

## Effects of dexamethasone on Flt3 receptor expression and proliferation induced by recombinant human Flt3 ligand in malignant hematopoietic cells<sup>1</sup>

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**KEY WORDS** recombinant proteins; recombinant human Flt3 ligand; receptor protein-tyrosine kinases; hematologic neoplasms; dexamethasone

### ABSTRACT

**AIM:** To investigate the effect of dexamethasone (DXM) on the expression of Flt3 receptor and the proliferation mediated by recombinant human Flt3 ligand (rhFL) in leukemia cells. **METHODS:** Eighteen malignant hematopoietic cell lines and 10 leukemia blasts from leukemia patients were examined by flow cytometry for the expression of Flt3 receptor before and after incubation with DXM 0.1  $\mu\text{mol/L}$  for 24 h. The effect of DXM on the proliferation of malignant hematopoietic cells was measured by MTT assay. **RESULTS:** (1) Expression of the Flt3 receptor in malignant hematopoietic cell lines and leukemia blasts was widespread and extremely heterogeneous; (2) The presence of receptor on the surface of malignant hematopoietic cell didn't necessarily imply a significant ligand-induced response, at least in terms of proliferation. Conversely, some Flt3 receptor-negative malignant hematopoietic cells responded to rhFL; (3) DXM down-regulated the expression of Flt3 receptor and inhibited the proliferation induced by rhFL in some malignant hematopoietic cell lines and fresh leukemia cells. **CONCLUSION:** DXM may down-regulate the expression of Flt3 receptor on the surface of malignant hematopoietic cells and inhibit the proliferation induced by rhFL. A combination of rhFL and DXM may serve to control hematopoietic defects in malignant hematopoietic diseases.

### INTRODUCTION

Hematopoiesis is a complex process in which cell growth and differentiation are controlled by a number of hematopoietic growth factors or cytokines, acting as either positive or negative regulators. Among them, class III receptor-type tyrosine kinases (RTKs) and their ligands seem to play an important role in hematopoiesis, especially in the early stages of the process<sup>(1)</sup>. The receptors for SCF (stem cell factor), M-CSF (macrophage colony-stimulating factor), and PDGF (platelet-derived growth factor) belong to the RTK class III. These receptors are involved in the proliferation and differentiation of hematopoietic and germ cells. Flt3 receptor (fms-like tyrosine kinase receptor III), another type of RTK, and its ligand, Flt3 ligand (FL), were identified in 1994<sup>(2)</sup>. Flt3 receptor is expressed in adult bone marrow and thymus, fetal thymus and fetal liver. The binding of FL to Flt3 receptor stimulates the proliferation of hematopoietic stem/progenitor cells and plays a critical role in normal hematopoiesis. FL may also stimulate the production of dendritic and natural killer cells, both *in vitro* and *in vivo*. Thus FL may find uses in the stimulation of hematopoiesis and in cancer therapy<sup>(3-5)</sup>.

However, many of the malignant hematopoietic cells are derived from the transformation of hematopoietic stem/progenitor cells. It may be reasonable to reveal the role played by FL and its receptor in the proliferation of those malignant cells. An investigation of potential effects of FL on malignant hematopoietic cells may be relevant to a clinical usage of the cytokine as an antitumor bioterapy factor or as a hematopoietic factor in dys-hematopoiesis of the patients. It will also be valuable to find a factor that prevents Flt3 receptor expression on malignant hematopoietic cells and inhibits the proliferation induced by FL. Thus, the aims of this study were: (1) to investigate the pattern of Flt3 receptor expression in leukemia cell lines and fresh acute adult leukemia sam-

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ples; (2) to evaluate the mitogenic potential of rhFL for the proliferation and colonial growth of leukemia cell lines and primary human acute leukemia cells; (3) to investigate the effect of dexamethasone (DXM) on the expression of the Flt3 receptor in malignant hematopoietic cells and the response of these cells to recombinant human Flt3 ligand (rhFL).

