Interleukin 10 Inhibits Growth and Granulocyte/Macrophage Colony-stimulating Factor Production in Chronic Myelomonocytic Leukemia Cells

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Summary

Autonomous release of hematopoietic growth factors may play a crucial role in the pathogenesis of certain hematological malignancies. Because of its cytokine synthesis-inhibiting action, interleukin 10 (IL-10) could be a potentially useful molecule to affect leukemic cell growth in such disorders. Chronic myelomonocytic leukemia (CMML) cells spontaneously form myeloid colonies (colony-forming units–granulocyte/macrophage) in methylcellulose, suggesting an autocrine growth factor–mediated mechanism. We studied the effect of recombinant human IL-10 (rhIL-10) on the in vitro growth of mononuclear cells obtained from peripheral blood or bone marrow of patients with CMML. IL-10 specifically binding to leukemic cells had a profound and dose-dependent inhibitory effect on autonomous in vitro growth of CMML cells. IL-10 significantly inhibited the spontaneous growth of myeloid colonies in methylcellulose in 10/11 patients, and autonomous CMML cell growth in suspension in 5/5 patients tested. Spontaneous colony growth from CMML cells was also markedly reduced by addition of anti-granulocyte/macrophage colony-stimulating factor (GM-CSF) antibodies, but not by addition of antibodies against G-CSF, IL-3, or IL-6. IL-10-induced suppression of CMML cell growth was reversed by the addition of exogenous GM-CSF and correlated with a substantial decrease in GM-CSF production by leukemic cells, both at the mRNA and protein levels. Our data indicate that IL-10 profoundly inhibits the autonomous growth of CMML cells in vitro most likely through suppression of endogenous GM-CSF release. This observation suggests therapeutic evaluation of rhIL-10 in patients with CMML.

Chronic myelomonocytic leukemia (CMML) is a hematopoietic malignancy of the elderly that is characterized by leukocytosis with monocytes and granulocytic cells in all stages of development, marked dysmyelopoiesis, a chronic course, and unresponsiveness to aggressive chemotherapy (1–3). CMML differs from chronic myeloid leukemia in several ways, including cytologic criteria, lack of Philadelphia chromosome, and as we have originally shown, spontaneous colony formation by CMML cells in semisolid medium (4). The growth of hematopoietic colonies without addition of exogenous growth factors raises the possibility that CMML cells secrete their own growth factors that might subsequently lead to the proliferation of leukemic cells through an autocrine growth factor–mediated mechanism in vitro and possibly also in vivo (5). Later work by others confirmed our original observation and suggested GM-CSF and IL-6 as candidate growth factors for CMML cells (6).

IL-10 is a 35-kD protein, originally identified by virtue of its ability to inhibit cytokine synthesis in T helper 1 clones (7, 8). It is primarily produced by mononuclear cells (MNC; 9) and possesses a wide range of activities on a number of cell types, including B cells (10), T cells (11), NK cells (12), mast cells (13), neutrophils (14), eosinophils (15), and monocytes (16). The main feature of this cytokine is a suppressive effect on cytokine expression. Thus, IL-10 profoundly suppresses the induced production of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and GM-CSF by human monocytes (16) and mouse peritoneal macrophages (17).

Because of its cytokine synthesis–inhibiting action, IL-10 could be a potentially useful molecule in hematological malignancies in which autostimulatory mechanisms may
Table 1. Clinical and Laboratory Data in Patients with CMML

| Patient | Age/sex | Duration (mo) | WBC ($\times 10^9$/liter) | Hb (g/dl) | Plt ($\times 10^9$/liter) | Mono (%) | LDH (U/liter) | Lysozyme (mg/ml) | Karyotype | CT |
|---------|---------|---------------|--------------------------|----------|-------------------------|---------|-------------|-----------------|-----------|----|
| JL      | 55/F    | 15            | 160                      | 9.3      | 125                     | 17      | 658         | 49              | 45XX,-7   | +  |
| PM      | 70/F    | 22            | 181                      | 8.4      | 118                     | 26      | 503         | 126             | 46XX,+2/11 | +  |
| RL      | 66/F    | 60            | 20                       | 14.2     | 387                     | 30      | 287         | 27              | 46XX      | -  |
| MR      | 68/M    | 1             | 55.8                     | 9.9      | 130                     | 23      | 1280        | 62              | 45XO      | -  |
| ML      | 59/F    | 8             | 8.5                      | 9.9      | 72                      | 23      | 138         | 49              | 46XX      | +  |
| HL      | 68/M    | 105           | 19.9                     | 9.7      | 87                      | 15      | 440         | 34              | NA        | +  |
| DH      | 88/F    | 5             | 7                        | 10.0     | 101                     | 27      | 520         | 114             | NA        | -  |
| MA      | 79/F    | 36            | 14.3                     | 8.5      | 130                     | 30      | 165         | 146             | 46XX      | -  |
| WM      | 81/F    | 6             | 9.8                      | 8.6      | 8                       | 29      | 94          | 51              | 46XX      | -  |
| FJ      | 83/M    | 1             | 39.8                     | 9.9      | 13                      | 55      | 373         | 106             | NA        | -  |
| KR      | 80/M    | 180           | 22.4                     | 14.7     | 345                     | 12      | 246         | 32              | NA        | -  |

