

# Tamoxifen Modulates Apoptotic Pathways in Primary Endometrial Cell Cultures

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## ABSTRACT

Clinical data indicate that tamoxifen (TAM) therapy may cause an increased risk of endometrial pathology in postmenopausal but not in premenopausal women. Molecular mechanisms of the uterotrophic activity of TAM have not been clearly established nor its relevance to apoptosis in endometrial cells. The present study was implemented to evaluate the apoptotic effect of TAM on primary endometrial cell cultures in the presence or absence of steroid hormones (SHs). A total of 14 primary endometrial cell cultures were established and maintained both with and without SHs. Cell cultures were treated for 24 h with either 20  $\mu\text{M}$  TAM or 10 nM estradiol. Apoptotic cells presented in a pre-G<sub>1</sub> peak and the expression of bcl-2 were studied using flow cytometry. All endometrial cell cultures maintained in a SH-containing environment, except one, responded to TAM by a significant increase ( $P = 0.03$ ) in the pre-G<sub>1</sub> cell fraction, indicating a proapoptotic effect. A significant ( $P = 0.03$ ) reduction in the pre-G<sub>1</sub> peak equivalent to an anti-apoptotic response was observed in 6 of 13 cell cultures maintained in a SH-deficient environment. In 4 of 10 cell cultures evaluated in both media, the pre-G<sub>1</sub> population was medium dependent. In 8 of 10 cultures evaluated for Bcl2 levels, no trend was found in either media, but a dependency on SH content was observed. Comparison between effects of TAM and estradiol demonstrated identical trends, regardless of the menstrual phase or SH content in cell environments. These results suggest that TAM acts as an estrogen agonist on endometrial tissue in both environments. We conclude that TAM modulates apoptotic pathways in primary endometrial cell cultures. The SH content in the cell environment influences the apoptotic effect of TAM and determines the propensity for a cell to undergo apoptosis or, on the contrary, to resist apoptotic death in response to

TAM treatment. This is in concordance with the observed clinical risk of endometrial pathologies in postmenopausal versus premenopausal women.

## INTRODUCTION

TAM<sup>2</sup> is a nonsteroidal compound that binds to ERs and elicits estrogen agonist or antagonist responses, depending on the target tissue (1). It is the most widely prescribed antineoplastic hormonal treatment agent today. Its effectiveness appears to be greatest in postmenopausal breast cancer patients with positive ER, but it is also effective in premenopausal patients (2). TAM is also currently being evaluated as a preventive agent for healthy women with increased risk for breast cancer (3).

There is sufficient clinical data to suggest that postmenopausal TAM therapy may cause an increased risk for the development of benign and malignant endometrial pathologies (4). Although in premenopausal women there is nearly no excess risk of endometrial adverse effects (3, 5), it has been hypothesized that in the low E<sub>2</sub> environment of menopause, TAM can function as an estrogen agonist on the human endometrial tissue and thus may cause endometrial pathologies (6). The hormonal dependencies of endometrial as well as breast cancers suggest that this difference may be attributed to the hormonal diversities of the pre- and postmenopausal hormonal milieu.

In breast tissue, the competitive binding of TAM to ER prevents the estrogen/ER-mediated gene transcription, DNA synthesis, and breast cancer cell growth (1). Various studies showed that the effect of TAM on breast cancer cell lines was mediated through apoptosis (7–11). Molecular mechanisms of the uterotrophic activity of TAM have not been clearly established nor its relevance to the active process of apoptosis in endometrial cells (1, 12). Thus, there is a need to understand the mechanisms by which TAM induces changes in uterine compartments. It is known that carcinogenesis often involves selective failure of apoptosis that prolongs the survival of cells after mutagenic DNA damage (13). One of the main mechanisms through which oncogenes promote carcinogenesis is by inhibition of apoptosis (14, 15). Acquired resistance toward apoptosis is a hallmark of most cancer types (14). Moreover, dysregulation of apoptosis has been observed in different neoplasms of the endometrium and is likely to be associated with genetic alteration, driving progression along the hyperplasia-atypia-carcinoma sequence (16).

