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Porphyromonas gingivalis gingipains cause G_1 arrest in osteoblastic/stromal cells

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Introduction: The program for mammalian cell growth and division consists of four successive phases; G_1, S, G_2, and M. *Porphyromonas gingivalis* may manipulate the host cell cycle to benefit bacterial virulence expression, which likely causes the cell and tissue tropism observed in chronic periodontal infections. We examined *P. gingivalis* for its effects on cell-cycle modulation in mouse ST2 osteoblastic/stromal cells.
Methods: Synchronized ST2 cells were infected with *P. gingivalis* ATCC33277 (wild-type, WT), gingipain-mutants [KDP136 (Δ*rgpA*Δ*rgpB*Δ*kgp*), KDP129 (Δ*rgpA*Δ*rgpB*), and KDP133 (Δ*kgp*)], and a fimbria-deficient mutant (KDP150) for 24 h, then the cell cycle was evaluated using flow cytometry. Cell-cycle-related molecule expression was examined with a microarray, as well as with quantitative real-time polymerase chain reaction and Western blotting assays.
Results: Both the WT and KDP150 strains significantly inhibited cellular proliferation and arrested the cell cycle in the G_0/G_1 phase, while the expression levels of the cell-cycle regulatory molecules cyclin D and cyclin E were also decreased. In contrast, KDP136 did not show any effects. G_1 arrest was also clearly induced by KDP129 and KDP133, with KDP129 being more effective.
Conclusion: The present findings suggest that *P. gingivalis* gingipains reduce cyclin expression and cause early G_1 arrest, leading to the inhibition of cellular proliferation.

Key words: cell cycle; cyclin D; cyclin E; G_1-arrest; gingipains; microarray; *Porphyromonas gingivalis*

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Adult marginal periodontitis is a chronic inflammatory disease of the supporting tissues of the teeth and is one of the most common infectious diseases encountered in humans (33). *Porphyromonas gingivalis*, a gram-negative, black-pigmented anaerobe that has been characterized as a bona fide periodontal pathogen, expresses a number of potential virulence factors, including fimbriae and gingipains (11). Fimbriae reportedly mediate bacterial adherence to and invasion of periodontal cells (1), while gingipains, which consist of arginine-X [Arg-gingipain A and B (RgpA and RgpB)]-specific and lysine-X [Lys-gingipain (Kgp)]-specific cysteine proteinases, contribute to the pathogenesis of periodontitis (12). Gingipains degrade both collagen and fibronectin and they inhibit interactions between host cells and the extracellular matrix. Gingipains also degrade various cytokines, resulting in a disturbance of the host cytokine network. These bacterial factors contribute differentially to the progression of overall inflammatory destruction in infected periodontal tissues.

The program for mammalian cell growth and division (proliferation) consists of four successive phases; G_1, S, G_2, and M (28). Checkpoints in G_1 and G_2 act as safety controls to prevent cells from proceeding to the next phase in response to cell injury or environmental signals. The transition that occurs at the G_1 checkpoint commits the cell to the proliferative cycle (4) and if the conditions that signal this transition are not present, then the cell enters a non-proliferative phase called G_0 (25). Progression through the cell cycle is controlled by complexes of cyclins and cyclin-dependent kinases. The complexes of cyclin D (D1, D2, and D3) and cyclin-dependent kinases 4/6 control the G_1 checkpoint, and are important regulators of the activated transition from the G_0/G_1 phase to the G_1/S phase (28). In late G_1, high levels of cyclin E/cyclin-dependent kinase 2 drive the cell through the G_1/S transition to the G_2 phase.

Several bacterial pathogens have been shown to manipulate the host cell cycle to benefit bacterial survival and virulence expression within the host. For example, *Helicobacter pylori* exotoxin vacA is known to trigger G_1 cell-cycle arrest, while cyto-lethal distending toxin, which is produced by various bacteria, causes cell-cycle arrest in G_2 (32). Enteropathogenic *Escherichia coli* was also found to produce a cycle-inhibiting factor that induced cell-cycle arrest in G_2 (24). These effects are thought to be linked to several DNA-damaging agents, such as bacterial exotoxins, and cell-cycle arrest has been shown to inhibit cellular proliferation and induce apoptosis (32). In contrast, *H. pylori* cytotoxin-associated antigen A moves cells into the S phase and increases the turnover of gastric epithelial cells, so increasing the risk for development of neoplastic clones (27).

Several recent studies have shown that osteoblasts and bone marrow stromal cells are responsible for the development of local inflammatory responses (10, 37). We previously profiled alterations of gene expression in a mouse ST2 stromal cell line with preosteoblastic characteristics after infection with *P. gingivalis* (29). Those results showed that the expression of a variety of genes for chemokines, cytokines, and other proinflammatory factors were upregulated following bacterial infection. However, there is not enough information regarding the influence of *P. gingivalis* infection on the cell cycle and the involved factors of *P. gingivalis*. If this pathogen induces cell-cycle arrest, then it may be a factor that causes local immunosuppression and destruction of periodontal tissues. In the present study, we analyzed the effects of *P. gingivalis* infection on cellular proliferation and the cell cycle. Based on our results, we identified novel features of *P. gingivalis* related to host cell-cycle modification caused by the downregulation of cyclin expression and the arrest of the cell cycle in G_0/G_1, which may provide an explanation for the cell and tissue tropism observed in chronic periodontal infections.

