Advances in Brief

Effects of All-trans Retinoic Acid and Antireceptor Antibodies on Growth and Programmed Cell Death of Human Myeloma Cells

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Abstract

In contrast to cytotoxic agents inducing rapid cell death, biological agents such as hormones, vitamins (e.g., retinoids), cytokines, and antireceptor antibodies act slowly and may alter ratios between cell growth and programmed cell death (apoptosis). We showed previously that anti-interleukin 6 (IL-6) and antitransferrin (Tf) receptor antibodies inhibited in vitro growth and induced death of myeloma cells. Retinoids also inhibit in vitro growth of human cancer cells and decrease IL-6 receptor display and autosecretion by some myeloma cells. Retinoids may also antagonize in vitro growth-promoting effects of iron and transferrin. To develop a novel strategy for treating myeloma, we examined antiproliferative and cytotoxic effects of retinoids in combination with anti-Tf or anti-IL-6 receptor antibodies. Myeloma cell lines were cultured with retinoids with or without anti-growth factor receptor monoclonal antibodies. Both all-trans retinoic acid (ATRA) and 13-cis-retinoic acid showed variable, dose-dependent inhibition of myeloma cell line growth. ATRA also induced significant down-regulation of myeloma IL-6 receptors and inhibited IL-6 autosecretion by myeloma cells. Antiproliferative effects of ATRA were increased by coculture with anti-Tf but not anti-IL-6 receptor antibodies. Colony-forming assays showed that antiproliferative effects of anti-Tf receptor antibodies were largely reversible, but 1 μM ATRA was cytotoxic to myeloma cells. To assess apoptosis, a flow cytometry assay detecting DNA damage was used. Using previously studied cell line models, flow cytometry detected programmed cell death induced by transforming growth factor β1 in leukemia cells and by anti-growth factor receptor antibody treatment of IL-6-dependent myeloma cells, treatments which caused only modest increases in the percentage of cells undergoing morphological apoptosis and increased internucleosomal DNA degradation. Flow cytometry analysis of ATRA and anti-Tf antibody-treated myeloma cells also showed evidence for apoptosis induced by ATRA, but not with anti-Tf receptor antibodies. These changes were apparent several days before detection of internucleosomal DNA degradation on agarose gels in 8226 cells but were not detected at any time in U266 cells, which underwent cell death but showed no DNA damage using flow cytometry or degradation on agarose gels. Retinoids merit further study as possible maintenance or chemoprevention therapies for clonal plasma cell disorders and for treating paraneoplastic disorders such as Castleman’s disease. Flow cytometry rapidly detects apoptosis induced by biological agents and may be useful for in vitro screening of novel biological therapies.

Introduction

ATRA and other retinoids inhibit in vitro growth of a variety of malignant human cells (1–4) and induce complete remissions in patients with acute promyelocytic leukemia (5, 6). ATRA also has clinical activity against tumor cells used alone (7) and in combination with other agents (8–10). Previous studies showed ATRA decreased the IL-6 receptor display by a malignant plasma cell line (11) and inhibited in vitro maturation of plasma cell precursors in patients with MGUS (12). ATRA also inhibits an iron-dependent membrane reductase which may regulate normal and malignant cell growth (13).

Combining agents which block effects of growth factors and direct chemical receptor, antagonists such as antireceptor antibodies, provides an innovative approach to cancer therapy (14). Because we recently showed anti-Tf and anti-IL-6 receptor antibodies inhibit growth and were cytotoxic to some myeloma cells (14), we examined in vitro antiproliferative effects of ATRA combined with these antibodies against human myeloma cell lines.

Control of PCD (apoptosis) has emerged as an important mechanism regulating normal and malignant cell growth (15–19). Genes which alter apoptosis play important roles in the pathogenesis of lymphomas and as mediators of anticancer drug resistance (15, 19). In some cells, apoptosis is induced by growth factor deprivation, while in others, receptors and ligands (e.g., fas) directly trigger apoptosis (16–19). Recent studies showed retinoids induced apoptosis, usually in conjunction with cell differentiation (20). Because of its importance in these multiple settings, modulation of apoptosis is a target for new anticancer therapies including antisense DNA and anti-growth factor receptor monoclonal antibodies (15, 16, 19).