The present study was implemented to elucidate the cellular mechanism through which TAM exerts its stimulatory effect on postmenopausal human endometrium. In simulation of the postmenopausal hormonal environment, we maintained cell cul-

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<sup>2</sup> The abbreviations used are: TAM, tamoxifen; ER, estrogen receptor; E<sub>2</sub>, estradiol; SH, steroid hormone; FACS, fluorescence-activated cell sorter.

tures in SH-devoid medium and compared these cultures to the same cells maintained in SH-containing medium. We evaluated the effect of TAM and the relevance of SH content in the medium to the effect of TAM on cultured endometrial cells, measured as the apoptotic cell population and as the levels of the main antiapoptotic protein Bcl2.

## MATERIALS AND METHODS

**Materials.** All culture media and supplements were purchased from Biological Industries (Beit Haemek, Israel).

**Tissues.** We obtained human endometrial samples by curetting the uterine cavity, after the removal of the uterus, from 20 randomly assigned patients admitted for hysterectomy because of benign pathology. Detailed information including medical data, history, and indications for surgery was recorded. All endometrial tissue samples underwent pathological evaluation, and abnormal samples were excluded from the study. Institutional and Israeli Health Ministry Helsinki approvals were obtained.

**Establishment of Primary Endometrial Cell Cultures.** Each endometrial sample was minced into small pieces (1–2 mm<sup>3</sup>) under aseptic conditions. The resulting pieces were incubated for 2 h in Leibovitz medium supplemented with 200 units/ml collagenase, 1000 units/ml hyaluronidase, and antibiotics at 37°C with an occasional vigorous shake. The suspension was centrifuged at 300 × *g* for 8 min, and after removal of the supernatant, the cellular pellet was resuspended in DMEM supplemented with 10% FCS, glutamine, and antibiotics. The cells were seeded in 25-cm<sup>2</sup> plastic culture flasks and incubated at 37°C under a humidified atmosphere. Twenty-four h later, the medium and the nonadherent cells were replaced with fresh medium.

**Immunocytochemistry.** The epithelial identity of cells in culture was confirmed by immunocytochemical staining with monoclonal antibodies (Dako, Copenhagen, Denmark) against specific markers: cytokeratin for epithelial cells and vimentin for mesenchymal cells. Cells were fixated and perforated in the 6-cm<sup>2</sup> Petri dishes with 100% cold methanol and incubated with anticytokeratin antibody, respectively, with antivimentin antibody. Immunostaining was performed using the biotin-streptavidin-immunoperoxidase method using the Histostain-*plus* kit (Zymed Laboratories, Inc., San Francisco, CA). Visualization was achieved by the addition of 3-amino-ethylcarbazol substrate (Zymed Laboratories Inc.). All cytoplasmic red staining was considered positive.

**Maintenance of Endometrial Primary Cell Cultures.** Cell cultures were grown in DMEM supplemented with 10% FCS and after ~2 weeks were split into two subcultures. The subcultures were maintained 7 days either in the presence (DMEM with 10% FCS) or absence (phenol red-free DMEM and 10% charcoal stripped FCS) of SHs in culture medium.

**Administration of TAM.** Twenty-four h before the experiment, cells maintained either in medium with SH or in SH-deficient medium were seeded in six-well plates (10<sup>5</sup> cells/well) in a medium containing DMEM-12/HAM (1:1) supplemented with 10% FCS, 1% glutamine, and antibiotics or in phenol-red free DMEM-12/HAM (1:1) supplemented with 10% charcoal-stripped FCS. On the day of the experiment, the me-

dium together with all nonadherent cells was removed and replaced with fresh medium. The optimal administered concentration of TAM was established by standard curve (5–25 μM) and set as the maximal nontoxic effect at 20 μM TAM (Sigma, St. Louis, MO). The cells were treated for 24 h with either 20 μM TAM or 10 nM E2 (Sigma). Ethanol was administered as a negative control. All experiments were carried out in duplicates. The suspensions were centrifuged at 300 × *g* for 8 min, perforated, and fixed with 70% cold methanol (–20°C for 40 min).