Materials and methods

Bacterial strains

Five strains of *P. gingivalis* — ATCC33277, KDP136 (a triple-deficient mutant for three gingipains, RgpA, RgpB, and Kgp of ATCC33277) (35), KDP150 (a fimbria-deficient mutant of ATCC33277) (39), KDP129 (RgpA- and RgpB-deficient mutant of ATCC33277) (26), and KDP133 (Kgp-deficient mutant of ATCC33277) (31) — were grown under anaerobic conditions in trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD) supplemented with yeast extract (0.1%; BBL), hemin (5 μg/ml; Sigma, St Louis, MO), and menadione (1 mg/ml; Sigma) as described previously (18). Activities of the gingipains, including Rgp and Kgp, of these strains were determined as described previously (13), and no loss of mutations was confirmed (20).

Bacterial cells were harvested by centrifugation, then extensively washed twice with phosphate-buffered saline (PBS) and resuspended in alpha minimum essential medium (αMEM; Gibco-BRL, Grand Island, NY) containing no antibiotics. The numbers of bacteria were determined with a spectrophotometer at an optical density of 600 nm, based on a standard curve established with a colony formation assay, as described previously (18).

Cell culture and infection protocol

Mouse ST2 cells (bone marrow cell line) were obtained from the Riken Bioresource Center (Tsukuba, Japan) and were grown at 37°C with 5% CO_2 in αMEM supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco-BRL) for normal growth or without FCS to induce starvation. Sub-confluent cultures of ST2 cells (1.0×10^5 cells/well of culture dish) were synchronized in the G_0/G_1 phase by serum starvation as described previously (6). Following synchronization, the cells were subsequently infected with various viable *P. gingivalis* strains at a multiplicity of infection (MOI) of 100 (1.0×10^7 cells/well of culture dish) at 37°C with 5% CO_2 in αMEM without FCS. Six hours after infection, aliquots of ST2 cells were washed with PBS and the total RNA was extracted from each to evaluate the gene expression profile; this was performed using an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. For other assays, the remaining ST2 cells were washed three times with PBS to remove external non-adherent bacteria and further incubated in fresh αMEM containing 10% FCS, gentamicin (300 μg/ml), and metroimidazole (200 μg/ml) for various time periods.

GeneChip microarray

The qualities of the RNA samples extracted from the cells 6 h after infection were analyzed using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). Samples (5 μg) of total RNA were then converted to first-strand complementary DNA (cDNA) by reverse transcription using T7-oligo(dT) Promoter Primer (Affymetrix Inc., Santa Clara, CA) and SuperScriptII RNase H-reverse transcriptase (Invitrogen Co., Carlsbad, CA). Double-stranded cDNA was synthesized using *E. coli* RNase H, *E. coli* DNA polymerase I, and *E. coli* DNA ligase (Invitrogen Co.), then purified using a GeneChip Sample Cleanup Module (Affymetrix Inc.). Biotinylated cRNA samples were amplified from double-stranded cDNA by *in vitro* transcription with 3′-Amplification Reagents for IVT Labeling (Affymetrix Inc.) and purified using a GeneChip Sample Cleanup Module.

After confirming the quantity and purity of the biotinylated cRNA spectrophotometrically, 20 mg cRNA was fragmented by mild alkaline treatment for 35 min at 94°C using a GeneChip Sample Cleanup Module. The fragmented cRNA (6.5 μg) was then hybridized using a Mouse Genome 430A 2.0 GeneChip Array (Affymetrix Inc.) according to the manufacturer's protocol, including a test chip. Briefly, 10 μg fragmented biotinylated cRNA was placed in a hybridization cocktail containing four different biotinylated hybridization controls (1.5 pM BioB, 5 pM BioC, 25 pM BioD, 50 pM Control Oligonucleotide B2, and 100 pM Cre; Affymetrix Inc.), 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated bovine serum albumin, and 10% dimethyl sulfoxide, then hybridized for 16 h at 45°C with constant rotation (60 r.p.m.). The arrays were washed first with non-stringent buffer (6× SSPE (3 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA, pH 7.4) and 0.01% Tween-20) and then stringent buffer (100 mM MES (2-Morpholinoethanesulfonic acid, monohydrate), 0.1 M NaCl, and 0.01% Tween-20), after which they were stained with a streptavidin–phycoerythrin-R conjugate (SAPE) (Invitrogen) using a GeneChip Fluidics Station 450 (Affymetrix Inc.).

The signals were amplified using goat biotinylated antistreptavidin antibody and stained again with SAPE. Finally, the arrays were scanned using a GeneChip Scanner 3000 (Affymetrix Inc.) and the scanned images were analyzed using GeneChip Operating Software Version 1.1. Functional categorization by gene ontology and bioinformatics analyses were performed with Genespring GX Ver7.3.1 according to the Kyoto Encyclopedia of Genes and Genomes nomenclature (http://www.genome.jp/kegg/).

