Effects of STAT3 antisense RNA on IL-6-induced differentiation and growth arrest of M1 leukemia cells*

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Abstract To investigate the biological roles of STAT3 in the regulation of growth and differentiation of leukemia cells, we modified a murine myeloid leukemia cell line M1 with STAT3 antisense RNA. The effects of STAT3 antisense RNA on the growth arrest and terminal differentiation of M1 cells induced by interleukin 6 (IL-6) were determined. It was found that STAT3 antisense RNA blocked the activation of STAT3, and reduced the growth arrest and terminal differentiation of IL-6-induced M1 leukemia cells. These results indicate that STAT3 activation is a necessary process for IL-6-induced growth arrest of M1 cells and for the differentiation of M1 cells into macrophage.

Keywords: IL-6, STAT3, antisense RNA, M1 cell line.

Interleukin 6 (IL-6) is a pleiotropic cytokine. It induces the differentiation of B cells to antibody-producing plasma cells, T cell growth and differentiation (in particular, Th2 development), the differentiation of myeloid leukemia cells into macrophages, and the neural differentiation of PC12 cells. IL-6 is also a growth factor for myeloma/plasmacytoma[1].

It is known that JAKs (Janus kinases) and STATs (signal transducers and activators of transcription) play essential roles in the signal transduction of IL-6. IL-6 activates STAT3 in all its target cells, and JAK1 is the main kinase that makes STAT3 tyrosine phosphorylated. In certain target cells, IL-6 activates STAT1 and STAT5 too[2].

Previously, we found that IL-6 could significantly activate STAT3 and the effect lasted over 24 hours in M1 acute myeloid leukemia (AML) cells, which in turn underwent growth-arrest and terminal differentiation in response to IL-6. In contrast, another IL-6-related signal transduction pathway, Ras/MAPK/NF-IL6 pathway, was activated transiently and only by high dose of IL-6[3]. These facts suggest that STAT3 may play an important role in the IL-6-induced regulation of differentiation and growth arrest of M1 cells. To confirm our speculation, in this study we constructed STAT3-antisense-RNA-modified M1 cells, and investigated the effects of STAT3 antisense RNA on the IL-6-induced growth arrest and terminal differentiation into macrophage of M1 cells, this will help to elucidate the mechanism of the signal transduction of IL-6.

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1 Materials and methods

1.1 Cells

The murine myeloid leukemia cell line M1 cells (ATCC, USA) were grown in RPMI1640 (Gibco) supplemented with 10% fetal bovine serum. M1-neo and M1-ST/Fan cells were constructed by transfecting pcDNA3 vector (Clontech) or pST/Fan recombinant plasmid into parental M1 cells with electroporation technique and the transformants were selected with G418. To make pST/Fan, a 1.2 kb STAT3 cDNA (a generous gift from Dr. FU X.Y. of Yale University) containing the start code was inserted into pcDNA3 vector in the opposite direction.

1.2 Cell proliferation assay

M1, M1-neo, M1-ST/Fan cells were seeded into 96-well plate with a density of 1×10^4 cells per well. Then different doses of rhIL-6 (10 μL) were added into the culture. The specific activity of rhIL-6 was determined by the proliferation of the IL-6-dependent 7TD1 mouse B cell hybridoma[4]. The cells were cultured for 72 h following rhIL-6 treatment, then 10 μL MTT (Thiazolyl blue, Sigma, 5 mg/mL) was added to each well, 4 h later, an equal volume of 10% SDS-0.01 mol/L HCl was added to dissolve the blue crystals of formazan. Then the samples were measured at $OD_{570\ nm}$ by an ELISA reader (Dynatech Laboratories, Inc. USA).

1.3 Wright's staining

It was completed as previously described[5].

1.4 Detection of CD11b expression

M1, M1-neo and M1-ST/Fan cells were washed with serum-free medium, and were incubated with 1 μg of FITC-conjugated anti-mouse CD11b (Immunotech) for 30 min on ice, then were analyzed by a fluorescence-activated cell sorter (Becton Dickinson FACS)[6].

