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Estrogen Receptor Blockade by the Pure Antiestrogen, ZM 182780, Induces Death of Pituitary Tumour Cells

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Our previous studies have shown that even in the absence of estrogen, the estrogen receptor (ER) is still involved in growth by way of its conversion to a transcriptionally active state by growth inducing cytokines. The following paper now provides evidence that under more physiological conditions, the ER within the GH_3 cell line used for the previous investigations, not only controls growth, but that transcriptional activity of the receptor is required for cell survival. Therefore when GH_3 cells, maintained under serum and steroid replete conditions, are exposed to the pure antiestrogen ZM 182780 (10 nM), marked cell death is observed 72–120 h after first exposure. Studies on the nature of this cell death suggested that it had some of the reported characteristics of apoptosis or programmed cell death. Removal of steroids from the culture medium also resulted in cell death and this was enhanced by the addition of the pure antiestrogen. Both steroid withdrawal and ZM 182780 induced cell death was completely reversed by the inclusion of estrogens in the steroid free culture medium. In contrast, the non-steroidal antiestrogen, 4-hydroxytamoxifen (4-OHT) was not able to enhance steroid withdrawal death and at 1 μM, this compound was shown to have marked ER agonist activity. Further studies on the addition of conditioned medium from high density GH_3 cell cultures, to low density steroid free cells, strongly suggested that the ER within these cells was responsible for the production of autocrine/paracrine survival factors.

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INTRODUCTION

For a number of cell types containing the nuclear transcription factor the estrogen receptor (ER), removal of estrogen or the pharmacological blockade of the receptor by antiestrogens markedly reduces growth[1–3]. The precise mechanism by which the ER facilitates a growth response is still poorly understood but three major ideas have been postulated: (a) a direct 'nuclear mechanism' involving the induction of growth related genes such as c-*fos*, c-*myc* [4], ornathine decarboxylase and the cyclins [5, 6]; (b) a paracrine/autocrine mechanism involving the stimulation of growth factor production [7]; (c) the reversal of the inhibitory effects of serum derived factors [8]. These three major mechanisms of action are not necessarily mutually exclusive and the degree to which they operate most likely depends on the cell type and its environment.

Of all the studies on the ER, it is without doubt that the observations on the role of estrogens on breast tumour cell proliferation have provided the drive for the search for compounds that block the synthesis of estrogens or that block the action of estrogen at the receptor. Therefore following the first and second generation aromatase inhibitors like aminoglutethimide [9] and 4-hydroxyandrostenedione [10], highly selective and potent compounds like CGS 20267 [11] and vorozole [12] are currently undergoing clinical studies. These compounds are able to reduce plasma estradiol concentrations to undetectable levels, even with the most sensitive assays available. In parallel with these studies on aromatase inhibitors, compounds are currently under development that block the conversion of estrone sulphate to estrone [13]. Estrone sulphate in the human is the major estrogen pool in the circulation [14] and although it is originally derived from androgen precursor, the levels of estrone appear to be more refractory to treatment with aromatase inhibitors [9].

With regard to pharmacological blockade of the ER, tamoxifen is currently the agent of choice for treatment of breast cancer as the consensus from a number of

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studies indicate that it is of benefit [15]. Tamoxifen, however, and other 'first generation' antiestrogens, suffer the disadvantage that they have a significant degree of estrogenic activity [16]. This in a number of *in vitro* and *in vivo* test systems is manifest by overt estrogenic activity in the absence of exogenous estrogen [17–20]. Therefore, in terms of our current understanding of the role of ligand for the transcriptional activation of steroid receptors [21], these compounds are still capable of activating transcription through the *n*-terminal transactivating function (TAF-1). Thus depending on cell type and promotor context, this may be capable of endowing the ER with a partial or full transcriptional ability [22]. For this reason the last 5 years has seen the development of compounds that more fully block the transcriptional capability of the ER. Therefore 'pure' steroidal antiestrogens such as ZM 182780 [23] and RU 58668 [24] are currently under development and these give a higher degree of growth inhibition in a number of *in vitro* and *in vivo* test systems. Although these compounds are based on the estradiol (E_2) backbone, their long aliphatic side chains would appear to result in a receptor conformation that is incapable of any significant degree of activation of ER responsive genes.