Real-time reverse transcription polymerase chain reaction (RT-PCR)

The RNA samples were reverse-transcribed in the presence of oligo$(dT)_{15}$ using the SuperScript first-strand synthesis system (Bio-RAD, Hercules, CA), according to the manufacturer's instructions. To quantify messenger RNA (mRNA), real-time PCR was performed using a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) with SYBR Green PCR reagent (Qiagen GmbH). Samples were subjected to 35 cycles of amplification at 95°C for 15 s, followed by 57°C for 20 s and 72°C for 25 s. The amplification reaction was performed with the following primers. For cyclin D1 the forward primer was 5′-GCCGAGAAGTTGTGCATC-TAC-3′ and the reverse primer was 5′-CTTCACATCTGTGGCACAGAG-3′, for cyclin D2 the forward primer was 5′-GTAAGCTGAAGTGGAACCTG-G-3′ and the reverse primer was 5′-CGG-ATCTTCCACAGACTTGGA-3′, for cyclin E2 the forward primer was 5′-CCAGTAACAGTCATCTCCTGG-3′ and the reverse primer was 5′-CCA-TTACACACTGGTGACAGC-3′, and for glyceraldehyde-3-phosphate dehydrogenase the forward primer was 5′-GTCTTCACCACCATGGAGAAG-3′ and the reverse primer was 5′-GTTGTCATG-GATGACCTTGGC-3′. Each assay was normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA as described previously (19). The normalized data are expressed as the fold increase from the mRNA level of uninfected cells. All assays were performed in triplicate on three separate occasions (n = 9).

Cell proliferation and cell-cycle assay

Six hours after infection, ST2 cells were further cultured in fresh medium as noted above. The numbers of viable cells were determined using a trypan blue dye exclusion method and cells were counted. ST2 cells were stained with propidium iodide using a CycleTest Plus DNA Reagent Kit (Becton-Dickinson, Franklin Lakes, NJ), and subsequently analyzed using a fluorescence-activated cell sorter (FACS; Becton-Dickinson), which acquired 10,000 events. Data were analyzed using ModFit 3.0 software (Verity Software House, Topsham, NH).

Immunoblotting

Twelve hours after infection, ST2 cells were washed with ice-cold PBS containing 10 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK; Wako, Tokyo, Japan) and then dissolved in Triton-lysis buffer [50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 10 mM TLCK, 6.25 mM NaF, 12.5 mM β-glycerophosphate, 12.5 mM *p*-nitrophenyl phosphate, 1.25 mM $NaVO_3$, and a 1% protease inhibitor cocktail (Complete Protease Inhibitor Cocktail; Roche Diagnostics, Basel, Switzerland)]. The soluble fractions were collected by centrifugation at 15,000 ***g*** for 5 min at 4°C, after which immunoblotting was performed as described previously (18). The membranes on which the cellular proteins were transferred were then incubated with monoclonal antibodies against cyclin D1 (Calbiochem, La Jolla, CA), cyclin D2 (Abcam, Inc, Cambridge, MA), and β-actin (Sigma), and with polyclonal antibodies against cyclin E2 (SantaCruz Biotechnology Inc., Santa Cruz, CA) and β-actin. The reacted protein bands were visualized using ECL plus reagent (GE Healthcare Life Sciences, Uppsala, Sweden).

Adhesion and invasion assays

Immortalized human gingival epithelial cells were kindly provided by Prof. Murakami (Osaka University, Japan) and maintained in Humedia KB-2 (Kurabo, Osaka, Japan) as described previously (22). Adhesion to and invasion of ST2 cells or immortalized human gingival epithelial cells by *P. gingivalis* were quantified using a scintillation counting assay, as described previously (18).

Statistical analysis

All data are presented as the mean ± SD. Statistical analyses were performed using an unpaired Student's *t*-test.

Results

Inhibition of cellular proliferation and arrest of cell cycle at G_1 by *P. gingivalis*

Synchronized ST2 cells were infected with viable *P. gingivalis* strains at an MOI of 100 in serum-free medium to avoid non-specific biological effects of growth factors or hormones contained in FCS. Six hours after infection, the cells were washed and further incubated in fresh αMEM containing 10% FCS and antibiotics. Ten hours after infection there were negligible differences in proliferation efficiency among cells infected with the various *P. gingivalis* strains (Fig. 1A). However, 24 h after infection, the wild-type (WT) and KDP150 (fimbria-deficient mutant) strains were found to significantly inhibit cellular proliferation compared to the control cells, whereas KDP136 (gingipain-deficient mutant) did not show an inhibitory effect.

Next, we used flow cytometry to examine whether *P. gingivalis* infection had an influence on the cell cycle. Ten hours after infection, there was no marked difference in cell-cycle progression among the control cells and various infected cells, and more than 90% of all cells remained in the G_0/G_1 phase (Fig. 1B). However, 24 h after infection, the numbers of KDP136-infected and control cells in the S and G_2/M phases were greatly increased, whereas the cells treated with the WT and KDP150 strains showed cell-cycle arrest in the

