Inhibition of Myeloma Cell Growth by Dexamethasone and All-Trans Retinoic Acid: Synergy Through Modulation of Interleukin-6 Autocrine Loop at Multiple Sites

By Yi-Hsiang Chen, Pratibha Desai, Rei-Tsang Shiao, Donald Lavelle, Abdul Haleem, and Jennie Chen

Interleukin-6 (IL-6)/IL-6 receptor (IL-6R) plays a major role in autocrine/paracrine growth regulation of myeloma cells. We investigated the effect of dexamethasone and all-trans retinoic acid, previously shown to modulate IL-6/IL-6R, on the in vitro growth of a human myeloma cell line, OPM-2. Both agents inhibited the clonogenic growth and thymidine incorporation in a concentration-dependent fashion. Isobologram and median effect analysis showed a strong synergy between these two agents with a combination index in the range of 0.2 to 0.6. Both agents decreased the labeling index and the cell fraction in S and G2/M phases, suggesting a block in G1-S phase transition. The clonogenic growth was stimulated by exogenous IL-6 and was inhibited by monoclonal antibody to IL-6, suggesting an autocrine function of IL-6. The effect of dexamethasone but not all-trans retinoic acid was completely reversed by exogenous IL-6. Dexamethasone increased, while all-trans retinoic acid reduced, IL-6R but not gp130 mRNA expression. Their combination caused a net reduction in IL-6R mRNA. Cellular IL-6R density was altered correspondingly without changes in the binding affinity. IL-6 mRNA expression was reduced by dexamethasone and the combination, but was not affected by retinoic acid alone. However, IL-6 secretion into culture supernatant was abolished by both agents. A survey of 4 additional human myeloma cell lines showed that 1 was sensitive to both, 1 was sensitive to one agent only, and 2 were resistant to both. The study demonstrates the possibility of regulating myeloma cell growth through modulation of IL-6/IL-6R autocrine/paracrine loop and the principle of achieving a synergistic effect by blocking this loop at multiple sites.

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THE TREATMENT of multiple myeloma has not progressed significantly since the introduction of melphalan and prednisone. Intensive chemotherapy with multiple cytotoxic agents has failed to substantially improve the outcome of the patients with this disease.1-4 Recent use of marrow ablative cytotoxic treatment supported by bone marrow (BM) and/or peripheral stem cell infusion and hemopoietic growth factors appears to be capable of inducing marked tumor cyoreduction, and may produce a prolonged remission in a proportion of patients.5-9 Whether this approach will lead to a cure remains unknown and its use is currently limited to selected young patients. Thus, for a large segment of patients other therapeutic options need to be explored.

Cumulated evidence indicates that interleukin-6 (IL-6) is the major growth factor for myeloma cells.10 Myeloma cells from approximately 50% of patients were found to be responsive to the stimulatory effect of IL-6 in vitro, and spontaneous proliferation of myeloma cells could be inhibited by antibody to IL-6.8 Similarly, the growth of a number of human myeloma cell lines was shown to be stimulated by IL-6 and inhibited by antibody to IL-6 and IL-6 antisense oligonucleotides.11,12 Furthermore, infusion of mouse monoclonal antibody (MoAb) to IL-6 was effective in inhibiting myeloma cell proliferation, reducing myeloma protein, and ameliorating hypercalcemia in a patient with myeloma.13 However, the secretion of IL-6 or the expression of IL-6 mRNA by myeloma cells could not be consistently shown.7,14 Such failure of detection might be due to technical factors, in case of extreme low level of expression, and it seems likely that IL-6 may function as an autocrine in some primary myeloma cells and myeloma cell lines. In addition, the secretion of IL-6 that function as paracrine has clearly been shown to occur with marrow stromal cells.15 In view of its crucial role in the growth of myeloma cells, modulation of IL-6 autocrine/paracrine loop may prove to be a means of regulating tumor growth.

Glucocorticoids (GC) are among the most effective agents in treating myeloma.16-19 In a recent report, dexamethasone alone achieved a response rate only slightly lower than the combined treatment with vincristine, adriamycin, and dexamethasone.20 The mechanism of their action is unclear. In vitro model using human myeloma cell lines, GC were found to reversibly inhibit the growth of myeloma cells.20 They had also been shown to downregulate the expression of IL-6 in myeloma and other cells.22,23 Multiple genetic elements that were responsive to GC, including glucocorticoid-response element, had been identified in the IL-6 regulatory region.24 These findings and the data to be presented in this report suggest that GC exert their effect through modulation of IL-6 expression. The regulation of other components of the IL-6 autocrine/paracrine loop is largely unknown. However, downregulation of IL-6 receptor (IL-6R) by all-trans retinoic acid had been reported.25-26 Herein we report our findings on the in vitro effect of combination of dexamethasone and all-trans retinoic acid on myeloma cell growth, showing the synergistic effect achieved by blocking the expression of IL-6 autocrine loop at multiple sites.

