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Synergistic effects of chlorambucil and TRAIL on apoptosis and proliferation of Raji cells

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Abstract. – OBJECTIVE: Tumor necrosis factor (TNF) related apoptosis inducing ligand (TRAIL) can induce the apoptosis of tumor cells, but leaving its effect on malignant lymphoma largely insignificant, as these tumors may develop drug resistance. Chlorambucil (CLB) had definitive treatment efficacy on low-malignant non-Hodgkin lymphoma (NHL), but with unclear efficacy on highly malignant Burkitt lymphoma. A study has been shown that CLB could enhance the sensitivity of chronic lymphatic leukemia cells against TRAIL. This work aims to investigate the effect of CLB combined with TRAIL on in vitro proliferation and apoptosis of Raji cells.

MATERIALS AND METHODS: TRAIL (0, 20, 40 and 80 ng/ml) or CLB (0, 2.5 5 and 10 μM) was used to treat Raji cells. Cell counting kit 8 (CCK-8) was used to test proliferation whilst flow cytometry was employed to examine the apoptosis. The effect of TRAIL or CLB treatment on expression of death receptor 4 (DR4) and DR5 was tested. Combined treatment (80 ng/ml TRAIL and 10 μM CLB) was adopted for observing Raji cell proliferation and apoptosis.

RESULTS: Single treatment of TRAIL or CLB has weak effects of inducing apoptosis or inhibiting proliferation. TRAIL concentration has no significant effects on DR4/DR5 expression in Raji cells, whilst CLB treatment elevated those gene expressions. Combined treatment of TRAIL and CLB had more potent effects regarding cell proliferation inhibition or apoptosis induction compared to single treatment.

CONCLUSIONS: TRAIL or CLB has weak inhibitor effects on Raji cell proliferation or induction of apoptosis. Via up-regulating DR4 and DR5 expression, CLB has synergistic effects with TRAIL to potentiate the apoptotic induction and proliferation inhibition role.

Key Words:
TRAIL, Chlorambucil, Burkitt lymphoma, Raji, Proliferation, Apoptosis.

Introduction

Non-Hodgkin lymphoma (NHL) is a group of malignant tumor of lymphatic-hematological system that occurred in lymphatic organs, including lymph node, spleen and thymus and/or extra-nodal lymphatic tissues or organs[1,2]. NHL can be classified into B cell type, T cell type and NK/T cell type based on different cell origination. B cell NHL occupies more than 80% of all cases[3]. Burkitt's lymphoma is a subtype of highly invasive B cell NHL originated from follicular biogenesis central cells, and occupies about 1-3% of all NHL cases[4,5]. Tumor necrosis factor Related Apoptosis Inducing Ligand (TRAIL) selectively induces tumor cell apoptosis, but has no significant toxicity or killing effects on normal cells, indicating promising insights in clinical application[6]. TRAIL mainly functions on Death Receptor 4 (DR4) and DR5 on cell membrane, further recruiting downstream signal molecules to form Death-Inducing Signaling Complex (DISC), for activating caspase cascade reaction and inducing cell apoptosis[7,8]. Although TRAIL induces the apoptosis of most tumor cells, it has no significant effects on most types of malignant lymphoma, whose drug resistance largely limits application range of TRAIL[9]. Chlorambucil (CLB) is a derivative of nitrogen mustards compound, and can deprive the function of complementary strands of DNA molecules via alkalization-induced cross interaction, thus inhibiting tumor cell proliferation[10]. CLB has a long history as the first line medication for treating low-malignant NHL with definitive efficacy, but has undetermined effects on treating highly-malignant Burkitt lymphoma[11]. Increasing studies showed the synergistic effects on tumor cell killing by TRAIL in conjunction with other

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chemotherapy reagents including aclarubicin and adriamycin, thus overcoming resistance against TRAIL of tumor cells, and providing possibilities for tumor treatment[12,13]. Current studies found that CLB enhanced TRAIL sensitivity of lymphoblastoid SKW6.4[14] and chronic lymphocyte leukemia cell line[15]. This study, thus, investigated the effect of CLB combined with TRAIL on *in vitro* proliferation and apoptosis of Burkitt lymphoma cell line Raji.

