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Involvement of Lamin A/C in Apoptosis of Human Monocytic Cells Induced by *Actinobacillus actinomycetemcomitans* Cytolethal Distending Toxin

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Abstract

Actinobacillus actinomycetemcomitans, an oral bacterium implicated in the etiology of periodontal and systemic diseases, produces cytolethal distending toxin (CDT) that induces G₂/M cell cycle arrest followed by cell death. To investigate the possible role of CDT in the induction of monocyte apoptosis, I analysed the molecular mechanisms involved in the apoptotic pathway purified recombinant CDT in human monocytic U937 cells treated with purified recombinant CDT. Treatment with CDT caused cellular distension and condensation of nuclei after 24 h. Flow cytometry analysis revealed that U937 cells treated with CDT were arrested in the G₂/M phase of the cell cycle after 18 h, followed by DNA fragmentation and breakdown of the cell membrane after 24 h. It is well known that the degradation of lamin A/C occurs during apoptosis. In the present study, a found that the degradation of lamin A/C was observed after 24 h of CDT treatment, and that the degradation was significantly reduced by an inhibitor of caspase-6, partially reduced by an inhibitor of caspase-9, but not affected by an inhibitor of caspase-3/7/8. In contrast, caspase inhibitors suppressed DNA fragmentation, but had no effect on the G₂/M arrest of CDT-treated U937 cells. These results suggest the importance of the apoptotic pathway in lamin degradation through the activation of caspases in CDT-induced apoptosis of human monocytes.

Key words: Cytolethal distending toxin/*Actinobacillus actinomycetemcomitans*/Apoptosis/
Lamin

Introduction

Actinobacillus actinomycetemcomitans is a Gram-negative, capnophilic, fermentative coccobacillus that has been implicated in the pathogenesis of various human systemic

diseases, such as endocarditis, periocarditis, meningitis, osteomyelitis, empyema, and subcutaneous abscess, as well as aggressive periodontitis¹⁻⁴⁾. This organism has putative virulence factors, such as LPS, and secretes several toxic proteins, including cytolethal distending toxin (CDT)⁵⁾. Recent studies have proposed that this bacterium produces immunosuppressive factors that allow it to slip through the host immune defence, and impair the host immune response⁶⁻⁸⁾.

CDT was initially isolated from *A. actinomycetemcomitans* as an immunosuppressive factor^{9,10)}. This heat-labile holotoxin is produced by a range of pathogenic Gram-negative bacteria, such as *Escherichia coli*, *Haemophilus ducleyi*, *Campylobacter jejuni*, and multiple *Shigella* species¹¹⁾. CDT contains three subunits, CdtA, CdtB, and CdtC, which appear to function as an AB₂ holotoxin^{12,13)}. CdtB, the active subunit of CDT and a homologue of DNase, is delivered into the cytosol of its target cells by means of receptor-mediated endocytosis. From there it is retrogradely transported to the ER through unknown pathways, and driven into the nuclei¹⁴⁻¹⁷⁾. Most recent studies have shown that CdtA and CdtC bind to the cell-surface moiety of gangliosides such as GM3, and play a role in transporting CdtB into target cells via cholesterol-rich rafts^{18,19)}.

In response to CDT, eukaryotic cells, except for some fibroblastic cells, are arrested irreversibly at the G₂/M phase and exhibit progressive cellular distension with subsequent apoptosis¹¹⁾. Recently, two groups proposed that CDT treatment causes a critical increase in the activity of initiator caspases (caspase-2, -8, -9) and effector caspases (caspases-3, -7), which are involved in the apoptotic pathway^{20,21)}. Interestingly, cells that overexpress Bcl-2 or various caspase inhibitors largely escape the apoptotic pathway, but not cell cycle arrest, upon CDT treatment²⁰⁾. Despite these findings, the role of caspases in CDT-induced apoptosis remains virtually unknown.

In this study, I investigated the effect of CDT on monocytic cell proliferation, and determined the correlation with the activity of various caspases. The present results showed that CDT induces apoptotic cell death in the U937 monocytic cell line via activation of caspases and lamin A/C degradation. I discuss the close link between caspase cascades and lamin A/C degradation in CDT-treated cells.

