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Secreted caveolin-1 enhances periodontal inflammation by targeting gingival fibroblasts

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ABSTRACT

Caveolin-1 (Cav-1) is a membrane protein. Recently, it has been reported that secreted Cav-1 induces angiogenesis in inflammatory microenvironment. However, it is unclear that Cav-1 regulates gingival inflammation. Therefore, we investigated the Cav-1 function to periodontal cells. Expression of Cav-1 in human periodontitis tissues was examined pathologically. Secretion of Cav-1 from human gingival fibroblasts (HGFs) or human periodontal ligament cells (HPLFs) treated with IL-1β and TNF-α was examined using Western blotting. Likewise, intracellular signals induced by Cav-1 were examined. Finally, we examined whether the secreted Cav-1 induces production of inflammatory mediators in HGFs using ELISA or qRT-PCR. Pathologically, high expression of Cav-1 was observed in human periodontitis tissues. Cav-1 secretion increased in both cultured HGFs and HPLFs treated with IL-1β and TNF-α. Cav-1 induced phosphorylation of JNK and ERK, but not Stat3 in HGFs. Furthermore, Cav-1 increased proMMP-1 and VEGF secretion in HGFs, and the VEGF secretion was statistically suppressed by JNK inhibitor SP600125, but not ERK inhibitor PD98059. ProMMP-1 secretion was suppressed statistically by both SP600125 and PD98059. In addition, Cav-1 increased significantly MMP-1, -10 and -14 mRNA expressions, whereas no increase of TIMPs mRNA was observed in HGFs treated with Cav-1. These data suggest that secreted Cav-1 derived from periodontal fibroblastic cells enhances inflammation-related several proteases and VEGF secretion in HGFs *via* MAPKs pathway, resulting in progression of periodontitis through induction of tissue degradation or angiogenesis.

Periodontitis is an infectious inflammatory disease. It has been well reported that various cytokines, growth factors and hormones are involved in periodontal inflammation (1, 10), and the disease may result in loss of teeth by bone resorption depending on inflammation-mediated proteases (7). In addition, pro-inflammatory cytokines IL-1β and TNF-α are activated at initial stage of periodontitis, and regulate downstream of gingival inflammation (10). Angiogenesis is also important feature of mid stage of periodontitis, and vascular endothelial growth factor (VEGF) is a potent inducer of the inflammatory responses including endothelial cell proliferation (6). In fact, VEGF level in inflamed periodontal lesions is co-related to severity of periodontitis, and can be considered a useful biomarker of periodontitis progression (6).

Caveolae are flask-shaped invaginations of cell membrane; their major structural protein, caveolin-1 (Cav-1) is a scaffolding/regulatory protein that interacts with subsequent signaling molecules in various cell types (13). The putative functions of Cav-1 are

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cholesterol transport (32) and endocytosis (18). In addition, Cav-1 seems to modulate the signal transduction by linking the signaling molecules, leading to regulating their downstream activity (33). Previously, we have reported that IL-6/soluble IL-6 receptor (sIL-6R) complex enhances lysosomal enzymes cathepsin B and L production *via* Cav-1-JNK-AP-1 pathway in human gingival fibroblasts (HGFs) (29), thus Cav-1 should be an attractive target of IL-6 signals in HGFs to clarify the pathophysiology of periodontal disease. On the other hand, it has been shown that virulent prostate cancer cell lines secrete biologically active Cav-1 protein *in vitro*, and the secreted Cav-1 promotes both cell viability and angiogenic activities of prostate cancer cells, resulting in higher metastasis of the cancer (26). However, the molecular mechanisms whether secreted Cav-1 promotes periodontal inflammation by targeting periodontal cells have been still unknown.

In the present study, we investigated the possibility of Cav-1 function on periodontal inflammation to contribute to clarify the pathophysiology of periodontitis.

MATERIALS AND METHODS

Reagents. Recombinant IL-1β and TNF-α were obtained from R&D Systems (Minneapolis, MN). Recombinant Cav-1 was purchased from Abnova (Taipei, Taiwan). Phospho-ERK, -JNK and -Stat3 antibodies were obtained from Cell Signaling Technology (Beverly, MA). Cav-1 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Cathepsin L antibody was obtained from BD Biosciences (San Jose, CA). ERK inhibitor PD98059 and JNK inhibitor SP600125 were obtained from Calbiochem (La Jolla, CA).

Pathological examination. Gingival specimens were collected during gingivectomy with informed consent of each subject. Periodontal tissues including normal gingiva (9 cases) and inflamed gingiva (11 cases) were used for pathological examination. The tissues were fixed in 10% buffered formalin, embedded in paraffin wax, and 5 μm-thick sections were cut for microscopic studies. Sections were stained with haematoxylin and eosin. Immunohistochemical detection of Cav-1 (1 : 100 for first antibody) was performed using Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, CA). Cell types were identified morphologically (fibroblastic cells: spindle shape; inflammatory cells: round shape), and counted 5–6 visual fields. Data were expressed as number of staining-positive cells per visual field. This study was approved by the Ethics Committee of Iwate Medical University (approval No.1181).

Cell culture. HGFs and human periodontal ligament cells (HPLFs) were isolated as previously reported (26, 29), and were maintained in appropriate medium (DMEM for HGFs, α-MEM for HPLFs; Invitrogen, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) and antibiotics (Invitrogen). Informed consent was obtained from each volunteer before participation, and the Ethics Committee of Iwate Medical University approved the research protocol (approval No.1126). The cells between the 8–15 passages were used for assays. Twenty-four hours before treatment the medium was changed to medium supplemented with 0.5% FBS and same concentrations of antibiotics. The human monocytic leukemia cell line THP-1 cells (a gift from Dr. M. Taira, Iwate Medical University) were maintained in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FBS and antibiotics (Invitrogen). THP-1 cells were differentiated by 100 nM phorbol 12-myristate 13-acetate (PMA: Sigma, St. Louis, MO).