In previous studies, we examined in vitro effects of growth factors and anti-growth factor receptor antibodies on the growth
of malignant hemopoietic cells (14, 21, 22). Antiproliferative effects of some of these biologically active agents in short-term cultures were modest, but long-term growth and colony assays showed extinction of cell growth (14, 21). These results were consistent with increased rates of apoptosis and attributed to relative decreases in the ratio of cells “born” in culture and to those undergoing apoptosis (14, 19). This caused us to evaluate a more rapid in vitro assay for detecting apoptosis induced by biologically active agents such as anti-receptor antibodies and ATRA.

Materials and Methods

Cell Culture. Factor-dependent human myelogenous leukemia cell lines UCSD/AML1, AML-OCI1, and AML-193 were grown as previously described in recombinant human GM-CSF (23). Factor-independent HL60 leukemia cells were grown with RPMI 1640 with 5% fetal bovine serum. Factor-independent myeloma cell lines 8226 and U266 and IL-6-dependent OCI-My4 myeloma cells were grown as described (14). We showed previously that 8226, U266, and OCI-My4 cells express receptors for IL-6 and that U266 cells contain IL-6 RNA and autosecrete and respond to IL-6 protein (14).

Materials. Recombinant human TGF-β1 was kindly provided by Dr. Patricia Segarini (Celtrix, Inc., Santa Clara, CA). Anti-Tf receptor monoclonal antibodies A27.15 and E2.3 and anti-IL-6 receptor p80 antibody AUK 64–7 were purified as described previously (14). ATRA and 13-cis-retinoic acid were obtained from Sigma (St. Louis, MO), dissolved in DMSO, and stored at −70°C shielded from light. Experiments using ATRA were performed under low light conditions using DMSO controls. TdT, biotin-labeled dUTP, and avidin-FITC were obtained from Boehringer Mannheim (Indianapolis, IN).

Cell Growth Assays. Cell growth was assessed using [³H]thymidine uptake or viable cell counts as described (21, 24). Cells were grown in the continuous presence of varying concentrations of ATRA or 13-cis-retinoic acid alone or with ATRA and 10 μg/ml each of anti-Tf and anti-IL-6 receptor antibodies A27.15 and E2.3 or 10 μg/ml anti-IL-6 receptor antibody AUK 64–7. ATRA concentrations spanned achievable plasma concentrations demonstrated in clinical trials (25). To assess cytotoxicity, 8226 or U266 cells were grown for 1 to 3 days in the presence of 1 μM ATRA alone or with anti-Tf receptor antibodies, washed, and plated in methylcellulose as described (14). 8226 and U266 cells were also grown with 1 μM ATRA with or without 100 units/ml IL-6 and plated in colony assays. Colonies of >40 cells were scored after 7–14 days.

Assay for IL-6 Receptors and IL-6. IL-6 receptors were detected using immunofluorescence staining with phycoerythrin-conjugated IL-6 (R&D Diagnostics, Minneapolis, MN; Ref. 14). IL-6 was assayed in U266-conditioned medium using an ELISA (R&D Diagnostics; Ref. 14). IL-6 was assayed in U266-conditioned medium using an ELISA (R&D Diagnostics; Ref. 14).

Assessment of DNA damage (“nicking”) consistent with apoptosis was performed using flow cytometry and a modification of the technique described by Del Bino et al. (26). UCSD/AML1 or AML-193 cells were incubated for 1 to 4 days with GM-CSF alone or with 1 ng/ml TGF-β1 (21). OCI-My4 cells were grown with phytohemagglutinin leukocyte-conditioned medium containing IL-6 (14) or with conditioned medium in the presence of 10 μg/ml anti-Tf receptor antibodies and 10 μg/ml anti-IL-6 receptor antibody for 1 to 3 days (14). These culture conditions were identical to those used to assess growth inhibition and apoptosis previously, except for substitution of phytohemagglutinin leukocyte-conditioned medium for IL-6 in OCI-My4 cultures (14, 21). 8226 and U266 cells were studied after incubation with 1 μM ATRA alone, 10 μg/ml each of the anti-Tf receptor antibodies, or anti-Tf receptor antibodies and ATRA for 1 to 4 days.

Control or treated cells were washed in PBS and fixed in 1% buffered formaldehyde (pH 7.5) for 15 min at 4°C. The fixed cells were washed in PBS and stored in 2 ml 70% ethanol at −20°C until labeled. To label damaged DNA (“nicks”) using TdT, cells were rehydrated in PBS and suspended in cacodylate buffer (0.2 M potassium cacodylate, 25 mM Tris-HCl, 0.25 mg/ml BSA, 2.5 mM CoCl₂ plus 0.5 nmol biotin-dUTP, and 25 units TdT). DNA nicks were detected by incubating with avidin-FITC (4× SSC, 0.1% Triton X-100, 5% nonfat dry milk, and 2.5 μg/ml avidin-FITC) for 30 min in the dark. The labeled nuclei were washed with PBS/0.1% Triton X-100 and counterstained with propidium iodide (5 μg/ml) for DNA content. Red/green fluorescence was detected by excitation at 488 nm using 525- and 620-nm filters for two-color analysis. Positive cells were identified using cells incubated with unlabeled dUTP as a control.

