Regulation of Apoptosis in Normal and Malignant Ovarian Epithelial Cells by Transforming Growth Factor β¹

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ABSTRACT

Previously, we found that transforming growth factor β (TGF-β) inhibits proliferation of normal human ovarian epithelial cells. In addition, although only 1 of 5 immortalized ovarian cancer cell lines was inhibited, TGF-β inhibited proliferation of 19 of 20 primary epithelial ovarian cancers. In this study, we examined whether TGF-β induces apoptosis in normal and malignant ovarian epithelial cells. Among 5 immortalized cell lines, only OVCA 420 is markedly growth inhibited by TGF-β, and this was the only cell line in which TGF-β elicited DNA fragmentation characteristic of apoptosis. Induction of apoptosis in OVCA 420 was time and concentration dependent and could be partially inhibited by concurrent treatment with an anti-TGF-β mAb. Although apoptosis was not seen in normal ovarian epithelial cells (n = 7), [3H]thymidine incorporation was inhibited in all cases (mean = 61.2 ± 7.2% (SD) of untreated control; P < 0.01). Similarly, TGF-β inhibited [3H]thymidine incorporation in all 10 primary ovarian cancers (mean = 40.4 ± 7.1% of control; P < 0.01), but only 3 of 10 (30%) were found to undergo apoptosis when treated with TGF-β. There was no relationship between p53 status of the ovarian cancers and the ability of TGF-β to elicit apoptosis. In conclusion, TGF-β inhibits proliferation but does not induce apoptosis in normal human ovarian epithelial cells. In contrast, some ovarian cancers that are growth inhibited by TGF-β also undergo apoptosis. These data are consistent with the hypothesis that malignant cells are more susceptible to apoptosis than their normal nontransformed counterparts.

INTRODUCTION

TGF-β₁, β₂, and β₃ are produced by humans and inhibit proliferation of a wide range of cell types including epithelial cells, hepatocytes, and hematopoietic cells (1). Although the signal transduction pathways involved are not yet well understood, it has been shown that the biological activity of TGF-β is dependent on the presence of type I and II serine/threonine kinase membrane receptors (2, 3). Binding of TGF-β to these receptors initiates a cascade of molecular events that are thought to culminate in decreased activity of cyclin-dependent kinase 4, which prevents progression from G₁ into S phase of DNA synthesis (4).

Previously, we examined the effect of TGF-β on proliferation of normal and malignant human ovarian epithelial cells in monolayer culture. Growth of primary cultures of normal ovarian epithelial cells was found to be inhibited by 40–70% (5). In contrast, similar to many other types of immortalized cancer cell lines, we found that four of five ovarian cancer cell lines were relatively resistant to the growth-inhibitory effect of TGF-β (5). More recently, however, we found that 19 of 20 (95%) primary epithelial ovarian cancer cell cultures obtained directly from ascites were inhibited by TGF-β (6). These data suggest that loss of responsiveness to TGF-β is involved in the process of immortalization of ovarian cancer cells in vitro but not in the development of human ovarian cancer.

In addition to inhibiting progression through the cell cycle, it has been shown that TGF-β can elicit programmed cell death (apoptosis) in both normal and malignant cells under certain circumstances. TGF-β has been found to induce apoptosis in endometrial cells (7), gastric carcinoma cells (8), normal and transformed hepatocytes (9, 10), acute myelogenous leukemia cell lines (11), and a number of other cell types. It is unclear, however, why TGF-β causes reversible growth arrest of some cells while others undergo apoptosis. In the present study, we have examined whether, in addition to inhibiting proliferation, TGF-β is able to induce apoptosis in normal and malignant ovarian epithelial cells.

MATERIALS AND METHODS

Ovarian Cancer Cell Lines. Four epithelial ovarian cancer cell lines previously established in our laboratory (OVCA 420, OVCA 429, OVCA 432, and OVCA 433) (12) and one commercially available cell line (OVCAR 3) were grown in monolayer culture in DMEM supplemented with 10% FCS, t-glutamine (2 mM), and penicillin/streptomycin (100 units/ml).

Primary Ovarian Cancer Cells. Ovarian cancer cells were grown in monolayer culture as previously described from the solid tumor or ascites of patients with stage III/IV epithelial ovarian cancer who underwent surgery at Duke University Medical Center between 1993 and 1994 (6). In some cases, cells were removed from ascites by centrifugation and stored over liquid nitrogen. For experiments, cells were thawed and washed in MCDB 105/M199 medium supplemented with 10% FCS, t-glutamine (2 mM), and penicillin/streptomycin (100 units/ml). Purification of ovarian cancer cells was accomplished using discontinuous gradient centrifugation with Percoll (Pharmacia, Piscataway, NJ) density gradients (1.070–1.023 g/cm³) (6).

