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Original Paper

Short- and Long-term Oestradiol Treatment Inhibits Growth and Alters Expression of p21$^{WAF1/Cip1}$ in an Endometrial Adenocarcinoma Cell Line Lacking Functional p53

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Oestrogen is assumed to play a significant role in cell cycle regulation of cells expressing the oestrogen receptor, although its mechanism of action is not yet well defined. To examine this, a mutant p53-expressing human endometrial adenocarcinoma cell line of the oestradiol-inhibited growth phenotype was treated with oestradiol for 2 weeks (short-term) and 6 months (long-term). With short-term treatment, cells were treated with increasing doses of oestradiol. The highest dose, 1 μM, was used in the long-term interval. The influence of the hormone on growth, proliferation and expression of some cell cycle and apoptosis-related proteins was evaluated. In cells treated for 2 weeks, there was a dose-dependent inhibition of both growth and proliferation with a significant decrease in labelling index (LI) and S-phase fraction (SPF) and a simultaneous increase in the fraction of cells in G0/G1. Extending the oestradiol treatment to 6 months showed further growth retardation and decreased proliferation with cells accumulating in G0/G1. Analysis of the expression of p21$^{WAF1/Cip1}$ showed a nearly 2-fold increase after 2 weeks treatment with 1 μM oestradiol, which was also observed after long-term treatment without any further increase in protein levels. Expression of the anti-apoptotic protein bcl-2 was not affected after short-term treatment but decreased significantly after 6 months treatment compared to control cells. Our results suggest the existence of a p53-independent pathway of oestradiol regulation of growth and proliferation in this human endometrial adenocarcinoma, resulting in accumulation of cells in G0/G1 through p21$^{WAF1/Cip1}$ induction and, after prolonged treatment, downregulation of bcl-2 protein. © **1997 Published by Elsevier Science Ltd.**

Key words: endometrial adenocarcinoma, *in vitro*, growth, proliferation
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INTRODUCTION

OESTRADIOL IS thought to play an important role in the development of endometrial adenocarcinoma, but knowledge of its influence on manifest tumours is limited. Experimental models have shown that the growth of hormone-regulated tumours such as human breast and endometrial carcinoma progresses from hormone dependency to hormone independence through an intermediate phase where the cells are independent of but responsive to the hormone [1–4]. This phase comprises two different subgroups, one stimulated and the other inhibited by the hormone [5].

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Interaction of oestradiol with its receptor triggers a cascade of events, leading ultimately to the regulation of cellular proliferation through cell cycle intervention. Although the mechanisms involved in the different effects of oestradiol on tumour growth are obscure, it is known that hormonal induction of growth factors and other cell cycle related factors, such as c-myc, contributes to the proliferative action of oestradiol, and growth inhibition may also be associated with changes in the levels of these different factors [6, 7].

The growth of experimental tumours *in vivo* is determined through variations in both the growth fraction and the cell loss factor [8, 9], inferring that signals which ultimately regulate the cell cycle must interact with cellular mechanisms that control cell death. The *bcl-2* proto-oncogene plays an important role in this process by preventing programmed cell

death, resulting in increased cell survival [10]. *bcl-2* has been studied predominantly in haematolymphoid malignancies but recent investigations have shown that the bcl-2 protein occurs in a number of other tissues and in benign and malignant processes of the endometrium [11]. Conversely, the p53 protein may induce programmed cell death, and we have previously demonstrated an inverse relationship between p53 and bcl-2 expression in response to oestradiol treatment in a human endometrial adenocarcinoma expressing wild type p53. In this tumour, grown in nude mice, oestradiol inhibits growth and increases the expression of p53 protein with a simultaneous decrease in bcl-2 levels, suggesting a link between these proteins in response to oestradiol treatment [12].

