Role of proliferation and apoptosis in net growth rates of human breast cancer cells (MCF-7) treated with oestradiol and/or tamoxifen

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Abstract. Studies on growth regulation *in vitro* to a large extent rely on comparison of growth curves. However, these do not discriminate between the relative contributions of the mitotic rate and the apoptotic rate to the net growth rate. In the present study, differential effects of 17β -oestradiol (E_2 , 10^{-8} M) and/or tamoxifen (TAM, 10^{-6} M) on proliferation and apoptosis have been examined and related to growth curves of a subline of the human breast cancer cell line MCF-7 adapted to grow at low serum concentrations. Counting of cells and scoring of labelling and apoptotic indices were performed at the start of the experiment and 3, 6 and 9 days after changing the experimental media. The results demonstrate that apoptosis in this subline is constitutively expressed, that E_2 protects (at least partly) against apoptosis and stimulates proliferation, resulting in an increased (net) growth rate and final cell pool size, and that TAM has a weak cytostatic effect and stimulates apoptosis strongly, resulting in a decreased (net) growth rate and final cell pool size. When E_2 and TAM are added simultaneously to the medium, the cytotoxic effect of TAM is partly counterbalanced by the protective role of E_2 , resulting in a reduced apoptotic rate that, however, is at a higher level than in cultures grown with E_2 only. As the cytostatic role of TAM is partly abolished by E_2 , the combined effect of E_2 and TAM results in a final (net) growth rate and cell pool size intermediary to cells grown with E_2 or TAM alone.

INTRODUCTION

Breast cancer is one of the most frequent cancers among women. Hormonal risk factors for breast cancer include oestrogen (Pike *et al.* 1993), and the anti-oestrogen tamoxifen is used in endocrine therapy for breast cancer patients (reviewed by Mouridsen *et al.* 1978, Santen *et al.* 1990, Lykkesfeldt 1997). The effects of oestrogen and tamoxifen on the growth of oestrogen-dependent breast cancer cells *in vitro* have therefore been studied intensively. The results of early studies were often conflicting, probably owing to the use of different cell lines and different growth conditions, e.g. lack of factors acting synergistically with oestradiol and the presence in the medium of the pH indicator phenol red, which has been found to have an oestrogenic activity of its own (Berthois, Katzenellenbogen & Katzenellenbogen 1986,

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Lykkesfeldt 1997). Furthermore, conclusions were to a large extent based upon growth curves that, however, show growth of breast cancer cells in terms of net growth and do not discriminate between mitotic rate and rate of cell loss. It took many years before the concept of cell death by apoptosis, introduced by Kerr, Wyllie & Currie (1973) as a homeostatic mechanism complementary to controlled cell division, was generally accepted by the scientific community as a 'universal mechanism with wide-ranging implications in tissue kinetics'. Since proliferation and apoptosis may be controlled differently, early studies focusing on proliferation without appreciating apoptosis may have blurred an understanding of the growth control of breast cancer cells. Attempts at analysing for breast cancer cells, in the same experiment, changes in the cell pool size, cell proliferation and cell loss by apoptosis as a function of time are few and limited to growth of inoculated human mammary adenocarcinoma cells (MCF-7) into ovariectomized female nude mice (Brünner *et al.* 1989, Kyprianou *et al.* 1991, Wärris *et al.* 1993).

It has been shown that newborn calf serum at high concentrations inhibits the growth of the human breast cancer cell line MCF-7 and that this inhibition appears to be mediated by serum albumin (Lykkesfeldt *et al.* 1986a, Laursen, Briand & Lykkesfeldt 1990). A pilot experiment was implemented in order to compare the growth inhibition by tamoxifen with that of 10% newborn calf serum and particularly whether apoptosis, which has been shown to be involved in tamoxifen-induced growth inhibition (Perry, Kang & Greaves 1995, Chen *et al.* 1996), is also involved in newborn calf serum-induced growth inhibition. The results prompted a study of concomitant changes of cell pool size, proliferation and apoptosis, as a function of time. Evidence is presented that in MCF-7 cells adapted to grow at low serum concentrations (MCF-7, subline 0.5, (Briand & Lykkesfeldt 1984)), apoptosis is constitutively expressed and that oestrogen stimulates proliferation and plays a protective role against constitutive apoptosis, resulting in an increased net growth rate and final cell pool size, while tamoxifen has a weak cytostatic effect and strongly stimulates apoptosis, resulting in a decreased net growth rate and final cell pool size. The results are briefly discussed in relation to the potential selective modulatory role on proliferation or apoptosis of differential secretion of oestrogen-stimulated growth regulators.

