Synergistic induction of apoptosis in B-cell chronic lymphocytic leukemia cells after treatment with all-trans retinoic acid in combination with interleukin-21 and rituximab

ABSTRACT

Aim: B-cell chronic lymphocytic leukemia (B-CLL) is the most common adult leukemia in the Western world and characterized by the progressive expansion of malignant B lymphocytes in peripheral blood. In spite of advances in sciences to recognize the number of effective agents for the treatment of chronic lymphocytic leukemia (CLL), this leukemia is thought as incurable one. Introducing a new therapy that has a direct effect on B-CLL lymphocytes and no cytotoxic effects on the other cells is a great wish.

Materials and Methods: Twenty-one patients with B-CLL were enrolled in the study. Peripheral blood mononuclear cells (PBMCs) were isolated from patients’ blood samples and were further treated with all-trans retinoic acid (ATRA), interleukin (IL-21), and rituximab at concentrations of 30 ng/ml, 25 ng/ml, and 4 µg/ml, respectively. ATRA, IL21, and rituximab were used alone or in various combinations and their effects on apoptosis were measured using annexin V-fluorescein isothiocyanate apoptosis detection kit.

Result: Treatment of the patients’ cells with IL21 and rituximab showed a synergistic effect on the induction of apoptosis, in comparison with untreated CLL cells (P < 0.05). The induction of apoptosis by ATRA in combination with IL21 and rituximab were significantly increased compared to untreated CLL cells as a negative control (P < 0.01).

Conclusion: Treatment of patients’ PBMCs by ATRA in combination with IL21 and rituximab and also IL21 in combination with rituximab showed synergistic induction of apoptosis compared to untreated CLL cells as a negative control (P < 0.01). It seems that ATRA in combination with IL21 and rituximab activate different pathways of apoptosis (extrinsic pathway, intrinsic pathway, and granzyme B pathway).

KEY WORDS: All-trans retinoic acid, apoptosis, B-cell chronic lymphocytic leukemia, Bcl-2 family, interleukin-21

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in the Western world and consisting of 25–30% of leukemia. It is described through the progressive accumulation of malignant B-cells in peripheral blood, bone marrow, and secondary lymphoid tissues. One of the important factors enhancing the survival of CLL cells is the overexpression of anti-apoptotic Bcl-2 protein that causes cells resistance to chemotherapy drugs. CLL is a disease of adulthood and its prevalence increases with aging and disease is diagnosed at the ages 64–70 years. Global incidence of CLL is between 1 and 5.5/100,000 people.

Despite scientific advances in the understanding of the effective factors for treatment of CLL, this leukemia is regarded as a disease that has no cure and it is necessary to explore specific and effective therapeutic agents that help to cure the disease. Currently, purine analogs and DNA alkylating compounds such as chlorambucil and fludarabine are used to treat CLL leukemia. Recently, rituximab (anti-CD20) along with fludarabine is used for the treatment of these patients. The results are satisfactory, but it has some side effects.

All-tans retinoic acid (ATRA), a derivative of vitamin A, has an intracellular receptor. ATRA is one of the main compounds in the treatment of patients with acute promyelocytic leukemia. ATRA dramatically reduces the expression of anti-apoptotic protein Bcl-2 in CLL cells and induces apoptosis in these cells through an internal pathway that is dependent on caspase-9 and eventually caspase-3.\(^7\)

Interleukin (IL-21), a member of the type I cytokines family, is expressed in activated CD4 + T cells. This cytokine has multiple effects on natural killer cells and cytolytic T lymphocytes and can increase the activity of anti-tumor antibodies. IL-21 receptor is moderately expressed on the surface of CLL cells.\(^8\) Under the effect of CD40 and CpG ligands, the expression of these receptors are increased and preapoptotic signal is sent into the cell.\(^9\) It has been shown that IL21 through the external pathway by the means of the preapoptotic proteins includin BIM and caspase-8 and eventually caspase-3 activation leads cells to apoptosis. In addition, IL21 induces production of granzyme B by B-cell CLL (B-CLL) cells which triggers apoptosis pathway through caspase10.\(^11\)

Rituximab is a chimeric monoclonal antibody against the CD20 surface molecule on the CLL cells that currently used as a monoclonal antibody in the treatment of CLL. CD20 is present on the surface of 95% of cancerous and normal B-cells and as a target for therapy with monoclonal antibodies. CD20 by binding to its anti-CD20 antibody leads to activation of different pathways of cell death. It causes the activation of initiator caspase9, and then executive caspase-3, 6, 7 and finally leading cells to death.\(^12,13\)

In the present study, the synergistic induction of apoptosis in B-CLL cells after treatment with ATRA in combination with IL21 and rituximab is explored. Considering that these three substances significantly induce apoptosis in CLL cells through different pathways, we hypothesized that the combined use of these compounds may have synergistic effects. Using ATRA in combination with IL-21 and rituximab may be a new way to treat CLL disease.

