All-trans retinoic acid (ATRA) has previously been shown to inhibit the growth of OPM-2 human myeloma cells. The growth inhibition was postulated to result from a transcriptional downregulation of interleukin-6 receptor α (IL-6Rα) with IL-6Rβ (gp130) unaffected. To formally test this hypothesis, an expression vector designed for constitutive IL-6Rα expression was constructed and used for transfection of OPM-2 cells. Six stable transfectants were cloned. The expression of IL-6Rα was shown by immunofluorescence with anti-IL-6Rα antibody and 125I-IL-6 binding. In five of six transfectant clones, cellular IL-6Rα was 1.5- to 6-fold higher than the parental cells, with the ligand binding affinity unchanged. While ATRA reduced IL-6Rα expression in the parental OPM-2 cells, it enhanced its expression in these five transfectants. The clonogenic growth of these transfectants, however, remained strongly inhibited by ATRA. Further analysis, comparing the parental OPM-2 cells and a representative transfectant, clone C5, showed that IL-6 caused rapid tyrosine phosphorylation of gp130 in both OPM-2 and C5 clones. Pretreatment with ATRA greatly reduced IL-6-induced gp130 phosphorylation in OPM-2 cells, reflecting a reduction in cellular IL-6Rα. In contrast, IL-6-induced gp130 phosphorylation was not reduced by ATRA pretreatment in C5 cells, indicating that the expressed IL-6Rα was functional. Similar to OPM-2 cells, C5 cells were sensitive to growth inhibition by dexamethasone, which was entirely reversed by exogenous IL-6, suggesting that the IL-6 postreceptor signal transduction remained intact. ATRA was further shown to upregulate p21WAF1 expression and cause dephosphorylation of the retinoblastoma protein (pRB) in both OPM-2 and C5 cells. Exogenous IL-6 also failed to reverse these effects of ATRA. Thus, the growth inhibitory activity of ATRA is not mediated through cellular IL-6Rα downregulation and is likely to result from a direct upregulation of p21WAF1 and consequent dephosphorylation of pRB.

We have previously shown that all-trans retinoic acid (ATRA) and dexamethasone (Dex) synergistically inhibited the growth of a number of human myeloma cell lines. The mechanism of growth inhibition was postulated to be the downregulation of both autocrine interleukin-6 (IL-6) secretion and cellular IL-6Rα expression. It was shown in OPM-2 myeloma cells that Dex downregulated the expression of IL-6 at the transcriptional level, while upregulating IL-6Rα expression. ATRA, on the other hand, downregulated IL-6Rα expression, while the signal-transducing gp130 (IL-6Rβ) was unaffected. Their combined use led to abrogation of both IL-6 and IL-6Rα expression. These findings were consistent with reports by others on the effect of Dex and ATRA on IL-6/IL-6R in myeloma. Because IL-6 is the major growth factor for myeloma cells, downregulation of IL-6 by Dex should lead to growth arrest. Similarly, a reduction in functional IL-6Rα by ATRA can conceivably disrupt growth signaling and inhibit the cell growth. Indeed, blocking of IL-6Rα either by anti-IL-6R blocking antibody or by IL-6R “super-antagonists,” the mutated IL-6 molecules with enhanced binding affinity to IL-6R but incapable of triggering gp130 signal transduction, was shown to inhibit the growth of myeloma cells. Exogenous IL-6 reverses entirely the growth inhibitory effect of Dex, strengthening the contention that Dex-induced suppression of IL-6 expression is the primary mechanism of growth inhibition. Analogous evidence for ATRA action, however, is lacking. If ATRA inhibited myeloma cell growth through downregulation of IL-6Rα expression, as it has been hypothesized, it should be expected that its inhibitory effect will be reversed by a reconstitution of the functional IL-6Rα. The present study formally tested this hypothesis. We show that functional IL-6Rα, downregulated in myeloma cells exposed to ATRA, can be effectively reconstituted by transfected IL-6Rα cDNA. However, this reconstitution fails to reverse the growth inhibitory effect of ATRA, indicating that the growth inhibition by ATRA is not mediated through the downregulation of IL-6Rα.