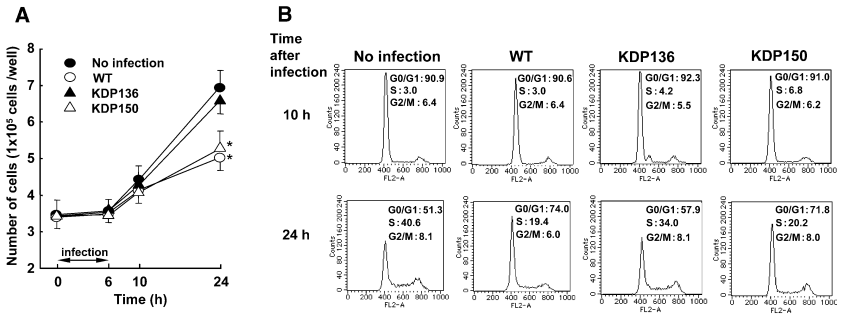

Fig. 1. Effects of gingipains on cellular proliferation and cell cycle. (A) Synchronized ST2 cells were infected with various *Porphyromonas gingivalis* strains at an MOI of 100 in serum-free medium. Six hours after infection, the cells were washed and further incubated in fresh αMEM containing 10% FCS and antibiotics. The numbers of viable cells were determined using a trypan blue dye exclusion method, as described in the text. WT, wild-type strain; KDP136, gingipain-deficient mutant; KDP150, fimbria-deficient mutant. *$P < 0.01$. (B) Six hours after infection, the cells were washed and further incubated in fresh αMEM containing 10% FCS and antibiotics. At 10 and 24 h after infection, cellular DNA contents were examined using flow cytometry. Insets indicate cell-cycle distribution (% of total cells).

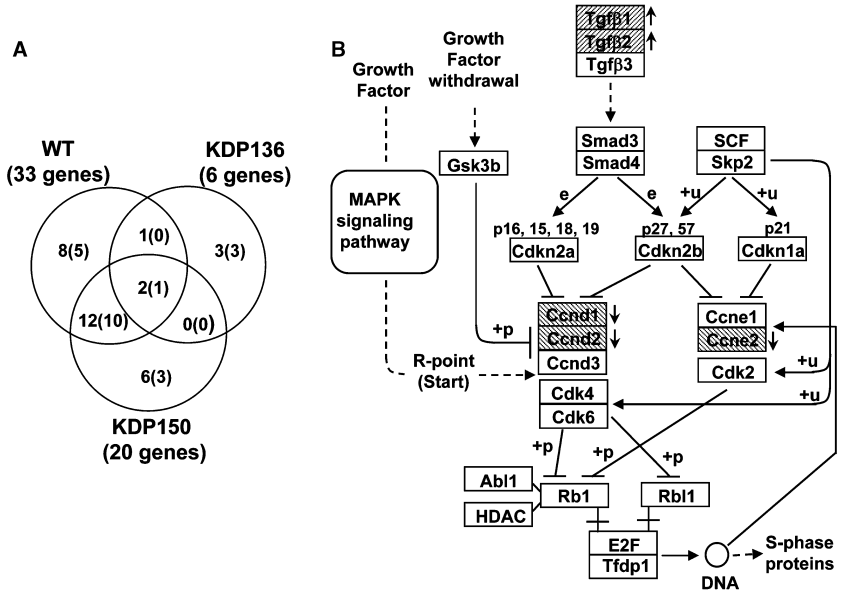

Fig. 2. Cell-cycle-related genes influenced by *Porphyromonas gingivalis* infection. (A) Venn diagram for schematic representation of cell-cycle-related genes influenced by infection with *P. gingivalis* stains. A total of 376 genes have been classified in the cell-cycle-related category by gene ontology. Of those, the numbers of influenced genes are shown, with the number of genes of which expression was decreased shown in parentheses. Genes were determined to be differentially expressed if there was at least a 2.0-fold difference in average expression value. (B) Cell-cycle-related pathway indicating *P. gingivalis*-influenced genes. Molecules marked were influenced as follows; ↑, upregulation; ↓, downregulation; +p, phosphorylation; −p, dephosphorylation; +u, ubiquitination; e, expression. Pointed arrow indicates molecular interaction resulting in cell-cycle progression and flat arrow indicates molecular interaction resulting in cell-cycle inhibition.

G_0/G_1 phase. These results suggest that gingipains have inhibitory effects on cellular proliferation and cell-cycle progression in the G_0/G_1 phase.

Microarray assays

To investigate further the effects of *P. gingivalis* infection on the cell cycle, microarray assays were performed to examine the expression levels of various cellular proteins involved in the cell cycle. A total of 376 genes have been classified in the cell-cycle-related category (GO7049) by gene ontology analysis and were determined to be differentially expressed when there was at least a 2.0-fold difference in average expression value. Among those 376 genes, WT infection altered the expression of 33 genes, while 20 genes showed a response to KDP150 infection (Fig. 2A). In contrast, KDP136 infection influenced only six of those genes. Figure 2B demonstrates the cell pathway that directly controls the cell cycle and, among the molecules listed there, the expression of only five was clearly influenced by *P. gingivalis* infection, as shown in Table 1. Expression of *Tgfβ1* and *Tgfβ2*, which exhibit antiproliferative effects (23), was increased, whereas that of *Ccnd1* (cyclin D1), *Ccnd2* (cyclin D2), and *Ccne2* (cyclin E2), which activate the cell cycle, was decreased. Other cell-cycle-related molecules shown in Fig. 2B were negligibly influenced (less than 2.0-fold). In contrast, KDP136 showed little effect on cell-cycle-related gene expression. These results also strongly suggest that *P. gingivalis* gingipains arrest the cell cycle in the G_0/G_1 phase.