**MATERIALS AND METHODS**

In this study, a total of 21 B-cell CLL (B-CLL) patients aged 43–78 years were enrolled. Prior to participation, informed written consent was obtained from all patients, and the study was approved by the Ethical Committee of Tehran University of Medical Sciences. Fifty milliliters of heparinized peripheral blood samples was collected from each participant. To minimize the negative effects of chemotherapy on their immune status, none of the patients was under treatment at the time of sample collection. Disease diagnosis was based on the clinical and immunophenotypic criteria outlined by the World Health Organization.\(^14\)

### Isolation of peripheral blood mononuclear cells and cell culture

Fifty milliliters of heparinized blood was taken from patients, and then peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll–Hypaque centrifugation. For separation of PBMCs, Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Invitrogen, UK) without fetal bovine serum (FCS) (Gibco, Invitrogen, UK) was added to peripheral blood in the same volume. Ficoll–Histopaque (Sigma, USA) was poured into a new falcon tube, and then diluted peripheral blood was carefully layered on it with sterile disposable Pasteur pipette. The samples were centrifuged at room temperature at 540 × g for 20 min, and then PBMCs were isolated using sterile disposable Pasteur pipette. After counting the cells, using Trypan blue method, they were cultured in 96-well plate in RPMI 1640 medium, supplemented with 10% FCS, at a density of 2 × 10^6 cells/ml in a humidified atmosphere at 37°C containing 5% carbon dioxide by PBMCs were treated with various textures by the means of ATRA (30 ng/ml), IL21 (50 ng/ml), and rituximab (4 µg/ml) in comparison to no treated PBMCs of patients as a negative control.

### B-cell enrichment by magnetic-activated cell sorting

B cells were isolated from PBMC using a magnetic-activated cell sorting negative selection kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of isolated B-cells was assessed by flow cytometry and was usually greater than 95%.

### Immunophenotyping of B lymphocytes using flow cytometry

B lymphocytes are purified from whole blood or fresh PBMC. The expressions of CD5 and CD19 on the cell surface of B-cells were evaluated using fluorescent antibody conjugated with PE and fluorescein isothiocyanate (FITC), respectively. The positive cut-off point of cell surface markers was considered more than 20%.

### Assessment of apoptosis and cell treatment

To quantitate the amount of apoptosis, after harvesting cells which were incubated by various textures in complete medium, cells were washed twice with washing buffer (phosphate buffered saline 0.15 M, 0.5% bovine serum albumin, 0.1% NaN3). Fresh B-cells were resuspended in 100 µl washing buffer and stained with appropriate fluorochrome-conjugated mAbs and incubated for 45 min at 4°C in the dark atmosphere to evaluate the amount of apoptosis by using Annexin V/Propidium iodide apoptosis kit (BD Biosciences, San Jose, CA, USA). Then 1000 cells/sample was acquired in an FAC Scan flow cytometer (Partec, Nuremberg, Germany). Moreover, the proportions of labeled cells were analyzed using Paint-A-Gate software (Becton and Dickinson, Sunnyvale, USA). Data analysis was performed using the Flomax software (Partec, Nuremberg, Germany).

### Statistical analysis

Data are expressed as mean ± standard error of the mean. Linear mixed-effects models used for test differences between groups. In this model, subject effect considered as clustering.
Abbaspadeh-Goudarzi, et al.: Induction of apoptosis in B-CLL cells by ATRA, IL21 and rituximab

effect that entered to model as random intercept. A $P = 0.05$
or less considered significant.