Quantitative RT-PCR. To examine Cav-1 mRNA expression in HGFs, cells were treated with rhIL-1β (1 ng/mL) or rhTNF-α (10 ng/mL) for indicated times. Next, to examine mRNA expression of various genes, cells were treated with Cav-1 (500 ng/mL) for 6 h. Total RNA was extracted with ISOGEN reagent (Nippongene, Tokyo, Japan), and first strand cDNA was synthesized by using the PrimeScript RT reagent kit (Takara Bio, Ohtsu, Japan). Quantitative RT-PCR (qRT-PCR) was performed using the Thermal Cycler Dice Real time System (Takara Bio) with SYBR Premix EX Taq II (Takara Bio) on cDNA generated from the reverse transcription of purified RNA. The mRNA expression was normalized against β-actin or GAPDH mRNA expression using the comparative cycle threshold method. Primer sequences for PCR reactions have been shown in Table 1.

Western blotting. After HGFs and HPLFs were treated with rhIL-1β (1 ng/mL) or rhTNF-α (10 ng/mL) for 24 h, both culture supernatants and total cell lysate were collected to examine the productivity of Cav-1. Next, after HGFs were treated with 100 or 1,000 ng/mL of Cav-1 for 10 min, the total cell lysates were collected to examine the intracellular signaling pathway. Total cell lysates were extracted with a lysis buffer: 10 mM Tris-HCl, pH 7.4, 50 mM

Table 1 *Primer sequences for qRT-PCR*

Target gene	Accession #	Primer	Target gene	Accession #	Primer
Cav-1*	-	F: TTCTGGGCTTCATCTGGCAAC	TIMP-3	NM_000362.4	F: GGCTTCACCAAGATGCCCCAT
		R: GCTCAGCCCTATTGGTCCACTTTA			R: ACAGCCCCGTGTACATCTTGC
MMP-1	NM_032006.3	F: AAGCAGCCCAGATGTGGAGTGCCT	Cathepsin B	NM_001908.3	F: AGCGCTGGGTGGATCTAGGA
		R: TGTGTTTGCTCCCAGCGAGGGTTCC			R: GTTGACCAGCTCATCCGACAGG
MMP-2**	-	F: GATAACCTGGATGCCGTCGTG	Cathepsin L	NM_001257972.1	F: AAGCAGCCCAGATGTGGAGTGCCT
		R: CAGCCTAGCCAGTCGGATTTG			R:TGTGTTTGCTCCCAGCGAGGGTTCC
MMP-3	NM_002422.3	F: TACAAGGAGGCAGGCAAGACAG	EGF	NM_001963.4	F: GTGCATCCACTTGCACAACCC
		R: GGATAGGCTGAGCAAACTGCCA			R: CACAGTGCTGCTGCAGTTTCC
MMP-10*	-	F: CTGGACCTGGGCTTTATGGAGA	IGF-1	NM_001111285.1	F: AGACCCAGAAGTATCAGCCCCC
		R: AGTTCATGAGCAGCAACGAGGA			R: TCTGTCCCCTCCTTCTGTTCCC
MMP-13	NM_002427.3	F: CCAGTTTGCAGAGCGCTACCT	IL-1ra	NM_173842.2	F: CCTTGCTGCAGTCACAGAATGG
		R: TTGCCAGTCACCTCTAAGCCG			R: GGATTTTCTCCCAGAGGGTCGG
MMP-14*	-	F: GGAACCCTGTAGCTTTGTGTCTGTC	IL-33	NM_033439.3	F: GCTCCGCTCTGGCCTTATGAT
		R: TGAGGGTCCTGCCTTCAAGTG			R: TTGACAGGCAGCGAGTACCAG
TIMP-1	NM_003254.2	F: TGAAAAGGGCTTCCAGTCCCG	β-actin*	-	F: ATTGCCGACAGGATGCAGA
		R: TGGACACTGTGCAGGCTTCAG			R: GAGTACTTGCGCTCAGGAGGA
TIMP-2	NM_003255.4	F: TGTGCCCTCCCAGGCTTAGTGT	GAPDH**	-	F: GCACCGTCAAGGCTGAGAAC
		R: GCTGGCGTCACATGCAGAAAGC			R: ATGGTGGTGAAGACGCCAGT

MMP, matrix metalloproteinase; TIMP, tissue inhibitors of matrix metalloproteinases.
*, purchased from Sigma; **, purchased from Takara Bio.

NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1% NP-40 and protease inhibitor cocktail (Complete™; Roche Diagnosis, Berkeley, CA). The total proteins (10 μg each) were separated in a denaturing 7.5–12% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) as described elsewhere. The membranes were then blocked with 5% skim milk in TTBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) and subsequently incubated with first antibodies. Immunoreactive proteins were detected using horseradish peroxidase (HRP)-conjugated secondary antibodies and Super Signal kits (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. β-actin was used as an internal control of loading proteins. Quantitation of the protein levels was performed by densitometric scanning of each band using Image J software (NIH, Washington DC, USA). Densitometry readings for lysate samples were normalized to β-actin levels and expressed as relative induction. In addition, to confirm the equivalent levels of loaded proteins in culture supernatant, blotted membrane was stained with coomassie-brilliant blue. Densitometry readings for supernatant samples were measured, and fold changes of each band were expressed as a ratio of control (untreated cells).

Cell viability. Cell viability was examined using MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium Bromide] assay as described previously (29). In short, HGFs were seeded in each well of a 96-well plate (Corning Costar, Cambridge, MA, USA) in a final volume of 100 μL of DMEM supplemented with 0.5% FBS. After cells reached subconfluence, Cav-1 was applied in a dose dependent manner and kept for 24 h. At the end of the treatments, MTT reagent (final concentration: 0.5 μg/mL) was added to each well and incubated for 4 h prior to the addition of 100 μL of acid solution (0.04 N HCl in isopropanol) into each well. The reaction mixture on each well was measured fluorometrically using an auto plate reader (Model 680; Bio-Rad Lab., Hercules, CA; excitation at 570 nm).