1.5 Measurement of phagocytic activity

M1, M1-neo and M1-ST/Fan cells were washed with serum-free medium; they were incubated for 4 h with polystyrene latex particles (average diameter 0.60 μm) at 37℃ in 5% CO_2. Afterwards, the cells were washed three times with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and the number of phagocytic cells among at least 200 cells was counted[6].

1.6 Electrophoresis mobility shift assay (EMSA)

M1-neo and M1-ST/Fan cells treated with rhIL-6 were collected and nuclear extracts were prepared as described[7]. Oligonucleotide probes were labeled by [α-^{32}P]dATP with Klenow enzyme. Nuclear extracts (10 to 20 μg of protein) were incubated with about 10 fmol (5 000 cpm) of the probe in gel shift incubation buffer for 20 min at room temperature. Then the protein-DNA complexes were resolved on a 4.5% non-denaturing polyacrylamide gel containing 15% glycerol in Tris-acetate-EDTA buffer at 4℃, and autoradiographed. The sequences of the probes used are: Stat3 5′CGATCTTC-

TGGGAATTCA3′[7] and NF-IL6 5′CTGAGATGACGTAGTTTTGGCGCT3′[8].

1.7 Cell cycle assay

M1, M1-neo, M1-ST/Fan cells were centrifuged and resuspended to a final density of 10^6 cells/mL in ice-cold 70% ethanol for a minimum of 18 h. After washing with PBS, the pellet was stained with propidium iodide (PI) (Sigma) at a concentration of 50 μg/mL in PBS containing 0.002% Triton X100 and 100 U/mL RNase A (Sigma), and incubated at room temperature in dark for 30 min. Then the cells were analyzed by a fluorescence-activated cell sorter (Becton Dickinson FACS).

2 Results

2.1 STAT3 antisense RNA blocks the IL-6-induced activation of STAT3

STAT3 antisense RNA expressed in M1 cells was expected to interfere the translation of STAT3 mRNA, resulting in reduced amount of STAT3 protein. And fewer tyrosine-phosphorylated STAT3 homodimers could get into the nucleolus where they bind the regulation sequence and regulate the expression of target genes. With electrophoresis mobility shift assay (EMSA), it was found that the DNA binding activity of STAT3 was much less in M1-ST/Fan cells than in mock control when the cells were induced by IL-6 (100 ng/mL), but the DNA binding activity of NF-IL6 was the same in M1-ST/Fan cells and in mock control (fig. 1), which indicates that STAT3 antisense RNA can specifically antagonize IL-6-induced activation of STAT3 in M1 cells as we expected.

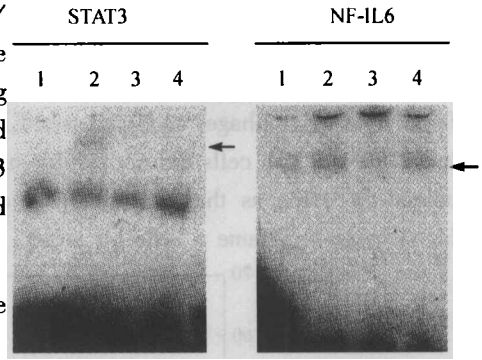

Fig. 1. Effect of STAT3 antisense RNA on the IL-6-induced activation of STAT3. 1, M1-neo; 2, M1-neo with 100 ng/mL rhIL-6; 3, M1-ST/Fan; 4, M1-ST/Fan with 100 ng/mL rhIL-6. The arrows indicate STAT3 and NF-IL6 homodimers respectively.

2.2 STAT3 antisense RNA partially reduces the inhibitory effect of IL-6 on the growth of M1 cells

The role of STAT3 antisense RNA in the inhibitory effect of IL-6 on M1 cells was studied. By incubation of M1 cells with IL-6 for 3 days, the growth of M1 cells was remarkably inhibited by IL-6. M1-neo cells responded to IL-6 in the same way. However, M1-ST/Fan cells responded quite differently. Low dose of IL-6 promoted the growth of M1-ST/Fan cells, while high dose of IL-6 inhibited the growth of M1-ST/Fan cells, but to a much less extent than it did in M1 and M1-neo cells (figure 2).