Therefore, we investigated the effect of ATRA on the postreceptor pathway and found that ATRA induced dephosphorylation of the retinoblastoma protein (pRB) in OPM-2 cells. This was in association with an upregulation by ATRA of the expression of p21WAF1 (p21), an inhibitor of CDK2 activity. These findings are consistent with the ATRA effect on the G1-S transit block we previously reported and provide a plausible mechanism for its growth inhibitory activity. To further investigate the role that IL-6Rα may play in ATRA action, we compared the effect of ATRA on the regulation of p21 and phosphorylation of pRB in OPM-2 and an IL-6Rα cDNA transfectant. We found that the pattern of p21 and pRB activation was identical in both clones. Exogenous IL-6 also failed to reverse the effect of ATRA on p21 upregulation, providing further evidence that growth inhibition by ATRA is independent of IL-6Rα modulation and is likely to be due to a direct action on p21 regulation.

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MATERIALS AND METHODS

Cell cultures. Human myeloma OPM-2 cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) with 10% fetal bovine serum (Intergen, Purchase, NY), 10 mM/L HEPES buffer, pH 7.2, 100 U/mL penicillin, and 100 µg/mL streptomycin (GIBCO) at 37°C in 5% CO2 in humidified air. Cells in logarithmic growth phase were exposed to various concentrations of ATRA or Dex (Sigma Chemical Co, St Louis, MO) for 2 to 3 days. ATRA, dissolved in dimethyl sulfoxide (Sigma) at 40 mM/L, was diluted with culture medium before use. The residual dimethyl sulfoxide was previously shown not to affect the growth of myeloma cells.1 Dex was dissolved in phosphate-buffered saline at 8 mM/L and diluted with medium for use in cultures.

Tyrosine phosphorylation of gp130 (IL-6Rβ). Cellular extracts were prepared from 10 to 20 × 10^6 myeloma cells in 1 mL of extraction phosphorylation lysis buffer consisting of 50 mM/L HEPES, pH 7.4, 150 mM/L NaCl, 1.5 mM/L MgCl2, 10 mM/L sodium pyrophosphate, 1 mM/L EDTA, 100 mM/L NaF, 100 mM/L sodium orthovanadate, 1% Triton X-100 (Boehringer Mannheim, Mannheim, Germany), 10% glycerol, 20 µg/mL aprotinin, and 0.5 mM/L phenylmethylsulfonyl fluoride (PMSF). Protein concentrations were determined by the Bradford method using the Biorad Protein Assay dye reagent (Biorad, Cambridge, MA). The immunoprecipitation and immunoblotting assays were performed as previously described.11,12 Briefly, the cell lysates with equal protein loads were immunoprecipitated with rabbit anti-human gp130 (Santa Cruz Biotech, Santa Cruz, CA) using protein-G sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ). After five washes with phosphorylation lysis buffer containing 0.1% Triton X-100, proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinyl difluoride filters (Immobilon; Millipore, Bedford, MA). The residual binding sites on the filters were blocked by incubating with TBST (10 mM/L Tris HCl, pH 8.0, 15 mM/L NaCl, 0.05% Tween 20) containing 20% BSA for 1 hour at room temperature. The filters were subsequently incubated with antiphosphotyrosine monoclonal antibody (4G10; Upstate Biotechnology, Lake Placid, NY) for 1 hour at 4°C, followed by overnight incubation with Protein A/G sepharose (Santa Cruz Biotech). The precipitated protein was washed with RIPA buffer (50 mM/L Tris, pH 7.4, 150 mM/L NaCl, 1 mM/L EDTA, 1% NP40, 0.25% sodium deoxycholate, 100 mM/L NaF, 5 µg/mL aprotinin, 1 µg/mL leupeptin, and 1 mM/L Na2VO4) and separated in 5% SDS-PAGE. Immunoblots, prepared as described above, were incubated with a second anti-RB antibody (sc-50-G; Santa Cruz Biotech) followed by peroxidase-conjugated anti-goat Ig (Santa Cruz Biotech) and visualized using ECL (enhanced chemiluminescence) reagents.

