Apoptosis in Cervical Cancer Cells: Implications for Adjunct Anti-Estrogen Therapy for Cervical Cancer

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Abstract. Background: Many tumors show dependence on estrogen for growth and establishment of drug resistance. We examined the effects of estrogen on cervical cancer cells exposed to apoptotic agents including drugs used for treatment. Materials and Methods: We tested the effect of estradiol on apoptosis in three cervical cancer cell lines. Apoptosis was measured by endonucleolytic degradation of DNA. Bcl-2 was measured by Western analysis. Results: Estradiol reduced the percentage of cells undergoing apoptosis after exposure to the DNA-damaging agents UVB, mitomycin-C and cisplatin. Protection against taxol-induced apoptosis was marginal. Protection was independent of HPV gene expression, and not specific to apoptosis induced by DNA damage, since estradiol significantly reduced the number of apoptotic cells produced after exposure to indole-3-carbinol (I3C), a non-genotoxic phytochemical effective in preventing HPV-induced tumors. Higher concentrations of I3C overcame the anti-apoptotic effect of estradiol. Treatment with I3C resulted in loss of the survival protein Bcl-2, and estradiol partially reversed this effect. Conclusion: Estrogen protects cervical cancer cells treated with DNA-damaging agents; UVB, mitomycin-C and cisplatin, from apoptotic death. For I3C, which induces apoptosis and is anti-estrogenic, the amount of apoptosis versus survival and the level of Bcl-2 depend on the I3C/estradiol ratio.

Induction and progression of cervical cancer are both estrogen-enhanced processes (1,2). In fact, one of the first indications that cervical cancer is estrogen-dependent came from the observation that these tumors typically arise in the most estrogen-responsive tissue, the "transformation zone"(3). This restricted localization occurs in spite of the fact that the viral co-factors for cervical cancer, certain highly oncogenic strains of human papillomavirus (HPV), are commonly found in cells from all regions of the cervix, in surrounding tissues and in the male genitalia (4,5). A clear indication of the involvement of estrogen in cervical cancer was that mice with HPV16 transgenes develop cervical cancer when given estradiol chronically (6).

The mechanism by which estrogen accomplishes this effect is not fully understood. Estrogen stimulates the growth of many tissues, such as breast and cervix (2,7,8), including anchorage-independent growth (2,9). However, the situation is more complex than simple growth stimulation by estradiol. An interplay between estrogen and HPVs occurs, each apparently amplifying the other's effects. In the transformation zone, estradiol has a high rate of conversion to 16a-hydroxyestrone (1), a metabolite that prolongs estrogen effects (10,11), and is genotoxic (2,9). This conversion is dramatically increased when cervical cells are immortalized with HPV16 (1). Whereas the virus increases estrogenic activity, the expression of integrated HPV oncogenes in transformed cervical cell lines is itself stimulated by estrogen (12,13). In highly oncogenic strains of HPV, these genes encode proteins that inactivate the cellular tumor suppressor proteins p53 (14) and Rb (15), respectively, abrogating normal cell-cycle checkpoints and leading to uncontrolled cell division.

Another potential mechanism by which estrogen could stimulate the development of cervical cancer involves interference with the process of programmed cell death, or apoptosis. Cell growth, whether in tumors, tissues, or cell culture, is now generally recognized to represent a balance between two actively regulated processes: cell division and cell death (16). All cells are thought to have the potential to kill themselves in a highly regulated fashion in response to appropriate developmental or distress signals. For example,
during the course of normal differentiation, keratinocytes undergo many of the morphologic changes associated with apoptosis (17,18). Fragmentation of DNA is one of the major cytopathologic hallmarks of apoptosis (17, reviewed by 19). In the pathological case, cells exposed to DNA damaging agents such as UVB will enter the apoptotic pathway via a mechanism involving p53 (20,21,22). Although the signaling pathways leading to apoptosis are diverse, they have several points of convergence, including the requirement that an apoptosis suppressor function be absent or inactivated. One of the most-studied of these apoptosis suppressors is a family of proteins that includes the bcl-2 gene and its protein product, Bcl-2 (reviewed by 27). A reduction in Bcl-2 protein, or its complete absence, often accompanies apoptosis. Investigators have reported that estrogen is anti-apoptotic in breast cancer cells, and that estrogen treatment is accompanied by increased Bcl-2 expression (23,24). Likewise, estrogen increases Bcl-2 in human keratinocytes (25) and neuronal cells (26), preventing oxidative stress and glutamate-induced apoptosis, respectively. Alternately, estrogen starvation in breast cells provokes apoptosis with only marginal changes in Bcl-2-related proteins (27).