MATERIALS AND METHODS

Cell lines and reagents. Human myeloma cell lines, OPM-2 and MM1.S, were kindly provided by Dr E.B. Thompson (University of Texas, Galveston) and Dr S. Rosen (Northwestern University, Chicago, IL), respectively. ARH-77 (CRL1621), HS-Sultan (CRL 1484), and RPMI-8226 (CLL 155) cell lines and IL-6-dependent murine hybridoma 7TD1 cells were obtained from American Type

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Culture Collection (Rockville, MD). Recombinant human IL-6 (rhIL-6, Escherichia coli origin) was obtained from Upstate Biotechnology, Inc (Lake Placid, NY); mouse MoAb to human IL-6 (IgG1), from Genzyme (Cambridge, MA); and purified mouse IgG1, from Sigma Chemical Co (St Louis, MO). 125I-labeled rhIL-6, 1.339 Ci/mmol, and 3H-thymidine, 5 Ci/mmol, were obtained from American, Searle Pharmaceuticals (Arlington Heights, IL). Mouse MoAb to bromodeoxyuridine (BU-1) was kindly provided by Dr J. Katzmann (Mayo Foundation, Rochester, MN).

**Cell cultures.** Myeloma cells were maintained in cultures in RPMI 1640 medium (GIBCO, Grand Island Biologicals, Grand Island, NY) supplemented with 5% fetal bovine serum (Intergen, Purchase, NY), 100 U/mL penicillin, and 100 μg/mL streptomycin (GIBCO) at 37°C in 5% CO2 in humidified air. All except for MM1.S line were in suspension cultures. The culture medium was replenished every 2 to 3 days. Cells in logarithmic growth phase were used in experiments. The stock dexamethasone 21-phosphate (Sigma) solution, 2 mmol/L, was prepared in phosphate-buffered saline, and all-trans retinoic acid solution (Sigma; 40 mmol/L) was in dimethyl sulfoxide (Sigma). These reagents, further diluted in culture medium, were added to the experimental groups at 10-5 to 10-4 mol/L at the start of an experiment.

**Tumor cell proliferation assays.** 3H-thymidine incorporation was determined in triplicate 4-day cultures of 5 x 104 viable cells pulsed with 0.5 μCi of 3H-thymidine for 4 hours. For clonogenic growth assay, 8 x 102 to 1 x 104 cells in 0.2 to 0.25 mL of Iscove's medium (Sigma), containing 30% heat inactivated human plasma, 0.8% methylenecelulose and 5 x 10-3 mol/L 2-mercapto-ethanol, were plated in 48-well or 24-well plates (Costar, Cambridge, MA). The growth of tumor cell colony (>50 cells) was scored after 2 to 4 weeks of culture at 37°C and 5% CO2.

**Labeling index and cell-cycle distribution analysis.** Myeloma cells were incubated at 106 cells/mL in medium containing 10 μmol/L of 5-bromo-2-deoxyuridine (BrDU) and 1 μmol/L of 5-fluoro-2-deoxyuridine for 4 hours. The labeled cells were detected with monoclonal anti-BrDU antibody,27 stained with RED613-conjugated goat-antimouse IgG (GIBCO), and counterstained with fluorescein isothiocyanate (FITC)-conjugated rabbit-antihuman IgG antibody (Sigma). At least 105 cells were counted under a fluorescence microscope. Flow cytometric DNA analysis28 involved sequential incubation of cells with trypsin and ribonuclease A and staining with propidium iodide. Samples were analyzed with an Epic V flow cytometer (Coulter Electronics, Inc, Hialeah, FL) with a 5-W argon laser (Coherent, Palo Alto, CA) operating at 488 nm, 200 mW, and a 570-nm long pass absorbance filter. Each fluorescence histogram accumulated 1 to 2 x 106 events and was analyzed using PAR 1 program (Coulter), which assumes the distribution of G0/G1 and M/ G2 to be gaussian, and the remaining cells to be in S-phase.