Analysis of retinoblastoma protein phosphorylation. Total cell extracts of OPM-2 and C5 cells were prepared with a high-salt RIPA buffer consisting of 50 mM/L Tris, pH 7.4, 300 mM/L NaCl, 1 mM/L EDTA, 1% NP40, 0.25% sodium deoxycholate, 100 mM/L NaF, 5 µg/mL aprotinin, 1 µg/mL leupeptin, and 1 mM/L Na2VO4. Retinoblastoma protein from extracts (200 µg protein) was immunoprecipitated by incubation with anti-RB antibody (OP28; Calbiochem, San Diego, CA) for 1 hour at 4°C, followed by overnight incubation with Protein A/G sepharose (Santa Cruz Biotech). The precipitated protein was washed with RIPA buffer (50 mM/L Tris, pH 7.4, 150 mM/L NaCl, 1 mM/L EDTA, 1% NP40, 0.25% sodium deoxycholate, 100 mM/L NaF, 5 µg/mL aprotinin, 1 µg/mL leupeptin, 100 µg/mL PMSF, and 1 mM/L Na2VO4) and separated in 5% SDS-PAGE. Immunoblots, prepared as described above, were incubated with a second anti-RB antibody (sc-50-G; Santa Cruz Biotech) followed by peroxidase-conjugated anti-goat Ig (Santa Cruz Biotech) and visualized using ECL reagents.

Analysis of p21WAF1 (p21), p27KIP1 (p27), CDK2, and CDK 4 expression. Nuclear extracts were prepared from 10 × 10^5 cells. Cells were first washed and incubated in 400 µL of buffer A (10 mM/L HEPES, pH 7.9, 1.5 mM/L MgCl2, 10 mM/L KCl, 0.5 mM/L dithiothreitol, 1 µg/mL aprotinin, 1 µg/mL leupeptin, 100 µg/mL PMSF, and 1 mM/L Na2VO4) on ice for 10 minutes. Cells were vortexed for 10 seconds and followed by a 10-second centrifugation. The pellets were resuspended in 50 µL of buffer C (20 mM/L HEPES, 320 mM/L NaCl, 1.5 mM/L MgCl2, 0.2 mM/L EDTA, 0.5 mM/L dithiothreitol, 25% vol/vol glycerol, 1 µg/mL aprotinin, 1 µg/mL leupeptin, 100 µg/mL PMSF, and 1 mM/L Na2VO4) incubated on ice for 20 minutes followed by a 2-minute centrifugation. The extracted proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes as described above. Immunoblots were sequentially stripped and probed with anti-p21 (sc-397-G; Santa Cruz Biotech), anti-p27 (sc-152-G; Santa Cruz Biotech), and anti-CDK2 (sc-150-G; Santa Cruz Biotech).
anti-p27 (sc-528-G), anti-CDK2 (sc-163-G), and anti-CDK4 (sc-260-G), followed by an appropriate anti-goat or anti-rabbit Ig second antibody (Santa Cruz Biotech), and visualized with ECL reagents.

Clonogenic growth assay and cell-cycle distribution analysis. The clonogenic growth assay was performed as previously described. Briefly, 800 cells in 0.2 mL of Iscove’s medium (Sigma), containing 30% prescreened, heparinized, normal human plasma, 0.8% methylcellulose, and $5 \times 10^{-5}$ M 2-mercaptoethanol, were plated in 48-well plates. Colony growth was scored after 2 to 3 weeks of incubation. Conventional flow cytometric DNA analysis with propidium iodide

**Fig 1.** IL-6Rα expression in OPM-2 parental line and “pooled” Rc/CMV-IL-6Rα cDNA transfectants. Histograms of unstained cells (top row), background fluorescence with antibody control (middle row), and cells stained with anti-IL-6Rα antibody (bottom row) were displayed. Fluorescence signals were acquired with logarithmic amplifiers.
staining was performed as previously described. Fluorescence intensity histograms were analyzed with ModFit program (Variety Software House, Inc, Sunnyville, CA).

RESULTS

IL-6Rα (gp80) expression in parental OPM-2 myeloma cells and selected transfectants. Flow cytometric analysis of cellular IL-6Rα expression showed increased expression of IL-6Rα in transfectants. Figure 1 displays the fluorescence intensity histograms of a representative analysis. The respective mean channel numbers (MCN) in linear scale of the unstained, antibody control (background), and stained cells were 30, 57, and 274 for the parental OPM-2 cells, and 25, 51, and 670 for “pooled” transfectants. Thus, the IL-6Rα density, expressed as MCN corrected for the background, was 217 and 619 for the parental cells and “pooled” transfectants, respectively. The analysis of the isolated stable transfectant clones are summarized in Table 1 (column A,a). IL-6Rα expression increased 1.5- to 6-fold in the transfectant clones except clone A1, where it was close to that of the parental OPM-2 cells, indicating loss or loss of function of the transfected IL-6Rα cDNA in this clone. A repeat experiment showed similar results.

