

# Tamoxifen Modulates the Expression of Ki67, Apoptosis, and Microvessel Density in Cervical Cancer

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## ABSTRACT

**Purpose:** The aim of the study was to investigate if a short-term administration of high-dose Tamoxifen (Tam) could affect the expression of biologically relevant biochemical parameters in cervical cancer tissue.

**Experimental Design:** The study was conducted in 24 patients with histologically confirmed cervical tumors. Biopsies were obtained by colposcopy on day 0 in all patients, who then received either 80 mg/die or 160 mg/die for 5 consecutive days until the second biopsy was obtained. Immunohistochemistry was performed with antiestrogen receptor (ER), anti-Ki67, anticaspase cleavage product of keratin 18 (M30), and anti-CD31 monoclonal antibodies.

**Results:** Eleven (45.8%) of 24 cervical tumors were ER positive. The percentage of Ki67-positive tumor cells in pre-Tam biopsies was significantly higher than the percentage in the corresponding posttreatment biopsies ( $z = 4.29$ ,  $P = 0.0001$ ). No difference in the pretreatment percentage of Ki67-positive cells according to ER status was found. The percentage of M30 positivity was higher in post-Tam than in pre-Tam biopsies. Microvessel density values in pre-Tam biopsies were significantly higher than corresponding values in posttreatment tissues ( $z = -3.72$ ,  $P = 0.0002$ ). The reduction in the percentage of Ki67-positive tumors was significantly ( $z = 3.58$ ,  $P = 0.0003$ ) higher in ER-positive than in ER-negative tumors, whereas no difference in Tam-induced reduction of microvessel density values according to ER status ( $z = -0.18$ ,  $P = 0.85$ ) was found. Tam treatment did not induce any change of M30 positivity in ER-positive tumors, whereas in ER-negative tumors, it produced a sig-

nificant ( $P = 0.015$ ) increase in the percentage of M30-positive cells in post-Tam versus pre-Tam biopsies.

**Conclusions:** A short-term treatment with Tam at doses 4–8-fold higher than those in conventional schemes is associated with modifications of biological parameters associated with tumor cell proliferation, apoptosis, and neoangiogenesis in cervical cancer.

## INTRODUCTION

Although cervical cancer is generally considered to be refractory to endocrine treatment, some evidences have been reported that cervical tumor tissue might be susceptible to respond to antihormonal manipulation, at least at a molecular level (1).

In particular, the possible biological and clinical role of the antiestrogen Tam<sup>2</sup> in human cervical cancer is still controversial: the presence of ERs in this neoplasia is well established (2–4), and *in vitro* data indicate that Tam can inhibit the growth of ER-positive and ER-negative cervical cancer cells (5–7). Nevertheless, Kavanagh *et al.* (8) failed to demonstrate any activity of the drug administered at conventional doses in patients with refractory adenocarcinoma of the cervix. Furthermore, no relevant modifications of the expression pattern of ER, progesterone receptors, HER-2/*neu*, p53, and proliferating cell nuclear antigen have been observed in cervical tumor biopsies after conventional Tam treatment *in vivo* (3), suggesting that Tam has minimal activity, if any, in cervical cancer.

It has to be taken into account that the biological activities exerted by Tam in cervical cancer cells *in vitro* are obtained at Tam concentrations achievable *in vivo*, after at least 4–8 weeks of treatment with conventional doses (9).

Therefore, it is at least conceivable that Tam's failure to modulate *in vivo* the expression of specific biological markers in cervical tumors could simply reflect the inability to reach active Tam concentrations, after a too brief period of treatment with conventional Tam doses.

Because it has been reported that high-loading doses of Tam allow to achieve biologically active plasma concentrations within a few hours (9), we were prompted at investigating if a short-term administration of high-dose Tam can affect the expression of biologically relevant biochemical parameters in cervical cancer tissue. In particular, Ki67 antigen was chosen as a marker of cell proliferative activity (10) and the caspase cleavage product of keratin 18, recognized by the M30 monoclonal antibody, as a marker of early apoptosis (11). Moreover, considering the tight relationship between neoangiogenesis and growth/apoptosis balance (12–14), we decided to measure the

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<sup>2</sup> The abbreviations used are: Tam, Tamoxifen; ER, estrogen receptor; MVD, microvessel density; *die*, every day.

