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Short communication

Topical application of *Aggregatibacter actinomycetemcomitans* cytolethal distending toxin induces cell cycle arrest in the rat gingival epithelium *in vivo*

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Background: Aggregatibacter actinomycetemcomitans is one of the etiological pathogens implicated in the onset of periodontal disease. This pathogen produces cytolethal distending toxin (CDT) that acts as a genotoxin to induce cell cycle arrest and cellular distension in cultured cell lines. Therefore, CDT is a possible virulence factor; however, the *in vivo* activity of CDT on periodontal tissue has not been explored. Here, CDT was topically applied into the rat molar gingival sulcus; and the periodontal tissue was histologically and immunohistochemically examined.

Materials and Methods: Recombinant purified *A. actinomycetemcomitans* CDT was applied to gingival sulcus of male Wistar rats and tissue samples were immunohistochemmically examined.

Results: One day after application, infiltration of neutrophils and dilation of blood vessels in the gingival connective tissue were found. At day three, desquamation and detachment of cells in the junctional epithelium was observed. This abrasion of junctional epithelium was not observed in rats treated with mutated CDT, in which a His274Ala mutation is present in the CdtB subunit. This indicates the tissue abrasion may be caused by the genotoxicity of CdtB. Expression of the proliferating cell nuclear antigen (PCNA), a marker for proliferating cells, was significantly suppressed using CDT treatment in the junctional epithelium and gingival epithelium.

Conclusion: Using the rat model, these data suggest CDT intoxication induces cell cycle arrest and damage in periodontal epithelial cells *in vivo*.

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Periodontal disease is a destructive inflammatory response affecting the tooth-supporting tissues. Etiologic studies have demonstrated that dental plaque – a composite of microorganisms and their products – plays a major role in the pathogenesis of periodontal disease. *Aggregatibacter actinomycetemcomitans* is one of the primary pathogens in the etiology of aggressive periodontal disease. The disease is characterized by a rapid and severe loss of alveolar bone and connective tissue supporting the teeth. *A. actinomycetemcomitans* produces a variety of virulence factors, including cytotoxins, proteases, Fc-binding proteins and an inhibitor of polymorphonuclear leukocyte function (1).

Cytolethal distending toxin (CDT) is a major cytotoxin of *A. actinomycetemcomitans* (2,3). The toxic mechanism of CDT has been extensively studied using *in vitro* culture cell systems. CDT inhibits the proliferation of cultured cells by arresting the cell cycle at G2/M (3,4). The intoxicated epithelial cell lineages show distension of the cell and of the nucleus, and eventually die. CDT induces programmed cell death in T-lymphocyte lineages (5–7). CDT is a heterotrimeric toxin in which the CdtB subunit is active and CdtA and CdtC are a heterodimeric subunit apparatus required to deliver CdtB into the cell (8–10). CdtB bears a structural similarity to members of the metalloenzyme superfamily, including nucleases and various phosphatases (11), and nuclear entry of the CdtB subunit is essential for cytotoxic activity (12–14). Mutations in amino acids of the DNase I active-site residues in CdtB abolish the cytotoxic activity of CDT (12,15). Upon entering the nucleus, CdtB induces DNA double-strand breaks, shown indirectly by the presence of phosphorylation of histone H2AX (16) and relocalization of the DNA repair complex, Mre11-Rad50 (17). DNA damage induced by CdtB may activate a checkpoint control, which results in the arrest of the intoxicated cells in G2 (18–20).

Few papers reporting an *in vivo* challenge of animals with CDT have been published. Intradermal injection of rabbits with *Haemophilus ducreyi* CDT induced pathogenic skin reactions, such as erythema with infiltration of inflammatory cells (21). Fox *et al*. (22) demonstrated that inoculation of *Campylobacter jejuni* produced gastroenteritis in nuclear factor-kappaB-deficient mice, whereas inoculation of *C. jejuni* lacking CDT did not result in gastoenteritis. However, the exact role of CDT in the pathogenesis of these bacteria is little understood.

Although detailed molecular dissection of CDT intoxication of cultured cells has been performed, the pathologic role of CDT in the etiology of periodontal disease has not been established. In this respect, one possible approach would be to construct a CDT-negative mutant strain and compare the effect of the mutant strain with the wild-type strain using an appropriate animal model. However, there is no good animal model with which to analyze the pathogenesis of *A. actinomycetemcomitans*, especially the initiation step of *A. actinomycetemcomitans*-induced destruction of the periodontal barrier. We therefore administered a topical preparation of purified CDT *in vivo* to rat periodontal tissues, to determine the toxicity of CDT in such tissues and to determine whether this could be used as a first step to determine the effect of CDT using an animal model approach.