Radioligand binding assays were also performed on parental, clone A1, C4, and C5 cells. Scatchard analysis of the binding data yielded an IL-6Rα density of 237 binding sites/cell and a dissociation constant of $1.5 \times 10^{-10}$ mol/L for the parental line. The respective values were 431 sites/cell and 1.2 $\times 10^{-10}$ mol/L for clone A1; 2,742 sites/cell and $2.2 \times 10^{-10}$ mol/L for clone C4; and 2,385 sites/cell and $2.4 \times 10^{-10}$ mol/L for clone C5, consistent with the relative IL-6Rα density detected by flow cytometry. The binding site estimate for parental OPM-2 cells was comparable to our previous measurement. The estimates of the dissociation constant, however, were higher in the present study. The reason for this discrepancy is not clear. Nevertheless, the dissociation constants were clearly of similar magnitude for the parental and transfected clones, indicating that the expressed IL-6Rα from the transfected IL-6Rα cDNA in transfectants were as avid as the endogenous IL-6Rα in specific ligand binding.

Effect of ATRA on IL-6Rα expression. The expression of IL-6Rα was analyzed before and after the exposure of cells to ATRA at $10^{-6}$ mol/L for 3 days. These conditions were previously shown to result in downregulation of IL-6Rα, but not of gp130, in OPM-2 cells. Flow cytometric analysis (Table 1, column A,b) showed that while IL-6Rα expression of the parental and A1 clones was reduced after ATRA exposure to 40% to 58% of the untreated control, it was increased to 134% to 220% of the control in the remaining transfectant clones. The effect of ATRA on clone C5 was also analyzed by the radioligand binding assay. The binding sites per cell and the dissociation constant were 2,663/cell and $2.8 \times 10^{-11}$ mol/L before and 3,783/cell and $3.7 \times 10^{-11}$ mol/L after ATRA treatment, an increase in binding sites to 142% of the untreated control. This magnitude of increase was almost identical to that measured by flow cytometry shown above (139% of untreated control). There were no significant changes in the dissociation constant with ATRA treatment. The increase in IL-6Rα expression with ATRA treatment most likely reflected the effect of ATRA on CMV promoter. It is of interest to note that, for clone A1, whose transfected IL-6Rα cDNA was apparently lost or nonfunctional, IL-6Rα expression was also reduced with ATRA exposure, reflecting the expression of the endogenous IL-6Rα gene.

IL-6–stimulated tyrosine phosphorylation of gp130 (IL-6Rβ) in parental OPM-2 cells and transfectant C5 clone. The binding of IL-6 to IL-6Rα initiates homodimerization and tyrosine phosphorylation of gp130, an early step in IL-6 signal transduction. To determine the functionality of the expressed IL-6Rα in parental and transected cells, IL-6–induced gp130 phosphorylation was determined. Ten to $10^5$ cells were washed twice with serum-free RPMI medium, and “starved” in 1 mL of serum-free medium at 37°C for 1 hour. Recombinant human IL-6 (Immunex, Seattle, WA) was then added to the final concentration of 100 ng/mL. After 10 minutes of incubation, cells were harvested and cell lysates prepared. Lysates of OPM-2 and C5 cells pre-exposed to $10^{-6}$ mmol/L of ATRA for 3 days were similarly prepared. The phosphorylation of gp130 was analyzed after immunoprecipitation. The results (Fig 2) showed that the parental OPM-2 and C5 transfectant clones contained comparable quantities of gp130 protein (anti-gp130 immunoblots, lanes 1, 2, 5, and 6). The level of gp130 was not greatly affected by pretreatment with ATRA (lanes 3, 4, 7, and 8). The addition of IL-6 triggered a rapid tyrosine phosphorylation of gp130 in parental and C5 cells (antiphosphotyrosine immunoblots, lanes 2 and 6). In OPM-2 cells pretreated with ATRA, however, IL-6–induced gp130 phosphorylation was greatly reduced (lane 4), consistent with ATRA-induced down-