ELISA. To examine the pro-matrix metalloproteinase (MMP)-1, VEGF, bFGF, IL-1ra and IL-8 secretion, cells were treated for 48 h with or without 500 ng/mL of Cav-1. To confirm the relation of MAPKs pathway on their secretion, the cells were also pretreated SP600125 (50 μM, JNK inhibitor) or PD98059 (50 μM, ERK inhibitor). HGFs were also treated with IL-6/sIL-6R (50 ng/mL each) as a positive control of VEGF secretion. Differentiated THP-1 macrophages were used as a positive control for IL-1ra secretion. Collected supernatants were stocked at −80°C until use. The amount of secreted proteins was measured using sandwich ELISA kits (R&D Systems) according to the manufacturer's instructions.

Statistical analysis. All experiments were repeated in triplicate at least. Statistical significances were determined by Student's *t*-test for *in vitro* assay or Mann-Whitney U test for pathological analysis. *P* value < 0.05 was considered statistically significant. All statistical analysis was performed with JMP 8.0.2 software (SAS Institute, Cary, NC).

RESULTS

Cav-1 expression is up-regulated in inflamed periodontal tissues compared to the healthy gingiva

By performing H-E staining, we observed infiltration of inflammatory cells such as mononuclear cells in human inflamed periodontal tissues (data not shown). Increased levels of Cav-1-positive cells were detected in inflamed periodontal tissues compared to that of healthy gingiva (Fig. 1A). Careful quantitative analysis using the described scoring system was performed, and we found both Cav-1-positive-fibroblastic and inflammatory cells were increased significantly in inflamed periodontal tissues relative to those of healthy gingiva as expected (fibroblastic cells: $P = 0.0056$; inflammatory cells: $P = 0.0040$, Mann-Whitney U test) (Fig. 1B).

Cav-1 is induced by pro-inflammatory cytokines in cultured periodontal fibroblastic cells

We found that Cav-1 mRNA expression increased significantly in HGFs treated with both IL-1β and TNF-α ($P < 0.05$, Student's *t*-test, compared to untreated cells) (Fig. 2A). As shown in Fig. 2B, production of intracellular Cav-1 was enhanced by IL-1β, but not TNF-α in HGFs. Importantly, Cav-1 secretion was enhanced dramatically by both IL-1β and TNF-α in culture supernatants of HGFs, compared to untreated cells (Fig. 2C). Furthermore, we demonstrated that IL-1β and TNF-α increased dramatically Cav-1 production of HPLFs in both cell lysate (Fig. 2D) and culture supernatant (Fig. 2E).

