Estrogen-Depleted Condition Induces Apoptosis of Rat Mammary Cancer Cells after Entering the S-phase of the Cell Cycle

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ABSTRACT. To elucidate the relationship of estrogen-depleted condition to apoptosis and tumor regression, 7,12-dimethylbenz[a]anthracene-induced mammary cancers of Sprague-Dawley rats were ovariectomized or treated with the anti-estrogenic agent epitiostanol after which proliferative activity and the incidence of apoptosis were investigated using the nick end labeling method, agarose gel electrophoresis of DNA, electron microscopy, the BrdU-labeling method and mitotic count. Tumor regression was found after 7-day treatment, and apoptosis induced by the agent on the 3rd day was clearly shown in both agarose gel electrophoresis of DNA and electron microscopy, which are two major methods used to judge apoptosis. The incidence of apoptosis revealed by the nick end labeling method reached its maximum, about threefold the control level, on the 3rd day of epitiostanol treatment compared with control tumors (P < 0.01). The incidence of the cells incorporating BrdU reached its maximum of 7.5% on the 2nd day of the treatment, while the incidence in tumors without treatment was 7.5% (P < 0.05). Subsequently, the incidence of apoptosis was reduced after 7-day treatment, and the incidence of BrdU-positive cells was significantly reduced to about 3% after 5-day treatment. The incidence of mitosis did not change until the 3rd day of the treatment and was reduced after 5-day treatment. Similarly, chronological changes of the incidences of BrdU-labeled cells, apoptotic cells and mitosis were observed in the tumors after ovariectomy. BrdU-labeled apoptotic bodies were detected in the tumors on the 3rd day in epitiostanol-treated rats that received a 6-hr bolus of BrdU before sacrifice. These findings indicate that, in a hormone-dependent rat mammary cancer model, treatment with this anti-estrogenic agent causes at least some cancer cells to fall into apoptosis after entering the S-phase of the cell cycle, resulting in the regression of mammary tumors.

Mammary cancer is one of the major causes of human death throughout the world. The incidence of mammary cancer in Japanese women is increasing year by year, probably due to westernization of the life style in Japan. Thus the establishment of an effective therapy for mammary cancer is a matter of urgency. Since one third of mammary cancer is considered to grow in a hormone-dependent fashion (12, 16), anti-estrogenic agents are frequently used for the treatment of mammary cancer. These agents are clinically shown to reduce the size of tumors, but cellular events that are induced in mammary cancer by anti-estrogenic agents are not fully understood.

Since it is well known that apoptosis occurs after depletion of hormones in hormone-targeted organs (2, 10, 19, 20, 22, 25), it seems plausible that anti-estrogenic agents cause apoptosis of mammary tumors by interfering with the hormone-dependent growth control mechanisms (8, 11, 18, 21). However, the relationship between anti-estrogenic effects on mammary cancer cells and apoptosis has not been settled yet.

Rat mammary cancer induced by 7,12-dimethylbenz[a]anthracene (DMBA) administration (7) is known to be dependent on estrogens for its development and growth (9). We observed that epitiostanol (2α,3α-epithio-5α-androstan-17β-ol) (13) induced morphological changes of human mammary cancer by a short term administration of the agent (14, 17). In this study, we examined in detail whether ovariectomy and an anti-estrogenic agent, epitiostanol, caused apoptosis in DMBA-induced rat mammary cancer by using the combination of nick end labeling method, electron microscopy and electrophoresis of DNA. We then studied the relation between apoptosis and DNA synthesis. The results showed that the mammary cancer cells entered the S
phase of the cell cycle prior to falling into apoptosis by estrogen-depletion, resulting in a reduction of tumor size.

**MATERIALS AND METHODS**

**Animals.** Female 50-day-old Sprague Dawley (SD) rats (Japan SLC Inc., Hamamatsu, Japan) were used for the experiments. All animals were kept under specific-pathogen-free conditions, fed on a standard diet and provided with drinking water ad libitum.

