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High spontaneous granulocyte/macrophage-colony formation in patients with myelofibrosis

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ABSTRACT

Unstimulated methylcellulose cultures in 25 myelofibrosis (MF) patients were performed to better understand the role of cytokines in the proliferation of MF cells. Compared to controls MF patients show a variable but highly increased spontaneous CFU-GM formation (66 vs $4.8/10^5$ PBMNC). There was a marked reduction of autonomous CFU-GM growth by the cytokine-synthesis-inhibiting molecule IL-10 as well as by antibodies against GM-CSF whereas antibodies against IL-3, G-CSF, M-CSF and IL-1β showed heterogeneous effects. Spontaneous CFU-GM growth $>100/10^5$ PBMNC predicted shorter survival. Constitutive release of GM-CSF seems to contribute to proliferation of MF cells in vitro and possibly in vivo.

1. Introduction

The ability of cancer cells to produce and to respond to their own growth factors (autocrine secretion) has become a central concept in the pathophysiology of malignant growth for many years. Oncogenes may confer growth factor autonomy on cells not only by coding directly for autocrine peptide growth factors or their receptors, but also by amplifying the mitogenic signals generated by a growth factor at its receptor [1]. In hematological malignancies the formation of colonies in semisolid medium without the addition of exogenous growth factors is considered as a surrogate in vitro phenomenon for this pathophysiological mechanism [2,3].

Drugs that interfere with growth factor production, with growth factor binding or with the intracellular signal transduction induced by growth factor receptors are of potential interest to impact on the proliferation and expansion of the malignant clone. The JAK2/1 inhibitor ruxolitinib may be a particular interesting molecule because it suppresses the expression of a variety of cytokines [4] and broadly blocks signal transmission from many growth factor receptors due to association of JAK2 with the majority of growth factor receptors [5] including the EPO receptor [6], the TPO receptor [6], the G-CSF receptor [7] as well the common β-chain [8] of the GM-CSF receptor and IL-3 receptor. Thus, the clinical efficacy of ruxolitinib in myelofibrosis (MF) patients not only with but also without the JAK2 mutation [9,10] has been attributed to a general dampening of cytokine signaling [11] and may be therefore considered as proof of principle for the effectiveness of cytokine inhibition as a therapeutic concept.

The role of cytokines and/or colony-stimulating factors (CSFs) in the proliferation of MF cells is not fully understood. To better understand the contribution of cytokines in the clonal expansion of MF cells we investigated the autonomous in vitro growth of primary cells from 25 patients with MF using methylcellulose cultures.

2. Patients and methods

Between February 1991 and April 2000, in vitro cultures assessing spontaneous colony-forming unit-granulocyte–macrophage (CFU-GM) growth were performed in 25 patients who met the diagnostic criteria of MF according to the Polycythemia Vera Study Group [12], cases of post-polycythemia vera (post-PV) or post-essential thrombocythemia (post-ET) myelofibrosis were not included. Peripheral blood was collected from routine clinical controls after obtaining informed consent. The median age of the patients was 66 years (range, 51–86). The median values (ranges) of blood picture parameters were 10.2×10^9/L (1.8–44.8) for white blood cell counts, 10.1 g/dL (7.6–14.3) for hemoglobin, and 224×10^9/L (15–1450) for platelet counts, respectively. All patients were being managed by best supportive care, no patient was treated by cytostatic drugs or interferon, respectively, at the time of study. For

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comparing autonomous colony formation in MF patients with that in normal individuals, in vitro data, which have been published previously by us [13], from seven healthy volunteers were used.

2.1. Preparation of cells

Peripheral blood (PB) was collected into sterile tubes containing EDTA. Mononuclear cells (MNCs) were isolated from PB of patients by Ficoll-Hypaque density gradient centrifugation (density 1.077 g/mL, 400 × *g* for 40 min). The low-density cells were collected from the interface between density solution and plasma, washed twice, and resuspended in Iscove's modified Dulbecco's medium (GIBCO, Paisley, Scotland).

2.2. Reagents

Antibodies directed against GM-CSF, IL-3, G-CSF, M-CSF and IL-1β were purchased from Genzyme (Cambridge, MA). Recombinant human IL-10 (rhIL-10; specific activity 1–2 × 10^6 U/mg) was kindly provided by Schering-Plough Corp. (Kenilworth, NJ) and rhGM-CSF and rhIL-3 by Sandoz (Basel, Switzerland). RhG-CSF was purchased from British Biotechnology (Oxan, UK) and M-CSF and IL-1β from Genzyme (Cambridge, MA).

2.3. Colony assay

PBMNCs were cultured in 0.9% methylcellulose, 30% fetal calf serum (FCS; INLIFE, Wiener Neudorf, Austria), 10% bovine serum albumin (Behring, Marburg, Germany), α-thioglycerol (10^{-4} mol/L) and Iscove's modified Dulbecco's medium with or without the addition of cytokines or anticytokine antibodies. Cultures were plated in triplicates at 7.5–120 × 10^3 MNC/mL. In some cases the numbers of MNC chosen in our experiments were based on the colony growth in prior cell cultures in the respective patient in order to optimize evaluation of CFU-GM formation. Neutralizing antibodies against GM-CSF, G-CSF, IL-3, M-CSF or IL-1β were used as recommended by the manufacturer. Plates were incubated at 37 °C, 5% CO_2, and full humidity. After a culture period of 14 days, cultures were examined under an inverted microscope. Aggregates with at least 40 cells were counted as CFU-GM.