| Table 1. IL-6Rα (gp80) Expression and Clonogenic Growth in OPM-2 Parental Line and Transfectant Clones With or Without ATRA Pretreatment |
|---|---|---|---|
| Cell Line | A. IL-6Rα (mean channel no.) | B. Clonogenic Growth (colony/well, n = 3) |
| | (a) Mean Channel No. | (b) Post/Pre-ATRA* (%) Control | + ATRA† % Inhibition |
| OPM-2 parental line | 217 (100%)‡ | 130/225 (58%) | 186 ± 55 | 33 ± 8 | 82 |
| Transfectants, pooled | 619 (267) | — | — | — | — |
| Clone A1 | 164 (76) | 108/267 (40%) | 155 ± 54 | 13 ± 10 | 92 |
| Clone C2 | 436 (201) | 714/324 (220) | 95 ± 28 | 4 ± 1 | 96 |
| Clone C3 | 315 (145) | 457/341 (134) | 125 ± 80 | 5 ± 3 | 96 |
| Clone C4 | 1,043 (480) | 853/636 (134) | 178 ± 62 | 4 ± 5 | 98 |
| Clone C5 | 1,282 (590) | 943/677 (139) | 355 ± 48 | 13 ± 11 | 96 |
| Clone D6 | 709 (326) | 1,190/700 (170) | 119 ± 21 | 11 ± 11 | 91 |

*Treatment with $10^{-6}$ mmol/L of ATRA for 3 days. 
† Treatment with $6.1 \times 10^{-7}$ mmol/L of ATRA (IC50). 
‡Percent of the parental OPM-2 cells.
regulation of functional IL-6Rα. In contrast, substantial IL-6–
duced gp130 phosphorylation was observed in C5 cells
exposed to ATRA (lane 8), indicating that the expressed IL-6R
were functionally equivalent to the endogenous receptors.
Similar results were obtained in a repeat study.

Effect of ATRA on the clonogenic growth of parental and
transfected clones. Table 1 (columns B) summarizes the
results of the clonogenic growth assays of myeloma cells in the
presence or absence of 6.1 × 10^{-7} mmol/L of ATRA, a
concentration previously shown to cause 99% suppression of the
growth of parental OPM-2 cells (IC_{99}). The growth of parental
cells was suppressed by 82%, while the growth of transfected clones was similarly suppressed, ranging from 91% to 98%. Similarly, ATRA at 9.9 × 10^{-8} mmol/L (IC_{50}) suppressed the growth of parental and transfected clones to a similar degree, ranging from 60% to 81% (data not shown).

Analysis of the cell-cycle distribution showed that exposure of
cells to ATRA at IC_{50} for 2 days resulted in a reduction of cells in S phase and an accumulation of cells in G0/G1 phases in the transfected clones studied (A1, C4, and C5), similar to the parental OPM-2 cells (Table 2). ATRA at IC_{10}, however, was less effective on C4 and C5 clones. The results indicate a block at the G1-S phase transition (Table 2).

The drug sensitivity of the representative transfected C5
close was further analyzed in detail. The dose-response analysis yielded an IC_{50} of 3.0 × 10^{-8} mol/L for ATRA, compared with 9.9 ± 4.6 × 10^{-8} mol/L (n = 5) for the parental OPM-2 line. Similar to the parental line, the inhibition of C5 cells by ATRA was not reversed by IL-6: the colony growth was 14 ± 8/well (n = 3) with ATRA at IC_{50}, as compared with the control of 155 ± 47 colony/well, and was not reversed by the addition of 10 ng/mL of IL-6 (0.3 ± 0.6/well). The sensitivity to Dex was also analyzed, showing an IC_{50} of 9.1 × 10^{-8} mol/L, compared with 5.6 ± 1.5 × 10^{-8} mol/L (n = 6) for the parental line. The inhibitory effect of dexamethasone was again reversible: the colony growth in culture with Dex at IC_{50} was 1 ± 1/well (n =
3), compared with the control of 59 ± 7/well. The addition of IL-6 at 10 ng/mL to the Dex–treated culture yielded 74 ± 9 colony/well, reversing the inhibition by Dex. Similarly, the drug sensitivity profile of clone A1 was identical to that of the parental OPM-2 cells (data not shown). Thus, clone C5 and A1 cells, the latter serving, in effect, as vector control, were fully sensitive to IL-6 in reversing the inhibitory effect of Dex, indicating that the IL-6 postreceptor transduction pathway was not altered by the transfection processes and remained functionally intact.

