Retinoic Acid Modulates a Bimodal Effect on Cell Cycle Progression in Human Adult T-Cell Leukemia Cells

Jamil Dierov, Bassel E. Sawaya, Misha Prosniaik, and Ronald B. Gartenhaus

ABSTRACT

Retinoids, the analogues of vitamin A, have a broad range of effects on different cell types. One biologically active form of vitamin A is all-trans-retinoic acid (ATRA), which binds to retinoic acid receptors, as does its intracellular metabolite, 9-cis-RA. Earlier studies have documented G1 cell cycle arrest and the induction of apoptosis in human adult T-cell leukemia cells after ATRA treatment. Previous work exploring the growth-inhibitory activity of ATRA in human malignancies has implicated several mechanisms that can arrest cells in the G1 phase of the cell cycle, including activation of p21Waf1 and inhibition of cyclin D1 expression. Therefore, we decided to examine the effects of ATRA exposure on G1 cell cycle components in human adult T-cell leukemia cells. Our data demonstrate a correlation between cyclin/cyclin-dependent kinase activity and subunit complex formation with duration of drug exposure. We also observed an increase in p53 protein levels that were not associated with an increase in p21Waf1 levels. Furthermore, we observed a differential effect on cell cycle progression that was temporally related to length of ATRA exposure. These observations, consistent with a bimodal effect of ATRA on cell cycle progression, may have important implications for the clinical application of ATRA.

INTRODUCTION

Retinoids, the analogues of vitamin A, have a broad range of effects on different cell types. They exert their biological effects primarily through intracellular receptors. These receptors fall mainly into two classes, the RARs and the retinoid X receptors (1). One biologically active form of vitamin A is ATRA, which binds to RAR, as does its intracellular metabolite 9-cis-RA; the retinoid X receptor preferentially binds 9-cis-RA (2). ATRA has been shown to possess both antiproliferative and differentiating properties in vitro and in vivo (3, 4). Previous work exploring the growth-inhibitory activity of ATRA in human malignancies has implicated several mechanisms that can arrest cells in the G1 phase of the cell cycle, including activation of p21Waf1 (5, 6) and inhibition of cyclin D1 expression (7, 8). Recent reports have also demonstrated that ATRA can have divergent effects on similar cell types, i.e., either stimulating or suppressing mitogenesis, depending on tissue source (9), culture conditions (10), or experimental design (11).

ATL is an aggressive T-cell malignancy etiologically linked to HTLV-1 (12, 13). Tax, the major viral transactivating protein, transforms human T cells in vivo and in vitro (12–14). Many cellular genes have been demonstrated to be transactivated by TAX, including some critical to cell proliferation (15). Several reports have examined the ability of RA to inhibit the growth of human adult T-cell leukemia cells (16–18). Additionally, it was reported that one putative mechanism may involve an imbalance in redox potential of treated cells (17). A recent report has documented the induction of apoptosis in human adult T-cell leukemia cells after ATRA treatment (16).

We have examined the temporal effects of ATRA treatment on G1 cell cycle components in human adult T-cell leukemia cells. Our data demonstrate a correlation between cyclin/cdk activity and subunit complex formation with duration of drug exposure. Furthermore, we observed a differential effect on cell cycle progression that was temporally related to length of ATRA exposure. These observations may have important implications for the clinical application of ATRA, particularly if it is used in combination with cell cycle-specific drugs.

