Effect of Retinoic Acid on DNA Cleavage and Cytotoxicity of Topoisomerase II-reactive Drugs in a Human Head and Neck Squamous Carcinoma Cell Line¹

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ABSTRACT

Evidence from several in vitro systems indicates that cellular responses to DNA topoisomerase II-reactive compounds (i.e., the epipodophyllotoxins and intercalating agents) may be affected by the relative rate of proliferation. Using a human head and neck squamous carcinoma cell line 183A, we have investigated the effect of β-all-trans-retinoic acid (RA), a substance with known antiproliferative effects, on the DNA cleavage and cytotoxic activities of etoposide and 4’-(acridinylamino)methanesulfon-m-anisidine which interact with topoisomerase II. The effect of RA treatment on the activity of X-irradiation and bleomycin, both of which produce free radical mediated effects, was also examined. RA treatment (10 to 20 μm for 72 h) does not significantly influence DNA cleavage induced by X-irradiation or bleomycin but decreases DNA cleavage and cytotoxicity mediated by etoposide and 4’-(acridinylamino)methanesulfon-m-anisidine. Further, this effect can be demonstrated at a dose of RA that is minimally growth inhibitory. The inhibitory effect of RA appears to be localized to the nucleus given that similar effects on drug-mediated DNA cleavage can be demonstrated in nuclei isolated from RA-treated cells. However, both drug-stimulated DNA cleavage activity and topoisomerase II catalytic activity are approximately equal in crude nuclear extracts of untreated and RA-treated cells. These data suggest that the resistance to topoisomerase II-reactive drugs induced by RA treatment of 183A cells is not mediated through a direct effect on the enzyme, but, instead, is related to other changes in the nuclear milieu occurring in the initial stages of quiescence such as altered chromatin conformation.

INTRODUCTION

Recent evidence substantiates the role of the nuclear enzyme DNA topoisomerase II as a critical intracellular target of a variety of antineoplastic agents, many of which are quite useful clinically. This includes the nonintercalative epipodophyllotoxins, etoposide and teniposide, and several different classes of intercalating agents, e.g., aminooxycocidines, anthracyclines, anthracenediones, and others (Ref. 1 for review). There are several studies which indicate that topoisomerase II activity, as well as sensitivity to topoisomerase II-reactive compounds, is decreased in quiescent versus cycling cell populations. Enzyme activity is increased in mitogenized lymphocytes (2) and in regenerating mouse liver tissue after partial hepatectomy (3). More recently it has been shown that enzyme content and sensitivity to etoposide-mediated strand-breaking activity are increased in peripheral blood lymphocytes stimulated with phytohemagglutinin and interleukin 2 (4). However, other data suggest that cultured cell lines are not uniform in their regulation of topoisomerase II content nor in their response to topoisomerase II-reactive agents in quiescence (5).

Retinoids are a group of metabolites and synthetic analogues of vitamin A (retinol) which have been shown to have both antiproliferative and differentiative effects on many human squamous cell lines in culture (6–8). In the present paper we have used a HHNSCC³ cell line 183A to investigate the effect of RA treatment on cellular responses to drugs that interact with topoisomerase II, namely, etoposide and m-AMSA. For comparison we examined the effect of RA treatment on the activity of bleomycin and X-irradiation, both of which produce free radical-mediated effects and are used quite commonly to treat head and neck cancer. We also determined topoisomerase II activity and drug-stimulated DNA cleavage activity in nuclear extracts of RA-treated cells. Our data indicate that RA treatment of 183A cells induces resistance to etoposide- and m-AMSA-induced DNA cleavage and cytotoxicity that does not appear to be mediated through a direct effect on the enzyme, and they suggest that other events occurring in the early stages of decreased proliferation can influence topoisomerase II-mediated DNA cleavage.

MATERIALS AND METHODS

Chemicals. Cell culture medium, fetal calf serum, trypsin, and Hanks’ balanced salt solution were purchased from Grand Island Biological Co. (Grand Island, NY). [3H]Thymidine (58 mCi/mmol), [methyl-3H]-thymidine (20 Ci/mmol), and 3H2O (1 mCi/g) were from New England Nuclear (Boston, MA); bleomycin and m-AMSA were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute; etoposide was provided by Bristol Myers (Syracuse, NY); [3H]Etoposide (200 mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA); [14C]-AMSA (19.6 mCi/mmol) was from SRI International (Menlo Park, CA); RA was provided by Dr. Reuben Lotan (Department of Tumor Biology, University of Texas M. D. Anderson Cancer Center, Houston, TX); tetrapropylammonium hydroxide was obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Lines and Culture Techniques. HHNSCC lines 183A and 1483 were grown in monolayer in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Details concerning the epithelial origin and characteristics of these cell lines have been published (9). Doubling time is approximately 48 h for 183A and 55 h for 1483. Mariner leukemia L1210 cells were grown in suspension culture in RPMI 1630 medium with 10% fetal calf serum. Cells were grown at 37°C in the presence of 5% CO2 with 2 mM glutamine, 50 μg/ml of penicillin, 50 μg/ml of streptomycin, and 100 μg/ml of neomycin added to the culture medium.