Normal Ovarian Epithelial Cells. Primary monolayer cultures of ovarian epithelial cells were established from surgical specimens of normal ovaries as described previously (5). Briefly, the surface of the ovary was scraped gently, and the epithelial cells were then plated in a 1:1 mixture of MCDB 105/M199 medium supplemented with 15% heat-inactivated fetal bovine serum and epidermal growth factor (10 ng/ml). Cells were cultured at 37°C in 5% CO₂ and 95% humidified air.

DNA Fragmentation Assay. TGF-β₁ (R&D Systems, Minneapolis, MN) was dissolved in 4 mM HCl-1 mg/ml BSA and stored at −20°C. Cells were grown to confluence on 6-well plates, and then treated with TGF-β₁ (10.0, 5.0, 1.0, 0.1, or 0 ng/ml) for 72 h. Then cells were harvested, centrifuged at 6000 × g for 10 min, and the resultant pellets were resuspended in 200 μl nuclei lysis buffer (5 mM guanidine thiocyanate-25 mM sodium citrate, pH 7.0-100 mM β-mercaptoethanol). DNA was precipitated with an equal volume of isopropanol at −70°C for 1 h. Samples were centrifuged for 30 min at 12,000 × g at 4°C, and the DNA pellets were washed in 70% ethanol at room temperature. Pellets were resuspended and incubated overnight at 37°C with 0.5 mg/ml RNase A (Sigma Chemical Co, St. Louis, MO). Pellets were again resuspended and an absorbance reading at 260 nm wavelength was obtained on a Perkin Elmer Cetus Lambda 3B UV/vis spectrophotometer to determine the concentration of DNA. Equal amounts of each DNA sample were then subjected to electrophoresis on a horizontal 1.5% agarose gel containing ethidium bromide and visualized under UV illumination. In cells in which apoptosis was not observed, the culture medium was also examined for the presence of floating cells, but these were not seen in significant numbers.

¹ The abbreviation used is: TGF-β, transforming growth factor β.

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[\textsuperscript{3}H]Thymidine Incorporation Assay. Cells were plated (2 \times 10^4 cells/well) in six replicate wells in 96-well culture plates. Cells were then allowed to grow to two-thirds confluence. Human recombinant TGF-\beta, (10.0, 5.0, 1.0, 0.1, or 0 ng/ml) was added, and cells were incubated at 37\(^\circ\)C for 48 h. During the last 6 h, 1 \muCi of [\textsuperscript{3}H]thymidine (6.7 Ci/mmol/liter) was added to each well. The culture medium was then removed, and cells were washed 3 times and harvested. Incorporated radioactivity was measured in a Packard liquid scintillation counter.

Immunohistochemistry. Primary ovarian cancer and normal ovarian epithelial cells of identical passage to those used for DNA fragmentation and thymidine incorporation experiments were used to prepare cytopsins for immunohistochemical staining. Staining was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). A panel of five mAbs [206F9, 317G5, 741F8 (from Perki Elmer Cetus), BT424.1, and BT8FF1.5 (developed in this laboratory)] shown to be reactive with surface markers on five epithelial ovarian cancer cell lines, but not with mesothelial and inflammatory cells from the benign peritoneal fluid of eight patients (6), was used to assess the purity of the ovarian cancer cells obtained from ascites and scraped tumor. mAb 1801 (Oncogene Science, Manhasset, NY) (5 \mug/ml) was used as the primary antibody for immunostaining of p53 as described previously (13).

Purified mAbs specific for human tissue (Coulter Immunology, Hialeah, FL), was used as a negative control. Anticytokeratin antibody AE1/AE3 (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used as a positive control. The slides were developed for 4 min with the enzyme substrate diaminobenzidine (0.5% diaminobenzidine in 0.05% Tris buffer-0.6% hydrogen peroxide). Slides were then rinsed in water, counterstained with methyl green, dehydrated, and mounted.

Statistics. The Student's t test was used to analyze experiments in which mean values were compared.

RESULTS

We examined whether TGF-\beta (10.0 ng/ml) induced apoptosis in immortalized epithelial ovarian cancer cell lines. Among the five cell lines studied, a DNA ladder consistent with apoptosis was seen on agarose gel electrophoresis only in the OVCA 420 cell line (Fig. 1). This result was reproducible under different experimental conditions in which the cell density (confluent versus subconfluent), serum content (10% versus serum-free), or incubation time (0, 6, 12, 24, 48, or 96 h) were varied. The induction of apoptosis in OVCA 420 cells by TGF-\beta was found to be time and concentration dependent (Fig. 2, A and B). Maximum DNA fragmentation was seen at 48–96 h using 5.0–10.0 ng/ml of TGF-\beta. Induction of apoptosis by TGF-\beta was partially reversed by concurrent treatment of OVCA 420 cells with anti-TGF-\beta mAb 1D11.16 (10 \mug/ml; Fig. 2C).