Methods

I Cells and culture conditions

The human monocyte cell line U937 was grown in RPMI1640 (GIBCO laboratories, Grand Island, NY) containing 2 mM L-glutamine, 10 % fetal calf serum, 100 µg/ml streptomycin, and 100 IU/ml penicillin in an atmosphere containing 5 % CO₂.

II Purification of recombinant proteins

Actinobacillus actinomycetemcomitans CDT was purified from *E. coli* carrying the *A. actinomycetemcomitans cdtABC* genes¹⁸⁾. *E. coli* transfected with a pET-28a expression vector (Novagen, Nottingham, United Kingdom) containing the *cdtA*, *cdtB*, or *cdtC* gene was grown

overnight in LB broth containing 30 $\mu\text{g/ml}$ kanamycin and 200 $\mu\text{g/ml}$ rifampicin, then diluted 1:20 in fresh broth, and incubated for a further 3 h at 37 °C. To stimulate gene expression, 1 mM isopropyl- β -D-thiogalactopyranoside was added and the culture was incubated for 6 h at 30 °C. The cells were harvested by centrifugation at 5,000 $\times g$ for 30 min, then resuspended and lysed for 10 min in the protein extraction reagent B-PER (Pierce, Rockford, IL). As the expressed proteins were contained in inclusion bodies, purification was performed as described by the B-PER manufacturer. The lysates were centrifuged, and the pellets were resuspended in the same volume of B-PER containing 100 $\mu\text{g/ml}$ lysozyme and incubated for a further 5 min at room temperature. Ten times the initial volume of B-PER diluted 1:10 was added to the lysates, and the inclusion bodies were collected by centrifugation at 15,000 $\times g$ for 20 min. After being washed twice in the same volume of diluted B-PER, the pellets were treated with 8 M urea in PBS, pH 7.5. The recombinant proteins were purified using Ni-nitrilotriacetic acid-agarose columns under denaturing conditions as specified by the manufacturer (Qiagen, Hilden, Germany), except that after the lysates were loaded onto the column, an additional column wash, consisting of 2.5 mg of polymyxin B per milliliter of wash buffer, was conducted to remove LPS. Refolding of the denatured proteins was performed as described previously.

III Detection of apoptotic cells

CDT-treated U937 cells were suspended in a hypotonic solution (0.1 % sodium citrate, 0.2 % NP-40, and 0.25 mg/ml RNase; pH 8.0), stained with 50 $\mu\text{g/ml}$ propidium iodide (PI), and analysed using an EPICS[®] XL flow cytometer (Beckman Coulter, Fullerton, CA). The cell cycle phases were examined using Multicycle for Windows (Phoenix Flow Systems, San Diego, CA).

For Annexin V/PI staining, U937 cells were washed with cold PBS (pH 7.2) and resuspended in a binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl_2 , pH 7.4). Fluorescein-conjugated Annexin V and PI solutions were added and mixed gently according to the manufacturer's instructions (Molecular Probes, Eugene, OR). Cells were incubated at room temperature and analysed using an EPICS[®] XL flow cytometer.

To observe apoptotic nuclei, U937 cells were fixed with 1 % glutaraldehyde for 1 h and washed with PBS. The samples were stained with 56 $\mu\text{g/ml}$ Hoechst dye 33342 (Molecular Probes), mounted on a glass slide, and nuclei were visualised by fluorescence microscopy with an excitation wavelength of 355 nm and an emission wavelength of 465 nm.

IV Immunoblot analysis

U937 cells were lysed in SDS lysis buffer (50 mM Tris-HCl and 2 % SDS, pH 6.8), and 15 μg samples of the protein extracts were subjected to electrophoresis in 12.5 % SDS-polyacrylamide gels and then electroblotted on PVDF membranes. After incubation with 5 % nonfat milk in PBS for 1 h, the membranes were reacted with primary antibodies for 8 h at 4 °C. Immunodetection was performed using an ECL Western blotting detection

system (Amersham Pharmacia Biotech, UK) according to the manufacturer's instructions. The protein contents of the samples were determined with a protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Blots were stained with Coomassie brilliant blue to confirm equal protein loading.