**Detection of IL-6, IL-6R, and gp130 expression by reverse transcriptase-polymerase chain reaction amplification (RT-PCR).** Total cellular RNA from 5 to 10 x 106 myeloma cells was isolated by conventional guanidium isothiocyanate method.29 The samples were coded and analyzed blindly during RT-PCR procedure. Briefly, 5 μg of total RNA was reverse transcribed in the presence of random hexanucleotides (Boehringer-Mannheim, Chicago, IL) as primers and Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD). The reaction mixture was incubated in 42°C for 1 hour and then at 75°C for 5 minutes to terminate the reaction. In PCR amplification of IL-6R, gp130, and β-actin, 5 μL of 1:8 dilution of the first-strand cDNA generated from 250 ng total RNA was amplified by Taq DNA polymerase (Perkin-Elmer, Norwalk, CT) in 50 μL vol, using the buffer condition recommended by the manufacturer. To increase the efficiency of IL-6 amplification, 1:2 dilution of cDNA was used, and additionally, 0.44 μL of TaqStart antibody (1.1 μg/μL, molar ratio of TaqStart antibody to Taq DNA polymerase = 28:1) was added into the PCR reaction mixture. For IL-6 RT-PCR, oligonucleotides homologous to position 34-56 and 640-661 of IL-6 cDNA sequence30 were used as primers, whereas for IL-6R RT-PCR, oligonucleotides homologous to positions 1143-1164 and 1372-1393 of IL-6R cDNA sequence32 were used. The sequence of upstream primer for gp130 was 5'-ACAGATGAAGTG GGAAAGGAT-3' and of downstream primer, 5'-AGATGACAT GCATGAAAGCC-3'.33 For amplification of β-actin transcripts as internal control, 5'-AAGATGCCAGCATGTTTGG-3' was the upstream, and 5'-AGGGAGCAATGACTGACATG-3', the downstream primer.34 For IL-6 and IL-6R, each PCR cycle consisted of 1 minute of denaturation at 94°C, 2 minutes of primer annealing at 60°C, and 3 minutes of extension/synthesis at 72°C. Thirty-five and 30 cycles were run for IL-6R and IL-6R, respectively. For gp130 PCR, 30 runs of amplification (94°C for 1 minute, 60°C for 1 minute, and 72°C for 2 minutes) were performed. For β-actin PCR, 30 rounds of amplification (94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute) were executed. PCR was performed with a DNA thermal cycler (Coy Lab Product Inc, MI). The TaqStart antibody and all primer sequences were obtained from Clontech Laboratories (Palo Alto, CA). Semi-quantitative determination of PCR products was performed following the procedures previously described.30,35,36. The products were subjected to electrophoresis in 3% agarose gels, and stained with ethidium bromide. The gels were then photographed under a UV light and the band intensities were measured on the negative films using a pdi densitometer. RT-PCR negative controls were parallel RT reactions without adding cellular RNA. The PCR products of IL-6, IL-6R, and gp130 were normalized in relation to β-actin.

**Radio-ligand binding assay for cell-surface IL-6R.** Conventional 125I-IL-6 binding assay was used.37 Before assay, myeloma cells, first depleted of nonviable cells by Ficoll-Hypaque gradient centrifugation when necessary, were washed twice with warm culture medium and washed once more after incubation at 4°C for 10 minutes in binding solution, consisting of Dulbecco's modified Eagle medium with 0.3% bovine serum albumin (BSA), 10 mmol/L HEPES, pH 7.2, and 0.1% sodium azide. 0.70 to 2 x 105 cells were incubated in graded concentration of 125I-IL-6 in 60 μL of binding solution in an ice-water bath with constant mixing on a rocking platform for 150 minutes. Ice-cold binding medium, 0.44 mL, was then added, and the whole volume was layered over a 0.5-mL cushion of the binding medium with 5% BSA and centrifuged at 3,000 rpm for 10 minutes. With the supernatant removed, the tube was cut just above the cell pellet, and the total (specific plus nonspecific) cell-bound radioactivity was counted in a gamma counter. The nonspecific binding was measured in assays in which 100× excess of unlabelled IL-6 was also added and was subtracted from the total bound radioactivity to yield specific cellular binding. The binding data were analyzed by Scatchard method.38

**IL-6 bioassay.** The thymidine incorporation by 2 x 104 7TD1 cells cultured in microtiter plate for 4 days and pulsed with 0.5 μCi of 3H-thymidine for 4 hours was used for bioassay of IL-6 in culture supernatant.39 3HIL-6 was the reference standard (specific activity, 1.9 x 106 cpm/μg).

**Statistical analysis.** Student's t-test was used for comparison of the control and experimental groups. For deriving parameters for isotherm and median effect analysis,40 dose-response relationship data were fitted to Hill's equation by linear regression analysis after appropriate logarithmic transformation.

**RESULTS**

Effect on the clonogenic growth and 3H-thymidine incorporation of OPM-2 cells. The effect of dexamethasone and all-trans retinoic acid (retinoic acid for simplicity), singly or combined, on the clonogenic growth of OPM-2 cells was...
examined in the range from $10^{-6}$ to $5 \times 10^{-9}$ mol/L. The plating efficiency of untreated OPM-2 cells ranged from 13% to 30%. Dexamethasone and retinoic acid inhibited the clonogenic growth in a concentration-dependent fashion, except at low concentration ($\leq 1$ to $2 \times 10^{-8}$ mol/L) where the effect was variable and appeared to be stimulatory in some experiments. The combination of both agents exerted a more profound inhibition. The results of a typical experiment are depicted in Figs 1 and 2. The sigmoidal dose-response curves, excluding the portion that appeared stimulatory, could be closely fitted to Hill’s equation with a correlation coefficient of $-0.963$, $-0.918$, and $-0.995$ ($P < 0.05$) for dexamethasone, retinoic acid, and the combination, respectively. The derived 20%, 50%, and 80% inhibitory concentration ($IC_{20}$, $IC_{50}$, $IC_{80}$) were $3.0$, $5.6$, and $10.5 \times 10^{-8}$ mol/L, respectively, for dexamethasone, and $6.1$, $11.4$, and $21.2 \times 10^{-8}$ mol/L, respectively, for retinoic acid. Two repeat experiments yielded similar results. The combined $IC_{20}$, $IC_{50}$, and $IC_{80}$ were $3.2 \pm 0.9$, $5.4 \pm 1.1$, and $9.3 \pm 1.7 \times 10^{-8}$ mol/L, respectively, for dexamethasone, and $8.6 \pm 2.3$, $13.2 \pm 1.5$, and $20.4 \pm 1.7 \times 10^{-8}$ mol/L, respectively, for retinoic acid. The combination of both agents was more inhibitory than each alone (vide infra). Dimethyl sulfoxide (DMSO) had no effect on cell growth: At the highest concentration used ($0.0025\%$, corresponding to that in $10^{-6}$ mol/L retinoic acid preparation), the clonogenic growth was $96\% \pm 8\%$ of control ($n = 12$).

