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ORIGINAL ARTICLE

The G1 cell cycle arrest of macrophages infected with *Aggregatibacter actinomycetemcomitans*

H Kasai[1,2], K Nakashima[2], M Yokota[2], T Nishihara[1]

[1]*Division of Infections and Molecular Biology, Department of Health Promotion, Kyushu Dental College, Kitakyushu, Japan;*
[2]*Division of Periodontology, Department of Cariology and Periodontology, Kyushu Dental College, Kitakyushu, Japan*

OBJECTIVES: Infection of murine macrophage cell line J774.1 with the periodontopathic bacterium *Aggregatibacter actinomycetemcomitans* induces apoptotic cell death. The infection induces cell cycle arrest in the G1 phase prior to the appearance of apoptotic cells. This study determined the involvement of various cell cycle-related signal molecules in *A. actinomycetemcomitans*-induced G1 cell cycle arrest.

MATERIALS AND METHODS: Cell cycle in J774.1 cells infected with *A. actinomycetemcomitans* was analyzed with a flow cytometer. Immunoblot analysis was also employed to determine the expression levels of intracellular signal molecules.

RESULTS: Flow cytometric analysis revealed that the percentage of cells in the G1 phase increased to 77.2% at 12 h after *A. actinomycetemcomitans* infection. Additionally, according to immunoblot analysis, expression levels of hyperphosphorylated forms of retinoblastoma protein (ppRb) declined in J774.1 cells following *A. actinomycetemcomitans* infection, whereas hypophosphorylated Rb (pRb) expression levels were elevated slightly. Expression levels of cyclin D1 and D2 in the cells decreased gradually postinfection; CDK2, CDK4, CDK6 and cyclin E levels were not changed. Furthermore, postinfection, $p21^{CIP1/WAF1}$ expression increased at 6 h, followed by a subsequent decrease.

CONCLUSION: These findings suggest that cyclin D1 and D2 and $p21^{CIP1/WAF1}$ participate in G1 cell cycle arrest in *A. actinomycetemcomitans*-infected J774.1 cells.*Oral Diseases* **(2010) 16,** 305–309

Keywords: *Aggregatibacter actinomycetemcomitans;* G1 arrest; macrophage

Correspondence: Tatsuji Nishihara, DDS, PhD, Division of Infections and Molecular Biology, Department of Health Promotion, Kyushu Dental College, 2-6-1 Manazuru, Kokurakita-ku, Kitakyushu 803-8580, Japan. Tel: +81 93 582 1131, ext. 8111, Fax: +81 93 581 4984, E-mail: tatsujin@kyu-dent.ac.jp
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Introduction

The gram-negative bacterium *Aggregatibacter actinomycetemcomitans* is involved in the pathogenesis of severe aggressive and chronic periodontitis; moreover, extracellular components from the bacterium are potent mediators of its adherence to human oral epithelial cells (Meyer and Fives-Taylor, 1993). The initial event in the pathogenesis of most bacterial diseases is microbial invasion of a host cell following the adherence of the microorganism to some host tissue. Many studies have demonstrated that *A. actinomycetemcomitans* can invade periodontal tissues (Saglie *et al*, 1982; Meyer *et al*, 1991). Sreenivasan *et al* (1993) also provided direct evidence of *in vitro* invasion of human epithelial cells by *A. actinomycetemcomitans*. We developed an *in vitro* cell culture infection model for *A. actinomycetemcomitans* and documented the participation of CD14 molecules in the phagocytosis of *A. actinomycetemcomitans* by macrophages, which resulted in the induction of apoptosis (Muro *et al*, 1997). *Shigella flexneri*, the etiologic agent of dysentery, *Bordetella pertussis*, the causative agent of whooping cough in humans, and the periodontopathic bacterium, *A. actinomycetemcomitans*, have been shown to induce apoptosis in macrophages (Zychlinsky *et al*, 1992; Khelef *et al*, 1993; Kato *et al*, 1995). Furthermore, in a series of associated studies, we identified the involvement of caspase activation through mitochondrial release of cytochrome *c* in apoptosis of macrophages infected with *A. actinomycetemcomitans* (Kasai *et al*, 2004).

