Flavopiridol Mediates Cell Cycle Arrest and Apoptosis in Esophageal Cancer Cells


ABSTRACT

Esophageal adenocarcinoma (SKGT-2, SKGT-4, and SKGT-5) and epidermoid carcinoma (HCE-4) cells containing variable retinoblastoma (Rb), cyclin D1, p16, and p53 expression patterns were exposed to the synthetic flavone, flavopiridol. The IC50 was approximately 100–150 nM for each of these cell lines. Exposure of esophageal carcinoma cells to 300 nM flavopiridol induced cell cycle arrest and apoptosis, resulting in a 90% inhibition of proliferation relative to that of nontreated cells after a 5-day exposure to the drug. Western blot analysis revealed diminution of cyclin D1, Rb, and p107 protein levels after flavopiridol exposure. Whereas cell cycle arrest and overall growth inhibition did not correlate in any obvious manner with the genotype of these cell lines, apoptosis seemed to be more pronounced in SKGT-2 and SKGT-4 cells that lack Rb expression. Pretreatment of esophageal cancer cells with 9-cis-retinoic acid did not substantially potentiate flavopiridol activity in these cell lines. Although the precise mechanism of flavopiridol-mediated cytotoxicity has not been fully defined, this drug is an attractive agent for molecular intervention in esophageal cancers and their precursor lesions; further evaluation of flavopiridol in this clinical context is warranted.

INTRODUCTION

Abrogation of G1 restriction point control is a common theme of malignant transformation in a variety of organ systems including the aerodigestive tract (1, 2). In normal tissues, progression through the restriction point is governed by highly orchestrated formation, activation, and degradation of cyclin-cdk2 complexes regulating phosphorylation of the Rb gene product (3, 4). Several mutations that perturb this aspect of cell cycle regulation are known to occur early during multistep esophageal carcinogenesis. Nearly 50% of esophageal cancers exhibit a loss of heterozygosity involving the Rb gene (5, 6), yet only 20% of these neoplasms lack Rb protein expression (7); therefore, in the majority of esophageal neoplasms, restriction point control is circumvented via overexpression of cyclin D1, loss of p16 expression, or both, often in the context of p53 mutations (1, 8).

Genetic events that disrupt cell cycle regulation are attractive targets for molecular intervention in esophageal cancers, the incidence of which continues to increase at an alarming rate. We previously reported a reciprocal relationship between Rb, cyclin D1, and p16 expression in esophageal cancer cell lines (9), similar to what has also been noted in a variety of other carcinomas (10, 11). Proliferation of esophageal squamous and adenocarcinoma cells can be inhibited by retroviral vectors expressing antisense cyclin D1 or wild-type p53 (12); however, the low titer of these viruses obtained with conventional packaging cell lines and the relatively low transduction efficiency of retroviral vectors diminish their utility with respect to gene therapy of esophageal neoplasms. Recombinant adenoviral vectors expressing p16 or p53 gene sequences can mediate G1 arrest in epidermoid esophageal cancer cells; however, esophageal adenocarcinomas are refractory to adenoviral transduction (9), hence the potential of adenoviral vectors for gene therapy of esophageal adenocarcinomas and Barrett’s esophagus seems limited.

Flavopiridol is a synthetic flavone that induces cell cycle arrest and apoptosis in a variety of transformed cells (13). This agent is known to inhibit cdk2 and cdk4 activity, diminish cyclin D1 expression, and induce apoptosis via p53-independent pathways, in part by decreasing Bcl-2 expression at the transcriptional level (14–16). At high concentrations, flavopiridol inhibits a variety of protein kinases (17).

Although the mechanisms of flavopiridol-mediated cytotoxicity have not been fully elucidated, it is conceivable that this agent may be efficacious in the treatment and prevention of esophageal cancers because it effectively achieves multigene targeting (p16 inactivation and cyclin D1 overexpression) without the complexities and limitations of viral gene delivery. In the present study, we examined the effects of flavopiridol in esophageal adenocarcinoma and squamous cell carcinoma cell lines of defined genotypes. In addition, we sought to determine if 9-cis-RA could enhance flavopiridol activity due to its ability to diminish cyclin D1 expression in cancer cells (18). We report herein that flavopiridol mediated growth inhibition via cell cycle arrest and apoptosis in esophageal cancer cell lines irrespective of histology and tumor suppressor gene status; 9-cis-RA alone had a minimal effect regarding the inhibition of proliferation of esophageal carcinoma cells and did not significantly enhance the activity of flavopiridol in these cells. The ability of flavopiridol to simultaneously target several different molecular defects in malignant cells of diverse histologies makes this an attractive agent for use in the treatment and possible prevention of esophageal cancers.