Table 1 Characteristics of the patients, ER status, and expression of Ki67, M30-recognized antigen, and MVD in pre and posttreatment cervical tumor biopsies

| No. | Age (yrs) | Stage | Histotype     | Grade | Tam      |    | Ki67         |               | M30          |               | MVD             |               |
|-----|-----------|-------|---------------|-------|----------|----|--------------|---------------|--------------|---------------|-----------------|---------------|
|     |           |       |               |       | (mg/die) | ER | Pretreatment | Posttreatment | Pretreatment | Posttreatment | Pretreatment    | Posttreatment |
| 1   | 42        | IIA   | Squamous      | 3     | 160      | —  | 85           | 65            | 0            | 0             | 98              | 75            |
| 2   | 36        | IB    | Squamous      | 3     | 160      | —  | 16           | 12            | 0            | 0             | 100             | 60            |
| 3   | 34        | IIB   | Squamous      | 3     | 160      | —  | 72           | 44            | 0            | +             | nd <sup>a</sup> | nd            |
| 4   | 50        | IIB   | Squamous      | 3     | 80       | —  | 40           | 32            | 0            | 0             | 82              | 25            |
| 5   | 46        | IIB   | Squamous      | 3     | 80       | —  | 82           | 30            | 0            | +             | 100             | 55            |
| 6   | 35        | IIB   | Squamous      | 2     | 80       | —  | 63           | 47            | 0            | +             | 93              | 78            |
| 7   | 38        | IIB   | Adenosquamous | 3     | 80       | —  | 33           | 30            | 0            | 0             | nd              | nd            |
| 8   | 46        | IIIA  | Squamous      | 3     | 80       | —  | 56           | 50            | 0            | 0             | nd              | nd            |
| 9   | 60        | IIB   | Adenosquamous | 3     | 80       | —  | 92           | 78            | 0            | +             | 119             | 102           |
| 10  | 56        | IIB   | Squamous      | 2     | 80       | —  | 35           | 12            | 0            | +             | 70              | 58            |
| 11  | 76        | IIIB  | Squamous      | 2     | 80       | —  | 79           | 45            | 0            | +             | nd              | nd            |
| 12  | 58        | IIB   | Squamous      | 2     | 80       | —  | 39           | 29            | 0            | 0             | 78              | 70            |
| 13  | 62        | IIB   | Adenosquamous | 2     | 80       | —  | 40           | 30            | +            | +             | 104             | 90            |
| 14  | 47        | IIIB  | Squamous      | 3     | 160      | +  | 70           | 32            | 0            | 0             | 70              | 50            |
| 15  | 62        | IB    | Squamous      | 3     | 160      | +  | 79           | 33            | +            | +             | 60              | 65            |
| 16  | 62        | IVA   | Squamous      | 2     | 160      | +  | 22           | 6             | 0            | 0             | nd              | nd            |
| 17  | 45        | IIIB  | Squamous      | 3     | 160      | +  | 35           | 4             | 0            | 0             | 88              | 45            |
| 18  | 72        | IIIA  | Adenosquamous | 2     | 160      | +  | 60           | 25            | 0            | 0             | nd              | nd            |
| 19  | 74        | IIIB  | Squamous      | 2     | 80       | +  | 22           | 2             | +            | +             | 45              | 36            |
| 20  | 53        | IIA   | Adenosquamous | 2     | 80       | +  | 53           | 31            | +            | +             | 159             | 144           |
| 21  | 70        | IVA   | Squamous      | 2     | 80       | +  | 42           | 20            | 0            | 0             | 65              | 27            |
| 22  | 46        | IIB   | Squamous      | 3     | 80       | +  | 38           | 8             | 0            | 0             | 98              | 90            |
| 23  | 61        | IVA   | Squamous      | 3     | 80       | +  | 59           | 36            | 0            | 0             | 66              | 16            |
| 24  | 53        | IIB   | Adenosquamous |       | 80       | +  | 42           | 25            | 0            | 0             | 67              | 53            |

<sup>a</sup> nd, not determined.