Recently this laboratory has reported that even in the complete absence of exogenous estrogens, the ER is still capable of acting as a transcription factor involved in growth [25–28]. The explanation given for this in these studies and those of other groups [29–34], is that growth factor activated signal transduction pathways convert the ER to a transcriptionally active state. Given these findings, theoretically, pure antiestrogens should be more effective in treatment as compared to aromatase inhibitors. The problem here may be that total growth suppression, while giving a short term benefit, may not result in a long-term advantage, as with time, adaption of tumour cells might occur, giving a phenotype in which the ER is no longer involved in growth. Preferentially, what is required is an endocrine agent capable of selectively killing the tumour cells. In this respect, some reports have indicated that death of breast tumour cell lines is induced following their exposure to antiestrogens [35, 36]. This death would appear to have some of the characteristics of programmed cell death or apoptosis [37].

During comparative studies of a number of steroidal and non-steroidal antiestrogens on the growth and function of the prolactin secreting pituitary tumour cell line GH_3, cell death was apparent when the pure antiestrogen ZM 182780 was given to these cells growing under serum containing conditions. Since in some experiments as much as 95% of the cell population was dead after 5 days of treatment with ZM 182780, these cells appeared to be a useful model system in which to study the factors governing antiestrogen induced cell death. This paper reports these findings.

EXPERIMENTAL

Chemicals

Materials and reagents, were obtained from Gibco (Kalsruhe, Germany), Flow (Mackenheim, Germany), Falcon (Heidelberg, Germany), Nunc (Weisbaden, Germany), Sigma (Deisenhofen, Germany), Boehringer (Mannheim, Germany), Dianova (Hamburg, Germany) and Aldrich (Steinheim, Germany). All estrogens used were dissolved as stock solutions in ethanol at 1 mg/ml. The pure steroidal antiestrogen ZM 182780 was obtained from Zeneca Pharmaceuticals (Cheshire, England). The non-steroidal antiestrogen 4-OHT was obtained from Besins Iscovesco (Paris, France). Both antiestrogens were dissolved as 20 mM stock solutions in ethanol and as for the estrogens, kept at 4°C. The purity of the estrogens and antiestrogens was checked, before higher dilutions were made, using HPLC an a C18 reverse phase column with methanol:water (90:10) as eluent. Each compound eluted essentially as one peak.

Cell culture

The ER containing, pituitary GH_3 cells, as previously described [26], were maintained in Dulbecco's modified Eagle's medium (DMEM) with antibiotics, and 10% foetal calf serum (FCS). These cells were routinely passaged every fifth day at a 1:5 split. For experiments, cells were seeded in the maintenance medium at densities between 10 and 40,000 cells per cm^2 in 48 well plates and medium was changed after 24 h to experimental medium consisting of phenol red free DMEM containing 10% charcoal stripped serum (SFCS), prepared as indicated in an earlier study [26]. For the majority of experiments where cells were seeded at around 10,000 per cm^2, cell numbers (as determined below) at the time of treatment ranged between 15 and 20,000 cells per well.

Cell number and determination of cell death and survival

Cell numbers were quantitated with the use of a Coulter counter or indirectly by the addition of the tetrazolium dye, MTT, again as we have previously described [26]. For cell numbers up to 200,000 cells per well, Fig. 1 shows the linearity of colour formation measured at 550 nm with the number of cells per well. As this method measures live cells (based on their mitochondrial metabolism of the dye, MTT) it can also be used to make an estimate of overall culture viability [38]. For this, at the end of the treatment period and 1–2 h after the addition of MTT, viable cells (showing blue cytoplasmic staining) were estimated as a proportion of all cells. In 48 well plates, the dead cells that failed to show any colour development remained attached to the plastic. Therefore, viable and dead cells were counted in 5 separate fields and the mean percentage live cells per well was used as a 'viability score'. (It should be noted that the attachment of the