MATERIALS AND METHODS

Cell Culture and Drug Treatment. The HTLV-1-transformed, IL-2-dependent cell line N1186 (19) was grown in RPMI 1640 containing 10% FBS, 50 mg/ml penicillin, 100 mg/ml streptomycin, 2 mM l-glutamine, and recombinant IL-2 (40 units/ml; Life Technologies, Inc., Grand Island, NY). HTLV-1-transformed, IL-2-independent cell lines C10MJ and MT-2 (Advanced Biotechnologies, Inc., Columbia, MD) were cultured in RPMI 1640 supplemented with 10% FBS, 50 mg/ml penicillin, 50 mg/ml streptomycin, 100 mg/ml neomycin, and 2 mM l-glutamine. Drug treatment was carried out by refedding cells every 3 days with culture medium supplemented with 4–40 nM ATRA (Wako Pure Chemical, Osaka, Japan) for 24–144 h. ATRA was dissolved in ethanol; the final concentration of ethanol was 0.1%. Controls included no drug or treatment with either 0.1% ethanol or 2–20 nM PMA (Sigma Chemical, St. Louis, MO), a phorbol ester with a bimodal effect that can acutely activate T cells through PKC activation or with chronic exposure down-regulate PKC activity (20).

Immunoprecipitation and Immunoblotting. Cell pellets were lysed with lysis buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.1% SDS, and 150 mM PMSF]. Total protein concentration in each sample was determined using a...
micro BCA method (Pierce, Rockford, IL), according to the manufacturer’s instructions. Whole-cell lysate protein (50–100 μg) was resuspended in 5 ml of TBS containing 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.01% PMSF, 0.01% N-tosyl-L-phenylalanine chloromethyl ketone, 0.01% Na-p-tosyl-L-lysine chloromethyl ketone, 0.1% sodium azide, and 1% NP40. Samples were precleared with protein G beads (Life Technologies, Inc., Grand Island, NY) and either normal rabbit or mouse ples were precleared with protein G beads (Life Technologies, Inc., Grand Island, NY) and either normal rabbit or mouse bodies: anti-cyclin D1, anti-p53, and anti-p21. All three cell lines demonstrated an increase in cyclin D1 protein after ATRA treatment. There was also an increase in p21Waf1 protein.

There was also an increase in p53 protein levels but without an increase in the level of p21Waf1 protein.

Fig. 1 Western blot analysis of three ATL cell lines treated with 40 nM ATRA for 72 h. Whole-cell lysates (25–50 μg) were blotted on supported nitrocellulose paper after separation by 8% SDS-polyacrylamide gels. The filters were sequentially incubated with the following antibodies: anti-cyclin D1, anti-p53, and anti-p21. The cells were then transferred to the microfuge tube (total volume in 500 μl of PBS). Immune complexes were precipitated with 1–5 μg of antibody and protein G beads (Life Technologies, Inc., Grand Island, NY) and either normal rabbit or mouse serum (1:1000 dilution). Immunoprecipitation with cyclin D1 was carried out for 12 h at 4°C. Immune complexes were precipitated with 1–5 μg of antibody and protein G-agarose, then heated at 95°C for 5 min in sample buffer containing 3% SDS, 1.1 mm β-mercaptoethanol, 0.01% bromphenol blue, and 15% glycerol. Eluant was analyzed on a denaturing, reducing SDS-PAGE gel and transferred to supported nitrocellulose paper by electroblotting. Immunoblot analysis was carried out by incubating filters with one of the following antibodies (1–5 μg): cyclin D1, cdk2, cdk4, cdk6, PCNA, p21, and p53. Chemiluminescence was then performed with ECL (Amersham Life Science, Arlington Heights, IL), according to the manufacturer’s instructions.

