Differential Retinoic Acid Radiosensitization of Cervical Carcinoma Cell Lines

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ABSTRACT

The potential of retinoic acid as a radiosensitizer was investigated using SiHa and CC-i human uterine cervical carcinoma cell lines, representative of high- and low-grade lesions, respectively. SiHa was significantly *P < 0.05* radiosensitized, whereas CC-i was not. Although 48 h of treatment with 5 µM 13-cis-retinoic acid prior to irradiation was sufficient to induce radiosensitization, continuation of treatment after irradiation significantly increased the effect *P < 0.05*. Three hypotheses were tested to explain the different responses of the two lines. One hypothesis was that SiHa is more sensitive to retinoic acid than CC-i. Measurement of growth revealed that SiHa was more sensitive to growth inhibition by retinoic acid than CC-i. The second hypothesis was that retinoic acid increases the proportion of G1-phase cells in SiHa but not in CC-i. This was found not to be true, because a retinoic acid treatment schedule that induced radiosensitization did not alter cell cycle distribution profiles in the absence of radiation. The third hypothesis was that retinoic acid alters the cell cycle response of SiHa but not CC-i to radiation. Postirradiation cell cycle profiles revealed that retinoic acid increased G1 delay in SiHa, whereas CC-i exhibited no significant G1 delay. Both lines exhibited G2 delays that were unaffected by retinoic acid. In conclusion, radiosensitization of SiHa but not CC-i may be explained by different sensitivities to retinoic acid and differences in postirradiation cell cycle responses. Radiosensitization at radiation doses used clinically was observed when retinoic acid was administered both before and after irradiation.

INTRODUCTION

Cancer of the uterine cervix is the number one worldwide cause of cancer death in women (1, 2). Primary radiation therapy is recommended for locally advanced disease (stage IIb or greater) but fails to control the tumors in 35–90% of patients (3). The 5-year survival for these patients ranges from 10 to 58% (4). To date, no effective salvage therapy has been developed. To improve survival rates of patients with locally advanced disease, combination chemotherapy regimens, neoadjuvant chemotherapy (drug prior to surgery or radiation), and chemoradiation have been evaluated. Response rates of up to 87% have been reported, but prolonged response is rare, and no clear survival benefit has been shown in any setting (5).

The response of cervical tumors to radiation therapy may be affected by HPV. HPV has been identified in the vast majority of cervical tumors and is believed to be an etiological agent in the development of this disease (6). Two HPV genes that are regularly expressed in tumors are the early E6 and E7 genes (7). The proteins encoded by the high-risk E6 and E7 oncogenes exert their effects on the cell by binding to host cellular proteins. The E6 protein binds to the p53 tumor suppressor protein and causes its degradation, resulting in altered cell cycle control, genomic instability, and altered cellular response to DNA damage (8). The E7 protein binds to the cellular retinoblastoma tumor suppressor gene product, resulting in altered cell cycle control and genomic instability (8). Retinoic acid has been shown to regulate expression of the E6 and E7 genes and, therefore, has potential in regulating the cellular response to radiation (9, 10).

Retinoic acid is the natural metabolite of vitamin A, which is required for the normal growth and differentiation of epithelium. Several normal geometric isomers of retinoic acid, all-trans, 13-cis, and 9-cis have the potential to shrink tumor size because they modulate growth, differentiation, apoptosis, and the immune system (11). 13-cis-retinoic acid has demonstrated some promise as a chemotherapeutic agent for the prevention and treatment of cervical cancer (12–18). In addition to down-regulation of HPV genes, retinoic acid exhibits other *in vitro* activities that support its potential as a chemotherapeutic agent for the treatment of cervical cancer. A greater effect on neoplastic and tumor cells over normal cells by retinoic acid is suggested by increased sensitivity of HPV-containing keratinocytes over normal keratinocytes to growth inhibition by retinoic acid (19). Possible radiosensitization is suggested by accumulation of cells in the G1 phase of the cell cycle in cultures that are growth inhibited by retinoic acid (20–22). The radiosensitivity of cells varies with the mitotic cycle. Cells in late G1 phase are more radiosensitive, although not as radiosensitive as cells in G2 or M phase (23). In this study, the potential of 13-cis-retinoic acid as a radiosensitizer was investigated using two cervical cell

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lines, SiHa and CC-1, which are representative of high- and low-grade carcinomas, respectively (24).