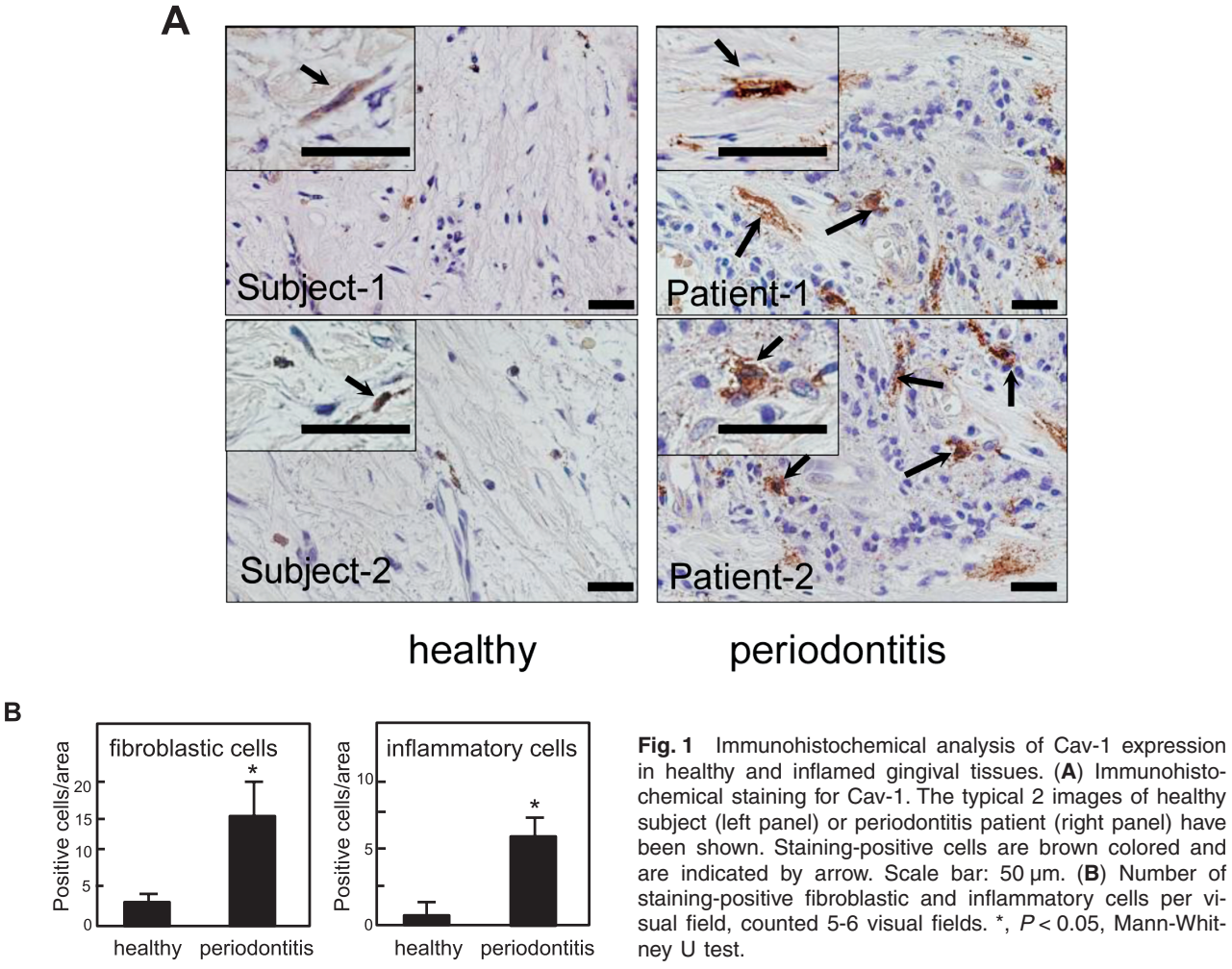

Fig. 1 Immunohistochemical analysis of Cav-1 expression in healthy and inflamed gingival tissues. (**A**) Immunohistochemical staining for Cav-1. The typical 2 images of healthy subject (left panel) or periodontitis patient (right panel) have been shown. Staining-positive cells are brown colored and are indicated by arrow. Scale bar: 50 μm. (**B**) Number of staining-positive fibroblastic and inflammatory cells per visual field, counted 5-6 visual fields. *, $P < 0.05$, Mann-Whitney U test.

Cav-1 has no effects on cell viability of HGFs

Cell viability of HGFs after Cav-1 stimulation was examined using MTT assay. No significant differences of the cell viability were observed in HGFs treated with any concentration of Cav-1 (Student's *t*-test) (Fig. 3A).

Cav-1 activates intracellular MAPKs signals in HGFs

To examine the effects of Cav-1 on intracellular signals, we performed Western blotting. Significant enhancement of phosphorylation of JNK was observed in HGFs treated with Cav-1 (1,000 ng/mL Cav-1: $P < 0.0001$; 100 ng/mL Cav-1: P = 0.0006, Student's *t*-test, compared to untreated cells) (Fig. 3B and 3C: middle panel). In addition, slight but statistical enhancement of phosphorylation of ERK was observed in HGFs treated with Cav-1 (1,000 ng/mL Cav-1: $P = 0.0215$; 100 ng/mL Cav-1: $P = 0.0872$, Student's *t*-test, compared to untreated cells) (Fig. 3B and 3C: bottom panel). Phosphorylation of Stat3 was not detected at any concentration of Cav-1 (Fig. 3B and 3C: upper panel).

Cav-1 induces the secretion of several inflammation-related molecules

To examine the effects of Cav-1 on protease secretion in HGFs, we focused matrix metalloproteinase (MMP)-1 as an important protease for gingival de-

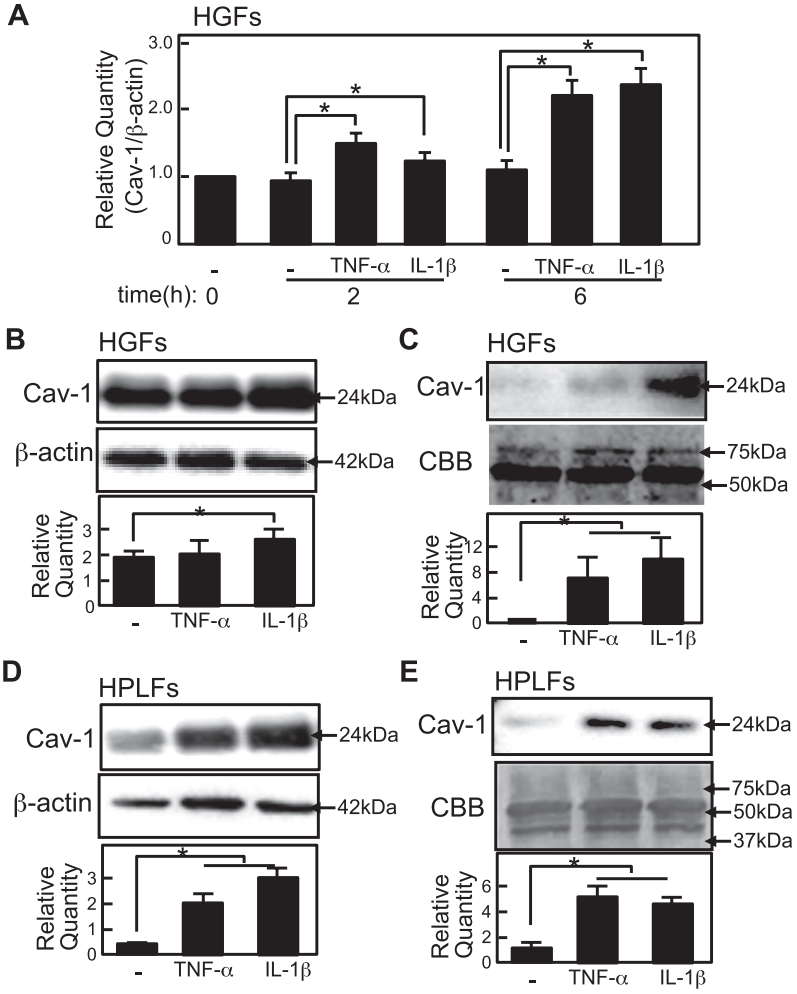

Fig. 2 Cav-1 production in periodontal fibroblastic cells. (**A**) Cav-1 mRNA expression of HGFs using qRT-PCR. Relative values of Cav-1 mRNA were normalized to that of β-actin levels and expressed as relative ratio. *, $P < 0.05$, Student's *t*-test. Production of (**B**) intracellular and (**C**) secreted Cav-1 in HGFs. Production of (**D**) intracellular and (**E**) secreted Cav-1 in HPLFs. Protein levels were analyzed using Western blotting. Results are shown as a representative data of 3 independent experiments. For quantitation of the protein levels in lysates, densitometry values were normalized to β-actin levels and expressed as relative induction. For quantitation of the protein levels in supernatants, fold changes of densitometry values were expressed as a ratio of control (untreated cells). *, $P < 0.05$, Student's *t*-test.