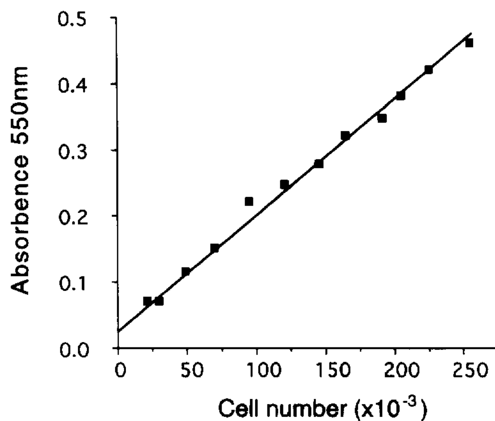

Fig. 1. Linearity between GH_3 cell number and absorbence at 550 nm. Cells were seeded at densities between 10,000 and 100,000 cells per well in 48 well plates and after 48 h, were harvested for counting or for the measurement of absorbence at 550 nm following the addition of MTT as described in the text.

dead cells was dependent on the type of culture plastic used.) In subsequent figures, values for overall viability are means for treatment replicates (3 or 4 wells) and in the majority of experiments shown, for clarity, they have been rounded to the nearest 5%. In some experiments, to check for membrane integrity following treatment of cells, culture medium was removed and 100 μl of a solution of acridine orange (1 μM) and ethidium bromide (25 μM) in phosphate buffered saline (PBS) was added to the cell monolayer. A red nuclear fluorescence under UV light indicated that membrane integrity was lost.

DNA breakdown (TdT-mediated dUTP nick end labelling-TUNNEL)

Cells seeded into two chamber slide flasks (Nunc) in DMEM with 10% SFSC at a density of 40,000 cells/cm^2 were made steroid free 24 h later and treated with 10 nM ZM 182780. After 48 or 72 h of treatment, medium was removed and cell monolayers were washed ($\times$3) with ice cold PBS. Cells were then fixed for 30 min at room temperature with 2% paraformaldehyde in PBS. Fixed monolayers were then washed ($\times$2) as above and cell membranes were made permeable by a 2 min exposure to a solution of 0.1% Triton X-100/0.1% sodium citrate at 4°C. Following this, cells were again washed ($\times$2) with PBS and then 50 μl of a solution in water of biotin-dUTP (0.5 nmol/50 μl), dATP (3 nmol/50 μl), $CoCl_2$ (1 nM), TdT (25 U/50 μl) and 10 μl TdT-buffer was placed on the cell monolayer and this was incubated for 60 min at 37°C in a humid atmosphere. This reaction was then stopped with 2 μl of 0.5 M EDTA and then one wash was made with PBS followed by a wash with PBS containing 1% Triton X-100 (PBS/Triton). Detection of the biotin end-labelled DNA fragments was then made by exposure of the monolayer for 45 min at room temperature to streptavidin-TRITC (Rhodamine) at a 1:500 dilution in PBS. Finally the excess detection reagent was removed by 3 washes with PBS followed by 3 washes with PBS/Triton and then again by 3 washes with PBS. 10% Glycerol was then placed on the monolayers followed by a cover slip. Photographs were then taken with a fluorescence microsope.

Transfection

Transfection of GH_3 cells was performed by electroporation using the reporter plasmids MTV-ERE-LUC and MTV-LUC and luciferase activity was measured 24 h after treatment as described in earlier studies [27].

RESULTS

Time-course and characteristics of antiestrogen induced cell death

Our previous studies on the role of the ER within pituitary GH_3 cells were conducted under steroid free conditions and in the absence of serum [25–28]. Under these conditions, serum and steroid independent growth was observed and this was blocked by the pure antiestrogen. In contrast, for the large majority of experiments reported here, treatment of GH_3 cells with 10 nM ZM 182780, 24 h after seeding at 10,000/cm^2 in DMEM containing 10% FCS, resulted in death, 4–5 days after treatment, as indicated by the time-course experiment of Fig. 2(a). Here cell numbers were measured with a Coulter counter and viability was estimated in parallel wells of cells by the addition of MTT [Fig. 2(b)]. Clearly dead cells (days 4 or 5), as evidenced by the cessation of mitochondrial metabolism, remained loosely attached to the culture well surface. The number of these cells could still be determined with the Coulter counter, although membrane integrity was poor as incubation with acridine orange/ethidium bromide gave red nuclear fluorescence under UV light. After a further 3 days, removal for counting by trypsinization, resulted in the complete break-up of the cells.