MATERIALS AND METHODS

Cell Culture and Retinoic Acid Treatment. SiHa and CC-1 cultures were maintained in Eagle’s MEM containing Earle’s salts and t-glutamine (Cellgro; Mediatech, Herndon, VA) supplemented with nonessential amino acids, sodium pyruvate, and 10% fetal bovine serum (Hycione Laboratories, Inc., Logan, UT). Only lots of fetal bovine serum that contain negligible quantities of retinoic acid as determined by high-performance liquid chromatography analysis were used. 13-cis-retinoic acid (Sigma Chemical Co., St. Louis, MO) was dissolved in ethanol at a concentration of 5 mM. Five ml of media inoculated with either 5 μl of retinoic acid stock (treated) or 5 μl of ethanol (solvent control) were allocated per tissue culture flask (25 cm²).

Radiation Survival Assay. Monolayer cell cultures in log phase were obtained by plating cells at a density of 5 × 10³ cells/25-cm² flask in the presence or absence of 5 μM 13-cis-retinoic acid. Forty-eight h later, the flasks were transferred to ice and irradiated with a 60Co radiation source. Each flask received a dose of radiation from 0.75 to 10 Gy. A control flask receiving no radiation was exposed to the same experimental conditions (mock irradiated). The cells were then trypsinized and plated in triplicate in the presence or absence of 5 μM 13-cis-retinoic acid. The density of cells plated were adjusted to achieve 50-100 surviving colonies per flask. All flasks were plated in triplicate. The flasks were incubated for 7-14 days, after which the colonies were fixed, stained, and counted. The surviving fraction was calculated using the following formula from Hall (25):

\[
\text{Surviving fraction} = \frac{\text{Average no. of colonies counted}}{(\text{No. of cells plated} \times \text{plating efficiency}/100)}
\]

All experiments were repeated three times, and the results are presented as averages and SDs. The DMFs were determined by dividing the dose required to obtain 90, 99, and 99.9% killing in the absence of retinoic acid by that in the presence of retinoic acid.

Growth Inhibition. Six-well tissue culture dishes were inoculated with a concentration of 4 × 10⁴ cells/well. Twenty-four h after plating, the medium was replenished and treatment was initiated. In the case of continuous treatment, media and drug were replenished every 2 days. In the case of 48-h treatment only, medium was replenished without drug every 2 days after the initial 48 h treatment. After 7 days, both continuously treated and 48-h-treated cultures were still in exponential growth. At this time, the number of cells per well in the treated and control cultures was determined using a particle counter (Coulter ZM, Miami, FL). The percentage of growth inhibition was determined by dividing the number of cells in the treated cultures by that in the control cultures and multiplying by 100.

Flow Cytometry. Cultures, prepared as described for the radiation survival assays, were either pretreated or not treated with retinoic acid and irradiated with a dose of 5 Gy of 60Co γ-rays. Similarly, control cultures either pretreated or not treated with retinoic acid were mock irradiated. Immediately after irradiation or mock irradiation, cultures were trypsinized and either replated or fixed in 70% ethanol. After fixation, cells were centrifuged, washed in PBS, and resuspended in a solution of 5 μg/ml PI and kept on ice for 30 min prior to immediate flow cytometric analysis. The PI dye was excited with an argon ion.
laser (Coherent, Palo Alto, CA) operating at 488 nm (100 mW), and cells were analyzed for PI fluorescence (DNA content) with a Becton Dickinson FACStar Plus cell sorter (San Jose, CA). Cell cycle distributions were analyzed using ModFit LT Listmode Analysis Software (Verity Software House, Topsham, ME).

**RESULTS**

**Radiosensitivity.** The effects of 13-cis-retinoic acid on the clonogenic survival of SiHa and CC-1 after exposure to a range of radiation doses were determined. Both cell lines were pretreated with either 5 μM 13-cis-retinoic acid or the same volume of ethanol vehicle for 48 h prior to irradiation. Forty-eight h was chosen because it was expected to be sufficient to induce detectable growth and differentiation effects. The doubling time of both cell lines is 48 h, and irreversible differentiation has been shown to be induced in cell lines treated with retinoic acid for 48 h (26, 27). The SiHa cell line exhibited a greater radiosensitivity that was enhanced by retinoic acid, whereas the lesser radiosensitivity of CC-1 was not enhanced by this agent (Fig. 1). The linear quadratic parameters of the SiHa and CC-1 radiation survival curves were different (Table 1). In the absence of treatment with retinoic acid, the SiHa curve exhibited a larger α component, and the CC-1 curve exhibited a much larger α component. Pretreatment with retinoic acid increased both the α and β components of SiHa. Although retinoic acid increased the β component in CC-1, the α component was actually decreased. Despite this, there was no significant difference in the two CC-1 survival curves with and without retinoic acid. Retinoic acid pretreatment slightly increased the plating efficiency of SiHa and significantly decreased that of CC-1.