Statistics. Differences between treated and control groups were detected using ANOVA or Student’s t test for paired variables.

Results

ATRA was reported to interfere with an iron-dependent membrane reductase required for malignant cell growth and induced IL-6 receptor down-regulation on malignant plasma cells (11, 12), while antibodies inhibiting the function of Tf or IL-6 receptors were previously shown to inhibit myeloma cell growth in vitro (14). To assess possible additive or synergistic inhibition by ATRA and anti-receptor antibodies on myeloma cells, we examined the effects of ATRA in combination with anti-Tf or anti-IL-6 receptor antibodies (Fig. 1) on myeloma cell growth. Consistent with results using many other malignant cells, ATRA caused variable dose-dependent growth inhibition of the myeloma cells. At concentrations up to 1 μM, ATRA and 13-cis-retinoic acid did not inhibit OCI-My4 cell growth (data not shown), and these cells were not studied further. ATRA caused dose-dependent inhibition of 8226 and U266 cells (Fig. 1), and similar or identical results were observed using 13-cis-retinoic acid (Fig. 1).

Consistent with results obtained previously (14), anti-Tf receptor antibodies also inhibited 8226 cell growth, but had little...
effect on the growth of U266 cells (Fig. 1). Similarly, the anti-IL-6 receptor antibody inhibited growth of the U266 cells which autosecrete IL-6 (Fig. 1A), but had no effect on the factor-independent 8226 cells (Fig. 1B; Ref. 14). Combining ATRA with anti-IL-6 receptor antibody did not appear to increase growth inhibition, but additive inhibition was observed when ATRA and anti-Tf receptor antibodies were used in combination (Fig. 1).

To assess cytotoxicity of ATRA combined with anti-Tf receptor antibodies, U266 cells were grown with 1 μM ATRA, anti-Tf receptor antibodies, or a combination and plated in colony-forming assays (14). Consistent with previous studies (14), growth inhibition of U266 cells by anti-Tf receptor antibodies was largely reversible (Fig. 2A). In contrast, ATRA caused loss of colony-forming capacity after 1–3 days. More pronounced ATRA activity was seen against 8226 cells (Fig. 2B). Although substantial cytotoxicity was observed against both 8226 and U266 cells, viable colony-forming cells were recovered each day, indicating cytotoxicity was incomplete and ATRA-resistant cells were present. Incubation of 8226 cells with ATRA plus IL-6 did not significantly alter cytotoxicity. Colony survival for 8226 cells at day 1 was 9 ± 3% with ATRA alone and 16 ± 10% with ATRA + IL-6 (n = 3; P, not significant).

Previous studies showed that ATRA inhibited IL-6 receptor display by a subline of U266 myeloma cells (11), and suggested that this activity of retinoids was important to growth inhibition of myeloma cells. We confirmed that ATRA induced down-regulation of IL-6 receptor display of 8226 and unselected U266 cells (Fig. 3; P = 0.007 for each cell line). We also found that the IL-6 receptor display fell with increasing cell density in culture. In more rapidly growing 8226 cells, an initial decrease in cell density after cultures were split resulted in a transient increase in IL-6 receptors (Fig. 3B). Effects of ATRA on IL-6

**Fig. 1** Growth of U266 (A) or 8226 (B) myeloma cells in the presence of various concentrations of ATRA alone (■) or 13-cis-retinoic acid (◇), or with anti-Tf receptor antibodies (●) or anti-IL-6 receptor antibody (□). Bars, SE.

**Fig. 2** Effects of ATRA or anti-Tf receptor antibodies on growth of U266 (A) or 8226 (B) colony-forming cells. Cells were grown under control conditions with DMSO and 1 μM ATRA (■), 10 μg/ml anti-Tf receptor antibodies (●), or a combination of ATRA and antibodies (□) for 1 to 3 days. Cells were then washed and plated in colony-forming assays. Results are the means of three to four experiments. Bars, SE.
receptor display were apparent on the first and second days of culture when there was no significant difference in cell density of control and ATRA-treated cultures. ATRA also suppressed autosecretion of IL-6 into conditioned medium by U266 cells [U266 control day 1, 27 ± 10 pg/10⁶ cells; ATRA-treated cells day 1, 15 ± 7 pg/10⁶ cells; U266 control day 2, 25 ± 12 pg/10⁶ cells; ATRA day 2, 13 ± 9 pg/10⁶ cells (mean ± SE, n = 5, P = 0.03)].