To determine whether prior to the loss of mitochondrial function, as evidenced by the lack of colour formation with the MTT assay, DNA breakdown occurred in the presence of 10 nM ZM 182780, DNA labelling was performed with the TUNNEL technique as described above. In contrast to control samples, where only a faint cytoplasmic fluorescence was detected [Fig 3(A and C)], a large number of cells treated for 48 or 72 h with antiestrogen showed an intense nuclear fluorescence [Fig. 3(B and D)]. By the addition of acridine orange/ethidium bromide to parallel wells of antiestrogen treated cells, no loss of membrane integrity was noted at this stage. It should be noted that for all experiments where cells were treated with ZM 182780, a marked cytoplasmic and nuclear shrinkage was observed 2–3 days after treatment. This observation was not only true of the cells maintained with

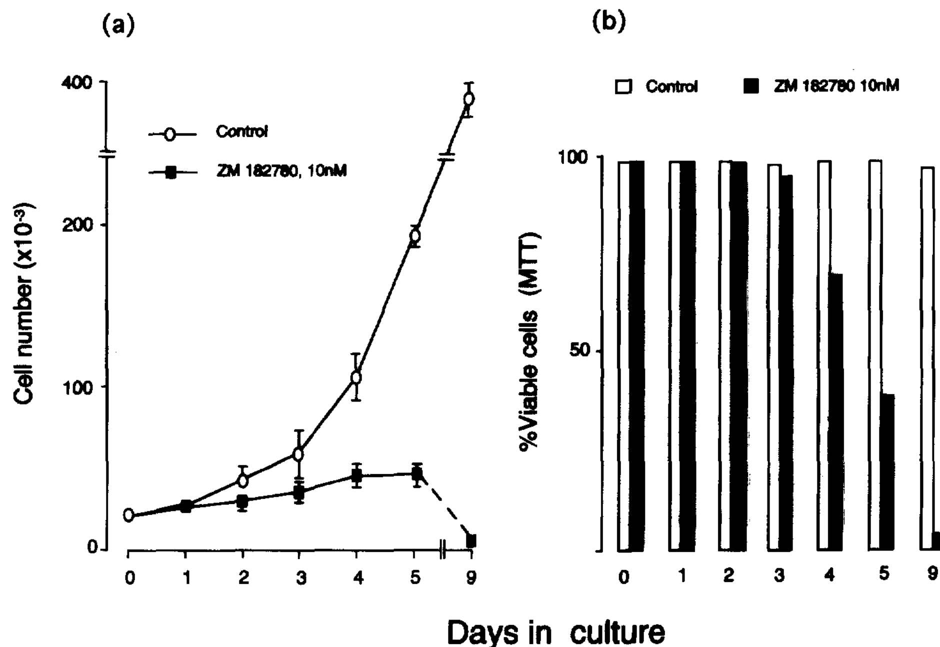

Fig. 2. Time-course for death of GH_3 cell in response to 10 nM ZM 182780. Following seeding of cells at a density around 10,000 cells in 48 well plates (1 cm^2 culture area) in DMEM with 10% FCS, medium was changed for the same, but to some plates, 10 nM ZM 182780 was added. At the time of treatment and on subsequent days thereafter, cell numbers were determined with a Coulter counter as shown in (a) and in parallel wells, viability was determined following the addition of MTT as shown in (b).

Fig. 3. DNA breakdown within GH_3 cells treated with 10 nM ZM 182780. Cells were seeded in 2 chamber slide flasks at a density of 40,000 per side in 1 ml of DMEM (10% FCS) and after 24 h medium was changed to phenol red free DMEM (10% SFCS) without (A and C) or with (B and D) ZM 182780. Following a 48 h (A and B) or 72 h (C and D) medium was removed and DNA breaks were labelled by the TUNNEL technique as described. For (A) and (B) an objective was used giving 200 × overall magnification, whereas (C) and (D) are pictures taken at 400 × overall magnification.

