Effects of the antioestrogen tamoxifen on the cell cycle kinetics of the human breast cancer cell line, MCF-7

A.E. Lykkesfeldt¹, J.K. Larsen², I.J. Christensen² & P. Briand¹

¹The Fibiger Laboratory, Ndr. Frihavnsgrde 70, DK-2100 Copenhagen, ²The Finsen Laboratory, The Finsen Institute, Strandboulevarden 49, DK-2100 Copenhagen, Denmark.

Summary The effect of 10^{-6} M tamoxifen on MCF-7 cells adapted to growth in 0.5% foetal calf serum has been studied. The growth inhibitory effect of this tamoxifen concentration was abolished by simultaneous addition of 10^{-8} M oestradiol, indicating that tamoxifen may exert its effect via binding to the oestrogen receptor. Flow cytometric cell cycle analysis of tamoxifen-treated cultures showed an increase in the proportion of cells in the G_1 phase of the cell cycle. By exposing the cells to BUdR before flow cytometry the growth fraction was determined and found to be dramatically decreased in tamoxifen-treated cultures. Cells were not only arrested in the G_1 phase but also in the G_2 phase of the cell cycle. A few colonies of MCF-7 cells were resistant to 10 days treatment with 10^{-6} M tamoxifen.

The antioestrogen tamoxifen is widely used for treatment of patients with breast cancer. In general, patients with oestrogen receptor-positive tumours have a response rate of 50%, whereas patients with oestrogen receptor-negative tumours have a response rate of only 10% (Mouridsen *et al.*, 1978; Rose *et al.*, 1980). The effect of tamoxifen has therefore been attributed to the presence of oestrogen receptors.

It is believed that tamoxifen acts by interfering with oestrogen receptor function at some level, though a specific antioestrogen receptor has been reported to exist in human breast cancer (Sutherland & Murphy, 1980), human endometrium (Sutherland *et al.*, 1980), rat liver (Sutherland *et al.*, 1980; Winneker & Clark, 1983), rat uterus (Winneker & Clark, 1983) and in human breast cancer cell lines (Miller & Katzenellenbogen, 1983). The antioestrogen receptor content is unrelated to the oestrogen receptor content, and the role of the antioestrogen receptor is still unknown. Miller & Katzenellenbogen (1983) suggest that the interaction with the oestrogen receptor and not with the antioestrogen receptor is most likely to be the mechanism underlying the growth inhibitory effects of antioestrogens. Rochefort *et al.* (1981) have found that tamoxifen binds specifically to the oestrogen receptor and not to other saturable proteins. However, they measured receptors in the cytosol and the nuclear KCl extract whereas the specific antioestrogen binding sites are said to be located in the microsome preparation. They conclude that tamoxifen blocks the growth of

human breast cancer by antagonizing the stimulatory effect of oestrogens. This mechanism cannot explain the growth inhibitory effect of tamoxifen on our MCF-7 cells since these cells do not respond to oestradiol (Briand & Lykkesfeldt, 1984). Depending on the concentration, tamoxifen inhibition was either oestrogen reversible or oestrogen irreversible. In the present work we have used 10^{-6} M tamoxifen, which is the highest concentration of tamoxifen, the effect of which can be abolished by oestradiol.

Sutherland and Taylor (1981) and Greene *et al.* (1981) have studied the effect of cytotoxic concentrations of tamoxifen on the cell cycle kinetics of MCF-7 and found an accumulation of cells in the G_1 phase. Two recent reports describe the use of lower concentrations of tamoxifen, where the effect can be reversed by oestradiol (Sutherland *et al.*, 1983; Osborne *et al.*, 1983). The tamoxifen treatment results in an accumulation of cells in the G_1 phase of the cell cycle and Sutherland *et al.* (1983) conclude that tamoxifen is a cell cycle phase-specific growth inhibitory agent. In this study we have investigated whether the accumulation of cells with a G_1 amount of DNA can be ascribed to non cycling cells, and we have also tested whether tamoxifen blocks cells in phases of the cell cycle other than the G_1 phase.