V Determination of caspase activity

Caspase-3, -6, and -9 activity in the cytosol of U937 cells treated with CDT was determined using Chemicon's Colorimetric Activity Assay Kit (Chemicon International, Temecula, CA). CDT-treated cells were lysed with a lysis buffer on ice for 10 min. Suspended cells were then centrifuged for 5 min, and the activity of each caspase in the cytosolic extracts was determined in each case according to the manufacturer's instructions.

Results

I CDT-induced apoptosis of U937 cells

To examine whether CDT could induce apoptosis in U937 cells, I used a combination of Annexin V and PI. As shown in Fig. 1A, flow cytometry revealed that the percentage of apoptotic U937 cells began to increase 18 h after treatment with 1 μ g/ml CDT and continued to increase until 24 h after treatment. Nuclear DNA accumulation indicating G₂/M arrest was observed 18 h after CDT treatment, based on the PI fluorescence. In addition, sub-G1 peak was detected when CDT-treated U937 cells were cultured for 24 h (Fig. 1B). I also investigated the morphological changes in CDT-treated U937 cells. After culture with 1 μ g/ml CDT for 24 h, U937 cells were stained with Hoechst dye and visualised under fluorescence microscopy. As shown in Fig. 1C, apoptotic cells were identified according to characteristic cell morphology, such as condensation and degradation of nuclei. In addition, treated cells were three to five times more distended than untreated U937 cells. These results strongly suggest that CDT induces apoptotic cell death in U937 cells. Immunoblot analysis revealed that CDT increased the amount of cleaved lamin A in a time-dependent manner (Fig. 2). The 28 kDa fragment was first detected 12 h after CDT treatment, and its intensity peaked 24 h after treatment.

II Caspase signalling pathways

To investigate the activity of caspase signalling pathways during CDT-induced apoptosis, I examined the effects of a variety of caspase inhibitors on CDT cytotoxicity in U937 cells using immunoblot analysis. Preincubation with 100 μ M Z-LEHD-FMK, an inhibitor of caspase-9, for 30 min partially reduced lamin A/C degradation in CDT-treated cells (Fig. 3). Preincubation with 100 μ M Ac-VEID-CHO, an inhibitor of caspase-6, for 30 min significantly reduced lamin A/C degradation in CDT-treated cells (Fig. 4). In contrast, preincubation with 100 μ M Ac-DEVD-CHO, an inhibitor of caspase-3/7/8, for 30 min had no effect on lamin A/C degradation (Fig. 5). In addition, I determined the percentage of apoptotic U937 cells and of G₂/M phase cells using PI fluorescence and flow cytometry to observe the nuclear

Fig. 1 Apoptosis of U937 cells induced by CDT.

Apoptotic cells exhibiting characteristic chromatin condensation were observed under fluorescence microscopy ($\times 200$). Bar; 50 μ m

Fig. 2 Cleavage of lamin A/C by CDT in a time-dependent manner.

Fig. 3 Effect of Z-LEHD-FMK on CDT-induced apoptosis.

The cells were analysed for expression of the 28 kDa fragment of lamin A/C by immunoblotting.

Fig. 4 Effect of Ac-VEID-CHO inhibitor on CDT-induced apoptosis. The cells were analysed for expression of the 28 kDa fragment of lamin A/C by immunoblotting.

Fig. 5 Effect of Ac-DEVD-CHO on CDT-induced apoptosis. The cells were analysed for expression of the 28 kDa fragment of lamin A/C by immunoblotting.

Fig. 6 Effects of various caspase inhibitors on CDT-induced apoptosis or G₂/M arrest. After 24 h, the cells were stained with PI and analysed by flow cytometry. The values shown indicate the percentage of the cell population in the G₂/M phase with error bars. The bars show standard error.

DNA. Inhibitors of caspase-3, -6 and -9 reduced CDT-induced DNA fragmentation to 62.5, 50.6, and 52.6 %, respectively, compared to a positive control (Fig. 6A). In contrast, these inhibitors had no effect on the G₂/M arrest induced by CDT (Fig. 6B).