MATERIALS AND METHODS

Cells

MCF-7 cells, subline 0.5 (Briand & Lykkesfeldt 1984) were kindly provided by Dr Anne Lykkesfeldt, Danish Cancer Society, Institute of Cancer Biology, Department of Tumor Endocrinology. Cells were propagated in standard medium in NUNC T25 plastic flasks (Life Technologies, Roskilde, Denmark). Cells were cultivated at 37°C in a 5% CO₂ atmosphere.

Media

The following media were used:

- A. Standard medium (SM): phenol red-free Dulbecco's MEM + Ham's F12 (1 : 1) supplemented with glutamine (2 mM), insulin (6 ng/ml) and heat inactivated fetal calf serum (1%).
- B. SM supplemented with oestradiol (E₂, 10⁻⁸ M).
- C. SM supplemented with tamoxifen (TAM, 10⁻⁶ M).
- D. SM supplemented with TAM (10⁻⁶ M) and E₂ (10⁻⁸ M).
- E. SM supplemented with newborn calf serum (NCS, 10%).
- F. SM supplemented with 10% NCS and E₂ (10⁻⁸ M).

Insulin, E₂ (17 β -oestradiol) and TAM were purchased from Sigma (St Louis, MO, USA).

The doses of E₂ and TAM were chosen because 10⁻⁸ M E₂ in previous experiments has been shown to weakly stimulate the growth of MCF-7 subline 0.5 and counteract the growth inhibitory effect of 10⁻⁶ M TAM, and because TAM in higher concentrations has been shown to have an unspecific cytotoxic effect (Briand & Lykkesfeldt 1984, Lykkesfeldt, Laursen & Briand 1989, Lykkesfeldt 1997).

Pilot experiment

MCF-7 cells (5.1×10^4) were seeded in each of six NUNC Lab-Tech flaskettes in standard medium (A), corresponding to 5×10^3 cells cm⁻². After 24 h, a total of at least 1000 cells were counted in an inverted microscope equipped with a calibrated net ocular, in at least 5 sites of each flask, and the numbers of cells per cm² were calculated. Subsequently the medium was changed to one of the six media A–F and the cells cultivated for another 7 days with a change of medium every second day. At the end of this period, cells were counted again and the average net growth rate during the period calculated (cells cm⁻² day⁻¹). Then the chambers were removed and the slides fixed in 4% phosphate-buffered paraformaldehyde and subsequently stained by the terminal deoxynucleotidyl transferase nick end-labelling (TUNEL) method (Oncor ApopTag *in situ* apoptosis detection kit (AH Diagnostics, Aarhus, Denmark), performed according to the manufacturer's instructions). Substituting distilled water for terminal deoxynucleotidyl transferase (TdT) served as a negative control. Slides with post-lactational regressing mammary gland (Oncor) served as positive control. The apoptotic index was expressed as the percentage of cells stained by the ApopTag kit. For each treatment, a total of at least 1000 cells were scored.

Main experiment

Protocol

In 24 NUNC Lab-Tech 4-chamber slides and in 12 NUNC flaskettes, MCF-7 cells were inoculated with a density of 5×10^3 cells cm⁻². Twenty-four hours later (at day 0 (control)), six slides were removed for analyses (see below). Then the medium in each of three flaskettes and in each of the remaining 18 4-chamber slides was changed to medium A, B, C or D, respectively, and these media were changed every second day during the rest of the experiment. Cells in the flaskettes were counted as described for the pilot experiment at each of the days 0, 3, 6, and 9. In addition, at each of these days, six slides were removed for doublet analyses of morphology, incidence of apoptosis (apoptotic index), and IdUrd incorporation during the S phase (labelling index) (see below).