Materials and methods

Cell culture

MCF-7 cells were kindly supplied from the Human Cell Culture Bank, Mason Research Institute, Rockville, Md., USA. They were propagated in plastic T flasks (Nunc, Denmark) in Dulbecco's

Correspondence: A.E. Lykkesfeldt

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MEM+Ham's F12 (1:1) supplemented with glutamine 2mM, insulin 6 ng ml⁻¹ and 0.5% heat inactivated foetal bovine serum. The cells had been adapted to growth at this serum concentration since passage 247 (Briand and Lykkesfeldt, 1984); cells from passage 336 were used in this study. In the experiments growth medium was replaced by the test medium one day after seeding: this consisted of growth medium supplemented with hydrocortisone (10⁻⁸ M) and prolactin (1 µg ml⁻¹). The flasks were divided into two groups. A tamoxifen group, which received 10⁻⁶ M tamoxifen dissolved in ethanol (final conc. 0.1%), and a control group to which ethanol was added at a final concentration of 0.1%. The medium was renewed daily during the experiment.

Growth curves

Three cultures were trypsinized and the cells counted in a Bürker-Türk chamber one day after seeding. These counts represent the cell number at day 0. Three cultures from each group were counted on designated days of the experiment.

Preparation of nuclei for flow cytometry

The cells were removed from the flasks with 1 mM EDTA in a buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) and washed twice with this buffer. They were lysed with 1% Triton X-100 in the buffer supplemented with 2 mM EGTA, 1.5 mM MgCl₂, pH 7.2 and citric acid added to a final concentration of 2% (Miller, 1979). Nuclei were pelleted by centrifugation at 800 g for 10 min, resuspended in the buffer with 2 mM EGTA, 1.5 mM MgCl₂ and 0.2% Triton X-100, pH 7.2 and stained with propidium iodide or mithramycin.

Flow cytometry

For conventional flow cytometric cell cycle analysis the samples were stained by addition of propidium iodide (50 µg ml⁻¹, Sigma) and RNase (0.1 mg ml⁻¹, Sigma type 1A) for at least 30 min. For determination of the growth fraction after incubation with BUdR the samples were stained by addition of mithramycin (20 µg ml⁻¹, Mithracin[®], Pfizer) and MgCl₂ (25 mM) (Swartzendruber, 1977). As internal DNA references stained chicken and trout erythrocyte nuclei were added to each sample. Batches of chicken and trout blood cells were kept frozen in buffer (250 mM sucrose, 40 mM sodium citrate, 5% dimethylsulfoxide, pH 7.6). Aliquots were thawed and lysis buffer added (10 mM Tris, 1 mM EGTA, 1% Nonidet P 40) prior to staining (Vindeløv *et al.*, 1982c). All stained samples were filtered through 30 µm nylon mesh before flow

cytometry. The flow cytometer used was a Becton Dickinson FACS IV Cell Sorter with a Spectra Physics 5 W argon laser nm, 488 mW for propidium iodide; 457 nm, 100 mW for mithramycin).

Determination of growth fraction

5-Bromodeoxyuridine, BUdR (final concentration 0.02 mM) was added to two T-25 flasks in each group at day 4. The cells were harvested 48 h later and nuclei prepared for flow cytometry. The growth of the cultures is unaffected by the presence of BUdR for at least two generation times. Cells which have not incorporated BUdR after administration for a generation time, may either be G₀ cells or cells with an unusually long cell cycle time. In this experiment cells were harvested after a period of approximately two generation times in the control culture. Cells with a G₁ amount of normal DNA are defined as G₀ cells but may include cells with a G₁ transit time longer than two generation times. The proportion of G₀ cells may be underestimated, because it is calculated as the proportion of cells with a G₁ amount of normal DNA out of the total number of cells which increases by cell division. Underestimation of the G₀ fraction caused by detachment of non cycling cells can be excluded since flow cytometry has shown that the non cycling cells are still present in the cultures after 96 h in BUdR medium.

Statistics

The phase fractions of the DNA histograms were estimated by fitting the observed distributions of fluorescence (deconvolution) by maximum likelihood using a statistical model described earlier (Christensen *et al.*, 1978; Vindeløv *et al.*, 1982b). The proportion of non cycling cells (G₀) in the flow cytometric histograms of DNA from BUdR treated cultures was estimated in a similar manner using mixtures of Gaussians to fit the additional peaks (Vindeløv *et al.*, 1982a).

