mean and standard deviation of time required for the half-maximal lysis of casein. Bars represent the mean ± standard deviation. $*p < 0.05$ and $**p < 0.01$ compared with untreated controls. CM, conditioned medium; GF, gingival fibroblasts; LYS, cell lysates; PDGF-AA, -AB, -BB, -CC; platelet-derived growth factor-AA, -AB, -BB, -CC; PDL, periodontal ligament fibroblasts; w/o, without.

to platelet-derived growth factor-BB and mediated the observed effects.

The activation of plasminogen is mainly attributed to urokinase-type plasminogen activator produced by periodontal ligament fibroblasts and gingival fibroblasts *in vitro* (13). These findings are in agreement with the casein zymography presented here and in other reports (31) and with recent observations that anti-urokinase-type plasminogen activator serum inhibits plasminogen activation of gingival fibroblast-conditioned medium (32). Tissue-type plasminogen activator activity did not reach the level of detection in our zymography and may require concentration of the conditioned medium (33). Periodontal ligament fibroblasts and gingival fibroblasts activated plasminogen although they released substantial amounts of plasminogen activator inhibitor-1. The large majority of plasminogen activator inhibitor-2 remains in the cytoplasm of these cells under serum-free conditions (34). The biological function of intracellular plasminogen activator inhibitor-2 requires further investigation.

We next examined the effects of platelet-derived growth factor isoforms on regulation of the plasminogen activation system in periodontal ligament fibroblasts and gingival fibroblasts. Platelet-derived growth factor-BB increased urokinase-type plasminogen activator mRNA levels in periodontal ligament fibroblasts. At the protein level, platelet-derived growth factor-BB increased urokinase-type plasminogen activator and plasminogen activator inhibitor-1 in both cell types. In the kinetic assay, activators and inhibitors reached an equilibrium in periodontal ligament fibroblasts, whereas in gingival fibroblasts the total plasminogen activation was weakly increased. These findings suggest that periodontal fibroblasts tend to maintain their plasminogen activation capacity by a balanced production of activators and inhibitors *in vitro*.

The relationship between plasminogen activation and the platelet-derived growth factor system is more complicated than our experiments might suggest. Platelet-derived growth factor-CC and -DD, which are expressed by periodontal ligament fibroblasts (25), are secreted as latent proforms that have to be proteolytically activated (23,24) by cleavage at the hinge region (23,24,35). Tissue-type plasminogen activator and plasmin can activate platelet-derived growth factor-CC (23,36), and the released CUB domain, in turn, inhibits the tissue-type plasminogen activator (37). The activation of platelet-derived growth factor-DD proforms is mediated by urokinase-type plasminogen activator and plasmin, suggesting similar mechanisms as for platelet-derived growth factor-CC (24,38). Periodontal ligament fibro-

blasts and gingival fibroblasts can therefore contribute mainly to the direct activation of platelet-derived growth factor-DD and to the activation of both isoforms by their ability to convert plasminogen into plasmin. As platelet-derived growth factor-CC stimulates revascularization (39), and platelet-derived growth factor-DD is expressed during periodontal mineralization (22), it is possible that these mechanisms are involved in periodontal tissue regeneration. However, our *in vitro* model is based on the active isoforms and therefore does not provide insight into a possible positive feedback regulation between the plasminogen activator system and platelet-derived growth factor-CC and -DD.

Another possible relationship exists that involves the interaction between the platelet-derived growth factor receptor-β and urokinase-type plasminogen activator/urokinase-type plasminogen activator receptor. The urokinase-type plasminogen activator receptor lacks a transmembrane domain and consequently urokinase-type plasminogen activator/urokinase-type plasminogen activator receptor signaling depends on mediators such as platelet-derived growth factor receptor-β, as observed in smooth muscle cells (40,41). This interaction also effects the responsiveness of the cells to the original ligand, platelet-derived growth factor-BB (40,41). Periodontal ligament fibroblasts (as we have shown) and gingival fibroblasts can form urokinase-type plasminogen activator/urokinase-type plasminogen activator receptor (14,42) and express the platelet-derived growth factor receptor-β, suggesting that this interaction may also be relevant in periodontal tissue. Our *in vitro* findings on the expression of single components of the plasminogen activation system in response to platelet-derived growth factor-BB, allows us to support the possibility of a functional relationship between the urokinase-type plasminogen activator/urokinase-type plasminogen activator receptor and the platelet-derived growth factor-BB/platelet-derived growth factor receptor-β in periodontal fibroblasts.

To understand which signaling pathways are involved in regulating urokinase-type plasminogen activator and plasminogen activator inhibitor-1 expression by platelet-derived growth factor-BB, the MAPK and Akt phosphorylation status was analysed. In agreement with recent reports (43,44), platelet-derived growth factor-BB increased the phosphorylation of ERK and p38 in periodontal ligament fibroblasts. Platelet-derived growth factor-BB also enhanced c-Jun N-terminal protein kinase and Akt phosphorylation, which has not been previously described for periodontal ligament and gingival fibroblasts. Despite the activation of MAPK and Akt signaling by platelet-derived growth factor-BB, only phosphoinositol-3 kinase was required to mediate the effect of platelet-derived growth factor-BB on plasminogen activator inhibitor-1 production. In other cell types, ERK and c-Jun N-terminal protein kinase also mediate the effect of platelet-derived growth factor-BB on plasminogen activator inhibitor-1 release (27,45). Our data further indicated that urokinase-type plasminogen activator release was not affected by either of the inhibitors raised against MAPK and Akt signaling. However, epidermal growth factor-stimulated urokinase-type plasminogen activator expression depends on ERK and c-Jun N-terminal protein kinase signaling (12), and c-Jun N-terminal protein kinase is required for urokinase-type plasminogen activator expression in response to transforming growth factor-β (31). All these lines of evidence support the notion that even though platelet-derived growth factor-BB activates MAPK and Akt in periodontal ligament fibroblasts and gingival fibroblasts, only phosphoinositol-3 kinase signaling is required for the increased release of plasminogen activator inhibitor-1.

The complex process of periodontal disease and its regeneration encompasses cell types other than fibroblasts, such as neutrophils, monocytes and lymphocytes, all of which are responsive to platelet-derived growth factor signaling and express components of the plasminogen activation system (46,47). Only limited information is available about the role of platelet-derived growth factor isoforms in the regulation of the plasminogen activation system in these cell types (48). *In vitro* studies on periodontal ligament fibroblasts and gingival fibroblasts offer restricted insights into the overall pathophysiologic role of platelet-derived growth factor isoforms on the plasminogen activation in periodontal disease and its regeneration. The *in vitro* model based on isolated periodontal ligament fibroblasts and gingival fibroblasts provides parts of a mosaic that help to explain the complex interaction of cells, signaling molecules and the extracellular matrix. We expect further insights into the role of the plasminogen activation systems from studies on mice deficient in components of the plasminogen activator system and induced for periodontal disease (6).

Based on our *in vitro* model, we conclude that the plasminogen activation capacity of periodontal ligament fibroblasts and gingival fibroblasts was not substantially altered by any of these platelet-derived growth factor isoforms. Our study underlines the importance of functional assays to detect the total plasminogen activation capacity and suggests that a compensatory regulatory mechanism exists in periodontal ligament fibroblasts and gingival fibroblasts where urokinase-type plasminogen activator and plasminogen activator inhibitor-1 are elevated in response to platelet-derived growth factor signaling.

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