Staining procedures

Morphology

Prior to staining, the chambers of two 4-chamber slides were removed, the slides washed in phosphate-buffered saline (PBS), fixed in Carnoy's fixative for 10 min and subsequently stained with Gomori's haematoxylin for 4 min

Apoptosis

Prior to staining, the chambers of two 4-chamber slides were removed and the slides washed in PBS. Following 10 min fixation in 4% phosphate-buffered paraformaldehyde, the slides were post-fixed in ethanol/acetic acid (2 : 1, -20°C, 7 min) and subsequently stained by the TUNEL method (Oncor ApopTag *in situ* apoptosis detection kit).

Substituting distilled water for TdT served as a negative control. Slides with post-lactational regressing mammary gland (Oncor) served as a positive control.

IdUrd

The medium in each chamber of two 4-chamber slides was replaced by the same medium, but containing 1 mM 5-iodo-2'-deoxyuridine (IdUrd) for 15 min and subsequently treated by a modified method of Aten *et al.* (1992). The chambers of the slides were removed, the slides washed in PBS, fixed in 70% ethanol for 15 min, and washed in PBS. Subsequently, DNA denaturation by formamide-citrate at 70°C for 1 h was followed by two rinses in 0.1 M borate buffer (pH 8.1) and PBS. Following pre-incubation in 20% BSA, slides were incubated in mouse anti-BrdU primary antibody (Becton-Dickinson (Brøndby, Denmark), 1 : 100 in PBS with 0.05% Tween-20 (PBT)). The second pre-incubation was in 20% normal rabbit serum, followed by incubation in horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG secondary antibody (DAKO (Copenhagen, Denmark), 1 : 20 in PBT). Pierce's metal-enhanced DAB was used as the chromogenic substrate. Substituting normal mouse serum for primary antibody served as a negative control.

Countings

At least 1000 cells were scored per preparation in a Nikon Microphot FXA equipped with a calibrated net ocular. The labelling index (LI) was expressed as number of labelled cells per 100 cells. The apoptotic index (AI) was expressed as number of cells stained by the TUNEL method per 100 cells. The cell pool size was expressed as number of cells per cm^2 .

RESULTS

Pilot experiment

Net growth rates

The final cell pool size and calculated average net growth rates (K_g) under the six growth conditions are shown in Table 1. The final cell pool size and the derived K_g increased under the influence of E_2 and decreased under the influence of TAM. The TAM-induced decrease in K_g was restored to normal levels by the concomitant presence of E_2 in the medium. Addition of 10% NCS to the standard medium increased the K_g and final cell pool size considerably, which was further increased by the addition of E_2 to the medium.

Table 1. Pilot experiment. Average net growth rate (K_g , $\text{cells cm}^{-2} \text{ day}^{-1}$), cell pool size (cells cm^{-2}) and apoptotic index (AI, %) of MCF-7 cells after 7 days under various growth conditions

	K_g^*	Cell pool†	AI
A. Standard medium (SM)	1.6×10^3	1.6×10^4	2.4
B. SM + E_2 (10^{-8} M)	3.7×10^3	3.1×10^4	1.7
C. SM + TAM (10^{-6} M)	0.9×10^3	1.1×10^4	3.8
D. SM + TAM (10^{-6} M) + E_2 (10^{-8} M)	1.6×10^3	1.4×10^4	2.9
E. SM + NCS (10%)	4.1×10^3	3.2×10^4	0.8
F. SM + 10% NCS + E_2 (10^{-8} M)	5.0×10^3	3.8×10^4	0.5

*Calculated as described in *Material and methods*.

†Initial cell pool size prior to changes of medium was $4.8 \times 10^3 \text{ cells cm}^{-2}$.

Figure 1. Apoptotic cells were found under all experimental conditions. ApopTag staining. Phase interference microscopy. Examples: (a) standard medium (SM); (b) SM + oestradiol (E_2 , $10^{-8}M$); (c) SM + E_2 + tamoxifen (TAM, $10^{-6}M$); (d) SM + 10% NCS + E_2 . Magnification $\times 312$.

