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Activation of apoptosis by caspase-3-dependent specific RelB cleavage in anticancer agent-treated cancer cells: Involvement of positive feedback mechanism

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ABSTRACT

DTCM-glutarimide (DTCM-G) is a newly found anti-inflammatory agent. In the course of experiments with lymphoma cells, we found that DTCM-G induced specific RelB cleavage. Anticancer agent vinblastine also induced the specific RelB cleavage in human fibrosarcoma HT1080 cells. The site-directed mutagenesis analysis revealed that the Asp205 site in RelB was specifically cleaved possibly by caspase-3 in vinblastine-treated HT1080 cells. Moreover, the cells stably overexpressing RelB Asp205Ala were resistant to vinblastine-induced apoptosis. Thus, the specific Asp205 cleavage of RelB by caspase-3 would be involved in the apoptosis induction by anticancer agents, which would provide the positive feedback mechanism.

1. Introduction

Most anticancer agents induce apoptosis in cancer and leukemia cells. Therefore, effective way of inducing apoptosis especially in drug-resistant cancer cells should be useful for future cancer therapy.

Adult T-cell leukemia (ATL) is caused by human T-cell leukemia virus (HTLV), and is highly resistant to chemotherapy [1,2]. MT-1 is a leukemic T-cell line derived from the ATL patient [3]. MT-1 is Tax-negative, and shows constitutively activated NF-κB [4].

NF-κB forms a family of transcription factors that activate expressions of many inflammatory cytokines and anti-apoptosis proteins [5,6]. The NF-κB family is composed of dimers of five members, including p65, RelB, c-Rel, p50, and p52 [7,8]. There are two signaling pathways leading to the activation of NF-κB known as the canonical pathway and the non-canonical pathway [9]. Canonical NF-κB mainly consists of p65 and p50, and has roles in immunity, general inflammation, and cancer progression. In one hand, non-canonical NF-κB mainly consists of RelB and p52, and often important in B-cell maturation and autoimmune diseases. However, it was recently reported that it would be involved in cancer and lymphoma [5].

DTCM-glutarimide (3-[(dodecylthiocarbonyl)methyl]glutarimide or DTCM-G) is a derivative of 9-methylstreptimidone, a known piperidine compound from *Streptomyces* [10]. It inhibits NF-κB, but its mechanism of inhibition has not been elucidated. It showed anti-inflammatory activity *in vivo* in mouse heart transplantation model [11,12].

In the present research we have studied anticancer activity of DTCM-G using ATL cells. In the course of this study, we found DTCM-G and common anticancer agents induced specific scission at Asp205 (D205) in RelB that is the site cleaved by caspase-3. We have then studied the role of D205 scission by preparation of scission-resistant cells by site-directed mutagenesis in human fibrosarcome HT1080 cells. Thus, we found a new positive feedback system in which RelB scission by caspase-3 would further activate apoptosis in anticancer-treated cancer cells.

2. Materials and methods

2.1. Materials

DTCM-G was synthesized in our laboratory as described before [10]. Ac-DEVD-CHO was purchased from Sigma–Aldrich (St. Louis, MO). Doxorubicin-HCl (adriamycin) and vinblastine sulfate were purchased from Enzo Life Sciences (Famingdale, NY).

2.2. Cell culture

MT-1 cells kindly supplied by Dr. R. Horie, Kitasato University, were cultured in RPMI containing 10% (v/v) heat inactivated FBS,