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2 The abbreviations used are: cdk, cyclin-dependent kinase; RA, retinoic acid.

3 D. S. Schrump, unpublished observations.
MATERIALS AND METHODS

Cell Lines. The SKGT-2 cell line, which was derived from an adenocarcinoma of the gastroesophageal junction, as well as the SKGT-4 and SKGT-5 cell lines, which were established from carcinomas arising in Barrett's mucosae, have been described previously (19). The HCE-4 epidermoid esophageal carcinoma cell line was obtained from Curtis Harris (National Cancer Institute, Bethesda, MD). Nuclear RA receptor α, β, and γ expression, as assessed by Northern blot techniques, is intact in all of these lines (20).4 These cell lines were maintained as described previously (9). All proliferation assays were conducted in RPMI 1640 containing antibiotics and 10% fetal bovine serum.

Materials. The synthetic flavone flavopiridol (formally designated L86-8275; Ref. 17) was obtained from Edward Sausville (Experimental Therapeutics Section, National Cancer Institute). 9-cis-RA was procured from Sigma. Both chemicals were diluted in absolute ethanol or DMSO to yield stock solutions from which the final drug concentrations were derived.

In Vitro Proliferation Assays. Esophageal cancer cells were plated in triplicate wells in complete RPMI 1640 at 1 × 10^4 cells/well in 6-well plates. Twenty-four h later, flavopiridol was added to appropriate wells, yielding the desired drug concentrations. For IC_{50} assays, cells were counted 3 days later using trypan blue exclusion techniques. For initial proliferation assays, cells were plated as described and counted at appropriate time points after flavopiridol exposure using similar techniques. The proliferation of cells after combined RA and flavopiridol treatment was analyzed using trypan blue exclusion techniques. In brief, cells were plated as described above and exposed to 10 μM 9-cis-RA for 3 days, at which time flavopiridol at a final concentration of 300 nM was added to appropriate wells with or without RA. The proliferation of cells was assessed on days 3, 4, 5, and 6 after initial RA treatment.

Western Blot Analysis. Whole cell lysates (50 μg) obtained from drug-treated and control cells were prepared as described previously (9), fractionated by PAGE, and electroblotted onto nitrocellulose membranes. Rb, p107, cyclin D1, and actin were detected with polyclonal or monoclonal antibodies obtained from Pharmingen (Rb), Santa Cruz Biotechnology (p107 and actin), and Upstate Biotechnology, Inc. (cyclin D1) using the appropriate secondary antibodies according to Abersham enhanced chemiluminescence protocols.

Cell Cycle Analysis. Cell cycle kinetics of nonsynchronized cells grown in the presence or absence of drug were analyzed by propidium iodide techniques (21, 22) using a FACScan apparatus (Becton Dickinson). Apoptosis in these cells was simultaneously evaluated by flow cytometry techniques using an Apo-BrdU kit (Pharmingen International) according to Pharmingen protocols.

RESULTS

We have previously demonstrated that an adenoviral p16 gene construct can induce profound cell cycle arrest without obvious apoptosis in HCE-4 and HCE-7 esophageal cancer cells (9). In addition, Zhou et al. (12) have reported that an antisense cyclin D1 construct delivered via a retroviral vector can diminish cyclin D1 expression and mediate growth inhibition of HCE-7 cells without inducing cell cycle arrest. These data suggested to us that flavopiridol might have activity in esophageal cancer cells, many of which harbor mutations involving p16, cyclin D1, or both.

In preliminary experiments, Rb, p107, cyclin D1, and p16 expression was verified in esophageal cancer cell lines using Western blot analysis. As previously reported by us (9) and summarized in Table 1, SKGT-2 and SKGT-4 cells lack Rb expression and have moderate levels of cyclin D1 and robust p16 expression. SKGT-5 cells express Rb, have moderate levels of cyclin D1, and lack p16 expression. HCE-4 cells express Rb, have elevated cyclin D1 levels due to the amplification and overexpression of the cyclin D1 proto-oncogene (7), and have negligible p16 expression. SKGT-2, SKGT-4, and SKGT-5 cells express a mutant p53 protein verified by single-strand conformational polymorphism and sequencing analyses; the HCE-4 cell line also harbors a p53 mutation (23).

