Inhibition of Proliferation and Induction of Apoptosis in Juvenile Myelomonocytic Leukemic Cells by the Granulocyte-Macrophage Colony-Stimulating Factor Analogue E21R

By Per O. Iversen, Robyn L. Rodwell, Lydia Pitcher, Kerry M. Taylor, and Angel F. Lopez

Juvenile myelomonocytic leukemia (JMML) is a malignancy that almost inevitably leads to death before adulthood. Chemotherapy has given disappointing results and a substantial number of patients relapse after bone marrow transplantation. A salient feature of this disease is that the JMML cells produce granulocyte-macrophage colony-stimulating factor (GM-CSF) spontaneously and survive and proliferate without exogeneous GM-CSF. Furthermore, JMML cells are hypersensitive to GM-CSF with addition of this cytokine leading to enhanced proliferation. We have recently generated a human GM-CSF analogue, E21R, that acts as a complete and selective GM-CSF receptor antagonist. We have now tested this molecule as a potential new agent to control the leukemic cell load in JMML with particular emphasis on its role in JMML cell survival. We found that E21R inhibited the spontaneous growth of JMML cells in vitro and caused their apoptosis in a dose- and time-dependent manner in seven of seven cases. In contrast, neither a neutralizing anti-GM-CSF monoclonal antibody (MoAb) nor a selective interleukin-1 (IL-1) receptor antagonist affected JMML cell survival. Furthermore, the apoptotic effect of E21R was seen even in the presence of interleukin-1β and tumor necrosis factor-α, which have also been implicated in the pathogenesis of JMML. The inhibitory effects of E21R on JMML cell growth and viability offer a novel approach to therapy in this lethal childhood leukemia.

© 1996 by The American Society of Hematology.

The effect of inhibiting GM-CSF action on the survival of JMML cells is unknown. Any GM-CSF-mediated effect on JMML cells would be exerted by the GM-CSF receptor, which is composed of a GM-CSF specific α chain, the major binding subunit, and a β chain that functions as the main signaling subunit. We recently constructed a GM-CSF analogue, E21R, that binds only to the α chain of the GM-CSF receptor. E21R is devoid of classical GM-CSF activity and behaves as a complete antagonist of GM-CSF in binding and biological assays. Moreover, E21R directly induces apoptosis (programmed cell death) in certain hematopoietic cells. These properties suggested a role for E21R in regulating JMML cell survival. We show here that E21R, like anti-GM-CSF antibodies, inhibits the spontaneous growth of JMML, but unlike an anti-GM-CSF blocking antibody, E21R induces generalized apoptosis of JMML cells. Given the exquisite sensitivity of JMML to GM-CSF, these results offer a new therapeutic approach to control JMML cell load.

MATERIALS AND METHODS

Patients

We studied seven children diagnosed with JMML according to criteria proposed by the International Juvenile Myelomonocytic Leukemia Working Group (Henrik Hasle, personal communication, November 1995). Table 1 summarizes the main laboratory data. No other diagnoses were given to these patients. Parental consent was given to collect cells, and the protocol was approved by the appropriate ethics committees.

Processing of Cells

Peripheral blood was collected from JMML patients who had been off any treatment for at least 3 weeks. JMML cells taken from bone marrow gave similar results as JMML cells taken from peripheral blood (data not shown). Polymorphonuclear cells were removed from the samples using density centrifugation after dextran sedimentation of erythrocytes. T lymphocytes were removed with an anti-CD3 MoAb coupled to a magnetic bead (Miltenyi Biotec, Gladbach, Germany). Using a MoAb raised against the GM-CSF receptor α chain, we isolated α chain-positive cells with a similar magnetic sorting procedure. These cells also expressed the GM-CSF receptor β chain as determined with a MoAb raised against the β chain.
Table 1. Laboratory Data From the JMML Patients at Time of Blood Sampling