Apoptosis has been shown to play important roles in immune control. This process is an active cell death and can be triggered by a variety of signals. Exposure of mammalian cells to several DNA-damaging agents evokes a complicated cellular response, including a reversible block in the cell cycle at the G1 and G2/M phases, and induces programmed cell death (Hartwell and Weinert, 1989). Cell cycle arrest at the G1 and G2/M phases reflects the fact that mammalian cells need time to repair damaged DNA. Following DNA damage,

the cell cycle stops at the transition from the G1 phase to the S phase and at the transition from the G2 phase to the M phase, with DNA complements of 2*n* and 4*n*, respectively. Transitions between different cell cycle phases reportedly are regulated at checkpoints controlled by cyclin-dependent kinases (CDKs), which are activated by cyclins (Lew *et al*, 1991). Despite the potential importance of apoptosis and cell cycle regulation in infected macrophages as a pathological mechanism, our understanding of its molecular basis and regulation is still minimal. In this study, we have focused on the involvement of various cell cycle-related molecules in *A. actinomycetemcomitans*-infected macrophages.

Materials and methods

Cell and bacterial strains

The murine macrophage cell line J774.1 was obtained from the Japanese Cancer Research Resources Bank. Cells were cultured in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY, USA) containing 10% heat-inactivated fetal calf serum (FCS), penicillin G (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) at 37°C in an atmosphere of 5% CO_2 in air. *A. actinomycetemcomitans* Y4 was selected as a representative strain of *A. actinomycetemcomitans* and grown in Brain Heart Infusion broth (Difco Laboratories, Detroit, MI, USA) supplemented with 1% (w/v) yeast extract at 37°C for 4 days in an atmosphere of 5% CO_2 in air.

Procedure for in vitro A. actinomycetemcomitans infection of macrophages

J774.1 cells were plated in a culture plate (Corning Glass Works, Corning, NY, USA) at a concentration of 1×10^6 cells ml^{-1} one day prior to the beginning of the experiment. *A. actinomycetemcomitans* Y4 bacterial cells were harvested by centrifugation and suspended in RPMI 1640 medium without antibiotics to an optical density of 0.55 at 550 nm, which corresponded to approximately 5×10^9 bacteria ml^{-1}. The bacterial suspension was added to the plates, which were centrifuged at 1000 $\times g$ for 10 min at 4°C prior to incubation at 37°C for 1 h. J774.1 cells infected with *A. actinomycetemcomitans* Y4 at a final bacterium/cell ratio of 5000:1 were washed three times with RPMI 1640 medium containing penicillin G, streptomycin and gentamicin (200 μg ml^{-1}) to remove extracellular bacteria. The infected J774.1 cells were then cultured in RPMI 1640 medium supplemented with 5% FCS and antibiotics.

Detection of G1 cell cycle arrest and apoptosis

To detect apoptotic nuclei and to analyze the cell cycle of J774.1 cells, cells (10^6) were suspended in hypotonic solution (0.1% sodium citrate, 0.2% NP-40, 0.25 mg ml^{-1} RNase; pH 8.0), stained with 50 μg ml^{-1} propidium iodide and analyzed with an EPICS XL (Beckman Coulter, Fullerton, CA, USA). The percentage of cells in each cell cycle phase was determined with MultiCycle for Windows (Phoenix Flow Systems, San Diego, CA, USA).

Immunoblot analysis

J774.1 cells infected with *A. actinomycetemcomitans* were lysed in SDS lysis buffer (50 mM Tris–HCl, 2% SDS; pH 6.8); subsequently, 20 μg of protein extracts were electrophoresed on 10%, 12.5%, or 15% SDS-polyacrylamide gels, electroblotted on PVDF membranes and reacted with primary antibodies. Immunodetection was performed employing an ECL Western blotting detection system (Amersham Pharmacia Biotech, Little Chalfont, UK). Blots were stained with Coomassie brilliant blue; similar protein extract content was confirmed for each lane. Anti-cyclin D1, anti-cyclin D2 and anti-$p21^{CIP1/WAF1}$ monoclonal antibodies and anti-CDK2, anti-CDK4, anti-CDK6 and anti-cyclin E polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-Rb monoclonal antibody was obtained from PharMingen (San Diego, CA, USA).

