INHIBITORY EFFECT OF ALL-TRANS RETINOIC ACID ON THE GROWTH OF FRESHLY ISOLATED MYELOMA CELLS VIA INTERFERENCE WITH INTERLEUKIN-6 SIGNAL TRANSDUCTION

By Atsushi Ogata, Norihiro Nishimoto, Yoshihito Shima, Kazuyuki Yoshizaki, and Tadamitsu Kishimoto

We showed the dose-dependent growth inhibition by all-trans retinoic acid (ATRA) of myeloma cells freshly isolated from patients. ATRA downregulated the cell surface expression of interleukin-6 receptor (IL-6R) and/or glycoprotein (gp) 130. The growth-inhibitory activity of ATRA was well correlated with that of anti-gp130 antibody in every sample. Furthermore, ATRA inhibited the production of IL-6 from both myeloma cells and marrow stromal cells, and recombiant IL-6 (rIL-6) could partially recover the myeloma cell growth that had been inhibited by ATRA. These data suggest that ATRA may inhibit the proliferation of myeloma cells both by the downregulation of IL-6R and gp130 expression on myeloma cells and by the inhibition of IL-6 production from myeloma and stromal cells. Prednisolone (PSL) and interferon-gamma (IFN-γ) also inhibited the myeloma growth, while their effects were different from those of ATRA on IL-6 R and gp130 expression, IL-6 production, and morphological change. The inhibitory effect of ATRA on myeloma cell proliferation was observed in 10 of 14 samples obtained from eight patients, which suggests that ATRA may be a potent new therapeutic agent for some myeloma patients.

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MATERIALS AND METHODS

Patients. Eight patients with advanced multiple myeloma (patients A through H) (three with extramedullary plasmacytoma [A, B, and E] and one with plasma cell leukemia [C]) were examined for this study. To obtain highly purified myeloma cells, BM aspiration and tissue biopsy of patients with advanced diseases (stage IIIA or IIIB) were performed for analysis after informed consent was obtained in accordance with policies established by the institutional review board. Early-stage myeloma patients were also examined, but we could not obtain satisfactorily purified myeloma cells. BM samples were obtained from the patients on different days and after chemotherapy or radiation therapy.

Reagents. Recombinant human IL-6 (rIL-6) was provided by Ajinomoto (Kawasaki, Japan). ATRA, retinol (vitamin A), and retinol were purchased from Sigma Chemical (St Louis, MO). Anti–IL-6 MoAb, PM-1 (γ1κ), and anti-gp130 MoAbs, AM64 and gp22 (γ1κ) were generously donated by Tosoh (Kanagawa, Japan). Isotype-matched MoAb, MPC.C2 (γ1κ) was provided by Cappel (West Chester, PA), and interferon-gamma (IFN-γ) was obtained from Hayashibara Biochemical Laboratories (Okayama, Japan).

Purification of myeloma cells. The purification procedure consisted of Iwato's method with some modification. Briefly, myeloma cells were isolated from BM aspirates, surgically removed tumors of plasmacytoma or venopunctured peripheral blood of a patient with plasma cell leukemia, followed by separation with Ficol-Caque density gradient (Pharmacia LKB, Uppsala, Sweden). The mononuclear cells (MNCs) at the interface were collected and washed (3 times) with RPMI-1640 medium (Nikken Bio-Medical Laboratories, Osaka, Japan) supplemented with 10% fetal calf serum (FCS). The cell concentration was adjusted to 1 × 10^6 cells/mL with RPMI-1640 medium.
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Table 1. DNA Synthesis of Each Myeloma Sample Cultured With rIL-6 or ATRA