Effect of ATRA on pRB phosphorylation and p21^{WAF1} expression. The above findings clearly indicate that the growth inhibition by ATRA is not mediated by modulation of IL-6R. Thus, we investigated the possibility that ATRA affects the IL-6 postreceptor pathway. We first examined its effect on pRB phosphorylation, as IL-6 has previously been shown to promote myeloma cell growth through pRB phosphorylation. Myeloma cells were exposed to 10^{-6} mol/L of ATRA. At 48 and 72 hours, aliquots of cells were obtained and total cellular proteins were extracted and immunoprecipitated. Western blot analysis (Fig 3) showed that pRB was constitutively phosphorylated (p-RB) in OPM-2 cells. Incubation with ATRA caused pRB dephosphorylation and dephosphorylated pRB was the predominant form after 72 hours of ATRA exposure. We next conducted Western blot analysis, screening for the expression of cell-cycle

<table>
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<th>Cell Lines</th>
<th>Treatment</th>
<th>G0/G1</th>
<th>S</th>
<th>M/G2</th>
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<tr>
<td>OPM-2</td>
<td>Medium</td>
<td>40</td>
<td>52</td>
<td>8</td>
</tr>
<tr>
<td>ATRA, IC_{99}</td>
<td>58</td>
<td>34</td>
<td>7</td>
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<tr>
<td>ATRA, IC_{90}</td>
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<tr>
<td>ATRA, IC_{99}</td>
<td>61</td>
<td>29</td>
<td>10</td>
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<td>ATRA, IC_{90}</td>
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<td>38</td>
<td>3</td>
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<td>ATRA, IC_{99}</td>
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<td>13</td>
<td>5</td>
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<tr>
<td>ATRA, IC_{90}</td>
<td>35</td>
<td>27</td>
<td>39</td>
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</tbody>
</table>

*Cells were cultured in medium with or without ATRA at 6.1 × 10^{-7}

designated as "IL-6R AND p21 IN ATRA EFFECT ON MYELOMA CELLS"
The activation of p21 and dephosphorylation of pRB is consistent with our previous finding that ATRA caused G1 arrest in OPM-2 cells.1 To determine whether the activation of p21 and dephosphorylation of pRB by ATRA is independent of IL-6R, we exposed C5 cells to 10^{-6} mol/L ATRA for 24 hours were analyzed by Western blot. The blots were sequentially stripped and probed with antibodies to p21, pRB, CDK2, and CDK4.

Figure 4 showed that p21 was upregulated by ATRA after 24 hours of ATRA exposure, while the expression of CDK2, CDK4, and p27 was not substantially affected. Kinetic studies of p21 upregulation showed that p21 was not or only minimally expressed in untreated cells, but was upregulated within 24 hours of ATRA exposure, and was markedly increased in 48 hours (Fig 5). The activation of p21 and dephosphorylation of pRB in OPM-2 and C5 cells was comparable to that of the parental OPM-2 cells. In contrast to the parental cells, ATRA treatment resulted in an increase in IL-6R expression in these transfectants. Thus, with the exception of clone A1, the transfected IL-6R cDNA in transfectants, which was not reversed by simultaneous treatment with IL-6, remained functionally intact. With functional IL-6R, the expressed IL-6R in transfectants, clone C5, which had the highest IL-6R expression, was analyzed in detail. It was shown that the gp130 content of C5 cells was comparable to that of the parental OPM-2 cells. In both OPM-2 and C5 cells, IL-6 induced rapid tyrosine phosphorylation of gp130, an early step in IL-6 signal transduction. Pretreatment of OPM-2 cells with ATRA greatly reduced the IL-6–induced phosphorylation of gp130, correlated with the downregulation of cellular IL-6R expression and the reduced binding of IL-6 shown previously.1 In contrast, C5 cells, pretreated with ATRA, continued to bind 125I-IL-6 with a dissociation constant comparable to that of the parental OPM-2 cells. In both parental and C5 clones, we found that both ATRA and Dex with comparable IC50. In both parental and C5 clones, IL-6 entirely reversed the inhibition by Dex but not by ATRA. Because evidence strongly indicated that Dex acted through downregulation of IL-6 expression, as discussed above, the retained Dex-sensitivity in C5 cells and its reversal by exogenous IL-6 strongly suggested that the IL-6 postreceptor signal transduction pathway was not altered by transfection and remained functionally intact. With functional IL-6R, reconstituted and an intact postreceptor transduction pathway, the

<table>
<thead>
<tr>
<th>CELLS</th>
<th>C5</th>
<th>OPM-2</th>
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<tbody>
<tr>
<td>ATRA</td>
<td>-</td>
<td>+</td>
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<tr>
<td>TIME (hr)</td>
<td>24</td>
<td>48</td>
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(a) Anti-p21WAF1
(b) Anti-CDK2