**Analysis of Apoptotic Cell Population.** Perforated cells were incubated for 15 min at 4°C with 400 μl propidium iodide diluted in PBS (1 mg/ml), and fluorescence was analyzed by a Coulter flow cytometer (EPICS-XL; Beckman Coulter, Bristol, United Kingdom). The apoptotic cells were presented in a pre-G<sub>1</sub> peak. At least 10,000 events were counted in each flow cytometry (FACS) analysis.

**Detection of Antiapoptotic Bcl2 Protein.** Perforated cells were incubated for 30 min at 4°C and in the dark with 10 μl of FITC-conjugated monoclonal mouse antihuman Bcl2 antibodies (Dako) and then washed and resuspended in 400 μl of PBS. Cell-associated fluorescence was analyzed by FACS. At least 10,000 events were counted in each FACS analysis.

**Statistical Analysis.** Statistical analyses of the data were performed using the Wilcoxon sign rank test. *P* < 0.05 was considered to reflect a significant difference from the corresponding control group.

## RESULTS

**Characteristics of Endometrial Samples.** The study included 15 primary endometrial cell cultures (numbered 1–15 consistently throughout the study). Clinical and pathological features of the patients' endometrial samples are presented in Table 1. All patients were premenopausal women ranging in age between 38 and 50 years (mean ± SD, 45.1 ± 3.5). Ten of the samples were in the proliferative stage of the menstrual cycle, and the other 5 were in the secretory stage. All cell cultures were negative for vimentin, ruling out the possibility of fibroblast contamination of the culture. All primary cultures were positive for staining with anticytokeratin antibodies, thus validating the epithelial nature of the culture (results not presented). Because of the variation between endometrial tissues on the one hand and the technical limitations in establishing and maintaining primary cultures on the other, not all samples were included in all aspects of the experiment.

**Maintenance of Endometrial Cells in an Environment Containing SHs.** Twelve of the 15 endometrial primary cultures included in this study were evaluated. All samples but one responded to TAM in a significant increase (*P* = 0.03) in the pre-G<sub>1</sub> peak (Fig. 1a). The increase in the apoptotic fraction of the cell population ranged between 11 and 277.7% (mean ± SE, 70.23 ± 22.05). The sample that reacted in an antiapoptotic fashion to TAM displayed a decrease of 32.66% in the pre-G<sub>1</sub> peak. Bcl2 levels were more variable, and an evident inverse relationship between pre-G<sub>1</sub> and Bcl2 levels was not obvious (data not shown).

**Endometrial Cells Maintained in an Environment Deficient of SHs.** A total of 13 endometrial cell cultures were studied in SH-deficient medium. Of 13 samples, 6 responded to

Table 1 Clinical characteristics of donors of endometrial samples<sup>a</sup>

	Menstrual cycle phase		Total
	Proliferative	Secretory	
No. of women	10	5	15
Age of women	39–50	42–50	39–50
Average age	44.8 ± 3.96	45.79 ± 3.19	45.13 ± 3.51
Indication for surgery	Fibroid uterus (9) Pelvic mass (1)	Fibroid uterus (3) Prophylactic (1) Menorrhagia Dysmenorrhea (1)	Fibroid uterus (12) Pelvic mass (1) Prophylactic (1) Menorrhagia Dysmenorrhea (1)

<sup>a</sup> Mean values are expressed as mean ± SD.

TAM in an antiapoptotic fashion with a significant reduction ( $P = 0.03$ ) in the pre-G<sub>1</sub> population ranging between 57.7 and 10.72% (mean ± SE, 31.30 ± 6.31). The other 7 samples reacted in an elevation of the pre-G<sub>1</sub> population between 12.5 and 264% (mean ± SE, 111.48 ± 33.57), indicating an apoptotic response (Fig. 1b). Of a total of nine cell cultures derived from proliferative endometrium, five responded in an antiapoptotic fashion with a decrease in the pre-G<sub>1</sub> peak ranging between 57.7 and 10.71% (mean ± SE, 30.76 ± 7.71) and four responded by apoptosis with an increase in the pre-G<sub>1</sub> peak in the range 12.5–264% (mean ± SE, 142.59 ± 53.45). Of the four secretory endometrial cell cultures, three had an increase in the pre-G<sub>1</sub> populations ranging between 18.75 and 102.28% (mean ± SE, 70.00 ± 25.91). One of the secretory endometrial cell cultures reacted in an antiapoptotic manner with 34% decrease in the pre-G<sub>1</sub> peak. High variability in Bcl2 levels was demonstrated, and no evidence for an inverse relationship between pre-G<sub>1</sub> and Bcl2 levels could be established (data not shown).