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Variable</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age (yr)</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>F</td>
<td>F</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>Hb (g/dL)</td>
<td>9.7</td>
<td>8.1</td>
<td>9.2</td>
<td>7.8</td>
<td>10.1</td>
<td>11.2</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>Fetal Hb</td>
<td>5</td>
<td>26</td>
<td>69</td>
<td>65</td>
<td>32</td>
<td>29</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>WBC (x10^9/L)</td>
<td>21</td>
<td>14</td>
<td>19</td>
<td>25</td>
<td>41</td>
<td>21</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Mono (%)</td>
<td>2</td>
<td>17</td>
<td>10</td>
<td>13</td>
<td>15</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Pit (x10^9/L)</td>
<td>42</td>
<td>55</td>
<td>29</td>
<td>71</td>
<td>89</td>
<td>93</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>Bone marrow</td>
<td>MH</td>
<td>MH</td>
<td>MH</td>
<td>MH</td>
<td>MH</td>
<td>MH</td>
<td>MH</td>
</tr>
<tr>
<td></td>
<td>Karyotype</td>
<td>46, XY</td>
<td>46, XX</td>
<td>46, XY</td>
<td>46, XX</td>
<td>46, XX</td>
<td>46, XY</td>
<td>46, XY</td>
</tr>
<tr>
<td></td>
<td>Hepato/ splenomegaly</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Abbreviations: M, male; F, female; Hb, hemoglobin; WBC, white blood cell count; PB, peripheral blood cell count; Mono, monocyte count; Pit, platelet count; MH, myeloid hyperplasia.

Cytokines and Monoclonal Antibody

We used recombinant GM-CSF (Genetics Institute, Cambridge, MA), recombinant interleukin-1β (R&D Systems, Minneapolis, MN); sensitivities > 0.5 pg/mL), L-glutamine (1.7 mmol/L), penicillin (10.5 µg/mL), and gentamicin (14 µg/mL) in a humidified atmosphere with 5% CO₂ in the air.

Determination of Cytokine Production

The concentrations of cytokines were assayed in supernatants collected from confluent JMML cells, and using the following enzyme-linked immunosorbent assay (ELISA) kits according to the instructions provided by the manufacturer: we performed triplicate measurements of GM-CSF (R&D Systems, Minneapolis, MN; sensitivities > 0.5 pg/mL), TNF-α (R&D Systems; sensitivities > 0.5 pg/mL); and IL-1β (R&D Systems; sensitivities > 0.5 pg/mL). The individual ELISA kits are highly specific to the corresponding cytokines and no cross-reactivity is seen.

Biological Assays

Colony formation. Colonies (>40 cells) of JMML cells (10⁵ per culture) were grown in methylcellulose as described, and triplicates were scored after 14 days.

Determination of apoptosis. The numbers of dead (apoptotic) cells were quantitated as reduced binding of propidium iodide to DNA using flow cytometry as previously outlined. Briefly, following incubation in the supplemented RPMI medium, cells were collected and kept in the dark at 4°C overnight in a buffer containing propidium iodide (50 µg/mL; Sigma, St Louis, MO) and Triton X-100 (0.1%; Sigma). Triplicate measurements were performed the day after using an EPICS-Profile II flow cytometer (Coulter Electronics, Hialeah, FL).

The fragmentation of chromosomal DNA was displayed with ul-
traviolet light on an ethidium bromide stained 1.2% agarose gel after
isolation of the DNA using an overnight incubation with lysis buffer
followed by extraction with organic solutions, as described else-
where.20

Statistics
We used the medians of triplicate measurements from a single
case to calculate the mean for the seven JMML cases. Values are
thus given as means and standard error of mean (SEM). Differences
were evaluated with the Kruskal-Wallis test with Bonferroni’s test,
as appropriate. Two-tailed tests were used. A significant difference
was assumed for \( P < .05 \).