CT, chemotherapy; Hb, hemoglobin; Mono, monocytes; NA, not available; Plt, platelets; WBC, white blood cells.

play a crucial role in pathogenesis. Here, we investigated the effect of IL-10 on the autonomous in vitro growth of MNC from patients with CMML. We found that IL-10 profoundly inhibits the spontaneous proliferation of CMML cells in methylcellulose and in suspension, most likely through suppression of spontaneous release of GM-CSF.

Materials and Methods

Patients. All 11 patients met the diagnostic criteria for CMML according to the FAB group, i.e., dyspoietic features of myelodysplastic syndromes associated with a blood monocytosis >109/μl, an increase in bone marrow (BM) monocyte precursors, and a blast cell percentage <5% in the peripheral blood (PB) and <30% in BM. Clinical and laboratory data of these patients are shown in Table 1. In four patients, cytoreductive chemotherapy was given before or at the time of the study.

Preparation of Cells. After informed consent, PB was collected into sterile tubes containing 4 ml EDTA. BM samples were obtained by aspiration into sterile tubes containing heparin with no preservative. PB MNC and BM MNC were harvested after a Ficoll-Hypaque gradient centrifugation (400 g, 40 min, 1.007 g/ml).

Reagents. Recombinant human IL-10 (rhlL-10; specific activity = 1-2 × 10⁶ U/mg) was kindly provided by Schering-Plough Corp. (Kenilworth, NJ). A neutralizing anti-IL-10 antibody was obtained from R.&D Systems Europe Ltd. (UK), antibodies directed against G-CSF, GM-CSF, IL-3, IL-6, and rhlL-6 were purchased from Genzyme Corp. (Cambridge, MA), rhGM-CSF and rhIL-3 were kindly provided by Sandoz (Basel, Switzerland), and rhG-CSF was purchased from British Biotechnology (Oxon, UK). Cytokines were added at the onset of the culture. The neutralizing antibody against IL-10 was preincubated with IL-10 for 2 h at room temperature. Neutralizing antibodies against G-CSF, GM-CSF, IL-3, and IL-6 were used as recommended by the manufacturer.

CFU-GM Assay. PB MNC or BM MNC were cultured in 0.9% methylcellulose, 30% FCS (INLIFE, Wiener Neudorf, Austria), and IMDM (Gibco, Paisley, Scotland) with or without the addition of cytokines or anticytokine antibodies. Cultures were plated in triplicate at 10–100 × 10³ MNC/ml. After a culture period of 14 d (37°C, 5% CO₂, full humidity) cultures were examined under an inverted microscope. Aggregates with at least 40 cells were counted as CFU-GM.

Suspension Cultures. PB MNC from CMML patients were cultured at 5 × 10⁵ per milliliter for 7 d in IMDM supplemented with 30% FCS with or without IL-10. Cell numbers were determined using a hemocytometer. For morphological examination, cytopsins were stained by May-Grünwald-Giemsa.

Radiolabeling of rIL-10. IL-10 was labeled with 125I using standard techniques (18). Briefly, 250 μg IL-10 (nmol), 2 mCi 125I-NaI, and 5 μg lactoperoxidase were added into a vial containing a magnetic stirrer. The reaction mixture was slowly stirred at pH 7 (0.01 M balance phosphate buffer) for 10 min and thereafter subjected to a preparative gradient HPLC (RP C18 column, MeCN gradient). The column eluent was passed through a scintillation radioactivity detector and a UV (280 nm) detector in series. The system was calibrated with unlabeled IL-10 and allowed collection of pure radioiodinated IL-10, separated from unlabeled IL-10, as well as from reagents and inorganic iodine species. 125I-IL-10 was isolated in a 40% yield at a specific activity of 2,000 Ci/mmol. The 125I-IL-10 product fraction was stabilized by addition of 2% human serum albumin and analyzed by analytic HPLC (same system as the preparative one), zone electrophoresis on 3MM paper (Whatman Chemical Co., Clifton, NJ), 0.1 M barbital buffer, pH 8.6, field 300 V for 10 min), and TCA precipitation (10% final TCA). Radiochemical purity was >97% and remained stable during >24 h.