## MATERIALS AND METHODS

**Cytokines and antibody** rhFL was expressed with a yeast expression system in our department<sup>(6)</sup>. DXM was obtained from Sigma (St Louis, USA). IL-6, GM-CSF, and IL-11 were purchased from Immunogen (Los Angeles, USA). Anti-Flt3-specific IgG1 monoclonal antibody SF1.340 was purchased from Immunotech (Marseille, France).

**Cell lines and fresh leukemia cells** Jurkat, Daudi, Raji, HL-60, U937, KG1- $\alpha$ , U266, TF1, Baf, 8266, and Wehi cell lines were from the ATCC, USA; the B9 cell line was donated by Dr Leony Arden from National Immunology Institute, Amsterdam, Netherland; B9-11, XG-1, XG-6, and XG-7 cell lines were a gift from Dr Bernard Klein of INSERM U475, Immunopathologie des Maladies Tumorales et autoimmunes, Montpellier, France. HSB2, JJhan, and Sub T1 cell lines were kindly provided by Prof YAO Kun, Nanjing Medical University, China. All cell lines in our laboratory were free from mycoplasma contamination. Cell lines were grown and maintained under routine conditions (at 37 °C in a humidified atmosphere with 5 % CO<sub>2</sub>) in RPMI 1640 supplemented with 10 % inactivated fetal bovine serum.

Fresh leukemia cells were collected from marrow aspirates or peripheral blood (PB) samples of 10 leukemia patients. Leukemia samples were classified according to the French-American-British (FAB) classification, based on morphologic and cytochemical criteria, and confirmed by phenotypic analysis. The mononuclear cells including leukemia cells were isolated by Ficoll-Hypaque from Pharmacia (Piscataway, USA) density-gradient centrifugation at 400  $\times$  g for 30 min.

**Flow cytometric analysis of Flt3 receptor expression** Expression of Flt3 receptors in human leukemia, lymphoma, and myeloma cell lines and fresh leukemia cells was determined by flow cytometric analysis. The cells were washed 3 times with PBS and counted. They were then adjusted to an appropriate concentration in a microtiter plate. The cells were incubated

for 30 min with the anti-Flt3-specific IgG1 monoclonal antibody SF1.340. For each cell line, an isotype irrelevant antibody was used as a negative control. The cells were washed and incubated with a fluoresceinated (fluorescein isothiocyanate) goat-anti-mouse IgG. After washing, the cells were fixed in 1 % paraformaldehyde PBS (in mmol/L: NaCl 137, KCl 2.7, Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O 4.3, and KH<sub>2</sub>PO<sub>4</sub> 1.4; pH 7.3) and analyzed on an EPICS flow cytometer (Coulter Electronic, Miami, USA).

**Proliferation assay of malignant hematopoietic cells** The proliferative effect of rhFL on malignant hematopoietic cell lines was tested. The cells were plated in 96-well plates at a density of 5  $\times$  10<sup>3</sup> cells per well for cell line cells and 2  $\times$  10<sup>5</sup> cells per well for fresh leukemia cells in a total volume of 150  $\mu$ L of RPMI 1640 containing 10 % FCS with or without rhFL 0 to 100  $\mu$ g/L. Wehi supernatant 1.5 % was added as a source of IL-3, required for the growth of the Baf cells. GM-CSF 10  $\mu$ g/L, IL-6 1  $\mu$ g/L, and IL-11 0.5  $\mu$ g/L were also added to TF1, XG-1, XG-6, XG-7, and B9-11 (B9) cell lines, respectively. After 72-h incubation, proliferation was measured by MTT assay as described by Carmichael *et al*<sup>(7)</sup>.

**Effects of DXM on Flt3 receptor expression and the proliferation induced by rhFL** Malignant hematopoietic cells incubated with DXM 0.1  $\mu$ mol/L for 24 h were harvested and analyzed for the expression of Flt3 receptor by flow cytometry. For studying the effect of DXM on the proliferation of malignant hematopoietic cells induced by rhFL, cells were harvested and plated in 96-well plates at a density of 5  $\times$  10<sup>3</sup> cells per well for cell line cells and 2  $\times$  10<sup>5</sup> cells per well for fresh leukemia cells. DXM 0.1  $\mu$ mol/L was added to the medium in addition to cytokines including rhFL as mentioned above. After 72-h incubation, cell proliferation was measured by MTT assay.