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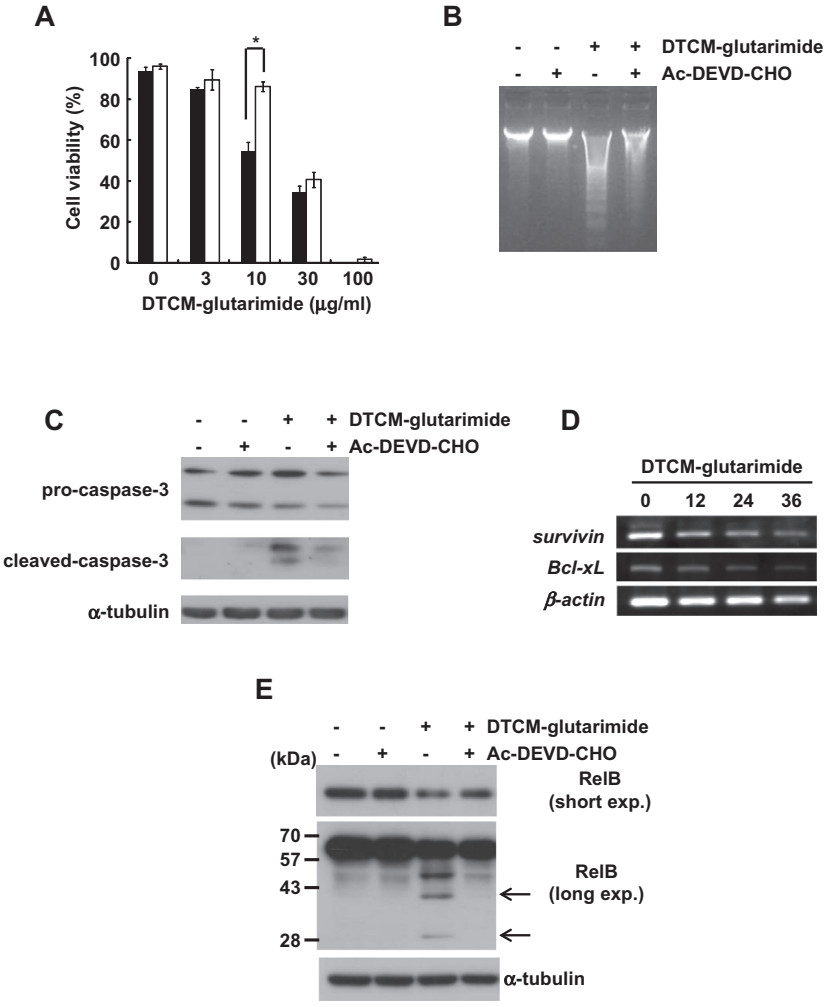

Fig. 1. Induction of apoptosis and RelB cleavage by DTCM-G in MT-1 cells. (A) Induction of cell death by DTCM-G. The cells were incubated for 24 h with DTCM-G with (white bars) or without (black boxes) 20 μM Ac-DEVD-CHO. Cell viability was assessed with trypan blue dye exclusion. (B) Induction of apoptosis by DTCM-G. The cells were incubated for 12 h. Cellular DNA was extracted and electrophoresed on 1% agarose gel. (C) Activation of caspase-3 by DTCM-G. The cells were incubated for 8 h. (D) Inhibition of apoptosis inhibitory protein expressions by DTCM-G. Cells were treated with 10 μg/mL DTCM-G for the indicated h. Expressions of survivin, Bcl-xL, and β-actin mRNA were analyzed by semi-quantitative PCR. (E) Caspase-3-mediated cleavage of RelB protein. Cells were incubated with DTCM-G for 8 h. Total cell lysate was subjected to SDS-PAGE and immunoblotted with anti-RelB or anti-α-tubulin antibodies.

100 units/mL penicillin G, 200 mg/L kanamycin, 600 mg/L L-glutamine, and 2.25 g/L $NaHCO_3$ at 37 °C in a humidified atmosphere with 5% CO_2. HT1080 cells, purchased from Japanese Cancer Research Resources Bank, were cultured in DMEM supplemented with 10% (v/v) FBS, 100 mg/L kanamycin, 100 units/mL penicillin G, 600 mg/L L-glutamine, and 2.25 g/L $NaHCO_3$ at 37 °C in a humidified incubator with 5% CO_2.

