Inhibition of AIDS-Kaposi’s Sarcoma Cell Proliferation following Retinoic Acid Receptor Activation

Wei-Xing Guo, Parkash S. Gill, and Tony Antakly

Department of Pathology, University of Montreal, C. P. 6128, Succursale, Centreville, Montreal, Quebec. Canada H3C 3J7 [W-X. G., T. A.], and Norris Cancer Center, University of Southern California, Los Angeles, California 90033 [P. S. G.]

ABSTRACT

Retinoids, a group of natural and synthetic vitamin A analogues, analogues the receptors of which belong to the superfamily of steroid receptors, can exert profound effects on growth and/or differentiation of embryonic and neoplastic cells. Kaposi’s sarcoma (KS), previously a rare multicentric neoplasm, has become epidemic with HIV infection, although the etiology of KS remains obscure. In the present study, the effects of two potent retinoids, all-trans-retinoic acid (RA) and 13-cis-RA, on the expression of retinoic acid receptor α and the growth of AIDS-related KS (AIDS-KS) cells were examined. The proliferation of AIDS-KS cells was significantly inhibited by RA and 13-cis-RA in a dose-dependent manner with 50% inhibitory concentration of 1.4 × 10^{-9} M and 4.7 × 10^{-8} M, respectively, which correlate with their potency. Growth inhibition was time dependent with maximal inhibition of 90% after 3 days of treatment with 10^{-8} M RA. Growth inhibition by RA was further potentiated by forskolin (1 μM), an intracellular cyclic AMP-inducing agent. Moreover, RA treatment blocked the proliferative effect of oncostatin M and tumor necrosis factor α, two major KS autocrine growth factors. The effects of RA were accompanied by a dramatic increase in nuclear staining for retinoic acid receptor α and in the relative number of strongly positive retinoic acid receptor α nuclei. Finally, RA induced morphological changes as KS cells became more flattened, better spread, and more adhesive to the substrate. These results suggest that retinoids inhibit proliferation of AIDS-KS cells and further support their utility as therapeutic agents in AIDS-KS.

INTRODUCTION

KS, once a rare tumor of mesenchymal origin, has become the most common tumor seen in HIV-infected individuals, and it remains one of the major clinical manifestations of AIDS (1–4). Unfortunately, the precise mechanism leading to the appearance and progression of KS lesions remains poorly understood. The recent establishment of long-term cultures of AIDS-KS spindle cells has provided experimental models for facilitating the study of the pathogenesis of AIDS-KS (5–8). It has been demonstrated that the in vitro proliferation of AIDS-KS cells is dependent on various cytokines and growth factors, such as OSM, IL-1β, IL-6, PDGF, basic fibroblast growth factor, and TNF-α, which serve as autocrine and paracrine modulators (5, 9–12). These insights in turn have provided the basis for new therapeutic approaches including the development of cytokines and growth factor inhibitors as well as AIDS-KS cell-specific cytokine receptor antagonists. For example, recombinant IL-6, which down-regulates IL-6 secretion by cultured AIDS-KS cells with a coordinate inhibition of AIDS-KS cells proliferation, is currently in phase I (3) and phase II (13) clinical trials.

Since growth and differentiation are mutually exclusive processes, agents and drugs that enhance cell differentiation could also be clinically relevant. For example, retinoids, a group of natural and synthetic vitamin A analogues, can exert profound effects on the growth and differentiation of normal, transformed, and tumor cells (14, 15). The remarkable ability of retinoids to inhibit tumor cell growth and to enhance the differentiation of certain malignant cells has led to their consideration as potential antitumor agents (16, 17). Although the mechanisms by which RA affect growth and differentiation are uncertain, it has been reported that RA treatment regulates expression of certain cytokines, growth factors, and their receptors. For example RA can modulate the growth-promoting effects of PDGF in normal rat kidney cells (17) and C3H 10T½ fibroblasts (18). It also up-regulates IL-2 receptor on activated human T lymphoblasts (19) and inhibit the growth of human myeloma cells partly by a down-regulation of IL-6 receptors (20). Moreover, RA can completely suppress the induction of HIV production by IL-6 in infected mononuclear phagocytes (21). The diverse effects of retinoids are mediated through two subfamilies of nuclear receptors RAR and RXR that belong to the superfamily of ligand-induced transcription factors, namely steroid receptors (reviewed in Refs. 22 and 23). The RAR family includes three subtypes, RARα, RARβ, and RARγ, along with their isoforms; the RARs are activated by all-trans- and 9-cis-RA. In contrast, the retinoid X receptors (RXRα, RXRβ, and RXRγ) are activated almost exclusively by 9-cis-RA (23, 24).

