Tamoxifen-induced Fluorescence as a Marker of Human Breast Tumor Cell Responsiveness to Hormonal Manipulations: Correlation with Progesterone Receptor Content and Ultrastructural Alterations

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ABSTRACT

The fluorescent binding of tamoxifen to eosin is used on Papanicolaou-stained smears as a marker of cell responsiveness to the antiestrogen molecule. Forty-two cases of human breast carcinomas were submitted to tamoxifen treatment between first diagnosis and surgery (4 to 30 days). Tamoxifen-induced fluorescence is observed in 17 of 42 cases (40%). There is a highly significant correlation between progesterone receptor content of the tumor and cellular fluorescence (0.01 > p > 0.001). Ultrastructural changes of such tumors (820 cells observed in 28 treated patients and 840 cells in 32 untreated controls) are observed in 42% of treated cells versus 10% of untreated cells. These ultrastructural alterations can be significantly correlated with cellular fluorescence induced by tamoxifen treatment and with progesterone receptor content of human breast cancers.

These data suggest that a short pretreatment with tamoxifen before surgery can give useful additional information at the biochemical, cytochemical, and ultrastructural levels regarding cell responsiveness to hormonal manipulation.

INTRODUCTION

Predictive tests of therapeutic responsiveness can be of great interest for the clinician dealing with the management of patients with breast cancer. During the last 10 years, biochemical approaches have been advocated, in which ER and PGR content of the tumor are measured (5, 8, 12, 18). The prognostic significance of the absence of such proteins has been emphasized as well (3, 15, 19, 28–30). However, when the proteins are present, there is still a large margin of uncertainty as to the response of such patients to hormonal manipulations (5, 12).

The present study uses the fluorescent binding of tamoxifen to eosin on Papanicolaou-stained smears as a marker of cell responsiveness to this widely used synthetic antiestrogen (2, 21, 22).

Fluorescence of breast tumor cells of patients submitted to a short treatment with tamoxifen before surgery is correlated to the ER and PGR content of the tumor and to therapeutic ultrastructural alterations of the tissue.

MATERIALS AND METHODS

Breast Tumors. Since November 1980, 58 patients with surgical breast cancers (T1 to T3M0) were submitted to tamoxifen treatment from 4 to 30 days before surgery (20 mg daily). The tumor tissue was submitted at time of surgery to electron microscopy, biochemical assays of steroid receptor content, and cytochemical analysis of tamoxifen-induced fluorescence within such treated tumor cells.

Sixteen patients were excluded from this study either because of insufficient tumor tissue for biochemical assays, a high level of plasma progesterone which precluded PGR analysis in tumor tissue, or unsatisfactory smears for cytochemical evaluation of tamoxifen-induced fluorescence. Thus, 42 patients were included in our trial.

Tamoxifen-induced fluorescence was also applied to 11 additional postsurgical patients unresponsive to antiestrogen therapy and who developed local recurrence, contralateral breast tumor, or pleural effusion in spite of a long course of tamoxifen treatment (8 to 16 months).

Cytology. In all cases, an initial cytological examination was performed by fine-needle aspiration before the onset of treatment. Controls after treatment were obtained in 47 cases either by fine-needle aspiration or by biopsies on tissue sections.

Smears were fixed immediately in equal parts of methanol and acetone. Drying before fixation was carefully avoided. The slides were submitted to an hypochromic Papanicolaou staining method (20).

Smears from the original description of Papanicolaou rest upon: (a) the composition of hematoxylin solution which is prepared with potassium alum as mordant (10%) (the stock solution is diluted 1:6 with the potassium alum aqueous solution); (b) differentiation in 0.5% aqueous HCl; (c) avoidance of any alkaline bath; and (d) composition of EA 50 solution (Merck).

Cells were observed comparatively with light and UV microscopy (Leitz microscope with Ploemopak illuminator). Tamoxifen-induced fluorescence detection was obtained with a 450 to 490 nm excitation filter and a 515 nm emission filter.