MVD expression in cervical cancer after immunostaining with CD31 monoclonal antibody.

## PATIENTS AND METHODS

**Patients.** Our study was conducted in a group of 24 patients with histologically confirmed cervical tumors, admitted at the Department of Gynecology, Catholic University of Rome.

Characteristics of the patients are shown in Table 1. Tumors were defined as stage IB to stage IVA, according to the International Federation of Gynecologists and Obstetricians classification. Histologically, 18 tumors were squamous cell carcinomas and 5 adenocarcinomas. One case was classified as adenosquamous. The degree of differentiation was defined according to the WHO criteria.

None of the patients had diseases that might contraindicate treatment with Tam. Informed consent according to our Institutional Committee guidelines was obtained for each patient. Cervical biopsies were obtained by colposcopy on day 0 in all patients ( $n = 24$ ). Sixteen patients were randomly assigned to receive either 80 mg/die or 160 mg/die for 5 consecutive days until the second biopsy specimens were obtained. Because the evaluation of this series demonstrated a similar range of response extent in both the 80-mg and 160-mg Tam dose groups, the subsequent 8 patients were administered 80 mg/die Tam for 5 days until the second biopsy was taken.

**Immunohistochemical Analysis.** Tumor biopsies were fixed in 10% neutral buffered formalin for 12 h, and standard dehydration and paraffin-wax embedding procedures were used on fixed tissues. Sections (3  $\mu$ m) mounted on organosilane-treated slides were dewaxed in xylene, rehydrated through de-

scending concentrations of alcohol, and, after three washings in tap and then distilled water, treated with 0.3% hydrogen peroxide in methanol for 5 min to block the endogenous peroxidase activity. The sections were then washed in PBS and subjected to heat-induced epitope retrieval in a microwave oven using the Dako ChemMate detection kit (Dako, Glostrup, Denmark), according to manufacturer's instructions. Incubations with anti-ER (clone 1D5; Ylem, Rome, Italy), anti-Ki67 (clone MIB1; Ylem), anticaspase cleavage product of keratin 18 (clone M30; Boehringer Mannheim GmbH, Mannheim, Germany), and anti-CD31 (clone JC/70 A; Dako) monoclonal antibodies (diluted 1/50) were carried out for 1 h at room temperature. In negative controls, preimmune mouse serum instead of primary antibodies was used.

Immunoreactions were revealed by the avidin-biotin complex technique using 3-amino-9-ethylcarbazole (AEC Substrate System; Dako) as a substrate. Endogenous biotin was saturated by a biotin-blocking kit (Vector Laboratories, Burlingame, CA). For CD31 immunostaining, DAB/NiCl<sub>2</sub> chromogen, without nuclear counterstaining, was used.

ER staining was scored in a minimum of 300 histologically identified neoplastic cells, showing nuclear reaction. Tumors showing positive nuclear immunostaining in >10% of tumor cells were defined as ER positive.

Scoring of Ki67 was expressed as the percentage of cells with positive nuclear immunostaining.

Relative to M30 immunoreactivity, samples were classified as positive (+) if >3% immunostained tumor cells were detected. Tumor MVD (MVD = number of vessels/mm<sup>2</sup>) was evaluated after CD31 immunostaining. The area of highest

vascular density was assessed by a two-headed microscope in four different random fields by using  $\times 20$  objective and  $\times 10$  ocular, equivalent to  $0.86 \text{ mm}^2$  in areas of highest neovascularization. The mean vessel count from these fields was used for MVD scoring.