Results

The growth curves of control and tamoxifen treated cultures are shown in Figure 1. The control cultures grow exponentially for the first 6 days with a doubling time of 20 h. Thereafter the doubling time increases gradually. The cell number increases in the tamoxifen treated cultures for the first 5 days, but with an increased doubling time compared to the control culture. After day 6 the cells detach from the flasks and at day 7 a great variation in the cell number is seen in the different flasks. By day 10 only a few flasks contained colonies of growing cells.

Figure 1 Growth curves for control cultures of MCF-7 cells (O) and cultures treated with 10^{-6} M tamoxifen (●). The lines are drawn between median cell number of 3 flasks. At day 10 two flasks from the tamoxifen treated cultures were empty and few cells were present in the third flask.

At days 3, 4, 5, 6, 7 and 10, cells were harvested from control and tamoxifen treated cultures and nuclei prepared and stained for flow cytometric analysis as described in **Materials and methods**. Figure 2 shows the DNA distribution in control and tamoxifen treated cultures at day 4. From such histograms the distribution of cells in the different phases of the cell cycle was calculated as described above. The number of cells analyzed in each sample was $>10,000$, apart from the tamoxifen culture at day 6, where only 5200 cells were analyzed. Even with the low number of cells, the standard error due to deconvolution of the DNA histograms has been shown to be $<2\%$ of the total number of cells (Vindeløv *et al.*, 1982b). The results of these analyses are shown in Figures 3A, 3B and 3C. The effect of tamoxifen on the proportion of cells with a G_1 amount of DNA is shown in Figure 3A and it may be seen that already after 3 days of treatment

Figure 2 DNA histograms from (a) control culture of MCF-7 cells and (b) tamoxifen (10^{-6} M) treated culture at day 4. The DNA was stained with propidium iodide and measured by flow cytometry with chicken and trout erythrocyte nuclei as internal references.

a greater proportion of the cells in the tamoxifen treated cultures had a G_1 amount of DNA. This difference in the proportion of cells with a G_1 amount of DNA was evident for the first 7 days but at day 10 no difference was observed. In Figure 3B the proportion of cells in the S phase is shown as a function of the treatment time and here a lower proportion of cells was seen in the cultures

Figure 3 Effect of 10^{-6} M tamoxifen on the cell cycle kinetic parameters of MCF-7 cells. The proportion of cells in the different phases of the cell cycle was calculated from DNA histograms as presented in Figure 2. Control cultures (○), tamoxifen cultures (●).

treated for 3–7 days with tamoxifen. At day 10 no difference was observed. Figure 3C shows the proportion of cells in G_2+M and after 4–7 days treatment fewer cells in the tamoxifen treated cultures were in G_2+M than in the control culture.

At day 10 no significant difference was observed between the proportion of cells in G_2+M in tamoxifen treated and control cultures.

The proportion of cells participating in cell cycle events was measured by the BUdR-technique. Figure 4 shows the DNA distribution in cells from

Figure 4 Flow cytometric mithramycin fluorescence histograms from (a) control and (b) tamoxifen cultures of MCF-7 cells. The cells were harvested after treatment from days 4–6 (48 h) with 0.02 mM BUdR. G_0 refers to the position for G_1 amount of normal DNA, G_1^* to G_1 amount of DNA with BUdR in one strand, G_1^{**} to G_1 amount of DNA with BUdR in both strands. Similar symbols are used for G_2 amounts of DNA.

a control culture and a tamoxifen treated culture exposed 48 h to BUdR in the period from days 4–6 of the experiment. During this period the median cell number in the control cultures increased from 3.2×10^5 to 12.0×10^5 . The peak to the left in the histogram, representing cells with a G_1 amount of DNA without BUdR, is indicated in the figure as G_0 and amounts to 2% of the total cell number. The next peak represents cells with a G_1 amount of DNA containing BUdR in one strand. In the tamoxifen cultures the median cell number during the time of exposure to BUdR increased from 3.6×10^4 to 8.5×10^4 . The peak representing cells with a G_1 amount of DNA without incorporated BUdR is indicated in the figure as G_0 and represents 43% of the total cell number. A peak with a G_2 amount of DNA without BUdR is present in the histogram of tamoxifen treated cells, but not in the control histogram.