In the present work, using long-term cultures of AIDS-KS cells, we demonstrated the presence therein of high amounts of RARα in AIDS-KS cells. Treatment with RA induced a dramatic increase in AIDS-KS cell nuclear staining. Furthermore, RA was found to exert an antiproliferative effect on AIDS-KS cells and to antagonize the growth-promoting effects of two principle growth factors implicated in AIDS-KS proliferation, namely oncostatin M and TNFα.

MATERIALS AND METHODS

Cell Culture. AIDS-KS derived spindle cells (KS 313B and KS 29B) were maintained as described previously (7). The AIDS-KS cells were grown on gelatin (1.5%)-coated plates or dishes in RPMI 1640 containing 15% activated fetal bovine serum, 2 mm l-glutamine, essential and nonessential amino acids, 1% Nutramed-HU, 100 units/ml penicillin, and 100 μg/ml streptomycin. All components of the culture medium were obtained from Sigma and Gibco. Human skin fibroblasts were obtained from Dr. P. Eydoux, Montreal Children’s Hospital, and rat hepatoma cells and mouse L cells were obtained from Dr. M. Nemer, Clinical Research Institute of Montreal and grown in DMEM containing 10% inactivated fetal bovine serum. The cells were incubated at 37°C in a humidified atmosphere consisting of 5% CO2 and 95% air.

Reagents. Stock solutions of OSM (R & D System) and TNFα (Genentech, Inc.) or 8-bromo-cAMP (Sigma) were dissolved in PBS; forskolin (Sigma), all-trans-RA (Sigma), and 13-cis-RA (Sigma) were dissolved in 95% ethanol as 10^{-2} M stock solutions in light-tight tubes at −20°C. All procedures involving retinoids were performed under subdued light. Before an experiment, stock solutions were diluted with 95% ethanol and the working solution was further diluted 1:1000 directly in the culture medium to obtain final concentrations containing only 0.1% ethanol. Control cultures (vehicle) received an equivalent amount of ethanol.

[{^{3}H}Thymidine Incorporation. Cells were plated at 4 × 10^{3}well(24-well plates) and treated with RA or 13-cis-RA at various concentrations or for
different times as indicated below. Prior to harvest (18 h), $[^{3}H]$thymidine (DuPont) was added into each well at 1 $\mu$Ci/ml ($1 \text{ Ci} = 37 \text{ GBq}$), and the incubation was continued for 18 h. The cells were then washed once with PBS, released with 0.5% trypsin/EDTA (GIBCO), and harvested onto glass fiber filters with a cell harvester (Skatron). Radioactivity was measured by liquid scintillation (Wallac 1409).

**Analysis of Growth Inhibition by Cell Counting.** AIDS-KS cells were seeded into 35-mm Petri dishes, treated with $10^{-6} \text{ M RA}$, and harvested on the 6th day with 0.5% trypsin/EDTA. The released cells were suspended in PBS by repeated pipeting to give a homogeneous cell suspension, and cell were counted using a hemocytometer. Viable cells were assessed by 0.1% trypan blue exclusion.

**Cell Detachment Assay.** Cell-substratum adhesiveness was determined in duplicate dishes by measuring the number of cells that detached or remained attached after treatment with trypsin/EDTA for varying time periods as described. Briefly, cells were grown in the presence or absence of $10^{-6} \text{ M RA}$ for 3 days. Then the cells were washed twice with PBS and incubated with 0.1% trypsin/EDTA at room temperature. During the incubation, the dishes were shaken at 120 rpm. The trypsin/EDTA added into the dishes was collected and replaced every 5 min. The number of cells released into the solution was determined with the hemocytometer. The percentages of the detached cells were calculated as

$$C_i \times 100\%$$

where $C_i$ is the number of the detached cells in a designated time (such as 5 min) and $C$ is the total number of the detached cells during the period of 30 min, i.e., $C = C_5 + C_{10} + \cdots + C_{30}$.