Scoring of each parameter was performed by two independent pathologists (F. O. R. and G. Sa.) without any knowledge of the receptor status, immunohistochemical results of other markers, treatment, and clinicopathological features. Disagreement between the pathologists was noted in two (8%) cases for Ki67, two (8%) cases for M30 staining, and three (12%) cases for MVD, and consensus was reached by joint reevaluation by three independent observers (F. O. R., G. Sc., and L. M. L.). Intra and interobserver variability were evaluated by twice repeat scoring of 20 randomly selected sections, by the same (F. O. R.) or two (L. M. L. and F. O. R.) observers. K statistics for intra- and interobserver variability were 0.90 ( $P < 0.05$ ) and 0.80 ( $P < 0.05$ ), respectively.

The issue of heterogeneity of basal expression of the markers was addressed by studying the expression of Ki67, M30 staining, and MVD in three biopsies from different areas in four different cervical tumors. We found a very low level of variability for Ki67 (percentage of difference  $< 20\%$  in each of the four tumors examined). Similarly, the three biopsies from each M30-negative tumor ( $n = 3$ ) were negative, whereas the three biopsies from the only M30-positive tumor were scored as positive. As far as MVD is concerned, we found, as expected, variable expression of CD31 staining in different areas, and MVD was properly scored in the area of the highest vascular density as assessed by a two-headed microscope ( $\times 200$ ) in four separate random fields.

**Statistical Analysis.** Wilcoxon's signed rank-sum test for paired samples was used to analyze the statistical significance of the differences in the percentages of Ki67-positive cells and MVD between pre and posttreatment biopsies. Fisher's exact test for proportion was used to analyze the distribution of M30-positive samples in pre- versus post-Tam cases. Mann-Whitney test was used to analyze the distribution of Ki67, M30-recognized antigen, and CD31 staining according to ER status and clinicopathological characteristics.

## RESULTS

As already pointed out in "Patients and Methods," the first 16 patients were randomly assigned to receive either 80 or 160 mg/die of Tam for 5 consecutive days. No difference in the clinicopathological characteristics or ER status of patients was found according to Tam dose assignment.

Because doses of 80- and 160-mg Tam induced similar modifications of Ki67, M30, and CD31 expression, the subsequent patients were administered 80 mg/die Tam for 5 days, and all of the data were pooled and examined together.

In the overall series, 11 (45.8%) of 24 primary cervical tumors revealed a positive nuclear immunostaining for ERs (Table 1).

Fig. 1 shows the immunoreaction with the MIB1, M30, and CD31 antibodies in pre and posttreatment cervical tumor biopsies from a Tam-responsive patient (number 5 in Table 1). In tumor samples obtained before Tam treatment, use of MIB1

antibody showed a nuclear immunostaining pattern for Ki67 which appears markedly reduced in biopsies obtained after Tam treatment (Fig. 1, A and B). Specific immunoreaction of M30-recognized antigen was undetectable in pre-Tam biopsies, whereas a cytoplasmic granular staining is evident in post-Tam tumor cells (Fig. 1, C and D). Cytoplasmic anti-CD31 immunoreactivity was present in several endothelial cells in pre-Tam biopsies (E), whereas it was reduced in post-Tam samples (F).

Table 1 also shows Ki67, M30-recognized antigen, and MVD levels in pre- and post-Tam biopsies. There were no correlations among the basal levels of the three markers.

The percentage of Ki67-positive tumor cells in pre-Tam biopsies ranged from 16 to 92% (median: 47.5%) and was significantly higher than the percentage in the corresponding posttreatment biopsies (median: 27%; range, 2–78;  $z = 4.29$ ,  $P = 0.0001$ ; Fig. 2).

No difference in the pretreatment percentage of Ki67-positive cells according to clinicopathological features and ER status was found: the median percentage of Ki67 positivity was 56% (16–92%) in ER-negative versus 42% (22–79%) in ER-positive cases ( $z = 0.84$ ,  $P = 0.4$ ).

Specific cytoplasmic staining for the caspase cleavage product of keratin 18, recognized by M30 monoclonal antibody, was detected in 4 of 24 (16.7%) pre-Tam biopsies and was distributed independently of clinicopathological characteristics and ER status. The percentage of M30 positivity was higher (10 of 24 cases, 41.7%) in post-Tam than in pre-Tam biopsies, although the difference was of borderline statistical significance ( $P = 0.055$ ).