serum, but was also apparent for cells maintained for several months under low serum conditions as indicated previously [25–28] and treated serum free or with 10% SFCS. Also in none of these experiments was there a loss of mitochondrial function or membrane integrity in response to antiestrogen. For the current study where cell death was observed, the rounding up of the cells in the presence of antiestrogen meant that the majority of the cell volume was occupied by the nucleus. In 25 experiments where clear cell death occurred in response to antiestrogen treatment, no evidence of cytoplasmic swelling occurred to indicate that death was induced by necrosis. Although following the loss of mitochondrial activity, acridine orange/ethidium bromide staining indicated a loss of cell membrane integrity, there was now a supercondensation of nuclear DNA giving an apparent nuclear size, around 1/5 that of the normal nucleus (not shown). Under the current definitions of cell death [37, 38], these observations suggested that antiestrogen treatment of these cells was inducing a process of cell death with more of the reported characteristics of apoptosis than those of necrosis.

Steroid withdrawal cell death and estrogen reversal

Since medium containing 10% FCS contained around 20 pM E_2 and a phenol red preparation, previously shown to provide an estrogenic stimulus [39], we next tested the effect of acute steroid withdrawal by changing medium, 24 h after seeding, to phenol red free DMEM, containing 10% SFCS. For 30 experiments conducted in this way, clear cell death by day 5 of treatment was observed in 25 and the overall proportion of surviving cells, taken as the range of mean values for individual experiments, was 40–90% (median 75%). The inclusion of 10 nM ZM 182780 further reduced overall viability in all these experiments such that the range was reduced from <5 to 50% (median 30%). The specificity of steroid withdrawal and antiestrogen treatment for the ER was confirmed by the inclusion in the steroid depleted medium of a dose range of estrogens alone [Fig. 4(a)] or a dose range of estrogens in combination with 10 nM ZM 182780 [Fig. 4(b)]. For the experiment shown in Fig. 4(a) overall viability on day 5 was increased from 50% to the maximum (98%) by the inclusion of 10 pM E_2 or 100 pM estrone (E_1) and the numbers of cells in each well was further increased by a 10-fold higher concentration of each of these estrogens (100 pM E_2 or 1 nM E_1). The data presented in Fig. 4(b) for an experiment where viability in the presence of 10 nM ZM 182780 was around 25% shows that now at least 10 nM E_2 was required to fully reverse the effects of the antiestrogen. Here it was apparent that in the presence of a suboptimal concentration of ZM 182780 alone (100 pM) or where the effects 10 nM ZM 182780 were partially reversed by estrogens [1 nM E_2; 100 nM E_1 or estriol