**Immunocytochemistry of RARa.** The general immunocytochemistry procedure was based on our previous report (25). Briefly, AIDS-KS cells cultured in 8-well slides (Miles) were washed once and then fixed in situ with a buffer of 4% paraformaldehyde (Baker, Inc.) for 30 min at room temperature. Following permeation with ethanol, a monospecific antibody to RARa (26) was applied at 1:500 dilution for overnight at 4°C. This antibody was characterized by ELISA and immunoblot procedures. The peptide was conjugated to either BSA or keyhole limpet hemocyanin before use as an immunogen. The procedures are essentially identical to those described earlier (27). Nonimmune or preimmune sera were used as controls. The bound antibodies were detected with the avidin-biotin complex (Vector Laboratories, Inc.) and the peroxidase activity was demonstrated by the diaminobenzidine cytochemical reaction. Preparations were examined under light microscopy by two different observers and the number of unstained, moderately stained, or strongly stained nuclei was empirically evaluated.

**Western Blot.** The procedures were followed essentially according to the instructions of the manufacturer (Promega). Briefly, KS cells were homogenized as described (27), and total protein was assayed by the method of Bradford (Bio-Rad, Richmond, Ca). The extracted protein from KS cells was electrophoresed [each gel lane was loaded with an identical amount of protein (100 $\mu$g)]. The electrophoresed proteins were transferred electrophoretically to polyvinylidene difluoride membrane (Bio-Rad). After blocking with 5% nonfat dry milk, the membrane was incubated overnight with a monospecific antibody to RARa (26) at 1:500 dilution at 4°C. The membrane was washed extensively and incubated with anti-rabbit IgG conjugated to alkaline phosphatase (Promega) for 30 min at room temperature. Finally, the membrane was washed...
Table 1 Effects of RA on cell proliferation

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<tr>
<th>Cell line</th>
<th>Untreated</th>
<th>RA $10^{-6}$ M</th>
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<tr>
<td>KS-29B</td>
<td>4,310 ± 149</td>
<td>3,304 ± 254a</td>
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<tr>
<td>Fibroblast</td>
<td>2,765 ± 43</td>
<td>1,766 ± 64a</td>
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<tr>
<td>HTCb</td>
<td>84,159 ± 6,340</td>
<td>71,926 ± 2,174</td>
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<tr>
<td>L</td>
<td>712 ± 42</td>
<td>1,165 ± 61a</td>
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a $P < 0.05$.

b HTC, hepatoma cells.

and developed with nitro blue tetrazolium (Promega) color development substrates.

**RESULTS**

**Retinoid-induced Growth Inhibition.** The effect of treatment of AIDS-KS cell with various concentrations of all-trans-RA or 13-cis-RA on cell growth as assayed by $[^{3}H]$thymidine incorporation is illustrated in Fig. 1. With both agents, there was dose-dependent growth inhibition with increasing dose of retinoid. The cells were very sensitive to both retinoids. Treatment of AIDS-KS cells with RA (1 $\mu$M) and 13-cis-RA (1 $\mu$M) for 3 days resulted in maximal inhibitions of 92 and 78%, respectively. Minimal inhibition was noted with RA and 13-cis-RA concentrations of about $1 \times 10^{-12}$ M and $1 \times 10^{-18}$ M, respectively. The concentrations of RA and 13-cis-RA required for 50% growth inhibition of AIDS-KS cells were $1.4 \times 10^{-10}$ M and $4.7 \times 10^{-9}$ M, respectively. In addition, the effect of RA on cell number was investigated. A dose-dependent inhibitory effect of RA on cell number was noted (not shown). There was a significant increase in the doubling time following a 5-day exposure to RA (1 $\mu$M) during a 6-day incubation period. The doubling time increased from 4 days in untreated cells to 6 days in RA-treated cells.

