

RESEARCH ARTICLE

Epirubicin Inhibits Soluble CD25 Secretion by Treg Cells Isolated from Diffuse Large B-cell Lymphoma Patients

Lan-Fang Li¹, Hua-Qing Wang¹, Xian-Ming Liu¹, Xiu-Bao Ren^{2*}

Abstract

Objective: To investigate the effect of epirubicin on soluble CD25 (sCD25) secretion by CD4+CD25+ regulatory T (Treg) cells isolated from diffuse large B-cell lymphoma (DLBCL) patients. **Methods:** Treg cells were isolated from the peripheral blood mononuclear cells isolated from the newly diagnosed DLBCL patients. The concentration of sCD25 in the supernatant was determined with a commercial sCD25 (IL-2R) enzyme-linked immunosorbent assay (ELISA) kit. The fluorescence intensity of CD25 was detected by flow cytometry. **Results:** Cell survival rate was significantly decreased along with the increase of epirubicin concentration after treatment for 24 h. There was also a significant difference in the concentration of sCD25 between the epirubicin group and the control group ($P < 0.01$). A positive correlation between the Treg cells survival rate and the concentration of sCD25 was detected ($r = 0.993, P < 0.01$). When equal numbers of CD4+CD25+ Treg cells of the epirubicin group and the control group were cultured for another 24 h without epirubicin the CD25 fluorescence intensity on the surface of Treg cells was obviously higher in the epirubicin group than that in the control group ($P < 0.01$), while the sCD25 concentration in the supernatant in the epirubicin group was significantly lower than that in the control group ($P < 0.05$). **Conclusion:** Epirubicin may improve the body's immune functions by inhibiting the sCD25 secretion by Treg cells in DLBCL patients.

Keywords: Diffuse large B-cell lymphoma - CD4+CD25+ regulatory T cells - epirubicin - soluble CD25

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Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of malignant lymphoma (Fan et al., 2012). Chemotherapy remains the main treatment method for DLBCL. Most DLBCL patients can be alleviated after standard chemotherapy regimens, but about 25~30% of DLBCL patients may be resistant to the chemotherapy drug and belongs to the refractory DLBCL (Liu et al., 2013). Although many new chemotherapeutic agents and chemotherapy regimens for refractory DLBCL have been reported in recent years, the outcome is not good yet.

CD4+CD25+ regulatory T (Treg) cells, characterized by coexpression of CD4 and CD25 markers, are recognized as a subpopulation of suppressor T cells that is able to suppress autoreactive T cells (Sakaguchi, 2000). In many patients with malignant diseases, such as ovarian, lung, breast, liver, gastric and esophageal cancers, the population of Treg cells are usually increased (Woo et al., 2001; Liyanage et al., 2002; Ichihara et al., 2003; Ormandy et al., 2005). Interestingly, there is also a tight correlation between Treg cells and DLBCL. Interleukin -2 (IL-2), an important cytokine in vivo, is able to induce T cell proliferation and activation after binding to its receptor,

IL-2 receptor (IL-2R) and then enhance the immune response in vivo. Soluble CD25 (sCD25), which is the free form of IL-2R α subunit, could competitively inhibit the binding of IL-2 with cell membrane IL-2R, and thus suppress the immune responses, resulting in low immune function. Many studies have shown that Treg cells can suppress the immune responses by releasing sCD25 in B-cell non-Hodgkin lymphoma patients. Therefore, many studies aimed at improving tumor immunosuppression, especially Treg cells have achieved good results (Chang et al., 2012; Felcht et al., 2012).

Epirubicin is one of the most common agents used in the chemotherapy of DLBCL. Although the cytotoxic effect of the agent has been widely studied, whether it may also affect the immune system through regulating the secretion of sCD25 by Treg cells has not been understood. Therefore, we observed the effect of epirubicin on sCD25 secretion in vitro.

Materials and Methods

CD4+CD25+ Regulatory T Cell Isolation

Peripheral blood was acquired from the newly diagnosed DLBCL patients in the Tianjin Medical

¹Department of Lymphoma, Sino-US Center of Lymphoma and Leukemia, Tianjin Medical University Cancer Hospital and Institute,

²Department of Immunology, Tianjin Key Laboratory of Cancer Prevention and Therapy, Tianjin Medical University Cancer Hospital and Institute, Tianjin, China *For correspondence: xbtianjin@163.com