Table 1. Cell cycle-related genes upregulated or downregulated in ST2 cells infected with various *Porphyromonas gingivalis* strains

Cell-cycle-related genes			Gene expression (fold)		
			P. gingivalis strains used		
Common name	GenBank accession no.	Description	WT	KDP136	KDP150
Tgfβ1	NM_011577	Transforming growth factor, beta 1	2.36	NS	2.19
Tgfβ2	BF144658	Transforming growth factor, beta 2	2.84	NS	2.52
Ccnd1	NM_007631	Cyclin D1	0.39	NS	0.29
Ccnd2	AV310588	Cyclin D2	0.33	NS	0.27
Ccne2	AF091432	Cyclin E2	0.33	NS	0.33

NS, difference was less than 2.0-fold.

Decreased expression of *cyclinD1*, *cyclinD2*, and *cyclinE2* by *P. gingivalis* gingipains

Since microarray analysis demonstrated that the expression of *cyclinD1*, *cyclinD2*, and *cyclinE2* was significantly reduced by infection with either WT or KDP150, those effects were confirmed using quantitative real-time PCR and Western blotting assays (Fig. 3). Gene expression for *cyclinD1*, *cyclinD2*, and *cyclinE2* was significantly reduced by infection with WT and KDP150, whereas the effect of KDP136 was negligible (Fig. 3A). Similar effects were observed at the protein level, i.e. the cyclin D1, cyclin D2, and cyclin E2 proteins were clearly inhibited by infection with WT and KDP150 infection, but not by infection with KDP136 (Fig. 3B). Thus, it was confirmed that *P. gingivalis* gingipains delayed cell-cycle progression.

Involvement of Rgp and Kgp in G1 arrest

As noted previously, gingipains have been classified into two isozymes, Rgp and Kgp (12). To examine which of these plays a critical role in cell-cycle arrest, the effects of KDP129 (an Rgp-deficient mutant) and KDP133 (a Kgp-deficient mutant) were studied. At 24 h after infection, both KDP129 and KDP133 were found to inhibit cellular proliferation, though the effect of KDP129 was slightly greater (Fig. 4A). Arrest of G_1 was also clearly induced by KDP129 and KDP133, with KDP129 being more effective, suggesting that Kgp arrests the cell cycle more efficiently than Rgp. The profiles of gene expression (Fig. 4B) and protein production (Fig. 4C) also indicated the involvement of Kgp and Rgp, with the effect of Kgp apparently more effective. Together, these results suggest that *P. gingivalis* reduces cellular protein levels of cyclin D and cyclin E by the activities of its gingipains, especially Kgp, and causes early G_1 arrest, which leads to the inhibition of cellular proliferation.

Bacterial interaction with ST2 and HE cells

P. gingivalis fimbriae are a critical factor in the mediation of bacterial adhesion to

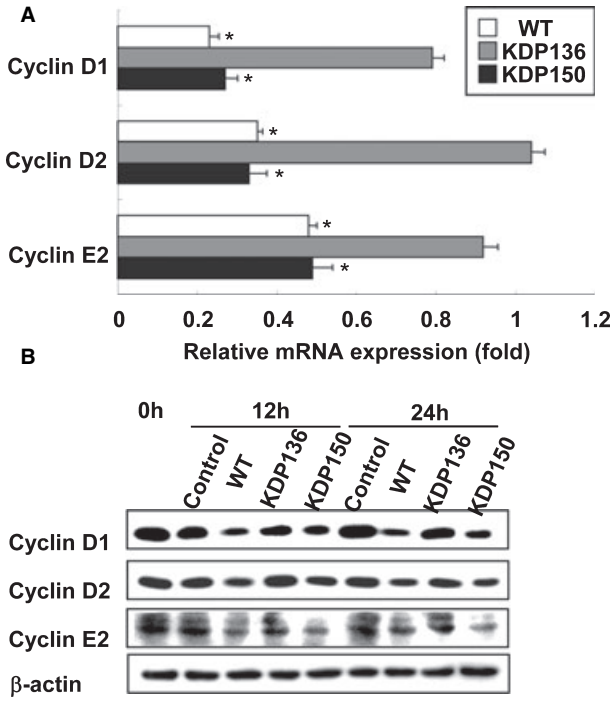

Fig. 3. Decreased expression of *cyclinD1*, *cyclinD2*, and *cyclinE2* by gingipain-positive *Porphyromonas gingivalis* infection. (A) Six hours after infection, RNA samples were prepared, and gene expression for *cyclinD1*, *cyclinD2*, and *cyclinE2* was assayed using real-time PCR. The gene expression level is shown as relative to the control (non-infection). WT, wild-type strain; KDP136, gingipain-deficient mutant; KDP150, fimbria-deficient mutant. $^*P < 0.01$. (B) At 12 and 24 h after infection, ST2 cells were dissolved and soluble fractions were subjected to immunoblotting. The protein bands shown were probed with antibodies against cyclin D1, cyclin D2, cyclin E2, and β-actin.

and invasion of various types of host cells and they contribute to the expression of bacterial virulence (3). However, the present results did not indicate the involvement of fimbriae in cell-cycle arrest. We therefore examined the adhesive/invasive efficiency of the *P. gingivalis* WT strain with ST2 and immortalized human gingival epithelial cells. When infected at an MOI of 100, 14.2 ± 0.4% of the bacteria adhered to human gingival epithelial cells and 12.6 ± 0.3% of those invaded the cells. In contrast, only 2.1 ± 0.2% of the bacteria adhered to ST2 cells and 1.4 ± 0.1% of those subsequently invaded. Thus, the role of *P. gingivalis* fimbriae in ST2 cell infection is likely very limited.