Finally, the presence of apoptosis in TGF-\beta-treated OVCA 420 cells was confirmed by electron microscopy (data not shown).

As described previously, we were able to obtain relatively pure populations of ovarian cancer cells directly from patients with advanced stage disease as evidenced by immunostaining with the panel of antibodies reactive with ovarian cancers (6). Greater than 90% of cells in cultures established from 10 patients were reactive with the panel of antibodies that recognize ovarian cancer-associated antigens (data not shown). TGF-\beta (10.0 ng/ml) induced DNA fragmentation characteristic of apoptosis in 3 of 10 (30%) primary ovarian cancers (Fig. 3A). In contrast, [\textsuperscript{3}H]thymidine incorporation of all 10 primary ovarian cancers was significantly inhibited by TGF-\beta.
Fig. 3. Effect of transforming growth factor β on programmed cell death in normal and malignant ovarian epithelial cells. (A) Apoptosis was seen in primary ovarian cancer TOV7 but not in TOV6. (B) Apoptosis was not seen in normal ovarian epithelial cell cultures OSE3 or OSE4.

(10.0 ng/ml; mean = 40.4 ± 7.1% of untreated control; P < 0.05; Fig. 4A). Significant inhibition of[^3H]thymidine incorporation was also seen when cells were treated with 1.0 ng/ml TGF-β (mean inhibition = 57.7 ± 11.0% of control) or 0.1 ng/ml TGF-β (mean inhibition = 77.6 ± 7.2% of control; P < 0.05).

Cells cultured from 7 normal human ovaries demonstrated immunostaining with anti-cytokeratin antibody AE1/AE3 consistent with their epithelial origin (data not shown). None of the normal ovarian epithelial cell cultures underwent DNA fragmentation associated with apoptosis following treatment with TGF-β (10.0 ng/ml; Fig. 3B). In contrast, TGF-β (10.0 ng/ml) inhibited[^3H]thymidine incorporation in all 7 of these normal ovarian cell cultures (mean = 61.2 ± 7.8% of untreated control; P < 0.05; Fig. 4B). Significant inhibition of[^3H]thymidine incorporation also was seen when cells were treated with 1.0 ng/ml TGF-β (mean inhibition = 57.7 ± 11.0% of control) or 0.1 ng/ml TGF-β (mean inhibition = 77.6 ± 7.2% of control; P < 0.05).

We also examined the relationship between p53 status and the ability of TGF-β to elicit apoptosis in ovarian cancers (Table 1). Previously, we had found that OVCA 432 has a missense mutation in codon 277 (cysteine to phenylalanine), while the other 4 immortalized cell lines have normal p53 genes. Although the one cell line with a mutant p53 gene (OVCA 432) did not undergo apoptosis, only 1 of 4 lines with normal p53 genes underwent apoptosis in response to TGF-β. Among the 10 primary ovarian cancers, in 7 cases cells were available for p53 immunostaining. Previously, our group and others have shown that approximately 90% of cancers in which p53 immunostaining is seen have point mutations in the gene (14, 15). Similar to prior studies (13), we observed p53 immunostaining in 3 of 7 (43%) primary ovarian cancers. As in the immortalized cell lines, there was no relationship between p53 status and the ability of TGF-β to elicit apoptosis. Apoptosis occurred in 2 of 4 cases in which p53 immunostaining was not seen and in 1 of 3 cases in which immunostaining was present.

Fig. 4. Transforming growth factor β (10.0 ng/ml) inhibits[^3H]thymidine incorporation in normal and malignant ovarian epithelial cells. (A) Primary ovarian cancers (P < 0.05). (B) Normal ovarian epithelial cells (P < 0.05).
DISCUSSION

TGF-β inhibits proliferation of many types of normal cells in primary culture, while most immortalized cancer cell lines usually are resistant to the growth-inhibitory effect of TGF-β (1). Resistance may be manifest as a requirement for a higher concentration of TGF-β to elicit growth inhibition, a lesser degree of inhibition relative to that seen in nontransformed cells, or complete lack of responsiveness to TGF-β. Studies of the effect of TGF-β on proliferation of normal ovarian epithelial cells and ovarian cancer cell lines are consistent with these studies in other cell types. We found that TGF-β consistently inhibits proliferation of normal human ovarian epithelial cells in monolayer culture by 40—70% (5). In contrast, only one of five immortalized ovarian cancer cell lines that we have examined is markedly growth inhibited by TGF-β (5). Likewise, studies by other groups have shown that proliferation of only 5 of 10 ovarian cancer cell lines were inhibited by greater than 25% by TGF-β (16—19).