Material and methods

Purification of *A. actinomycetemcomitans* CDT

CDT holotoxin was prepared using the pQE 60 (C-terminal histidine tag) protein expression system in M15 *Escherichia coli* (Qiagen, Tokyo, Japan) (7). Briefly, expression of the *cdtABC* gene by pQE*cdtABC* (which carries the *A. actinomycetemcomitans cdtABC* gene) was induced by adding isopropyl-β-D-1-thiogalactopyranoside (1 mM; Sigma-Aldrich, St Louis, MO, USA) to a culture of *E. coli* at an optical density at 660 nm of 0.5–0.7. After induction for 4 h, the culture supernatant was harvested by centrifugation at 5000 *g* for 5 min; the crude proteins were then precipitated by gentle stirring in ammonium sulfate (80% saturation) for at least 4 h. The precipitates were recovered by centrifugation at 15,000 *g* for 20 min, dissolved in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4), then dialyzed overnight against PBS. Nickel-chelated agarose beads (His-Select Nickel Affinity Gel; Sigma-Aldrich) were added to the dialyzed solution and gently shaken for at least 1 h, then the extracted proteins were applied to a chromatography column. The column was washed with washing buffer [50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 20 mM imidazole] and eluted with elution buffer [50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 250 mM imidazole]. The eluted CDT holotoxin was dialyzed against PBS and concentrated using Centricon 10 concentrators (Millipore, Bedford, MA, USA). The lipopolysaccharide (LPS) concentration in the CDT preparation was measured using the endotoxin signal test from Wako using Limulus amebocyte lysate (Wako Pure Chemical Industries, Ltd, Osaka, Japan). The amount of LPS contaminant in the purified CDT was 1853 ± 127 pg/mL.

Animal experiment

The experimental protocol described below was approved by the Animal Care Committee of Hiroshima University. Twenty-four, 7-wk-old male Wistar rats (of about 210 g in weight) were used. They were divided into eight groups of three rats each. Using intraperitoneal anesthesia with 20% ethyl carbamate (100 mg/100 g body weight), a rat was fixed on its back on an experimental stand. The rats received a topical application of 100 ng/μL of purified wild-type CDT or mutated CDT (in which His274 was changed to Ala in the CdtB subunit) (23), suspended in PBS, pH 7.4. Two microliters of CDT solution was applied six times into the palatal gingival sulcus of both maxillary molars (every 10 min during a 1-h time-period). In total, ca. 1.7 fmole of CDT was administered. Rats were killed, 1, 2 or 3 d after treatment with CDT or mutant CDT, using an overdose of ethyl ether. Three rats were used as an untreated control group.

As a positive control for PCNA immunoexpression, a further three rats were treated with LPS from the gingival sulcus. A cotton roll (2 mm in diameter and 1 cm in length), saturated with 5 mg/mL of LPS from *E. coli* (Sigma-Aldrich) in sterile physiological saline (Otsuka Med., Tokyo, Japan), was placed on palatal marginal periodontal regions of both maxillary molars for 1 h. The cotton roll was changed every 20 min during the 1-h treatment period. Two days after the application of LPS, rats were killed as described previously.

Tissue samples were resected *en bloc* from the right and left upper molar regions and fixed for 8 h in a periodic-lysine paraformaldehyde solution at 4°C. The samples were cut into two parts (about 2 mm thick ea.), which included the first or the second molar, at the buccopalatal plane parallel to each distopalatal root. They were then decalcified in 10% ethylene-diaminetetraacetate in PBS for 5 d at 4°C. The decalcified tissue blocks were embedded in paraffin. Serial sections (4.5 mm), parallel to the long axis of the tooth and including the root apex, were cut and applied to glass slides.