However, unlike most cervical cancers, the breast cancer cells used in many of the studies contain a functional p53 and are not infected by HPV. We report here that estrogen also protects cervical cancer cells from apoptosis, suggesting that interference with apoptosis, possibly through a regulatory mechanism that bypasses p53 and increases Bcl-2 expression, may be a general pathway by which estrogen stimulates tumor cell growth in hormone-responsive cancers. We show and discuss the possible use of an anti-estrogen in overcoming the anti-apoptotic effect of estrogen.

Materials and Methods

Reagents. 17β-estradiol (E2), indole-3-carbinol, mitomycin C and paclitaxel were purchased from Sigma (St. Louis, MO, USA). Cis-diamminedichloroplatinum was purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA).

Cell lines and cell culture. The cervical cancer cell lines CaSkI (containing multiple copies of integrated HPV16 DNA) and C33A (HPV-negative, mutant p53) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). C33A/E6 cells were stably-transfected with the HPV16 E6 gene (pLXSN16E6 from D. Galloway, Fred Hutchinson Cancer Research Center, Seattle, WA, USA) and express low levels of E6 transcripts. All cells were grown in monolayer at 37°C, 5% CO₂ in RPMI medium with glutamine and bicarbonate (GIBCO-BRL, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum, penicillin and streptomycin.

Cell viability was assessed by trypan blue exclusion. Trypsinized cells were incubated with 0.2% trypan blue (GIBCO-BRL) in phosphate-buffered saline (PBS) and examined microscopically. The percentage of cells excluding trypan blue was determined by counting 500 - 1500 cells.

UV irradiation. For cells growing in monolayer the medium was replaced with PBS, and the cultures were exposed for 15sec to 576 J/m² of UVB from a G30T8 30 watt germicidal lamp (General Electric). The UVB output was monitored with a shortwave ultraviolet measuring Meter (Blak-rat J-225, UVP, Inc., Upland, CA, USA) at a distance of 25 cm. After exposure, culture medium was replaced, and the cells were grown for 18 h.

Protein was quantitated with the MicroBCA kit (Pierce, Inc. Rockford, IL, USA) using a BSA standard.

Western blotting. Cells treated with I3C or vehicle controls, with or without estradiol, were lysed at room temperature in buffer containing 10 mM NaHPO₄, 2% triton X-100, 1.2% SDS, 1.0% DOC supplemented just before use with 2 μg aprotinin, 100 μM phenylmethylsulfonyl fluoride (PMSF) and 1mM EDTA, boiled for 2 min, and centrifuged for 10 min at 12,000 × g at 4°C. Supernatant solutions were stored at -80°C until use. One hundred μg of extract protein in sample buffer (125 mM Tris-HCl, pH6.8, 1% SDS, 2% β-mercaptoethanol and 0.01% bromophenol blue) was loaded onto a 12% SDS-polyacrylamide gel. After electrophoresis at 32V for 3 h at room temperature, protein bands were transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA USA) by electroblotting overnight in Transfer Buffer (192mM glycine, 25mM Tris and 20% methanol). Before incubation with antibodies, the membrane was blocked with TBST/milk (20mM Tris-HCl, 137mM NaCl, 1.5% nonfat dry milk and 0.1% Tween20, pH7.6) for 1h. Bcl-2 was detected with mouse monoclonal anti-Bcl-2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) by incubating for 1h. After washing in TBST/milk, the filters were incubated with HRP-conjugated antimouse IgG antibody (Santa Cruz) at 1/2000 dilution for 1h at room temperature. Antibody bound to protein was detected using the enhanced chemiluminescence system (Amersham LIFE Science, Piscataway, NJ, USA).

TUNEL assay used a kit (in situ cell death detection kit, POD, from Boehringer Mannheim Inc. Indianapolis, IN, USA). Cells were grown for 24 h in 8-well chamber slides seeded with 10⁵ cells per well, treated and incubated of 37°C, for 1 or 2 days depending on the treatment. The slides were washed in PBS and fixed with 4% paraformaldehde for 30 min at room temperature. Fixed cells were washed in PBS, permeabilized with sodium citrate buffer containing 0.1% Triton X-100 for 2 min on ice, and then incubated with terminal deoxynucleotidyl transferase for 1 h at 37°C. After rinsing with PBS, the slides were treated with converter-POD (conjugated with horseradish peroxidase) at 37°C for 30 min and mounted with a glass coverslip. At least 200 cells/well were evaluated for staining.

