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Original Article

Overexpression of IL-9 induced by STAT6 activation promotes the pathogenesis of chronic lymphocytic leukemia

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Abstract: Chronic lymphocytic leukemia (CLL) is a common leukemia in adults, but its pathogenesis is still poorly understood. Recently, extensive evidence suggests that the malignant cells of CLL patients secrete a range of cytoprotective cytokines including interleukin-4 (IL-4). IL-4 induced the rapid phosphorylation(p) and activation of the signal transducer and activator of transcription (STAT)-6 transcription factor in CLL cells in vitro. Interleukin-9 (IL-9) is not expressed by Th2 and Th9 cells in the absence of STAT6 expression. To elucidate whether there was a function link between IL-9 and STAT6 in CLL, MEC-1 cells were analyzed using RT-PCR, and western blot. Interestingly, when added with recombinant human IL-4 (rIL-4) in culturing MEC-1 cells, expressions of p-STAT6 and IL-9 in MEC-1 cells increased at a time-dependent manner and their expressions could be inhibited by STAT6 inhibitor. Our data indicated that the upregulation of IL-9 induced by pSTAT6 may be involved in the pathogenesis of CLL.

Keywords: pSTAT6, chronic lymphocytic leukemia, prognosis

Introduction

B-cell CLL continues to be a more common leukemia with no obvious curative approaches [1, 2]. CLL is characterized by a dynamic imbalance between the proliferation and apoptosis of leukemia cells and by the accumulation of neoplastic B lymphocytes co-expressing CD5 and CD19 antigens [3-6]. Nevertheless, the pathogenesis of CLL is still poorly understood.

Previously, STAT6 has been reported to be constitutively activated in HLderived cell lines. Recurrent mutations of STAT6 DNA binding domain strongly support the involvement of STAT6 in the pathogenesis of the aggressive Bcell lymphoma [7-9]. In recent years, a resurgence of interest in IL-9 has been spurred due to an expanded identification of its receptor on various immune cells [10, 11]. A series of observations have pointed to this cytokine as a factor promoting oncogenesis, especially lymphomagenesis [12, 13]. The dysregulated expression of IL-9 can be detected in biopsies and serums from patients with Hodgkin's disease (HD), CLL, anaplastic large cell lymphomas (ALCL) as well as nasal natural killer (NK)/T-cell lymphoma [14-20]. The present study is aimed to investigate whether there was a function link between IL-9 and STAT6 in CLL.

Materials and methods

Cells culture

The human CLL cell line MEC-1 was purchased from the American Tissue Culture Collection (Manassas, VA, USA) and maintained at 37°C in 5% carbon dioxide. It was cultured in Iscove's modified Dulbecco's medium (IMDM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA).

Co-treatment or coculture experiments

To explore the effects of extracellular IL-4 on pSTAT6 and intracellular IL-9 expression in MEC-1 cells, recombinant human IL-4 (rIL-4) was added into the medium of MEC-1 cells, and the final concentration of rIL-4 was 10 ng/ml.

Table 1. Primer sequences

Gene Name	Sequence
IL-9	5'-CTCTGTTTGGGCATTCCCTCT-3' 5'-GGGTATCTTGTTTGCATGGTGG-3'
β-actin	5'-CATTAAGGAGAAGCTGTGCT-3' 5'-GTTGAAGGTAGTTTCGTGGA-3'

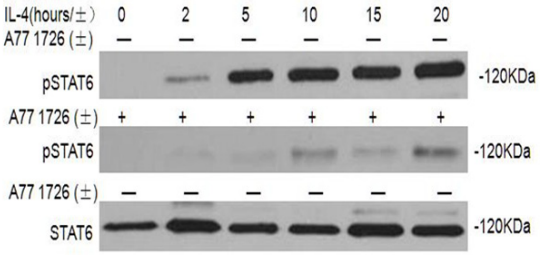

Figure 1. Effects of rIL-4 on pSTAT6 in MEC-1 cells. MEC-1 cells were pretreated with or without A77-1726 (10 nM) for 48 hours and then were cocultured with rIL-4 for different times. rIL-4 promotes STAT6 phosphorylation and IL-9 production in MEC-1 cells at a time-dependent manner. A77-1726, STAT6 inhibitor, could abolish the effects of rIL-4 on MEC-1 cells significantly.