Discussion

Bacteria can gain considerable advantages by causing cellular phenotypic changes in the host cells that arrest the cell cycle of those host cells, possibly resulting in increased bacterial survival (27, 32). Cell-cycle arrest of immune cells causes local immunosuppression as well as inhibition of cellular proliferation. Many studies have shown that infection with gram-negative bacteria causes an upregulation of cyclins, thereby arresting the cells in G_2/M and inhibiting cells re-entering G_1, leading to high levels of mitotic activity and malignancies (5, 7, 24, 36). However, that is not the case with *P. gingivalis* infection. Our present findings are the first known report of cyclin downregulation and G_1 arrest as a result of *P. gingivalis* infection.

Arrest at G_0/G_1 by *Trypanosoma brucei* (34) and *Neisseria gonorrhoeae* (16) infections has been reported, and was considered to account for reduced cell responsiveness during chronic infection. It is tempting to speculate that these pathogens can develop long-term asymptomatic chronic infections by arresting target cells in a non-proliferating phase (16). G_1 arrest would also enable *P. gingivalis* to block the ability of cells to reach the important G_1/S checkpoint, which is essential for cellular growth. Further, low levels of metabolism in non-cycling cells may reduce the levels of innate immune response, such as cytokine production

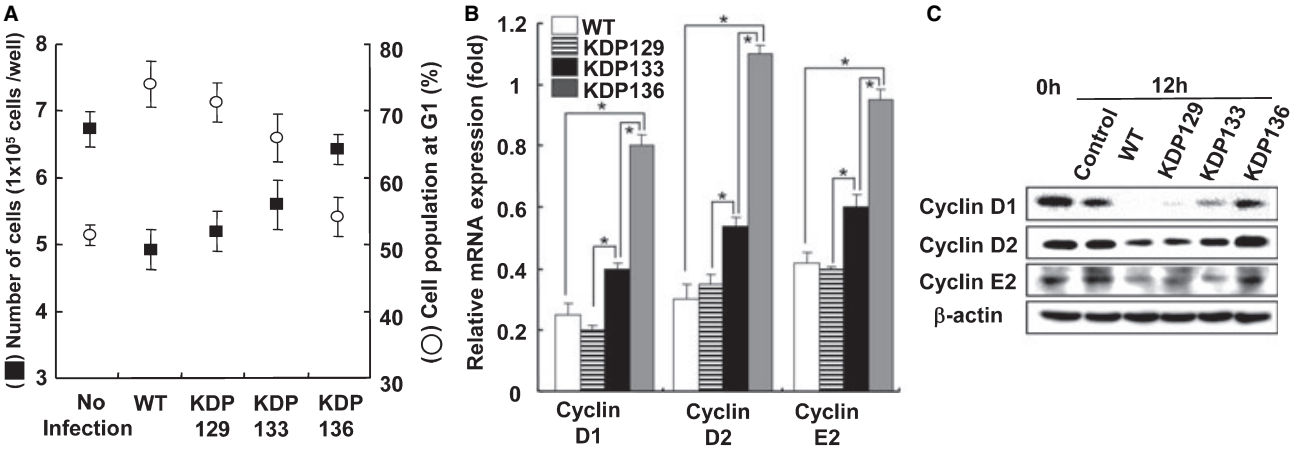

Fig. 4. Involvement of Rgp and Kgp in modulation of cell cycle. (A) The effects of *Porphyromonas gingivalis* infection on cellular proliferation and cell-cycle distribution were examined 24 h after infection, in the same manner as in Fig. 1. WT, wild-type strain; KDP129, Rgp-deficient mutant; KDP133, Kgp-deficient mutant; KDP136, gingipain-deficient mutant. (B) Effects of gingipain-deficient mutant on gene expression of cyclins. The gene expression of *cyclinD1*, *cyclinD2*, and *cyclinE2* was assayed using real-time PCR, in the same manner as in Fig. 3A. $^*P < 0.01$. (C) Twelve hours after infection, ST2 cells were dissolved and subjected to immunoblotting in the same manner as in Fig. 3C.

and intracellular lysosomal killing (14), possibly resulting in prolonged local bacterial survival within the cells.

Gingipains were shown to be involved in G_1 arrest. Gingipains are secreted proteins, associated with extracellular vesicles, found on the bacterial cell surface and in the culture supernatant (12). Thus, these molecules would modulate gene expression by host cells with or without the direct interaction of *P. gingivalis*. Gingipains reportedly degraded various cellular components including focal contact components, i.e. focal adhesion kinase, adherens junction proteins such as catenins, and adhesion signaling molecules such as Src tyrosine kinase (11, 12). Transmembrane proteins, such as integrins, also undergo proteolysis by gingipains (18). Thus, these proteolytic actions of gingipains might have caused arrest in G_1; however, it is also possible that adhesion domains of gingipains (12) affect G_1 arrest. In addition, the involvement of protease-activated receptors is another possibility. Our previous study suggested that Rgp and Kgp likely stimulate osteoblasts through protease-activated receptor signals (30). Rgp was found to stimulate the production of hepatocyte growth factor through protease-activated receptors in human gingival fibroblasts (38). However, other factors may also induce G_1 arrest. It was previously reported that the exogenous addition of butyric acid, an extracellular metabolite from various periodontopathic bacteria including *P. gingivalis*, arrested the cells at the G_1 phase (21). In addition, Rgp acts not only as a direct virulence factor, but also makes a significant contribution as a major processing enzyme with bacterial virulence factors (17). Rgp-null mutants (KDP129 and KDP136) failed to express several virulence factors on the bacterial surface, such as major and minor fimbriae. Therefore, we cannot exclude the possible involvement of other bacterial factors in G_1 arrest. However, an Rgp-null mutant (KDP129) apparently suppressed the cell cycle (Fig. 4) so the bacterial surface components that are processed with Rgp may not be involved in G_1 arrest. Further studies are needed to elucidate how *P. gingivlias* causes G_1 arrest, as well as the possible involvement of protease-activated receptors.