We have established several human endometrial adenocarcinomas expressing the oestradiol-independent but inhibited phenotype in nude mice, and have recently reported on an *in vitro* cell line derived from one of these tumours. This cell line, called GL-18, still expresses the inhibited phenotype in response to oestradiol but contains two point mutations of the *p53* gene. We have recently observed that oestradiol causes a redistribution of the cells to the G1 phase, suggesting a p53-independent ability of the hormone to block progression from G1 [13]. p21$^{WAF1/Cip1}$ is known to be induced by p53 and to cause a G1-block, but other studies suggest the existence of pathways activated by growth factors that also induce p21$^{WAF1/Cip1}$ independent of p53 [14].

In the present study, we analysed growth, cell kinetics and the expression of p21$^{WAF1/Cip1}$ and bcl-2 in GL-18 cells after short- (2 weeks) and long-term (6 months) treatment with oestradiol to evaluate its effect on the inhibited growth phenotype.

MATERIALS AND METHODS

Cell culture

The GL-18 endometrial adenocarcinoma cell line used was previously established in our laboratory [13]. The growth of the cell line was characterised as oestradiol independent but responsive (inhibited), expressing low concentrations (3–6 fmol/mg protein) of oestrogen receptors (ER). The cells overaccumulated p53 protein due to point mutations at codon 175 on both alleles and codon 248 on one allele, which renders the protein inactive and incapable of binding DNA. Cells were routinely cultured in RPMI 1640 supplemented with 10% FCS (fetal calf serum), penicillin 100 U/ml and streptomycin 100 mg/ml. Incubation was at 37°C in an atmosphere of 5% CO_2 in air. Cells were passaged with the use of trypsin–EDTA when just confluent.

Cell growth experiments

To determine the effect of oestradiol on cell growth and proliferation, GL-18 cells were pre-incubated in phenol red free RPMI 1640 medium supplemented with 10% charcoal-dextran-treated FCS and 17-β-oestradiol (10 nM, 100 nM and 1 μM) for 2 weeks. In the long-term experiment, cells were treated with the addition of 1 μM 17-β-oestradiol for 6 months. Cells were passaged once every week and the medium was changed three times a week. At the end of each time period cells were seeded, grown, and measured in 96-well plates using crystal violet staining [15]. Briefly, each day, cells in one plate were fixed by the addition of 11% glutaraldehyde solution, washed, air-dried and stained by adding crystal violet dissolved in 200 mM MES (2-[*N*-Morpholino] ethane-sulphonic acid), pH 6.0. Excess dye was removed by washing and the plates were air-dried prior to dye solubilisation in 10% acetic acid. The optical density (OD) of dye extract was measured directly in plates using a microplate reader. Dual wavelength was set at 590–405 nm. The mean OD value for each plate was plotted versus time, comparing these results with those from cells grown under control conditions (ethanol vehicle 0.1%). To evaluate the hormone dependence, the growth of cells that had been treated for 6 months was analysed after 2 weeks in oestrogen depleted medium compared to cells remaining in medium with 1 μM 17-β-oestradiol.

5-bromo-2'-deoxyuridine (BrdU)-labelling

BrdU (1 mM stock solution in PBS, phosphate-buffered saline) was added to the medium of exponentially growing cells to a final concentration of 1 μM. The cells were incubated for 30 min at 37°C and the pulse-labelling was blocked by rinsing with culture medium. After the end of pulse-labelling, cells were harvested at time 0 and 4 h by trypsinisation, centrifuged and fixed in ice-cold 70% ethanol. Samples were stored at −20°C.

Flow cytometry analysis

The method for preparation and staining has been described previously and further modified by Wilson and associates [16]. The analysis was performed using a Cytoron Absolute (Ortho Diagnostic System Inc., New York, U.S.A.). Approximately 20 000 nuclei per sample were analysed. The S-phase fraction (SPF) was calculated with the Multicycle software package (Phoenix Flow System, California, U.S.A.). For evaluation of cell data, three regions were set according to Begg and associates [17] in the bivariate BrdU versus DNA cytogram. The movement of undivided BrdU-labelled cells relative to G1 and G2 was calculated according to Begg and associates, and defined as RM (relative movement). Tumour (cell) potential doubling time, T_{pot}, was determined without regard to cell loss (CL), with the equation [18]:

$$T_{pot} = T_s \times \text{lambda/LI}$$

where lambda, a correction factor for the non-linear distribution of cells through the cell cycle, was set at 0.8. T_s is the DNA synthesis time calculated from the RM, and LI is the labelling index.