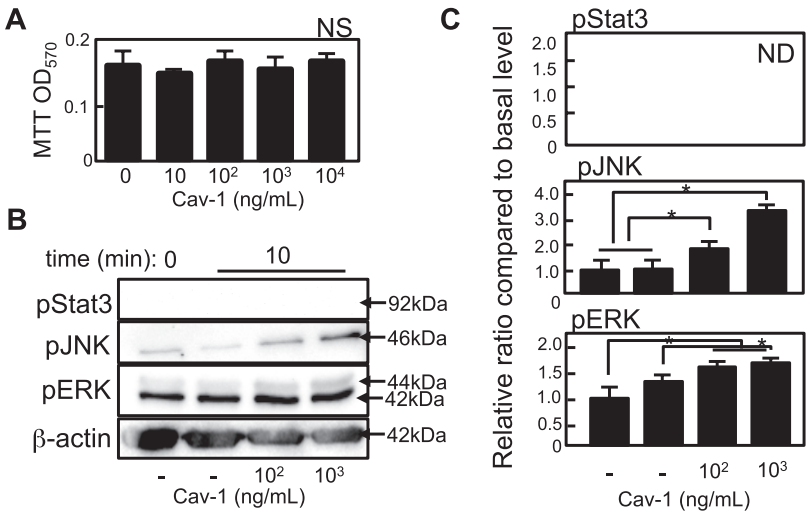

Fig. 3 Effects of Cav-1 on cell viability and intracellular signals in HGFs. (**A**) Cell viability was measured using MTT assay. Each sample was tested in 4 individual wells and repeated 3 times. Representative findings were shown as the mean ± SD of assays. NS, no significant (Student's *t*-test, compared to the data of untreated cells). (**B**) Detection of phosphorylated molecules (pStat3, pJNK and pERK) by Western blotting. Results are shown as a representative data of 3 independent experiments. (**C**) Densitometric analysis of the protein levels (pStat3, pJNK and pERK). Densitometry values were normalized to β-actin levels, and fold changes of each band were expressed as a ratio of control at 0 h. ND, not detected. *, $P < 0.05$, Student's *t*-test.

struction and measured proMMP-1 levels in supernatant using ELISA methods. As shown in Fig. 4A, Cav-1 increased significantly proMMP-1 secretion in HGFs ($P < 0.0001$, Student's *t*-test, compared to untreated cells). This enhancement of proMMP-1 secretion was suppressed significantly by pretreatment of both SP600125 (JNK inhibitor) and PD98059 (ERK inhibitor) in HGFs treated with Cav-1 (SP: $P = 0.0023$; PD: $P = 0.0004$, Student's *t*-test, compared to un-pretreated cells). In addition, in physiological condition (Cav-1 untreated), pretreatment of PD98059 slightly, but not statistically suppressed proMMP-1 secretion in HGFs ($P = 0.11$, Student's *t*-test, compared to un-pretreated cells).

Next, to examine the effects of Cav-1 on secretion of angiogenic factors in HGFs, we focused VEGF and bFGF as important factors for gingival angiogenesis. Cav-1 increased significantly VEGF secretion in HGFs ($P = 0.032$, Student's *t*-test, compared to untreated cells) (Fig. 4B). The increase of VEGF secretion by Cav-1 was suppressed statistically by SP600125, but not PD98059 in HGFs (SP: $P = 0.0002$; PD: $P = 0.85$, Student's *t*-test, compared to un-pretreated cells). VEGF levels in HGFs treated with IL-6/sIL-6R was enhanced significantly compared to that of Cav-1 treated-cells ($P < 0.0001$, Student's *t*-test). Additionally, in physiological condition (Cav-1 untreated), VEGF secretion was also inhibited by SP600125, but not PD98059 in HGFs (SP: $P = 0.047$; PD: $P = 0.42$, Student's *t*-test, compared to un-pretreated cells). On the other hand, Cav-1 had no significant effects on bFGF secretion in HGFs (Fig. 4C).

Finally, to examine the effects of Cav-1 on secretion of inflammation-related molecules in HGFs, we selected IL-1ra and IL-8. Interestingly, we found that Cav-1 increased significantly IL-1ra secretion in HGFs ($P = 0.0006$, Student's *t*-test, compared to untreated cells) (Fig. 4D), although the secreted levels of HGFs were quite low compared to PMA-treated THP-1 macrophages. In addition, enhancement of IL-1ra by Cav-1 was not suppressed statistically by both SP600125 and PD98059 (SP: $P = 0.29$; PD: $P = 0.95$, Student's *t*-test, compared to un-pretreated cells). Whereas, in physiological condition (Cav-1 untreated), IL-1ra secretion was inhibited by PD98059, but not SP600125 (SP: $P = 0.26$; PD: $P = 0.0001$, Student's *t*-test, compared to un-pretreated cells). On the other hand, Cav-1 has no significant effects on IL-8 secretion in HGFs (Fig. 4E).

Cav-1 induces mRNA expression of various inflammation-related molecules

To examine the effects of Cav-1 on expression of various mRNA in HGFs, we performed qRT-PCR (Fig. 5). Expression of various MMPs mRNA increased significantly in HGFs treated with Cav-1 (MMP-1, $P = 0.040$; MMP-10, $P = 0.0011$; MMP-13,