Because of the difficulty in obtaining and culturing material directly from patients, few studies have examined the effect of TGF-β on primary human cancer cells. Recently, we reported that in 19 of 20 cases (95%), proliferation of ovarian cancer cells purified from the ascites of patients with advanced stage disease was significantly inhibited by TGF-β (6). As in the present study, there was no difference in the magnitude of growth inhibition, or the concentration of TGF-β required to achieve maximal growth inhibition between normal ovarian epithelial cells and primary ovarian cancers. Similarly, Daniels et al. (20) found that TGF-β inhibited colony formation of 7 of 9 fresh ovarian cancers in soft agar. Studies that have examined proliferation of leukemia cells obtained directly from patients have shown these cells are also growth inhibited by TGF-β (21, 22). These studies in ovarian cancer and leukemia are among the few that have examined the effect of TGF-β on proliferation of early passage cancer cells from patients. They suggest that while loss of responsiveness to TGF-β often occurs in vitro during the process of immortalization, it may not commonly occur during the development of human cancers. Although immortalized cell lines are useful models for cancer research, most human cancer cells are not immortal, and the alterations in growth regulatory pathways that occur during the process of immortalization may be unrelated to those involved in the process of tumorigenesis.

In addition to arresting progression through the cell cycle, it has been shown that TGF-β can trigger apoptosis in normal cells under certain circumstances. Apoptosis has been observed in the context of withdrawal of trophic hormones in the postpartum uterus (23), in atretic ovarian follicles (24), and in the postcastration prostate (25).

Although TGF-β primarily inhibits proliferation of normal ovarian epithelial cells, we were unable to demonstrate that these cells undergo apoptosis in response to TGF-β. It is possible that apoptosis could have been induced in a small fraction of cells, but that this was below the threshold of detection of the DNA fragmentation assay. Among the five immortalized ovarian cancer cell lines, the only line that is profoundly growth inhibited by TGF-β (OVCA 420) also underwent apoptosis. Apoptosis in OVCA 420 cells was dose and time dependent, and can be partially reversed by concurrent treatment with anti-TGF-β mAb. We also examined 10 primary ovarian cancers from patients with advanced stage disease. In all 10 cases, proliferation was inhibited by TGF-β, but apoptosis occurred in only 3 cases. There was no relationship between the magnitude of inhibition of proliferation and whether apoptosis occurred.

It some cell lines, loss of p53 function due to mutations and/or deletions of this gene during the process of malignant transformation has been associated with the development of resistance to the growth-inhibitory effect of TGF-β (26). Previously, however, we had found that some ovarian cancers with mutant p53 protein were responsive to TGF-β, suggesting that the growth-inhibitory effect was not dependant on the presence of functional p53 (6). It also has been shown, in some cell types, that DNA damage due to radiation can lead to apoptosis and that this process is dependent on the presence of normal p53 protein (27). It is thought that this serves as a surveillance mechanism for elimination of cells with genetic damage, which are more likely to undergo malignant transformation. Similarly, transfection of normal p53 genes into cancer cells that lack normal p53 induces apoptosis in some cases (28, 29). Since p53 is altered in approximately one-half of epithelial ovarian cancers (13) and normal p53 appears to play a role in inducing apoptosis under certain circumstances, we examined whether there was a relationship between the status of p53 and the ability of TGF-β to induce apoptosis in ovarian cancers. The absence of such a relationship in these ovarian cancers suggests that TGF-β induced apoptosis occurs via molecular pathways that are independent of p53. Similarly, another group has shown that transfection of a mutant p53 gene into a hepatoma cell line did not negate the ability of TGF-β to induce apoptosis of these cells (30).

Presently, it is unclear why only a fraction of ovarian cancers undergo apoptosis in response to TGF-β, whereas proliferation of almost all of these cancers is arrested by this growth inhibitor. Although it has been postulated that cancer cells may be more susceptible to apoptosis than their normal counterparts due to the presence of genetic damage, most advanced ovarian cancers are aneuploid (31). Thus, if a significant load of genetic damage is the primary factor involved in an increased propensity to undergo apoptosis, a larger fraction of ovarian cancers would have been expected to undergo apoptosis in response to TGF-β. Further studies that elucidate the specific molecular pathways involved are needed to determine why only a minority of ovarian cancers that are growth inhibited by TGF-β undergo apoptosis.

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APOTOPSIS IN OVARIAN CANCER


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