The inhibitory effect was also evident in $^3$H-thymidine incorporation of OPM-2 cells in the range from $10^{-6}$ to $10^{-9}$ mol/L. The results of a typical experiment are depicted in Fig 3. DMSO, from $0.0025$ to $0.0000025\%$, corresponding to that solvent’s concentration in retinoic acid preparations, had no effect. The degree of suppression was less than that observed with clonogenic assay (Fig 1). For example, both dexamethasone and retinoic acid at $10^{-8}$ mol/L suppressed $^3$H-thymidine uptake by approximately 50%, whereas a nearly total suppression of clonogenic growth was achieved by both agents at this
and lower concentrations. The combination again exerted enhanced inhibition. However, the dose-response data could not be fitted to a simple model, such as Hill's equation, and were not further analyzed.

Interaction between dexamethasone and all-trans retinoic acid. The drug interaction in clonogenic assay was examined by isobologram and median effect analysis. Figure 4 shows the results of a typical analysis using data depicted in Fig 1. The derived IC\textsubscript{50}, IC\textsubscript{30}, and IC\textsubscript{10} for dexamethasone and retinoic acid (vide supra) are plotted on y- and x-axis, respectively, with straight lines representing the expected equi-effective combinations, were two agents noninteracting. The observed equi-effective drug combinations were greatly deviated from the straight lines (Fig 4). The combination of dexamethasone and retinoic acid at 0.75 and 1.5 \times 10^{-8} \text{mol/L}, 1.0 and 2.0 \times 10^{-8} \text{mol/L}, and 1.35 and 2.7 \times 10^{-8} \text{mol/L} effected a 20\%, 50\%, and 80\% inhibition, respectively. Thus, the respective combination index (CI = \Sigma IC_{single agent}/IC_{combination}) was 0.496, 0.354, and 0.256, indicating a synergy between these two agents. The combined CIs at IC\textsubscript{20}, IC\textsubscript{50}, and IC\textsubscript{80} were 0.608 \pm 0.172, 0.474 \pm 0.160, and 0.388 \pm 0.132, respectively, significantly (P < .05, n = 3) lower than the expected mean value of 1.0 for noninteracting agents.

Effect on labeling index and cell-cycle distribution. Table 1 summarizes the drug effect on the labeling index and the cell-cycle distribution of OPM-2 cells. Both dexamethasone and retinoic acid suppressed the labeling index. The cell-cycle analysis showed a reduced proportion of cells in S and M/G\textsubscript{2} phases but an increased proportion in G\textsubscript{0}/G\textsubscript{1} phase, suggesting a block at G\textsubscript{1}-S phase transition. Similar results were obtained in a repeat experiment. The combination of two agents showed no significant enhancement in inhibition in these measurements.

Exogenous rhIL-6 stimulates cell growth and reverses the inhibitory effect of dexamethasone but not all-trans retinoic
Table 1. Effect of Dexamethasone and All-trans Retinoic Acid on Cell-Cycle Distribution and Labeling Index

<table>
<thead>
<tr>
<th>Drug Exposure*</th>
<th>G2/G1 (%)</th>
<th>S (%)</th>
<th>M/G0 (%)</th>
<th>Labeling Index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>59.3 ± 1.5</td>
<td>30.3 ± 0.6</td>
<td>11.3 ± 2.1</td>
<td>47.7 ± 7.1</td>
</tr>
<tr>
<td>Dexamethasone 10^(-6) mol/L</td>
<td>64.3 ± 0.6</td>
<td>26.3 ± 2.1</td>
<td>9.3 ± 1.51</td>
<td>13.1 ± 0.8</td>
</tr>
<tr>
<td>Dexamethasone 10^(-7) mol/L</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>20.5 ± 0.9</td>
</tr>
<tr>
<td>Retinoic acid 10^(-6) mol/L</td>
<td>71.3 ± 1.5</td>
<td>20.0 ± 1.0</td>
<td>8.7 ± 1.5</td>
<td>26.7 ± 9.0</td>
</tr>
<tr>
<td>Retinoic acid 10^(-7) mol/L</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>40.1 ± 1.01</td>
</tr>
<tr>
<td>Dexam. + retinoic acid 10^(-6) mol/L</td>
<td>73.0 ± 1.0</td>
<td>19.0 ± 0</td>
<td>8.0 ± 1.0</td>
<td>19.2 ± 3.0</td>
</tr>
<tr>
<td>Dexam. + retinoic acid 10^(-7) mol/L</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>27.6 ± 1.1</td>
</tr>
</tbody>
</table>

* 2-day exposure to drugs.
† The effect of drugs was significant (P < .05) except those marked.