Biochemical Assays. At the time of surgery, aliquots of tumors were trimmed from fat and stored in liquid nitrogen. Cytosol ER and PGR contents were processed within 3 months by the dextran-coated charcoal assay (31).

Forty-two treated tumors were compared to the same number of untreated controls.

Electron Microscopy. Tissue fragments were doubly fixed with glutaraldehyde and osmium tetroxide. They were embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate. Observations were made with a Philips 300 electron microscope.

Tissue sections of tamoxifen-treated patients were compared to tumor sections of a similar number of untreated patients, and 820 malignant treated cells were compared to 840 cells of malignant untreated controls.

Statistical Analysis. Comparison between the groups was performed by the $\chi^2$ test, and the analysis of mean values was determined by Student’s test. Differences were considered as significant at $p = 0.05$ or less.

1 Supported by the Ligue Nationale Française contre le Cancer, the Fondation pour la Recherche Médicale et le Comité Espoir de l'île.

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: ER, estradiol receptor; PGR, progesterone receptor.

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RESULTS

Tamoxifen-induced Fluorescence. Fluorescence microscopy of tamoxifen-treated cells has been described previously (21, 22) and is summarized here. In control cells before treatment, under the conditions of fixation and staining procedure used, benign or malignant mammary epithelial cells are not fluorescent. The cytoplasm is dark green, while the nucleus appears as a black sphere. Nuclei may sometimes be recognized as faintly fluorescent yellow spherules.

With the fixative used, RBC show a brilliant yellow fluorescence. Pericellular mucous casts and some intracellular mucoid material such as that seen in intracytoplasmic duct-like vacuoles fluoresce in the orange yellow range.

After tamoxifen treatment, fluorescent yellow intracytoplasmic granules are observed scattered within the cytoplasm of epithelial cells. They may be located preferentially in the Golgi zone or surrounding the nucleus. In some cases, nuclei are highly fluorescent. Such observations are more easily done on isolated cells on the smears than on clumps of cells which may overlap.

The intensity and degree of fluorescence vary from cell to cell, suggesting a heterogeneous malignant population. Using this technique, a careful confrontation of light and UV data was obtained (Figs. 1 to 3).

When well-defined yellow fluorescent intracytoplasmic granules, regardless of association with nucleolar fluorescence, are found in more than 25% of the malignant cells, the tumor is considered fluorescence positive.

Fine-needle aspiration controls before the onset of treatment were performed in 46 patients. No fluorescence was observed in malignant epithelial cells.

Intracellular fluorescence has been observed on cytological specimens in 17 of 42 treated patients at the time of surgery (40%).

In 11 cases, with relapse during a long course of treatment, fluorescence was negative.

Biochemical Correlation. A correlation was established with the steroid receptor content of the tumors.

The presence of both cytosol ER and cytosol PGR in 42 control patients without tamoxifen treatment prior to surgery was compared to ER and PGR content in treated patients (Table 1).

As expected in the tamoxifen-treated group, ER was negative at less than 20 fmol/mg cytosol protein in 37 cases compared to 23 control cases. PGR was positive in 10 of 33 ER-negative samples versus 1 of 20 controls. The mean value of PGR was 661 ± 256 (S.E.) fmol/mg protein in 16 treated patients, whereas it was 207 ± 42 fmol/mg protein in 11 control patients (Table 1). The difference is significant.

PGR has been considered as indicative of functional ER and correlated with tamoxifen-induced fluorescence (Table 2).

A good correlation is found between fluorescence and PGR values (0.01 > p > 0.001).

Electron Microscopy. Ultrastructural controls of presurgical tamoxifen-treated breast tumors have allowed visualization of therapeutic damage at a cellular level. These controls were performed on 31 of our treated patients. In 3 cases, tumor cells were too scarce in the samples to allow any reliable evaluation. The 28 treated patients were compared to a similar number of untreated patients.