The sensitivity of esophageal cancer cells to flavopiridol was initially evaluated in a 3-day assay depicted in Fig. 1. The IC_{50} for the four esophageal cancer cell lines was approximately 100-150 nM, results of which were in accordance with those reported previously for nonesophageal cancer cell lines (24). Subsequent studies revealed that at a concentration of 300 nM, flavopiridol inhibited the proliferation of SKGT-2, SKGT-4, SKGT-5, and HCE-4 cells by approximately 95% relative to control cells after 5 days of continuous drug exposure (Fig. 2). Although cell cycle arrest seemed to mediate the response to flavopiridol in SKGT-4 and SKGT-5 cells during the first 24 h of drug exposure, cell death was eventually noted in all cell lines such that by day 5, cell counts were reduced by 80, 70, 60, and 66% for SKGT-2, SKGT-4, SKGT-5, and HCE-4 cells, respectively, relative to initial cell counts. The extent of growth inhibition mediated by flavopiridol did not correlate in any manner with Rb, cyclin D1, p16, or p53 status in these cell lines.

Propidium iodide and Apo-BrdU analyses of cell cycle kinetics and apoptosis were performed to further characterize the mechanism(s) of flavopiridol activity in esophageal cancer cells. As shown in Table 2, flavopiridol primarily induced a G2-M-phase block in nonsynchronized cells within 24 h after drug exposure that remained pronounced after 3 days of continuous drug exposure. Apo-BrdU analysis of the same cell populations revealed a marked increase in apoptotic cell activity in all four cell lines after flavopiridol exposure. Interestingly, apoptotic activity seemed more pronounced in SKGT-2 and SKGT-4 cells that lack detectable Rb expression and retain high level p16 expression.

In an effort to further characterize the activity of flavopiridol in esophageal cancer cells, we evaluated cyclin D1, Rb, and p107 protein levels in SKGT-2, SKGT-5, and HCE-4 cells grown in the absence or presence of flavopiridol (these three cell lines were chosen because they represented the three predomi-
Fig. 1 Analysis of the dose-related inhibitory activity of flavopiridol in esophageal cancer lines. Cells were exposed to varying concentrations of flavopiridol for 3 days and counted (triplicate wells) by trypan blue exclusion techniques. Representative experiments for each cell line are depicted.

Fig. 2 The proliferation of esophageal cancer cells after flavopiridol exposure is shown. Cells were plated at $1 \times 10^5$ cells/well and exposed 24 h later to either media alone or media containing 300 nM flavopiridol. The proliferation of cells in the presence and absence of drug was assessed at the designated time points by trypan blue exclusion techniques. Representative experiments for each cell line are depicted.

nant genotype patterns outlined in Table 1). Cyclin D1 expression was markedly decreased in SKGT-2 cells after flavopiridol exposure (a representative experiment is depicted in Fig. 3); less pronounced reductions in cyclin D1 expression were observed in SKGT-5 and HCE-4 cells after similar treatment. Interestingly, rather than observing mobility shifts consistent with altered phosphorylation states of Rb and p107 (which were expected due to the ability of flavopiridol to inhibit the activity of cdk4 and cdk6; Ref. 14), levels of both of these tumor suppressor gene products seemed diminished in all cell lines after flavopiridol exposure. Although flavopiridol has been reported to inhibit Bcl-2 expression in leukemia cells (16), no obvious

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A. Senderowicz, personal communication.
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Table 2  Propidium iodide and Apo-BrdU analysis of cell cycle kinetics and apoptosis in flavopiridol-treated esophageal cancer cells