Immune Complex Protein Kinase Assay (cdk2) Kinase Assay. Cells were sedimented by centrifugation. The cell pellet was washed once with ice-cold PBS, and 50 μg of cell extract were transferred to the microtube [total volume in 500 μl in lysis buffer with inhibitors (10 mm sodium phosphate (pH 7.2), 150 mm NaCl, 1% NP40, 1% EDTA, 5 mm β-glycerophosphate 2 mm DTT, 5 mm sodium fluoride, 2.5 mm PMSF, 120 kmborg IU (KIU/ml aprotinin), 10 μg/ml leupeptin, and 1 mm sodium vanadate)]. Immune complexes were precipitated by incubation overnight at 4°C with 1:100 (5 μl) of rabbit polyclonal cdk2 (M2) antibody, followed by incubation for 4 h with 25 μl of protein A-agarose beads. Precipitated protein pellets were washed three times with ice-cold kinase lysis buffer and then resuspended in 20 μl of ice-cold histone H-1 kinase buffer [20 mM HEPES (pH 7.3), 80 mM β-glycerophosphate, 20 mM ethylene glycol bis (β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), 50 mM MgCl2, 5 mM MnCl2, 1 mM DTT, 2.5 mM PMSF, 60 KIU/ml aprotinin, 10 μg/ml leupeptin, and 10 μM cyclin AMP-dependent protein kinase-inhibitory peptide]. Twelve-μl of reaction mix containing 10 μCi [γ-32P]ATP (~3000 Ci/mmol; Amersham), 1 mM unlabeled ATP, and 30 μg of histone H1 protein (Life Technologies, Inc., Grand Island, NY) was resuspended in 5 ml of reaction mix containing 1 μg/ml leupeptin, 1 μg/μl aprotinin, 0.01% PMSF, 0.01% N-tosyl-L-phenylalanine chloromethyl ketone, 0.01% Na-p-tosyl-L-lysine chloromethyl ketone, 0.1% sodium azide, and 1% NP40. Samples were precored with protein G-agarose beads (Life Technologies, Inc., Grand Island, NY) and either normal rabbit or mouse serum (1:1000 dilution). Immunoprecipitation with cyclin D1 was carried out for 12 h at 4°C. Immune complexes were precipitated with 1–5 μg of antibody and protein G-agarose, then heated at 95°C for 5 min in sample buffer containing 3% SDS, 1.1 mm β-mercaptoethanol, 0.01% bromphenol blue, and 15% glycerol. Eluant was analyzed on a denaturing, reducing SDS-PAGE gel and transferred to supported nitrocellulose paper by electroblotting. Immunoblot analysis was carried out by incubating filters with one of the following antibodies (1–5 μg): cyclin D1, cdk2, cdk4, cdk6, PCNA, p21, and p53. Chemiluminescence was then performed with ECL (Amersham Life Science, Arlington Heights, IL), according to the manufacturer’s instructions.

Antibodies. The monoclonal and polyclonal antibodies anti-cyclin D1 (HD11), anti-PCNA (PC10), anti-p21 (F-5), anti-p53 (DO-1), anti-cdk2(M2), anti-cdk4 (H-303), and anti-cdk6 (H-230) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Flow Cytometric Analysis. At 24, 48, 72, and 144 h, asynchronously growing cells were collected and analyzed for DNA content by flow cytometry. Cells were fixed and stained with propidium iodide with 0.6% NP40 and 2 mg/ml RNase. Fluorescence data were collected with the Coulter Epics XL-MCL flow cytometer, and the percentage of cells within the G1, S, and G2-M phases of the cell cycle were determined by analysis with the software program MultiCycle (Phoenix).

---

**Clinical Cancer Research 2541**

---

Downloaded from clincancerres.aacrjournals.org on October 16, 2017. © 1999 American Association for Cancer Research.
Modulation of G1 Cyclin/cdk Complexes. Because earlier studies implicated cyclin D1 as a potential downstream target of ATRA (7–11), we examined what effect ATRA would have on cyclin D1 protein levels in HTLV-1-transformed human T-cell lymphocytes. Incubation of cells with 40 nM ATRA resulted in the increased level of cyclin D1 protein at 72 h, as shown by Western blot analysis (Fig. 1). This was accompanied by an increase in p53 protein but with no increase in the p21WAF1 protein level (Fig. 1). After 24 h exposure to the DNA-damaging agent, Adriamycin, all cell lines showed increased expression of p21WAF1 (data not shown). Because both cdk4 and cdk6 associate with cyclin D1 during G1-S cell cycle progression, we investigated the complex formation of these molecules using coimmunoprecipitation analysis. We examined the ability of ATRA to modulate the subunit complex formation of G1 cyclin/cdk complexes in two IL-2-independent (MT-2 and C10MJ) and one IL-2-dependent (N1186) HTLV-1 transformed lymphocyte cell lines. As demonstrated in Fig. 3, there was increased subunit complex formation after 72 h in ATRA-treated cell lines relative to controls. Furthermore, we analyzed the physical interaction of PCNA with these complexes. Coimmunoprecipitation of cyclin D1 with PCNA revealed an increase in this interaction as well (Fig. 2). Analysis of these ternary complexes after 144 h of ATRA exposure revealed a lower level of ternary complex formation in ATRA-treated cell lines compared with untreated controls (Fig. 2).