To evaluate the transition of cells from G1 to S-phase, the appearance of BrdU-non-labelled cells in the S-phase during the postlabelling time was estimated. The number of non-labelled cells were expressed as a proportion of the total number of cells in S-phase and the difference between time 0 and 4 h after labelling was compared.

Western blot analysis

At the end of each time period, cells at subconfluence were harvested, rinsed, pelleted and resuspended in ice-cold PBS. Sodium dodecyl sulphate (SDS) at a final concentration of 5% was added and the samples were boiled for 10 min, centrifuged for 10 min at 14 000 rpm and the protein content in the supernatant was determined by Bio-Rad microassay. Samples (75 μg) were boiled for 5 min in loading buffer, separated on an SDS polyacrylamide gel, and transferred to nitrocellulose membranes. After blocking, membranes were incubated sequentially with primary polyclonal antibody and a bridging anti-rabbit IgG antibody conjugated to horseradish peroxidase. To detect bcl-2 protein and p21$^{WAF1/CIP1}$

Table 1. Cell kinetic parameters after 2 weeks treatment with oestradiol

Parameters measured	Control ($n=10$)	10^{-8} M ($n=10$)	10^{-7} M ($n=10$)	10^{-6} M ($n=10$)
Cell doubling time	22.5 h	24 h	26.4 h	30 h
Labelling index	52.7 ± 0.3%	46.6 ± 0.6%	45.2 ± 0.7%	44.6 ± 0.4% ($P<0.0001$)*
S-phase fraction	49.4 ± 1.5%	45.4 ± 1.7%	42.5 ± 3.4%	41.3 ± 1.2% ($P<0.0001$)
G0/G1	36.7 ± 1.1%	41.9 ± 1.4%	42.8 ± 2.3%	44.1 ± 2.0% ($P<0.0001$)
G2/M	13.9 ± 1.3%	12.7 ± 1.5%	14.7 ± 1.4%	14.6 ± 1.4% (n.s.)
DNA synthesis time (T_s)	14.8 ± 0.9 h	13.8 ± 0.6 h	11.7 ± 0.4 h	12.2 ± 0.8 h ($P=0.001$)
Potential doubling time (T_{pot})	22.4 ± 1.2 h	23.7 ± 1.3 h	20.8 ± 0.5 h	21.8 ± 1.5 h (n.s.)

*P values represent comparison of control cells and cells treated with 1 μM (10^{-6} M) oestradiol for 2 weeks. Values are presented as mean ± S.D. n.s., not significant.

protein, rabbit polyclonal antibodies were used (Santa Cruz Biotechn, Inc. U.S.A.). Finally, the membranes were incubated in Enhanced Chemiluminescense (ECL) reagents (Amersham), exposed to ECL hyperfilm and developed as usual for autoradiography.

A software package (Quantity One, pdi, New York, U.S.A.) was used for semiquantitative measurements of oncoproteins. In this computer program the area and OD of each band in scanned films was measured and expressed as OD × mm^2.

Statistical analysis

Differences in cell kinetic variables were analysed by Student's t-test. This was also the case when evaluating the differences in proportions of unlabelled cells in S-phase between treated and control cells. Differences in expression of the bcl-2 and p21$^{WAF1/CIP1}$ proteins were also analysed by Student's t-test.

When comparing sub-G1-fractions between different groups, the Mann–Whitney test was used.