Binding Assay. In saturation studies, intact cells (5 × 10⁵ cells per tube) were incubated in the assay buffer containing 50 nmol/liter Tris-HCl (pH 7.4) and 5 nmol/liter MgCl₂ with increasing concentrations of 125I-IL-10 (0.01-25 nmol/liter) in the absence (total binding) and the presence of unlabeled IL-10 (1 μmol/liter, nonspecific binding) for 45 min at 4°C. Displacement studies

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increasing concentrations of unlabeled IL-10 (0.01-1 nM/liter, mean values from triplicates). Assays were performed with 5 nM/liter 125I-IL-10 (total binding) and increasing concentrations of unlabeled IL-10 (0.01-1 nM/liter, nonspecific binding). After incubation, the reaction mixture was diluted 1:10 with assay buffer (4°C) and centrifuged (5,000 g, 10 min, 4°C) to separate the membrane-bound from free ligand. The resulting pellet was washed twice in buffer and counted in a gamma counter for 1 min. Binding data were calculated according to Scatchard (19).

Figure 1. Dose-dependent inhibitory effect of IL-10 on spontaneous CFU-GM growth of CMML cells in patient JL. PB MNC were cultured in methylcellulose with medium alone or with increasing concentrations of IL-10. Colony growth was assessed after 14 d. Results represent the mean values from triplicates.

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Results and Discussion

Inhibitory Effect of IL-10 on Spontaneous Growth of CMML Cells in Methylcellulose and Suspension. As has been found previously by us (4) and by others (6), MNC obtained from PB or BM of CMML patients form a high number of mye-
Table 2. Inhibitory Effect of IL-10 on Spontaneous CFU-GM Growth in Methylcellulose in CMML Patients

| Patient | Source | MNC/dish \( \times 10^3 \) | GFU-GM ± SD/dish | Percent of inhibition | \( P \) |
|---------|--------|-----------------------------|----------------|----------------------|--------|
| JL      | PB     | 10                          | 130.0 ± 12.5  | 22.3 ± 11.5          | 83     | <0.001 |
| PM      | PB     | 15                          | 124.3 ± 7.5   | 28.0 ± 14.9          | 77     | <0.001 |
| RL      | PB     | 100                         | 23.7 ± 2.9    | 9.3 ± 4.2            | 61     | <0.01  |
| MR      | BM     | 50                          | 77.7 ± 1.5    | 1.0 ± 1.0            | 99     | <0.001 |
| ML      | PB     | 34                          | 75.0 ± 1.7    | 7.0 ± 2.0            | 91     | <0.001 |
| HL      | PB     | 50                          | 19.3 ± 4.2    | 8.3 ± 2.3            | 57     | <0.05  |
| DH      | PB     | 50                          | 54.0 ± 12.0   | 6.0 ± 2.0            | 89     | <0.005 |
| MA      | PB     | 50                          | 9.7 ± 3.5     | 6.7 ± 1.5            | 31     | >0.05  |
| WM*     | PB     | 20                          | 154.7 ± 23.5  | 6.0 ± 1.0            | 96     | <0.001 |
| FJ      | PB     | 25                          | 45.3 ± 6.1    | 13.0 ± 6.9           | 71     | <0.005 |
| KR      | PB     | 16                          | 130.0 ± 1.4   | 3.0 ± 2.7            | 98     | <0.001 |

PB MNC or BM MNC from CMML patients were cultured as described in Materials and Methods with or without 10 ng/ml IL-10 (* in patient WM, 5 ng/ml IL-10 was used). Colony growth was assessed after 14 d. Results represent mean values ± SD from triplicates.

Preparations contained only small amounts of normal cells as compared to malignant cells, it was unlikely that the inhibitory effects of IL-10 on cell growth were mediated by normal lymphocytes and/or monocytes. To further prove the direct action of IL-10 on the leukemic clone, it was important to examine whether CMML cells expressed receptors for IL-10. Cell preparations from three patients (FJ, JL, and PM) containing ≥90% malignant cells, as determined by morphological examination, were used for binding studies with \(^{125}\)I-IL-10. A typical binding isotherm is shown in Fig. 5. In all three patients, significant binding of \(^{125}\)I-IL-10...
to intact cells was found. The linear Scatchard transformation suggested a single class of specific binding sites. CMML cells of these patients expressed \(5.6, 5.0,\) and \(5.2 \times 10^4\) IL-10-binding sites per cell with \(K_a\) of \(1.8, 1.2,\) and \(1.2\) mmol/liter, respectively. The corresponding inhibitory constants (IC\(_{50}\)) were 2.1, 1.8, and 2.4 mmol/l.