RESULTS

Patient characteristics
Twenty-one CLL patients were enrolled in this study. Table 1 provides a summary of the demographic and laboratory characteristics of the study population. The age range of patients was 43–78 years with an average of 61 years. Six patients were female and fifteen were male. Moreover, patients were classified either in indolent ($n = 18$) and progressive ($n = 3$). Disease progression was identified according to the National Cancer Institute Working Group criteria. Of the 21 patients with CLL, 16, 4, and 1 were classified into stages 0, I, and IV, respectively, based on Rai staging system. Major characteristics of the patients with CLL are shown in Table 1. Of the 21 patients with CLL recruited to our study, only four patients were previously treated. These patients received their last treatment at least 12 months before enrollment in the study, to minimize the negative effects of chemotherapy on their immune status.

CD5 and CD19 expression in chronic lymphocytic leukemia patients
The most diagnostic parameter of the CLL patient is simultaneous expression of CD5 and CD19. In 21 CLL patients, the amount of CD5 and CD19 cell surface marker expression were examined by the means of PE-conjugated anti-CD5 mAb and FITC-conjugated anti-CD19 mAb, and positive cut-off was considered more than 20%. As demonstrated in Figure 1, patients for both markers are shown, confirming that patients were diagnosed with CLL the expression of CD5 and CD19 markers on B-CLL patients shown in Figure 1.

The effect of factors studied alone and in combination on the amount of B-cells apoptosis
ATRA in combination with IL21 and rituximab showed a synergistic effect on the induction of apoptosis which is statistically significant ($P < 0.01$) [Figure 2]. Significant difference was demonstrated ($P > 0.05$) in overall apoptosis of cells treated with rituximab in combination with IL21 and rituximab alone compared to the negative control group [Figure 2].

The effect of factors studied alone and in combination on the presence of viable B cells in patients with chronic lymphocytic leukemia
According to the results of this study, ATRA in combination with IL21 and rituximab showed a significant reduction on the percent of viable cells compared to negative control ($P > 0.05$) [Figure 3]. A significant decrease was obtained on the viability of cells treated with rituximab and rituximab in combination with IL21 ($P > 0.05$) compared to negative control.

As shown in Figure 3, the least amount of viable cells is seen in the group treated with combination of ATRA, IL21, and rituximab.

The effect of factors studied alone and in combination on the presence of necrotic B cells in chronic lymphocytic leukemia patients
After 20 h culture, the amount of B cells necrosis in CLL patients showed no significant difference at the exposure of ATRA, IL21, and rituximab alone and combinations of binary and ternary compared to negative control group. This evidence shows the lack of difference compared to negative control group that these factors alone and in combination did not induce necrosis. In Figure 4, information about the amount of necrosis induced by various factors on B cells in CLL patients is stated.

Table 1: Biological and some clinical characteristics of the CLL patients studied

<table>
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<th>Patients code</th>
<th>Prognosis</th>
<th>Age (year)</th>
<th>Sex</th>
<th>WBC x10$^3$</th>
<th>Lymphocyte (%)</th>
<th>Hemoglobin (g/dL)</th>
<th>Treatment</th>
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CLL=Chronic lymphocytic leukemia, WBC=White blood cell
Abbaszadeh-Goudarzi, et al.: Induction of apoptosis in B-CLL cells by ATRA, IL21 and rituximab

**DISCUSSION**

B-CLL is the most prevalent form of adult leukemia. At this time, treatment options for B-CLL are limited, and progressive disease is typically fatal. Recently, one of the drugs used to treat CLL patients is rituximab (anti-CD20) that is administered along with fludarabine. The results are satisfactory, but it has some side effects. It is obvious that new treatment options are needed for these patients. In this study, for the first time, combined effects of ATRA, IL21, and rituximab on the amount of B-cells apoptosis in B-CLL patients were explored.

ATRA induces apoptosis through an internal pathway that is dependent on caspase-9 and eventually caspase-3. It has been shown that IL21 induces apoptosis through the external pathway by the means of the proapoptotic proteins including BIM and caspase-8 and eventually caspase-3 activation, IL21 also induces production of granzyme B by B-CLL cells triggers apoptosis pathway through caspase-10. CD20 itself by binding to anti-CD20 (rituximab) antibody leads to activation of different pathways of cell death. It causes the activation of initiator caspase-9, and then executive caspase-3, caspase-6, caspase-7, and finally leading cells to death. These three factors are inducing apoptosis through different pathways, and we expect that the combined use of these compounds may have synergistic effects.

Based on a literature review, for the first time, this study has evaluated the effects of ATRA in combination with IL-21 and rituximab induction of apoptosis. The results of this study showed a significant increase in apoptosis in combined treated group compared with controls. These three factors are inducing apoptosis through different pathways, and we expect that the combined use of these compounds may have synergistic effects.