STAT3 antisense RNA also influenced the G_0/G_1 phase arrest of M1 cells that had been induced by IL-6. After incubated with 100 ng/mL IL-6 for 4 days, more M1-neo cells (64.18% ± 0.97%) were arrested in G_0/G_1 phase when compared with M1-ST/Fan cells (45.05% ± 10.94%); while more M1-ST/Fan cells were in S phase (38.12% ± 9.14%) than mock control (22.68% ± 0.51%). The difference between M1-ST/Fan and mock control was of statistical significance ($P < 0.05$, two-tail test). These results indicate that STAT3 activation is necessary for the growth arrest of M1 cells induced by IL-6.

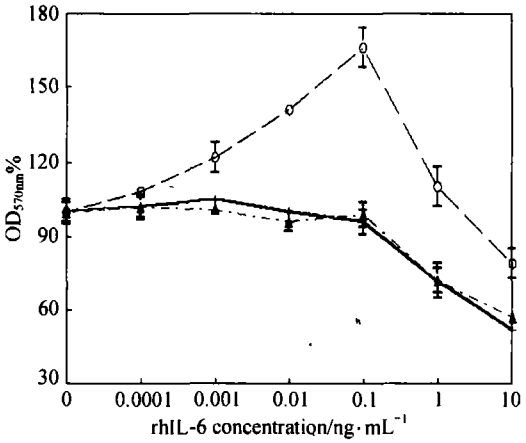

Fig. 2. STAT3 antisense RNA antagonizes the inhibitory effect of IL-6 on the growth of M1 cells. —+— M1, —○— M1-ST/Fan, —▲— M1-neo.

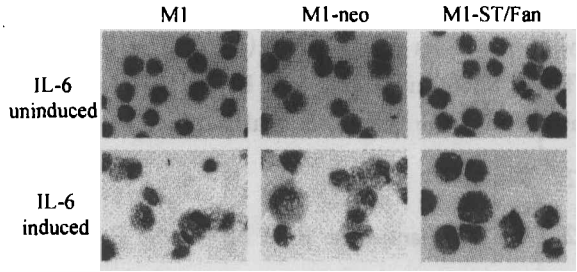

Fig. 3. Effect of STAT3 antisense RNA on morphological changes of M1 cells induced by IL-6.

2.3 STAT3 antisense RNA reduces the differentiation of M1 cells induced by IL-6 into macrophage

The morphological changes and enhanced CD11b expression in IL-6-induced M1 cells were observed. It was found that M1 and M1-neo cells exhibited morphological characteristic of terminal differentiated macrophages and expressed CD11b after 3—4 days treatment of IL-6. In contrast, IL-6-induced M1-ST/Fan cells did not exhibit such morphological changes (fig. 3) and CD11b expression remained the same as that in uninduced cells (fig. 4). However, the size of M1-ST/Fan cells induced by IL-6 became a little bit larger.

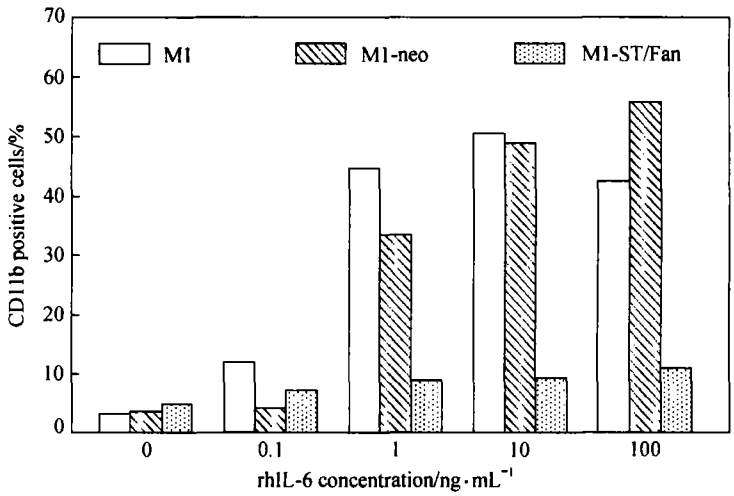

Fig. 4. Effect of STAT3 antisense RNA on CD11b expression induced by IL-6 in M1 cells.