<table>
<thead>
<tr>
<th>Patient</th>
<th>Stage</th>
<th>Sample*</th>
<th>% Myeloma Cells</th>
<th>rIL-6 (cpm)</th>
<th>3H-TdR Uptake</th>
<th>IFN-γ (Δcpm)</th>
<th>ATRA (cpm)</th>
<th>% Inhibition†</th>
<th>IL-6R</th>
<th>gp130</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>III-A</td>
<td>1</td>
<td>99</td>
<td>17,188 ± 451</td>
<td>31,641 ± 857</td>
<td>1.84</td>
<td>1,188 ± 5</td>
<td>99.3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>99</td>
<td>4,624 ± 1,224</td>
<td>7,343 ± 335</td>
<td>1.59</td>
<td>230 ± 4</td>
<td>95.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>B</td>
<td>III-B</td>
<td>3</td>
<td>99</td>
<td>54,825 ± 2,141</td>
<td>72,523 ± 3,082</td>
<td>1.32</td>
<td>15,141 ± 1,703</td>
<td>73.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C</td>
<td>III-B</td>
<td>4</td>
<td>70</td>
<td>20,164 ± 950</td>
<td>27,804 ± 689</td>
<td>1.38</td>
<td>1,223 ± 44</td>
<td>93.1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>80</td>
<td>28,169 ± 584</td>
<td>35,101 ± 561</td>
<td>1.25</td>
<td>7,302 ± 442</td>
<td>74.1</td>
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<td>+</td>
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<td>6</td>
<td>99</td>
<td>6,209 ± 454</td>
<td>20,140 ± 323</td>
<td>3.24</td>
<td>1,676 ± 175</td>
<td>73.0</td>
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<td>ND</td>
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<tr>
<td>D</td>
<td>III-B</td>
<td>7</td>
<td>80</td>
<td>3,344 ± 155</td>
<td>5,259 ± 35</td>
<td>1.57</td>
<td>2,067 ± 80</td>
<td>30.9</td>
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<td>+</td>
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<tr>
<td>E</td>
<td>III-A</td>
<td>8</td>
<td>70</td>
<td>2,670 ± 81</td>
<td>3,887 ± 230</td>
<td>1.35</td>
<td>2,116 ± 191</td>
<td>24.5</td>
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<td>+</td>
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<tr>
<td>F</td>
<td>III-A</td>
<td>9</td>
<td>421 ± 31</td>
<td>598 ± 57</td>
<td>1.42</td>
<td>287 ± 11</td>
<td>31.7</td>
<td>ND</td>
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<td></td>
<td></td>
<td>10</td>
<td>455 ± 20</td>
<td>528 ± 57</td>
<td>1.16</td>
<td>308 ± 12</td>
<td>32.3</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>99</td>
<td>2,422 ± 343</td>
<td>2,243 ± 88</td>
<td>-0.8</td>
<td>2,692 ± 195</td>
<td>-11.1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>G</td>
<td>III-B</td>
<td>12</td>
<td>99</td>
<td>22,366 ± 561</td>
<td>42,288 ± 561</td>
<td>1.89</td>
<td>30,061 ± 377</td>
<td>-34.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13</td>
<td>99</td>
<td>76,563 ± 2,039</td>
<td>86,473 ± 2,008</td>
<td>1.13</td>
<td>81,425 ± 2,316</td>
<td>-6.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>H</td>
<td>III-A</td>
<td>14</td>
<td>70</td>
<td>11,666 ± 703</td>
<td>12,373 ± 709</td>
<td>1.06</td>
<td>11,962 ± 182</td>
<td>-0.1</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.
* Samples were obtained from the patient on different days before and after chemotherapy or radiation therapy.
† SI (stimulation index) = 3H-TdR uptake with IL-6/3H-TdR uptake without IL-6.
‡ % inhibition = (1 - [3H-TdR uptake with ATRA]/3H-TdR uptake without ATRA]) × 100.

To ascertain the inhibitory effect of ATRA on the growth of myeloma cells, purified myeloma cells from patients were cultured for 72 hours with ATRA from 10^{-6} to 10^{-4} mol/L. Representative data (sample 1) are shown in Fig 1. As shown in Fig 1A, ATRA inhibited the proliferation of freshly isolated myeloma cells in a dose-dependent manner, and the cell growth was almost completely inhibited at the 10^{-4} mol/L.