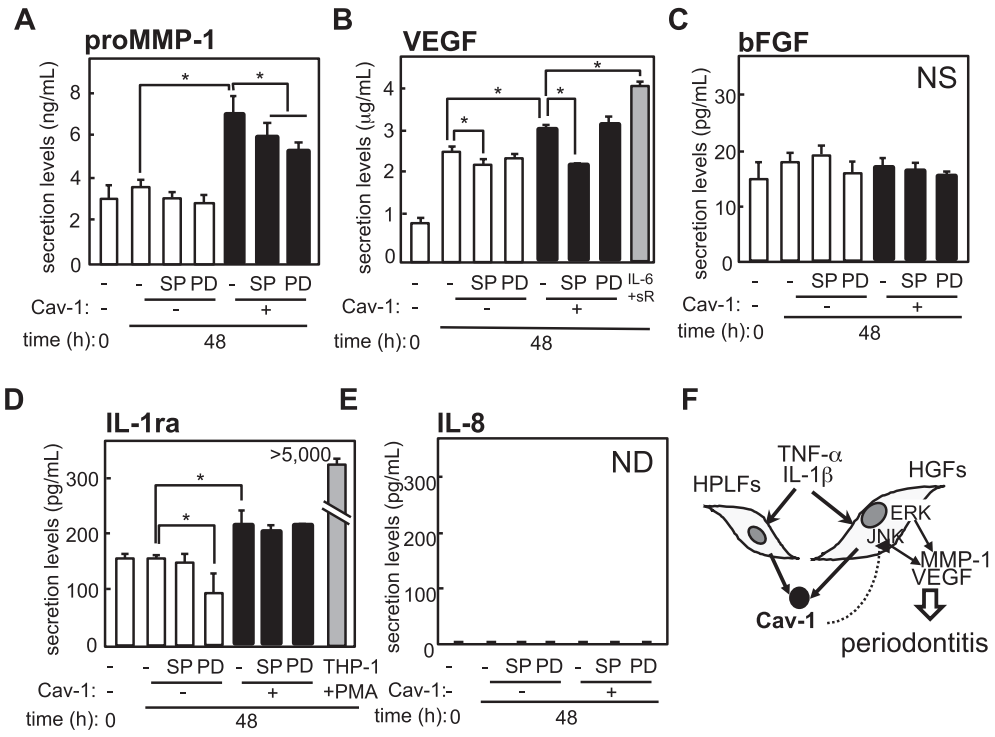

Fig. 4 Effects of Cav-1 on secretion of inflammatory molecules in HGFs. Levels of various molecules in the supernatants were measured using ELISA method. (**A**) proMMP-1, (**B**) VEGF, (**C**) bFGF, (**D**) IL-1ra, (**E**) IL-8. Data are expressed as means ± SD of triplicate cultures. ND, not detected. *, $P < 0.05$ (Student's *t* test). (**F**) Schematic representation of Cav-1 auto/paracrine loop surrounding HGFs. Proinflammatory cytokines, IL-1β and TNF-α induce Cav-1 secretion in periodontal fibroblastic cells. Secreted Cav-1 enhances secretion of various inflammatory mediators such as cathepsins, proMMP-1 and VEGF in HGFs *via* MAPKs pathway, resulting in severe periodontitis.

$P = 0.0001$, Student's *t*-test). Furthermore, MMP-2 mRNA expression was slightly up-regulated by Cav-1 ($P = 0.0933$, Student's *t*-test). No significant differences were observed in mRNA expression of both MMP-3 and MMP-14. Importantly, mRNA of tissue inhibitor of MMP (TIMP)-1, -2, -3, known as inhibitors of MMPs, also had no change in HGFs treated with Cav-1. Cysteine proteinases cathepsin L mRNA increased significantly ($P = 0.0190$, Student's *t*-test), and cathepsin B mRNA increased slightly but not statistically ($P = 0.0986$, Student's *t*-test). Angiogenic factor, insulin-like growth factor (IGF)-1 mRNA increased significantly ($P = 0.0055$, Student's *t*-test), whereas expression of EGF mRNA had no change in HGFs treated with Cav-1. Finally, we found Cav-1 did not increase inflammation-related cytokine IL-1ra and IL-33 mRNA expression.

DISCUSSION

Periodontitis is an infectious inflammatory disease (7). Several inflammatory mediators, *i.e.*, proteases, growth factors and inflammatory cytokines play an important role in periodontal inflammation (22). These molecules directly or indirectly stimulate connective tissue cells such as leukocytes, fibroblastic cells, mast cells and endothelial cells (8). Especially, elevated inflammatory cytokines-mediated fibroblasts contribute to the development of periodontitis (28). Fibroblasts such as HGFs are the most abundant cells in gingival connective tissues and play an important role in the control of inflammation (28, 29). Recently, we demonstrated that VEGF expression is significantly induced by IL-6 in the presence of sIL-6R in HGFs (20). Our finding suggests that IL-6 promotes angiogenesis *via* VEGF expression by HGFs, resulting in progression of periodontitis. Thus, although many researchers have reported about regulation of periodontal inflammation by various cytokines, the biological regulation by HGFs remains unclear.

Cav-1 is a multifunctional protein and major component of caveolae, serving important functions such as signal transduction, endocytosis, transcytosis and molecular transport (13). Tahir *et al.* (27) has reported that virulent prostate cancer cells secrete