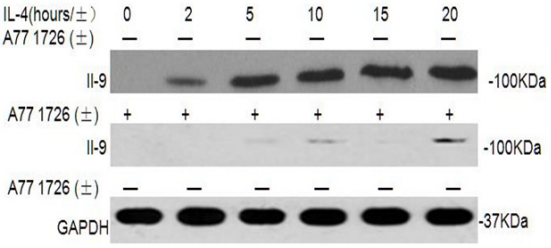

Figure 2. Effects of rIL-4 on IL-9 production in MEC-1 cells. MEC-1 cells were stimulated with 10 ng/ml rIL-4 for different times followed by treatment of A77-1726 (10 nM) for 48 hours. Western blot analysis of IL-9 protein levels in MEC-1 cells. GAPDH was loaded as control.

For experiments with STAT6 inhibitor treatment, MEC-1 cells were pretreated with A77-1726 (Scbt, 10 nM) for 48 hours and then were incubated with rIL-4. When cells were cocultured with rIL-4 for 0, 2, 5, 10, 15, and 20 hours, expression of pSTAT6 and intracellular IL-9 was measured using Western blot.

Western blot analysis

Total protein was extracted from MEC-1 cells using RIPA and 1% PMSF (Shenergy Biocolor, China). The measurement of protein concentrations and detailed procedures of immunoblot analysis were described previously [16]. GAPDH antibody (1:800) was purchased from SANTA. IL-9 antibody was obtained from Boster. pSTAT6 antibodies was from Abcam.

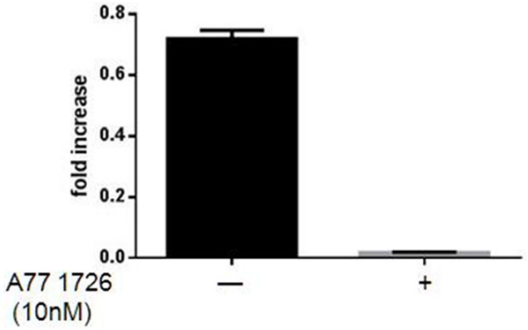

Figure 3. Expression of IL-9 mRNA in CLL cells. MEC-1 cells were pretreated with 10 ng/ml IL-4 for 20 hours. RT-PCR analysis of IL-9 expression in pretreated cells treated with or without A77 1726.

Real-time quantitative polymerase chain reaction (RT-PCR)

Total RNA was extracted from MEC-1 cell lines using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The reverse transcription reaction and RQ-PCR analyses were performed as described previously [17]. Specific primers for RT-PCR were obtained from Biosune (Shanghai, China), and the primer sequences were shown in **Table 1**.

Assessment of cell apoptosis

MEC-1 cells were seeded into 96-well plates (5×10^3/well) and were pretreated with or without rIL-9 (20 ng/ml) for 120 minutes At indicated time, apoptotic cells and necrotic cells were analyzed by staining the cells with fluorescein isothiocyanate (FITC)-annexin V and propidium iodide (PI), according to the manufacturer's instructions (Neobioscience, Shenzhen, China). Briefly, an aliquot of 10^6 cells were incubated with annexin V-FITC and PI for 10 minutes at room temperature in the dark. Cells were then immediately analyzed with FACS can flow cytometer (Beckman Coulter, Chicago, USA). Viable cells are not stained with annexin V-FITC or PI. The necrotic cells were annexin V-FITC and PI-positive, whereas apoptotic cells were annexin VFITC positive and PI negative.

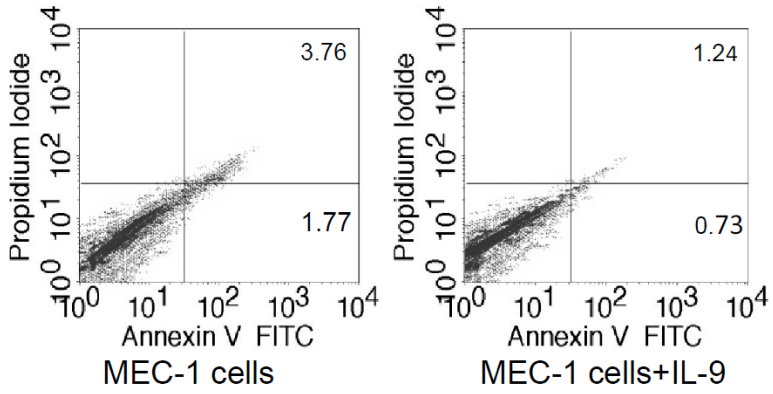

Figure 4. Effects of IL-9 on apoptosis of MEC-1 cells. MEC-1 cells were pretreated with or without rIL-9 (20 ng/ml) for 120 minutes. Cell apoptosis and necrosis were determined using an annexin V-FITC and PI apoptosis detection kit.