Results

Cell cycle arrest in the G1 phase and apoptosis induced by A. actinomycetemcomitans infection

J774.1 cells, which were infected with *A. actinomycetemcomitans* Y4 at a bacterium/cell ratio of 5000:1, were analyzed for cell cycle distribution with a flow cytometer. Upon 12-h culture of *A. actinomycetemcomitans*-infected J774.1 cells, the percentage of cells in the G1 phase increased from 34.2% to 77.2%, whereas the percentage of cells in the S phase dropped from 51.3% to 3.6%. The percentage of cells in the sub-G1 phase representing apoptotic cells increased to 35% at 24 h postinfection (Figure 1). As shown in Figure 1, cultivation with *A. actinomycetemcomitans* time-dependently increased the percentage of cells in the G1 phase but reduced the percentage in the S phase for 12 h. The increased percentage of *A. actinomycetemcomitans*-infected J774.1 cells in the G1 phase might represent G1-arrested cells. Additionally, these results indicated that the percentage in the sub-G1 phase increased gradually until 30 h after infection with *A. actinomycetemcomitans* (data not

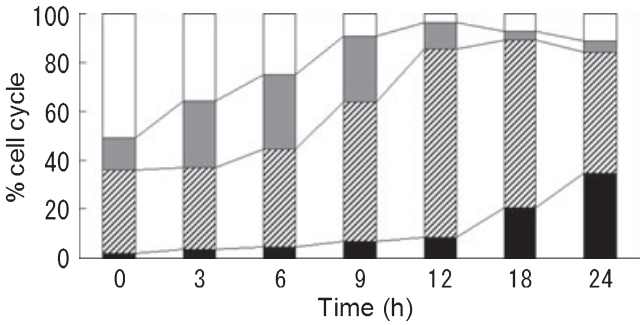

Figure 1 Cell cycle analysis of J774.1 cells infected with *A. actinomycetemcomitans*. Infected J774.1 cells were stained with propidium iodide, which were analyzed using a flow cytometer. DNA content was analyzed at the times indicated., G1 phase; S phase; G2/M phase; sub-G1 phase. Data are representative of an experiment conducted three times with similar results.

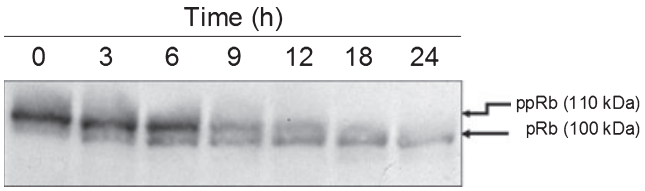

Figure 2 Expression of Rb in J774.1 cells after infection with *A. actinomycetemcomitans*. Proteins extracted from infected J774.1 cells were electroblotted on PVDF membranes and analyzed in terms of Rb expression by immunoblotting. pRb, hypophosphorylated Rb; ppRb, hyperphosphorylated Rb

shown). In contrast, uninfected J774.1 cells showed no change in the cell cycle distribution until 30 h (data not shown).

Rb hypophosphorylation induced in J774.1 cells by infection with A. actinomycetemcomitans

In order to examine the mechanism by which *A. actinomycetemcomitans* induces G1 cell cycle arrest in J774.1 cells, the phosphorylation state of Rb was evaluated by immunoblot analysis. Hyperphosphorylated forms of Rb (ppRb) and minimal levels of hypophosphorylated forms of Rb (pRb) were detected in uninfected J774.1 cells (data not shown). ppRb levels gradually decreased and pRb levels slightly increased in cells infected with *A. actinomycetemcomitans* (Figure 2). However, uninfected cells showed no change in both ppRb and pRb levels until 24 h (data not shown).