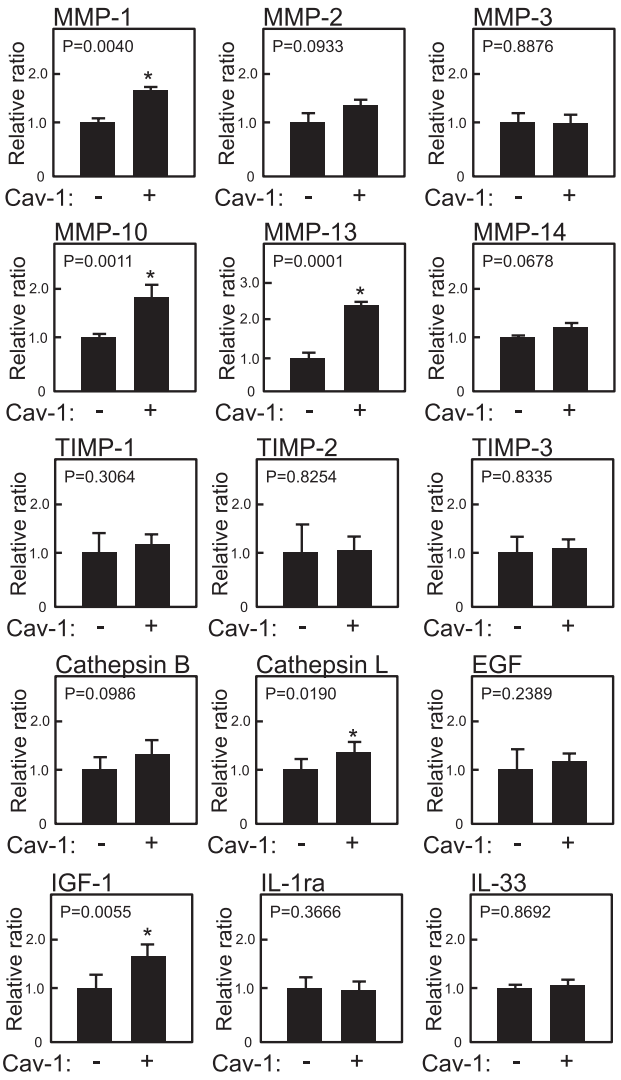

Fig. 5 Effects of Cav-1 on mRNA expression of various inflammation-related factors in HGFs. Expression of mRNA was examined by qRT-PCR. Relative values of each mRNA was normalized to that of GAPDH levels and expressed as relative ratio. *, $P < 0.05$, Student's *t*-test.

biologically active Cav-1 that is produced by endothelial cells, leading to stimulation of specific angiogenic activities. In the report, they have discussed that secreted Cav-1 might lead to the metastatic progression of prostate cancer. Therefore, we hypothesized that periodontal Cav-1 also plays an important role in pathophysiology of periodontitis. Interestingly, increased levels of Cav-1 were observed in inflamed periodontal tissues compared with healthy gingiva (Fig. 1). These findings encourage us that Cav-1 is involved in pathophysiology of periodontitis, although the precise function of Cav-1 remains unknown. Therefore, we investigated the possible mechanisms of Cav-1-mediated periodontitis more in depth.

Secretion of Cav-1

At first, we investigated whether Cav-1 is secreted from periodontal fibroblastic cells treated with pro-inflammatory cytokines, although Cav-1 is known as one of components of caveolae. As shown in Fig. 2A, expression of Cav-1 mRNA increased significantly in HGFs treated with IL-1β and TNF-α, thus Cav-1 production by these cytokines should be regulated at the gene expression levels. Furthermore, we demonstrated that intracellular Cav-1 expression promoted slightly (Fig. 2B), and both IL-1β and TNF-α also induced dramatically the Cav-1 secretion from HGFs (Fig. 2C). We have considered that the result is reasonable, because the detected band shown in Fig. 2B might be possible to be masked for highly expressed intracellular Cav-1 in HGFs. On the other hand, enhancement of both intracellular (Fig. 2D) and secreted (Fig. 2E) Cav-1 by IL-1β and TNF-α was observed in HPLFs. A series of results indicate for the first time that periodontal fibroblasts treated with pro-inflammatory cytokines such as IL-1β and TNF-α might be a source of Cav-1 in inflamed periodontal lesions. Secreted Cav-1 should be functional at initial stage of periodontal inflammation.

To clarify the function of secreted Cav-1 in periodontal lesions, we investigated the effects of Cav-1 on HGFs. First, we examined the effects of Cav-1 on cytotoxicity of HGFs using MTT assay. As shown in Fig. 3A, no significant differences were observed in cell viability of HGFs treated with any concentration of Cav-1. Next, we examined whether intracellular proteins are phosphorylated in HGFs treated with Cav-1. Interestingly, we found that 100 ng/mL of Cav-1 induced phosphorylation of JNK, but not Stat3 in HGFs, and 1,000 ng/mL of Cav-1 enhanced phosphorylation of JNK dramatically (Fig. 3B). In addition, Cav-1 enhanced phosphorylation of ERK slightly in HGFs. Although Cav-1 might activate intracellular MAPKs family in HGFs, it remains unknown whether Cav-1 binds specific receptor protein on cell surface. Li *et al*. has reported that overexpression of Cav-1 in Cav-1-negative cells using adenovirus vector system resulted in significantly increased levels of phosphorylated Akt (15). If effects of Cav-1 on HGFs depend on non-specific binding, secreted Cav-1 might accumulate in several caveolae domain of cell surface and lead to activation of downstream signaling such as MAPKs. Anyway, to clarify the molecular mecha-

nisms of secreted Cav-1 binding to HGFs, further experiments will be needed in future.

Proteases

To evaluate the effects of Cav-1 on HGFs function, we focused proteases, angiogenic factors and inflammatory factors. Degradation of extracellular matrix proteins is caused by various proteases including members of the following protease classes: matrix metalloproteinases (*e.g.* MMP-1, 2, 3, 10, 13 and 14), cysteine proteases (*e.g.* cathepsin B, C, and L), and serine proteases (*e.g.* plasmin) (2, 14). Finally, the process of degradation is likely to take place particularly within a lysosomal apparatus after phagocytosis of the partial digestion of matrix proteins (3). As shown in Fig. 4A, Cav-1 increased proMMP-1 secretion in HGFs, corresponding to the result of mRNA expression (Fig. 5). It is well known that MMPs are observed in inflamed lesions such as rheumatoid synovial tissues, and MMPs work at neutral pH (16). However, the local area in inflamed lesion has an acidic pH at attachment sites of macrophages and osteoclasts (9). Since chronic periodontitis is one of local inflammatory diseases with bone resorption, local acidic conditions may increase the action of cathepsins rather than MMPs. As shown in Fig. 5, Cav-1 increased significantly cathepsin L mRNA expression in HGFs. Cathepsin L is a cysteine protease with broad specificity that is responsible for degradation of structural proteins such as type I collagen, laminin and proteoglycans (4). Of course, cathepsin L degrades directly gingival collagen fibers because type I collagen is dominant in gingival connective tissue (34). Furthermore, Ikeda *et al*. have reported that cathepsin L not only directly degrades type I collagen, but also converts pro-urokinase into urokinase (11). Urokinase activates plasminogen to plasmin, and plasmin activates MMPs (11). Thus, cathepsin L is extracellularly released into the inflamed periodontal tissue, and may be involved in the destruction of connective tissue by directly degrading collagens or by activating the fibronolytic protease cascade. Therefore, our finding might indicate that Cav-1 induces tissue degradation as seen in chronic periodontitis by producing cathepsin L from HGFs. Next, we examined intracellular signaling that regulates Cav-1 mediated-protease production in HGFs. ProMMP-1 induction by Cav-1 was found to be inhibited dramatically by both SP600125 and PD98059 in HGFs. Thus, Cav-1 might induce proMMP-1 secretion, at least in part, *via* JNK and ERK pathway in HGFs. In addition, we found that Cav-1 increased significantly mRNA expression of both MMP-10 and MMP-13 in HGFs (Fig. 5). Importantly, since mRNA expression of all type of TIMPs, inhibitors of MMPs, did not change in HGF treated with Cav-1, effects of MMPs should be significant in degradation of periodontal tissues.