2.3. Construction of RelB expression plasmids

Wild-type FLAG-RelB-myc cDNA subcloned into pCMV-Tag2B vector (Stratagene, La Jolla, CA, USA) was constructed previously [13]. We substituted the Asp205 (D205) residue in RelB with Ala (A) residues by PCR site-directed mutagenesis with overlap extension technique [13]. The sequences of primers used for the mutagenesis were as follows: 5′-CAGCCTCGTGGGGAAAGACTGCAC CGCCGG-3′ (forward) and 5′-CCCTGCAGATGCCGGCGGTGCAGTCTT TCC-3′ (reverse). The sequences of primers used for D262A and R85A were follows: D262A 5′-CATCAGGAAGTAGCCATGAATGTGGT GAGG-3′ (forward) and 5′-GAAGCAGATCCTCACCACATTCATGGCT AC-3′ (reverse), and R85A 5′-GCCACGCCTGGTGTCTGCCGGGGCTG CGTC-3′ (forward) and 5′-GACGCAGCCCCGGCAGACACCAGGCGTG GC-3′ (reverse). For the preparation of RelB (D205E) mutant, the sequences of primers used were as follows: 5′- GGGAAAGACTGCAC CGAGGGCATCTGCAGG-3′ (forward) and 5′- CCGCACCCTGCAGATG CCCTCGGTGCAGTC-3′ (reverse).

2.4. Establishment of RelB-overexpressing cell lines

A permanent cell line expressing wild-type RelB (HT1080-FLAG-RelB-myc) was established previously [13]. Permanent cell lines expressing mutant FLAG-RelB-myc were established by transfecting the vectors into HT1080 cells, followed by 400 μg/mL G418 (Roche Applied Sciences, Indianapolis, IN) selection. The clone cells that expressed high levels of FLAG and myc-tagged RelB (D205A) was designated HT1080-FLAG-RelB-myc (D205A) cells. The cells transfected with pCMV-Tag2B vector were designated HT1080-Neo [13].

2.5. Semi-quantitative RT-PCR

Total RNAs were extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) as described in the manufacturer's method, and solutions containing 1 μg of total RNAs were taken for the reverse