Because ATRA is metabolized from retinol through retinal stage,16 retinol, retinal, and ATRA were examined for their capability to inhibit the growth of myeloma cells. Figure 1B clearly shows that both retinol and retinal could not inhibit the proliferation of the cells even at a dose of 10^{-6} mol/L. However, ATRA showed a strong inhibitory effect, which suggests that myeloma cell growth inhibition is not a common characteristic of vitamin A derivatives.

To compare the growth-inhibitory effect of ATRA with that of other inhibitory agents, the purified myeloma cells were cultured with the optimal doses of IFN-γ and PSL, as well as ATRA, for 72 hours. IFN-γ (100 U/mL) and PSL (10^{-7} mol/L) inhibited the cell growth less than ATRA did (Fig IC).

Modulation of IL-6R and gp130 expression by ATRA. Because ATRA was reported to downregulate IL-6R expression on a myeloma cell line,17 the modulation of IL-6R and gp130 on ice for staining. Isotype-matched MoAb, MOPC-21 (γ1κ), served as the negative control. Flow-cytometric analysis was performed on a FACSscan (Becton Dickinson, Mountain View, CA).

RESULTS

Inhibition of myeloma cell growth. To ascertain the inhibitory effect of ATRA on the growth of myeloma cells, purified myeloma cells from patients were cultured for 72 hours with ATRA from 10^{-10} to 10^{-6} mol/L. Representative data (sample 1) are shown in Fig 1. As shown in Fig 1A, ATRA inhibited the proliferation of freshly isolated myeloma cells in a dose-dependent manner, and the cell growth was almost completely inhibited at the 10^{-4} mol/L.

Preparation of stromal cells. BM MNCs prepared as described earlier were cultured at 1 × 10^7/mL with RPMI-1640 medium supplemented with 10% fetal calf serum and 1 × 10^{-5} mol/L 2-mercaptoethanol (2-ME; Nakarai Tesque, Kyoto, Japan) in 24-well culture plates (Costar) for 24 hours. After removal of nonadherent cells, adherent cells on plastic plates were used as freshly isolated stromal cell fractions for testing IL-6 production. A BM stromal cell line derived from multiple myeloma, MM3-M5, was established by transfecting the plasmid for expression of SV40 large T Ag, and was kindly provided by Dr T. Kaihara (Biomedical Research Center, Osaka University).16

Preparation of culture supernatants and detection of IL-6. Purified myeloma cells were cultured at a density of 1 × 10^5/mL on 24-well plates for 48 hours with ATRA, IFN-γ, or PSL. Freshly isolated stromal cells derived from 1 × 10^7 MNCs and a stromal cell line, MM3-M5, at a density of 1 × 10^4/mL were cultured for 48 hours with ATRA, IFN-γ, or PSL. IL-6 in the culture supernatant was measured by a dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA).17

Phenotypic analysis. Cell surface IL-6R and gp130 of freshly isolated myeloma cells and of cells cultured with 10^{-6} mol/L ATRA, 100 U/mL IFN-γ, or 10^{-7} mol/L PSL for 48 hours were identified by biotinylated PM1 and AM64 or gpX22 MoAb, respectively, in conjunction with phycoerythrin-labeled streptavidine. A quantity of 2 × 10^6 cells was incubated with 0.5 mg of antibody for 20 minutes
expression on freshly isolated myeloma cells by ATRA was examined by means of flow-cytometric analysis using anti-IL-6R MoAb, PM1, and anti-gp130 MoAbs, AM64 and gp22. A representative fluorescence-activated cell sorter (FACS) profile is shown in Fig 2. Before culture with ATRA, all of the freshly isolated myeloma cells expressed gp130 and all except patient D expressed IL-6R on their cell surface, regardless of IL-6-dependent growth. Mean fluorescence intensity detected by FACS was different from patient to patient (Fig 2). As shown in Table 1, IL-6R or gp130 expression was partially downregulated by ATRA in seven of nine myeloma samples. The expression of IL-6R was downregulated by ATRA in four of nine samples. Furthermore, ATRA also downregulated the expression of gp130 in four of nine samples. One of these samples showed downregulation of both receptors. No downregulation of IL-6R or gp130 was observed in two samples. These data suggest that the downregulation of IL-6R and/or gp130 by ATRA may be one of the causes of cell growth inhibition.