<table>
<thead>
<tr>
<th></th>
<th>SKGT-2</th>
<th>SKGT-4</th>
<th>SKGT-5</th>
<th>HCE-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G0-G1</td>
<td>49.9</td>
<td>53.9</td>
<td>48</td>
<td>36</td>
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<tr>
<td>S phase</td>
<td>40.1</td>
<td>27.3</td>
<td>37</td>
<td>34</td>
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<tr>
<td>G2-M</td>
<td>10.0</td>
<td>18.8</td>
<td>13</td>
<td>30</td>
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<td></td>
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<tr>
<td>Day 3</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>G0-G1</td>
<td>54.7</td>
<td>59.2</td>
<td>46</td>
<td>38</td>
</tr>
<tr>
<td>S phase</td>
<td>31.3</td>
<td>23.6</td>
<td>41</td>
<td>16</td>
</tr>
<tr>
<td>G2-M</td>
<td>14.0</td>
<td>17.2</td>
<td>13</td>
<td>46</td>
</tr>
<tr>
<td>% apoptosis</td>
<td>0.45</td>
<td>31.51</td>
<td>1.7</td>
<td>26.8</td>
</tr>
</tbody>
</table>

* NM, normal media.
* Flavo, flavopiridol.

diminution of Bcl-2 protein levels was detected after flavopiridol treatment of esophageal cancer cells, possibly related to a lower dose of drug used in the present study (data not shown).

Because 9-cis-RA reduces cyclin D1 expression in breast cancer cells (18), subsequent experiments were performed to determine if this drug could inhibit the growth of esophageal cancer cells and potentiate the effects of flavopiridol. To investigate this, esophageal cancer cells were exposed to 10 µM 9-cis-RA for 3 days followed by flavopiridol (300 nM) in the presence of RA. 9-cis-RA had a negligible inhibitory effect (0–10%) on SKGT-2, SKGT-4, and SKGT-5 adenocarcinoma cell lines (data not shown) and had only a modest effect (approximately 20–25% inhibition of proliferation after 6 days of exposure) in HCE-4 squamous carcinoma cells (Fig. 4). Pretreatment of esophageal cancer cells with RA did not significantly enhance cytotoxicity mediated by flavopiridol in adenocarcinoma cells; a slight but reproducible additive effect (20%) was observed after similar treatment of HCE-4 cells. Additional experiments in which 9-cis-RA and flavopiridol were added simultaneously to HCE-4 cells revealed no obvious additive effect after 5 days of continuous drug exposure. Flavopiridol-mediated apoptosis was not enhanced in HCE-4 after pretreatment with 9-cis-RA; furthermore, although 9-cis-RA has been reported to diminish cyclin D1 expression in breast cancer cells, Western blot analysis revealed that this agent, by itself or in conjunction with flavopiridol, had no appreciable effect regarding cyclin D1 expression in HCE-4 cells (data not shown).

**DISCUSSION**

Esophageal cancers arise via the stochastic accumulation of mutations that disrupt cell cycle regulation in mucosal tissues of the aerodigestive tract (1, 2). For reasons that are as yet unclear, the incidence of esophageal adenocarcinomas has risen faster than that of any solid malignancy during recent years (25). These neoplasms and their precursor lesions can be detected endoscopically, thus enabling the systematic analysis of early molecular events associated with esophageal carcinogenesis and the evaluation of interventions designed to abort this process.

Significant cell cycle derangements have been identified in preneoplastic lesions adjacent to esophageal carcinomas. Mobilization of quiescent cells into G0 with subsequent accumulation of cells in G2-M phase occurs in association with increasing aneuploidy during histologic progression to cancer in Barrett’s esophagus (26); similar cell cycle perturbations have been observed recently in preneoplastic tissues adjacent to esophageal squamous cell carcinomas (27). The outgrowth of clonal populations within the cancerization field correlates with sequential genetic events that are known to disrupt cell cycle regulation (2).

Specifically, overexpression of cyclin D1 has been observed in 30% of biopsy specimens obtained from precancerous Barrett’s mucosa (24). Furthermore, a strikingly high frequency of mutations involving 17p, 9p, and 13q (presumably targeting loci encoding p53, p16, and Rb tumor suppressor genes, respectively) have been detected in flow cytometrically isolated clonal populations from Barrett’s mucosa by PCR/loss of heterozygosity and DNA sequencing techniques (28–30). Transgenic mice overexpressing cyclin D1 spontaneously develop severe esophageal dysplasia (31), and antisense cyclin D1 or wild-type p16 or p53 gene sequences delivered via retroviral or adenoviral vectors mediate growth inhibition, cell cycle arrest, and apoptosis, respectively, in esophageal carcinoma cells expressing the Rb protein (9, 12). Collectively, these data indicate that cyclin D1, p16, Rb, and p53 mutations are early, frequent, and critical events during esophageal carcinogenesis; as such, these mutations are appropriate targets for molecular intervention in esophageal cancers and their precursor lesions.