Materials and Methods

Major Reagent, Materials and Instrument

Burkitt lymphoma cell line Raji was purchased from Cell Bank, China Academy of Science. Roswell Park Memorial Institute 1640 (RPMI 1640) culture medium, fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco Co., Ltd., (Grand Island, NY, USA). Rabbit anti-cleave caspase-3 monoclonal antibody was purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). FITC-labeled Ki67 antibody was purchased from eBioscience (San Diego, CA, USA). Mouse anti-human DR4/DR5 polyclonal antibody was purchased from Santa Cruz Biothech. (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-coupled goat anti-mouse and goat anti-rabbit secondary antibody were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Annexin V/propidium iodide (PI) apoptotic assay kit was purchased from Shanghai Yusheng Enterprise Development Co. Ltd. (Shanghai, China). Caspase-3 activity kit was purchased from Beyotime Biotech. (Shanghai, China). Recombinant human TRAIL was purchased from RD Systems (Minneapolis, MN, USA). CLB was purchased from Sigma-Aldrich (St. Louis, MO, USA). FC 500MCL flow cytometry was purchased from Beckman Coulter Inc. (Brea, CA, USA). Micro-plate reader was purchased from Bio-Tek Inc. (Winooski, VT, USA). CFX96 fluorescent quantitative PCR cycler was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Cell Culture and Treatment

Raji cell was incubated in Roswell Park Memorial Institute 1640 (RPMI 1640) medium containing 10% fetal bovine serum (FBS) and 1% streptomycin-penicillin, and was kept in a 37°C chamber with 5% CO_2. Culture medium was changed every other day. Cells at log-growth phase were collected for experiments. Cells were then treated with TRAIL at 0, 20, 40 and 80 ng/ml or CLB at 0, 2.5, 5 and 10 μM for 48 h.

CCK-8 Assay for Cell Proliferation Activity

Cultured cells at log-growth phase were digested by trypsin into single cell suspension and were seeded into 96-well plate at 1000 per well density. The plate was placed in a 37°C chamber with 5% CO_2. After attached growth for 24 h, cells were treated with TRAIL at 0, 20, 40 and 80 n g/ml or CLB at 0, 2.5, 5 and 10 μM for 48 h. 10 μl CCK-8 reagent was added to each well, followed by incubation at 37°C for 4 h. Absorbance (A) values at 450 nm were then measured by a micro-plate reader. Six parallel samples were performed in each treatment group. Cell proliferation rate (%) = mean value of experimental group/mean value of control group × 100%.

qRT-PCR for Gene Expression

cDNA was synthesized in a 10 μl system including 1 μg total RNA, 2 μl RT buffer (5×), 0.5 μl oligo dT + random primer mix, 0.5 μl RT enzyme mix, 0.5 μl RNase inhibitor, and ddH_2O. The reaction conditions were: 37°C for 15 min, followed by 98°C for 5 min. cDNA products were kept at -20°C fridge. Using cDNA as the template, PCR amplification was performed under the direction of TaqDNA polymerase using primers ($DR4P_F$: 5'-GTGTG GGTTA CACCA ATGCT T-3'; $DR4P_R$: 5'-AGTTC CTGGT TTGCA CTGAC A-3'; $DR5P_F$: 5'-ATGGA ACAAC GGGGA CAGAA C-3'; $DR5P_R$: 5'-TTAGG ACATG GCAGA GTCTG CATTA C-3'; β-actinP_F: 5'-GAACC CTAAG GCCAA C-3'; β-actinP_R: 5'-TGTCA CGCAC GATTT CC-3'. In a PCR system with 10 μl total volume, we added 4.5 μl 2× SYBR Green Mixture, 1.0 μl of forward/reverse primer (at 2.5 μm/l), 1μl cDNA, and 3.0 μl ddH_2O. PCR conditions were: 95°C for 15 s, 60°C for 30 s and 74°C for 30 s. The reaction was performed on Bio-Rad (Hercules, CA, USA) CFX96 fluorescent quantitative PCR cycler for 40 cycles to collect fluorescent data.