**Statistical analysis** Student's *t*-test was used to compare the variance between two different groups.

## RESULTS

**Flt3 receptor expression in malignant hematopoietic cell lines and leukemia blasts** A total of 18 leukemia, lymphoma, and multiple myeloma cell lines were screened for Flt3 receptor expression by flow cytometric analysis. The anti-Flt3-specific IgG1 monoclonal antibody SF1.340 was used to recognize the extracellular epitope of the Flt3 receptor. Of the 18 cell

lines tested, 5 contained Flt3 receptor-positive cells. The levels of Flt3 receptor expression were different in the positive cell lines. In Raji, Daudi, and 8266 cells, a high level of reactivity with anti-Flt3 antibody was seen, whereas HL-60 and XG-6 cells showed relatively low fluorescence intensity (Tab 1).

The percentage of malignant hematopoietic cells in peripheral blood or bone marrow in 10 acute leukemia patients was 25 % to 90 %. Of the 10 acute leukemia patients, 6 expressed Flt3 receptor without any correlation with FAB subtype (Tab 2).

**Effect of rhFL on the proliferation of malignant hematopoietic cell lines and leukemia blasts**

rhFL at the concentrations from 10 to 100 µg/L stimulated the proliferation of Raji and HL-60 cells ( $P < 0.01$ ), but had no effect on Jurkat, U937, KG1-α, U266, TF1, Baf, B9, B9-11, XG-1, XG-7, HSB2, JJhan, Sub T1, Daudi, 8266, and XG-6 cells even though the Flt3 receptor was expressed in the Daudi, 8266, and XG-6 cell lines ( $P > 0.05$ ). Interestingly, FL elicited a significant growth response in HL-60 cells which weakly expressed Flt3 receptors (Fig 1).

**Tab 1. Effect of DXM on the expression of Flt3 receptor on the surface of some malignant cell lines.  $n = 6$ .  $\bar{x} \pm s$ .  $^c P < 0.01$  vs Control.  $^f P < 0.01$  vs Positive.**

Cell line	Cell type (Original disease)	Receptor expression/%		
		Control	Positive	Positive + DXM
Raji	lymphoid (B type)	2.1 ± 0.3	36 ± 4 <sup>c</sup>	14.7 ± 2.1 <sup>f</sup>
Daudi	lymphoblastoid (B type)	2.7 ± 0.4	13.4 ± 2.2 <sup>c</sup>	6.7 ± 1.9 <sup>f</sup>
Sub-T1	lymphoid (T type)	0.7 ± 0.4	0.4 ± 0.3	0.50 ± 0.24
JJhan	lymphoid (T type)	1.4 ± 0.7	1.3 ± 0.3	1.2 ± 0.3
HSB2	lymphoid (T type)	0.8 ± 0.5	1.2 ± 0.7	1.2 ± 0.9
Jurkat	lymphoid (ALL T)	0.8 ± 0.4	0.7 ± 0.6	1.2 ± 0.6
HL-60	myeloid (AML M2)	1.1 ± 0.8	4.1 ± 0.5 <sup>c</sup>	4.9 ± 0.3
KG-1α	myeloid (AML)	1.6 ± 0.7	1.8 ± 0.9	1.2 ± 0.3
TF-1	erythroid (AML M6)	1.8 ± 0.4	2.5 ± 0.5	2.8 ± 0.5
U937	monocytic (Histiocytic lymph)	1.7 ± 0.4	3.8 ± 0.6	1.9 ± 0.9
8266	myeloma	0.7 ± 0.6	46 ± 4 <sup>c</sup>	23 ± 4 <sup>f</sup>
U-266	myeloma	2.0 ± 0.3	3.8 ± 0.7	2.2 ± 0.8
XG-1	myeloma	1.8 ± 0.6	1.7 ± 0.4	1.5 ± 0.7
XG-6	myeloma	2.5 ± 0.3	6.3 ± 0.5 <sup>c</sup>	4.3 ± 0.3 <sup>f</sup>
XG-7	myeloma	1.93 ± 0.18	3.0 ± 0.5	4.0 ± 0.4
B9	murine lymphoid (B type)	1.8 ± 0.3	1.4 ± 0.3	1.2 ± 0.5
B9-11	murine lymphoid (B type)	2.2 ± 0.4	1.9 ± 0.3	2.0 ± 0.4
Baf	murine pro B-cell	1.1 ± 0.4	1.2 ± 0.6	1.2 ± 0.7