Discussion

Monocytes play a central role in inflammatory reactions caused by pathogenic bacteria. Many pathogens have evolved specific mechanisms for avoiding, altering or disabling the antimicrobial effects of monocytes. I have previously reported that *A. actinomycetemcomitans* CDT induced cell cycle arrest at G₂/M in the human monocyte cell line U937¹⁸⁾. The present study demonstrated the apoptotic cell death of U937 cells following *A. actinomycetemcomitans* CDT intoxication. Lamins are intranuclear intermediate filament proteins that form a fibrous layer interposed between the inner nuclear membrane and the chromatin²²⁾. Lamins are also present in the nuclear interior, where they have been implicated in organising transcription, replication, and DNA repair²³⁾. The proteolytic degradation of lamin A occurred via activation of caspase signalling pathways during apoptosis, resulting in nuclear disregulation and cell death. The present results demonstrated that lamin A/C degradation occurs in CDT-induced apoptosis. It is well-known that lamin A is one of the major substrates of caspase-6, and this results strongly suggest that cleavage of lamin A, resulting in nuclear disregulation, is caused by caspase-6 via a classical caspase pathway.

Many studies have demonstrated that a G₂/M arrest precedes the onset of apoptosis after approximately 24 h in CDT-treated lymphocytes^{15, 21, 24)}; however, these two sequential events are independent, since overexpression of an anti-apoptotic protein, Bcl-2, blocks apoptosis but not the cell cycle arrest of CDT-treated cells²⁰⁾. This study suggest that G₂/M arrest and apoptotic cell death are independent events in CDT intoxication.

It remains unclear whether the characteristic DNA degradation during apoptosis is directly caused by endogenous nucleases, such as CAD or CdtB, localized in the nuclei. Previous reports have indicated that nuclear-localised CdtB induced double-stranded DNA breaks¹⁶⁾ and activated the DNA-damage checkpoint response via ATM kinase in treated cells²⁵⁾. It has also been shown that CDT causes the phosphorylation of the histone H2AX as early as 1 h after treatment in an ATM-kinase-dependent manner²⁶⁾, and this phosphorylation is inhibited by overexpression of Bcl-2 in lymphocytes²⁴⁾. Moreover, it was reported that lamin A is involved in the regulation of ATM and histone H2AX function and localization²⁷⁾. Further analysis of the interaction of lamins and the DNA-damage checkpoint during CDT treatment will clarify the mechanism of CDT action.

In conclusion, these findings indicate that *A. actinomycetemcomitans* CDT induces apoptosis in U937 cells and that the cleavage of lamin via activation of caspase-6, but not caspase-3, is involved in this apoptosis. Defection of the immune system by CDT-induced apoptosis occurs in periodontal tissue during chronic infection with *A. actinomycetemcomitans*. Elucidation of the cellular and molecular mechanisms of *A. actinomycetemcomitans* CDT-induced human monocyte apoptosis will contribute to facilitating identification of specific

molecular targets in periodontal disease therapy.

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CDT が誘導するアポトーシスにおける Lamin A/C の解析

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抄 録

Actinobacillus actinomycetemcomitans は歯周病の原因と考えられている口腔内細菌で、感染した免疫細胞にアポトーシスを誘導することが知られており、このことが歯周疾患の慢性化の一因と考えられている。本研究では、この免疫細胞を障害する因子として本菌が産生するタンパク毒素 cytolethal distending toxin (CDT) に注目し、ヒト単球細胞 U937 細胞を用い、CDT による細胞障害機序を分子生物学的な手法を用いて解析した。CDT を作用させた U937 細胞では、18 時間後に G₂/M 期で細胞周期が停止し、24 時間後には顕著な細胞膨張とアポトーシス特有の核濃縮、および染色体 DNA の断片化と細胞膜の崩壊が観察された。また、CDT 処理後 24 時間で、核膜成分である lamin A/C の開裂が観察された。この開裂は caspase-6 の活性を阻害すると著しく減弱され、caspase-9 を阻害すると部分的に減弱された。しかし、caspase-3/7/8 を阻害しても変化はみられなかった。なお、caspase-3, -6, -9 のいずれを阻害しても、細胞周期の停止に変化は見られなかった。以上のことから、CDT はヒト単球細胞をアポトーシスにより死滅させるが、このアポトーシスでは、caspase の活性化と、それに引き続き lamin A/C の開裂が誘導されることが示唆された。

キーワード : Cytolethal distending toxin/*Actinobacillus actinomycetemcomitans*/アポトーシス/Lamin