Acid. As summarized in Table 2, rhIL-6 stimulated ³H-thymidine uptake of OPM-2 cells in a dose-dependent fashion at 0.1 to 10 ng/mL. It lessened the suppression by 10^(-5) mol/L dexamethasone at as low a concentration as 0.1 ng/mL (from 76% suppression to 43%), and totally reversed the suppression at 10 ng/mL. The suppression by retinoic acid and dexamethasone plus retinoic acid was not reversed by rhIL-6. Similarly, rhIL-6, at as low as a concentration of 0.5 ng/mL, maximally stimulated the clonogenic growth of OPM-2 cells (84% increase in colony formation) and totally reversed the inhibition by dexamethasone (Table 2). It slightly but significantly increased the colony count of cultures exposed to retinoic acid and retinoic acid plus dexamethasone, but only minimally reversed their suppressive effect. In addition, the clonogenic growth of OPM-2 cells was totally inhibited by 10 μg/mL of mouse MoAb to human IL-6, while the control mouse IgG1 at the same concentration reduced the clonogenic growth by only 23%.

Effect of drugs on IL-6, IL-6R, and gp130 expression. The RT-PCR procedure used for detecting IL-6, IL-6R, and gp130 mRNA appears suitable for semi-quantitative measurement of their expression. Figure 5 depicts the quantitative relationship between the final PCR products, measured densitometrically, and the starting dilution of cDNA used for PCR. It shows an approximate log-linear proportionality between the input cDNA and the PCR end products in the range of cDNA dilution used for RT-PCR. Furthermore, the RT-PCR products of IL-6, IL-6R, and gp130 mRNA were normalized in relation to β-actin internal control. Figure 6 shows that the expression of IL-6 mRNA decreased in cultures treated with dexamethasone and dexamethasone plus retinoic acid (both at 10^(-5) mol/L) at 17 hours, but was unchanged at 48 hours. IL-6 mRNA expression was not changed by retinoic acid alone. On the other hand, IL-6R mRNA expression increased after 17 hours of incubation with dexamethasone but did not change with retinoic acid or dexamethasone plus retinoic acid. After a 48-hour incubation, in addition to an increase in IL-6R mRNA with dexamethasone, IL-6R mRNA was markedly reduced with retinoic acid and with retinoic acid plus dexamethasone. The expression of gp130 mRNA was not changed by drug treatment.

Radio-ligand binding assay for cell surface IL-6R, using a commercial 125I-rhIL-6 preparation with a trichloroacetic acid precipitability of 80% and a maximum cellular binding capacity of 75%, showed the corresponding alterations of IL-6R expression after drug treatment. The Scatchard plots (Fig 7) show that control OPM-2 cells possess high affinity IL-6R with a kd of 6.7 × 10^(-11) mol/L at a density of 183 binding sites/cell. In three assays, kds were 7.8 ± 2.8 × 10^(-11) mol/L and the binding sites were 181 ± 40/cell. With a 2-day incubation at 10^(-6) mol/L, dexamethasone increased the density of the binding sites approximately threefold, whereas retinoic acid reduced the binding sites by about 70%. The affinity of the receptors was not significantly altered by drug treatment (Fig 6). With a 4-day incubation at 5 × 10^(-7) mol/L, retinoic acid also reduced high-affinity (kd = 5.3 × 10^(-11) mol/L) binding sites to 55/cell. There were insufficient cells for analysis in culture with dexamethasone after 4 days of treatment. However, with a 1-day exposure at 10^-6 mol/L, dexamethasone raised the binding sites to 697/cell (kd = 2.8 × 10^(-11) mol/L), consistent with RT-PCR analysis, where enhanced IL-6R mRNA expression was already evident after 17 hours of dexamethasone treatment (Fig 6). There were insufficient viable cells in cultures with both agents for accurate radioligand binding assay.

The supernatant was harvested from 2- to 5-day cultures of OPM-2 cells (starting cell density = 3 × 10^5 cells/mL) and assayed for IL-6 bioactivity. To facilitate the concentration process anticipated to be necessary for bioassay of superna-

Table 2. IL-6 Reverses the Inhibitory Effect of Dexamethasone But Not All-trans Retinoic Acid on the Growth of OPM-2 Cells