**Comparison between Changes in TAM-induced pre-G<sub>1</sub> Peaks in Endometrial Cells Maintained in SH-containing Environments versus SH-deficient Environments.** Ten samples were evaluated in both media (with or without SHs; Fig. 1c). In four of them, the pre-G<sub>1</sub> population was medium dependent, whereas the SH content of the medium did not influence the other six cultures. All three (100%) cultures originating from secretory endometrium responded in the same fashion, regardless of the contents of the medium, whereas in cultures originating from proliferative endometrium, three of seven (42%) were not affected by steroidal content, and four of seven (58%) were affected.

**Comparison between Responses to TAM versus E<sub>2</sub>, Both in Endometrial Cells Maintained in SH-containing Environments and SH-deficient Environments.** E<sub>2</sub> was administered to five endometrial cultures maintained in SH-containing medium and to five cell cultures maintained in SH-deficient medium. The apoptotic response, measured as the pre-G<sub>1</sub> peak, was assayed. All endometrial cell cultures (10 of 10), regardless of the menstrual cycle phase, responded to TAM and E<sub>2</sub> in the same manner. We observed an increase in the pre-G<sub>1</sub> peak in both TAM- and E<sub>2</sub>-treated cultures maintained in medium containing SHs and a decrease in the pre-G<sub>1</sub> population in 80% of cultures maintained in a SH-deficient environment (Fig. 2).

## DISCUSSION

TAM therapy of postmenopausal women has been shown to be associated with various endometrial pathologies, including endometrial cancer (4, 5), yet the molecular pathways involved have not been fully elucidated (1, 12). Although in breast cancer cells TAM treatment has been shown to activate apoptotic pathways (7–11), the association between TAM treatment and apoptosis in human endometrial tissue has not yet been evaluated.

Our results demonstrate that administration of TAM to primary endometrial cell cultures modulates apoptotic pathways. A significant ( $P = 0.03$ ) proapoptotic effect of TAM was detected in the majority of cell cultures grown in a steroid-containing medium. This effect was not dependent on the menstrual phase of the originating endometrial samples. These results are consistent with the clinical observations in premenopausal TAM-treated breast cancer patients, in which endometrial pathologies are uncommon (3, 5). It was assumed that menstrual shedding, by evacuation of the uterine cavity of the possibly affected cells, eliminates the risk of endometrial pathologies. Our results suggest that TAM treatment activates apoptotic pathways in the endometrium of premenopausal women, and therefore, the lack of carcinogenic effect in hormonally active endometrium is possibly attributable to an active anticancerous process and not the result of menstrual shedding alone. The possibility that apoptosis serves as a barrier to cancer was first raised in 1972 (17) and was further supported by numerous studies (14). The clinical observation that TAM treatment might reverse endometrial hyperplasia in premenopausal women (18) further supports our assumption, that in the presence of high endogenous estrogen levels, TAM acts in a protective manner.

Two opposing trends (proapoptotic and antiapoptotic) were observed in cells maintained in a steroid-deficient medium. Of the examined samples, 46% demonstrated a significant antiapoptotic response ( $P = 0.03$ ). This effect is consistent with the clinically known, estrogenic-like agonistic effect of TAM in the low estrogenic environment of menopause. This effect may explain why a relatively high percentage (25–30%) of postmenopausal TAM-treated patients develop different endometrial pathologies, including endometrial carcinoma (4, 5).