On the contrary, PSL upregulated IL-6R or gp130 in seven of nine samples. IFN-γ downregulated IL-6R in five of nine samples, while it affected gp130 in two samples.

Inhibition of IL-6 production both from myeloma cells and from stromal cells by ATRA. Because it is known that IL-6 mediates autocrine and paracrine regulation of myeloma cell growth, the question whether ATRA inhibits IL-6 pro-
ATRA INHIBITS THE GROWTH OF MYELOMA CELLS

ATRA inhibits the growth of myeloma cells. ATRA (10^{-8} mol/L) also inhibited the production of IL-6, whereas IFN-γ (100 U/mL) enhanced it. These results suggest that the growth inhibition of myeloma cells by ATRA may be partially caused by the inhibition of IL-6 production from both myeloma cells and stromal cells.

IL-6 partially reactivated myeloma cell growth that was inhibited by ATRA. To confirm that the decrease in IL-6 production plays a part in the growth-inhibitory effect of ATRA, we examined whether exogenous IL-6 was able to rescue the myeloma cell growth that had been inhibited by ATRA. In fact, the growth inhibition by ATRA was partially counteracted by additional rIL-6 as shown in Fig 5, indicating that the inhibition of IL-6 production by ATRA may be partly responsible for the inhibitory effect of ATRA on the proliferation of myeloma cells.

Morphologic analysis of cultured myeloma cells. Morphologic analysis of myeloma cells was performed before and after the in vitro culture with ATRA, IL-6, PSL, and IFN-γ for 48 hours. Cultured cells and their nuclei without any reagent (Fig 6B) were larger than freshly isolated myeloma cells (Fig 6A). However, the cells cultured with ATRA (Fig 6C) were small with a dense nucleus, which suggests that they were dormant, while some of the cells had died. On the other hand, the cells with rIL-6, PSL, or IFN-γ appeared to be the same as those without any reagent (Fig 6D, E, and F).

Correlation between the antiproliferative effect of ATRA and of anti-gp130 MoAb on myeloma cells. Table 1 summarizes the inhibitory effect of ATRA on the proliferation of 14 myeloma cell samples obtained from eight patients. In addition, Table 1 shows the augmentation of their growth caused by rIL-6. ATRA strongly inhibited the growth of myeloma cells in six of 14 samples, whereas a less inhibitory effect was observed in four samples. In contrast, no inhibitory effect was observed in four samples, and one showed augmented growth by ATRA. These different effects of ATRA were closely related to the growth-inhibitory effect of anti-gp130 MoAb (Fig 7). The myeloma cells in which proliferation was inhibited by greater than 50% by ATRA showed greater than 30% growth inhibition by anti-gp130 MoAb. On the other hand, the myeloma cells in which proliferation was inhibited by less than 50% by ATRA showed less than 30% growth inhibition by anti-gp130 MoAb.

Fig 4. IL-6 production by stromal cells derived from myeloma patients. (A and B) Stromal cells freshly isolated from 1 x 10^6/mL of BM MNC were cultured for 48 hours. (C) SV40-transformed stromal cell line, MM3-3 cells (1 x 10^4/mL per well) were cultured for 48 hours with varying concentrations of ATRA. IL-6 in the culture supernatant was measured by the DELFIA method.

Fig 5. Additional rIL-6 partially recovered the myeloma cell growth that was inhibited by ATRA. Myeloma cells (sample 5) were cultured with 10^{-8} mol/L ATRA in the presence or absence of 10 ng/mL rIL-6. DNA synthesis was measured by 3H-TdR incorporation after a 3-day culture.
over, the rate of inhibition by ATRA closely correlated with that by anti-gp130 MoAb \( (y = 0.79x - 11.2) \), where \( y \) is the percent inhibition by anti-gp130 MoAb, and \( x \) is the percent inhibition by ATRA; \( r = .78 \). These data indicate that the inhibitory effect of ATRA on the growth of myeloma cells varied from patient to patient. However, because the inhibitory effect of ATRA closely correlated with that of anti-gp130 MoAb, growth inhibition of myeloma cells by ATRA may be caused by blocking the growth signal transduction of IL-6.