RESULTS

Inhibition of GM-CSF Decreases JMML Colony
Formation

We tested cells from seven patients with diagnosed
JMML, and Table 1 provides a summary of the main labora-
tory findings in these patients. Culturing of JMML cells
in the absence of any exogeneous growth factor led to a
spontaneous formation of colonies, a feature typical of these
cells (Fig 1A). We first studied the effect of exogeneously
adding GM-CSF, TNF\( \alpha \), or IL-1\( \beta \) on JMML cell growth.
Figure 1A shows that GM-CSF increased JMML colony
numbers in a dose-dependent manner, reaching a maximum
value of about four times the spontaneous growth rate. TNF\( \alpha \)
also increased JMML colony numbers, but less markedly
than GM-CSF. In contrast, IL-1\( \beta \) had no effect (\( P > .05 \)).

We then examined the effect of inhibiting either GM-CSF
or IL-1 activity on the colony formation of JMML cells.
Both the GM-CSF analogue E21R and the neutralizing anti-
GM-CSF MoAb 4D4 blocked colony growth in a dose-
dependent manner (Fig 1B). The addition of the IL-1 recep-
tor antagonist IL-1Ra did not affect colony formation (\( P > .05 \)).

Control experiments showed that both IL-1\( \beta \) and IL-Ra
were active. In these experiments, IL-1\( \beta \) stimulated colony
growth of cells from adults with chronic myeloid leukemia,
and IL-1Ra inhibited this growth (data not shown).

The GM-CSF Analogue E21R Causes Apoptotic Cell
Death in JMML

To test whether cytokine inhibitors could affect the sur-
vival of JMML cells, we determined the extent of apoptosis
(programmed cell death) among these cells. Figure 2 shows
an electron microscopic picture of a JMML cell grown in
either medium only (Fig 2A) or E21R (Fig 2B). Cells treated
with E21R displayed features consistent with apoptosis such
as initial blebbing of the plasmalemma, decrease in size,
and condensation of chromatin.21 A molecular hallmark of
apoptosis, a ladder-like pattern of fragmented DNA bands,22
was identified from cells grown with E21R (Fig 2C). Cells
incubated with IL-1Ra did not show signs of apoptosis (Fig
2C), nor did cells incubated with the MoAb 4D4 (data not
shown).

Quantitation of cells undergoing apoptosis was performed
using staining of DNA with propidium iodide. The dose-
response curves depicted in Fig 3A show that E21R induced
apoptosis of more than 80% of the JMML cells at a concen-
tration of 10 \( \mu g/mL \), while neither MoAb 4D4 nor IL-1Ra
affected cell survival in any significant way. Furthermore,
the time courses given in Fig 3B show that a maximal degree
of apoptosis was achieved with a 48-hour incubation of the
JMML cells with E21R.
APOPTOSIS OF JMML CELLS

E21R Does Not Affect Endogeneous Production of GM-CSF, IL-1β, or TNF-α in JMML Cells

To examine whether the E21R-induced apoptosis could be due to a down regulation of cytokines produced by the JMML cells, we measured cytokine concentrations in supernatants collected from JMML cells grown in liquid culture with or without E21R. It is evident from Table 2 that, over 24 hours, E21R did not affect the production of either GM-CSF, TNFα, or IL-1β by JMML cells. These experiments also show that E21R was not toxic to the cells.

DISCUSSION

The central role of GM-CSF in stimulating the growth of malignant cells in JMML is well documented, but nothing is known about the role of inhibition of GM-CSF on the survival of these cells. Clearly, understanding the regulation of JMML cell survival is essential to control its development and progression.

The main and novel finding of the present study is that the GM-CSF analogue E21R induces cell death (apoptosis) of JMML cells, demonstrating the pivotal role of GM-CSF in JMML cell survival and thus offering a new approach for the control of this disease.

The induction of apoptosis was confirmed with three independent methods, namely (1) identification of known morphological features of apoptosis, (2) DNA fragmentation, and (3) reduced DNA binding of propidium iodide. Importantly, E21R induced apoptosis even in the presence of the cytokines TNF-α and IL-1β, both of which have been implicated as stimulators of JMML growth. Collectively, these data support the notion that E21R might be beneficial in reducing not only the growth, but also the cell load in JMML patients despite the presence of other hematopoietic growth factors in the microenvironment.