Opposing effects of TAM on the apoptotic cell population of the primary cell cultures were observed as a consequence of different media. Our results suggest that TAM modulates the

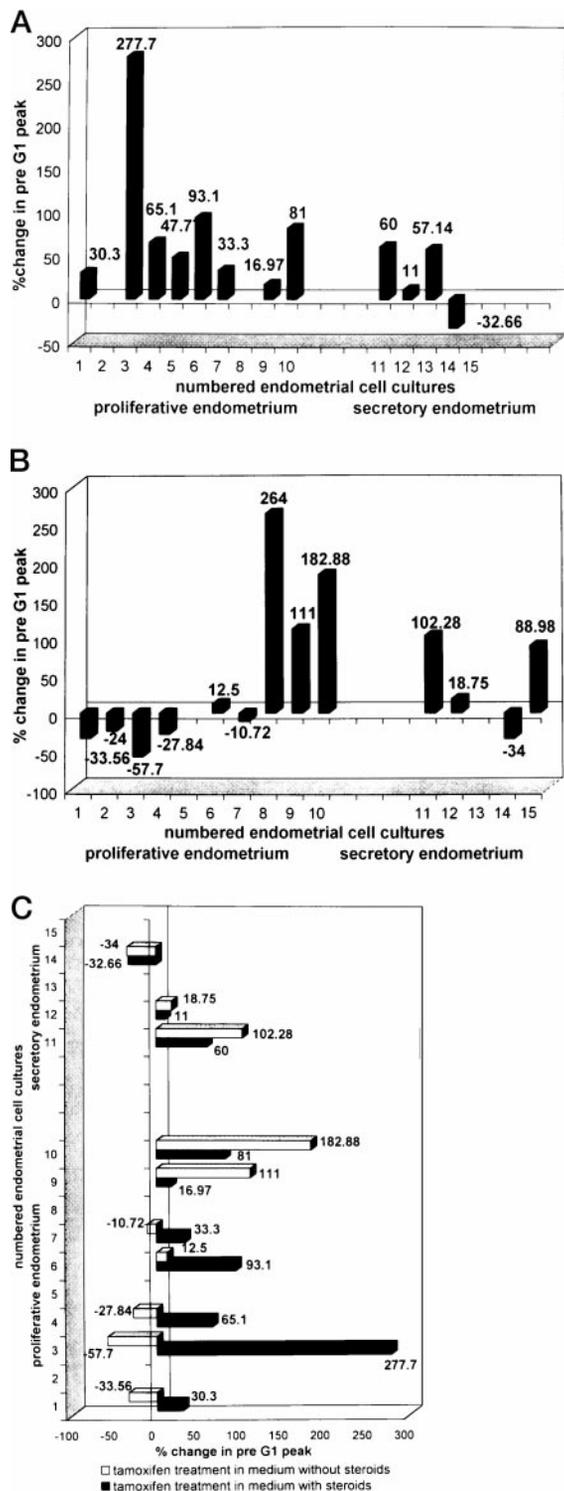


Fig. 1 The modulation of apoptotic pathways by TAM in endometrial primary cultures (originating from proliferative or secretory phase respectively) in different hormonal environments. a, TAM-induced changes (compared with an ethanol-administered control) in pre-G<sub>1</sub> cell populations in endometrial primary cultures maintained in SH-containing medium. b, TAM-induced changes (compared with ethanol-administered control) in pre-G<sub>1</sub> cell populations in endometrial primary cultures maintained in SH-deficient medium. c, comparison of TAM-induced changes in pre-G<sub>1</sub> cell populations in endometrial cells maintained in SH-containing medium versus SH-deficient medium.

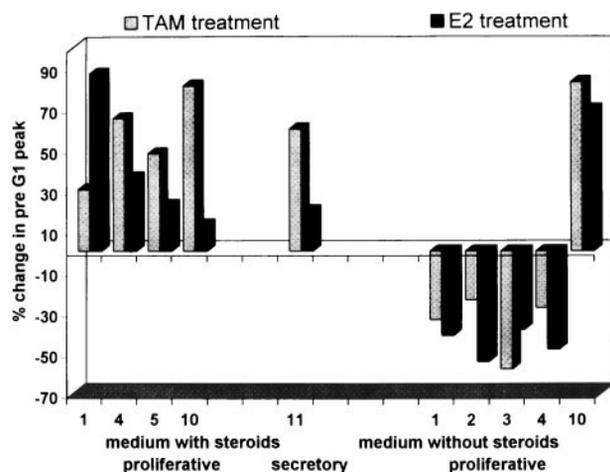


Fig. 2 Comparison of endometrial cells responses (measured as the pre-G<sub>1</sub> peaks) to treatment with TAM versus E<sub>2</sub> treatment.