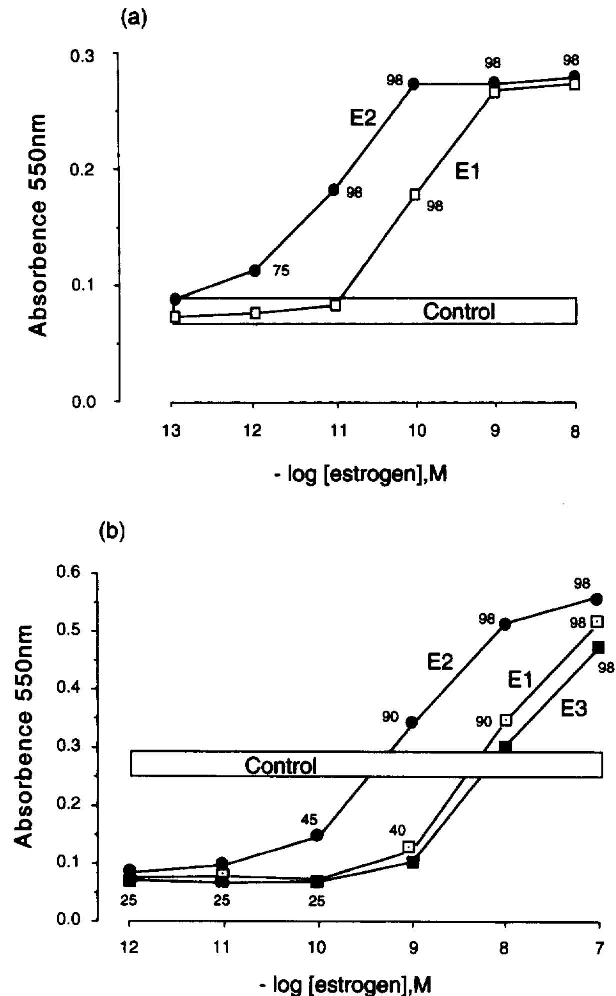

Fig. 4. Estrogen reversal of steroid withdrawal (a) and antiestrogen induced death, (b). For both experiments, cells seeded at around 10,000 cells per well were made steroid free after 24 h and treatments were made with dose ranges of estrone (E_1) or estradiol (E_2) alone as shown in (a), or in the additional presence of 10 nM ZM 182780, in (b). In (b) estriol (E3) was also co-incubated with the antiestrogen. For clarity error bars have been omitted, but as for all other growth experiments presented, the CV at each data point was less than 10%. Since each data point represents colour formation by live cells on day 5 of treatment, the estimated (as described in Experimental) proportion these cells constituted of the total population (live and dead), is given by the numbers next to data points. Viability for the untreated control cells, was around 50% in (a) and 75% in (b).

(E_3)] cell death was more apparent at the edges of the wells, where cells were at higher density. [At seeding in 48 well plates there was a tendency for cells to cluster at higher density at the edges of the wells. For Fig. 4(b), 90% overall viability is the nominal score given to reflect this observation.]

Comparison of ZM 182780 with 4-OHT

Initial studies under steroid containing conditions indicated that no marked death of GH_3 cells could be induced on incubation with 10 nM 4-OHT over a 5 day

period. To confirm these observations under steroid free conditions, cells were seeded at 10,000 per cm^2 from stock conditions and after 24 h, treatments were made with a concentration range of ZM 182780 or 4-OHT in phenol red free DMEM containing 10% SFCS. For the experiment represented in Fig. 5(a), survival of cells in medium without treatment was around 50% and the pure antiestrogen dose-dependently decreased survival to around 25% at 10 nM and above. In contrast, no enhancement of cell death was observed with any concentration of 4-OHT and contrary to ZM 182780, 4-OHT increased cell survival to around 75% at a concentration of 1 μM.

In order to test whether the high dose effect of 4-OHT represented a true estrogenic response to the compound, cells grown to around 75% confluence in 75 cm^2 culture flasks, were washed with three changes of phenol red free medium containing 10% SFCS over a 2 h period and then transfected with the reporter plasmids ΔMTV-ERE-LUC and ΔMTV-LUC as previously described [27]. Treatments were made with a dose range of ZM 182780 or 4-OHT and luciferase activity was measured in cell lysates 24 h later. Figure 6 shows data from one of three identical experiments. As we have reported previously for serum free conditions [26–28], even in the absence of estrogen, the ER was clearly able to enhance transcription from this promotor and this was blocked by the pure antiestrogen. In contrast, 4-OHT was less effective than the pure antiestrogen at concentrations of 10 and 100 nM and it showed a clear agonist effect at a concentration of 1 μM. The other two experiments gave qualitatively similar results.

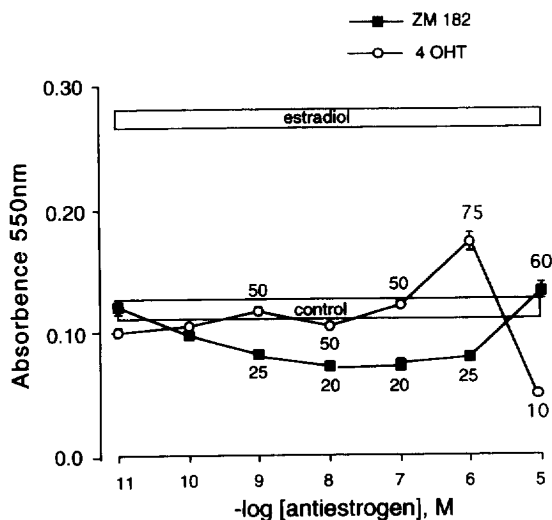

Fig. 5. Comparison of the abilities of ZM 182780 and 4-OHT to enhance steroid withdrawal death of GH_3 cells. Cells were seeded as described in Fig. 4 and MTT estimates of survival were made on day 5 of treatment. Again numbers next to data points represent nominal percentage survival. For the no-treatment control cells, around 45% survival was noted, whereas in the presence of E_2 (100 pM) maximal survival was observed (98%).