---

**RESULTS**

**Modulation of G1 Cyclin/cdk Complexes.** Because earlier studies implicated cyclin D1 as a potential downstream target of ATRA (7–11), we examined what effect ATRA would have on cyclin D1 protein levels in HTLV-1-transformed human T-cell lymphocytes. Incubation of cells with 40 nM ATRA resulted in the increased level of cyclin D1 protein at 72 h, as shown by Western blot analysis (Fig. 1). This was accompanied by an increase in p53 protein but with no increase in the p21WAF1 protein level (Fig. 1). After 24 h exposure to the DNA-damaging agent, Adriamycin, all cell lines showed increased expression of p21WAF1 (data not shown). Because both cdk4 and cdk6 associate with cyclin D1 during G1-S cell cycle progression, we investigated the complex formation of these molecules using coimmunoprecipitation analysis. We examined the ability of ATRA to modulate the subunit complex formation of G1 cyclin/cdk complexes in two IL-2-independent (MT-2 and C10MJ) and one IL-2-dependent (N1186) HTLV-1 transformed lymphocyte cell lines. As demonstrated in Fig. 2, there was increased subunit complex formation after 72 h in ATRA-treated cell lines relative to controls. Furthermore, we analyzed the physical interaction of PCNA with these complexes. Coimmunoprecipitation of cyclin D1 with PCNA revealed an increase in this interaction as well (Fig. 2). Analysis of these ternary complexes after 144 h of ATRA exposure revealed a lower level of ternary complex formation in ATRA-treated cell lines compared with untreated controls (Fig. 2).
**Fig. 4** Time course analysis of G1 cdk kinase activity. Cells were cultured with 40 nm ATRA up to 144 h and shown to have altered G1 cdk kinase activity in a time-dependent manner. A representative experiment shows an initial increase in catalytic activity of cdk2 (H1) and cdk4 (pRb), followed by a gradual decrease in ATL cell lines using kinase assays as described in Fig. 3.

**G1 cdk Kinase Activity.** Because increased G1 cyclin/cdk subunit complex formation was observed in those cell lines treated with ATRA for 72 h, we were interested in assaying the catalytic activity of cdk4 and cdk6 in these same cell extracts. As shown in Fig. 3, a markedly increased ability to phosphorylate Rb substrate is observed when in vitro immune complex kinase assays were carried out with either cdk4 or cdk6 immunoprecipitated from the ATRA-treated cell lines. We also analyzed the kinase activity of cdk2, another kinase molecule involved in G1/S transition from ATRA-treated cell lines using the H1 histone kinase assay. At 72 h, G1 cdk kinase activity was increased in ATRA-treated cell extracts relative to untreated control cell extracts (Fig. 3). In a temporal manner, cdk4 activity was shown to initially increase then to decrease back to baseline after prolonged ATRA exposure (Fig. 4). This was also true for cdk6 activity (data not shown). Similar results were obtained when examining the catalytic activity of cdk2, except that its activity peaked earlier, reaching a maximum after 72 h of ATRA treatment (Fig. 4).