**Figure 4.** May-Grünwald-Giemsa staining of CMML cells cultured for 7 d with medium alone (A) or with 10 ng/ml IL-10 (B) in patient PM.

**Inhibitory Effect of Anti-GM-CSF Antibody on Autonomous CFU-GM Growth from CMML Cells.** To elucidate the possible mechanism of the inhibitory action of IL-10, we first tried to identify the factor responsible for autonomous colony growth by adding neutralizing antibodies against GM-CSF, G-CSF, IL-3, and IL-6 to cell cultures from three pa-

**Figure 5.** Binding of \(^{125}\text{I}-\text{IL-10}\) to CMML cells in patient FJ. The left panel shows the saturation curve and the right panel shows the Scatchard plot. Specific binding was determined as total binding − nonspecific binding. In this patient, the binding capacity amounted to 93 fmol/10\(^6\) cells (i.e., 56,000 sites per cell) with a corresponding \(K_a\) of 1.8 mmol/liter.

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patients with excessive colony growth. A previous study has suggested GM-CSF and IL-6 as candidate growth factors in CMML, since both cytokines were detectable in conditioned media from CMML cells, and antibodies against GM-CSF and IL-6 could partially inhibit spontaneous colony formation by CMML cells (6). In our patients, only antibody against GM-CSF, however, reproducibly inhibited autonomous CFU-GM growth, suggesting GM-CSF as a major autocrine growth factor (Fig. 6).

Effect of Exogenous Growth Factors on IL-10 Induced Suppression of CFU-GM Growth from CMML Cells. The anti-proliferative action of anti-GM-CSF antibody in CMML cells and the fact that IL-10 has been shown to inhibit cytokine synthesis, including GM-CSF, in human monocytes...
(16), led us to hypothesize that inhibition of CMML growth by IL-10 was secondary to IL-10–induced suppression of endogenous GM-CSF release. If this was the case, one would expect that exogenous addition of GM-CSF could largely reverse growth inhibition by IL-10. In contrast, restoration of colony growth by exogenous growth factors would not be observed if IL-10 had a direct cytotoxic effect on CMML cells. In fact, exogenous GM-CSF was able to completely overcome IL-10–induced suppression in two patients tested (Fig. 7). Not surprisingly, G-CSF, IL-3, or IL-6 were ineffective or only moderately effective in correcting IL-10–induced growth inhibition.

**Inhibitory Effect of IL-10 on GM-CSF Production by CMML Cells.** To confirm the inhibitory effect of IL-10 on GM-CSF production in CMML cells, supernatants from CMML cell suspension cultures obtained at different time points were analyzed for GM-CSF by an immunoenzymometric assay (EASIA) in two patients. As shown in Fig. 9, CMML cells cultured in medium alone secreted GM-CSF spontaneously, with levels peaking at day 2 and gradually declining thereafter. In contrast, GM-CSF secretion by CMML cells was almost completely abrogated in the presence of IL-10 (10 ng/ml) at all time points studied.

In summary, we demonstrate here a hitherto unknown profound inhibitory effect of IL-10 on the in vitro growth of CMML cells. IL-10 not only inhibited growth of CFU-GM in semisolid medium, but also clearly inhibited the growth of CMML cells in suspension. IL-10 was remarkably effective in the majority of patients tested. The demonstration of specific binding of IL-10 to CMML cells suggests a direct effect of IL-10 on the leukemic cell population. Although the exact mechanism of the inhibitory action of IL-10 on CMML cells remains to be shown, our data provide strong evidence that a modulation of endogenous GM-CSF release in CMML cells by IL-10 plays a major role. Therefore, growth inhibition by IL-10 may represent a novel strategy to interrupt autostimulatory loops in hematological malignancy. Administration of IL-10 as a therapeutic option should be harmless to normal hematopoietic cells, since we have not observed any inhibitory effect of IL-10 on normal CD34+ cells (data not shown).

Regardless of the mechanism involved the profound inhibitory effect of IL-10 on CMML cell growth in vitro has clear clinical implication. Currently, patients with CMML appear to have a poor prognosis with conventional cytotoxic drugs (3). Recently, IL-10 has been administered in healthy volunteers and serum levels of the cytokine that are effective in vitro were achieved at tolerable administered doses (25). Thus, biologic chemotherapy of CMML with IL-10 is feasible and our findings provide experimental support for clinical trials of IL-10 in patients with CMML.

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