Western Blot

RIPA buffer was used to lyse cells. The supernatant was saved and quantified for protein concentration. Total of 50 μg protein samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (45 V for 180 min), and were transferred to polyvinylidene difluoride (PVDF) membrane (300 mA for 100 min). The membrane was

blocked in 5% defatted milk powder for 1 h, followed by primary antibody (anti-cleaved caspase-3, DR4, DR5 and β-actin) incubation at 4°C overnight. After PBST washing (5 min × 3 times), HRP-labelled secondary antibody was added for 1 h incubation. After phosphate buffered saline tween (PBST) rinsing for three times (5 min each), enhanced chemiluminescence (ECL) reagent was added for 2 min dark incubation. The membrane was then exposure in dark. Quantity One image analysis software (Bio-Rad, Hercules, CA, USA) was used to analyze relative grey density of bands.

Spectrometry for Caspase-3 Activity

Standard dilutions of 0, 10, 20, 50, 100 and 200 μM pNA were prepared from 10 mM stock. Absorbance values at 405 nm wavelengths were measured by a microplate reader to plot a standard curve with pNA concentration against A405 value. Attached cells were digested in trypsin, and were collected into culture medium for 4°C centrifugation for 5 min at 600×g. Supernatant was carefully removed and washed out by phosphate buffer solution (PBS). 100 μl lysis buffer was added for every 2×10^6 cells. Cells were lysed at 4°C for 15 min, and were centrifuged at 18000×g at 4°C for 10 min. Supernatants were saved for further use. Ac-DEVD-pNA was placed on ice, mixed with buffer and test samples, with 10 μl Ac-DEVD-pNA. The mixture was incubated at 37°C for 2 h. A405 value was measured when color changed significantly.

Flow Cytometry for Cell Apoptosis

Cells were collected by centrifugation and were then washed in PBS twice. 100 μl Binding Buffer was used to re-suspend cells. The mixture was then mixed with 5 μl Annexin V-FITC and 5 μl PI, and incubated in dark for 10 min, with the addition of 400 μl Binding Buffer for re-suspension. Beckman FC 500MCL flow cytometry was used to test cell apoptosis.

Flow Cytometry for Ki-67 Expression

Cells were collected from all groups, and were rinsed twice in PBS containing 2% FBS. After fixation in 4% paraformaldehyde for 30 min, cells were treated using PBS containing 0.1% Triton X-100. FITC labeled Ki-67 antibody was added at 4°C dark incubation for 40 min, followed by twice rinsing in PBS containing 2% FBS. Cells were loaded for online testing in Beckman FC500MCL flow cytometry apparatus (Brea, CA, USA).

Statistical Analysis

SPSS18.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Measurement data were presented as mean ± standard deviation (SD). Student *t*-test was used for comparison of measurement data between groups. A statistical significance was defined when $p<0.05$.

Results

Effects of TRAIL or CLB Treatment on Raji Cell Proliferation and Apoptosis

Flow cytometry results showed no significantly elevated apoptotic rate of Raji cells under the treatment of different concentrations of TRAIL (Figure 1A). CLB treatment also had weak effects on Raji cell apoptosis (Figure 1B). CCK-8 assay result showed that either TRAIL (Figure 1C) or CLB (Figure 1D) treatment had weak effects on proliferation activity of Raji cells. High concentration of TRAIL (80 ng/ml) or CLB (10 μM) had about 20% inhibition rate on cell proliferation activity. These results showed certain drug resistance of Raji cells against TAIL or CLB treatment.

Effects of TRAIL or CLB Treatment on DR4, DR5 mRNA/Protein Expression in DISC of Raji Cells

qRT-PCR results showed no significant effects of TRAIL at various concentrations on DR4 or DR5 mRNA expression in Raji cells (Figure 2A). qRT-PCR results showed that CLB treatment at different concentrations significantly elevated DR4 and DR5 mRNA expression in Raji cells (Figure 2B). Western blot results showed that different concentrations of TRAIL weakly up-regulated caspase-3 activity, but had no significant effects on DR4 or DR5 protein expression in Raji cells (Figure 2C). Western blot results showed that different concentrations of CLB weakly enhanced caspase-3 activity, but remarkably up-regulated DR4 and DR5 protein expression in Raji cells (Figure 2D).