**Cell Cycle Effects of ATRA Treatment.** Because cyclin D1 through its association with cdk4 and cdk6 is instrumental in driving cells through G1 into S phase, we asked whether the increased subunit complex formation and kinase activity correlated with an increase in the percentage of ATRA-treated cells in S phase. To explore this association, we examined the effects of ATRA treatment on the cell cycle in exponentially growing cell cultures by flow cytometric analysis. After ATRA exposure (4–40 nm) for 24–144 h, the cells were collected for cell cycle analysis. The C10MJ cells showed an increase in the percentage of cells in S phase at 72 h. At 144 h of ATRA treatment, there was a significant increase in the percentage of cells (87.8%) arrested in G1 (Fig. 5). They also exhibited an increase in apoptotic cells, as evidenced by the fragmented DNA appearing to the left of the G0/G1 peak (Fig. 5). MT-2 cells showed a similar cell cycle profile, with almost 50% of cells in S phase at 72 h, and ~70% of cells arrested in G1 after 144 h of ATRA treatment (Fig. 5). However, in contrast to the C10MJ cells, there was minimal apoptosis present in the MT-2 cells at 144 h. All assays yielded reproducible results in independent experiments (n = 2).

**DISCUSSION**

Despite the administration of aggressive combination chemotherapy, the reported median survival of patients with ATL is still <1 year (22). Several groups have independently carried out in vitro studies examining the growth-inhibitory effects of ATRA on ATL cells with various sensitivities, depending on the cell lines used (16–18). However, there is little data regarding the effect of ATRA on other components of cell cycle regulation in lymphoid malignancies. Distinct cyclin/cdk complexes are formed at different phases of the cell cycle with activation of their kinase activities. The D-type and E-type cyclins are involved in regulating G1 phase progression. D-type cyclins interact with cdk4 and cdk6, whereas cyclin E associates with cdk2 and is maximally active during the G1-S phase transition (23). We decided to focus on the effects of ATRA on G1 cyclin/cdk complexes to elucidate the mechanisms associated with either growth inhibition or stimulation after ATRA treatment of lymphoid malignancies.

We exposed three ATL cell lines to ATRA for 24–144 h and examined for cyclin D1 protein levels, G1 cyclin/cdk complex formation, and kinase activity. Consistent with a previous report (11), we observed an increase in cyclin D1 protein after 72 h of ATRA exposure. In contrast to that study, we did not observe a concomitant increase in the expression of p21 protein. Because cyclin D1, through its physical interaction with cdk4 and cdk6, is a critical regulator of G1-S cell cycle progression, we investigated the cyclin/cdk subunit association. We also examined the ternary complex formation with PCNA, the auxiliary protein of DNA polymerase δ and ε, involved in DNA replication and repair (24). In all 72-h, ATRA-treated cell lines, there was a striking increase in the ternary complex formation between cyclin D1, cdk4/cdk6, and PCNA. Because previous studies have established that p21 can act as a universal inhibitor of cyclin/cdk kinase activity (25, 26) through a quaternary complex formation, the barely detectable p21 protein in the ATRA-treated cell lines supported our findings of a significantly increased cdk4 and cdk6 kinase activity.