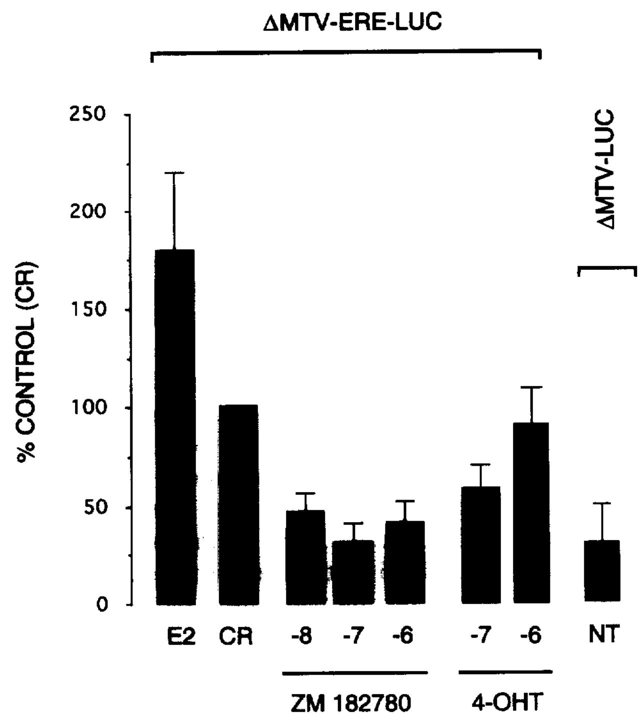

Fig. 6. Luciferase expression by GH_3 cells transfected with the reporter plasmid ΔMTV-ERE-LUC in response to 24 h treatment with ZM 182780 and 4-OHT as indicated. Treatments were made in phenol red free DMEM containing 10% SFCS after a steroid free 'washout' of the cells. All values, including those for transfections conducted with the ΔMTV-LUC control plasmid, are expressed as a percentage (based on mean, $n = 3$) of the untreated control transfection (CR) with the ΔMTV-ERE-LUC reporter plasmid. Errors are given as CV for untransformed data. NT = no treatment.

Cell death as a function of culture density and the effects of steroid free autocrine conditioned medium (CM)

As steroid depletion alone was less effective at inducing the death of these cells than when antiestrogen was

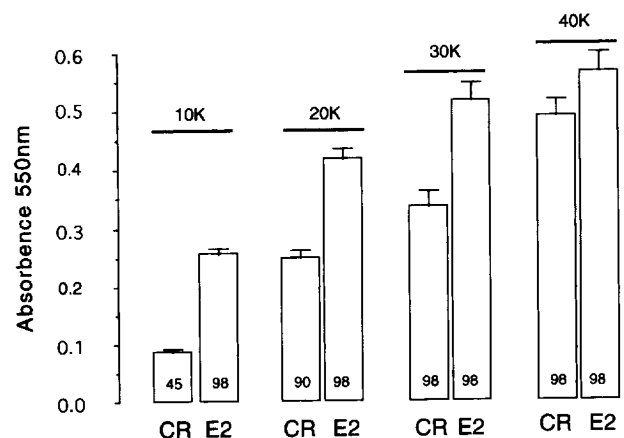

Fig. 7. Steroid withdrawal death as a function of seeding density. Cells seeded at the density indicated were made steroid free after 24 h and to some wells at each seed density, E_2 (100 pM) was added. A visual estimate of the proportion of surviving cells was made (number at the bottom of bars), followed by the measurement of dye absorbence at 550 nm.