DNA fragmentation was assessed directly by gel electrophoresis. After treatment, the cells were harvested, centrifuged at 500xg for 5 min and washed with PBS. The cell pellet was lysed in 200 μl of lysis buffer containing 50 mM Tris-HCl, 20 mM EDTA and 1% NP-40, and centrifuged at 1600xg for 5 min. The supernatant solution was incubated with (5 μg/ml) RNase A for 2 h at 56°C.
and then digested with proteinase K (2.5 μg/ml) at 37°C for 16 h. DNA was precipitated with an equal volume of 10M ammonium acetate and 2.5 volumes of ethanol. The precipitates were rinsed with 70% ethanol, air dried, dissolved in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), electrophoresed through a 1.5% agarose gel, stained with ethidium bromide and photographed on a UV transilluminator.

Results

Estradiol increases cell survival and decreases apoptosis after UV irradiation. Ultraviolet light at 254nm (UVB) induces apoptosis in many cell types, including keratinocytes (22). This process results in a rate of cell killing greater than that expected from the genotoxic effects of UV. We asked whether estradiol could protect cells from killing by UVB (Figure 1a). When CaSki cells growing in monolayer were UV-irradiated, cell death (measured by trypan blue exclusion) was dependent on the intensity of UV exposure, with 40% survival 18 h after at a standard dose of 576 Joules/m². After treatment with 10 μM estradiol (E2) immediately following UV irradiation, 18-h survival was increased to nearly 60%. Even at E2 concentrations as low as 100 nM, cell survival was significantly improved. Estradiol did not alter the proportion of viable cells in un-irradiated cultures. The effect of E2 on cell survival after treatment with another genotoxic apoptotic inducer, mitomycin C, was qualitatively similar to its effect on UV-irradiated cells (Figure 1b).

Because cell death after moderate UVB exposure is known to be apoptotic, we next asked whether increased survival of CaSki cells after irradiation was reflected by a decrease in the number of cells undergoing apoptosis. Cell cultures were irradiated and then exposed to varying concentrations of estradiol as before. DNA fragmentation was assessed by agarose gel electrophoresis 18 h after irradiation (Figure 2A) and also by TUNEL assay (Figure 2B). Nucleosomal 'laddering' of cell DNA characteristic of apoptosis was clearly observed in irradiated cells (Figure 2A, lane 3), and degradation was decreased in a dose-dependent manner by E2 (Figure 2A, lanes 4-6). No laddering was observed in DNA from estradiol-treated, un-irradiated cells (Figure 2A, lane 7). These results were confirmed more quantitatively by TUNEL assay (Figure 2B). At 10 μM E2, the fraction of cells containing multiple DNA strand breaks 18 h after UV exposure (16%) was significantly reduced compared to irradiated, untreated cells (22%). E2 did not alter the percentage of un-irradiated cells undergoing apoptosis (7%). Because the anti-apoptotic effect of E2 in CaSki cells might have been due to its effect on expression of the extensive HPV DNA sequences resident in this cell line (28), we tested the ability of E2 to protect UVB-induced apoptosis in C33A, another cervical cancer cell line believed to be void of HPV sequences. The results were identical to those for CaSki cells except that the C33A cells were more sensitive to UVB-induced apoptosis (results not shown).
Effect of estradiol on apoptosis induced by cisplatin and taxol. Two anti-cancer drugs in current clinical use, cisplatin and taxol, have been shown to induce apoptosis in a number of tumor cell lines in vitro (29-32). In particular, taxol induces apoptosis in breast tissue which, like the cervix, is estrogen-responsive. We tested the effect of E2 on apoptosis in the C33A cervical cancer cell line treated with each of these drugs (Table I). E2 inhibited the induction of apoptosis by cisplatin to a small but significant extent. Although the systematic error (p=0.054) in the TUNEL assay did not allow us to conclude that E2 reduces taxol-induced apoptosis in CaSki cells, a trend in the E2 dose response in taxol-treated cells suggests that E2 may affect this case also. Estradiol reduces apoptosis of cervical cancer cells induced by indole-3-carbinol in vitro. We have determined that indole-3-carbinol (I3C) is a potent apoptotic inducer in human cervical cancer cell lines in vivo and in vitro using multiple indicators of apoptosis (33). Others have observed that I3C induced apoptosis in breast cancer cell lines (34, 35). Unlike UV, cisplatin, taxol and mitomycin C, however, I3C does not damage DNA directly. We therefore tested whether E2 could also counteract the apoptotic effect of I3C. In this experiment, we used CaSki, C33A and C33AE6

