Original Article

Enhanced expression of mRNA for FLT3 in bone marrow CD34+ cells in rheumatoid arthritis

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***Objective*: We have recently demonstrated that the generation of pre-plasmacytoid dendritic cells (pre-pDC) from bone marrow (BM) CD34+ cells is increased in rheumatoid arthritis (RA) compared with osteoarthritis (OA) in correlation with the degree of synovial proliferation. It has been shown that FLT3 ligand promotes the differentiation of pDC through its interaction with FLT3 on pDC precursors. We explored the expression of FLT3 mRNA in BM $CD34^+$ cells in RA to delineate the mechanism for their abnormal differentiation into pre-pDC.**

***Methods*: CD34+ cells were purified from BM samples obtained from 46 RA patients and from 29 OA patients during joint operations via aspiration from the iliac crest. The expression of FLT3 mRNA was examined by quantitative RT-PCR.**

***Results*: The expression of FLT3 mRNA was significantly higher in RA BM CD34+ cells than in OA BM CD34+ cells (FLT3/β-actin: $[0.686 \pm 0.152] \times 10^{-3}$ and $[0.252 \pm 0.053] \times 10^{-3}$ [mean ± SEM], respectively; p=0.0269). FLT3 mRNA expression was not correlated with serum CRP or with administration of methotrexate or oral steroid. Finally, TNF-α did not enhance FLT3 mRNA expression, but rather decreased it, in BM CD34+ cells from normal individuals.**

***Conclusions*: These results indicate that FLT3 mRNA expression is upregulated in RA BM CD34+ cells independently of the systemic inflammation or treatment regimens. The data therefore suggest that abnormal FLT3 mRNA expression in BM CD34+ cells might lead to the expansion of immature pDC in RA BM, supporting the enhanced output of pDC into the inflamed synovium in RA.**

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Table 1 Demographic features of the patients

	RA	OA
n	46	29
male:female	7:39	3:26
Age (mean±SD)	58.1±11.0	71.4±7.3
methotrexate (MTX)	23	0
Steroid	34	0
Biologicals	1	0
CRP	2.39±2.40	0.38±0.64

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by hyperplasia of synovial lining cells, consisting of macrophage-like type A synoviocytes and fibroblast-like type B synoviocytes[1]. Type A synoviocytes, also called intimal macrophages, have been shown to be derived from monocyte precursors in the bone marrow (BM)[2, 3]. On the other hand, type B synoviocytes, also called fibroblast-like synoviocytes, have the morphologic appearance of fibroblasts as well as the capacity to produce and secrete a variety of factors, including proteoglycans, cytokines, arachidonic acid metabolites, and matrix metalloproteinases (MMPs), and play an important role in the destruction of joints[1].

Although the precise origin of type B synoviocytes has been unclear[4], accumulating studies have disclosed that type B synoviocytes are also derived from BM. Thus, it has been demonstrated that BM CD34+ cells from RA patients have abnormal capacities to respond to tumor necrosis factor-α (TNF-α) and to differentiate into fibroblast-like cells (FLC), producing MMP-1[5]. Moreover, we have revealed that RA BM plasmacytoid DC (pDC) as well as OA BM pDC comparably differentiated into FLC, expressing cadherin-11, a marker for type B synoviocytes, especially in the presence of TNF-α[6].

On the other hand, we have recently disclosed that the generation of pre-pDC from BM CD34+ cells is increased in RA in correlation with the degree of synovial proliferation[7]. It has been found that FLT3 ligand promotes the differentiation of pDC through its interaction with FLT3 on pDC precursors[8]. It is thus possible that the increased generation of pre-pDC from BM CD34+ cells in RA might involve interactions between FLT3 and FLT3 ligand. The current studies were therefore undertaken to explore the expression of FLT3 mRNA in BM CD34+ cells in RA in order to delineate the mechanism for their abnormal differentiation into pre-pDC.

Materials and Methods

1)Patients and samples

BM samples were obtained from 46 patients with RA (7 males and 39 females: mean age 58.1 years) who satisfied the American College of Rheumatology 1987 revised criteria for RA[9] and gave informed consent in accordance with the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. The samples were taken during joint operations via aspiration from the iliac crest under anesthesia. As a control, BM samples were similarly obtained from 29 patients with osteoarthritis (OA) (3 males and 26 females: mean age 71.4 years), who gave informed consent. The demographic features of the patients are summarized in Table 1.

2)Preparation of BM CD34+ cells

BM mononuclear cells (BMMNC) were isolated by centrifugation of heparinized BM aspirates over sodium diatrizoate-Ficoll gradients. CD34+ cells were purified from BMMNC by positive selection with magnetic beads (CD34 progenitor cell selection system; Dynal, Oslo, Norway), as previously described (purity >95%, as stated in manufacturer's description)[5]. In addition, 2 preparations of CD34+ cells derived from bone marrow aspirates from the iliac crests of healthy individuals (purity >95%) were purchased from BioWhittaker (Walkersville, MD).