<table>
<thead>
<tr>
<th>Drugs (10^-6 mol/L)</th>
<th>0</th>
<th>0.1</th>
<th>1</th>
<th>10.0</th>
<th>0</th>
<th>0.5</th>
<th>5.0</th>
<th>50.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(cpm/2 × 10^6 cells)*</td>
<td>(cpm/2 × 10^6 cells)*</td>
<td>(cpm/2 × 10^6 cells)*</td>
<td>(cpm/2 × 10^6 cells)*</td>
<td>(colony/10^6 cells)*</td>
<td>(colony/10^6 cells)*</td>
<td>(colony/10^6 cells)*</td>
<td>(colony/10^6 cells)*</td>
</tr>
<tr>
<td>0</td>
<td>20,512 ± 2,121</td>
<td>30,351 ± 7,695</td>
<td>36,082 ± 2,370</td>
<td>36,782 ± 5,930</td>
<td>293 ± 24</td>
<td>538 ± 85</td>
<td>421 ± 19</td>
<td>445 ± 42</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>4,925 ± 1,846</td>
<td>11,608 ± 2,331</td>
<td>15,809 ± 1,129</td>
<td>32,560 ± 6,764</td>
<td>23 ± 8</td>
<td>455 ± 34</td>
<td>367 ± 20</td>
<td>393 ± 26</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>12,581 ± 3,615</td>
<td>5,056 ± 1,958</td>
<td>8,622 ± 2,645</td>
<td>12,537 ± 2,692</td>
<td>17 ± 3</td>
<td>43 ± 9</td>
<td>56 ± 11</td>
<td>48 ± 14</td>
</tr>
<tr>
<td>Dexamethasone + retinoic acid</td>
<td>5,707 ± 1,472</td>
<td>4,861 ± 1,087</td>
<td>3,721 ± 1,591</td>
<td>3,552 ± 456</td>
<td>1 ± 1</td>
<td>34 ± 8</td>
<td>35 ± 11</td>
<td>54 ± 12</td>
</tr>
</tbody>
</table>

* Mean ± SD of triplicate cultures.
† In these cultures with rhIL-6, the ³H-thymidine uptake or colony count was increased significantly (P < .05), compared with respective controls without drugs (first row).
‡ In these cultures with drugs (dexamethasone, retinoic acid, or both), the ³H-thymidine uptake or colony count was reduced significantly (P < .05), compared with respective cultures without rhIL-6 (first column).
SYNERGY BETWEEN DEXAMETHASONE AND RETINOIC ACID

Fig 5. Semiquantitative relationship between input cDNA and PCR products. PCR was performed using serial 1:2 dilution of reverse transcription products from 250 ng of total RNA derived from OPM-2 cells using the primers for IL-6 (A), IL-6R (B), gp130 (C), and β-actin (D). Methods are detailed in Materials and Methods.

Fig 3. Some cell lines did not form tight colonies, making enumeration of colony count less precise. MM1.S line was found to be as sensitive as OPM-2 lines to both agents. Similarly, both agents acted synergistically in this cell line, at least at high concentration (Table 3). On the other hand, ARH-77 cells were quite sensitive to retinoic acid and yet highly resistant to dexamethasone. HS-sultan and RPMI-8226 cells were resistant to both agents.

DISCUSSION

We have studied in detail the effect of dexamethasone and retinoic acid on the in vitro growth of a human myeloma cell line. OPM-2 cells, using ³H-thymidine incorporation, tumor colony growth, the labeling index, and cell-cycle distribution as measures of the cellular proliferative activity. The results show clearly in all parameters that dexamethasone and retinoic acid inhibited the growth of myeloma cells in a concentration-dependent fashion. The estimated IC₅₀ were in the order of 5 × 10⁻⁸ and 15 × 10⁻⁸ mol/L for dexamethasone and retinoic acid, respectively. Isobologram and median effect analyses show a strong synergy between these two agents, especially at high concentration. At low concentration, these agents, particularly dexamethasone, might have a stimulatory effect on clonogenic growth. Others also observed an apparent growth promoting effect of a medium containing growth factors and hydrocortisone in culturing myeloma cells and in establishing cell lines. This phenomenon might account for decreasing synergy observed in low drug concentrations. The inhibitory effect of these agents appeared less pronounced when measured by ³H-thymidine incorporation.

Sensitivity of various human myeloma cell lines to dexamethasone and all-trans retinoic acid. A survey was made of the sensitivity of some other human myeloma cell lines using clonogenic assay. The results are summarized in Table 3. Some cell lines did not form tight colonies, making enumeration of colony count less precise. MM1.S line was found to be as sensitive as OPM-2 lines to both agents. Similarly, both agents acted synergistically in this cell line, at least at high concentration (Table 3). On the other hand, ARH-77 cells were quite sensitive to retinoic acid and yet highly resistant to dexamethasone. HS-sultan and RPMI-8226 cells were resistant to both agents.