Morphological cellular changes as regards necrosis or necrosis were obvious in the tamoxifen-treated patients. The earlier alterations were nuclear, consisting of a gradient of morphological events which affected most if not all of the cells of a given area. Clumping of chromatin was the earliest cytological change. The thin layer of chromatin along the nuclear envelope was thickened. This event was followed by the condensation of heterochromatin in compact masses. Nuclei finally appeared smudged, imparting a rather diffuse density to the spaces between the clumps.

The nuclear membrane appeared indented, separated from the cytoplasm by an enlarged perinuclear space (Figs. 4 to 6).

While in some cases cytoplasmic structures were preserved, in others the cytoplasmic events consisted essentially of the occurrence of numerous empty vacuoles of different sizes, partly due to the fragmentation and dilution of endoplasmic reticulum, partly to altered swollen mitochondria which had lost their cristae, and also to lysosomes (Fig. 7).

These cellular alterations were taken into account when they affected most of the cells of the section. Isolated cells surrounded by well-preserved malignant cells were not counted.

A total of 820 malignant epithelial cells has been classified according to cellular alterations and compared with 840 cells in untreated malignant breast tumors (Table 3).

Forty-two % of tamoxifen-treated cells versus 10% of untreated tumor cells displayed a morphology similar to that which is seen in the process of cellular death (Table 3). This observation at an ultrastructural level can be significantly related to antiestrogen therapy.

The ultrastructural alterations observed in the selected group of 28 treated patients have been correlated with tamoxifen-induced fluorescence and with PGR content (Table 4). According to these data, there is a significant correlation between cellular alterations induced by tamoxifen treatment, positive fluorescence, and the PGR content of the tumor.

In this preliminary study, the absence of morphological

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Table 3

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<th>Ultrastructural alterations observed in 28 tamoxifen-treated breast cancers (820 cells) and 32 untreated controls (640 cells)</th>
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<td>Total no. of cells</td>
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a, b vs. c, significantly different.
Numbers in parentheses, percentage.

Table 4

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<th>Ultrastructural alterations (820 cells) related to tamoxifen-induced fluorescence and to PGR content in 28 treated human breast cancers</th>
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PGR

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<td>212 (63)</td>
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<td>125 (37)</td>
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Numbers in parentheses, percentage.

Table 5

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<th>Ultrastructural control of 28 patients as a function of the length of presurgical tamoxifen treatment and tumor PGR content</th>
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<td>Tamoxifen treatment (days)</td>
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Numbers in parentheses, percentage.

DISCUSSION

Several cytochemical techniques have been proposed to trace the estradiol and progesterone content of breast tumor cells at a cellular level (1, 6, 7, 10, 16, 17, 24, 27, 35). These methods have added useful data to biochemical techniques, particularly as to the heterogeneity of the tumor tissue. However, controversy has arisen regarding the specificity of binding proteins thus marked (4, 7, 25, 26).

The present study takes advantage of the binding of tamoxifen to eosin recently demonstrated in vitro (2). The fluorescence of such a complex is used in Papanicolaou-stained smears as a marker of therapeutic responsiveness. Routine fine-needle aspirations of breast tumors, stained with a hypochromic Papanicolaou staining procedure, were used for diagnosis and controls. Four to 30 days of presurgical treatment with tamoxifen (20 mg/}

changes at an ultrastructural level does not seem to be related to the length of treatment before surgery (Table 5). Cellular alterations are obvious after 7 days of tamoxifen and do not increase with the duration of presurgical therapy.

The pharmacokinetics of tamoxifen in treated patients has been thoroughly studied (11, 14). Fabian et al. (9) have shown that in treated patients after a single dose the peak blood level is reached in 6 hr, the half-life varying from 9 to 12 hr (9). After loading doses of 20 mg/sq m daily, steady state values were reached in 3 of 4 patients within 1 week. Individual variations of tamoxifen metabolism as a result of liver or kidney impairment may interfere with the blood level of the molecule (11, 13).

In the present study, pretreatment with tamoxifen from 4 to 30 days resulted in an adequate impregnation of the tumor tissue and a predictive test as to cell response. The procedure described using stained smears enables a careful analysis of the cell morphology and a close control of the fluorescent spots by alternate light and UV observation. An immediate fixation of the smears is essential inasmuch as poorly fixed cells are fluorescent.