A recent report has documented ATL cells arresting in the G1 phase of the cell cycle after exposure to ATRA, 9-cis-RA,
Fig. 5 Effects of ATRA exposure on cell cycle progression. Cell lines were treated with 4 or 40 nM ATRA for up to 144 h, and aliquots of cells were collected every 24 h and analyzed for DNA content by flow cytometry. Fluorescence data were collected with the Epics Coulter flow cytometer, and the percentage of cells within the G_1, S, and G_2-M phases of the cell cycle were determined by analysis with the software program MultiCycle (Phoenix). A, histogram of 72-h, ATRA-treated MT-2 and C10MJ. There is approximately a 2-fold increase in the percentage of cells in S phase after ATRA treatment relative to the no treatment controls. B, histogram of 144-h, ATRA-treated MT-2 and C10MJ. Both cell lines showed a return of cells to G_1 after prolonged exposure to ATRA. Cells exposed to 0.1% ethanol showed the same cell cycle profile as the no drug arm (data not shown). Similar flow cytometry results were obtained in independent experiments (n = 2).
Fig. 5  Continued.
and 13-cis-RA (16). In that same study, the p21Waf1 and p27Kip1 cell cycle inhibitors were analyzed after ATRA treatment. Although the p27Kip1 protein was not detected, even after ATRA treatment, there was an induction of p21Waf1. Interestingly, in that report the increased expression of p21Waf1 was associated with a reduction of p53 protein. Liu et al. (6) have described previously the transcriptional activation of the p21Waf1 gene through the RAR in a p53-independent manner. In contrast to these findings, we observed an increase in the levels of p53 after ATRA exposure but with no associated induction of p21Waf1. Although it has been reported that p21Waf1 contains an RA-responsive element, the induction of p21Waf1 transcription by ATRA is dose dependent, requiring higher levels than those used in our experiments (6, 11). However, the observed increase in the p53 protein level in ATRA-treated cell lines would be expected to induce the p21Waf1 protein. We and others have reported previously that p53 protein in ATL cell lines may be functionally inactive (27–29), consistent with our findings of barely detectable p21 protein, despite increased p53 protein levels. In fact, we have demonstrated previously the p53-independent induction of p21WAF in some HTLV-1 transformed lymphocyte cell lines after exposure to the DNA-damaging agent, Adriamycin (30). The regulation of p21 expression in these cell lines is complex and likely to be multifactorial.

We also observed an increase in cdk2 kinase activity after 72 h of ATRA treatment. However, after prolonged ATRA exposure (144 h), the increase in G1 cyclin/cdk complex formation and associated kinase activity decreased to below that of untreated samples. This temporal relation between duration of ATRA exposure and G1 cyclin/cdk complex association prompted us to examine the impact of ATRA on cell cycle progression. Our studies revealed a correlation between G1 cyclin/cdk complex association and the percentage of cells in S phase after a 72-h ATRA exposure. This was in striking contrast to the percentage of cells arrested in G1 after 144 h of ATRA exposure. These latter results showing profound G1 cell cycle arrest are consistent with those of Fujimura et al. (18) in which even resistant ATL cell lines demonstrated growth inhibition after 120 h of ATRA treatment. However, they did not report a bimodal effect on cell cycle progression as demonstrated in our study. This initial growth-stimulatory phase observed in our experiments is likely due in part to the increased cyclin D1 protein levels without the accompanying increase in p21Waf1 protein usually found at higher ATRA concentrations (11). The observed decrease in G1 cyclin/cdk activity after chronic exposure is consistent with the decreased cell cycle kinetics at 144 h in ATRA-treated cells.

It is important to note that analogous results were obtained after PMA treatment, consistent with its ability to activate T-cells after short-term exposure but suppression after chronic exposure due to PKC depletion. It has been reported by Kizaki et al. (31) that HTLV-1 immortalized lymphocytes express the RAR. In addition to ATRA working through its cognate receptors, other growth-regulatory molecules may be suppressed or expressed in response to ATRA treatment. The increased cyclin D protein levels observed in our experiments as well as a prior report documenting increased transcription of cyclin D1 after short-term ATRA exposure (11) supports such a model.

The ability of retinoids to have different effects on similar cell types i.e., either stimulating or suppressing mitogenesis, depending on experimental design have been reported previously (9–11). However, this time-dependent impact on cell cycle kinetics is the first documentation, to our knowledge, of a divergent effect on cell cycle progression using a similar ATRA dose on lymphoid cells. The present interest in the clinical utility of ATRA in the treatment of ATL requires careful design of protocols, especially the duration of drug exposure as well as drug dose due to its potential bimodal effect on cell cycle progression and mitogenesis. Whether this effect is true for other retinoids requires further investigation.

REFERENCES


Retinoic Acid Modulates a Bimodal Effect on Cell Cycle Progression in Human Adult T-Cell Leukemia Cells

Jamil Dierov, Bassel E. Sawaya, Misha Prosniak, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/5/9/2540

Cited articles
This article cites 27 articles, 10 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/5/9/2540.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/5/9/2540.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.