RESULTS

Cell growth, cell kinetics and expression of p21$^{WAF1/Cip1}$

GL-18 cells in the cell-growth assay showed a dose-dependent growth retardation when incubated with 17-β-oestradiol for 2 weeks (Figure 1). There was a parallel decrease in proliferation parameters through a significant decrease in LI, SPF and a concomitant increase in the number of G0/G1 cells even at the lowest dose of 10^{-8} M (Table 1). Ts was unchanged at the lowest dose but decreased significantly at 0.1 and 1 μM. There were no significant differences in T_{pot} after short-term treatment with different doses as both the T_s and LI decreased. Extended treatment for 6 months in medium containing 1 μM 17-β-oestradiol resulted in further growth retardation (Figure 2) and redistribution of the cells in the cell cycle with lower LI and SPF and higher G0/G1 fraction compared to control cells in the same passage (Table 2). Western blot analysis of p21$^{WAF1/Cip1}$ (Figure 3) showed a significant increase in expression after 2 weeks of treatment with 1 μM oestradiol, which was maintained at 6 months. The fraction of cells in G0/G1 showed a dose–response relationship, but the dose–response of p21$^{WAF1/Cip1}$ increase was not evaluated. With long-term treatment, the decrease in T_s between control and treated cells remained significant, whereas T_{pot} was significantly increased as a result of the further decrease in LI in this group (Table 2). Evaluating the appearance of non-BrdU-labelled cells in S-phase during postlabelling time showed that in cells treated with 1 μM oestradiol for 2 weeks, there was an increase of 1% compared with 1.8% in control cells ($P=0.008$). The same was seen in cells treated for 6 months where the increase was 0.5% compared with 1.3% in control cells ($P=0.001$).

To evaluate possible changes in the hormone independence of this cell line after long-term treatment, oestradiol

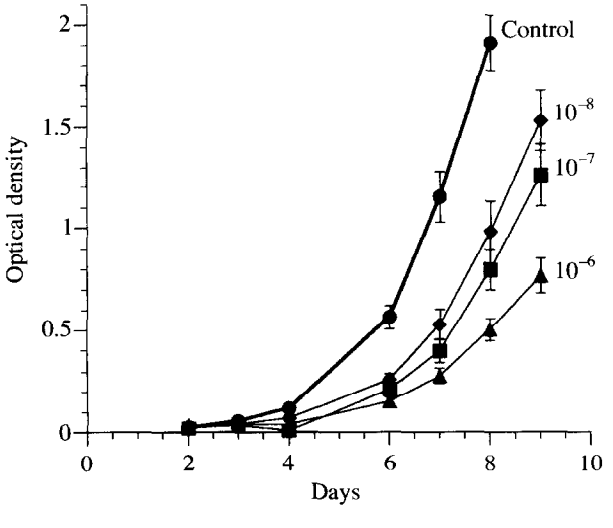

Figure 1. Effect of 17-β-oestradiol on cell growth in GL18 cell line after 2 weeks treatment with the indicated concentration (Molar) of the hormone. Each point represents the mean of 60 samples ± S.D.

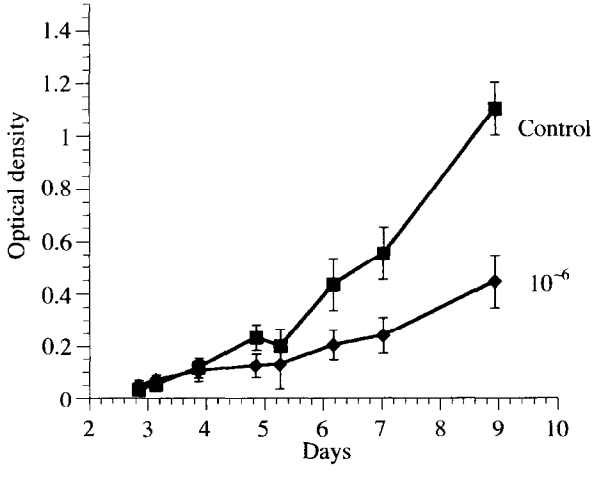

Figure 2. Effect of 17-β-oestradiol on cell growth after 6 months treatment with 1 μM. Each point represents the mean of 60 samples ± S.D.