Apoptosis as revealed by the TUNEL *in situ* staining method was, by the end of the experiment, seen under all growth conditions (Figure 1) although with different frequencies (Table 1). The AI under the six growth conditions was SM + TAM > SM + TAM + E_2 > SM > SM + E_2 > SM + NCS > SM + NCS + E_2 (Figure 2). There was a negative linear correlation between the AI and the final cell pool size (Figure 2) and consequently also between the AI and the derived average K_g (described by $y = -0.000725x + 4.06$, correlation

Figure 2. Negative linear correlation between cell pool size and apoptotic index 7 days after change to experimental media (pilot experiment). □, SM + TAM; △, SM; ○, SM + 10% NCS; closed symbols, + E₂. The curve is the regression line described by $y = -1.07 \times 10^{-4}x + 4.56$, $r = 0.97$.

coefficient $r = -0.97$). The staining reaction was similar to that of apoptotic cells in positive controls (regressing mammary gland). No staining reaction was seen in negative controls.

Main experiment

Changes in the cell pool sizes during exposure to the experimental media are shown as growth curves (Figure 3). With E₂ added to the standard medium (SM), the net growth rate (K_g) and the final cell number (at day 9) was slightly higher than under standard conditions. With TAM present in the SM, K_g was strongly inhibited and the cell numbers did not exceed 2–3 times that of starting controls. Addition of E₂ to SM + TAM increased the K_g compared to medium with TAM alone, but did not restore the K_g to that seen in SM, and the final cell pool size only increased to about 10-times that of controls compared to an 80-times increase in the pool size of cells grown in SM.

Changes in the labelling index (LI) during exposure to the experimental media are shown in Figure 4. In all media, there was a decrease in LI with time. A slower initial rate of decrease and higher LI were noted in cultures grown in the presence of E₂ (Figure 4b,d) than in cultures grown without (Figure 4a,c). Compared to cells grown in SM alone (Figure 4a), the LI was, at all days, higher for cells grown in the presence of E₂ (Figure 4b,d) and lower for cells grown in the presence of TAM (Figure 4c). There was no significant difference between LI at days 3, 6 and 9 of cultures grown in SM + E₂ and that of cultures grown in SM + TAM + E₂. No staining reaction was seen in negative controls.

Changes in the AI during exposure to the experimental media are shown in Figure 5. In the SM, there was a linear decrease of the AI with time, while the AI in SM supplemented with E₂ reached a minimum level after 3 days. TAM induced a strong increase in the AI which peaked at day 3, but at day 6 and 9 it was still higher than in any of the other media. Concomitant addition of E₂ to the SM + TAM decreased the AI but to a higher level than SM + E₂ without TAM. As MCF-7 cells undergoing apoptosis may detach from the substrate (Wilson *et al.* 1995) and thus be lost with the spent medium, the 'cumulative AI' provide a rough comparison of the minimal cell loss by apoptosis under different growth

Figure 3. Growth curves for MCF-7 cells grown in SM (Δ) or SM + TAM ($\square$). Closed symbols, + E₂. Mean values and sd of triplicate cultures.

Figure 4. Changes in labelling indices with time under different growth conditions. (a) SM; (b) SM + E₂; (c) SM + TAM; (d) SM + TAM + E₂. Mean $\pm$ sd.

Figure 5. Changes in apoptotic index with time under different growth conditions. (a) SM; (b) SM + E₂; (c) SM + TAM; (d) SM + TAM + E₂. Mean $\pm$ SD.

conditions. Thus Figure 6 shows the strong impact of TAM upon apoptosis. As for the pilot experiment, the staining reaction was similar to that of apoptotic cells in post-lactational regressing mammary gland (positive control) and no staining was seen in the negative controls.

Figure 6. Cumulative apoptotic index under different growth conditions. (a) SM; (b) SM + E₂; (c) SM + TAM; (d) SM + TAM + E₂.

Changes in the morphology in the TAM-treated cultures included condensation of chromatin in the centre, or in the periphery, of the nucleus, particularly at days 3 and 6. This pattern of nuclear condensation was not observed in cultures grown in SM or SM + E₂ (data not shown).