**Experimental procedure.** The rats were administered DMBA (Sigma Chemical Co., St. Louis, MO, USA) at a dose of 100 mg/kg body weight in 2 ml of sesame oil by gastric tube. They were palpated once a week for the detection of mammary tumors. When a tumor became palpable, its size was measured using calipers, rectangularly crossing 2 diameters of the tumor. After the longest diameter of the tumor reached about 1 cm, the rats were intramuscularly injected with the anti-estrogenic agent, epitostanol (Shionogi & Co., Ltd., Osaka, Japan), at a dose of 4 mg/kg body weight in 2 ml of sesame oil daily for 1, 2, 3, 5 and 7 days. As a control study, the rats were injected with the same volume of the vehicle without the agent. As a positive control, some of the rats in this study, the rats were injected with the same volume of the vehicle. After the longest diameter of the tumor reached about 1 cm, the rats were intramuscularly injected with the anti-estrogenic agent, epitostanol (Shionogi & Co., Ltd., Osaka, Japan), at a dose of 4 mg/kg body weight in 2 ml of sesame oil daily for 1, 2, 3, 5 and 7 days. As a control study, the rats were injected with the same volume of the vehicle without the agent. As a positive control, some of the rats were ovariectomized, and sacrificed 1, 2, 3, 5 or 7 days after the operation.

One hour before sacrifice, the rats were injected i.p. with 30 mg 5-bromo-2-deoxyuridine (BrdU)/kg of body weight. Several rats were injected with the same dose of BrdU 6 hr before sacrifice. When multiple mammary tumors developed in the same rat, each tumor was regarded as an independent one. Excised tumors were immediately treated as follows. For histopathological examination and assessment of apoptosis by the nick end labeling method, transversal sections of the mammary tumor less than 2 mm thick were fixed in 10% formalin at room temperature (RT) for 24 hr, and then embedded in paraffin. For assessment of the mitotic rate and proliferation by BrdU staining, sections less than 2 mm thick were fixed in 4% paraformaldehyde at 4°C for 2 hr. For electron microscopy, tumors cut into approximately 1 × 1 × 1 mm in size were fixed in a mixture of 1% glutaraldehyde-4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4. They were then postfixed with 1% osmium in the buffer, dehydrated and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and 2% bovine serum albumin were deparaffinized and immersed in methanol containing 0.3% H2O2 for 30 min, denatured by 2 N HCl for 15 min at 37°C, rinsed with borate buffer, and then treated with 0.05% pronase E (Sigma Chemical Co.) for 1 hr. After incubation with normal rabbit serum at RT for 10 min, the sections were incubated with a 1:20 dilution of anti-BrdU monoclonal antibody (DAKO Co., Glostrup, Denmark) in phosphate-buffered saline (PBS) at RT for 1 hr. Following incubation with biotinylated anti-mouse IgG + IgA + IgM (Nichirei Co., Tokyo, Japan) at RT for 10 min, the sections were incubated with peroxidase-conjugated streptavidin (Nichirei Co.) at RT for 5 min. After washing in PBS, the sections were stained using 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Katayama Chemical Co., Osaka, Japan), and then counterstained with hematoxylin. About 1,000 cells per section were counted and the BrdU-positive cells were expressed as a percent.

**Nick end labeling method.** DNA nick end labeling of tissue sections was performed as described by Gavrieli et al. (4). Two-micrometer paraffin-embedded sections were applied to slides pretreated with poly-L-lysine. After deparaffinization and hydration, sections were incubated with 20 µg/ml proteinase K (Sigma Chemical Co.) at RT for 15 min, and then inactivated by covering the sections with 2% H2O2 at RT for 5 min. The sections were rinsed with distilled water, and immersed in TdT buffer (30 mM Tris base, pH 7.2, 140 mM sodium cacodylate, and 1 mM cobalt chloride). The units of terminal deoxynucleotidyl transferase (Boehringer Mannheim GmbH, Mannheim, Germany) and 1 nmol biotin-16-dUTP (Boehringer Mannheim GmbH) in 50 µl of TdT buffer were then added to cover the sections, which were incubated in a humid atmosphere at 37°C for 60 min. The reaction was terminated by transferring the slides to TB buffer (300 mM sodium chloride, 30 mM sodium citrate) at RT for 15 min. After incubation with 2% bovine serum albumin at RT for 10 min, the sections were covered with peroxidase-conjugated streptavidin (Nichirei Co.) at RT for 5 min. After washing in PBS, the sections were stained using DAB at RT for 5 min, then counterstained with methyl green. The numbers of positive cells were counted with a microscope at high magnification (×1,000). As a positive control, deparaffinized sections were pretreated with 1 µg/ml DNase I (Sigma Chemical Co.) in DN buffer (30 mM Tris base, pH 7.2, 140 mM potassium cacodylate, 4 mM MgCl2 and 0.1 mM DTT) at RT for 10 min. The apoptotic rate was determined as the absolute number of nick end labeling-positive cells per 1 mm² of the section.
Analysis of DNA fragmentation. An excised tumor weighing about 0.5 g stored at -80°C was dropped into liquid nitrogen in the stainless-steel cup of a Waring blender, and blended until the tissue was ground to a powder. DNA extraction was carried out following the method described by Nicoletti et al. (15) with slight modification. The powdered tissue was dissolved in approximately 10 volumes of hypotonic lysis buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, 1% SDS and 200 μg/ml proteinase K, pH 7.5) at 50°C for 3 hr. DNA was extracted from the sample with an equal volume of phenol equilibrated with 0.5 M Tris-HCl (pH 8.0), and extracted twice with a mixture of phenol, chloroform and isoamyl alcohol (25:24:1). The samples were treated with DNase-free RNase A (100 μg/ml) (Sigma Chemical Co.) at 37°C for 1 hr. Next, DNA was extracted with phenol twice, and then with a mixture of phenol, chloroform and isoamyl alcohol. DNA was recovered by centrifugation at 5,000 g at 4°C for 10 min after precipitation at -20°C overnight in 2 volumes of absolute ethanol in the presence of 10 mM MgCl₂ and 0.3 M sodium acetate. The pellet was air dried, and then dissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 4°C. The DNA concentration was determined by absorbance at 260 nm with a U-3200 spectrophotometer (Hitachi Ltd., Tokyo, Japan). Three micrograms of DNA was electrophoresed with the Mupid-2 system (Advance Co., Tokyo, Japan) through a 1.8% agarose gel (SEA KEM GTG agarose; FMC BioProducts, Rockland, USA) containing 0.5 μg/ml ethidium bromide. DNA was visualized with a UV transilluminator and photographed with a Polaroid camera.