2.4. Statistical analysis

CFU-GM data were expressed as mean values from duplicate or triplicate cultures. The Wilcoxon two sample test was used to compare differences with regard to spontaneous CFU-GM growth in normal individuals and patients with MF. The Student's *t*-test and the paired Student's *t*-test, respectively, were used to compare colony growth between cultures containing IL-10 or anti-cytokine-antibodies and control cultures, respectively. A Kaplan–Meier plot was used to visualize the differences in cumulative probability of death between patients with low and high colony growth using a cut-off of 100. Survival time was defined as the period between the time of in vitro culture and the time of death. Surviving patients were censored at the time of the last follow up. Univariate and multivariable Cox regression analyses were used to calculate the risk of death. The variable of main interest was GFU-GM (either as continuous or as binary variable), further tested variables were age (continuous and binary variable, cut-off 65 years), hemoglobin (continuous and binary variable, cut-off 10 g/dL), white blood cell count (continuous and binary variable, cut-off 25,000) and blast count (continuous and binary variable, cut-off 1%). The multivariable Cox regression analysis was further adjusted for variables that were shown to be statistically significant in univariate analyses. A p value of <0.05 was considered as statistically significant. Statistical analysis was performed with IBM SPSS Statistics 21.

3. Results

3.1. Spontaneous CFU-GM formation in MF patients

In vitro cultures assessing autonomous CFU-GM formation were performed in 25 patients with MF. The number of colonies growing without the addition of exogenous growth factors ranged from 0 to 1050/10^5 PBMNC (median, 66) among these patients. In contrast, autonomous colony growth in normal volunteers (n = 7) ranged from 3.5 to 8.5 with a median number of 4.8/10^5 PBMNC as reported previously by us [13]. As shown in Fig. 1, colony growth in these MF patients was not normally distributed. Using a cut-off level of 100 CFU-GM/10^5 PBMNC, 10 patients had colony numbers exceeding 100/10^5 MNC. These patients were considered as individuals with high colony growth, whereas colony numbers below this cut-off level were called low growth. Interestingly, blast cell counts ≥10% in PB were found in both patients with the highest CFU-GM numbers as shown in Fig. 1 but only in 1 of the 23 other patients.

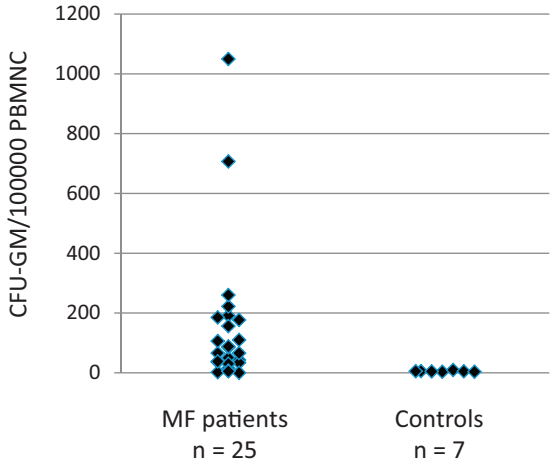

Fig. 1. CFU-GM numbers per 10^5 PBMNC in 25 patients with myelofibrosis (MF) and 7 control subjects. PBMNC from MF patients were cultured as described in Section 2. Results represent mean values from triplicate or duplicate cultures without exogenous growth factors.

3.1.1. Inhibitory effect of IL-10 on spontaneous CFU-GM growth in patients with MF

We and others have shown that IL-10 is a cytokine-synthesis-inhibiting cytokine suppressing the release of a large variety of cytokines and growth factors [14,15]. The effect of 10 ng/mL IL-10 on autonomous CFU-GM growth was investigated in PBMNCs from 2 patients with MF. As shown in Fig. 2, autonomous formation of myeloid colonies was significantly inhibited by IL-10 in both patients. Furthermore addition of colony stimulating factors including GM-CSF, IL-3 and G-CSF restimulated colony growth in IL-10 suppressed cultures arguing against a direct cytotoxic effect of this cytokine.

3.1.2. Effect of anticytokine antibodies on spontaneous CFU-GM growth from MF cells

In some of the patients we tried to identify the factors responsible for autonomous CFU-GM formation by adding neutralizing antibodies against GM-CSF, IL-3, G-CSF, M-CSF and IL-1β. As shown in Table 1 antibodies against GM-CSF significantly inhibited spontaneous CFU-GM formation in 4 patients tested whereas antibodies against the other growth factors showed heterogeneous effects.

3.1.3. Spontaneous CFU-GM growth and survival

Kaplan–Meier analysis revealed that MF patients with high CFU-GM growth had a significantly shorter survival than patients with low CFU-GM growth at presentation (p < 0.05). The probability of survival after 5 years was 45% for patients with low colony growth and 20% in those with high colony formation (Fig. 3). From the remaining variables of interest (age, hemoglobin, white blood cell count, blast count) only low hemoglobin levels were of a predictive value for a shorter survival when calculated as binary variable (cut-off 10 g/dL; HR 3.94, 95% CI 1.35–11.54). When combining GFU-GM and hemoglobin in multivariate analysis, statistical significance was lost for both parameters (HR 1.80, 95% CI 0.64–5.03, p = n.s. and HR 3.08, 95% CI 0.97–9.78, p = n.s.).

4. Discussion

In patients with MF the number of peripheral blood hematopoietic progenitor cells which can be grown in semisolid cultures containing conditioned medium or exogenous growth factors has been reported to be always consistently higher than in control subjects [16–24] but autonomous colony formation in these patients