DISCUSSION

Surprisingly, in the pilot study, the addition of 10% NCS to the standard medium did not inhibit K_g of MCF-7 over a period of 7 days as shown previously (Lykkesfeldt *et al.* 1986a, Laursen *et al.* 1990) but did, on the contrary, stimulate K_g, – an effect that was further augmented by E₂. The reason for this discrepancy may be a result of a higher level of oestrogen in the batch of NCS used in this study, combined with the high serum albumin content of 10% NCS, as serum albumin has been shown to inhibit MCF-7 cell growth but that this inhibition can be abrogated by E₂ (Laursen *et al.* 1990). The finding, however, presented an opportunity to study the incidence of apoptosis over a wide range of growth rates.

MCF-7 net growth rates

In the pilot study as well as in the main experiment, a stimulatory effect of E₂ and an inhibitory effect of TAM was observed when compared to growth rates in standard medium without addition of these substances. These results are consistent with results previously obtained in MCF-7 and other oestrogen receptor-positive breast cancer cell lines (reviewed by Lykkesfeldt 1997). Contrary to the results of the pilot experiment and those of others (Sutherland *et al.* 1983, Bardon *et al.* 1984, Briand & Lykkesfeldt 1984), E₂ in the main experiment was not able to abolish the growth inhibitory effect of TAM. Similar growth curves for MCF-7 subline 0.5 have been published by, e.g. Briand & Lykkesfeldt (1984), Lykkesfeldt *et al.* (1984) and Lykkesfeldt *et al.* (1994). In principle the effects on net growth may be caused by different rates of proliferation and/or different rates of cell death and inter-experimental variations in either of these may cause different K_g and final cell numbers (see below). The results of the pilot experiment were obtained from cultures grown for 7 days, and although the average K_g and final cell numbers do give some information on the net growth of MCF-7 under various conditions, the pilot experiment provides neither information on the relative importance of proliferation and apoptosis for net growth nor on the time course of changes in proliferation and apoptosis. The main experiment was implemented in order to elucidate in greater detail these aspects of growth regulation.

Apoptosis

There has been some controversy concerning the ability of the MCF-7 cell line to undergo apoptosis *in vitro*. Thus cell death with apparent apoptotic morphology but without internucleosomal DNA degradation has been shown in response to TAM or cisplatin (Otto *et al.* 1996) while MCF-7 cells, detached in response to oestrogen-withdrawal, or the oestrogen antagonist ICI 182,720, exhibited DNA fragmentation but a morphology that was not consistent with apoptosis (Wilson *et al.* 1995). Perry *et al.* (1995) and Chen *et al.* (1996) observed DNA fragmentation of MCF-7 cells as revealed by agarose gel electrophoresis and *in situ* end-labelling in TAM-induced cell death in addition to apoptotic morphology. Moreover, the latter authors found a substantial increase in the amount of TRMP-2 mRNA after treatment with TAM, which is probably involved, although not exclusively, in apoptosis

(Tenniswood *et al.* 1994). These discrepancies may relate to the definition of apoptosis (Zakeri *et al.* 1995, Bursch *et al.* 1996), the subline of MCF-7 cells, the growth conditions and/or the period of time to which the cells have been exposed to the agents.

In MCF-7 subline 0.5 (the present study), DNA fragmentation as revealed by TUNEL-staining was observed under all growth conditions and an apoptotic or apoptotic-like morphology was observed in cultures with TAM present in the medium, consistent with the observations of Perry *et al.* (1995), Zakeri *et al.* (1995) and Chen *et al.* (1996). In cultures with E₂ present in the medium or without ('oestrogen withdrawal'), morphological manifestation of apoptosis was lacking, supporting the suggestion that in a given cell type, several pathways leading to active cell death may coexist (Bursch *et al.* 1996).