3)Culture medium, cytokines and stimulation of BM CD34+ cells from healthy individuals

RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with L-glutamine (0.3 mg/ml) and 10% fetal bovine serum (Life Technologies) was used for all cultures. Recombinant human TNF-α was purchased from Pepro Tech EC, London, UK. Highly purified BM CD34+ cells from healthy individuals were cultured in the presence of TNF-α (10 ng/ml) for 24 h, in which the expression of NFkB1

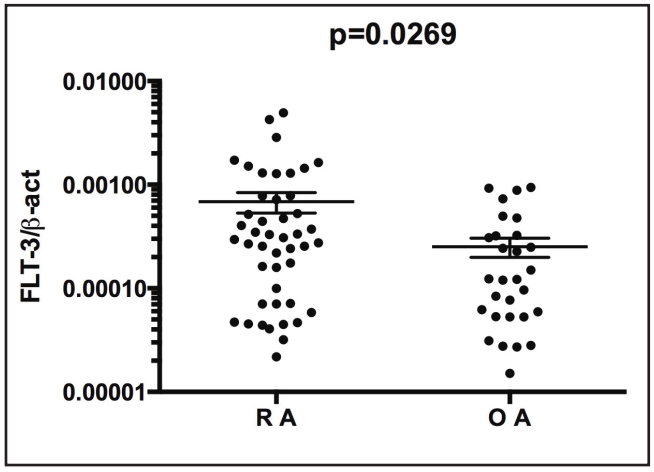

Fig. 1 Quantitative analysis of the expression of mRNA for FLT3 in BM CD34+ cells

Total RNA was isolated from purified BM CD34+ cells from 46 RA patients and 29 OA patients. The expression of mRNAs for FLT3 and β-actin was evaluated by real-time quantitative PCR. The data are expressed as the ratio of the mRNA copy numbers to those of β-actin. Horizontal lines and whiskers indicate the mean values and SEM, respectively. Statistical analysis was performed by Mann Whitney *u* test.

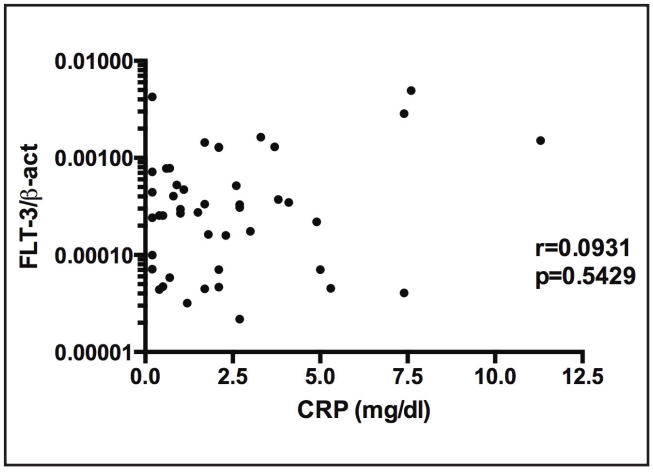

Fig. 2 Lack of correlation of FLT3 mRNA in BM CD34+ cells with serum CRP in RA patients

The expression of mRNAs for FLT3 and β-actin was evaluated by real-time quantitative PCR. The data are expressed as the ratio of the mRNA copy numbers to those of β-actin. Statistical significance was evaluated by Spearman's rank correlation test.

mRNA was markedly upregulated[10]. After the cultures, the expression of mRNAs for FLT3 was examined.

4) RNA isolation and real-time quantitative polymerase chain reaction (PCR)

Total RNA was isolated from purified bone marrow CD34+ cells or cultured normal CD34+ cells using the Trizol reagent (Life Technologies) according to the manufacturer's application. cDNA samples were prepared from 1 μg of total RNA using the SuperScript reverse transcriptase preamplification system (Life Technologies) with oligo (dT) primer and subjected to PCR. Real-time quantitative PCR was performed using LightCycler rapid thermal cycler system (Roche Diagnostics, Lewes, UK) with primer sets for FLT3 or β-actin (Search LC, Heidelberg, Germany), and Light Cycler-Fast Start DNA master SYBR Green I (Roche Diagnostics). Quantitative analysis was performed using LightCycler Software v.4.1. PCR reaction condition was denaturing at 95°C for 10 min for 1 cycle, followed by 35 cycles [FLT3] or 40 cycles [β-actin] of denaturing (10 sec at 95°C), annealing (10 sec at 68°C [FLT3] or 62°C [β-actin]), and extension (16 sec [FLT3] or 10 sec [β-actin] at 72°C).