M1-ST/Fan did not display a phagocytic activity as strong as M1 and M1-neo did in response to IL-6. After incubated with 10—100 ng/mL IL-6 for 4 days, most of M1 or M1-neo cells could phagocytize polystyrene latex particles, however the number of phagocytic M1-ST/Fan cells was much smaller. Also phagocytic M1-ST/Fan cells had much fewer polystyrene latex particles in them than in phagocytic M1 or M1-neo cells (fig. 5). It might be due to the fact that STAT3 antisense RNA

blocked IL-6-induced up-regulation of CD11b and FcγR, the latter is the target gene of STAT3[7]. Our results indicate that STAT3 activation is a necessary process for terminal differentiation of IL-6-induced M1 cells.

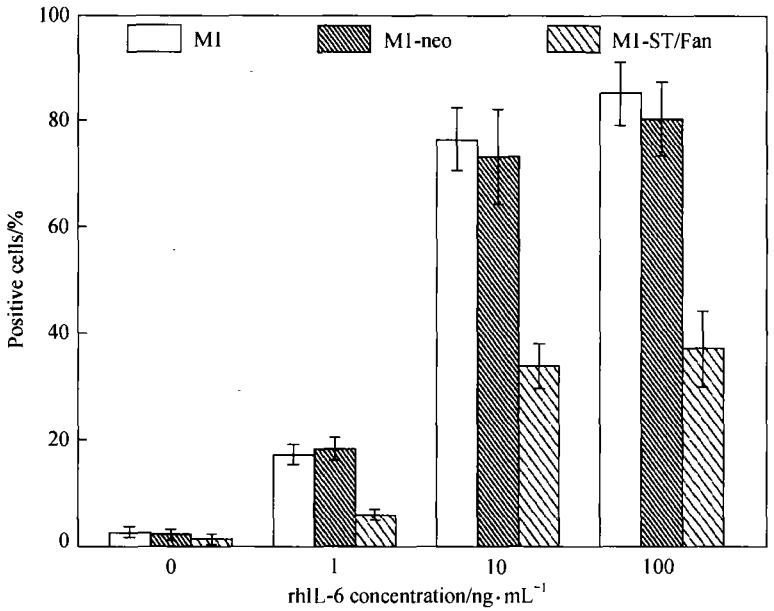

Fig. 5. Effect of STAT3 antisense RNA on phagocytic activity of IL-6-induced M1 cells.

3 Discussion

To date, six members of the STAT family have been identified[2]. One or more STAT family members are activated in response to different cytokines, which is assumed to contribute to the diversity of cytokine responses. In the case of IL-6 signal transduction, IL-6 induces tyrosine phosphorylation of STAT3, STAT1 and STAT5, but which STAT family member affects cell proliferation and differentiation remains unknown[2].

In our previous study, IL-6 caused sustained STAT3 activation in M1 cells and this activation pattern was not observed in other lineages of cells. Our results indicate that the expression and activation of STAT3 is absolutely necessary for IL-6-induced growth arrest and terminal differentiation of M1 cells. And this is in accordance with the results of Nakajima et al., who showed that the mutant forms of STAT3 inhibited both IL-6-induced growth arrest and macrophage differentiation in the M1 transformants[9]. However, the mutant STAT3 might function differently from wild type STAT3. For example, STAT3 β which lacks the C terminal of STAT3 could functionally interact with c-Jun, while the full-length STAT3 could not[10]. Therefore the conclusion of Nakajima et al.'s remains to be confirmed.

Is STAT3 activation sufficient for macrophage differentiation? We would say no, because G-CSF activates predominantly STAT3 in AML cell lines but the cells do not differentiate in response to GCSF[10]. It is highly possible that STAT3 needs other components to function together to induce differentiation of AML cells to macrophage, although STAT3 activation is necessary for macrophage

differentiation. This conclusion is also supported by the fact that STAT3 antisense RNA only partially but not completely antagonizes the IL-6-induced growth arrest and phagocytic activity of M1 cells.

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