The findings above suggested that ATRA might induce death of myeloma targets in vitro through apoptosis. In previous studies, we showed that treating UCSD/AML1 leukemia cells with TGF-β1 or OCI-My4 myeloma cells with antireceptor antibodies induced PCD (apoptosis; Refs. 14 and 21). In these previous studies, increases in the percentage of cells undergoing apoptosis detected by light and electron microscopy were modest in contrast to dramatic increases often seen when cells are incubated with cytotoxic agents such as VP-16 (26). Furthermore, serial culture in vitro or colony-forming assays were required to show that short-term treatment with TGF-β1 or antireceptor antibodies caused sufficient apoptosis to “extinguish” these cultures. Because all assays currently in use for apoptosis have some limitations for detecting biochemical and genetic changes accompanying PCD (15, 17, 26), we felt it was important to verify that an assay for apoptosis detected PCD using culture conditions evaluated previously. Therefore, the same culture conditions and cell line targets incubated with TGF-β1 or antireceptor antibodies were used to validate the ability of flow cytometry to detect apoptosis.

Fig. 4 shows flow cytometry identified significantly increased numbers of apoptotic cells after TGF-β1 treatment of leukemia cells (P = 0.005) and myeloma cells treated with antireceptor antibodies (P = 0.04). For UCSD/AML1 cells, quantitative results were strikingly similar to previous studies using morphology and electron microscopy to identify apoptotic cells (21). Increased DNA labeling was not detected in HL-60 or AML-193 cells which are resistant to TGF-β1-induced apoptosis or AML-OCI1 cells which show low levels of apoptosis after treatment with TGF-β1 (21). These results indicate that flow cytometry detects apoptosis induced by biologically active agents such as antibodies and cytokines, but does not detect DNA damage in all cells undergoing apoptosis.

![IL-6 Binding (Percent Positive Cells)](image1.png)

Fig. 3 Down-regulation of IL-6 receptors induced by ATRA in myeloma cells. U266 (A) or 8226 (B) cells were grown for varying periods with medium (■) or 1 μM ATRA (▲), and IL-6 receptors were detected using flow cytometry and phycoerythrin-labeled IL-6. Results are the means of four experiments. Bars, SE.
We then used flow cytometry to detect ATRA-induced apoptosis in myeloma target cells. After exposure for 3 days, ATRA induced increased DNA labeling in 8226 cells consistent with apoptosis (Figs. 5A and 6). In contrast, anti-Tf receptor antibodies did not increase DNA damage (Fig. 6). Consistent with previous studies suggesting DNA nicking precedes other evidence of apoptosis (27), increased DNA labeling detected by flow cytometry (Figs. 5A and 6) was present before internucleosomal DNA degradation was detected on day 4 (Fig. 5B). In contrast, despite cytotoxicity for U266 cells, increased DNA labeling by TdT was not observed (data not shown, three experiments). Internucleosomal DNA degradation was also not observed in U266 cells cultured with ATRA for up to 7 days.

Discussion

On the basis of preclinical studies (14, 22, 24) and Phase Ia trials (28, 29), anti-growth factor receptor monoclonal antibodies provide an innovative approach to anticancer therapy. The present studies using anti-Tf and anti-IL-6 receptor antibodies in combination against myeloma cells (Fig. 1B) document the ability of these agents to induce apoptosis (14). However, it seems probable that anti-growth factor therapies, like anticancer drugs, must be used in combination to obtain antitumor responses and avoid normal tissue toxicity. Thus, antireceptor antibodies might be combined with chemicals inhibiting intracellular signal transduction or which down-regulate surface growth factor receptors. Candidate prototype drugs inhibiting signal transduction are under investigation (30, 31).

Known chemical structures might serve similar roles in cancer therapy. Retinoids inhibit in vitro growth of many malignant cells, and were effective in clinical prevention trials of squamous cell cancer (32, 33). As noted above, retinoids reduced IL-6 receptor expression and iron-dependent membrane reductase activity in human tumor cells (11, 12). The toxicities of retinoids are known (33, 34), and they present an attractive model for testing combinations of biologically active agents and antireceptor antibodies.