5) Statistical analysis

The results were analyzed for statistical significance by Mann Whitney *u* test and Spearman's rank correlation test, where appropriate.

Results

1) Expression of mRNA for FLT3 in BM CD34+ cells

It has been shown that FLT3 ligand promotes the differentiation of pDC through its interaction with FLT3 on pDC precursors[8]. Initial experiments were therefore undertaken to examine the expression of FLT3 mRNA in BM CD34+ cells. The expression of mRNA for FLT3 in BM CD34+ cells is shown as the ratio of the copy numbers to those of β-actin mRNA. As shown in Fig. 1, the expression of FLT3 mRNA was significantly higher in RA BM CD34+ cells than in OA BM CD34+ cells (FLT3/β-actin: [0.686 ± 0.152] $\times 10^{-3}$ and [0.252 ± 0.053] $\times 10^{-3}$ [mean ± SEM], respectively, p=0.0269). It should be also noted that the expression of FLT3 mRNA in BM CD34+ cells was not significantly correlated with serum CRP levels in RA patients (Fig. 2). In addition, there were no significant differences in FLT3 mRNA expression between patients with methotrexate and those without methotrexate or between patients with steroids and those without steroids (Fig.3). The data indicate that the expression of FLT3 mRNA in BM CD34+ cells is upregulated in RA independent of the activity of systemic inflammation or treatment regimens.

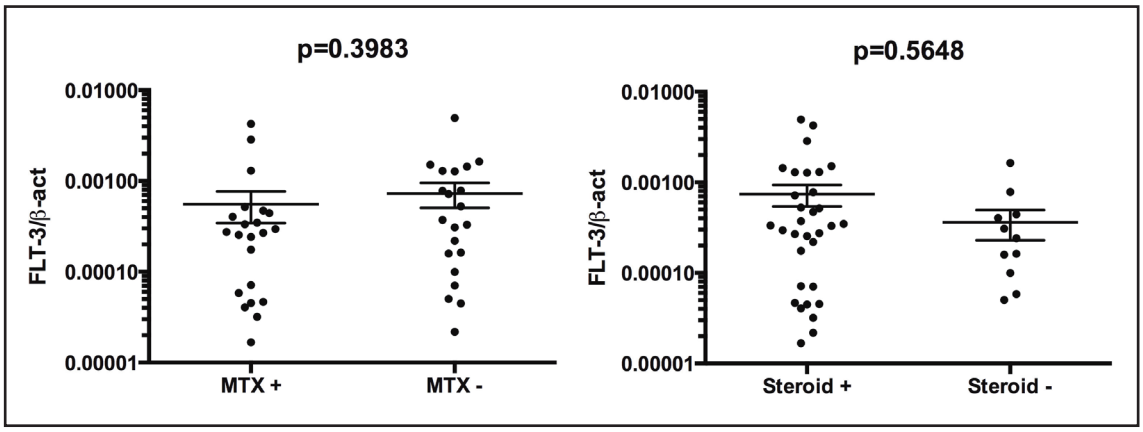

Fig. 3 The relevance of treatment with the expression of mRNA for FLT3 in BM CD34+ cells

Total RNA was isolated from purified BM CD34+ cells from 46 RA patients. The expression of mRNAs for FLT3 and β-actin was evaluated by real-time quantitative PCR. The data are expressed as the ratio of the mRNA copy numbers to those of β-actin. Effect of treatment with methotrexate (MTX) or oral steroids (PSL) was evaluated by Mann-Whitney's *U* test. Horizontal lines and whiskers indicate the mean values and SEM, respectively.

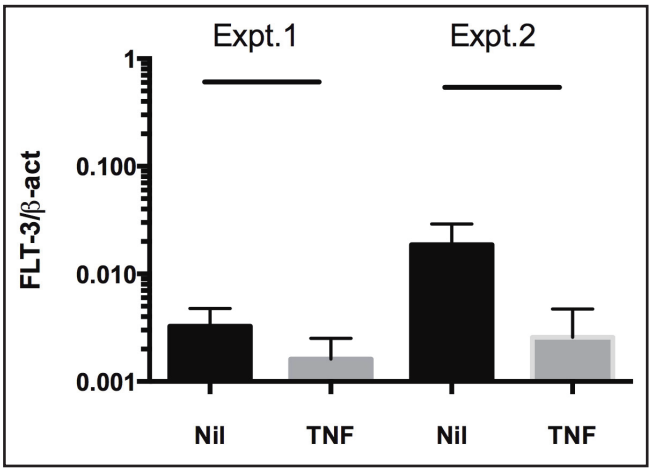

Fig. 4 Effect of TNF-α on the expression of mRNA for FLT3 in BM CD34+ cells

BM CD34+ cells from healthy individuals were incubated in culture medium with or without TNF-α (10 ng/ml) for 24 h. After the incubation, total RNA was isolated for evaluation of the expression of mRNAs for FLT3 and β-actin by real-time quantitative PCR. The data are expressed as the ratio of the mRNA copy numbers to those of β-actin. Error bars denote SD of triplicated determinations. Representative data of 2 different experiments.