Effect of A. actinomycetemcomitans infection on expression of G1 cyclins and CDKs

To clarify Rb phosphorylation, expression of G1 cyclins and CDKs was examined in *A. actinomycetemcomitans*-infected J774.1 cells by immunoblot analysis. Expression levels of subtypes of D-type cyclins in J774.1 cells, cyclin D1 and cyclin D2, were decreased following *A. actinomycetemcomitans* infection. In contrast, those levels in uninfected cells were similar to the levels in infected cells at 0 h until 24 h (data not shown). Levels of CDK4 and CDK6 were not changed in uninfected or infected cells (Figure 3). Moreover, immunoblot analysis revealed that infection with *A. actinomycetemcomitans* did not influence the expression of cyclin E and CDK2 in J774.1 cells (Figure 4).

Effect of A. actinomycetemcomitans infection on expression level of p21$^{CIP1/WAF1}$

Following the infection of J774.1 cells with *A. actinomycetemcomitans*, the expression level of an inhibitor of cyclin-dependent kinases, p21$^{CIP1/WAF1}$, was assessed by immunoblot analysis. p21$^{CIP1/WAF1}$ in uninfected J774.1 cells was scarcely detectable (data not shown). Expression levels of p21$^{CIP1/WAF1}$ were elevated in cells at 6 h after infection with *A. actinomycetemcomitans*; however, the expression level subsequently decreased (Figure 5). In contrast, uninfected cells showed no change in p21$^{CIP1/WAF1}$ levels until 24 h (data not shown).

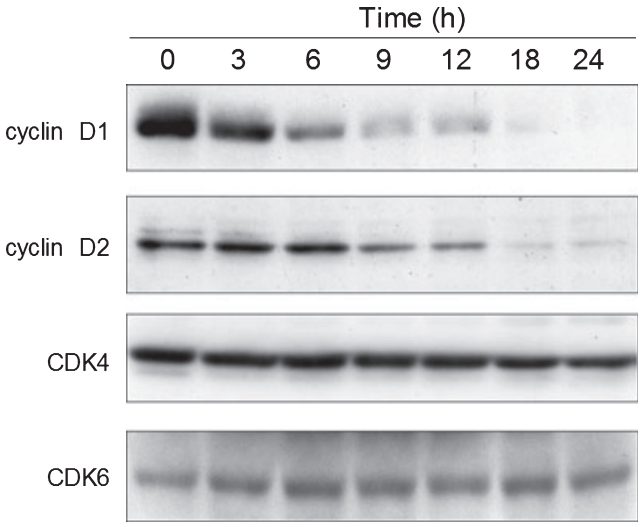

Figure 3 Effect of infection with *A. actinomycetemcomitans* on expression of cyclin D1, cyclin D2, CDK4 and CDK6 in J774.1 cells. J774.1 cells were cultured following infection for the times indicated

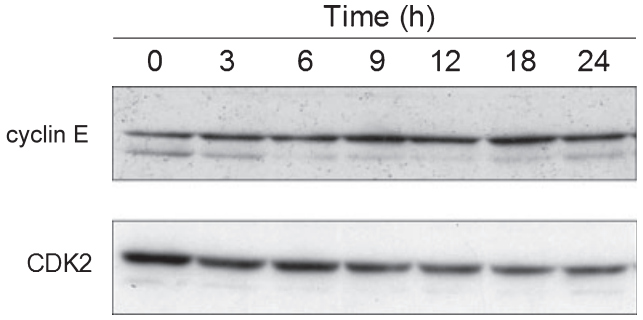

Figure 4 Expression of cyclin E and CDK2 in J774.1 cells after infection with *A. actinomycetemcomitans*. J774.1 cells were cultured following infection for the times indicated

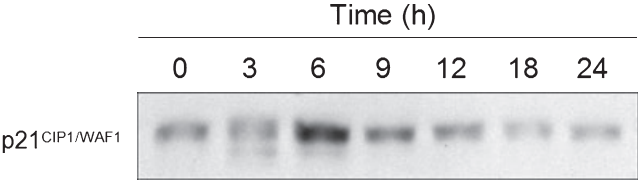

Figure 5 p21$^{CIP1/WAF1}$ expression in *A. actinomycetemcomitans*-infected J774.1 cells. Proteins extracted from infected J774.1 cells were analyzed with respect to expression of p21$^{CIP1/WAF1}$ by immunoblotting