**Tab 2. Expression of Flt3 receptor in acute leukemia cells and proliferative response to rhFL (100 µg/L) and rhFL (100 µg/L) + DXM (0.1 µmol/L).  $n = 6$  wells.  $\bar{x} \pm s$ .  $^c P < 0.01$  vs control (Medium).  $^f P < 0.01$  vs rhFL.**

Acute leukemia cells	Malignant cells/%	Receptor expression/%		Proliferative response (MTT A <sub>595</sub> )		
		Positive	Positive + DXM	Medium	rhFL	rhFL + DXM
ALL (B) PB	59.5	46.1	69.5	1.22 ± 0.27	1.83 ± 0.29 <sup>c</sup>	1.27 ± 0.17 <sup>f</sup>
M2 PB	35	1.9	1.1	1.01 ± 0.15	1.19 ± 0.13	0.85 ± 0.11
MM BM	80	15.8	19.1	1.53 ± 0.19	1.48 ± 0.25	1.41 ± 0.21
M1 BM	90	1.3	2.0	1.22 ± 0.15	1.90 ± 0.20 <sup>c</sup>	1.07 ± 0.20 <sup>f</sup>
M5 PB	25	6.9	13.2	0.88 ± 0.17	0.85 ± 0.15	0.97 ± 0.16
M5b BM	76	24.8	18.8	1.43 ± 0.24	1.64 ± 0.23 <sup>c</sup>	1.23 ± 0.20 <sup>f</sup>
M1 PB	76	1.2	0.9	1.19 ± 0.19	1.21 ± 0.17	1.01 ± 0.14
M1 BM	74	2.4	1.6	0.93 ± 0.17	0.85 ± 0.14	0.87 ± 0.14
M4 PB	35	21.3	15.2	1.46 ± 0.24	1.57 ± 0.22	1.21 ± 0.19 <sup>f</sup>
M3 PB	45	16.5	18.1	0.72 ± 0.14	0.76 ± 0.16	0.66 ± 0.14

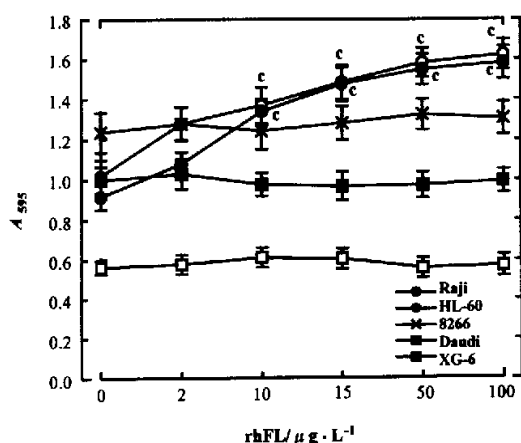


Fig 1. Proliferative effect of rhFL in malignant hematopoietic cell line cells expressing Flt3 receptors.  $n = 3$ .  $\bar{x} \pm s$ .  $^*P < 0.01$  vs control (without rhFL).

FL stimulated the proliferation of leukemia blasts in 3 out of 10 cases of acute leukemia. Of the three cases with a strong proliferative response to rhFL, two expressed Flt3 receptors and one was Flt3-negative. Conversely, in some Flt3-positive cases there was no response to rhFL (Tab 2).