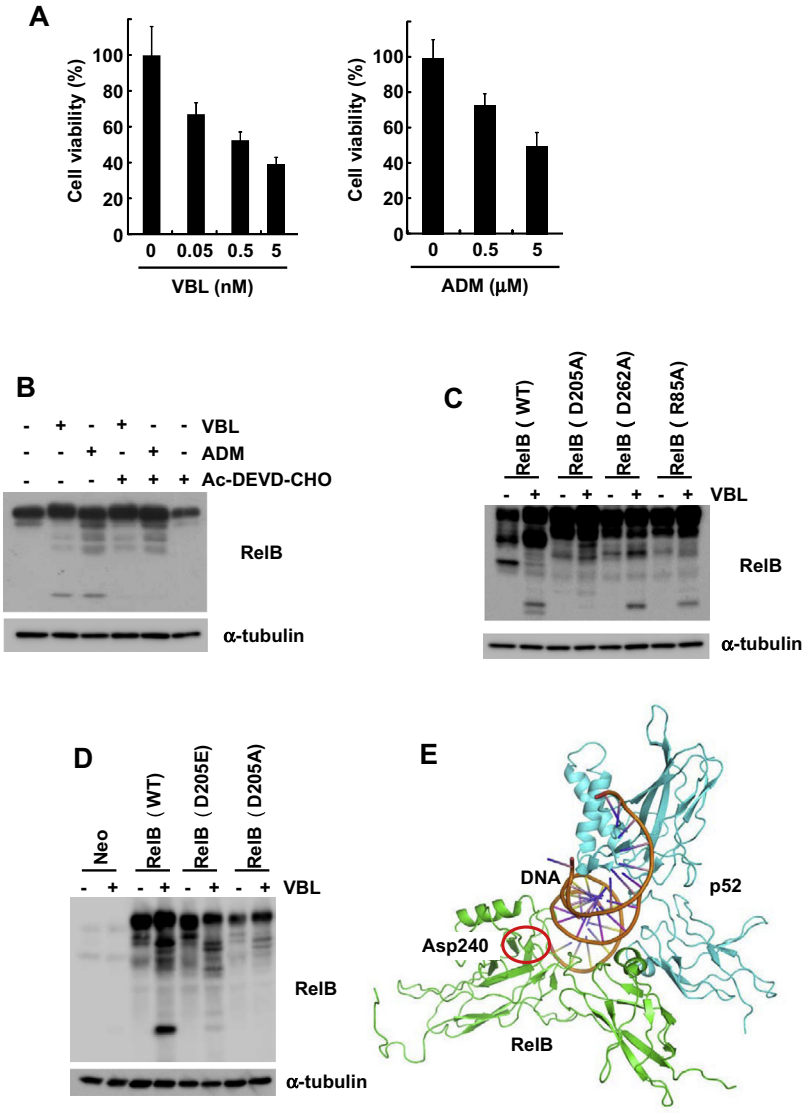

Fig. 2. Limited RelB cleavage in anticancer agent-treated HT1080 cells. (A) Cytotoxicity of vinblastine (VBL) and adriamycin (ADM) in HT1080 cells. Cells were treated with anticancer agents for 24 h, and cell viability was assessed by trypan blue dye exclusion. Each value represents the mean plus/minus standard deviation for 4 samples. (B) Induction of caspase-dependent RelB cleavage by anticancer agents. Cells were incubated for 24 h. Total cell lysate was subjected to SDS-PAGE and immunoblotted with anti-RelB or anti-α-tubulin antibodies. (C) Identification of cleavage site in RelB in vinblastine-treated HT1080 cells. The cells were over-expressed with FLAG-tagged RelB (wild type, D205A, D262A, and R85A), then treated with 1 nM vinblastine for 24 h. Total cell lysate was subjected to SDS–PAGE and immunoblotted with anti-RelB or anti-α-tubulin antibodies. (D) Inhibition of vinblastine-induced RelB scission by D205E mutation. The cells were treated with 5 nM vinblastine for 24 h. (E) X-ray structure of mouse RelB/p52/DNA complex (PDB; 3DO7). The images were rendered by using PyMol software. DNA, mouse RelB, and human p52 are drawn by orange, green, and cyan, respectively. The mouse Asp240 site shown by red circle is identical to human Asp205. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

transcription reaction that was performed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA), as described previously [14]. The sequences of the primers used in the semi-quantitative RT-PCR were as follows: survivin; 5′-CAGC CCTTTCTCAAGGACCA-3′ (forward) and 5′-GCACTTTCTCCGCAGT TTCC-3′ (reverse), Bcl-xL; 5′-GGAGCTGGTGGTTGACTTTC-3′ (forward) and 5′-CTGCTGCATTGTTCCCATAG-3′ (reverse), and β-actin; 5′-GATGACGACGATAAGAGCCC-3′ (forward) and 5′-GAAGCCGTT CTCCTTGATGT-3′ (reverse). PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide, and visualized under a UV illuminator.

2.6. Apoptosis analysis by flow cytometry

HT1080 cells (2.3×10^5) grown on 60-mm dishes were treated with vinblastine for 36 h. After the incubation period, cells were trypsinized and mixed supernatant, then total cells were fixed with 70% EtOH. Subsequent to being treated with PBS containing RNase (10 μg/mL), the fixed cells were incubated for 30 min at 37 °C. Cells were resuspended in propidium iodide (50 μg/mL) solution. The stained cells were then analyzed by flow cytometry (BECKMAN COURTER, Cytomics FC 500). DNA content of the cells at different phases of cell cycle was determined.