Statistical analysis. Due to the nonnormal distribution of the data, a nonparametric test, the Mann-Whitney U test, was adopted for statistical evaluation of the results.

<table>
<thead>
<tr>
<th>treatment</th>
<th>tumor number</th>
<th>relative tumor weight&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>sesame oil for 7 days&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21</td>
<td>2.5±1.5</td>
</tr>
<tr>
<td>epitiostanol for 7 days</td>
<td>25</td>
<td>1.2±1.2&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>7 days after ovariectomy</td>
<td>11</td>
<td>0.5±0.3&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Relative tumor weight at 0 day was regarded as 1.0.  
<sup>b</sup> regarded as control.  
<sup>*</sup> P<0.01 compared with the corresponding value for the control group.

RESULTS

Histological classification of mammary tumors induced by DMBA. The first tumors in the rats became palpable about 30 days after DMBA-treatment. The mean number of mammary tumors per rat was 6.0 in the control, 5.4 in the epitiostanol-treated group and 5.6 in the ovariectomized rats. Histopathological examination revealed that most tumors were adenocarcinomas distinctly separated from surrounding tissues and characterized by varying proportions of tubular and papillary growth patterns. A few benign tumors, fibroadenomas, adenomas, fibromas and cysts, and non-epithelial malignant tumors, were also induced. These tumors were excluded from analysis in our experiment.

Changes in estimated relative tumor weight of DMBA-induced mammary cancers after epitiostanol treatment and ovariectomy. The estimated relative tumor weight changed from 1.0 (day 0) to 2.5±1.5 on the 7th day of the control experiment. On the other hand, the values of the tumors of epitiostanol-administered

Fig. 1. Histopathological appearance of DMBA-induced rat mammary cancers. Tumor without treatment (a), tumor treated with epitiostanol for 3 days (b), and for 7 days (c). Arrows show apoptotic cells. Hematoxylin-eosin staining. (× 340)
and ovariectomized rats on the 7th day of the observation were 1.2±1.2 and 0.5±0.3, respectively. These results clearly showed that DMBA-induced rat mammary cancer cells were sensitive to epitiostanol and ovariectomy (Table I). Vaginal smears were taken from the rats to assess the phase of the estrous cycle. All of the vaginal smears from epitiostanol-treated and ovariectomized rats indicated that they were in the diestrus state (data not shown).

**Histopathological changes of mammary cancer induced by epitiostanol.** Initially, the mammary cancer grew expansively with a tubulopapillary pattern. Necrosis and apoptotic bodies were unremarkable (Fig. 1a). After 3-day treatment with epitiostanol, many apoptotic bodies were found (Fig. 1b). After 7-day treatment with epitiostanol, mammary cancer tissues become cystic (Fig. 1c), probably as a result of the decrease in the number of cancer cells. Similar changes in cancer tissues were observed in ovariectomized rats (data not shown).