The role of *P. gingivalis* fimbriae in ST2 cell infection is likely very limited. It was previously reported that the maximum level of *P. gingivalis* adhesion to primary mouse osteoblasts at an MOI of 100 was approximately 3%, while the invasion efficiency reached approximately 0.8% (30). Cells with osteoblastic characteristics may therefore faintly express receptor molecules for *P. gingivalis* fimbriae. *P. gingivalis* fimbriae are classified into six genotypes (types I–V, and Ib) based on the different nucleotide sequences of the *fimA* genes encoding the fimbrial subunits (3). Our previous reports strongly suggested that type II fimbriae are the most adhesive to and invasive of host cells among the six fimbrial variants; in contrast, type I fimbria clones are not so invasive and virulent (2). *P. gingivalis* ATCC33277 used in this study is a type I clone, and other *fimA* clones, especially type II, might cause cellular responses that are different from the present results.

The cell-cycle phase during which blocking is induced is dependent on cell type (8, 9, 15). For example, it was reported that *Haemophilus ducreyi* arrested epithelial cell lines (HeLa and Hep-2) and normal keratinocytes in the G_2 phase, while primary human fibroblasts were blocked in the G_1 phase (9). Thus, other cell types, such as gingival epithelial cells, may respond differently to ST2 cells. The effects of *P. gingivalis* on the cell cycle of several other cell types will be examined in the near future to achieve a better understanding of the cell cycle and its role in periodontal pathogenesis.

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References

1. Amano A. Molecular interaction of *Porphyromonas gingivalis* with host cells: Implication for the microbial pathogenesis of periodontal disease. J Periodontol 2003: **74**: 90–96.
2. Amano A. Disruption of epithelial barrier and impairment of cellular function by *Porphyromonas gingivalis*. Front Biosci 2007: **12**: 3965–3974.
3. Amano A, Nakagawa I, Okahashi N, Hamada N. Variations of *Porphyromonas gingivalis* fimbriae in relation to microbial pathogenesis. J Periodontal Res 2004: **39**: 136–142.
4. Boonstra J. Progression through the G1-phase of the on-going cell cycle. J Cell Biochem 2003: **90**: 244–252.
5. Ceelen LM, Decostere A, Ducatelle R, Haesebrouck F. Cytolethal distending toxin generates cell death by inducing a bottleneck in the cell cycle. Microbiol Res 2006: **161**: 109–120.
6. Chen CJ, Makino S. Murine coronavirus replication induces cell cycle arrest in G0/G1 phase. J Virol 2004: **78**: 5658–5669.
7. Chiou CC, Chan CC, Kuo YP, Chan EC. *Helicobacter pylori* inhibits activity of cdc2 kinase and delays G2/M to G1 progression in gastric adenocarcinoma cell line. Scand J Gastroenterol 2003: **38**: 147–152.
8. Cortes-Bratti X, Karlsson C, Lagergard T, Thelestam M, Frisan T. The *Haemophilus ducreyi* cytolethal distending toxin induces cell cycle arrest and apoptosis via the DNA damage checkpoint pathways. J Biol Chem 2001: **276**: 5296–5302.
9. Frisan T, Cortes-Bratti X, Thelestam M. Cytolethal distending toxins and activation of DNA damage-dependent checkpoint responses. Int J Med Microbiol 2002: **291**: 495–499.
10. Gasper NA, Petty CC, Schrum LW, Marriott I, Bost KL. Bacterium-induced CXCL10 secretion by osteoblasts can be mediated in part through toll-like receptor. Infect Immun 2002: **70**: 4075–4082.
11. Holt SC, Ebersole JL. *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*: the "red complex", a prototype polybacterial pathogenic consortium in periodontitis. Periodontol 2000 2005: **38**: 72–122.
12. Imamura T. The role of gingipains in the pathogenesis of periodontal disease. J Periodontol 2003: **74**: 111–118.
13. Inaba H, Kawai S, Nakayama K, Okahashi N, Amano A. Effect of enamel matrix derivative on periodontal ligament cells *in vitro* is diminished by *Porphyromonas gingivalis*. J Periodontol 2004: **75**: 858–865.
14. Jacinto A, Martinez-Arias A, Martin P. Mechanisms of epithelial fusion and repair. Nat Cell Biol 2001: **3**: 117–123.
15. Johnson WM, Lior H. A new heat-labile cytolethal distending toxin (CLDT) produced by *Campylobacter* spp. Microb Pathog 1988: **4**: 115–126.
16. Jones A, Jonsson AB, Aro H. *Neisseria gonorrhoeae* infection causes a G1 arrest in human epithelial cells. FASEB J 2007: **21**: 345–355.
17. Kadowaki T, Nakayama K, Yoshimura F, Okamoto K, Abe N, Yamamoto K. Arg-gingipain acts as a major processing enzyme for various cell surface proteins in *Porphyromonas gingivalis*. J Biol Chem 1998: **273**: 29072–29076.
18. Kato T, Kawai S, Nakano N et al. Virulence of *Porphyromonas gingivalis* is altered by substitution of fimbria gene with different genotype. Cell Microbiol 2007: **9**: 753–765.