Drug Treatment. Etoposide, m-AMSA, and RA were dissolved in DMSO at 10 mM, stored at −20°C, and diluted with medium as

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3 The abbreviations used are: HHNSCC, human head and neck squamous cell carcinoma; RA, β-all-trans-retinoic acid; m-AMSA, 4’-(acridinylamino)methanesulfon-m-anisidine; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; kDNA, kinetoplast DNA; SDS, sodium dodecyl sulfate.
necessary. Control cultures were treated with DMSO alone. Bleomycin was made fresh and dissolved in water at 1 mg/ml. Cells were pretreated with RA for 72 h unless otherwise noted and washed with PBS before subsequent treatment with drug or X-radiation. Drug treatment was for 30 min at 37°C.

Cytotoxicity. Cells treated with RA and drug or X-radiation as described above were washed with ice-cold PBS, trypsinized, and seeded into 100-mm dishes. Cells treated in this way were consistently ≥95% viable by trypan blue dye exclusion. After 18 days of incubation, medium was removed and colonies were stained with 2% crystal violet in methanol. Colonies containing more than 50 aggregated tumor cells were counted. Plating efficiency was 3 to 4%.

Alkaline Elution. Single strand breaks in DNA were assayed by the technique of alkaline elution with proteinase K as previously described (10, 11). Cells were labeled with [³²P]thymidine (0.01 μCi/ml) for 72 h followed by 24-h incubation in label-free medium. In experiments with RA, cells were incubated in label-free medium for 5 h prior to the addition of RA. L1210 cells labeled with [³²P]thymidine and X-irradiated with 300 or 1000 rads were included on each filter as internal standards. Low break frequencies (≤0.5 x 10⁶ nucleotides) were quantified by eluting at 0.03 to 0.04 ml/min for 15 h. Higher break frequencies were quantified by eluting at 0.16 to 0.20 ml/min for 35 min.

Drug Transport. Steady-state concentrations of [³Hj]etoposide and [³C]m-AMSA were assayed as previously described (12). Approximately 10⁶ 183A cells were incubated in the presence of 1 nM [³C]m-AMSA or 10 μM [³H]etoposide. Tritiated water added to the cells treated with [³C]m-AMSA was used to quantify cell volume.

Isolation of Nuclei and Preparation of Nuclear Extracts. Nuclei were isolated from 183A cells in monolayer as previously described (13). All procedures were performed at 0–4°C. Phenylmethylsulfonyl fluoride, 0.1 mM (from 100 mM stock in isopropanol alcohol), was added to all buffers used in the preparation of nuclear extracts. Cells were harvested by trypsinization, sedimented, and washed 2 times with cold nucleus buffer containing 150 mM NaCl, 1 mM KH₂PO₄, 5 mM MgCl₂, 1 mM EGTA, 0.1 mM dithiothreitol, and 10% glycerol, pH 6.4. After resuspension in one volume of nucleus buffer, nine volumes of nucleus buffer containing 0.3% Triton X-100 were added to the cells, and the preparation was gently rotated for 10 min. Nuclei were sedimented at 3000 x g for 10 min, washed 5 times in detergent-free buffer, and used in alkaline elution or extracted in buffer containing 0.35 M NaCl on ice for 30 min. After extraction, the preparation was centrifuged at 3000 rpm for 20 min. The protein concentration in the supernatant was determined using the method of Bradford (14). The supernatant was stored in 50% glycerol at −20°C.

Preparation of kDNA. Kinetoplast DNA was purified from sarkosyl extracts of Crithidia fasciculata trypanosomes by cellulose chloride-ethidium bromide density centrifugation as described (15). kDNA was labeled with [³H]thymidine by adding 1 mCi of the isotope to 300 ml of exponentially growing trypanosome cultures 24 h before purification.