While there are several publications on the effects of E₂ and TAM on proliferation kinetic data, the effect of these agents on the kinetics of cell death/survival of breast cancer cells are scarce and largely restricted to *in vivo* models, in which oestrogen receptor-positive cells are inoculated into ovariectomized female nude mice and treated with TAM and/or E₂ (Osborne, Hobbs & Clark 1985, Osborne, Coronado & Robinson 1987, Brnner *et al.* 1989, Kyprianou *et al.* 1991, Wrri *et al.* 1993). In the pilot experiment, apoptotic cells, as revealed by *in situ* end-labelling, after 7 days were present under all culture conditions, whether grown in SM, SM plus TAM (10⁻⁶ M), SM plus 10% NCS, or in any of these media supplemented with E₂ (10⁻⁸ M), however, with different incidences. For each medium, the AI was lower with E₂ added than without (Table 1). These observations indicate that in MCF-7 subline 0.5 (Briand & Lykkesfeldt 1984), apoptosis is constitutively expressed, and that E₂ partially protects the cells from undergoing apoptosis. This interpretation is supported by results of the main experiment which found an almost linear decrease in the AI from the start to day 9 in cells grown in SM, while a low basal level was already present at day 3 when E₂ was added to the medium (Figure 5a,b). Similarly, a high TAM-induced AI was counterbalanced by E₂ (Figure 5c,d), although at a higher basal level than in cultures grown in SM + E₂ without TAM (Figure 5d,b). An apoptosis-protective role of E₂ in MCF-7 cells has also been suggested by Wang & Phang (1995). In the pilot experiment, a strong negative correlation was found between the AI and the final cell pool size and the derived average K_g. These correlations might by themselves indicate that the K_g and the cell pool size are primarily determined by apoptosis. However, a number of reports indicate that the proliferation of breast cancer cell lines is also affected by E₂ (Weichselbaum *et al.* 1978, Reddel & Sutherland 1984, Lykkesfeldt *et al.* 1986a, Leung & Potter 1987, van Der Burg *et al.* 1989) as well as by TAM (Osborne *et al.* 1983, Sutherland *et al.* 1983, Lykkesfeldt *et al.* 1984). The effect of TAM and/or E₂ on proliferation is discussed below.

Proliferation

The use of LI as a proliferation parameter relies on certain assumptions and has potential pitfalls (see Wright & Alison 1984). To the best knowledge of the author, there are no experimental data to indicate that in breast cancer cells a change in the LI reflects an altered duration of the S phase rather than an altered rate at which cells enter DNA synthesis or to suggest an altered enzyme activity involved in DNA precursor metabolism or uptake. Thus the LI, although a static parameter, is considered a reliable estimator of the proliferative rate.

In each of the four culture conditions applied in the main experiment, there was a decrease in the LI with time. Since MCF-7 cells grown with SM or SM + E₂ at day 9 were still undergoing exponential growth (Figure 3) at day 9, this decrease in the LI with time may at first seem surprising. The reduction in LI with time is not easily explained in terms of the cultures becoming confluent as the cultures (at least in cultures grown in SM or SM + E₂) at

day 9 are still in an exponential growth phase (see Figure 3). As the growth curves represent the net growth rate, a positive net growth can be achieved with a decreased proliferation only through a decreased rate of cell death. Since apoptosis (as shown in the present paper) is constitutively expressed and decreases with time (Figure 5), this fact is the most likely explanation for this apparent 'growth paradox'. Thus it is reasonable to assume that exponential growth under these experimental conditions, in spite of a decreased proliferative rate, should be explained by a simultaneous decrease in the rate of apoptotic cell death as is indicated by the decreasing AI (Figure 5a,b). An initial delay in the decrease of the LI (from start to day 3) was observed in cultures grown in the presence of E_2 and at days 6 and 9 the LI was higher in cultures with (Figure 4b,d) than without E_2 added (Figure 4a,c). This is consistent with published data on MCF-7 cells (Weichselbaum *et al.* 1978, Lykkesfeldt *et al.* 1986a) and on T47D cells (Reddel & Sutherland 1984). These authors found a higher fraction of S phase cells in cultures with oestrogen addition than in the cultures without. There is only a small difference in growth fraction between MCF-7 cells grown with E_2 and without E_2 : 98% and 92%, respectively (Lykkesfeldt *et al.* 1986a, Lykkesfeldt, Larsen & Christensen 1986b) and hence it is likely that the higher LI in cultures grown with, compared to without, E_2 reflects a higher rate of proliferation. The oestrogen effect on proliferation is probably a shortening of cell cycle duration resulting from a reduction in the G_1 phase time (Weichselbaum *et al.* 1978, Lykkesfeldt *et al.* 1986a, Leung & Potter 1987). The mechanism by which oestrogen stimulates breast cancer cell proliferation may be indirectly through the stimulation of autocrine growth factors (Lykkesfeldt, Laursen & Briand 1992) or directly through gene activation by oestradiol (Kasid *et al.* 1986, van der Burg *et al.* 1989).