19. Kato T, Okahashi N, Kawai S et al. Impaired degradation of matrix collagen in human gingival fibroblasts by the anti-epileptic drug phenytoin. J Periodontol 2005: **76**: 941–950.
20. Kato T, Tsuda T, Omori H, Kato T, Yoshimori T, Amano A. Maturation of fimbria precursor protein by exogenous gingipains in *Porphyromonas gingivalis* gingipain-null mutant. FEMS Microbiol Lett 2007: **273**: 96–102.
21. Kurita-Ochiai T, Ochiai K, Fukushima K. Volatile fatty acid, metabolic by-product of periodontopathic bacteria, induces apoptosis in WEHI 231 and RAJI B lymphoma cells and splenic B cells. Infect Immun 1998: **66**: 2587–2594.
22. Kusumoto Y, Hirano H, Saitoh K et al. Human gingival epithelial cells produce chemotactic factors interleukin-8 and monocyte chemoattractant protein-1 after stimulation with *Porphyromonas gingivalis* via toll-like receptor 2. J Periodontol 2004: **75**: 370–379.
23. Lee KY, Bae SC. TGF-beta-dependent cell growth arrest and apoptosis. J Biochem Mol Biol 2002: **35**: 47–53.
24. Marches O, Ledger TN, Boury M et al. Enteropathogenic and enterohaemorrhagic *Escherichia coli* deliver a novel effector called Cif, which blocks cell cycle G2/M transition. Mol Microbiol 2003: **50**: 1553–1567.
25. McHugh PJ, Sarkar S. DNA interstrand cross-link repair in the cell cycle: a critical role for polymerase zeta in G1 phase. Cell Cycle 2006: **5**: 1044–1047.
26. Nakayama K, Kadowaki T, Okamoto K, Yamamoto K. Construction and characterization of arginine-specific cysteine proteinase (Arg-gingipain)-deficient mutants of *Porphyromonas gingivalis.* Evidence for significant contribution of Arg-gingipain to virulence. J Biol Chem 1995: **270**: 23619–23626.
27. Nougayrede JP, Taieb F, DeRycke J, Oswald E. Cyclomodulins: bacterial effectors that modulate the eukaryotic cell cycle. Trends Microbiol 2005: **13**: 103–110.
28. Obaya AJ, Sedivy JM. Regulation of cyclin-Cdk activity in mammalian cells. Cell Mol Life Sci 2002: **59**: 126–142.
29. Ohno T, Okahashi N, Kawai S et al. Proinflammatory gene expression in mouse ST2 cell line in response to infection by *Porphyromonas gingivalis*. Microbes Infect 2006: **8**: 1025–1034.
30. Okahashi N, Inaba H, Nakagawa I et al. *Porphyromonas gingivalis* induces receptor activator of NF-**κ**B ligand expression in osteoblasts through the activator protein-1 pathway. Infect Immun 2004: **72**: 1706–1714.
31. Okamoto K, Nakayama K, Kadowaki T, Abe N, Ratnayake DB, Yamamoto K. Involvement of a lysine-specific cysteine proteinase in hemoglobin adsorption and heme accumulation by *Porphyromonas gingivalis*. J Biol Chem 1998: **273**: 21225–21231.
32. Oswald E, Nougayrede JP, Taieb F, Sugai M. Bacterial toxins that modulate host cell-cycle progression. Curr Opin Microbiol 2005: **8**: 83–91.
33. Pihlstrom BL, Michalowizc BS, Hohnson NW. Periodontal diseases. Lancet 2005: **366**: 1809–1820.
34. Sacco RE, Hagen M, Donelson JE, Lynch RG. B lymphocytes of mice display an aberrant activation phenotype and are cell cycle arrested in G0/G1A during acute infection with *Trypanosoma brucei*. J Immunol 1994: **153**: 1714–1723.
35. Shi Y, Ratnayake DB, Okamoto K, Abe N, Yamamoto K, Nakayama K. Genetic analyses of proteolysis, hemoglobin binding, and hemagglutination of *Porphyromonas gingivalis.* Construction of mutants with a combination of rgpA, rgpB, kgp, and hagA. J Biol Chem 1999: **274**: 17955–17960.
36. Shirin H, Sordillo EM, Oh SH et al. *Helicobacter pylori* inhibits the G1 to S transition in AGS gastric epithelial cells. Cancer Res 1999: **59**: 2277–2281.
37. Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. Nat Rev Immunol 2007: **7**: 292–304.
38. Uehara A, Muramoto K, Imamura T et al. Arginine-specific gingipains from *Porphyromonas gingivalis* stimulate production of hepatocyte growth factor (scatter factor) through protease-activated receptors in human gingival fibroblasts in culture. J Immunol 2005: **175**: 6076–6084.
39. Ueshima J, Shoji M, Ratnayake DB et al. Purification, gene cloning, gene expression, and mutants of Dps from the obligate anaerobe *Porphyromonas gingivalis*. Infect Immun 2003: **71**: 1170–1178.