3. Results

3.1. DTCM-glutarimide induced apoptosis and RelB cleavage in MT-1 cells

As shown in Fig. 1A, DTCM-G induced cell death in MT-1 cells at 10 μg/mL in 24 h, which was partly canceled by a caspase-3 inhibitor Ac-DEVD-CHO. Apoptosis induction was shown by DNA ladder

formation as shown in Fig. 1B. DTCM-G also induced the cleavage of pro-caspase-3 in 8 h, which was inhibited by Ac-DEVD-CHO (Fig. 1C). Thus, DTCM-G induced apoptosis in MT-1 cells. DTCM-G lowered expressions of survivin and Bcl-xL in 12–24 h (Fig. 1D). MT-1 cells have been confirmed to possess RelB [15]. Next, we found that DTCM-G decreased the protein level of RelB in 8 h. Moreover, the RelB degradation should be due to the limited protein cleavage producing limited number of fragments, as shown in Fig. 1E. A caspase inhibitor Ac-DEVD-CHO inhibited the specific cleavage of RelB, therefore, the cleavage was suggested to be dependent on caspase-3. These results suggest that DTCM-G induces apoptosis through RelB cleavage, thereby decreased NF-κB-dependent survivin and Bcl-xL mRNA expressions.

3.2. Limited RelB cleavage at Asp205 by anticancer agents in HT1080 cells

For the characterization of RelB cleavage, we employed human fibrosarcoma HT1080 cells, in which gene manipulation is easier. Moreover, we employed vinblastine and adriamycin for the apoptosis-inducing agents. Vinblastine lowered the viability of HT1080 cells at 0.05–5 nM in 24 h, while adriamycin at 0.5–5 μM (Fig. 2A). As shown in Fig. 2B, RelB cleavage was also observed by vinblastine and adriamycin in HT1080 cells. These RelB cleavages were inhibited by a caspase-3 inhibitor Ac-DEVD-CHO. Caspase-3 is known to recognize the specific amino acid sequence such as conserved DXXD consensus tetrapeptide motif [16]. We have then selected the mutation site at Asp205 and Asp262. In addition, RelB is known to be cleaved by Malt1 at R85 [17]. Then, we introduced point mutation of D205, D262, and R85 into alanine. As a result, RelB (D205A) was resistant to cleavage, while wild type RelB, RelB (D262A) and RelB (R85A) mutant proteins were sensitive (Fig. 2C). We have also prepared RelB (D205E) mutant, since glutamic acid (E) has acidic character as D. This mutant-expressing cells were also resistant to vinblastine on the cleavage (Fig. 2D). Then, these results would indicate that caspase-3 cleaves the Asp-Cys-Thr-Asp (D)205-Gly206 motif of RelB. D205 should be the site of caspase-3 cleavage, and located near the DNA binding region. Fig. 2E shows the X-ray structure of mouse RelB/p52/DNA complex [18], in which D240 is identical to D205 in human RelB.