Discussion

In a previous study, we established the involvement of caspase activation through mitochondrial release of cytochrome *c* during apoptosis of macrophages infected with *A. actinomycetemcomitans*. We documented the participation of mitochondrial cytochrome *c* release and activation of caspase-1, -3 and -9 in the induction of apoptosis in macrophages infected with *A. actinomyce-*

temcomitans (Nonaka *et al*, 2001; Kasai *et al*, 2004). In addition, we reported that caspase-9, -6 and -3/7 inhibitors block induction of apoptosis in macrophages infected with *A. actinomycetemcomitans*. We also determined that *A. actinomycetemcomitans* infection induces apoptosis in macrophages through activated caspase-6, after which cleavage of lamin A/C occurs. Furthermore, immunoblot analysis revealed cleavage of PARP, whereas caspase-3/7 inhibitor blocked cleavage in macrophages infected with *A. actinomycetemcomitans* (Okinaga *et al*, 2007). It is well known that DNA damage in proliferating cells induces a complex intracellular response that includes perturbation of the cell cycle and apoptotic cell death. The loss of cell cycle control and the inability of cells to repair DNA at cell cycle checkpoints result in propagation of genetic lesion.

Retinoblastoma protein (Rb) controls cell cycle progression at the G1 to S transition in response to some signals for growth inhibition (Weinberg, 1995). Hypophosphorylated Rb binds to the E2F transcription factor and neutralizes its ability to activate genes required for entry into the S phase. Phosphorylation of Rb abrogates its binding to E2F, which allows E2F to activate target genes (Nevins, 1992). Rb is phosphorylated by catalytic subunits of CDK4/CDK6 and CDK2 complexed with specific regulatory subunits of cyclins D and E, respectively.

The current study demonstrated that *A. actinomycetemcomitans* infection can inhibit macrophage cell growth by arresting the cell cycle in the G1 phase, subsequently inducing apoptosis. *Bordetella pertussis*, the etiological agent of whooping cough in humans, induced apoptosis in J774.1 cells after the infection at the bacterium to cell ratios 100:1 (Khelef *et al*, 1993). J774.1 cells hardly produced cytokines after incubation with viable cells of some lactococcal strains at the bacterium to cell ratios of 5000:1, in which case the percentage of J774.1 cells double-stained with the FITC-labeled annexin V and propidium iodide was significantly increased (Suzuki *et al*, 2008). Unfortunately, we could not include these bacteria as positive references in the present study. Therefore, we should to use these strains as positive reference in the future experiment. *A. actinomycetemcomitans* infection inhibits Rb phosphorylation and cyclin D expression in J774.1 cells; consequently, it is highly possible that *A. actinomycetemcomitans* infection causes G1 arrest by blocking cyclin D-CDK4/6-mediated Rb phosphorylation, which is crucial to progression of the cell cycle at the G1 to S transition (Figures 2 and 3). The Rb kinase activity of CDK4/6 is positively regulated by D-type cyclins and negatively regulated by CDK inhibitors, including $p16^{INK4a}$, $p15^{INK4b}$, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ (Sherr and Roberts, 1995; Weinberg, 1995). We demonstrated that J774.1 cells express cyclin D1 and D2 as the regulatory subunit of CDK4 and CDK6; subsequently, we showed that *A. actinomycetemcomitans* infection suppresses cyclin D1 and D2 by immunoblot analysis (Figure 3). However, expression of CDK4, CDK6, cyclin E and CDK2 were not inhibited in cells infected with *A. actinomycetemcomitans* (Figure 4). Cyclin E-CDK2 complex is unable to phosphorylate pRb in the absence of prior phosphorylation by cyclin D-CDK4/6 complexes. In fact, complete phosphorylation of Rb protein, inactivation of E2F binding and activation of E2F transcription occurs only after the sequential action of at least two distinct G1 cyclin kinase complexes (Lundberg and Weinberg, 1998). These findings suggested that hypophosphorylation of Rb through suppression of cyclin D1 and D2 actively participates in G1 arrest in macrophages infected with *A. actinomycetemcomitans*.