To further evaluate the radiosensitization of SiHa, the effects of retinoic acid after irradiation were evaluated in the presence and absence of pretreatment (Fig. 2). The survival of SiHa cells that received no pretreatment was not affected by the presence of retinoic acid in the medium. This is despite the decrease in plating efficiency from 89% in untreated cultures to 54% in posttreated cultures (Table 1). The combination of pre- and posttreatment significantly radiosensitized the SiHa cell line. The α component was greatly increased, and the β component was decreased. The plating efficiency of cultures receiving both pre- and posttreatment was intermediate between that observed in the presence of pre- or posttreatment only. The DMFs were moderate in cultures receiving pretreatment only but significantly increased in cultures receiving both pre- and posttreatment (Table 2). In both cases, the DMFs decreased with increasing radiation dose.

To determine whether the differences in surviving fractions of SiHa cultures exposed to the various retinoic acid treatments were statistically significant, the surviving fractions at individual radiation doses were tested with a one-way ANOVA (Fig. 3). The survival of cultures exposed to combined pre- and posttreatment was significantly less than either pre- or posttreatment alone and appeared additive at 7.5 Gy. Pretreatment alone resulted in significant decreases in survival at doses of 5 Gy and higher. Posttreatment significantly decreased survival only at doses above 5 Gy. The combination of pre- and posttreatment resulted in significant decreases in the surviving fraction at all radiation doses tested. At doses above 5 Gy, the surviving fractions of all three retinoic acid treatment schedules were significantly different from each other.

**Proliferation.** One possible explanation for the radiosensitization of SiHa and not CC-1 by retinoic acid is that the SiHa line is more responsive to retinoic acid. To test this hypothesis, the effects of 13-cis-retinoic acid on SiHa and CC-1 growth were evaluated over a range of concentrations. Over a 1-week period, the effects of retinoic acid on the growth rate of cultures were dependent on the cell line and the concentration of drug used (Fig. 4). At a concentration of 10−9 M, no significant growth effects were observed for either cell line. The growth...
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Fig. 3 Comparison of surviving fractions resulting from different retinoic acid treatment schedules at individual radiation doses. Surviving fractions that were significantly less \((P < 0.05)\) than irradiated cultures not exposed to retinoic acid, as determined by a one-way ANOVA, are indicated by an asterisk \((P < 0.05)\). At 5 Gy and above, all retinoic acid treatment schedules were significantly different from each other. Bars, SD.

Fig. 4 Comparison of retinoic acid effects on the growth rate. Logarithmically growing cultures of SiHa (■) and CC-1 (□) cell lines were treated with 10-fold increasing concentrations of retinoic acid. After 7 days, the growth rate was determined by dividing the number of cells in the treated cultures by that in control cultures treated with the same volume of solvent. Bars, SD.

rate of CC-1 was slightly increased at \(10^{-8} \text{ M}\) and decreased at higher concentrations in a dose-dependent manner. In contrast, no increase in the growth rate was observed for the SiHa cell line at any concentration.

The effect of the 48-h pretreatment schedule was evaluated on both cell lines. After the 48 h, the retinoic acid containing medium was removed and replenished with fresh medium. The growth of each cell line was monitored over 7 subsequent days and found to be identical to untreated control cultures (48 h doubling time, data not shown).

Cell Cycle Progression. A potential explanation for the different responses of the two cell lines is that retinoic acid causes an accumulation of cells in \(G_1\) in SiHa but not CC-1 cultures. To test this hypothesis, the effects of retinoic acid on cell cycle progression were evaluated by flow cytometric analysis (Fig. 5). No significant differences were observed in the cell cycle distribution of either cell line after 48 h of pretreatment (Fig. 5, 0 h). As a control, both cultures were pretreated for 48 h with retinoic acid and mock irradiated; the cell cycle distribution was monitored daily for 9 days. No significant effects of retinoic acid pretreatment on the cell cycle distribution were observed in either cell line (data not shown).