Combined Effects of TRAIL and CLB for Synergistic Facilitation on Raji Cell Apoptosis and Inhibition on Proliferation

We used single treatment of TRAIL (80 ng/ml) or CLB (10 μM) or their combined treatment on Raji cells, whose proliferation and apoptosis were observed. Flow cytometry results showed significantly lower proliferation ability of Raji cells

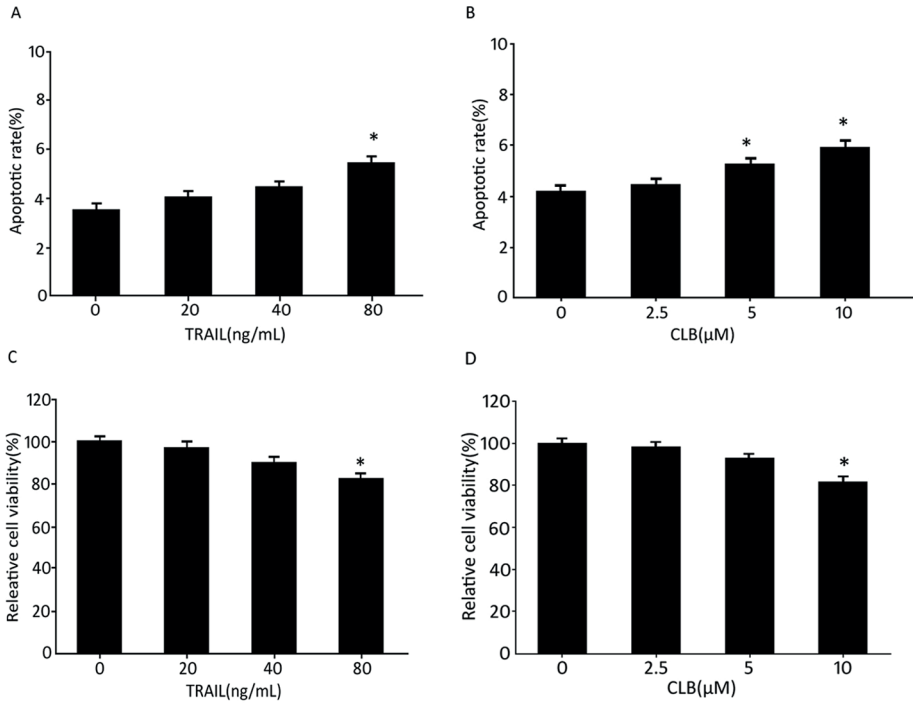

Figure 1. Weak effects of TRAIL or CLB treatment on Raji cell proliferation. ***(A)*** Flow cytometry for TRAIL effects on Raji cell apoptosis. ***(B)*** Flow cytometry for CLB effects on Raji cell apoptosis. ***(C)*** CCK-8 assay for TRAIL effects on Raji cell proliferation. ***(D)*** CCK-8 assay for CLB effects on Raji cell proliferation. *, $p<0.05$ compared to untreated group.

under combined treatment compared to single treatment group (Figure 3A). CCK-8 assay also showed remarkably lower proliferation ability of Raji cells under combined treatment compared to single treatment group (Figure 3B). The single treatment of TRAIL or CLB did not significantly enhance caspase-3 activity (Figure 3C-D), or elevated apoptosis of Raji cells. The combined treatment of both drugs, however, remarkably induced Raji cell apoptosis (Figure 3E).