No association between MVD basal levels and clinicopathological characteristics or ER status was found. MVD values in pre-Tam biopsies ranged from 45 to 159 and were significantly higher than corresponding values in posttreatment tissues ( $z = -3.72$ ,  $P = 0.0002$ ; Fig. 2).

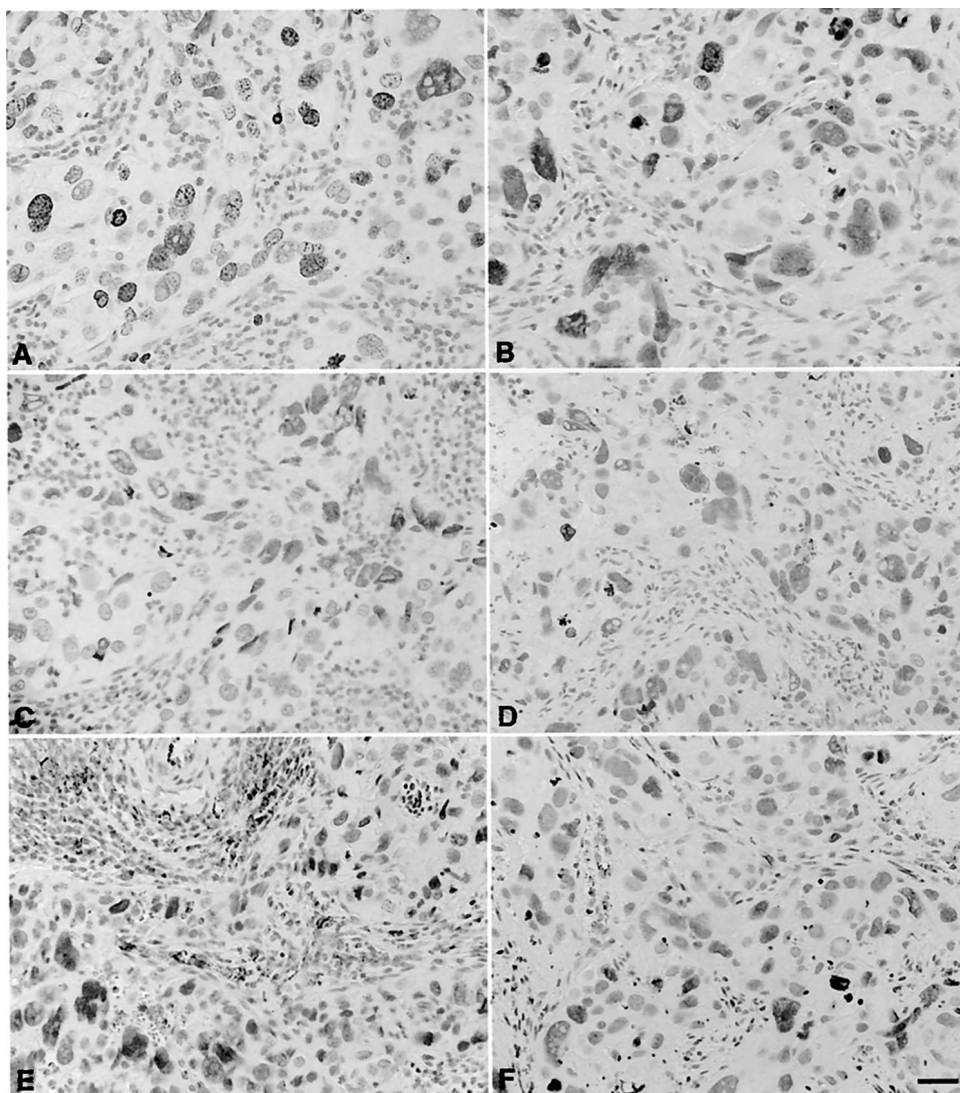
We were then prompted at evaluating if the difference in pre versus posttreatment values of the markers examined could vary according to ER status. As shown in Table 2, the reduction in the percentage of Ki67-positive tumors was significantly ( $z = -3.58$ ,  $P = 0.0003$ ) higher in ER-positive than in ER-negative tumors. On the contrary, there was no difference in Tam-induced reduction of CD31 immunostaining between ER-positive and ER-negative cases ( $z = -0.18$ ,  $P = 0.85$ ; Table 2). Relative to M30 immunostaining, Tam treatment did not induce any significant change of M30 positivity in ER-positive tumors, whereas in the subgroup of ER-negative tumors, it produced a significant ( $P = 0.015$ ) increase in the percentage of M30-positive cells in post-Tam (7 of 13 cases, 53.8%) versus pre-Tam (1 of 13 cases, 7.6%).