<table>
<thead>
<tr>
<th>Cis-platinum</th>
<th>Taxol</th>
<th>Estradiol</th>
<th>% Apoptotic Cells (± s.d.)</th>
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<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9.7 ± 1.7</td>
</tr>
<tr>
<td>10 μM</td>
<td>0</td>
<td>0</td>
<td>19.6 ± 2.1</td>
</tr>
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<td>10 μM</td>
<td>0</td>
<td>0.1 μM</td>
<td>19.6 ± 2.5</td>
</tr>
<tr>
<td>10 μM</td>
<td>0</td>
<td>1.0 μM</td>
<td>15.8 ± 1.0</td>
</tr>
<tr>
<td>10 μM</td>
<td>0</td>
<td>10.0 μM</td>
<td>13.6 ± 0.6</td>
</tr>
<tr>
<td>0</td>
<td>10.0 μM</td>
<td>0</td>
<td>22.3 ± 3.3</td>
</tr>
<tr>
<td>0</td>
<td>10.0 μM</td>
<td>0.1 μM</td>
<td>17.8 ± 1.2</td>
</tr>
<tr>
<td>0</td>
<td>10.0 μM</td>
<td>1.0 μM</td>
<td>18.1 ± 1.5</td>
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<td>10.0 μM</td>
<td>10.0 μM</td>
<td>16.3 ± 2.5</td>
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<tr>
<td>0</td>
<td>0</td>
<td>0.1 μM</td>
<td>9.3 ± 1.4</td>
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<tr>
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<td>0</td>
<td>1.0 μM</td>
<td>9.0 ± 1.4</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>10.0 μM</td>
<td>9.8 ± 1.8</td>
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C33A cells were treated for 24 h with cis-platinum or taxol, with and without estradiol, and apoptosis was evaluated by TUNEL. Results are expressed as mean ± SD.
As shown in Figure 3, exposure to I3C for 24 h (untreated controls) increased the percentage of cells scoring positive in the TUNEL assay for all of the cell lines (C33A, 35% > C33AE6, 21%, > CaSki, 13%). E2 partially blocked this I3C-induced apoptosis (28%, 18% and 9% for C33A, C33AE6 and CaSki, respectively). The inhibition was dependent on the dose of E2 (not shown), and the effect of E2 at 10 μM was significant for C33A and C33AE6 but not CaSki (p≤0.05). HPV sequences did not contribute either to the induction of apoptosis by I3C or to the protective effect of E2. However, C33A cells, without the viral oncogenes, were the most sensitive to induction of apoptosis by I3C.

Relative amounts of I3C and estradiol determine the amount of I3C-induced apoptosis. I3C induces multiple anti-estrogenic activities (13,36-38) separate from inducing apoptosis. Therefore, two opposing activities (anti-estrogenic versus estrogenic and apoptotic versus anti-apoptotic) occur when both I3C and estrogen coexist. We then asked about how the relative amounts of I3C and estradiol affect apoptosis (Table II). Higher concentrations of I3C partially abrogated the protective effect of estradiol. Conversely, higher concentrations of estradiol are more effective in preventing the apoptotic effect caused by I3C.

Estradiol partially restores Bcl-2 levels in indole-3-carbinol-treated cervical cancer cells. Apoptosis in a number of cell types and circumstances correlates with reduced intracellular levels of the Bel-2 gene product. We found that I3C reduces Bel-2 expression in cervical cancer cell lines (33). It was therefore reasonable to ask whether E2 could restore Bel-2 levels in I3C-treated CaSki cells, and this proved to be the case (Figure 4). Whereas the steady-state content of Bel-2 determined by Western analysis was reduced approximately 10-fold by 200 μM I3C, 100 nM E2 restored Bel-2 levels to 50% of untreated controls, and 1 μM E2 raised Bel-2 levels even further.