**DISCUSSION**

We demonstrated that ATRA inhibited the growth of freshly isolated myeloma cells in vitro. ATRA is a well-known therapeutic agent that can differentiate APL cells into mature granulocytes. APL is often associated with the translocation t(15;17) and the gene encoding the retinoic acid receptor-\( \alpha \) (RAR-\( \alpha \)) maps on chromosome 17q21. Because the translocation alters the transacting properties of RAR-\( \alpha \), only a high dose of ATRA can differentiate APL cells into mature granulocytes. In human multiple myeloma, such translocation has not been reported. Therefore, the mechanism of ATRA action on myeloma cells seems to be different from that on APL cells.

To analyze the mechanism of the antiproliferative activity of ATRA on myeloma cells, we examined the effects of ATRA on both the expression of IL-6R and gp130 by myeloma cells, as well as the production of IL-6 by myeloma cells and stromal cells.

In 1991, Sidell et al. showed the antiproliferative activity of ATRA on a myeloma cell line, AF10, which grew autocrinely in an IL-6-dependent manner. They demonstrated that the antiproliferative activity of ATRA might be a result of the downregulation of IL-6R expression on myeloma cells. Their observation is consistent with our result that the expression of IL-6R was downregulated by ATRA in four of nine cases. Furthermore, ATRA also downregulated the expression of gp130 in four of nine cases. In short, IL-6R and/or gp130 expression was partially downregulated by ATRA in seven of nine myeloma samples. Although downregulation of these receptors was also observed in some myeloma cells in which growth was independent of IL-6 by in vitro assay, ATRA could not inhibit their growth. These data suggest that the antiproliferative activity of ATRA may be the result of the downregulation of IL-6R and/or gp130 only in the myeloma cases in which growth might be dependent on IL-6. This idea was also supported by the observation of a strong correlation between the antiproliferative effect of ATRA and that of anti-gp130 MoAb in every sample \( (r = .78, P < .001; \text{Table 1 and Fig 7}) \). However, the question still remains why the growth stimulation by additional IL-6 apparently did not correlate with the inhibitory effect of
ATRA inhibits the growth of myeloma cells

ATRA. One possible answer is that in vivo stimulation of cells by IL-6 may decrease the response of freshly isolated myeloma cells to the additional IL-6 in vitro. Another is that in vitro production of IL-6 in the myeloma cell fractions may increase the background response, thereby decreasing the stimulation index of the additional IL-6. A third reason may be the redundancy among IL-6, leukemia inhibitory factor (LIF), and oncostatin M (OSM), which use the same signal transducer, gp130. Recently, we and another laboratory identified the cases that could respond to LIF, OSM, and IL-6.24,25 Because ATRA downregulates gp130, ATRA may inhibit the growth of myeloma cells stimulated by LIF and OSM, as well as by IL-6 in some samples.

We also showed the inhibition of IL-6 production by ATRA in myeloma cell fractions. It is known that IL-6 is an autocrine growth factor for myeloma cells,1 whereas Klein et al2 reported the paracrine but not autocrine regulation of the growth of myeloma cells by IL-6, which was secreted from the stromal cells in the BM of their patients. Recently, Uchiyama et al26 showed that the adhesion of myeloma cell lines to normal BM stromal cells triggered IL-6 production by stromal cells, rather than by myeloma cell lines. We showed the inhibition of IL-6 production in both freshly isolated BM stromal cells and SV40-transformed BM stromal cells established from a patient with multiple myeloma. This suggests that ATRA may inhibit the IL-6 production from stromal cells, as well as myeloma cells. Therefore, even though the purified cell fraction was contaminated with a few stromal cells, ATRA must have inhibited their IL-6 production and ultimately blocked the autocrine and paracrine growth of myeloma cells. Furthermore, the finding that the growth inhibition of myeloma cells by ATRA was partially counteracted by the reconstitution of exogenous rIL-6 through the residual receptors confirmed that some of the growth inhibition by ATRA was caused by the inhibition of IL-6 production. The rest of the growth inhibitory effect may be caused by the partial downregulation of IL-6R and gp130.