## DISCUSSION

We demonstrated that in cervical cancer, a short-term treatment with Tam at doses 4–8-fold higher than those in conventional schemes is associated with relevant modifications of the expression pattern of biological parameters associated with tumor cell proliferation, apoptosis, and neoangiogenesis in cervical cancer. The schedule of Tam administration we chose was designed on the basis of previous observations by Fabian *et al.* (9), who showed that high-loading doses of p.o.-administered



**Fig. 1** Specific reaction with MIB1, M30, and CD31 monoclonal antibodies in primary human cervical carcinoma before (A, C, and E) and after (B, D, and F) Tam treatment. Intense nuclear immunostaining for Ki67 antigen is evident in A, whereas it appears markedly reduced in biopsies obtained after Tam treatment (B). Specific immunoreaction of M30-recognized antigen is undetectable in pre-Tam biopsies (C), whereas a cytoplasmic granular staining is evident in post-Tam tumor cells (D). MVD is detectable in pre-Tam biopsies (E), whereas it is reduced in post-Tam samples (F). Scale bar, 100  $\mu$ m.



Tam allowed to achieve in a few days plasma concentrations of the drug ( $\leq 400$  ng/ml) obtainable only after  $\geq 4$ –8 weeks of conventional doses. Because most of the biological activities exerted by Tam *in vitro* are exerted at micromolar concentrations of the drug, the adoption of a high-loading dose Tam schedule could be more appropriate, if the short-term modulation of biochemical markers critically relevant for cancer cell biology is to be detected. Schedules of high-dose Tam alone or in combination with cytotoxic agents have been explored in solid tumors, demonstrating the feasibility and safety of this schedule and some activity (15–17).

In our series, the pretreatment basal levels of Ki67 and MVD expression were comparable with those reported by others in cervical cancer (12, 14) and were not related to ER status or to clinicopathological characteristics of the patients. Similarly, the low percentage of M30-positive cases in the pretreatment biopsies was consistent with previous studies demonstrating, although by different techniques, a low spontaneous apoptotic index in cervical cancer cells (18, 19). In cervical carcinomas, a

statistically significant decrease in the percentage of Ki67-positive cells together with an increase in the percentage of M30-positive ones were observed after Tam treatment, suggesting that Tam exposure is able both to reduce the proliferative potential and to possibly trigger the apoptotic pathways in this neoplasia. Our results, therefore, suggest that once the schedule administration allows the rapid attainment of biologically active Tam concentrations, Tam is effective both to inhibit the proliferative activity and to induce cancer cell apoptosis.

Our study also provides the first demonstration that Tam exposure can reduce the MVD expression in cervical cancer. Although the inhibition of angiogenesis has been reported to be strictly associated with limited tumor growth because of elevation of the incidence of apoptosis in experimental models (13) and in human tumors (14, 20), we observed that cases showing a reduction in MVD values did not exhibit a parallel increase in M30 positivity, suggesting the effects of Tam on angiogenesis and apoptosis processes may be independent. We also observed a selective activity of Tam to modulate Ki67, M30-recognized