To assess the specificity of action of RA on cell proliferation, the effect of RA on the growth of other cell lines was determined (Table 1). The growth rate of KS-29B, another cell line derived from an AIDS-KS patient, was significantly inhibited in the presence of 1 $\mu$M RA for 2 days. Interestingly, the growth of the human fibroblast cells was also inhibited by RA. In contrast, the growth of mouse fibroblasts (L cells) was stimulated under the same conditions while the growth of rat hepatoma cells was not significantly altered. At all concentrations used, retinoid did not affect cell viability as determined by trypan blue exclusion.

**Time Course of RA-induced Growth Inhibition.** The effect of RA on AIDS-KS cell proliferation was measured over a 5-day period. The growth curves of AIDS-KS cells in the presence of retinoids are shown in Fig. 2. Maximal inhibition was seen after 2 days of exposure to RA. Growth inhibition reached 90% with $10^{-8}$ M RA following a 4-day exposure. Growth inhibition, although to lesser extent, was also observed in cells treated with 13-cis-RA ($10^{-7}$ M). It should be noted that short-term (24 h) exposure to retinoids resulted in some experiments in a small but marginally significant proliferative effect.

**Effect of RA on Cell Morphology and Adhesion.** The morphological changes of AIDS-KS cells after exposure to RA was examined by phase-contrast light microscopy. As shown in Fig. 3, RA-treated cells are more flattened and better spread on the substrate than are untreated cells.

During routine passage of AIDS-KS cells with trypsin, it was observed that cells grown in the presence of RA were more adhesive to the tissue culture flask than cells growing in the absence of RA. Thus the detachment of AIDS-KS cells by trypsin was measured. Fig. 4 shows that when AIDS-KS cells without treatment of RA were incubated with trypsin for 5 min, 10.17% of cells detached. In the presence of RA, only 3.2% of cells detached with a significant decrease (69% less; $P < 0.05$) relative to untreated cells. The numbers of RA-treated cells that detached rose to only 63% of control after 10 min incubation and become similar to that of the control group after longer trypsin treatments.

**Antagonistic Effect of RA on OSM- and TNF-induced Proliferation of AIDS-KS Cells.** Since OSM and TNF have been demonstrated to act as autocrine growth factors in initiating and maintaining AIDS-KS cell growth, we explored the possibility that RA may inhibit the proliferation of AIDS-KS cells via interference with the actions of OSM or TNF. Fig. 5 demonstrates that in AIDS-KS cells exposed to OSM (10 ng/ml) or TNF (5 ng/ml), cell proliferations were increased to 178 and 128%, respectively. However, when the cells were simultaneously treated with RA plus OSM or TNF, the stimulatory effect of OSM or TNF on AIDS-KS cell proliferation was significantly reduced. In fact, the proliferation rate of cells treated with OSM plus RA decreased by 60% relative to cells treated only with OSM and was vertically back to control level (Fig. 5). In cells treated with RA plus TNF, RA inhibited not only TNF-induced growth but also "basal" growth rate of KS-29B, another cell line derived from an AIDS-KS patient,
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antibody to RARα for immunostaining. As shown in Fig. 7a, AIDS-KS cells exhibited a positive and moderate level of nuclear staining in the absence of RA; cells are heterogeneous with respect to their staining intensity. When AIDS-KS cells were treated with RA (10⁻⁶ M), there was a noticeable increase in nuclear staining (Fig. 7b). The results from Western blot further demonstrated the up-regulation of RARα in presence of RA. A marked increase of RARα in KS cells exposed to RA (10⁻⁶ M) for 24 and 48 h was detected (Fig. 8). The RARα-immunoreactive band (M₇, 50,000–55,000) corresponds to the known molecular weight of RARα (14). Using immunocytochemistry, the time course of RARα up-regulation in KS cells was semi-quantitatively evaluated. An increased nuclear staining was observed with increased time of exposure to RA. Following a 72-h incubation with RA, 92% of KS cells showed apparent nuclear staining compared with 24% of untreated cells (Fig. 9).

proliferation, which decreased to 60% of control (vehicle)-treated cells.