Type II Topoisomerase Assays. The reaction mixture for all assays contained 50 mM Tris-Cl (pH 7.5), 10 mM MgCl₂, 5 mM dithiothreitol, 0.5 mM EDTA, 0.03 mg/ml of bovine serum albumen, 1 mM ATP, and KCl concentration varying from 0 to 85 mM to keep the final total [KCl] + [NaCl] (with the addition of extract) between 85 to 150 mM. Quantitative analysis of decatenating activity in extracts was assayed using [³H]kDNA as described by Sahai and Kaplan (16). [³H]kDNA (0.35 μg) was incubated with varying extract concentrations in a final volume of 50 μl at 30°C for 30 min. The reaction was terminated by the addition of SDS to 1% and proteinase K at 0.1 mg/ml. After an additional 30-min incubation, samples were centrifuged at 12,000 x g in a microcentrifuge for 10 min. Then 80% of the volume was carefully removed and analyzed as supernatant, containing free minicircles, and the remainder was analyzed as pelleted networks.

% of decatenating activity = dpm of supernatant
dpm of supernatant + dpm of pellet

Results from the quantitative assay correlated well with those of duplicate samples loaded onto agarose gels and electrophoresed through 1% agarose as described (17). Drug-stimulated DNA cleavage activity was assayed qualitatively by the generation of Form III (linearized) DNA from supercoiled (Form I) SV40 DNA as detailed (17).

RESULTS

Initial experiments confirmed that etoposide and m-AMSA produced protein-linked DNA breaks in 183A and 1483; i.e., drug-induced single strand breaks were completely concealed in the absence of treatment with SDS and proteinase K (data not shown). The cytostatic effect of RA on 183A cells is shown in Fig. 1. Note that a relatively high concentration of RA is required to demonstrate growth inhibition. After 72-h treatment, the fraction of control growth was 0.9 ± 0.08 (SD) for 10 μM RA and 0.46 ± 0.10 for 20 μM RA. In separate experiments, growth curves generated by cell counts at 24, 44, and 72 h show exponential growth of 183A cells throughout the period of RA treatment (10 μM). Fractional growth inhibition at these time points, respectively, is 0.97 ± 0.03, 0.95 ± 0.01, and 0.94 ± 0.06. RA treatment alone produces no detectable DNA strand breaks but significantly inhibits strand-breaking activity of both etoposide and m-AMSA (Fig. 2). Although inhibition is nearly complete at 20 μM RA, a significant decrease in strand breaks is seen at 10 μM RA for both drugs (50% reduction in etoposide-treated cells and 33% in m-AMSA-treated cells). The inhibitory effect of RA treatment on etoposide and m-AMSA DNA cleavage activity can be demonstrated over a wide range of drug concentrations (Fig. 3) and is time dependent, requiring 72 h...
to become apparent (Fig. 4). This effect is not related to decreased drug accumulation in RA-treated cells as shown in Table 1. Further, similar results are obtained when nuclei isolated from RA-treated cells are exposed to etoposide or m-AMSA (data not shown).

X-ray-induced single strand breaks are also partially inhibited by RA treatment, but this effect is much less profound than the effect on etoposide and m-AMSA strand-breaking activity (Fig. 5). In contrast, RA treatment did not significantly alter the frequency of strand breaks induced by bleomycin (Fig. 5).

RA treatment does alter the colony-forming efficiency of 183A cells as shown in Fig. 6. When the data are corrected for the cytotoxic effect induced by RA alone, partial protection from etoposide and m-AMSA-mediated cytotoxicity can be demonstrated in RA-treated cells (Fig. 6, B and C). The doses of etoposide producing a 50% reduction in colony formation in control and RA-treated cells (10 μM for 72 h) were, respectively, 16 μM and 31 μM. Corresponding doses for m-AMSA are 2 μM (control) and 5 μM (RA 10 μM). This is well correlated with RA-induced reduction of drug-induced DNA cleavage. The colony-forming efficiency of X-irradiated cells is not altered by RA treatment, whereas the cytotoxic effect of bleomycin is enhanced (data not shown).

Type II topoisomerase catalytic activity in crude 0.35 M NaCl nuclear extracts from RA-treated (10 μM for 72 h) and control 183A cells was assayed by decatenation of [3H]kDNA using the method of Sahai and Kaplan (see “Materials and Methods” and Ref. 16). Fig. 7 demonstrates that, at equivalent amounts of total protein, decatenating activity in extracts from RA-treated and control cells is nearly equal.

Drug-stimulated DNA cleavage activity, as assayed by Form III generation (linearized DNA) from supercoiled double-stranded SV40 DNA, is difficult to demonstrate with either extract (Fig. 8) despite the use of extremely high concentrations of etoposide and testing over a broad range of protein concentrations from 0.1 to 10 μg of protein. Similar results were obtained in experiments with m-AMSA at 10 to 250 μM (data not shown). Fig. 8 does demonstrate one difference between the two extracts in that the catenating activity was consistently greater in the extract from RA-treated cells.