Discussion

Although part of NHL patients obtained long-term remission by routine treatment, there were still over 60% patients who did not response to normal treatment or had post-remission recurrence[16]. In recent years, incidence of NHL is rising by years, and has occupied 7th position among all malignant tumors[17]. Burkitt lymphoma is one of common malignant tumors in childhood, and occupies about 30-50% of all lymphoma in children [18,19]. Burkitt lymphoma is a highly malignant tumor with rapid progression and unfavorable prognosis. It is commonly occurred in extra-nodal sites or presents as acute leukemia. Burkitt lymphoma is composed of single type B cells with medium size, with strong proliferation ability and short doubling time. The translocation of myc gene related chromosomes[20,21] and EB virus infection[22] are closely correlated with Burkitt lymphoma. Immune therapy using rituximab can improve patient's survival rate to certain extents, but is largely limited in clinics due to high medical costs[23]. Therefore, chemotherapy is still the major treatment for Burkitt lymphoma [24]. TRAIL is an important member of tumor necrosis factor (TNF) super-family, and is a newly discovered apoptosis inducing factor functioning on DR[25]. TRAIL can selectively function on tumor cells, transforming cells and viral infected cells to induce their apoptosis, but invade the killing effect on normal cells, thus making it as one next-generation anti-tumor drug with promising foresight[6,26]. TRAIL can bind with death receptor DR4 or DR5 on the membrane to form ligand-receptor trimer complex, which further induces the binding between death domain (DD) of cytoplasm and C-terminal DD of Fas-associated death domain (FADD). FADD further uses its death effector domain (DED) at N-terminus to bind with

procaspase-8 to form DR4/DR5-FADD-Procaspase-8 death inducing signal complex, to facilitate the auto-digestion of procaspase-8 to form caspase-8 as one active apoptosis initiator and eventually caspase cascade reaction to activate executor molecule caspase-3, which can directly degrade structural/functional proteins, eventually leading to cell apoptosis[7]. Previous study[9] showed insignificant effects of TRAIL on most types of malignant lymphoma, possibly due to the higher frequency of drug resistance and tolerance against TRAIL. CLB is a compound of nitrogen mustard derivatives with dual alkalization functions. As a non-specific cell cycle drug, it can impede DNA synthesis via cross-interaction with cellular DNA, thus inhibiting tumor proliferation and exerting anti-tumor effects[10]. As the first line treatment, CLB has satisfactory effects on chronic lymphocyte leukemia and low-malignant NHL, but with unclear treatment efficacy on highly malignant Burkitt lymphoma[11]. Increasing evidence showed the synergistic effects between common chemotherapy reagent, including aclarubicin and Adriamycin, and TRAIL to potentiate its killing effects on tumor cells, thus providing new insights for clinical treatment of tumors[12,13]. Previous studies showed that CLB could enhance sensitivity on TRAIL in lymphoid cell SKW6.4 cell line[14] and chronic lymphocyte leukemia cell line[15]. This study thus investigated the effect of CLB combined with TRAIL treatment on *in vitro* proliferation and apoptosis of Raji cells.

Results showed weak effects on Raji cell apoptosis by single application of TRAIL or CLB, along with low inhibitory effects on Raji cell proliferation. Moreover, results showed certain resistance of Raji cells against TRAIL or CLB treatment. Further tests showed no significant effect of different concentrations of TRAIL on DR4/DR5 mRNA or protein expressions in Raji, along with weak induction effects on caspase-3 enzyme activity. CLB treatment also had lower effects of caspase-3 activity induction, but significantly up-regulated DR4, and DR5 mRNA/protein expressions inside Raji cells. Combined treatment between TRAIL and CLB had more potent effects regarding inhibition on cell proliferation and induction of apoptosis compared to single treatment of TRAIL or CLB,