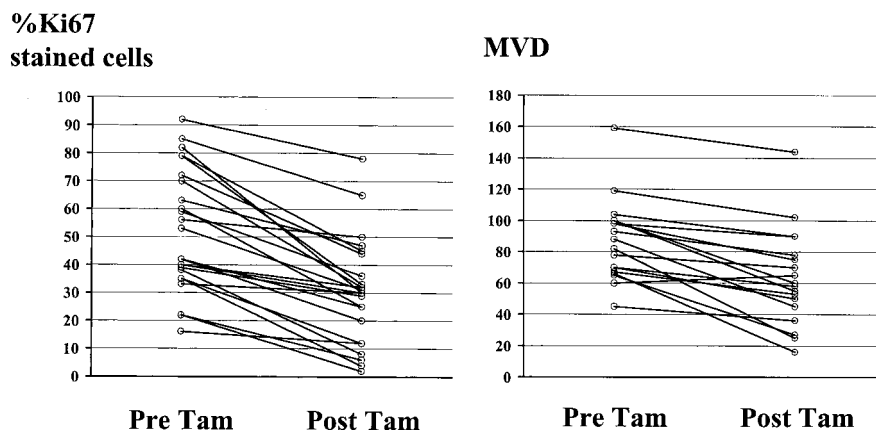


Fig. 2 Expression of Ki67 and MVD in pre-Tam and post-Tam cervical tumor biopsies.

Table 2 Difference (%) in Ki67 and MVD values between pre and posttreatment, according to ER status

| Marker | ER positive<br>(mean $\pm$ SD) | ER negative<br>(mean $\pm$ SD) | <i>P</i> |
|--------|--------------------------------|--------------------------------|----------|
| Ki67   | 61 $\pm$ 18 <sup>a</sup>       | 27 $\pm$ 15                    | 0.0001   |
| MVD    | 28 $\pm$ 24                    | 30 $\pm$ 26                    | 0.9      |

<sup>a</sup> Results were calculated as:  $\frac{\text{pre-Tam values} - \text{post-Tam values}}{\text{pre-Tam values}} \times 100$ .

antigen, and MVD, according to ER status. In particular, a Tam-induced decrease in Ki67 values was higher in ER-positive than in ER-negative tumors. This finding could be explained considering that the cytostatic effects of Tam are mainly mediated by its antiestrogenic activity. Moreover, the observation that the induction of apoptosis almost exclusively occurred in ER-negative tumors suggests that Tam-induced apoptosis is not mediated by ER-mediated mechanisms, probably involving kinase inhibition as reported in *in vitro* systems (21, 22).

We found that Tam-induced reduction in MVD values is independent of ER status of cervical tumor cells. Normal and hemangioma-derived endothelial cells have been shown to express ER and to exhibit a stimulatory response to estradiol in terms of DNA synthesis and expression of vascular endothelial growth factor and its receptors (23, 24). Moreover, in the same models, Tam was shown to counteract the estradiol-promoted activities (23, 24). Therefore, it is conceivable that, independently of ER status of tumor cells, Tam could affect the microvessel structure through the antagonism with endothelial cells ER. On the other hand, it seems unlikely that a direct effect of Tam on tumor cells can somehow influence the finally resulting activity on microvessels, because it has been reported that Tam can mimic the estradiol-induced increase in vascular endothelial growth factor mRNA and protein in ER-positive MCF-7 cells, whereas it has no effect on ER-negative MDA-MB 231 cells (25). In this context, the simultaneous assessment of proangiogenic, as well as antiangiogenic, factors could be helpful in elucidating the complex tumor/stroma/endothelium interactions.

In conclusion, our data, although preliminary, emphasize the need to look at the supposed lack of sensitivity of cervical

cancer to Tam in light of the dose schedule administered and prompted at reevaluating the potential clinical usefulness of the drug in this neoplasm.

In particular, the ability of Tam to reduce MVD expression could be exploited in the context of an adjuvant treatment of cervical cancer patients with high basal levels of MVD (26) and, therefore, at high risk of recurrence. Finally, because Tam has been reported to synergize with cisplatin and possibly radiosensitize tumor cells (27–29), schedules combining high-dose Tam with classical cytotoxic agents and/or radiation could be explored in proper animal models.

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