Interestingly, while both the GM-CSF analogue E21R and the neutralizing anti-GM-CSF MoAb 4D4 blocked GM-CSF-mediated JMML cell growth, only E21R induced apoptosis. This difference may be due to an accessibility phenomenon whereby MoAb 4D4 can neutralize extracellular excess GM-CSF, but cannot dislodge the autocrine-produced GM-CSF bound to its receptor in the endoplasmic reticulum. A limitation to the use of neutralizing antibodies has also been noted in human myeloma cell lines. These cells produce IL-6 in an autocrine manner and anti-IL-6 antibodies fail to inhibit proliferation, yet IL-6 antisense oligonucleotides do.

The molecular mechanism of the E21R-induced apoptosis in JMML cells is not known. One possibility is that apoptosis is the result of antagonizing autocrine-produced GM-CSF. Alternatively, E21R may induce apoptosis of JMML cells through a direct effect following interaction with the GM-CSF receptor. This active mechanism can be seen in cells not producing GM-CSF and requires phosphorylation and stimulation of gene transcription and translation. A normal, functional GM-CSF receptor appears crucial for survival of myeloid cells, as truncations of the β-chain lead to shortened survival, possibly mediated by deactivation of ras proteins involved in maintaining cellular viability. Furthermore, there is an increased frequency of mutations in the ras genes in JMML patients.

Table 2. Cytokine Production in JMML Cells Incubated for 24 Hours in Medium Only or With E21R

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>+Medium</th>
<th>+E21R</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>34.0 ± 4.1</td>
<td>35.3 ± 3.0</td>
</tr>
<tr>
<td>TNFα</td>
<td>21.5 ± 3.2</td>
<td>20.9 ± 4.1</td>
</tr>
<tr>
<td>IL-1β</td>
<td>14.5 ± 3.8</td>
<td>15.0 ± 2.9</td>
</tr>
</tbody>
</table>

Values are expressed as pg/mL and are given as means ± SEM from the 7 JMML cases. There were no significant differences between concentrations of cytokines assayed in supernatants of cells grown in medium only or with E21R (10 μg/mL).
Despite previous results supporting the role of IL-1 in JMML growth, our data could not substantiate this, neither could we attribute any survival effect to IL-1. Hence our observations support earlier studies failing to demonstrate any suppression of JMML colony growth after incubation with polyclonal anti-IL-1 antibodies, or antisera against IL-1. We cannot, however, rule out the possibility that IL-1 indirectly affects JMML growth and survival, eg, by induction of cytokines.

TNFα has been shown to promote JMML growth, and anti-TNFα antibodies block JMML colony formation. With the use of a neutralizing anti-TNFα MoAb, we similarly observed a decline in colony formation, but it had no effect on JMML survival (data not shown).

JMML is a rare and complicated leukemic disorder with high mortality and current treatment modalities have mostly proven unsuccessful. A growing body of evidence has identified GM-CSF as mandatory for growth in JMML. The ability of E21R to inhibit JMML cell growth and viability offers a new approach for the treatment of JMML.

ACKNOWLEDGMENT

E21R was a gift from BresaGen, GM-CSF was a gift from Genetics Institute, IL-β was a gift from Hoffmann-La Roche, and TNFα was a gift from Genentech Inc. Dr H. Hasle kindly provided the diagnostic criteria proposed by the International JMML Working Group. We thank Dr M. H. Freedman for helpful discussions during the course of this study.

REFERENCES

22. Inhorn RC, Carlesso N, Durst N, Frank DA, Griffin JD;


Inhibition of proliferation and induction of apoptosis in juvenile myelomonocytic leukemic cells by the granulocyte-macrophage colony-stimulating factor analogue E21R

PO Iversen, RL Rodwell, L Pitcher, KM Taylor and AF Lopez