**Agarose gel electrophoresis.** The pattern of DNA fragmentation in DMBA-induced rat mammary cancer was determined using agarose gel electrophoresis (Fig. 2). The characteristic ladder of oligonucleosomal DNA fragmentation was found in the tumors of anti-estrogenic-agent-treated rats and ovariectomized rats. However, the ladder formation was also observed in DNA obtained from tumors without any treatment, though the formation was very weak compared with epitiostanol-treated and ovariectomized rats. The formation of the characteristic ladder pattern was the strongest after 3-day epitiostanol administration and 3 days after ovariectomy. DNA isolated from normal rat liver did not show this characteristic pattern.

**Incidence of apoptotic cells.** To evaluate the frequency of apoptosis, we counted the positive cells stained by the nick end labeling method. In mammary cancers without any treatment, the apoptotic index was about 52.5±28.8. In the cancer treated with epitiostanol, the index reached a maximum of 161.6±115.5 after 3-day treatment and even on the 7th day the index was higher than in the control. Changes of the indices of the tumors of ovariectomized rats were very similar to those of epitiostanol-treated rats (Table II). Typical areas stained by the nick end labeling method are shown (Fig. 2).

<table>
<thead>
<tr>
<th>Tumor number</th>
<th>Apoptosis (nick end labeling)</th>
<th>Proliferation (BrdU %)</th>
<th>Mitosis a</th>
</tr>
</thead>
<tbody>
<tr>
<td>control b</td>
<td>18</td>
<td>52.5±28.8</td>
<td>7.5±2.1</td>
</tr>
<tr>
<td>Epitiostanol-treatment</td>
<td>24</td>
<td>99.7±88.8</td>
<td>8.1±3.2</td>
</tr>
<tr>
<td>1 day</td>
<td>21</td>
<td>113.9±73.1*</td>
<td>9.7±2.9*</td>
</tr>
<tr>
<td>2 days</td>
<td>27</td>
<td>161.6±115.5**</td>
<td>8.8±3.1</td>
</tr>
<tr>
<td>3 days</td>
<td>26</td>
<td>126.2±59.9**</td>
<td>2.7±2.7**</td>
</tr>
<tr>
<td>5 days</td>
<td>25</td>
<td>71.5±44.7</td>
<td>3.2±3.2**</td>
</tr>
<tr>
<td>7 days</td>
<td>10</td>
<td>91.6±78.0</td>
<td>8.4±5.4</td>
</tr>
<tr>
<td>Ovariectomy</td>
<td>10</td>
<td>143.0±80.6*</td>
<td>7.9±3.2</td>
</tr>
<tr>
<td>2 days</td>
<td>12</td>
<td>132.1±58.3*</td>
<td>11.9±5.5*</td>
</tr>
<tr>
<td>3 days</td>
<td>13</td>
<td>122.2±85.8*</td>
<td>2.2±2.3**</td>
</tr>
<tr>
<td>5 days</td>
<td>11</td>
<td>100.8±60.6*</td>
<td>1.7±1.2**</td>
</tr>
</tbody>
</table>

a) Number of positive cells per mm²

b) Tumors without any treatment

* P<0.05 compared with the corresponding value for the control group.

** Table II. Effects of epitiostanol and ovariectomy on apoptosis and proliferation of DMBA-induced mammary cancer cells of SD rats. **
Estrogen depletion causes apoptosis of mammary cancer

in Figs. 3a, b and c.

Cell-proliferation activity. In mammary cancers of control rats, the incidence of the cells incorporating BrdU was 7.5 ± 2.1% during the experimental period. In the tumors treated with epitiostanol, the incidence reached 9.7 ± 2.9% after 2-day treatment with epitiostanol and then significantly dropped to about 3%. This increase in the incidence was significantly different from the control value (P < 0.05). In the cancers of ovariectomized rats, the changes of the incidence were very similar to those of epitiostanol-treated tumors (Table II).

Immunohistochemical staining of typical areas in mammary cancer is presented in Figs. 4a, b and c. Epitiostanol-treated rats with mammary cancer that received a 1-hr bolus of BrdU before sacrifice were clearly stained. When BrdU was administered 6 hrs before sacrifice, some fragmented nuclei in apoptotic bodies in epitiostanol-treated rats with mammary cancer were positively stained by BrdU (Figs. 5a and b).

The incidence of mitosis without any treatment was 20.7 ± 17.9. Significant differences were not shown until 3-day treatment, and then the incidence significantly
dropped after 5-day treatment \((P < 0.01)\). A similar tendency was observed in the cancers of ovariectomized rats (Table II).