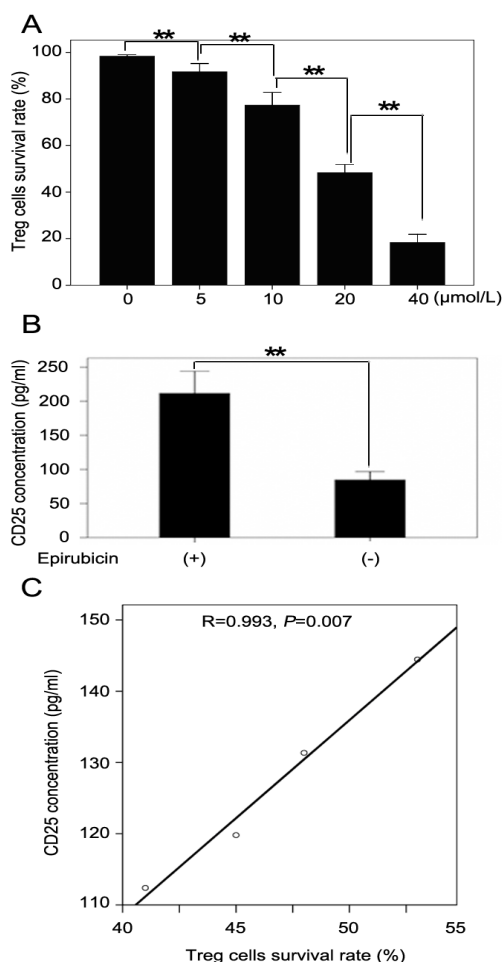


Figure 1. Epirubicin Decreased the Survival Rate of Treg Cells and the Secretion of sCD25 by Treg Cells. CD4+CD25+ Treg cells were incubated with epirubicin at different concentrations (5, 10, 20, 40 μmol/L) for 24 h, and then (A) the survival rate of Treg cells was examined. (B) After 24 h incubation, the effect of epirubicin (20 μmol/L) on the sCD25 concentration in the supernatant was determined by ELISA; C: sCD25 concentration was positively correlated with the survival rate of Treg cells. ** P<0.01

University Cancer Hospital and Institute. Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood by Ficoll density gradient centrifugation. CD4+CD25+ Treg cells were isolated from the PBMCs using a CD4+CD25+ Regulatory T Cell Isolation Kit (Miltenyi-Biotec, Bergisch Gladbach, Germany).

Screening of epirubicin concentration

Two hundred microliter CD4+CD25+ Treg cells were seeded into a U- bottom 96-well plate in triplicates at a density of 1×10⁶/ml. The cells were incubated with epirubicin at different concentrations (5, 10, 20, 40 μmol/L) for 24 h. Then the cell survival rate was calculated.

CD25 release test

The isolated CD4+CD25+ Treg cells were re-suspended and seeded into a U- bottom 96-well plate in triplicates (200 μl/well). The cells were incubated with epirubicin at indicated concentration for 24 h. Then the cell survival rate was calculated.

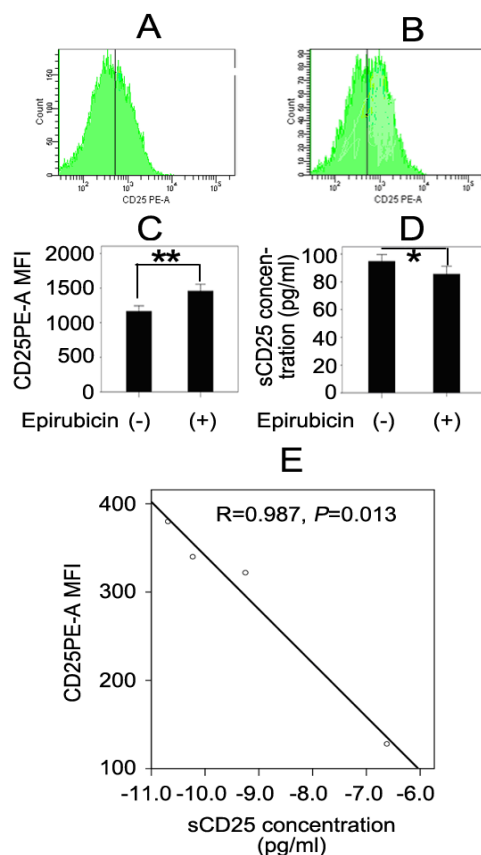


Figure 2. Effect of Epirubicin on the sCD25 Secretion by Treg Cells. In (A) the epirubicin group and (B) the control group, CD25 fluorescence intensity on the surface of Treg cells was determined by flow cytometry (C) after epirubicin treatment for 24 h. According to the survival rate, equal numbers of CD4+CD25+ Treg cells were taken from the epirubicin group and the control group and cultured for another 24 h, and then sCD25 concentration in the supernatant was determined by ELISA (D); correlation between the CD25 fluorescence intensity and the sCD25 concentration (E). *P<0.05; **P<0.01

According to the survival rate, equal numbers of CD4+CD25+ Treg cells were taken from the epirubicin group and the control group and cultured for another 24 h. The cells were centrifuged and the supernatant was kept in the -80 °C for future examination. The cells were placed into the U- bottom 96-well plate and incubated with CD4 PerCP/CD25 PE/CD127 APC cocktail fluorescent antibody (Biolegend, San Diego, CA, USA). The fluorescence intensity of CD25 was detected by flow cytometry.