Discussion
To test the hypothesis that ATRA inhibited the growth of myeloma cells by downregulation of IL-6Rα expression, we constructed an IL-6Rα expression vector, in which IL-6Rα cDNA was linked to the CMV promoter. Transfection of OPM-2 myeloma cells with this vector yielded stable transfectants expressing high levels of IL-6Rα. In all six stable transfectants, except clone A1, high levels of cellular IL-6Rα were shown both by immunofluorescence with specific anti-human IL-6Rα antibody and 125I-IL-6 binding assay. The expressed IL-6Rα in these transfectants was shown to bind 125I-IL-6 with a dissociation constant comparable to that of the parental OPM-2 cells. In contrast to the parental cells, ATRA treatment resulted in an increase in IL-6Rα expression in these transfectants. Thus, with the exception of clone A1, the transfected IL-6Rα cDNA in transfectants, clone C5, which had the highest IL-6Rα expression, was analyzed in detail. It was shown that the gp130 content of C5 cells was comparable to that of the parental OPM-2 cells. In both OPM-2 and C5 cells, IL-6 induced rapid tyrosine phosphorylation of gp130, an early step in IL-6 signal transduction. Pretreatment of OPM-2 cells with ATRA greatly reduced the IL-6–induced phosphorylation of gp130, correlated with the downregulation of cellular IL-6Rα expression and the reduced binding of IL-6 shown previously.1 In contrast, C5 cells, pretreated with ATRA, continued to bind 125I-IL-6 with high affinity and the addition of exogenous IL-6 caused intense gp130 phosphorylation (Fig 2). Clearly, the expressed IL-6Rα in C5 cells in the presence of ATRA were functionally equivalent to endogenous receptors, capable of binding ligand and initiating gp130 phosphorylation. Comparing the drug sensitivity of the parental and C5 clones, we found that both clones were equally sensitive to the growth inhibitory effect of ATRA and Dex with comparable IC50. In both parental and C5 clones, IL-6 entirely reversed the inhibition by Dex but not by ATRA. Because evidence strongly indicated that Dex acted through downregulation of IL-6 expression, as discussed above, the retained Dex-sensitivity in C5 cells and its reversal by exogenous IL-6 strongly suggested that the IL-6 postreceptor signal transduction pathway was not altered by transfection and remained functionally intact. With functional IL-6Rα reconstituted and an intact postreceptor transduction pathway, the

Fig 5. Kinetic studies of ATRA effect on p21 expression in OPM-2 and C5 cells. Nuclear extracts from control cells and cells treated with 10^{-6} mol/L ATRA for 24 and 48 hours were analyzed by Western blot. The blots were sequentially stripped and probed with antibodies to p21 and CDK2.
(1) **CELL CYCLE DISTRIBUTION ANALYSIS:**

![Cell cycle distribution graphs](Fig 6)

(1) Control
- G0/G1: 45%
- S: 43%
- M/G2: 13%

(2) ATRA
- G0/G1: 56%
- S: 34%
- M/G2: 10%

(3) IL-6
- G0/G1: 33%
- S: 53%
- M/G2: 15%

(4) ATRA + IL-6
- G0/G1: 54%
- S: 36%
- M/G2: 10%

**FLUORESCENCE INTENSITY**

(2) **WESTERN BLOT ANALYSIS:**

![Western blot analysis](Fig 6)

A.  
B.  
C.
clonogenic growth of these transfectants, however, was still strongly inhibited by ATRA. Thus, in OPM-2 myeloma cells, the growth inhibitory effect of ATRA is clearly independent of the downregulation of IL-6Rα.

Studies on the postreceptor pathway showed that, in the parental OPM-2 cells, ATRA upregulated p21, but not CDK2, CDK4, or p27 expression, and caused dephosphorylation of pRB. Our previous studies have shown that the increased level of p21 was sufficient to inhibit CDK2 activity by 60%.16 Thus, upregulation of p21 and consequent dephosphorylation of pRB may be a key mechanism of ATRA effect in the G1 arrest and growth inhibition of myeloma cells. The role that IL-6R modulation may play in the activation of p21 and pRB by ATRA was further investigated by comparing the effect of ATRA in the parental OPM-2 and C5 clones. As shown above, the pattern of p21 upregulation and pRB dephosphorylation was identical between OPM-2 and C5 clones, indicating that their activation is independent of IL-6R expression. Reconstitution of functional IL-6R in C5 clone also failed to impart the reversibility of ATRA effect by IL-6, providing further evidence that the ATRA effect is not mediated through downregulation of IL-6R.