Potentiation of RA-induced Growth Inhibition by cAMP-inducing Agent. cAMP is an important signaling messenger in the regulation of cell growth and differentiation. To test whether cAMP is involved in the antiproliferative effect of RA in AIDS-KS cells, we tested the effect of forskolin, a cAMP-inducing agent, on growth of AIDS-KS cells. Results demonstrated that treatment of AIDS-KS cells with 1 μM forskolin significantly (P < 0.01) potentiated the antiproliferative effect of RA (Fig. 6). Similarly, when 8-bromo-cAMP (5 μM) was simultaneously added with RA (10⁻⁹ M) for 2 days, potentiation of RA-induced inhibition of AIDS-KS proliferation was also observed (data not shown).

Expression and Regulation of RARα. To determine whether retinoic acid receptor is expressed in KS cells, we used a monospecific

Fig. 3. Morphology of AIDS-KS cells (KS 313B) cultured for 6 days in the absence or presence of 10⁻⁶ M RA. Phase-contrast shot of live cells. × 120.

Fig. 4. Effect of RA on cell adhesiveness of AIDS-KS cells (KS 313B). Cells were seeded at 3 x 10⁴ cells/35-mm dish and treated with 10⁻⁶ M RA in 0.1% ethanol or with 0.1% ethanol (control). After 3 days of exposure, cell adhesion was determined via counting the detached cell numbers as described in “Materials and Methods.” Points, mean of triplicate dishes; bars, SE; *, P < 0.05, RA versus control.

Fig. 5. Antagonistic effect of RA on cytokine-induced cell proliferation. AIDS-KS cells (KS 313B) were seeded at 4 x 10⁴ cells/well. After 24 h, RA(10⁻⁶ M), OSM (10 ng/ml), RA plus OSM, TNFα (5 ng/ml), and RA plus TNFα were added into culture media. On day 5, cells were harvested for detecting [³H]thymidine uptake. Columns, mean of quadruplicates; bars, SE. Compared with control, **, P < 0.05; and # P < 0.01; *, P < 0.01 in TNFα versus TNFα + RA; OSM versus OSM + RA.

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DISCUSSION

The data presented in this paper demonstrated that AIDS-KS cells possess RARα and are responsive to retinoids in culture. The antiproliferative effect of both RA and 13-cis-RA on AIDS-KS cells was achieved at low and noncytotoxic concentrations with a dose-dependent manner and was detectable only after the cells had been exposed to retinoids for 48 h or longer. Analysis of the growth curve shows that RA altered the doubling time, causing growth inhibition. Growth inhibition was also observed, although to lower extent, with the less potent retinoid 13-cis-RA. The decrease in growth rate following RA treatment was accompanied by morphological changes in cell shape and adhesiveness, suggesting that RA might also alter AIDS-KS differentiation.

Treatment of AIDS-KS cells with RA also caused morphological changes. The flattened morphological appearance induced by RA is similar to those observed in other sarcoma cell lines (28) and mouse fibroblasts (29). In addition, AIDS-KS cell adhesiveness was also enhanced following RA treatment. It has been suggested that the increase of some cell surface components, such as glycosaminoglycan and large external transformation-sensitive glycoprotein, may be involved in enhancing the adhesiveness and the flattening of mouse fibroblasts treated with RA (29). Thus, RA may be able to modify the cell surface glycoconjugates in AIDS-KS cells, and morphological changes may result from alterations of the cell surface glycoproteins.

When AIDS-KS cells were treated with RA or 13-cis-RA for short term (24 h), a small but marginally significant increase in proliferation was observed in some experiments whereas a time-dependent inhibition was invariably observed at longer exposures to retinoids (>24 h). This phenomenon is consistent with the report by Marth et al. (30) that in the early stage of RA treatment of human breast cancer cells, DNA synthesis was enhanced as evidenced by an increased number of cells in S phase, whereas after 2—4 days there was a decrease in cell proliferation and cells accumulated in G0-G1. However, the mechanism underlying the early stimulatory effect of RA remains obscure.