Our study showed that in the group who were exposed to combine treatment of IL21 and rituximab, the rate of apoptosis were increased compared to controls. IL21 and rituximab have synergistic effects in the induction of apoptosis, and this is due to activation of different pathways of...
apoptosis. We know that IL21 induce production of granzyme B and also to enable the external apoptosis through activation caspase-8 and caspase-3, and anti-CD20 (rituximab) antibody produce granzyme B and inducing apoptosis by the activation of internal pathway through caspase-9 and caspase-3. Using them in combination showed the effects of synergy. The results of this study are consistent with Gowda et al. study that showed rituximab and IL21 had synergistic effects in inducing B cells apoptosis in CLL patients.\[11\]

This study showed that group received only anti-CD20 (rituximab) antibody, a statistically significant increase in apoptosis has occurred in comparison to control [Figure 2]. Our results are consistent with the results of Byrd et al. (2001) showed that rituximab induces apoptosis in B-CLL cells isolated from CLL patients.\[13\]

Our study showed that groups that exposed to the combination of ATRA and IL21 showed an increase in apoptosis than ATRA and IL21 alone [Figure 2], but this increase is not statistically significant. Perhaps by increasing the number of samples and using V-bottom microtiter plates, providing an effective and better interaction between B-CLL cells, we may observe statistically significant increase in apoptosis rate. ATRA in combination with IL21 showed synergistic effects on induction of apoptosis compared to the effects of ATRA and IL21 alone because, by the combined treatment of ATRA and IL21, different pathways of apoptosis are activated.

Contrary to our expectation, the results indicated that ATRA in combination with rituximab-induced less apoptosis compared to rituximab alone. As we know both ATRA and rituximab can activate internal pathway of apoptosis, but why these two compounds inhibited each other’s, needs further investigation.

In our study, we found that ATRA by itself has no effect on apoptosis induction in comparison with control. This finding is not consistent with the results of Pepper et al. who demonstrated that ATRA induces apoptosis in all samples of CLL by reducing the expression of molecules of anti-apoptotic Bcl-2. In contrast, the proapoptotic molecules including Bax, P21, and p53 were not changed.\[10\] The incubation time in our study was 20h but in the Chris Peper’s study, this time was 48 h. Perhaps by increasing the incubation time, induction of apoptosis by ATRA is increased.

Our results showed that in the IL21 treated group, apoptosis was greater than controls, but the amount of increase was not statistically significant; this finding was not consistent with the results reported by Daniela de Toteroand (2006). In this study, IL21 induced apoptosis through activation of caspase-8 and 3.\[9\] Although they treated the cells with IL21 in the same concentration as we did, they worked on 31 patients, and we enrolled 21 in our study; then if the sample size is increased the result of our study for the induction of apoptosis by IL21 may become significant.

CONCLUSION

The results of this study confirmed previous reports on the induction of apoptosis by rituximab alone and synergistic effects of IL21 and rituximab in CLL (B-CLL). The results showed that ATRA on induction of apoptosis with IL21 and rituximab have synergistic effects on B-CLL cells. According to the results of our research on the synergistic effects of ATRA plus IL21 and rituximab on apoptosis rate of B-CLL cells and the results of various studies to evaluate the effect of ATRA on CLL disease in the past two decades, it seems that ATRA in combination with IL21 and rituximab can be utilized for the treatment of patients with CLL and reduce the side effects by the rituximab.

Suggestions

1. Effects of vitamin D with IL21 and rituximab on B lymphocytes of CLL patients induced apoptosis in in vitro
2. Evaluation the expression of promoter and inhibitor of apoptosis on B lymphocytes of CLL patients which have taken effect by ATRA plus IL21 and rituximab
3. Find the time of incubation and more varied doses of ATRA and IL21 as well as the use of V-shaped floor plates with increasing the levels of drug-cell contact.

Acknowledgments

This study was supported by grants obtained from Tehran University of Medical Sciences with the number of 10,902 and Imam Khomeini Hospital to provide samples for doing this project.

Financial support and sponsorship

Tehran University of Medical Sciences with the number of 10902 and Imam Khomeini hospital to provide samples for doing this project.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

Abbaszadeh-Goudarzi, et al.: Induction of apoptosis in B-CLL cells by ATRA, IL21 and rituximab