The exact mechanism of ATRA growth inhibition, however, requires further investigation. A disruption and, thus, a loss of IL-6 growth signaling remains a possibility, as a block at a site further downstream of IL-6 postreceptor signal pathway may still be present, even though the initial step of gp130 phosphorylation in IL-6 signal transduction is apparently unaffected by ATRA, as shown above. Because multiple alternative signaling paths exist subsequent to gp130 activation,17 it is also conceivable that ATRA may “redirect” IL-6 signaling away from growth, perhaps, toward differentiation signaling. Whether a loss of IL-6 growth signaling can lead to p21 upregulation is not known, although IL-6 has been shown to suppress Dex-induced p21 expression in IL-6–responsive myeloma cells.18 Alternatively, ATRA activation of p21 could be separate and independent of its effect on IL-6 signal transduction, and represents the triggering of a negative growth signal. This appears likely as it has been shown in U937 myelomonoblastic cells that ATRA induction of p21 was dependent on a retinoic acid response element in the p21 promoter.19 Thus, p21 could be directly upregulated through the family of RAR and RXR nuclear receptors. Furthermore, ATRA has been shown to upregulate STAT-1 in U937, NB4 promyelocytic, and MCF-7 breast cancer cells,20-22 raising the possibility that ATRA may additionally upregulate p21 through the induction of STAT1 that subsequently activates a STAT-responsive element in the p21 promoter.23 Further studies on the effect of ATRA on the IL-6 postreceptor signaling should be facilitated by the use of stable transfectants with constitutive IL-6Rα expression.

The present study is also of particular interest and relevance to the general concept of growth regulation through modulations of the expression of cytokine/growth factor receptors. There are many examples of changes in cytokine/growth factor receptors accompanying growth modulation by various agents. In myeloma, in addition to ATRA, interferons have also been shown to inhibit myeloma growth while downregulating IL-6Rα in some IL-6–dependent cell lines.24,25 In a variety of cell types, the inhibition of cell growth by retinoic acid is associated with modulations of a number of cytokine/growth factor receptors.26 For example, the growth arrest of an epidermoid carcinoma cell line27,28 and sensitive glioma cells29 is associated with reduced expression of the epidermal growth factor receptor (EGFR). On the other hand, RA-induced cellular differentiation and growth inhibition in embryonal carcinoma,30 neuroblastoma,31 and HL-60 leukemia cell lines32 is accompanied by an upregulation of transforming growth factor β receptors (TGFβR) and an induced-susceptibility to the growth inhibition by TGF-β, suggesting the activation of negative growth regulatory pathways. Other cytokines and hormones, such as TGF-β,33 tumor necrosis factor-α,34 interferon-γ,35 IL-2,36 and dihydrotestosterone,37 can also modulate various cytokine/growth factor receptors, including receptors for hematopoietic growth factors, TGF-β, and EGF, in cell types ranging from hematopoietic stem cell lines to prostate and hepatocellular carcinoma cell lines. Thus, despite the keen awareness of the complex, pleiotropic effects of cytokines and hormones and the recurring question of whether the alterations in receptors might be the consequence of cellular phenotypic changes associated with cell growth and differentiation,26 the modulation of cytokine/growth factor receptors has been widely implicated as a simple and yet elegant mechanism of the growth regulation by these agents.25,26,38 This concept, to our knowledge, has not been critically analyzed, and the present study represents the first attempt at formally testing this model. In our case, the failure of the reconstituted functional IL-6Rα to reverse the growth inhibition by ATRA is clearly inconsistent with the simple model of growth regulation through quantitative modulation of cytokine/growth factor receptors. Our study points out the need for careful validation of this concept in various systems, where modulations of cytokine/growth factor receptors have been implicated as the mechanisms of growth regulation by biologic and pharmacologic agents.

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REFERENCES

6. Shiao R-T, McLeskey SB, Khera SY, Wolfson A, Freter CE: Mechanisms of inhibition of IL-6-mediated immunoglobulin secretion
by dexamethasone and suramin in human lymphoid and myeloma cell lines. Leuk Lymphoma 21:293, 1996

Growth Inhibition of a Human Myeloma Cell Line by All-trans Retinoic Acid Is Not Mediated Through Downregulation of Interleukin-6 Receptors but Through Upregulation of p21WAF1

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