Discussion

We have demonstrated that 10^{-6} M tamoxifen inhibits growth *in vitro* of the human breast cancer cell line, MCF-7 by reducing the growth fraction. Furthermore, we have shown that cells were arrested not only in the G_1 phase of the cell cycle as previously described (Osborne *et al.*, 1983; Sutherland *et al.*, 1983), but also in the G_2 phase. The inhibitory effect of 10^{-6} M tamoxifen can be abolished by simultaneous addition of 10^{-8} M oestradiol and no effects on cell cycle kinetics are observed under these conditions. Lower concentrations of tamoxifen also inhibit growth, though to a smaller extent. In agreement with others (Horwitz & McGuire, 1978) we have also found that tamoxifen binds to the oestrogen receptor and the oestrogen receptor-tamoxifen complex translocates to the nucleus. The oestrogen receptor-negative cell line, HBL-100 originating from cells in normal human milk is completely insensitive to tamoxifen concentrations up to 2×10^{-6} M (Briand & Lykkesfeldt, 1984) indicating that the oestrogen reversible effect of tamoxifen in MCF-7 cells is mediated via the oestrogen receptor mechanism.

The inhibition of cell proliferation was mainly due to an accumulation of cells with a G_1 amount of DNA. This accumulation of cells in the G_1 phase of the cell cycle occurs already after 3 days of treatment with tamoxifen. Prolonged treatment leads to further increase in the G_1 population. However, the DNA distribution after 10 days of treatment is very similar to the DNA distribution in the control culture. This reflects the very dramatic changes which occur from days 7–10, *viz* that the majority of the cells in the cultures have died and disappeared and only a few culture flasks contained

colonies of live cells. The DNA measurements were done on cells from these selected flasks with viable cells and their DNA distribution indicates that they may be proliferating. This is supported by our observation of the presence of mitoses in the viable colonies. We conclude that the majority of the MCF-7 cells are tamoxifen sensitive and the cell kinetic observations from days 3–7 reflect the effect of tamoxifen on sensitive cells. A minor fraction of the MCF-7 cells are apparently tamoxifen resistant since they still proliferate after 10 days of treatment. We have now treated MCF-7 cultures with 10^{-6} M tamoxifen for up to one month and isolated growing colonies from such cultures. These MCF-7 cell sublines are now under investigation and they can be subcultured several times in the presence of 10^{-6} M tamoxifen, indicating that they are tamoxifen resistant. Usually, MCF-7 cells cannot be subcultured in tamoxifen medium after one week of treatment with 10^{-6} M tamoxifen. Resistance to tamoxifen treatment has been shown in the cell kinetic work of Osborne *et al.* (1983), and two human breast cancer cell sublines resistant to tamoxifen have been isolated and characterized (Nawata *et al.*, 1981, Horwitz *et al.*, 1982).

Sutherland *et al.* (1983) describe tamoxifen as a cell cycle phase specific growth inhibitory agent, which blocks cells in the G_0/G_1 phase of the cell cycle, and Osborne *et al.* (1983) suggest that tamoxifen inhibits cell proliferation by invoking a transition delay or block in the early-to-mid- G_1 phase of the cell cycle. By the BUdR-mithramycin method applied in this study it is possible to identify the cells which do not participate in cell cycle events. We find that tamoxifen treatment reduces the growth fraction considerably and both cells with a G_1 amount of DNA and cells with a G_2 amount of DNA are growth arrested. The BUdR-mithramycin technique does not allow a determination of cells arrested in the S phase.

This is the first study to demonstrate that both cells with a G_1 amount of DNA and a G_2 amount of DNA are growth arrested after tamoxifen treatment. By conventional flow cytometry an accumulation of cells in one compartment may indicate a single block in the cell cycle. However, identification of more than one block in the cell cycle is more difficult, since an accumulation in one compartment may be hidden by a decreased entry of cells into the compartment due to a block in another compartment. The BUdR-mithramycin technique is therefore superior to conventional flow cytometry because both non cycling cells with a G_1 amount of DNA and a G_2 amount of DNA can be identified.

If the MCF-7 model is representative of human breast cancer, the results of these experiments may give valuable information to clinicians for the

treatment of this disease. Tamoxifen inhibits growth by reducing the growth fraction and non cycling cells may survive in the tumour for a rather long period of time before they die, indicating the need for long term treatment with tamoxifen. The finding of tamoxifen resistant cells suggests that combined endocrine and cytotoxic treatment may give better overall survival than tamoxifen alone. Knowledge about the effect of tamoxifen on cell cycle kinetic parameters may be useful for the

choice and scheduling of combined treatment regimes.

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