The mechanisms of flavopiridol activity in cancer cells are extremely complex, as well as the diminution of cyclin D1 and Bcl-2 expression (14, 16, 17). Recent data indicate that RNA and protein synthesis is required for flavopiridol activity in noncycling lung cancer cells (32); furthermore, apoptosis in leukemia cells does not directly correlate with the expression of p53, Bcl-2, or Bax, and cell cycle arrest mediated by this flavone may be separated from its capacity to induce apoptosis in these cells (33).

In this study, cell cycle arrest and apoptosis were observed in esophageal epidermoid and adenocarcinoma cells after flavopiridol treatment. Continuous drug exposure induced G2-M arrest and apoptosis in all cell lines, irrespective of Rb, p16, or p53 protein status. Interestingly, although no dramatic differences regarding cell cycle parameters or intrinsically rates of apoptosis were observed in these cell lines, apoptosis was more pronounced in SKGT-2 and SKGT-4 cells that lack detectable Rb protein expression. These observations are consistent with previously published data involving a variety of cancer cell lines that have demonstrated that Rb protein expression enhances resistance to apoptosis mediated by diverse stimuli including.
E7, E1A, IFN-γ, ceramide, radiation, and chemotherapeutic agents (34–38).

Although flavopiridol seems to diminish cyclin D1 expression in breast cancer cells by transcriptional regulation,5 the mechanisms underlying flavopiridol-mediated changes in cyclin D1 protein levels in esophageal cancer cells have not been conclusively defined. Cyclin D1 is known to be a mediator of a variety of mitogenic signals during G1 (39, 40), and it is intriguing that cyclin D1 expression was markedly diminished in SKGT-2 cells exhibiting pronounced apoptosis after flavopiridol exposure. IFN-γ-induced apoptosis in Rb-deficient cells can be enhanced by serum starvation (36), and ectopic overexpression of cyclin D1 in cells retaining Rb expression facilitates promiscuous S phase entry and apoptosis under similar conditions (41). DNA synthesis and apoptosis can be induced in growth-arrested SAOS-2 cells expressing a temperature-sensitive Rb construct upon shift to a nonpermissive temperature (42). Antisense cyclin D1 and adenoviral p16 constructs diminish Rb expression in malignant cells via protein destabilization and transcriptional regulation, respectively; these events precede the onset of apoptosis in these cells (43–45). Interestingly, reduced Rb and p107 protein levels were consistently observed after flavopiridol treatment of esophageal cancer cells. Conceivably, flavopiridol-induced diminution of cyclin D1 expression achieves the same effects as serum starvation in Rb-deficient cells, thus potentially accounting for the enhanced apoptotic activity observed in the SKGT-2 cell line. The molecular mechanisms underlying diminished cyclin D1, Rb, and p107 expression and the relative significance of each of these complex, interrelated events during flavopiridol-mediated apoptosis in esophageal carcinoma cells are currently under investigation.

Data presented in this study may have potential implications regarding pharmacologic intervention in esophageal cancers and their precursor lesions. Specifically, 9-cis-RA seemed to have minimal inhibitory effects on esophageal carcinoma cell lines, suggesting that this retinoid may be of limited potential in the treatment and chemoprevention of esophageal cancers, particularly adenocarcinomas that arise in the context of Barrett’s esophagus (1). On the other hand, flavopiridol mediated impressive cytotoxicity in all cultured esophageal cancer cell lines irrespective of histology and tumor suppressor gene status, indicating that this drug may be therapeutically efficacious in esophageal cancer patients. Equally important, given its ability to target genetic events that are known to occur early during aerodigestive tract carcinogenesis and its documented efficacy in the setting of small tumor burden in vivo (17), flavopiridol should be considered as a potential chemoprevention agent. Conceivably, flavopiridol may be highly efficacious in patients with a prior history of primary aerodigestive tract malignancy or Barrett’s esophagus as well as individuals who by geographic location or cultural practices are at risk for the development of highly lethal esophageal cancers.

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