Table 2. Cell kinetic parameters after 6 months treatment with 1 μM oestradiol

Parameters measured	Untreated 6 months ($n = 10$)	Treated 6 months ($n = 10$)
Cell doubling time	25 h	44 h
Labelling index	49.5 ± 0.7%	39.2 ± 1.2% ($P < 0.0001$)*
S-phase fraction	45.6 ± 2.2	33.3 ± 0.6% ($P < 0.0001$)
G0/G1	40.4 ± 0.6%	52.6 ± 0.8% ($P < 0.0001$)
G2/M	14.0 ± 1.7%	14.1 ± 0.4% (n.s.)
DNA synthesis time (T_s)	12.7 ± 1.0 h	11.3 ± 0.4 h ($P < 0.02$)
Potential doubling time (T_{pot})	20.6 ± 1.5 h	23.0 ± 0.5 h ($P < 0.01$)

*P values represent comparison of control cells with cells treated with 1 μM oestradiol for 6 months. Values are presented as mean ± S.D. n.s., not significant.

was withdrawn from the cells and the growth analysed. The oestrogen depleted cells showed slightly faster growth than treated cells, although this difference was not significant. Cell kinetic parameters showed no significant differences (data not shown).

Apoptosis-related protein expression and analysis of sub-G1 cells

To evaluate apoptosis in oestradiol treated cells, expression of bcl-2 and analysis of the percentage of cells in the DNA range below G1, (sub-G1), considered to represent apoptotic cells [19], was calculated. bcl-2 Did not show any differences after short-term treatment, independently of the oestradiol dose used, but was significantly decreased after 6 months treatment with 1 μM oestradiol (Figure 4). The percentage of cells in sub-G1 showed no differences after short-term treatment, but was significantly increased in cells treated with oestradiol for 6 months compared to control cells at that time point, suggesting an increased number of cells undergoing apoptosis (Table 3). This increase in sub-G1 fraction parallels the decreased bcl-2 expression and more pronounced growth retardation seen in these cells.

DISCUSSION

In this study we analysed the effect of different doses of oestradiol on growth and cell kinetics *in vitro* in a human endometrial adenocarcinoma with low oestrogen receptor (ER) concentration. The oestradiol treatment was also extended for 6 months to investigate how continued exposure to oestrogens affects growth and proliferation in a cell line with an oestradiol-inhibited growth phenotype and a non-functioning p53 protein.

With short-term treatment, growth retardation was dose-dependent, starting at physiological concentrations (10^{-8}) [20] and the cell kinetic parameters showed a parallel successive dose-dependent redistribution toward an increasing G0/G1 fraction and a decreasing SPF.

Dose dependence and consequent changes in cell kinetics in our experiment suggest that the inhibition seen in our cell line is a true receptor-mediated effect of oestradiol, although the receptor content was low. These results thus indicate the existence of the oestradiol-inhibited growth phenotype *in vitro*. This phenotype has previously been described in tumours growing in nude mice [13, 21, 22]. Increasing doses of oestradiol have been reported to result in a biphasic response in MCF-7 cells, with inhibition at doses greater than 10^{-6} M [20]. It has been suggested that inhibition by high concentrations of oestrogens may be a non-specific effect not mediated by receptor interaction. Others have proposed the contrary; using even higher doses they found a cell-cycle specific effect and cell cycle independent cytotoxicity in dose ranges greater than 10^{-6} M [23]. The role of the ER in mediating this high dose effect was not obvious, but significant differences in sensitivity between an ER$^+$ and ER$^-$ breast carcinoma cell line were found.

After prolonged treatment with oestradiol, the initial growth retardation was maintained and even further retarded. When oestrogen was withdrawn there was only a slight acceleration of growth which was not significant. The cells treated long term are therefore considered to represent subpopulations of GL-18 cells that have retained their hormone independence in the presence of oestrogen inhibition without returning to their original proliferation, i.e. proliferation before any oestrogen treatment.