A comparative study of ATRA, PSL, and IFN-γ suggested that IFN-γ inhibited the growth of freshly isolated myeloma cells by the downregulation of IL-6R. Previous reports have presented contradictory data on the modulation of IL-6R expression by IFN-γ.27,28 Portier et al29 demonstrated that IFN-γ downregulated IL-6R, whereas Jernberg-Wiklund et al30 maintained it did not. Our data support the former. However, the therapeutic application of IFN-γ must be performed carefully, because IFN-γ stimulated the IL-6 production from myeloma cells in vitro. PSL showed similar effect to that of ATRA on IL-6 production. The myeloma cells cultured in vitro appeared larger in size and their cytoplasm was more abundant than that of freshly isolated cells, which suggests that the former may have been stimulated by IL-6 secreted from myeloma cells or stromal cells in vitro. The myeloma cells cultured with PSL and IFN-γ appeared morphologically identical to the cells cultured with or without rIL-6. However, the myeloma cells cultured with ATRA appeared to be dormant or dead, which suggests that the mechanism of ATRA action may be different from that of PSL or IFN-γ.

Generally, retinol is incorporated in target cells and converted to retinoic acid (RA) through the retinal stage.14 This converted RA was shown to inhibit the proliferation and IL-6 production in normal B cells.8 However, activated B cells, which possess the receptors for RA, could not convert retinol into RA.29 Our data show that ATRA, but not retinol or retinal, inhibits the growth of myeloma cells. Therefore, it is suggested that retinol may not be converted to RA in myeloma cells such as activated B cells, and that retinol and retinal are not able to inhibit the growth of myeloma cells. We did not analyze the effect of 13-cis RA on myeloma cells, but 13-cis RA may also have an inhibitory effect similar to that of ATRA.

In this report, we showed that ATRA inhibits myeloma cell growth in vitro. ATRA can interfere with the autocrine and paracrine loop of IL-6–dependent myeloma cell growth both by downregulation of IL-6R and gp130 expression and by inhibition of IL-6 production. This suggests that ATRA is a potential therapeutic agent for IL-6–dependent myeloma. We analyzed the inhibitory effect of ATRA on myeloma cells only from the patients with advanced disease to obtain highly purified myeloma cells. Thus, we do not know whether myeloma cells from early stages can respond to ATRA. Recently, Vesole et al31 tested the therapeutic efficacy of ATRA in 11 myeloma patients in vivo but could not

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**Fig 7. Correlation between the antiproliferative effect of ATRA and that of anti-gp130 MoAb.** Purified myeloma cells were cultured with 10⁻⁶ mol/L ATRA or 10 μg anti-gp130 MoAb. DNA synthesis was measured by [³H-TdR incorporation after a 3-day culture. % inhibition = [1·(³H-TdR uptake with ATRA or anti-gp130 MoAb/³H-TdR uptake without reagent)] × 100.
obtain any positive data.\textsuperscript{30} Taken together with the recent report, myeloma cells from advanced cases could respond to IL-6.\textsuperscript{31} The myeloma patients reported by Vesole et al might be with early stage myeloma and their myeloma cells might not show the IL-6-dependent growth. Therefore, it is important to check the efficacy of ATRA in every myeloma patient before clinical application, while further studies are required to ascertain the clinical utility of ATRA.

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REFERENCES

Inhibitory effect of all-trans retinoic acid on the growth of freshly isolated myeloma cells via interference with interleukin-6 signal transduction [see comments]

A Ogata, N Nishimoto, Y Shima, K Yoshizaki and T Kishimoto