DISCUSSION

We have studied in detail the effect of dexamethasone and retinoic acid on the in vitro growth of a human myeloma cell line. OPM-2 cells, using ³H-thymidine incorporation, tumor colony growth, the labeling index, and cell-cycle distribution as measures of the cellular proliferative activity. The results show clearly in all parameters that dexamethasone and retinoic acid inhibited the growth of myeloma cells in a concentration-dependent fashion. The estimated IC₅₀ were in the order of 5 × 10⁻⁸ and 15 × 10⁻⁸ mol/L for dexamethasone and retinoic acid, respectively. Isobologram and median effect analyses show a strong synergy between these two agents, especially at high concentration. At low concentration, these agents, particularly dexamethasone, might have a stimulatory effect on clonogenic growth. Others also observed an apparent growth promoting effect of a medium containing growth factors and hydrocortisone in culturing myeloma cells and in establishing cell lines. This phenomenon might account for decreasing synergy observed in low drug concentrations. The inhibitory effect of these agents appeared less pronounced when measured by ³H-thymidine incorporation.
incorporation, suggesting a differential drug-sensitivity of various tumor cell subpopulations; clonogenic cells being more sensitive than nonclonogenic cells. The latter, capable of, perhaps, limited proliferation, might also account for the failure to observe the synergistic effect, when the drug effect was assayed by labeling index and cell-cycle distribution analyses (Table I), as these measurements might reflect predominantly the changes in nonclonogenic cell populations.

The mechanisms of action of these agents are not clear. Both agents reduced the labeling index and the cell fractions in S and M/G2 phases, indicating a block at G1-S phase transition. Similar observation was made with other myeloma cell lines. Dexamethasone was previously shown to downregulate the expression of IL-6 in various cell types including myeloma cells. With highly sensitive RT-PCR the expression of IL-6 mRNA by OPM-2 cells was also found to be reduced after 17 hours of dexamethasone treatment. Surprisingly, IL-6 mRNA expression after 48 hours of drug exposure was not reduced. This may suggest a transient effect of dexamethasone. Because the number of viable cells decreased greatly in cultures at 48 hours, a more likely alternative explanation is that the normal level of IL-6 mRNA expression at this time point reflected the presence of a small surviving GC-resistant subpopulation. This explanation is supported by the finding that bioactive IL-6 activity in the culture supernatant was reduced to an undetectable level. The combination of dexamethasone and retinoic acid had the same effect. Although retinoic acid alone had no effect on IL-6 mRNA expression, no IL-6 bioactivity was detectable in the culture supernatant. A downregulation of IL-6 in primary myeloma explants by retinoic acid had recently been reported. In view of the findings that the growth of OPM-2 cells was stimulated by exogenous IL-6 and was strongly inhibited by mouse MoAb to rhIL-6, and that the inhibitory effect of dexamethasone could be totally reversed by exogenous IL-6, it is most likely that dexamethasone inhibits myeloma cell growth through abrogation of endogenous IL-6 production. On the other hand, the inhibition of clonogenic growth by retinoic acid was not reversed by exogenous rhIL-6, suggesting additional effect of retinoic acid besides the modulation of IL-6 secretion.

The expression of IL-6R was markedly altered by retinoic acid and dexamethasone at mRNA and surface receptor level, increased by dexamethasone and reduced by retinoic acid. Because the 125I-IL-6 preparation used was only about 80% precipitable by trichloroacetic acid and bindable by cells, the estimates of binding sites and affinity constants obtained in our assays should be considered only approximate. Scatchard plots (Fig 7) may also suggest the presence of more than one class of receptors with different binding affinity, similar to that observed by others in some U266 cells. However, the binding datum points in each assay were too few to allow detailed analysis for multiple receptor classes, and we chose to fit the data to a single line. Despite these technical reservations, the pattern of changes induced...
by these agents was clearly evident. Dexamethasone increased the binding sites threefold to fourfold after as short as 24 hours of exposure. Retinoic acid, on the other hand, reduced the binding sites by 70%, which may require 48 hours of drug treatment, as suggested by IL-6R mRNA analysis. The binding affinity appeared unchanged, consistent with the finding that the expression of gp130 mRNA, whose protein product is essential for high-affinity ligand binding, was unaffected by either agents.24 Despite extensive washing of cells before ligand-binding assay, endogenous IL-6 secretion might affect the number of IL-6R available for 125I-IL-6 binding. However, the observed changes in cellular IL-6R after drug exposure are unlikely to result from a simple shift in the proportion of unoccupied receptors, as both dexamethasone and retinoic acid inhibited the secretion of IL-6, yet their effect on the assayable IL-6R was exactly the opposite of each other. The corresponding changes in the IL-6R mRNA level induced by these agents also argue strongly for a modulation of IL-6R expression. Similar findings of the retinoic acid effect on IL-6R were previously reported,25,26 although reduced expression of gp130 was also noted in some primary myeloma explants.27 Thus, it is plausible that retinoic acid inhibits myeloma cell growth through additional mechanism of IL-6R downregulation, as was previously suggested.25,26 Conceivably, a lack of functional IL-6R could also render retinoic acid–treated cells refractory to the stimulation of exogenous rhIL-6, as was observed in our study. The altered expression of IL-6R mRNA by retinoic acid seemed to take a longer time to manifest than by dexamethasone, suggesting that the effect of retinoic acid might be an indirect one. It is of interest to note that dexamethasone upregulates IL-6R expression as well as downregulates IL-6 expression. Its net effect would likely depend on the extent of modulation on each component. It is possible that dexamethasone at low concentration might effect an increase in IL-6R without a significant reduction in IL-6 production, resulting in a stimulation in myeloma cell growth, as it seemed to occur in our study (Fig 1). Nevertheless, addition of retinoic acid effectively abrogated the IL-6R upregulating effect of dexamethasone, resulting in a net reduction in IL-6R (Fig 6). Thus, the combined use of dexamethasone and retinoic acid, by blocking IL-6 autocrine loop at both the cytokine and its receptor level, can be expected to produce a synergistic effect. The observations presented here are in support of this hypothesis.