Immunohistochemistry

Immunostaining was performed using a Histofine Simple Stain Rat MAX-PO (Multi) kit (Nichirei Inc., Tokyo, Japan) using a PCNA monoclonal antibody (clone PC10, diluted 1 : 100; DAKO Japan, Tokyo, Japan). After dewaxing and rehydration, the sections were incubated in 0.3% hydrogen peroxide in methanol for 60 min at room temperature to quench endogenous peroxidase activity. After incubation in protein block (DAKO Japan) for 30 min at room temperature, the sections were incubated with the primary antibody at 4°C overnight. Then, the specimens were rinsed twice with PBS for 5 min and incubated with HISTOFINE simple stain MAX-PO for 30 min. Color was developed using 0.025% 3, 3′-diaminobenzidine tetrahydrochloride in Tris–HCl buffer with 0.3% hydrogen peroxide (DAKO Japan). The sections were counterstained with hematoxylin, dehydrated, cleared and mounted. Specificity was determined by comparison, substituting the target antibody with PBS and normal mouse serum.

Histomorphometric analysis of neutrophil infiltration in the junctional epithelium

For histomorphometric analysis, more than eight representative specimens from a different area of each experimental group at 1 and 2 d and from the control group were selected.

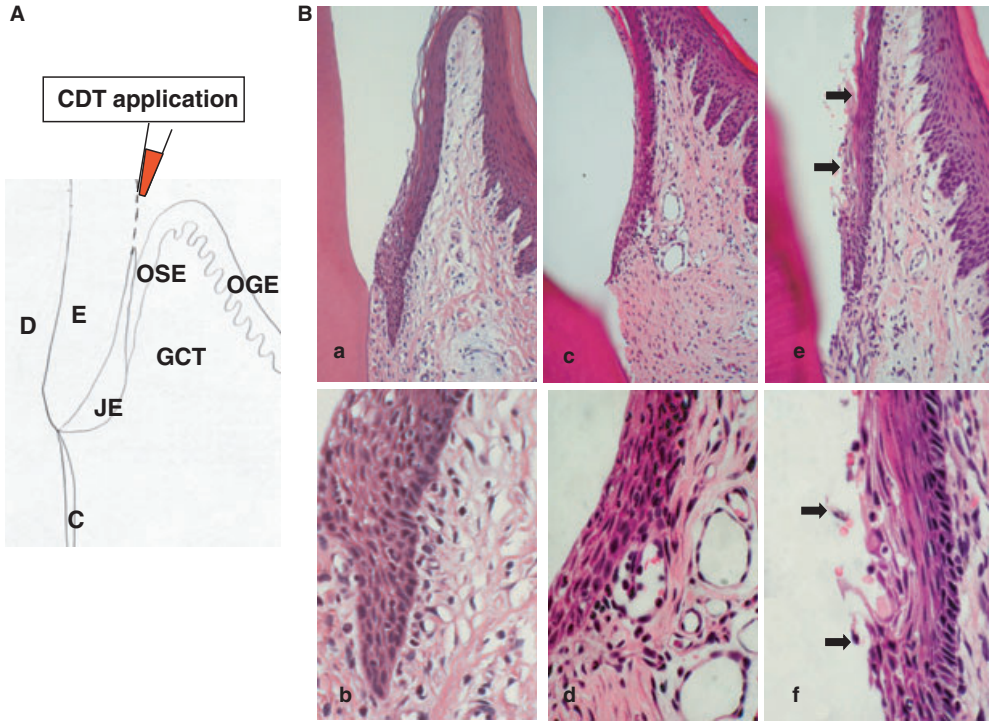

Fig. 1. Hematoxylin and eosin stain of rat periodontal tissue treated with cytolethal distending toxin (CDT). (A) Anatomical representation of the tooth and the gingival tissues. C, cementum; D, dentin; E, enamel; GCT, gingival connective tissue; JE, junctional epithelium; OGE, oral gingival epithelium; OSE, oral sulcular epithelium. CDT (a total of 1.7 fmole) was applied into the gingival sulcus next to the molar tooth surface through the tip of a pipette. (B) After 1, 2 and 3 d, the gingival tissues were examined histologically. In normal gingival tissue (a, b), only a few neutrophils were observed in the junctional epithelium (JE). On day 2 (c, d), markedly dilated blood vessels were seen in the sub-JE area. Numerous neutrophils infiltrated into the JE and sub-JE areas. On day 3 (e, f), in addition to infiltration of neutrophils into the gingival tissue, degeneration, cellular distension and desquamation or detachment were evident in the coronal portion of the JE (arrows). Upper panels, magnification 40 ×; and lower panels,, magnification 200 ×.