We obtained similar results in experiments with another HNSCC line, 1483. Although this line is more sensitive than 183A cells to the cytostatic effects of RA in long-term culture (9), growth inhibition by RA at 10 and 20 μM for 72 h is the same for both lines. Similarly, etoposide and m-AMSA strand-breaking activity in whole cells is inhibited to the same extent by RA treatment.

Table 1. Uptake of [[H]etoposide (10 μM for 30 min) or [14C]m-AMSA (1 μM for 30 min) in 183A cells treated with RA (20 μM for 72 h)

<table>
<thead>
<tr>
<th>Drug</th>
<th>No retinoic acid</th>
<th>+ Retinoic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]Etoposide</td>
<td>12.7 ± 7.0μM</td>
<td>15.8 ± 1.7μM</td>
</tr>
<tr>
<td>[14C]m-AMSA</td>
<td>17.0 ± 2.1μM</td>
<td>16.2 ± 2.8μM</td>
</tr>
</tbody>
</table>

* Mean ± SE.

Fig. 4. Time course of RA effect on etoposide and m-AMSA-induced DNA single strand breaks. 183A cells were treated with 10 μM RA for 1, 2, or 3 days before treatment with drug and alkaline elution as shown for Fig. 2.

Fig. 5. Effects of RA treatment on DNA single strand breaks produced by X-radiation and bleomycin. 183A cells were treated with varying doses of RA for 72 h before X-radiation or bleomycin treatment. DNA was eluted at a pump speed of 0.03 to 0.04 ml/min for a total elution time of 15 h. Points, mean of 3 experiments; bars, SD.

Fig. 6. A, inhibition of colony-forming activity of 183A cells by RA treatment for 72 h. B and C, inhibition of colony-forming activity of RA-treated cells by etoposide and m-AMSA. In B and C, data were corrected for cytotoxic effects induced by RA alone. Points, mean of 3 separate experiments; bars, SE.

Fig. 7. Decatenation of [3H]kDNA by 0.35 M NaCl nuclear extracts from untreated and RA-treated 183A cells. Decatenation was quantified as described in “Materials and Methods.”

Fig. 8. Effect of etoposide in stimulating DNA cleavage activity in 0.35 M NaCl nuclear extracts from untreated and RA-treated 183A cells. Numbers refer to etoposide concentration in micromolar units. Protein concentration was held constant at 7.5-μg/50-μl reaction mix. CTL, no nuclear extract.
DISCUSSION

RA treatment of 183A cells induces modest cytotasis that is accompanied by decreased cellular sensitivity to DNA cleavage and cytotoxic activities of etoposide and m-AMSA, both of which interact with topoisomerase II. We did not observe significant inhibition of cellular responses to free radical-mediated events, suggesting that the RA effect is relatively specific for topoisomerase II-mediated drug activity. Our data also provide further support for the concept that etoposide and m-AMSA share a common intracellular target and that drug-induced DNA cleavage is an important component of the cytotoxic effects of these agents.

Although the mechanism of RA's inhibitory effect remains undefined, our data suggest that it is not mediated through altered drug transport nor through changes in 0.35 M NaCl extractable nuclear topoisomerase II decatenating or DNA cleaving activity. We did not examine enzyme content in whole cells, but the fraction of topoisomerase II we studied is known to constitute both etoposide- and m-AMSA-interactive enzyme in isolated nuclei (18, 19). The assay we used to measure enzyme activity, decatenation of kDNA, is known to be sensitive to inhibition by high ionic strength and other substances that promote aggregation of DNA (3). This can lead to problems in quantitative analysis of crude nuclear extracts. In early experiments with extracts from 183A cells we found decatenating activity inhibited at a total protein concentration >1 μg/50 μl (20). This occurred despite strict control of ionic strength and was observed equally in extracts from retinoid-treated and control cells. Repeated study of two additional extracts, however, revealed no evidence of significant inhibitory activity up to a protein concentration of 10 μg/50 μl (Fig. 7), allowing comparison of decatenating and drug-stimulated cleavage activity over a much broader range of total protein. It was at these higher protein concentrations that we observed increased catenane formation in the extract from RA-treated cells (Fig. 8). If this finding is related to the inhibition of drug activity in whole cells, at least two separate mechanisms may be operative. RA may alter the functional state of the enzyme such that catenane formation is favored over cleavage. Alternatively, RA may enhance expression of another factor in the nucleus which promotes condensation of DNA and thus decreases accessibility of DNA to drug.