Another explanation for the different responses of the cell lines could be that retinoic acid alters the cell cycle response of SiHa but not CC-1 to radiation. After irradiation, the cell cycle distributions of the two cell lines changed with time (Fig. 5). The SiHa cell line exhibited a slight transient increase in \(G_2\), followed by a slight increase in \(G_1\). Retinoic acid pretreatment enhanced the increase in \(G_1\) with a corresponding decrease in S-phase cells and no effect on the percentage of cells in \(G_2\).

The cell cycle response of CC-1 to radiation was different from the response of SiHa. By 24 h after irradiation, a significant \(G_2\) arrest was evident; the percentage of cells in \(G_2\) more than doubled, and no S-phase cells were present. Retinoic acid treatment did not induce significant changes in the CC-1 profile within this time period. By 96 h, however, an increase in the percentage of cells in \(G_1\) with a corresponding decrease in S phase was evident. No effect of retinoic acid on the percentage of cells in \(G_2\) was observed.

DISCUSSION

The ability of 13-cis-retinoic acid to radiosensitize tumor cells was evaluated using two human cervical carcinoma cell lines, SiHa and CC-1. The two cell lines exhibited intrinsic differences in their response to and survival of irradiation. Survival curves with broad shoulders and small \(\alpha\) components, as observed in SiHa, indicate that the mode of cell death is mostly due to sublethal damage (28). Conversely, survival curves with minimal shoulders and large \(\alpha\) components, as observed in CC-1, indicate that cell killing is mostly due to irreparable damage (28). The \(G_2\) arrest and complete loss of S-phase cells in postirradiated CC-1 cultures may account for the greater resistance of this line to the lethal effects of radiation.
Fig. 5 Effects of retinoic acid on the cell cycle distribution of SiHa and CC-1 cultures. Cultures were either untreated (□) or pretreated (■) with retinoic acid for 48 h. At time zero, they were irradiated and were fixed immediately or after the indicated time period (hours). Fixed cells were evaluated for DNA content using flow cytometric analysis to determine the percentage of cells in the various phases of the cell cycle as indicated.

Arrest of cells in G₂ phase has been shown to be important for repair of DNA damage and cell survival (29). Although the plating efficiencies vary considerably between cell lines and treatments in this study, the research of others has indicated that the plating efficiency does not correlate with radiosensitivity in SiHa and other lines (30).

Varying degrees of differentiation may contribute to the different radiosensitivities of these two cell lines. A higher degree of differentiation in CC-1 is indicated by expression of the squamous cell differentiation marker, involucrin, in organotypic cultures of CC-1 but not SiHa (24). In bladder tumors, variations in radiosensitivity have been correlated with the degree of differentiation, and increased radioresistance has been associated with squamous differentiation (31, 32). In a study of six ovarian carcinoma cell lines, the two cell lines derived from well-differentiated tumors appeared more radioresistant than the other cell lines (33).

Although SiHa is more sensitive than CC-1 to the antiproliferative effects of retinoic acid, growth inhibition cannot be solely responsible for radiosensitization. This is because the 48 h of pretreatment is sufficient to radiosensitize SiHa but is not sufficient to decrease the growth rate. The continued presence of retinoic acid in the media past 48 h is required to slow the growth rate; therefore, cultures receiving retinoic acid posttreatment will be growth inhibited. Because posttreatment alone radiosensitizes SiHa at high radiation doses and combined pre- and posttreatment schedules radiosensitizes SiHa better than pretreatment alone, it is possible that the antiproliferative effects might contribute to radiosensitization.

A potential mechanism of radiosensitization is through accumulation of cells in G₂, shown to be induced by retinoic acid in several different types of cells (20-22). In this study, 13-cis-retinoic acid did not induce alterations in the cell cycle distribution of either cell line in the absence of radiation. Radiosensitization by concentrations of retinoic acid that do not affect the cell cycle distribution has also been observed in bladder and lung carcinoma cell lines (34). Therefore, cell cycle
arrest in SiHa cells prior to irradiation is not a likely mechanism for the radiosensitization observed in this study.