The number of neutrophils that infiltrated into the junctional epithelium (JE) was statistically analyzed. For the histomorphometric analysis, the number of neutrophils in the JE area was counted from specimens stained with hematoxylin and eosin. The small round cells that had a nucleus with lobes were judged as neutrophils. The palatal gingival tissue of each selected specimen was photographed under a magnification of 100. On the color prints, the number of neutrophils seen in the JE area was counted. The JE area was traced onto translucent paper using the same prints, and the traced area was measured using IMAGE J software (http://rsbweb.nih.gov/ij/). The number of neutrophils in a unit area (1 mm^2) was calculated.

The mean ± standard deviation values were determined. Differences in the numbers of neutrophils between each group were evaluated using the Mann–Whitney *U*-test. Probabilities of < 0.05 were considered to be significant.

Results and Discussion

Cellular distension and abrasion of CDT-intoxicated junctional epithelial cells

CDT was applied into the palatal gingival sulcus of both maxillary molars (Fig. 1A). Figure 1B shows the histology of the gingival tissue after topical application of CDT. In normal gingival tissue from the untreated control group, a small number of neutrophils were observed in the JE. Inflammatory reactions in the sub-JE area (including edema and dilation of blood vessels) were not obvious (Fig. 1B, a and b). It has been reported that in normal rat gingival tissue, a low level of neutrophil infiltration is present in response to the normal flora and to CXC chemokines, including monocyte/macrophage inflammatory protein-2 (24). cytokine-induced neutrophil chemoattractant-2, constitutively expressed in the coronal portion of the JE, may contribute to the recruitment of neutrophils in JE areas under physiological conditions. On day 2 after treatment with CDT, many capillary vessels were dilated, resulting in increased permeability and increased infiltration of neutrophils (Fig. 1B, c and d). On day 3, infiltration of neutrophils was seen throughout the JE and the sub-JE area. At the coronal portion of the JE, some cells showed degeneration, cellular distension and desquamation or detachment (Fig. 1B, e and f, arrows). An intraepithelial cleft

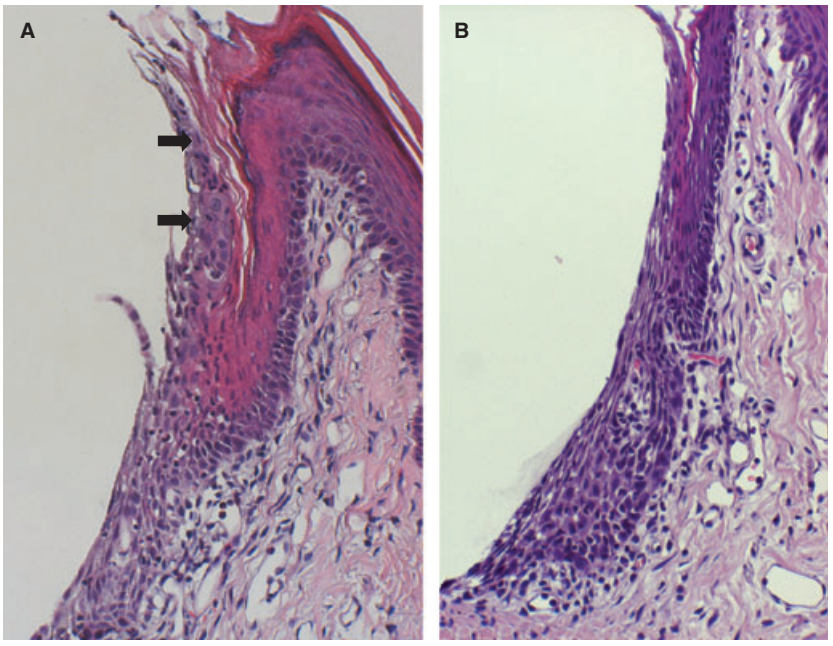

Fig. 2. Effect of the mutated cytolethal distending toxin (CDT) carrying the His274Ala mutation in the CdtB subunit. A CDT-expression plasmid with the site-directed mutation was constructed in the *cdtB* gene, corresponding to a His274Ala mutation in the CdtB subunit that is known to be the catalytic site of the CDT DNase-like enzymatic activity. The mutated CDT holotoxin carrying the His274Ala mutation of CdtB was prepared using the same method as the wild-type holotoxin. The purified CDT and the mutated CDT were introduced into the rat gingival sulcus. After 3 d, periodontal tissues were examined after staining with hematoxylin and eosin. Infiltration of neutrophils was observed in animals treated with both wild-type (A) and mutant (B) CDT. However, epithelial damage at the coronal portion of the junctional epithelium was seen only in the animals treated with wild-type CDT (A). Magnification 100×.