Enzyme-linked immunosorbent assay

The concentration of sCD25 in the supernatant was determined by a commercial sCD25 (IL-2R) enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer’s instructions (Dioclone, France).

Statistical analysis

Statistical significance was determined using SPSS 16.0 for Windows. Measurement data are expressed as means ± S.D. Student’s t-test was performed to detect any differences between the two groups. Linear correlation was used to validate the correlation between the level of

Treg cells and the concentration of sCD25. Differences were deemed significant if $P < 0.05$.

Results

Epirubicin inhibited the viability of Treg cells in vitro

As shown in Figure 1, after epirubicin treatment for 24 h, cell survival rate was significantly decreased along with the increase of epirubicin concentration. The survival rates were $98.33 \pm 0.58\%$, $91.67 \pm 3.06\%$, $77.33 \pm 4.73\%$, $48.33 \pm 3.09\%$ and $18.33 \pm 3.12\%$ at the concentration of 0, 5, 10, 20 and 40 $\mu\text{mol/L}$. Since the survival rate was about 50% at the concentration of 20 $\mu\text{mol/L}$, the concentration of 20 $\mu\text{mol/L}$ was used for the following experiments. After epirubicin treatment for 24 h, there was also a significant difference in the concentration of sCD25 between the epirubicin group and the control group ($P < 0.01$). There was a positive correlation between the Treg cells survival rate and the concentration of sCD25 ($r = 0.993$, $P < 0.01$).

Effect of epirubicin on the sCD25 secretion by Treg cells

We next observed the effect of epirubicin on the sCD25 secretion by Treg cells. After epirubicin treatment for 24 h, equal numbers of CD4+CD25+ Treg cells of the epirubicin group and the control group were cultured for another 24 h without epirubicin. The CD25 fluorescence intensity on the surface of Treg cells was obviously higher in the epirubicin group than that in the control group ($P < 0.01$). In contrast, the sCD25 concentration in the supernatant in the epirubicin group was significantly lower than that in the control group ($P < 0.05$). A negative correlation between the CD25 fluorescence intensity and the sCD25 concentration in the supernatant was detected ($P < 0.05$).

Discussion

Epirubicin, a cell cycle nonspecific chemotherapeutic drug, is commonly used in the treatment of DLBCL. Epirubicin forms a complex with DNA by intercalation of its planar rings between nucleotide base pairs, with consequent inhibition of nucleic acid (DNA and RNA) and protein synthesis. It also inhibits topoisomerase II activity by stabilizing the DNA-topoisomerase II complex, preventing the religation portion of the ligation-religation reaction that topoisomerase II catalyzes. In the present study, we found that epirubicin may also affect the systematic immune system by downregulating the level of Treg cells and the sCD25 secretion from Treg cells, which may provide a new insight into the mechanisms of the antitumor effect of epirubicin.

Circulating sCD25 can competitively bind to IL-2 with IL-2R, and then inhibit the proliferation of lymphocyte and downregulate the activity of NK cells, resulting in lower immune function. Therefore, circulating sCD25 level is considered as an indicator of the immune inhibition degree (Goto et al., 2005). Bien and Balcerska (Bien and Balcerska, 2008) suggest that in most patients with haematological malignancies, tumor cells continuously producing a large number of sCD25 is the main reason for the upregulation of sCD25. But in a recent study, the

level of sCD25 is significantly higher in patients with B cell non-Hodgkin lymphoma than that in normal people and Treg cells is able to release sCD25 and inhibit the proliferation of T cells in vitro (Lindqvist et al., 2010). In the present study, the results showed that epirubicin not only reduced the number of Treg cells, but also downregulated the level of sCD25, further confirming that Treg cells are capable of releasing sCD25. In addition, when the same number of Treg cells in the two groups was cultured for another 24 hours, the concentration of sCD25 in the epirubicin group was lower than that in the control group, while the CD25 fluorescence intensity of the epirubicin group on the surface of Tregs was higher than that of control group, indicating epirubicin is able to inhibit the release of sCD25 from Treg cells. Therefore, in addition to directly killing tumor cells, epirubicin may also improve the body's immune suppression.

In conclusion, our study demonstrated that epirubicin could reduce the number of Treg cells and downregulate the sCD25 secretion from Treg cells obtained from DLBCL patients, indicating epirubicin may improve the body's immune functions. However, this is just an in vitro study, and whether epirubicin may also exert the same effect on DLBCL patients should be further investigated.

Acknowledgements

The author(s) declare that they have no competing interests.

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