The enhanced expression of FLT3 mRNA might lead to the increased expression of FLT3 on RA BM CD34+ cells, thus accounting for the accelerated differentiation of immature pDC in RA BM.

2) Lack of enhancement of FLT3 mRNA expression in normal BM CD34+ cells by TNF-α

Previous studies have demonstrated that TNF-α plays a critical role in the pathogenesis of RA[4]. It is therefore possible that the up-regulation of FLT3 mRNA in BM CD34+ cells might be secondary to the action of TNF-α. Next experiments were therefore carried out to test this possibility. As shown in Fig. 4, treatment of BM CD34+ cells with TNF-α did not upregulate the expression of FLT3 mRNA, but rather decreased it. These results indicate that the increased expression of FLT3 mRNA in RA BM CD34+ cells is not caused by the action of TNF-α, but might reflect a intrinsic abnormality.

Discussion

It has been demonstrated that BM pDC, irrespective of their origin from RA BM or OA BM, have prominent capacity to differentiate into type B synoviocyte-like cells, expressing cadherin-11 and producing MMP-1, especially under influences of TNF-α[6]. We have recently disclosed that RA BM CD34+ cells contain greater percentages of immature pDC expressing CD34 and BDCA2 than OA BM CD34+ cells[7]. The expansion of immature pDC in RA BM might account for the enhanced capacity of RA BM CD34+ cells to differentiate into type B synoviocyte-like cells[5].

The results in the current studies have demonstrated that the expression of FLT3 mRNA in RA BM CD34+ cells was higher than OA BM CD34+ cells. FLT3 ligand- FLT3 interactions play a critical role in the differentiation of pDC[8]. It is therefore suggested that the expansion of immature pDC within BM CD34+ cells might be due to the enhanced FLT3 mRNA expression in BM CD34+ cells.

Since there was no significant correlation between the expression of FLT3 mRNA in BM CD34+ cells and serum CRP, the enhanced expression of FLT3 mRNA in BM CD34+ cells might not be a result of systemic inflammation. Of note, a number of studies have shown that TNF-α plays a pivotal role in the pathogenesis of RA[11-13]. However, TNF-α did not increase the expression of FLT3 mRNA in BM CD34+ cells from normal healthy individuals. Taken together, these results suggest that the enhanced expression of FLT3 mRNA in BM CD34+ cells might not be secondary to inflammatory process of RA, but rather be a primary intrinsic abnormality in RA.

FLT3 (CD135) is a member of type III receptor tyrosine kinase, and plays an important role in the proliferation and differentiation of hematopoietic cells[14, 15]. Previous studies have demonstrated that the overexpression of FLT3 due to the internal tandem duplication mutation of FLT3 gene is found in 30-40% of patients with acute myeloid leukemia (AML), resulting in an increased relapse and reduced survival[16]. Moreover, it has been also shown that FLT3 expression levels were significantly correlated with a worse survival in AML patients with or without FLT3 gene mutation[17]. On the other hand, recent studies revealed that the overexpression of FLT3 increases the expression of IL-6 through activation of NFkB in BaF3 cells[18]. Abnormal responses to TNF-α have been observed in RA BM CD34+ cells[5, 10]. It is therefore likely that the upregulated expression of FLT3 mRNA might play a role in the abnormal responses of RA BM CD34+ cells to TNF-α. In this regard, it is likely that an inhibitor of FLT3 tyrosine kinase quizartinib, which has been shown to be beneficial in the treatment of AML[19], might also have effects in RA.

The limitation of this study is that we did not study the effects of TNF-α on BM CD34+ cells from RA patients. Since abnormal responses to TNF-α have been observed in RA BM CD34+ cells[5, 10], it is also possible that TNF-α might stimulate RA BM CD34+ cells to express abnormally high levels of FLT3 mRNA. Moreover, it is possible that other cytokines, such as IL-6 and IFN-α, might be involved in the activation of pDC[20]. It is therefore necessary to examine the effects of these cytokines in addition to TNF-α on RA BM CD34+ cells.

The mechanism for the abnormal expression of FLT3 mRNA in RA BM CD34+ cells remains unclear. In this regard, previous studies have disclosed the abnormal expression of mRNA for several genes in RA BM CD34+ cells, including NFkB1, KLF-5, and FKBP5[10, 21, 22]. Further studies to delineate the relationship of FLT3 with such genes would be helpful for an understanding not only of the mechanism of abnormalities in RA BM CD34+ cells, but also of the etiopathology of RA.

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Conflict of interests

As to the conflict of interest, all the authors declare none.

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