The involvement of cAMP in the regulation of cell growth and differentiation (31) is well established. Interestingly, the effects of retinoids on gene expression and cellular differentiation could be modulated by cAMP intracellular signaling (32—40). Of particular relevance is the finding that RARα, which is presumably mediating RA effects in AIDS-KS cells, is phosphorylated by protein kinase A (40); furthermore, this is correlated with a potentiation of RARα transcriptional activity by transient cotransfection of the catalytic subunit of protein kinase A. In this respect, it is noteworthy that Ambrus et al. (41) reported increased cAMP phosphodiesterases in F5 lesions and suggested that the high levels of cAMP phosphodiesterases which result in low cAMP level may contribute to the pathogenesis of KS (42). Together with our preliminary data showing that
cAMP-inducing agents, such as forskolin, potentiated the antiproliferative effect of RA, it is intriguing to speculate whether increased cAMP phosphodiesterase in AIDS-KS cells interferes with RA-dependent or RA-sensitive pathways of growth and differentiation.

Recently, it has been proposed that the development of AIDS-KS might be initiated and maintained by the autocrine and paracrine secretions of several cytokines and growth factors, such as IL-1, IL-6, OSM, TNFα, and PDGF. TNFα appears to be the most important pathogenic factor of AIDS, which is considered as a cytokine or TNF disease (42). TNFα selectively enhanced the replication of HIV-1 (43, 44) and induced its own expression as well as that of other cytokines, such as IL-1 (45). In the case of AIDS-KS, it has also been demonstrated that KS expresses TNFα which significantly promotes the growth of AIDS-KS cells (43). OSM, originally found to be secreted by macrophages and T lymphocytes, has been revealed to be another important growth factor in the initiation and progress of AIDS-KS cells. The presence of specific OSM receptor on AIDS-KS cell lines was confirmed (46) and its signaling transduction pathway has also been investigated (47, 48). Our results demonstrate that RA can antagonize OSM or TNFα proliferative activity on AIDS-KS cells while recently, RA inhibition of KS cell growth was found to be independent of cytokine regulation (49). Since TNFα is produced locally by AIDS-KS cells, inhibition by RA of “basal” proliferation might in fact reflect interference with TNFα action. While the exact mechanism of action of RA will need to be investigated, it is noteworthy that RA inhibition of growth in other systems has been associated with interference with cytokines and growth factor receptors including down-regulation of the IL-6 receptor (20) and the epidermal growth factor receptors (50).

Currently, retinoids are widely used in cancer therapy due to their dramatic antitumor effects and to the significant clinical response obtained in patients with dermatological malignancies and patients with squamous cell carcinomas of the head and neck where a combination of 13-cis-RA with interferon α gives a high response rate (51). In the present study, RA is demonstrated to be growth inhibitory and to antagonize the effect of OSM- and TNFα-stimulated proliferation in AIDS-KS cells. Our results provide a valuable scientific basis for further consideration of retinoids as potential therapeutic agents for AIDS-KS.

![Fig. 8. Up-regulation of RARα in AIDS-KS cells by RA as demonstrated by Western blot analysis. Cells were treated with RA (10^{-8} M) for 48 h (Lanes 1 and 5), 24 h (Lane 2), and 8 h (Lane 3). Lane 4, cells treated solely with the vehicle (0.1% ethanol); Lanes 1-4, cells incubated with monoclonal antibody of RARα; Lane 5, cells incubated with nonimmune serum. Arrow, specific RARα band. Note that in cells untreated or treated with RA for 8 h, the specific RARα band was extremely weak and difficult to see in the micrograph.](image1)

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![Fig. 9. Time course of up-regulation of RARα by RA in AIDS-KS cells (KS 313B). AIDS-KS cells were treated with either RA 10^{-8} M for 24 or 72 h or the vehicle (control) before fixation and subsequent immunochemical localization of RARα. Nuclear staining was evaluated by counting the number of stained nuclei. A time-dependent increase in nuclear staining of RARα is shown.](image2)

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**REFERENCES**


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