The specificity of such a test rests upon the absence of intracellular fluorescence in untreated patients; intracytoplasmic fluorescent granules have been observed in 17 treated patients.

The best predictive value of tamoxifen-induced fluorescence is the clinical course of the disease. One patient developed a metastatic pleural effusion; 10 patients who had local recurrence of the disease under tamoxifen treatment showed no tamoxifen-induced fluorescence.

These observations have been correlated with steroid receptor content of tamoxifen-treated tumors. Namer et al. (23) and Waseda et al. (36) have demonstrated a decrease or absence of ER content in tumor tissue of tamoxifen-treated patients to be associated with an increase in PGR content. Our observations support their findings. Nine of 42 treated patients had ER content in their tumor tissue versus 22 of 42 in the control group. The mean PGR value rose from 207 ± 42 fmol/mg protein in untreated patients to 661 ± 256 in treated ones. Such increased PGR values after tamoxifen treatment have been considered as a predictive test of functional ERs (23) and are correlated in this study with tamoxifen-induced fluorescence.

A significant correlation between tamoxifen-induced fluorescence and PGR content of the tumor is observed in 31 of 42 patients.

This result may be compared with several recent studies which have shown that clinical response to hormonal manipulations occurs in approximately 75% of the patients with both estradiol- and progesterone-positive results (12).

Ultrastructural study of treated tumors has permitted a better understanding of such observations. A statistically significant correlation has been obtained between tamoxifen-induced fluorescence and the morphological events occurring during the process of cellular death. They have been thoroughly described in hepatic cells by Trump et al. (32–34).

Only 10% of untreated malignant cells and 20% of treated nonfluorescent malignant cells show morphological alterations of necrosis or necrosis at an ultrastructural level, while 69% of fluorescent cells are affected.

No specific intracytoplasmic structures may be ascertained as the origin of intracytoplasmic fluorescent granules observed by UV microscopy, although swollen degenerative vacuoles, lysosomes, or secretory granules may be suggested. Untreated necrotic cells with pyknotic nuclei do not fluoresce.
Whatever the basis of such fluorescence, it correlates strongly with tamoxifen-induced cell necrosis and thus has a predictive value as to therapeutic response.

Thus, pretreatment of breast tumors with tamoxifen during the time between the first examination of the patient and surgery (4 to 30 days) gives additional information as to tumor cell behavior when submitted to hormonal manipulations.

Tamoxifen-induced fluorescence, PGR content of the tumor, and ultrastructural examination correlate well in the prediction of cell responsiveness to hormonal therapy.

ACKNOWLEDGMENTS

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REFERENCES

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Fig. 1. Tamoxifen-treated breast tumor for 8 days before surgery. PGR-negative Papanicolaou-stained imprint. Fluorescence is negative. Nucleoli can be recognized. x 1,250.

Fig. 2. Tamoxifen-treated breast tumor for 23 days before surgery. PGR, 670 fmol/mg protein. Papanicolaou-stained imprint. Intracytoplasmic fluorescent granules. Nucleoli are not seen. x 1,250.

Fig. 3. Tamoxifen-treated breast tumor for 30 days before surgery. PGR, 216 fmol/mg protein. Papanicolaou-stained imprint. Intracytoplasmic fluorescent granules associated with fluorescent nucleoli. x 1,250.
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Fig. 4. Untreated malignant breast tumor. × 8,500.

Fig. 5. Tamoxifen-treated malignant breast tumor for 8 days before surgery. Tamoxifen-induced fluorescence is negative. PGR = 0. × 8,300.
Fig. 6. Tamoxifen-treated malignant breast tumor for 10 days before surgery. Tamoxifen-induced fluorescence is positive. PGR = 0. × 9,500.

Fig. 7. Tamoxifen-treated malignant breast tumor for 3 days before surgery. Tamoxifen-induced fluorescence is positive. PGR, 570 fmol/mg protein. The numerous secretory granules and lysosomes may be responsible for the fluorescence. × 13,400.
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