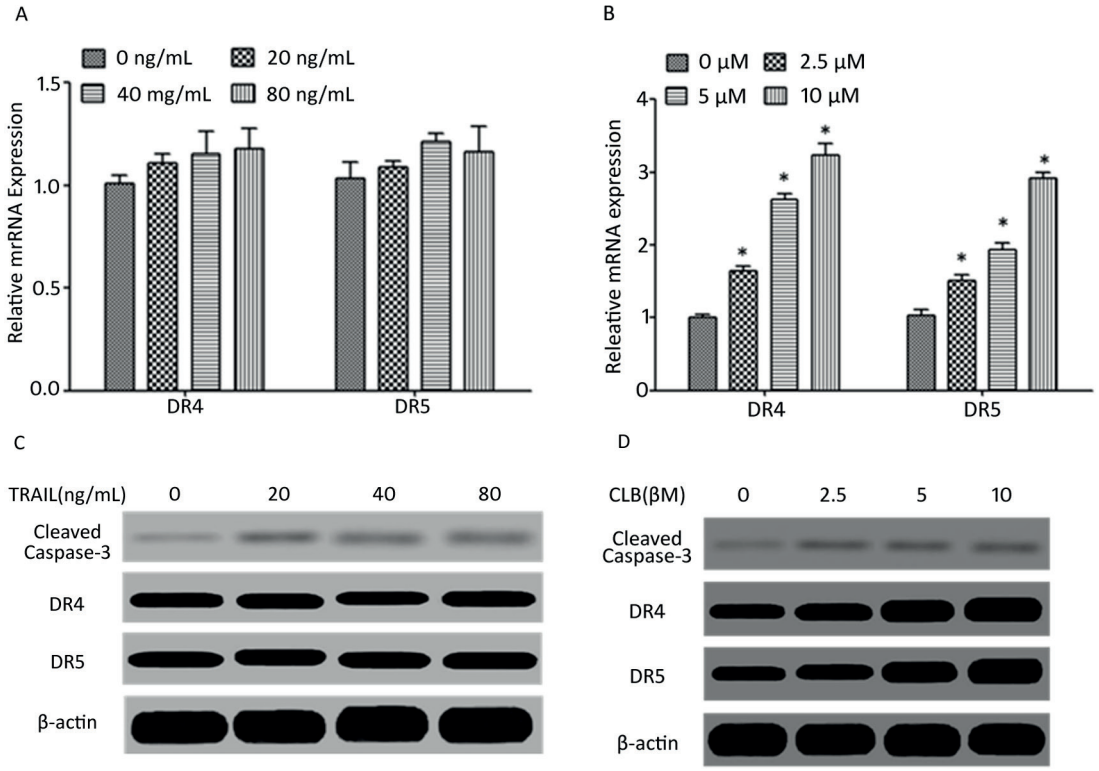

Figure 2. Effects of TRAIL or CLB treatment on DR4/DR5 expression in Raji cells. (A) qRT-PCR for TRAIL effects on DR4 and DR5 mRNA expression. (B) qRT-PCR for CLB effects on DR4 and DR5 mRNA expression. (C) Western blot for TRAIL effects on DR4 and DR5 protein expression. (D) Western blot for CLB effects on DR4 and DR5 protein expression. *, $p<0.05$ compared to untreated group.