Postirradiation cell cycle effects, however, may contribute to the mechanism of retinoid radiosensitization. Normal cells exposed to ionizing radiation arrest their growth at several points in the cell cycle to allow for repair of DNA damage (35). Retinoic acid increased the G1 delay that occurred in SiHa cultures within the first 48 h after irradiation. CC-1 did not exhibit a G1 delay, and no effect of retinoic acid on G1 was observed until 96 h after irradiation. Retinoic acid had no effect on the G2 delay observed in each cell line. Therefore, the increase in G1 delay induced by retinoic acid within the first 48 h after irradiation may be responsible for radiosensitization in SiHa cells. The lack of G2 delay in CC-1 may explain the lack of radiosensitization in this line.

An increase in G1 delay, however, is not consistent with a radiosensitization effect. The radiosensitization observed in this study may be due to accumulation of treated SiHa cells in late G1 as opposed to early G1. This is because in cell lines that have G1 phases of appreciable length, cells in early G1 are more radiosensitive, whereas those in late G1 are more radiosensitive (23). Early and late G1 cannot be deciphered with the experimental conditions used in this study.

Cell cycle regulation in SiHa and CC-1 is most likely affected by the presence of high-risk HPV types 16 and 18, respectively. The high-risk HPV E6 and E7 gene products interact with cellular proteins that are involved in radiation-induced G1 delay and thereby alter the cellular response to radiation (36, 37). Down-regulation of E6 expression, therefore, may be the mechanism by which retinoic acid induces an increase in G1-phase cells in irradiated SiHa cultures (9, 10). Decreased expression of E6 in SiHa cells would result in a relief of the inhibition of cellular p53 protein by E6 and a corresponding increase in G1 delay.

Another potential mechanism of retinoid radiosensitization is through increased apoptosis in treated cultures. This is unlikely, however, because 13-cis-retinoic acid has been shown not to induce apoptosis in SiHa cells (38). SiHa cells are capable of apoptosis, however, because another retinoid, N-(4-hydroxyphenyl)retinamide (4-HPR), does induce this activity in this cell line (38).

The major objective for clinical use of a radiosensitizer is to sensitize tumor cells more than normal cells. Because HPV-infected cells are more sensitive to retinoic acid than uninfected cells, it seems highly likely that retinoic acid will differentially radiosensitize normal and tumor cells. In a study of retinoic acid effects on recovery from potentially lethal damage, a greater radiosensitization of malignant over normal cell lines was observed (39). Furthermore, clinically beneficial effects of retinoic acid are indicated by the observation that peripheral blood lymphocytes are actually radioprotected by retinoic acid (40).

The DMFs of 1.3—1.9 in SiHa cultures pre- and posttreated with retinoic acid indicate that this drug could allow patients to be treated with lower radiation doses without compromising the clinical outcome. In animal models, a DMF of 1.8 dramatically improves the proportion of tumors controlled when radiation is given in a single dose (41). Conventional radiotherapy, however, involves multifractionated doses and, therefore, the clinical effectiveness of retinoic acid may be different from what would be predicted from in vitro experiments.

The dose per fraction administered clinically, 2 Gy or less, corresponds to the low-dose portion of the radiation survival curve where the α component exerts the dominating effect (25). The dose at which survival of cells irradiated in vitro best relates to the radioresponsiveness of the corresponding tumor has been shown to be 2 Gy (42—45). At this dose, the combined pre- and posttreatment of SiHa induced a significant decrease in the surviving fraction, further demonstrating promise for the clinical effectiveness of retinoids as radiosensitizers. In a similar study of four different cancer cell lines, increases in the α component by paclitaxel (Taxol®) treatment corresponded with the degree of radiosensitization (46). Consistent with our findings, the cell line exhibiting the highest α component in their study was not radiosensitized.

In conclusion, the potential effectiveness of 13-cis-retinoic acid as a radiosensitizer is indicated by potentiation of the lethal effects due to radiation in SiHa cultures treated with this agent. The greatest radiosensitization was observed when retinoic acid was present both before and after irradiation and occurred at clinically relevant radiation doses. The lack of radiosensitization in CC-1 cultures does not deter from this potential, because primary radiation therapy is routinely used for locally advanced disease only and CC-1 is representative of a low-grade tumor. The different responses of SiHa and CC-1 may be explained by differences in sensitivity to retinoic acid and in postirradiation cell cycle responses. Future studies comparing such differences should gain further insight into the mechanism of radiosensitization by retinoic acid.

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