ATRA and 13-cis-retinoic acid inhibited in vitro growth of some myeloma cell lines, and ATRA showed increased effects when combined with anti-Tf receptor antibodies in cell growth assays. However, when cytotoxicity was assessed, the combination of ATRA and anti-Tf receptor antibodies was not more effective than ATRA alone. These results confirm the importance of testing for cytotoxicity as well as growth inhibition when screening biological anticancer agents (14, 21).
In past studies, retinoids showed in vitro and in vivo antitumor activity against a variety of human tumors, including U266 myeloma (11). A previous study suggested that inhibitory activity of retinoids on a U266 variant cell line was linked to IL-6 receptor down-regulation (11). We confirmed effects of ATRA on IL-6 receptor display and showed ATRA suppressed IL-6 autosecretion by U266 cells. However, ATRA and 13-cis-retinoic acid were more active against factor-independent 8226 than U266 cells and nearly inactive against IL-6 dependent OCI-My4 cells. Synergistic or additive growth inhibition was also not observed against any of these cells using an anti-IL-6 receptor antibody and ATRA. These findings suggest non-IL-6-receptor-dependent mechanisms of ATRA growth inhibition in myeloma.

Apoptosis has become an increasingly important area for new drug and biological developmental therapeutics (15, 19). Available evidence suggests ATRA killed a subpopulation of myeloma cells by inducing apoptosis (Figs. 5 and 6). The potential importance of genes which alter apoptosis in drug resistance and carcinogenesis has recently been reviewed (15, 19). PCD induced by cytotoxic drugs is frequently accompanied by easily detected in vitro growth inhibition and internucleosomal DNA degradation (19, 26). In contrast, apoptosis induced by biological agents may induce relatively subtle changes in ratios of cell “birth” to death (14, 18, 19, 21). This may occur physiologically in organs such as the thymus and during ontogeny (16, 18, 19) as well as in cancer therapy. Anticancer agents acting by such mechanisms may require prolonged administration, but can result in substantial antitumor effects (e.g., hormonal therapy in breast and prostate cancer; Refs. 19, 35, and 36).

Using in vitro hematological tumor models such as those shown in Fig. 4 (14, 21), we showed previously that some agents inducing in vitro apoptosis caused substantial cell killing detected only in long-term cell cultures. Thus, clonogenic capability or in vitro self-renewal were lost early after short-term exposure to these agents. Rather subtle changes in the number of cells undergoing morphological evidence of apoptosis or increased internucleosomal DNA degradation were present under these culture conditions. These alterations stand in contrast to the more pronounced and easily demonstrated effects of in vitro exposure to many anticancer drugs on apoptosis of similar cell targets (18).

Long-term and colony-forming cell cultures are time consuming and expensive as in vitro screens, and we sought a more rapid, semiquantitative assay for PCD induced by biologically active agents. As shown in the present study (Figs. 4 and 6), flow cytometry detects early evidence of apoptosis induced by biologically active agents in some cells and shows promise for this purpose. However, responses by individual cells to PCD are variable and no single assay defines this process in all cells (14, 19). In the present study, ATRA was cytotoxic to U266 cells, but induced neither DNA labeling nor internucleosomal DNA degradation. Some cells undergoing apoptosis show DNA degradation only to large (>50 kb) fragments detected using pulsed field gels (17, 18). Similar changes were not examined here in U266 cells, but in the final analysis, it is loss of proliferative and self-renewal capabilities of target cells that is important in therapy and not demonstrating the type of DNA damage.

The present studies suggest ATRA should be evaluated in malignant plasma cell disorders, perhaps, as a maintenance therapy or treatment for early-stage disease. ATRA was active against factor-independent myeloma cells, but its action on IL-6 receptors makes it an attractive candidate for use in early and paraneoplastic plasma cell disorders which may be IL-6 dependent, such as Castleman’s disease (37, 38). Finally, progression of MGUS to malignant plasma cell disorders occurs in 10–20% of patients at 10 years (39). This approaches the rate of second epithelial tumors in smokers with aerodigestive tract tumors (33), suggesting retinoids might find a role in preventing plasma cell cancers in MGUS patients. Although very long-term trials would be required to assess ATRA effects in preventing these cancers, MGUS is an attractive model since retinoid effects can be monitored in short-term studies by effects on paraprotein levels or on abnormal B-cell precursors in the peripheral blood of MGUS patients (12, 40).

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References

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