Results

IL-4 treatment of CLL cells results in the rapid tyrosine phosphorylation of STAT6, which is inhibited by A77 1726

Phosphorylation of STAT6 was undetectable in unstimulated CLL cells, but was rapidly and strikingly induced by the addition of 10 ng/mL IL-4 at a time-dependent manner. IL-4-induced STAT6 phosphorylation was completely abolished by the JAK3-selective inhibitor, A77-1726 (**Figure 1**).

IL-4 treatment of CLL cells results in up-regulation of IL-9

We measured the level of IL-9 in the medium of culturing MEC-1 cells, although no detectable IL-9 was found (data were not shown). Then, MEC-1 cells were cultured with rIL-4. As shown in **Figure 2**, expressions of IL-9 in MEC-1 cells were measured using Western blot at indicated time. We found that expressions of IL-9 increased at a time-dependent manner. Furthermore, this phenomenon could be inhibited by A77-1726, which is a STAT6 inhibitor. These results indicate that rIL-4 could enhance MEC-1 cells to produce IL-9 through STAT6 phosphorylation.

Overexpression of IL-9 mRNA induced by pSTAT6 in CLL patients

To confirm these data, the expressions of IL-9 mRNA were determined in MEC-1 cells using RT-PCR. As illustrated in **Figure 3**, there is a high expression of IL-9 mRNA in CLL cells pretreated with IL-4 for 20 hours. A77-1726 could abolish IL-9 production in cells induced by pSTAT6.

IL-9 inhibited MEC-1 cell apoptosis

To determine the role of extracellular IL-9 in MEC-1 cell growth, we examined the role of rIL-9 in CLL cell apoptosis and necrosis. As shown in **Figure 4**, rIL-9 could decrease cell apoptosis to approximately 60% of the baseline level.

Discussion

In B cells, STAT6 is required for IL-4-stimulated proliferation, similar to the previously described role of T cells [21]. In addition, apoptosis is prevented in B cells by IL-4 in a STAT6dependent manner [22]. Extensive evidence suggests that the malignant cells of CLL patients are in close contact with activated T lymphocytes, which secrete a range of cytoprotective cytokines including IL-4. IL-4 induced the rapid phosphorylation and activation of STAT6 in CLL cells in vitro. IL9 is not expressed by Th2 and Th9 cells in the absence of STAT6 expression. Collectively, our results indicate a novel “extracellular IL-4-pSTAT6 intracellular IL-9” mechanisms involved in CLL pathology.

IL-9 is a member of the common γ-chain family of cytokines, using this receptor in combination with the cytokine-specific receptor IL-9 receptor-α (IL-9Rα) [23]. Besides its role during immune responses, its growth factor and anti-apoptotic activities on multiple transformed cells suggest a potential role in hematological malignancies. It can induce the proliferation of various lymphoid and hemopoietic cells. The dysregulated expression of IL-9 has been detected in biopsies or serum of patients with some hematological malignancies, such as ATL, HD, CLL, ALCL and NKT-cell lymphoma, which provides clinical evidence for its possible involvement in pathogenesis of hematological malignancies [14-20]. Our previous study has indicated that IL-9 participated in pathogenesis of B-cell NHL through up-regulation of immunosuppression mediated by Treg cells and mast cells [16, 17]. Many studies have shown that the IL-9Ralpha chain promoted JAK1 mutant phosphorylation and STAT activation, including STAT1, STAT3, and STAT5 [24-26], and IL-9 was not expressed by Th2 and Th9 cells in the

absence of expression of STAT6 [27, 28]. Based upon the previous study, we strive to prove that IL-9 directly take part in the development of CLL. We found that expressions of IL-9 increased in pretreated cells at a time-dependent manner. Furthermore, this phenomenon could be inhibited by A77-1726, which is a STAT6 inhibitor. Our findings suggest that rIL-4 could enhance MEC-1 cells to produce IL-9 through STAT6 phosphorylation.

To further investigate the potential function of IL-9 in CLL pathogenesis, we detected the effects of rIL-9 on CLL cell apoptosis. After treatment with rIL-9, the apoptosis of MEC-1 cells decreased. The upregulation of IL-9 may be involved in the pathogenesis of CLL.

In summary, the results clearly indicate that IL-9 is markedly overexpressed in CLL cells which pretreated by IL-4 compared to their counterparts. A77-1726 could inhibit the IL-9 production. The apoptosis of MEC-1 cells decreased after treatment with rIL-9. Our findings suggest a new explanation of the possible molecular mechanism in the regulation of IL-9 production in CLL. It helps us to get a deeper understanding about the molecular mechanism of CLL and could be served as a potentially therapeutic target for CLL patients in the future.

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Disclosure of conflict of interest

The authors declare that they have no competing interests.

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