A survey on four additional cell lines showed variable drug sensitivity: MM1.S cells were sensitive to both agents, whereas ARH cells were resistant to dexamethasone yet highly sensitive to retinoic acid. HS and RPMI-8226 cells were resistant to both. The estimated IC50 for sensitive lines were 2 to 5 × 10^-8 mol/L for dexamethasone and 1 to 15 × 10^-8 mol/L for retinoic acid. Combination of both agents again exhibited a synergistic effect in MM1.S line. Thus, 40% to 60% of the cell lines studied are sensitive to either or both agents. The causes for this variability among myeloma cell lines, likely to be multifold, await further investigation. Both agents presumably act through their respective cellular receptors. Any abnormalities in receptors and postreceptor signal pathways are likely to result in a loss of drug sensitivity. An example of such mechanisms is the occurrence of differentiated progenies of limited proliferative potential. Although substantial evidence suggests a growth factor activity of IL-6,27-30 its role in myeloma in vivo is still being debated. In both the freshly isolated myeloma cells and the established myeloma cell lines, IL-6 has been shown to stimulate the 3H-thymidine incorporation and to increase the S phase fraction in a proportion of cells. Such effect cannot be readily shown in clonogenic assay.31,32 Furthermore, neither IL-6 secretion and IL-6 mRNA expression nor in vitro growth inhibition by antibody and antisense oligonucleotide to IL-6 can be consistently shown.32,33,34 Current laboratory technologic limitation is likely to be partly responsible: primary myeloma explants give rise to poor clonogenic growth.35,36 The low level of IL-6 expression may be difficult to detect.11 The principal difficulty, however, appears intrinsic to myeloma cell biology due to tumor cell heterogeneity and the low frequency of clonogenic stem cells, still incompletely characterized.49 In this regard, recent findings provide some insight: it was shown that among tumor cells of plasma cell morphology, the less mature CD45+ fraction frequently expressed IL-6 and IL-6R, suggesting that the more immature stem cells are likely to express these genes also.52 Furthermore, "lymphoid" myeloma precursor cells were shown to proliferate under the combined stimulation of IL-3 and IL-6. Subsequently monotypic myeloma cells of low proliferative activity emerged.38 Thus, although clonogenic stem cells may be responsive to IL-6 and other cytokines, the majority of myeloma cells obtained from patients consists of differentiated progenies of limited proliferative potential. Use of such heterogenous cell preparations may contribute to the inconsistency in various observations. On the other
hand, given the multi-functionality of IL-6 and the heterogeneity of tumor cells, it is conceivable that IL-6 may have different functional activities on subpopulations of tumor cells.

In considering the clinical relevance of our study, we noted that the effective drug concentrations in vitro are achievable clinically. For example, a daily dose of 40 mg dexamethasone, the dose used in vincristine-adrarnycin-dexamethasone regimen for myeloma, may yield a mean plasma concentration of $3 \times 10^{-7}$ mol/L based on pharmacokinetic parameters. The measured peak plasma retinoic acid level exceeded $10^{-6}$ mol/L in leukemia patients receiving 45 mg/m$^2$/d, although its half-life was less than 1 hour. Furthermore, our preliminary study suggests that the effect of retinoic acid is long-lasting so that prolonged continuous therapy may not be essential. Thus, a proper dose scheduling may avoid the progressive shortening of plasma half-life and the resultant low plasma level associated with its prolonged use. The effectiveness of dexamethasone in myeloma treatment has long been established. However, early trial with retinoic acid failed to show a therapeutic benefit. This result is not surprising, as the patients in trial were all with refractory disease.

The theoretical advantages of combination use of both agents appear evident: The synergistic effect may produce a greater cytoreduction. A subpopulation of myeloma cells may be only sensitive to one of the agents. Finally, the secretion of autocrine and paracrine IL-6 in vivo is subjected to a multitude of modulating influences: IL-1, transforming growth factor-$\alpha$ (TGF-$\alpha$), TGF-$\beta$, interferon-$\alpha$, and granulocyte-macrophage colony-stimulating factor have all been shown to stimulate IL-6 secretion by such cells as macrophages, fibroblasts, endothelial cells, and marrow stromal cells. Among these, the stimulatory effect of IL-1 had been shown to be blocked by all-trans retinoic acid. In such a complex inter-connected system, it seems likely that for effective control of cell growth through modulation of cytokine network, blocking at multiple sites is not only beneficial but also mandatory.

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REFERENCES


Inhibition of myeloma cell growth by dexamethasone and all-trans retinoic acid: synergy through modulation of interleukin-6 autocrine loop at multiple sites

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