The accumulation of cells in the G0/G1 phase and the simultaneous increase in p21$^{WAF1/Cip1}$ observed in this cell line in response to both short- and long-term oestradiol

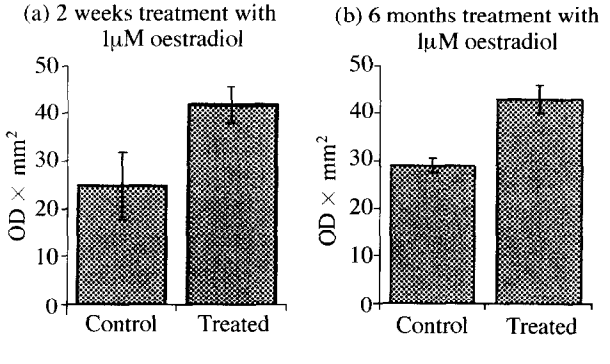

Figure 3. Expression of p21$^{WAF1/Cip1}$ in cells treated with 1 μM 17-β-oestradiol for 2 weeks (a) and 6 months (b). Data were obtained by measuring Western blots and each column represents the mean OD × mm² of three bands (samples) ± S.D.

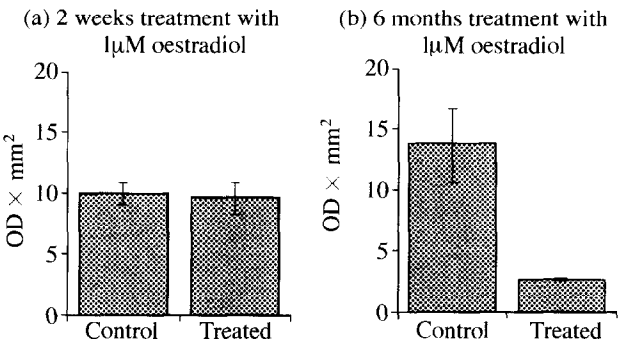

Figure 4. Expression of bcl-2 in cells treated with 1 μM 17-β-oestradiol for 2 weeks (a) and 6 months (b). Data were obtained by measuring Western blots and each column represents the mean OD × mm² of three bands (samples) ± S.D.

Table 3. Sub-G1 fraction

	Dose	Control ($n=10$)	Treated ($n=10$)	
2 weeks	10^{-6}	2.9 ± 1.1	3.3 ± 1.6	n.s
6 months	10^{-6}	1.8 ± 0.8	2.8 ± 0.2	$P=0.009$

Values are presented as mean ± S.D. Comparison between control and treated cells at each time point. n.s., not significant.

treatment is an interesting phenomenon, especially since the cell line carries *p53* mutations resulting in a non-functioning protein.

Transient induction of $p21^{WAF1/Cip1}$ independently of p53 has been described in response to serum and purified growth factors [14]. There are also reports of p53-independent $p21^{WAF1/Cip1}$ induction in response to physiological and chemical inducers of differentiation [24]. It is quite clear from these reports that $p21^{WAF1/Cip1}$ gene expression is tightly controlled and that the protein product plays a central role in connecting different external signals to cell proliferation. We can only speculate as to the role oestradiol plays, since it has the ability to act directly on the genetic programme that controls cell proliferation through the nuclear ER itself. Studies from our laboratory on *in vivo* growing tumours with an oestradiol-inhibited growth phenotype have shown a more differentiated appearance of tumours in response to oestradiol treatment [2]. Further analysis of the immediate response to oestrogen of this inhibited phenotype *in vitro* would be of interest.

In a recent report, oestradiol was shown to induce inhibition of a breast carcinoma cell line transfected with wild type ER [25], and the inhibition was followed by a 3- to 5-fold increase in $p21^{WAF1/Cip1}$ expression. The increase observed in our cell line was barely 2-fold which could be a result of the low receptor number. Still, this finding indicates that the accumulation of cells in the G0/G1 phase could be due to a lengthening of the G1 phase. Evaluating the proportion of unlabelled cells entering S-phase during the postlabelling time may give us some information as to what extent the transition of cells across the G1 checkpoint differs between control cells and cells treated with oestradiol. Indeed, both in cells treated for 2 weeks with oestradiol and cells treated in the long term, fewer non-BrdU-labelled cells enter the S-phase compared to control cells. Even if the differences between treated and control cells are small, this could reflect the fact that the treated cells are in one way or another delayed in G1. Finally, there also exists the possibility of cells being withdrawn from the potentially cycling fraction, but this cannot be evaluated with this method.