Angiogenesis

Angiogenesis consists of multiple processes such as degradation of vascular basement membranes surrounding extracellular matrix, migration and proliferation of endothelial cells (14). The angiogenic responses play an important role in developing chronic inflammation as seen in inflamed periodontal lesions (31). Although several angiogenic mediators have been reported, VEGF is known to be a powerful regulator of angiogenesis (23). It has been generally accepted that VEGF plays an important role in the regulation of inflammatory periodontal disease (12). Recently, it was reported that both Cav-1 and VEGF co-expressed in prostatic intraepithelial neoplasia (30). In addition, Liu *et al.* has reported that overexpression of Cav-1 accelerated tubule formation, a prerequisite step in the process of angiogenesis, in endothelial cells (17). Furthermore, it has been shown that VEGF-induced ERK1/2 activation is compartmentalized in the placental endothelial cell caveolae, which is important for endothelial cell migration, proliferation and differentiation (5). Thus, Cav-1 may co-relate to VEGF in inflamed lesions, leading to angiogenesis. Previously, we have reported that IL-6/sIL-6R promotes VEGF secretion in HGFs, and it is considered that HGFs possess the ability of VEGF production (20). Therefore, we examined whether secreted Cav-1 induces VEGF secretion in HGFs. As shown in Fig. 4B, Cav-1 induced significantly VEGF secretion in HGFs. This finding indicates for the first time that secreted Cav-1 should be a direct inducer of VEGF production in HGFs. The increase of VEGF by Cav-1 was relatively low compared to that of IL-6/sIL-6R stimuli. The finding might depend on the differences of biological potential between inflammatory cytokine and cell membrane component. Previously, we have reported that VEGF production in HGFs is regulated by JNK-AP-1 pathway mainly (20). Since Cav-1 induced JNK phosphorylation in HGFs (Fig. 3B), we examined whether VEGF secretion induced by Cav-1 is inhibited by JNK inhibitor SP600125. Importantly, the VEGF induction by Cav-1 was inhibited statistically by SP600125, but not PD98059 in HGFs (Fig. 4B). These findings indicate the Cav-1 induces VEGF secretion *via* JNK pathway in HGFs.

Whereas we obtained promoter sequences of VEGF gene from GenBank (accession No. AF0957851), and searched possible binding sequences of AP-1, exiting in downstream of JNK, on their promoter using SIGSCAN version 4.05. As expected, several consensus sequences including binding sites for AP-1 was found in the promoter of the VEGF gene. These results encourage our finding that phospho-JNK induced by Cav-1 activates its downstream transcription factor AP-1, resulting in VEGF secretion in HGFs. On the other hand, no significant differences were observed in bFGF production in HGFs treated with Cav-1 (Fig. 4C). Therefore, Cav-1-mediated angiogenesis in periodontitis lesions might be regulated by VEGF rather than bFGF.

Cytokines

Antagonists/agonists against inflammatory molecules are intermingled in periodontal lesions (28). When we examined the effects of Cav-1 on cytokines production, Cav-1 enhanced significantly secretion of IL-1ra, antagonist of IL-1, in HGFs (Fig. 4D), although the mRNA expression did not change in HGFs treated with Cav-1 for 6 h (Fig. 5). To detect the increase of IL-1ra mRNA expression, HGFs must be maintained on different culture period. These results might indicate that Cav-1 is possible to be a negative regulator of IL-1 in periodontitis lesions. However, since we found that PMA-differentiated THP-1 macrophage produced over 100-fold IL-1ra compared to HGFs, it has been unclear whether IL-1ra secreted by HGFs down-regulates sufficiently the IL-1 function.

According to recent studies, periodontitis, persistent low-grade infection of Gram-negative bacteria, is associated with increased atherosclerosis, cardiovascular disease, diabetes mellitus, and other systemic diseases through the blood stream (19). Indeed, in consistent with clinical studies, periodontitis patients have elevated systemic inflammatory markers caused by immune responses, such as C-reactive protein and IL-6 (24). On the other hand, postoperative prostate cancer patients with high level of serum Cav-1 seem to be experienced significantly higher risk of recurrence than those with low levels (25). Although Cav-1 derived from periodontal fibroblasts might possible to be significant risk of cancer metastasis clinically, further investigation will be needed in future.

Taken together, secreted Cav-1, as known a membrane structure protein, might be a potent inducer of gingival inflammation (Fig. 4F). The secreted Cav-1 might be possible to contribute to establish a unique concept of periodontitis progression following angiogenesis or tissue degradation. Autocrine or paracrine loop of Cav-1 surrounding HGFs may lead to severe periodontitis, and might be attractive target for regulation of the disease.

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