**Ultrastructures of apoptosis.** To confirm the findings of hematoxylin-eosin staining and the nick end labeling method, we further investigated the ultrastructures of the mammary cancer tissues. Apoptotic bodies were most frequently observed in the tissues treated with epitiostanol for 3 days. Figure 6a shows chromatin condensation around the periphery of the nucleus and Figure 6b shows an apoptotic body within a neighboring cell. Apoptotic bodies that detached themselves from their neighbors and formed multiple, condensed and fragmented chromatin spheres can be seen in Figure 6c, in which swollen and degenerated mitochondria can also be observed.
Estrogen depletion causes apoptosis of mammary cancer

DISCUSSION

Present experiments clearly showed that apoptosis of rat mammary cancer cells most frequently occurred within a few days after estrogen depletion, and that the mammary cancer cells fell into apoptosis after entering the S-phase of the cell cycle. Thus, the size of mammary tumors was significantly reduced by the administration of epitiostanol and by ovariectomy.

Apoptosis is generally identified by agarose gel electrophoretic demonstration of fragmented DNA (23) and electron microscopic finding of apoptotic bodies with characteristic chromatin condensations (24). However, these methods have certain limitations in that gel electrophoresis cannot identify the cells in apoptosis and the electron microscopy cannot be quantitative. In the present study, we added the nick end labeling method to agarose gel electrophoresis and electron microscopy, enabling us to identify the cells at the light microscopic level in a quantitative manner.

The results obtained clearly showed that ovariectomy and the administration of epitiostanol induced apoptosis in mammary cancer tissues and that apoptosis of mammary cancer cells occurred the most frequently on the 3rd day of estrogen-depletion. On the other hand, it was found that the incidence of BrdU incorporation into mammary cancer cells also increased on the 2nd and 3rd day in estrogen-depleted rats and markedly decreased after the 5th day of the treatment. To elucidate the biological implications of this transient increase in BrdU incorporation, we assessed whether the increase in BrdU-positive cells correlated with the cells in mitosis. The results showed that the increase in the incidence of BrdU-positive cells did not correlate with the mitotic index, strongly suggesting that BrdU incorporation does not simply mean cells undergoing mitosis. Furthermore, we found that a considerable number of apoptotic bodies in mammary cancer under estrogen-depleted condition was BrdU-positive, suggesting that some BrdU-positive cells underwent apoptosis. Thus, this is the first report showing that mammary cancer cells fell into apoptosis after entering the S-phase of the cell cycle by short term estrogen depletion, resulting in a reduction in tumor size. Our observations are consistent with the report that prostatic glandular cells of rats fell into apoptosis after entering the S-phase of the cell cycle during involution induced by castration (1).

Changes in the tumor size reflect the balance between cell proliferation and death. It has been accepted that anti-estrogenic agents cause the suppression of DNA synthesis by competitive inhibition against the forming complex of estrogen and estrogen receptor, possibly resulting in tumor regression. In other words, anti-estrogenic agents are considered to show static action against the tumor. However, our results clearly showed that anti-estrogenic agent induced the death of cancer cells within a few days after treatment, consistent with earlier reports that the agents induce apoptosis of mammary cancer cells in vitro. Thus, the conflicting ideas about the action of anti-estrogenic agents on mammary cancer may be attributed to the fact that apoptosis occurs within a very short period upon anti-estrogen-treatment followed by rather slow regression in tumor size. Even in a recent report (8) describing apoptosis induced by trenfene, changes in the early phase of the treatment were not fully examined. In our experiments, the incidence of BrdU-positive cells was significantly low after the 5th day of treatment. The low incidence of BrdU-positive cells in tumors treated with the agent lasted at least until the 14th day, and tumor size was simultaneously decreased by epitiostanol and ovariectomy (unpublished observation). Thus, the pharmacological effects of anti-estrogenic agents seem to be complex, i.e. the agent causes apoptosis as an acute effect and also suppression of cell proliferation as a subacute effect.

Estrogen receptor-positive mammary cancer represents two thirds of human mammary cancer. Clinically only half of the receptor-positive cancer responded to the agents (16). Thus the assay of the estrogen receptor of mammary cancer may not be essential for hormone therapy. However, our results suggest that it may become possible to judge whether a mammary cancer will respond to the agents without the receptor assay if, by using biopsied or aspirated materials, we can detect apoptosis induced by the agents in mammary cancer.

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