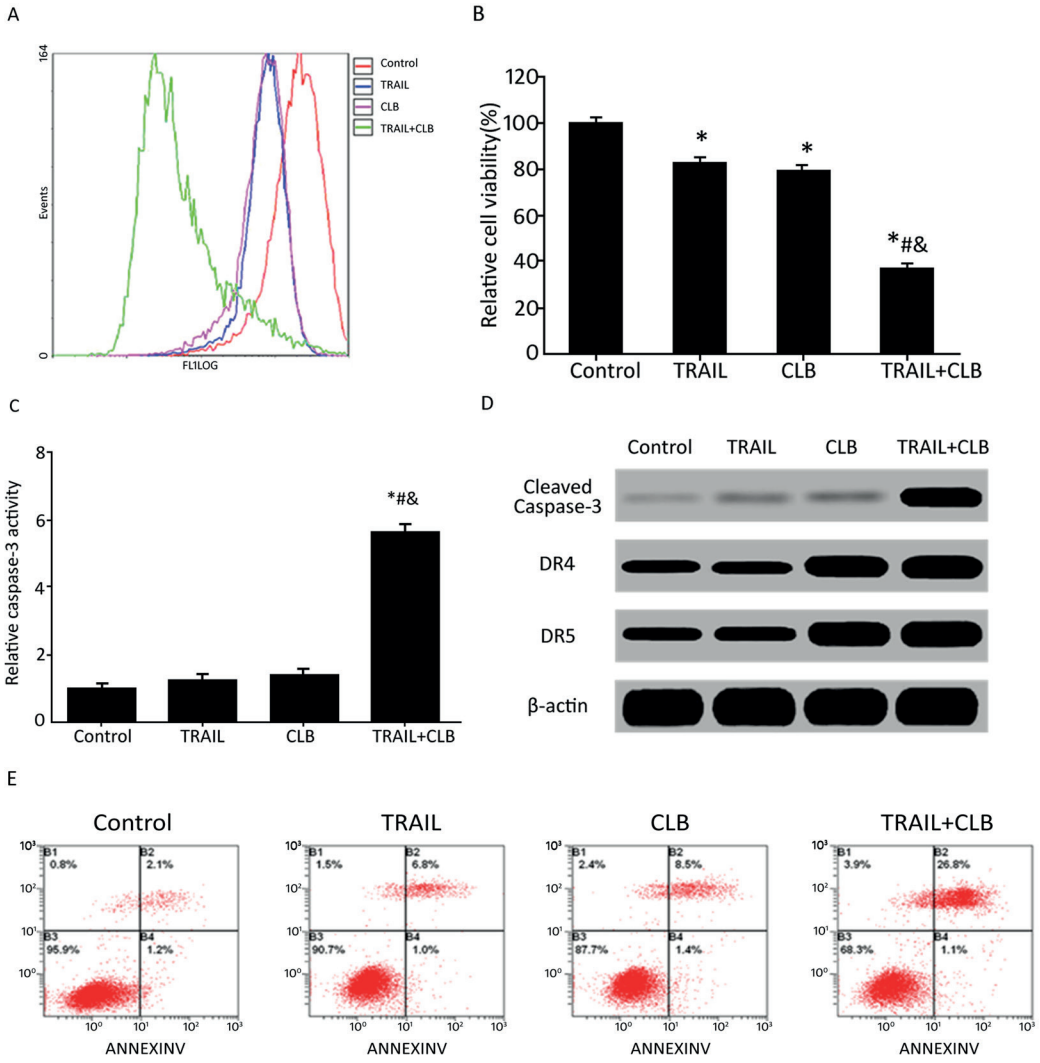

Figure 3. Combined effects of TRAIL and CLB on facilitation apoptosis and inhibition of proliferation of Raji cells. ***(A)*** Flow cytometry for Ki-67 expression. ***(B)*** CCK-8 assay for cell apoptosis. ***(C)*** Spectrometry for caspase-3 activity. ***(D)*** Western blot for protein expression. ***(E)*** Flow cytometry for cell apoptosis. *, $p<0.05$ compared to control group; #, $p<0.05$ compared to TRAIL; $p<0.05$ compared to CLB.

indicating the combined usage of TRAIL and CLB exerted synergistic effects to significantly induce Raji cell apoptosis and to inhibit cell proliferation. Johnston et al[15] found that CLB had synergistic effects with TRAIL to significantly potentiate sensitivity of chronic lymphocyte leukemia cell against TRAIL. Barbarotto et al[14] found that CLB could significantly enhance pro-apoptotic role of TRAIL on lymphoid cell SKW6.4. This study showed that combined usage of TRAIL and CLB produced synergistic effects to significantly induce Raji cell apoptosis whilst inhibit their proliferation. CLB treatment significantly up-regulated expression of death receptor DR4 and DR5 expression, forming one possible mechanism among others for the enhancement of sensitivity against TRAIL-induced apoptosis by Raji cells. Previous studies[16-27] found that certain DNA toxic drugs including doxorubicin or other alkalization reagents significantly facilitated apoptosis of chronic lymphocyte leukemia cells via up-regulating Fas ligand and Fas expression. This study observed that the enhancement of TRAIL-induced apoptosis in lymphoma cells by CLB worked via up-regulating DR4 and DR5. Whether it can affect the sensitivity of lymphoma cells of TRAIL-induced apoptosis via affecting Fas ligand and Fas expression, however, requires further study.

Conclusions

Single treatment of TRAIL or CLB has weak effects on inhibition of proliferation activity and induction of apoptosis of Raji cells. CLB significantly up-regulates DR4 and DR5 expression in Raji cells, and has synergistic effects when combined with TRAIL to significantly potentiate TRAIL-induced apoptosis induction and proliferation inhibition functions.

Conflict of interest

The authors declare no conflicts of interest.

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