Fig. 4 demonstrates the reduced clonogenic potential of RA-treated 183A cells despite minimal fractional growth inhibition at 72 h when RA exposure is ended. This suggests that the RA-mediated inhibition of drug activity is related to changes within the nuclear milieu occurring early during slowed proliferation before a dramatic decrease in cell number or topoisomerase II activity is observed. Previous work with other cultured cell lines has shown that responses to quiescence induced by nutrient depletion are not uniform (5). Growth arrested Chinese hamster ovary cells and human leukemia cells (CCRF) exhibit decreased topoisomerase II-mediated drug sensitivity accompanied by decreased topoisomerase II content assayed by whole cell immunoblots. In contrast, L1210 cells do not possess reduced enzyme content in quiescence despite partial protection from etoposide-induced cytotoxicity. It also should be noted that Chinese hamster ovary cells show a decreased sensitivity to m-AMSA- and etoposide-induced DNA cleavage activity that is progressive from mid-log into plateau phase (21). Thus, changes associated with approaching quiescence can influence responsiveness to topoisomerase II-interactive drugs, despite continued exponential growth. Additionally, treatment of HL-60 cells with growth-inhibitory concentrations of 1-β-d-arabinofuranosylcytosine enhances sensitivity to both etoposide and m-AMSA in parallel with an increase in DNA strand break frequency. Similar to results we report here, enhancement of drug effect is independent of decatenating and drug-stimulated DNA cleavage activity in 0.35 M NaCl extracts (22).

It is conceivable that both RA and 1-β-d-arabinofuranosylcytosine mediate their effect on m-AMSA and etoposide activity by affecting the target of the drug-topoisomerase II interaction, cellular chromatin, albeit most likely in different ways. Although several possible mechanisms may be hypothesized whereby alterations in chromatin could interfere with topoisomerase II-mediated drug activity, little information exists regarding this question. In general, eukaryotic topoisomerase II cleavage sites appear to be closely associated with nuclease-sensitive regions of chromatin and areas of active gene expression (23–25). Additional data indicating that nucleoprotein organization of DNA may influence accessibility to enzyme come from the observation that topoisomerase II-mediated cleavage of chromatin in isolated Drosophila nuclei is markedly diminished in the histone repeat unit of the 87A7 heat-shock locus compared with cleavage of the same locus in naked DNA (26). Although the mechanism by which retinoids modulate cellular proliferation and differentiation is unknown, the available data indicate that they may alter nucleoprotein organization or induce other changes in chromatin with subsequent effects on gene expression.

Recently, a high affinity nuclear receptor for RA has been identified which appears to be a member of the steroid/thyroid receptor family (27). RA-induced transcriptional activation has been demonstrated using a chimeric construct of the RA receptor and the DNA-binding region of the estrogen receptor (27). Interaction of this receptor–RA complex with chromatin could also conceivably influence topoisomerase II binding or DNA cleaving activity. Obviously, only speculation is possible until more data are available concerning both the mechanism of retinoic acid effects as well as the determinants of topoisomerase II binding to DNA and subsequent cleavage reactions, with or without drug.

Whereas m-AMSA and etoposide are not useful for treating patients with head and neck cancer, bleomycin and X-radiation are used quite commonly. cis-Retinoic acid is useful for treating dysplastic lesions of the oral cavity (28) and is currently being evaluated at our institution as adjuvant treatment in patients with localized head and neck cancer after primary treatment with either surgery, radiotherapy, or both. Retinoids may eventually be incorporated into induction chemotherapy regimens or in the treatment of metastatic disease from primary tumors in the head and neck. Our data suggest that the therapeutic efficacy of bleomycin or X-radiation will not be diminished by such an approach.

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5. Sullivan, D. M., Latham, M. D., and Ross, W. E. Proliferation dependent events, suggesting that the RA effect is relatively specific for topoisomerase II interaction, cellular chromatin, albeit most likely in different ways. Although several possible mechanisms may be hypothesized whereby alterations in chromatin could interfere with topoisomerase II-mediated drug activity, little information exists regarding this question. In general, eukaryotic topoisomerase II cleavage sites appear to be closely associated with nuclease-sensitive regions of chromatin and areas of active gene expression (23–25). Additional data indicating that nucleoprotein organization of DNA may influence accessibility to enzyme come from the observation that topoisomerase II-mediated cleavage of chromatin in isolated Drosophila nuclei is markedly diminished in the histone repeat unit of the 87A7 heat-shock locus compared with cleavage of the same locus in naked DNA (26). Although the mechanism by which retinoids modulate cellular proliferation and differentiation is unknown, the available data indicate that they may alter nucleoprotein organization or induce other changes in chromatin with subsequent effects on gene expression.

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