**DXM down-regulated the expression of Flt3 receptor and inhibited the proliferation induced by rhFL** After incubation with DXM  $0.1 \mu\text{mol/L}$  for 24 h, the expression of Flt3 receptor was down-regulated significantly in all 5 Flt3 receptor-positive cell lines ( $P < 0.01$ , Tab 1). The proliferative effect of rhFL on HL-60 and Raji was inhibited after incubation with DXM  $0.1 \mu\text{mol/L}$  or 72 h ( $P < 0.01$ , Fig 2).

Among six Flt3-positive leukemia blasts, the expression of Flt3 receptor in two cases was down-regulated after incubation with DXM  $0.1 \mu\text{mol/L}$  for 24 h, whereas in 4 cases there was an increase after the incubation. DXM inhibited the proliferation of leukemia blasts induced by rhFL in all the 3 cases ( $P < 0.01$ , Tab 2 and Fig 3). But there was no correlation between FAB subtype and the response to rhFL and down-regulation by DXM.

## DISCUSSION

FL acting through its tyrosine kinase receptor Flt3 has pleiotropic and potential effects on hematopoietic cells. It can stimulate the proliferation and colony formation of human hematopoietic progenitor cells.

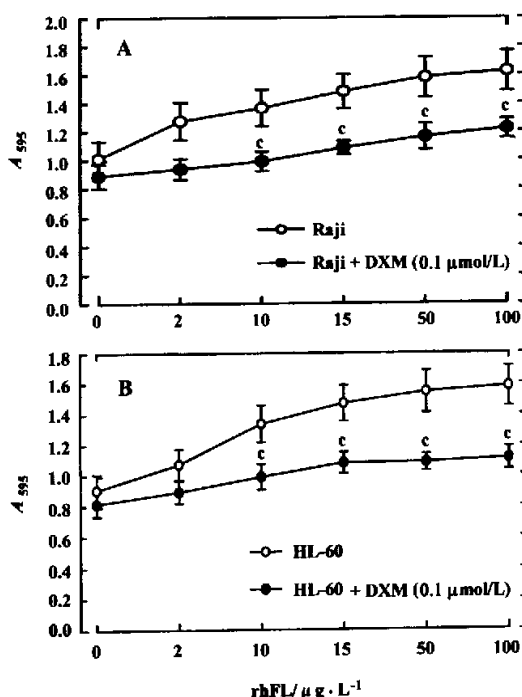


Fig 2. Inhibitory effect of DXM on the proliferation induced by rhFL in Raji and HL-60.  $n = 3$ .  $\bar{x} \pm s$ .  $^*P < 0.01$  vs control (without DXM). (A): Raji cell line; (B) HL-60 cell line.

Synergy was reported for co-stimulation with G-CSF, GM-CSF, M-CSF, IL-3, and SCF<sup>(8)</sup>. The well-documented involvement of this ligand-receptor pair in physiological hematopoiesis raised the question whether FL and Flt3 receptor also have a role in the pathogenesis of leukemia. Several investigators have focused their attention on this subject, but there were many disputes. Drexler<sup>(9)</sup> found that the vast majority of primary acute myeloid leukemia (AML) cells and continuous human myeloid leukemia cell lines were Flt3-positive. Among the myeloid cell lines, predominantly the monocytic and myelocytic cell lines were Flt3-positive whereas the erythrocytic and megakaryocytic cell lines were Flt3-negative. They also found that some leukemia cell lines displayed both ligand and receptor, and stimulated the proliferation of themselves by autocrine, intracrine, and paracrine. However, Siitonen and his colleagues found that FL did not induce the growth of malignant hematopoietic cells in myeloproliferative disorders<sup>(10)</sup>. In the present study, Flt3 receptors were expressed on the surface of certain cell lines derived from B type lymphoma