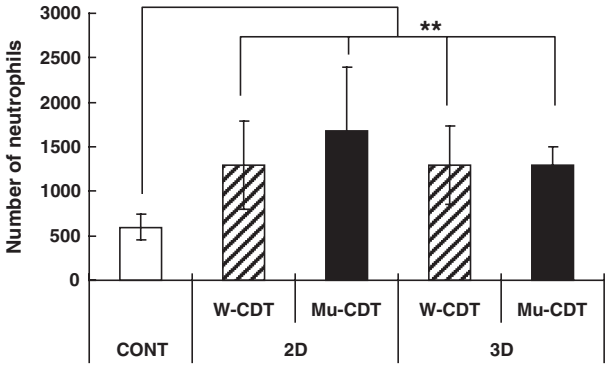

Fig. 3. Effects of wild-type cytolethal distending toxin (CDT) (W-CDT) and mutated CDT (Mu-CDT) on the number of neutrophils infiltrating into the junctional epithelium. Small round cells that had a nucleus with lobes were defined as neutrophils. Both W-CDT and Mu-CDT induced a significant increase in the numbers of neutrophils at 2 d (2D) and 3 d (3D). Data are shown as mean ± standard deviation. $^{**}p < 0.01$.

Table 1. Epithelial damage, induced by cytolethal distending toxin (CDT) from *Aggregatibacter actinomycetemcomitans*, of the coronal portion of the junctional epithelium

Days post-treatment with CDT	Treatment	Specimens showing epithelial damage	
		Number showing damage/total	Percentage
	Control	0/10	0.00
Two days	W-CDT	6/17	35.00
	Mu-CDT	0/8	0.00
Three days	W-CDT	5/11	45.50
	Mu-CDT	0/8	0.00

Mu-CDT, mutated CDT; W-CDT, wild-type CDT.

in the JE was also observed as a result of desquamation of degenerative epithelial cells.

CDT induced damage at the junctional epithelium

To determine if CDT was able to trigger subgingival inflammation and epithelial damage through its genotoxic activity, we histologically examined samples treated with wild-type CDT and the mutated CDT. The mutated CDT lacks genotoxic activity *in vitro* because its CdtB component carries a site-directed mutation (His274Ala) in the amino acid necessary in the catalytic site for the CDT DNase-like enzymatic activity. In both sections, infiltration of the inflammatory cells was observed, suggesting that the inflammatory effect might not be a result of the genotoxicity of CdtB (Figs 2A, B and 3). In support of this, Akifusa *et al.* (25) reported that the CdtC subunit could be the possible inducer of inflammation. Cell damage at the JE (Fig. 2A, arrows) was seen only in the sections treated with wild-type CDT, suggesting that epithelial abrasion was induced by the genotoxic activity of CDT (Fig. 2A, Table 1).

Cell cycle block of the CDT-treated basal cells

To determine the effect of CDT on the cell cycle of periodontal tissue cells, an immuno-histopathology study was performed. Several antibodies relevant to DNA double-strand breakage and cell cycle blockage, including anti-(phospho-histone H2AX) and the anti-(phospho-Cdc2 cyclin B complex) that we frequently used to observe the *in vitro* cellular effects of CDT, showed no immunoreactivity. In addition to the normal tissue-fixation procedure, histology examination of periodontal tissue requires the tooth to be decalcified before staining. This additional process may have affected the antigenicity of the sections. The antibody that we found to be reactive with our immunostained specimens was raised against the PCNA, which is known as a cofactor for DNA polymerase and a marker for dividing cells (26). In the untreated control sections, PCNA-positive cells were relatively common in the basal cell layers (Fig. 4A). In the sections of cells analyzed 3 d after treatment with CDT, PCNA-positive cells were absent from the gingival sulcus, JE regions and gingival epithelium (Fig. 4B arrows). As a positive control, LPS treatment induced increased expression of PCNA in both the JE and the gingival epithelium, as previously reported (27) (Fig. 4C). This is in contrast to the effect of CDT intoxication in cell culture. Our observations suggest that *in vivo* treatment with CDT induced cell cycle block in the basal cells in the JE and the gingival epithelium, leading to desquamation and detachment of the JE. The JE is a unique tissue through which supramolecules, such as LPS or protein, can be easily transported (28–30).