2 weeks of oestradiol treatment had no effect on T_{pot}, a parameter that is calculated from T_s and LI. With short-term treatment, the decrease in T_s was in proportion to the decrease in LI resulting in an unaltered T_{pot}. However, with long-term treatment, the LI was further decreased, whereas the difference in T_s was preserved, giving an increase in T_{pot}.

We can only theorise about how oestradiol treatment accelerates DNA synthesis. One explanation could be that the simultaneous decrease in T_s and increase in G0/G1 through p21 are parallel but separate effects of the hormone at the postreceptor level. Alteration of postreceptor interactions during progression could probably lead to dissociation of the hormonal effects, explaining why the same hormone has conflicting actions. If p53 protein is a key regulator at this level, mutation of this protein could explain our finding. Another explanation may be that when cells are arrested in G1, fewer cells enter S-phase, measured as a lower SPF and LI. The cells arrested in G1 could represent cells with a higher hormonal responsive capacity (i.e. ER content). Cells entering S-phase may have a faster DNA synthesis due to lack of ER. What we did not see was an accumulation of cells in G2/M indicating a disturbance of mitosis, that has been described by others as an effect of oestradiol inhibition [23]. There is a possibility that the decrease in T_s could be an artefact caused by cells residing longer in G2/M and in this way weighting the mean DNA content to a higher value interpreted as a shorter T_s. If this is the case it would most probably show in the distribution of cells between the compartments as an increased G2/M. We therefore consider this unlikely.

The finding that long-term oestrogen treatment decreased the expression of bcl-2 in these hormone-responsive cells could explain the accentuated growth retardation, seen only with prolonged time treatment, as caused by increased apoptosis. The observation that the fraction of cells in sub-G1 phase also increased significantly in treated cells further suggests the increased apoptotic activity in this cell population, most likely among cells arrested in G1. We recently reported similar effects of oestradiol on bcl-2 expression *in vivo* in an endometrial adenocarcinoma of the inhibited growth phenotype expressing wild type p53. The mechanism by which oestradiol regulates this anti-apoptotic protein has been proposed to involve an indirect pathway and we have suggested a possible link between oestradiol, p53 and bcl-2 in regulating proliferation and cell loss in some human endometrial adenocarcinomas [12]. Moreover, others have also suggested that bcl-2 could be an oestrogen-related protein. The expression of bcl-2 protein has been positively correlated with ER content in human breast cancer [26], and oestradiol has been shown to upregulate the expression of bcl-2 mRNA in MCF-7 cells correlating to the stimulated phenotype [27]. Since the p53 pathway is disrupted in the cell line used in our experiments, some alternative mechanism is likely to exist, possibly a direct transcriptional alteration through an oestrogen response element (ERE) in the *bcl-2* gene. There are no perfect consensus sequences for an ERE in the published *bcl-2* gene sequence [28]. However, the *bcl-2* gene promoter contains several Sp1-binding sites which have been shown to confer oestradiol responsiveness [29]. We propose that the decreased expression of bcl-2 protein in response to oestradiol seen in our experiment is a direct action of the hormone–receptor complex and is related to the inhibited phenotype. Again, the low receptor concentration may contribute to the slow regulatory response for this protein.

In summary, we found that oestradiol causes a dose-dependent inhibition of growth in an endometrial adenocarcinoma cell line expressing mutant p53 by $p21^{WAF1/Cip1}$ induction and G1 arrest. Prolonged exposure to the hormone further retards the growth and also decreases the expression of bcl-2 thus allowing apoptosis. This is also detected as an increased proportion of cells in sub-G1. Long-term treatment results in a hormone-independent phenotype with altered growth kinetics. This model provides further possibilities to study the inhibited growth phenotype and its regulation and progression.

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