The effect of TAM on proliferation appears to be through a decrease of the growth fraction with arrest of cells not only in the G_1 phase but also in the G_2 phase of the cell cycle (Lykkesfeldt *et al.* 1984, Bruno *et al.* 1988). This may explain the rapid and linear decrease of the LI in cultures grown in SM with TAM added (Figure 4). The mechanism by which TAM inhibits cell proliferation is not well known. Originally it was proposed that anti-oestrogens have an indirect action by antagonizing the oestrogen action when bound to oestrogen receptors (Lippmann, Bolan & Huff 1976). According to Cai & Lee (1996), TAM has some anti-proliferative effects through the inhibition of DNA synthesis by blocking the nucleoside salvage pathway. Other investigations have implied that TAM may exert a direct growth inhibitory effect mediated via the oestrogen receptor, possibly through down-regulation of cyclin D1 expression (Watts *et al.* 1994) or by mechanisms not involving the oestrogen receptor but through action as a calmodulin antagonist (Lam 1984, Gulino *et al.* 1986, Frankfurt *et al.* 1995) and thus interfering with several signal transduction pathways (Lykkesfeldt 1997).

Proliferation and apoptosis in E_2 and TAM-influenced net growth – conclusions and perspectives

Rates of proliferation as well as apoptosis are important in determining (net) growth rates of the human breast cancer cell line MCF-7. The present study provides evidence that in MCF-7 subline 0.5 adapted to growth at low serum concentration, E_2 increases proliferation and protects against apoptosis, resulting in an increased K_g and final cell pool size. TAM has a slight inhibitory effect on proliferation (cytostatic) and a strong stimulatory effect on apoptosis (cytotoxic), resulting in a decreased K_g and low final cell pool size, surviving cell colonies eventually giving rise to a TAM-resistant cell line (Lykkesfeldt & Briand 1986, Lykkesfeldt, Madsen & Briand 1994). When TAM and E_2 are added simultaneously to the growth medium, the cytotoxic effect of TAM is partly counterbalanced by the protective role of E_2 , resulting in a reduced apoptotic rate that, however, is maintained at a higher level than

in cultures grown with E_2 only. As also the the cytostatic effect of TAM is partly abolished by E_2 , the combined effect of TAM and E_2 is a K_g and final cell pool size higher than when grown with TAM alone and lower than when grown with E_2 alone.

These effects may be direct or indirect. It has been shown that in MCF-7 cells the secretion of several proteins, e.g. procathepsin D (Cavaillès *et al.* 1988, Lykkesfeldt *et al.* 1992), α_1 -antitrypsin (Lykkesfeldt *et al.* 1992), and α_1 -antichymotrypsin (Laursen & Lykkesfeldt 1992) are stimulated by E_2 while a 42-kDa protein of unknown nature is down-regulated in response to E_2 and it has been suggested that the three former proteins are positive growth regulators while the 42-kDa protein has a negative growth regulatory function (Lykkesfeldt *et al.* 1989). This suggestion is supported by the observation that steady state levels of mRNA of the former are stimulated by E_2 (Tamir *et al.* 1990; Lykkesfeldt *et al.* 1994). Furthermore, it has been shown that TAM stimulates the secretion of procathepsin D and the 42-kDa inhibitory protein (Lykkesfeldt *et al.* 1994). However, the potential role of this differential pattern of secretion following E_2 or TAM treatment has only been studied with regard to net growth as assessed by growth curves. In order to gain further insight into regulation or modulation of MCF-7 (net) growth, it will be important to study the specific effect(s) exerted by oestrogens and anti-oestrogens and mediated via each of these secreted proteins on rates of proliferation and/or apoptosis.

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