apoptotic pathways in endometrial cells, but the progression toward apoptosis or carcinogenesis is influenced by the hormonal environment of the cell. The significance of the hormonal environment is further supported by studies carried out on cell lines (19, 20). In a recent article (21), immunohistochemical studies of the Ki67 index also indicate that TAM exerts an antiestrogenic effect on the endometrium in the presence of endogenous estrogen secretion and an estrogenic effect in the absence of estrogen secretion. This mechanism is further supported by Cross *et al.* (22), who describes the occurrence of complex atypical hyperplasia in an ovariectomized premenopausal breast cancer patient undergoing TAM treatment. A similar modulation of the effect of TAM by the hormonal milieu was described previously in bone tissue (23).

We analyzed the effect of TAM on apoptosis of the endometrial cultures according to the menstrual cycle from which they originated. Secretory endometrium was insensitive to changes in the SH content in the medium. Proliferative endometrium was most often affected by the hormonal content. A possible explanation is preconditioning of the endometrium removed at the secretory phase, thus rendering it unresponsive to the SH content.

Two opposing trends in Bcl2 expression were observed, regardless of the steroidal content of the medium and of the menstrual phase of the originating endometrium. Moreover, a lack of consistent association is demonstrated between Bcl2 expression and the anti- or proapoptotic effect of TAM on endometrial cells. The association between overexpression of Bcl2 and endometrial carcinogenesis has not been demonstrated yet. Contradictory findings have been described with regard to Bcl2 expression in neoplastic endometrial tissue (24–27). Thus, the association between Bcl2 expression and apoptosis in normal and neoplastic endometrial tissue is not conclusive (27–29). Although Bcl2 is highly expressed in normal proliferative and hyperplastic endometrium, it is significantly down-regulated in atypical hyperplasia and adenocarcinoma. It was suggested that the role of Bcl2 in preventing cell death is bypassed in the progression of atypical hyperplasia to carcinoma by other fac-

tors impeding apoptosis (24, 25). Marked reduction in Bcl-2 protein levels, although rendering cells more prone to apoptosis, are by themselves often insufficient to cause cell death (30). The Bcl2:Bax ratio is considered to be a more important checkpoint in the modulation of the apoptotic process than the absolute level of either Bcl2 or Bax (31); therefore, the expression of Bax should be studied and might shed light on this dilemma.

TAM has been shown to have an estrogen-like agonistic effect on the endometrium in animal and human studies (32, 33). Our results show a complete correlation between TAM and E<sub>2</sub> responses in all cell cultures, regardless of the menstrual phase of tested endometrium or steroid content in the medium. The results obtained in the steroid-free medium support the mechanism of an estrogen-like effect of TAM and the hypothesis that TAM affects the endometrial cell through the ER pathway, similar to that of estrogen itself. Different studies have shown that ER recognizes a similar estrogen response element with TAM, as with estrogen itself, and that TAM and E<sub>2</sub> induces similar proto-oncogenes (1, 34). A recent study provides further documentation that the uterotrophic effect of TAM on postmenopausal endometrium may be attributable to overexpression of both ERs and progesterone receptors (35). However, this approach cannot explain the results seen in the steroid-containing medium. Because E<sub>2</sub> is known to have an antiapoptotic effect (36, 37), we would expect that the addition of E<sub>2</sub> to the medium would further reduce the pre-G<sub>1</sub> population. But an opposite effect was observed in our experiments.

Unraveling the mechanistic aspects of the effect of TAM on the endometrium may have applicable consequences as well. Our model may serve as an *ex vivo* prognostic assay for the risk of endometrial carcinoma development after TAM treatment in postmenopausal women. Moreover, because TAM is used as a chemopreventive agent in healthy patients, there is a clear need to elucidate the mechanism of the action of TAM in the reproductive tract. A better understanding will enable prediction of which groups of patients are more susceptible to develop endometrial pathologies.

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