**Table II. Effect of relative amounts of indole-3-carbinol and estradiol on apoptosis.**

<table>
<thead>
<tr>
<th>Indole-3-carbinol</th>
<th>Estradiol</th>
<th>% Apoptotic Cells (± s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>13.2 ± 1.8</td>
</tr>
<tr>
<td>50 μM</td>
<td>0</td>
<td>24.6 ± 1.8</td>
</tr>
<tr>
<td>100 μM</td>
<td>0</td>
<td>28.6 ± 3.2</td>
</tr>
<tr>
<td>50 μM</td>
<td>10 μM</td>
<td>21.6 ± 4.7</td>
</tr>
<tr>
<td>100 μM</td>
<td>1 μM</td>
<td>25.8 ± 4.7</td>
</tr>
<tr>
<td>100 μM</td>
<td>10 μM</td>
<td>23.9 ± 1.2</td>
</tr>
<tr>
<td>100 μM</td>
<td>100 μM</td>
<td>22.3 ± 1.3</td>
</tr>
<tr>
<td>200 μM</td>
<td>10 μM</td>
<td>27.5 ± 2.8</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>14.4 ± 4.2</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>15.4 ± 3.3</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>15.8 ± 4.0</td>
</tr>
</tbody>
</table>

C33A cells were treated with varying concentrations of indole-3-carbinol and estradiol for 48 h. Apoptosis was determined by TUNEL. Results are expressed as mean ± SD.
Discussion

This study not only identifies a mechanism whereby E2 could increase transformation of cervical cells, but also has important implications for the treatment of cervical cancer with cisplatin or other agents that induce apoptosis.

Our data clearly show that E2 significantly diminished the apoptotic response in three human cervical cancer cell lines exposed to a variety of agents, including UVB, mitomycin-C, cisplatin and I3C. Inhibition of the taxol-induced apoptosis was highly suggestive but not statistically significant. Our results were generally in accord with those obtained for MCF-7 breast cancer cells, except for taxol, where E2 was highly effective for inhibiting taxol-induced apoptosis (24). If the taxol result represents an underlying tissue type difference, this may have therapeutic significance for the use of taxol for cervical cancer. Separately, it is worth noting that a substantial background of apoptotic cells was observed in untreated cultures. The lack of effect of E2 on this population of cells suggests that a different mechanism may be at work to induce untreated cells in culture to undergo apoptotic cell death.

Since apoptotic agents such as cisplatin are used as chemotherapy for cervical cancer and many other cancers, our data suggest that anti-estrogens should be an adjunct treatment in order to prevent abrogation of apoptosis by estrogen and survival of tumor cells. Because the phytochemical I3C is an anti-estrogenic compound as well as an inducer of apoptosis, it is logical that the relative amounts of E2 and I3C would affect apoptosis in cervical cells (Figures 4,5). E2 could inhibit I3C-induced apoptosis and, conversely, increased concentrations of I3C could abrogate the inhibitory effect of E2 on I3C-induced apoptosis. The level of protection from estrogen was consistent with the overall increase in the cell survival protein Bcl-2, whose levels increase after estrogen treatment (38,39). This supports the idea that most, if not all, of the protective effect of E2 might be due to its effect on Bcl-2. As with apoptosis, I3C decreased the level of Bcl-2 and E2 could abrogate this effect of I3C. Interesting, perhaps because of its anti-estrogenic activities, I3C has become a treatment strategy for both laryngeal papillomatosis (benign tumors with an HPV etiology) (40,41) and, more recently, for cervical dysplasia (42). In vitro, high concentrations of I3C need to be used since this compound is slowly converted into active condensation products, e.g. diindolylmethane, compounds rapidly formed in acid environments as in the stomach (43) whereas in vivo benefits for cervical disease occur at concentrations obtainable from eating cruciferous vegetables (6, 42).

Interference with apoptosis by E2 probably occurs downstream from or independently of the DNA damage-sensing pathway that involves p53, because the phytochemical I3C has not been shown to damage DNA. Additionally, I3C inactivates many carcinogens by inducing phase I and phase II enzymes, and directly reduces DNA damage by reducing free radicals (reviewed by 44,45). The I3C-induced apoptosis in breast cancer does not depend on p53 (34). This conclusion is consistent with the fact that one of the HPV early genes expressed in two of the cell lines (C33AE6 and CaSki) would inactivate p53, and the other cell line, C33A, contains a mutant p53. Inactivation of p53 should abrogate the normal UVB-stimulated apoptotic response pathway. Our results with cervical cancer cell lines are generally in accord with those obtained in breast cancer lines, and the protective action of E2 in breast cancer cells (23,24) might also be independent of p53 status, although this remains to be determined. Finally, our studies suggest that HPV genes, which are expressed in most cervical cancers, may make them more resistant to apoptosis. However, the E2 inhibition of apoptosis is clearly independent of the HPV status.

Clearly, the results of this study indicate the need to consider estrogen status in the treatment of cervical cancer, since estrogen has the potential to interfere with the effectiveness of certain treatment modalities.

Acknowledgements

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