The small G protein Ras regulates many aspects of cell growth and differentiation. Expression of oncogenic forms of Ras protein leads to induction of cell cycle progression, causing quiescent cells to exit G0 and passage through at least the G1 and S phases in most cell types (Downward, 1997). Moreover, Ras exerts, depending on cellular context, both positive and negative effects on cell growth. In this investigation, suppression of cyclin D1 and D2 and the transient increase in $p21^{CIP1/WAF1}$ are thought to be causally related to Ras regulation or some signal proteins. Additional studies are required to determine the mechanism governing this process.

$p21^{CIP1/WAF1}$ is a well-known negative regulator of CDKs (Xiong *et al*, 1993). Expression of $p21^{CIP1/WAF1}$ is regulated by various anti-mitogenic signals, e.g., members of the transforming growth factor b superfamily and p53 (Dulić *et al*, 1994; Zeng and el-Deiry, 1996). In recent years, several observations have raised the possibility that $p21^{CIP1/WAF1}$ plays important roles at several stages of the cell cycle (Xiong *et al*, 1993). The current investigation demonstrated that infection with *A. actinomycetemcomitans* enhances $p21^{CIP1/WAF1}$ expression in J774.1 cells (Figure 5). $p21^{CIP1/WAF1}$ abolishes the kinase activities of cyclins D-CDK4/6 and cyclin E-CDK2 via direct binding to these catalytically active kinase complexes. Expression of $p21^{CIP1/WAF1}$ is induced by physiological agents including p53. This study revealed that *A. actinomycetemcomitans* infection elevates levels of $p21^{CIP1/WAF1}$ in the cells at 6 h of culture; however, expression subsequently decreased. It has been reported that $p21^{CIP1/WAF1}$ protein is rapidly induced but selectively cleaved during the apoptotic response. Caspase-like activity may be responsible for this cleavage (Gervais *et al*, 1998; Levkau *et al*, 1998). Following cleavage, $p21^{CIP1/WAF1}$ loses its nuclear localization sequence and exits the nucleus, which induces apoptosis. $p21^{CIP1/WAF1}$ may act as an important checkpoint regulator for both cell cycle arrest and apoptosis in macrophages infected with *A. actinomycetemcomitans*.

The result of cytolethal distending toxin (CDT) activity can differ somewhat depending on the eukaryotic cell types affected. Epithelial cells, endothelial cells, and keratinocytes undergo G2 cell cycle arrest; fibroblasts undergo G1 and G2 arrest; and immune cells including macrophages undergo G2 arrest followed by apoptosis (Smith and Bayles, 2006). It is recently reported that CDT of *A. actinomycetemcomitans* induce apoptosis in macrophages via G2 cell cycle arrest (Rabin

et al, 2009). In the present study, live *A. actinomycetemcomitans* infection induced cell cycle arrest in the G1 phase prior to apoptosis. We previously demonstrated that the invasion of live *A. actinomycetemcomitans* into macrophages is essential for the induction of apoptosis (Kato *et al*, 1995). Therefore, live *A. actinomycetemcomitans* may initiate the apoptosis pathway which is different from CDT.

These findings suggested that *A. actinomycetemcomitans* infection induces cell cycle arrest in the G1 phase prior to the appearance of apoptotic cells in macrophages; induction of $p21^{CIP1/WAF1}$ upregulation, cyclin D downregulation and the ensuing hypophosphorylation of Rb may play important roles in the intracellular signaling mechanism. In other words, our data suggested that reduction in the amounts of cyclin D-CDK4/6 complexes and CDK inhibitor upregulation in *A. actinomycetemcomitans*-infected cells leads to diminished CDK activities and to sufficient hyperphosphorylation of Rb, which results in G1 cell cycle arrest.

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