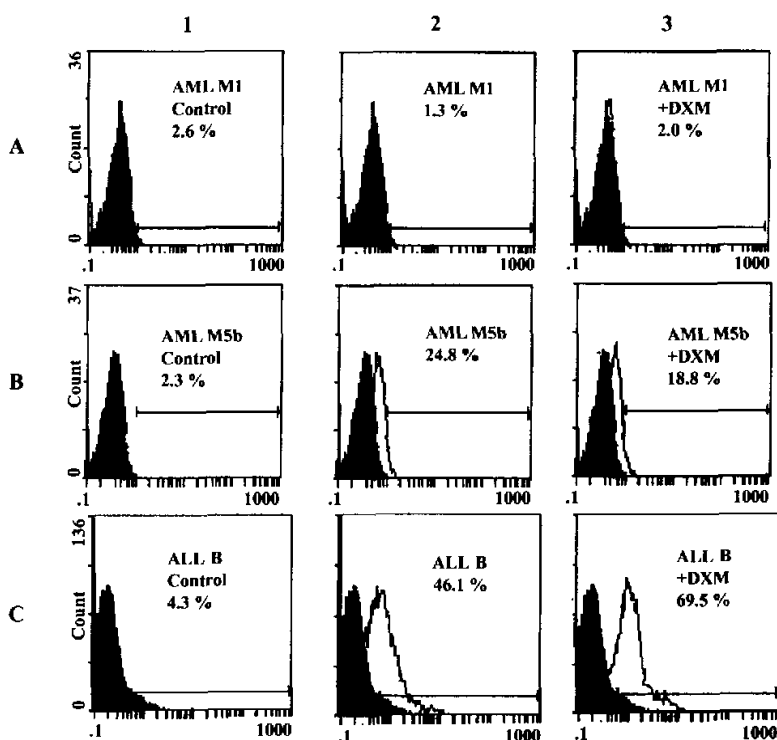


Fig 3. Flow cytometric detection of Flt3 receptor expression on the surface of leukemia cells from 3 patients with acute leukemia. Cells were stained with specific anti-Flt3 receptor monoclonal antibody SF1.340. The background staining (shaded curve) using an irrelevant antibody of the same isotype can be discerned from the specific staining (open curves). (A) Patient AML M1; (B) Patient AML M5b; (C) Patient ALL B. (1) Negative control; (2) rhFL (100 µg/L)-treated; (3) rhFL (100 µg/L) + DXM (0.1 µmol/L)-treated.

(Raji and Daudi), multiple myeloma (8266 and XG-6), and acute myeloid leukemia (HL-60). There was, however, no obvious correlation between the stimulating effect of FL and the occurrence of Flt3 receptor; thus Raji (high Flt3) responded to rhFL, and so did HL-60 (low Flt3). Most cell lines, such as Jurkat, U937, KG1 $\alpha$ , U266, TF1, Baf, B9, B9-11, XG-1, XG-7, HSB2, JJhan, and Sub T1 cells showed neither significant expression of Flt3 receptor nor a response to FL.

We also found that Flt3 receptor was expressed in 60 % cases of acute leukemia throughout the different morphological subtype (M0-M7). This finding was comparable to the positive percentage by the others. In various studies, Flt3 was detected in 64 % – 100 % (on average 86 %) of the acute myeloid leukemia at the level of mRNA by Northern blot and RT-PCR analysis, and in 62 % – 88 % (on average 72 %) at the level of protein by flow cytometry or Western blot<sup>[11,12]</sup>. But only 3 out

of 10 cases responded to the stimulation of rhFL. Interestingly, Flt3 receptor could not be observed at the detectable level in cases that exerting a significant response to rhFL. A possible explanation for this finding could be that the receptor expression was masked by the presence of the ligand, which could be expressed by the cells. Another possibility could be that these cells might express a very low number of high-affinity receptors, below the sensitivity threshold of flow cytometer, but highly responsive to the effect of the ligand. Third, it has also been reported recently that Flt3 receptor expression was strictly related to cell cycle phases, and that only cycling blasts were Flt3-positive<sup>[13]</sup>. Furthermore, using the colony assay, Stacchini *et al*<sup>[14]</sup> found that FL was able to induce the growth of clonogenic precursors in the Flt3-negative cases and to increase the colony number when used in combination with G-CSF, GM-CSF, IL-3, and SCF. It suggests that FL may up-regulate other growth