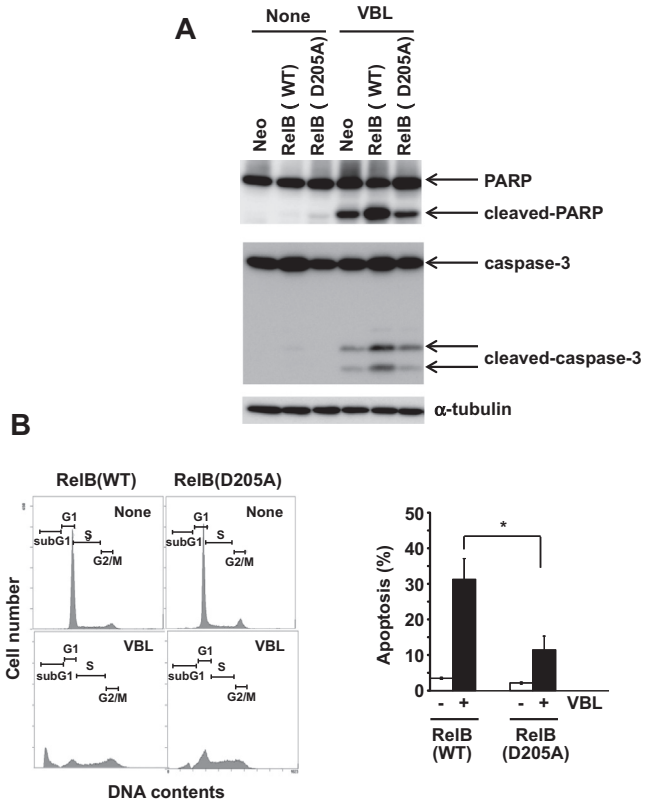

Fig. 3. Suppression of vinblastine-induced apoptosis by expression of RelB (D205A) mutant. (A) Effect on PARP and caspase-3 scissions. The cells were treated with 5 nM vinblastine (VBL) for 24 h. Total cell lysate was subjected to SDS–PAGE and immunoblotted. (B) Effect of D205A mutation on vinblastine-induced apoptosis detected by flow cytometry. Each cell lysate from the cells treated with 5 nM vinblastine (VBL) for 36 h was subjected to flow cytometry stained with propidium iodide. Data are shown as the mean ± SD of 3 independent experiments. $^{*}P < 0.05$, vs. the wild type cells.

3.3. Inhibition of vinblastine-induced apoptosis by RelB D205A mutation

Next we studied the role of RelB cleavage in vinblastine-treated HT1080 cells. Apoptosis was monitored by PARP scission and caspase-3 activation. In both analyses, apoptosis induction was lowered by D205A mutation. Compared with RelB (WT) expression, RelB (D205A) mutant also showed lower apoptosis induction in vinblastine-treated cells assessed by flow cytometric analysis, as shown in Fig. 3B. Thus, RelB degradation by caspase-3 was suggested to be important for apoptosis induction. Taken together, our results indicated that RelB was cleaved by caspase-3, which positively enhanced apoptosis induction.

4. Discussion

We have found that DTCM-G induces apoptosis selectively in Tax-free ATL cell lines including MT-1 and TL-Om1 (data not shown). Since the patient ATL cells are all Tax-free, it may be useful to suppress ATL in future. DTCM-G can be synthesized easily.

Caspase-3 recognizes DXXD motif of apoptotic substrates [16]. In case of p65, caspase-3 cleaves at the position after DCRD97 [19]. It was reported that casapase-3 cleaved p65 at Asp97 (D97) during apoptosis [19]. Caspase-3-mediated cleavage of p65 potentiated naphthoquinone analog-induced apoptosis [19]. In another report, after the caspase-3-mediated cleavage of p65, the carboxy-terminal fragment is formed and it inhibits IκBα and enhances NF-κB and HIV-I replication in human T lymphocytes [20]. The C-terminal fragment loses most of DNA binding domain, nuclear localization signal (NLS), and transcription-activating domain, and cannot bind to DNA. However, since it binds to IκBα, the fragment enhances the activity of full-length p65.

Malt1 cleaves RelB after Arg85. In case of Malt1-dependent RelB cleavage, the cleavage promotes canonical NF-κB activation in lymphocytes and lymphoma cell lines [17].

RelB is known to be degraded during the T-cell activation [21]. In this case, RelB is phosphorylated at Tyr84 and Ser552 resulting in the N-terminal scission and proteasome-dependent degradation. Anticancer agent-induced degradation of RelB may also involve phosphorylation of RelB.

Apoptosis induction in cancer cells is the most important function of anticancer agents. In this report we suggest that anticancer agent-induced apoptosis may often involve positive feedback mechanism with the RelB cleavage by caspase-3.

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