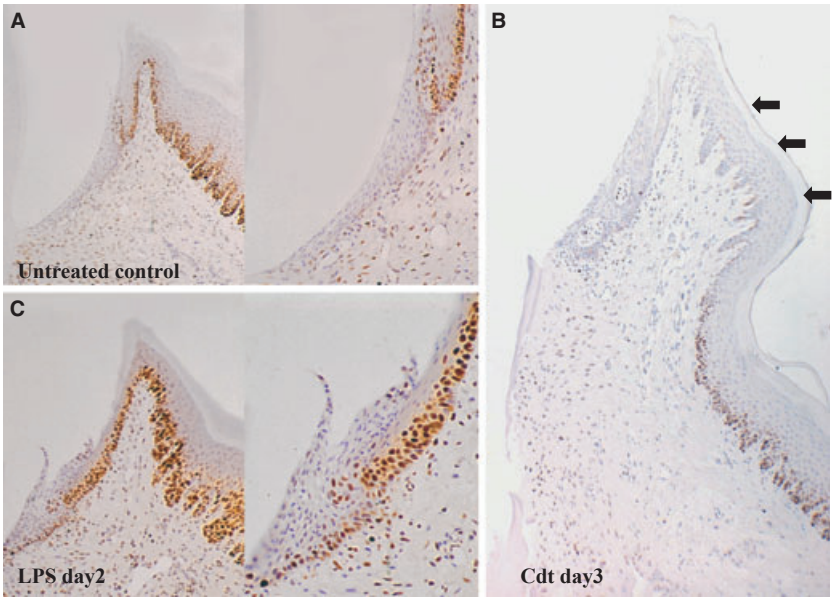

Fig. 4. Expression of the proliferating cell nuclear antigen (PCNA) in cytolethal distending toxin (CDT)-treated rat gingival tissues. To detect cell cycle arrest, an immunohistochemistry study against the PCNA was performed. PCNA is a known cofactor for DNA polymerase. We used mouse anti-PCNA IgG (PC-10) and anti-mouse IgG conjugated with horseradish peroxidase. In the untreated control animals, PCNA-positive cells were relatively frequent in the basal cell layers of the oral sulcular and oral gingival epithelium. Several PCNA-positive cells were also seen in the basal cell layer of the junctional epithelium (A). In the gingival tissue, 3 d after CDT treatment (B), PCNA-positive cells had disappeared from the gingival sulcus and junctional epithelium and had also disappeared from the gingival epithelium adjacent to the gingival sulcus (arrows). A positive control, using LPS-treated gingival tissue, showed an increase of PCNA-positive cells in these epithelia. (A) and (C) left panels, and (B), magnification 40×; (A) and (C) right panels, magnification 100×.

Our data further confirmed this characteristic and showed that topical application of very small amounts of CDT into the sulcus induced profound effects on periodontal tissues. Moreover, abrasion of the sulcus epithelium may further enhance the entry of such supramolecules into periodontal tissue and facilitate the adherence and invasion of periodontopathogenic bacteria.

A. actinomycetemcomitans CDT is implicated in the pathogenesis of localized aggressive periodontitis (1,31,32). CDT production from *A. actinomycetemcomitans* clinical isolates was observed in more than 86% of 50 strains from a Swedish group, 85% of 34 strains from a Brazilian group and 89% of 45 strains from a Japanese group (33–35). The biological effect of CDT on cells from periodontal tissue origin has been investigated using primary cell culture. CDT stimulates RANKL, but not osteoprotegerin, in gingival fibroblast culture cells (36). It has been suggested that RANKL activation is the primary factor in acute alveolar bone absorption in aggressive periodontitis. Gingival fibroblast cells also produced interleukin (IL)-6, and this is probably related to the inflammation of the periodontal tissues, but not to RANKL expression, because neutralizing antibodies for IL-6 did not inhibit RANKL expression. CDT induces expression of IL-6, IL-1β and IL-8 in peripheral blood mononuclear cells (25). However, there is no *in vivo* experimental data to support these observations. Furthermore, *A. actinomycetemcomitans* produces a variety of virulence factors other than CDT (1). The lack of 'whole' bacteria in our experiment is a limitation of our study. Further detailed *in vivo* studies might be necessary to elucidate the pathophysiological role of CDT intoxication in the etiology of *A. actinomycetemcomitans*-induced progressive periodontitis.

This study is the first *in vivo* demonstration, using an animal model, to show that *A. actinomycetemcomitans* CDT induces cell cycle arrest in periodontal tissues. Therefore, CDT-induced cell cycle arrest may be one of the initiation pathways that results in the onset of periodontitis.

Acknowledgements

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