factor receptors, and make the leukemic precursors become more responsive to the additional cytokines or the factors secreted by them. In FL-responsive cells, stimulation with FL usually led to tyrosine phosphorylation of the Flt3 receptor and the other protein, and finally resulted in the proliferation of the responsive cells. But in some Flt3-positive cases there was no response to rhFL. There are five possibilities: (1) the cells may be truly non-responsive; (2) the cells may produce endogenous ligand (and are thus refractory to exogenously added factor); (3) the cells express a defective receptor incapable of binding the ligand or of transmitting an intracellular signal<sup>[15]</sup>; (4) in certain cases the growth factor drives differentiation, but not proliferation; and (5) the growth factor inhibits apoptosis thus promoting survival. Therefore, a considerable exploration must be taken when an acute leukemia blast has not response to the stimulation of FL.

We also found that the expression of the Flt3 receptor in the 5 Flt3 receptor-positive cell lines decreased after the cells were incubated with DXM 0.1  $\mu$  mol/L. DXM apparently inhibited the proliferation induced by rhFL through the down-regulation of the Flt3 receptor in malignant hematopoietic cell lines (Tab 1). But in fresh acute leukemia blasts, DXM inhibited proliferation of the leukemia cells; meanwhile, the expression of Flt3 receptor on the surface of those cells could not be down-regulated by DXM. DXM may inhibit the proliferation of acute leukemia blasts mediated by FL via other way, eg, accelerating the apoptosis of the leukemia blasts. It has been reported that FL can inhibit apoptosis and promote survival of leukemia cells<sup>[16]</sup>. Thus, DXM may directly or indirectly inhibit the effects mediated by FL when they are used simultaneously. The precise mechanism of DXM in FL-induced proliferation of leukemia cells is under investigation, it might be clinically relevant to combine FL with DXM in those patients who need to down-regulate the expression of Flt3 receptors and inhibit the proliferation induced by FL. It must be stressed, however, that we carried out our study with leukemia, lymphoma, and myeloma cell lines and fresh acute leukemia blasts limited *in vitro*; it is essential to study the effects of the recombinant FL on more fresh tumor samples and to survey closely its effects *in vivo*.

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**地塞米松对恶性造血细胞 Flt3 受体表达及重组人 Flt3 配体诱导的细胞增殖的影响<sup>1</sup>**

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**关键词** 重组蛋白质类; 重组人 Flt3 配体; 酪氨酸激酶受体; 血液肿瘤; 地塞米松

**目的:** 研究地塞米松 (DXM) 对恶性造血细胞表面 Flt3 受体表达及重组人 Flt3 配体 (rhFL) 介导的恶性

造血细胞增殖的影响。方法: 采用流式细胞仪分析 18 株恶性造血细胞系细胞和 10 例新鲜白血病原代细胞表面 Flt3 受体的表达, 及与 DXM 0.1 μmol/L 共育 24 h 后 Flt3 受体的表达, 用 MTT 法测定 DXM 对 rhFL 介导的恶性造血细胞增殖的影响。结果: (1) 恶性造血细胞系细胞和白血病原代细胞 Flt3 受体的表达广泛并呈多样性。 (2) 恶性细胞表面 Flt3 受体的表达与 rhFL 介导的恶性细胞增殖无一定相关性, rhFL 促进部分 Flt3 受体阴性细胞的增殖。 (3) DXM 下调 Flt3 受体的表达, 抑制 rhFL 介导的部分恶性造血细胞系细胞和新鲜白血病原代细胞的增殖。结论: DXM 可下调恶性造血细胞表面 Flt3 受体的表达, 抑制 rhFL 介导的恶性造血细胞的增殖。DXM 和 rhFL 联合应用, 可使 rhFL 在恶性造血系统疾病的造血功能缺乏治疗时更为安全。

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