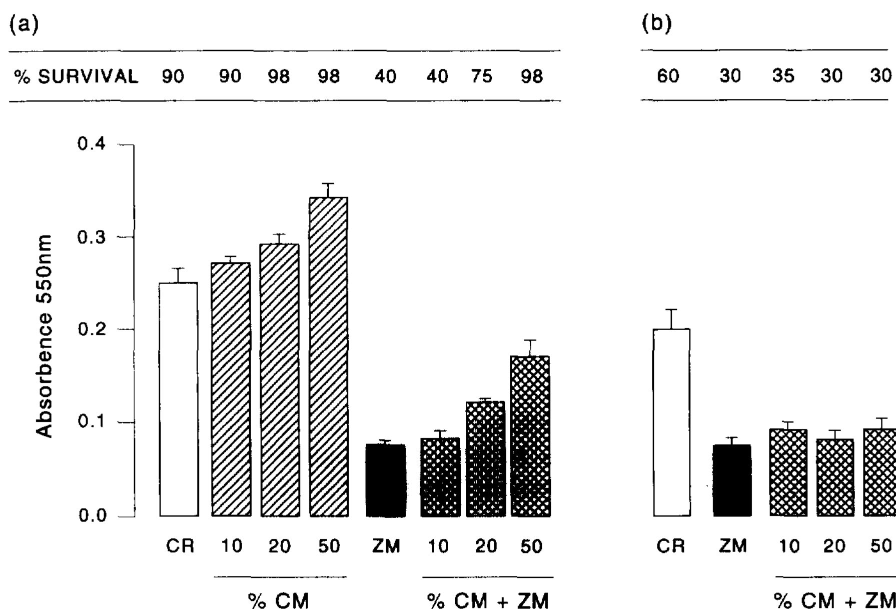

Fig. 8. (a) Survival of low density treated, steroid deprived GH_3 cells, in response to conditioned medium (CM) from high density, untreated steroid free cultures and (b) survival of low density treated cells in response to CM from high density steroid free cultures maintained with 10 nM ZM 182780 as described in the text. CM was added to cells in the proportions indicated mixed with fresh phenol red free DMEM containing 10% SFCS. Where indicated ZM 182780 was added at a concentration of 10 nM. Estimates of live cells and overall viability were made after 5 days of culture.

included in the medium and since we have previously shown that autocrine factors are responsible for basal transcriptional activation of the ER [28], we investigated steroid withdrawal death as a function of initial treatment density. Following the seeding of cells at 10, 20, 30 and $40,000/cm^2$ in medium containing steroids, medium was replaced 24 h later with 10% SFCS. Figure 7 shows that for seeding densities above $10,000/cm^2$, cell death failed to occur in the absence of steroids. The addition of E_2 to cells seeded at $10,000/cm^2$ protected these cells from steroid withdrawal death. At higher densities, E_2 had a clear effect on the growth rate of these cells, however, this effect became minimal at the highest treatment density tested.

Although these observations were consistent with the ability of autocrine factors to enhance the transcriptional state of the ER, we also sought to determine whether the transcriptionally activated ER was responsible for the production of autocrine survival factors. For the first of these experiments, we chose to use 24 h steroid-free CM from cells seeded 24 h earlier at high density ($100,000/cm^2$) in medium containing 10% SFCS. Following centrifugation to remove any non-attached cells, this CM was added back to cells seeded 24 h earlier in 48 well plates at 10,000 cells/cm^2 in DMEM containing 10% FCS. After 5 days, control cells in phenol red free medium containing 10% SFCS showed a marked loss of viability at the edges of the wells where density was highest, however, the addition of 20 or 50% steroid free CM completely protected these cells, giving a viability similar to those treated with 100 pM E_2 [Fig. 8(a)]. In addition this CM dose-dependently blocked death induced by the pure antiestrogen, leaving only a reduction in the rate of growth of these cells.

In order to distinguish between the possibilities that the transcriptionally activated ER was responsible for the production of autocrine survival factors or that survival factors were produced by ER independent pathways, a similar experiment as presented in Fig. 8(a) was conducted on CM obtained from high density cells (100,000 cell/cm^2) maintained for 72 h in the presence of 10 nM ZM 182780. Phenol red free medium containing 10% SFCS and the antiestrogen was changed every 24 h following seeding and the final medium change was collected as 24 h CM. In this way it was assured that the medium would be largely depleted of ER-induced survival factors. Although the CM now contained ZM 182780, survival factor production, independent of ER activity (but proportional to cell number), should have been detected on addition of this medium to low density, steroid depleted cells, under the protocol of the experiment in Fig. 8(a). As indicated in Fig. 8(b), no survival advantage, in comparison to cells treated alone with ZM 182780, was apparent. These observations strongly suggested that the production of autocrine survival factors was dependent on the transcriptional activity of the ER and not just dependent on culture density.

DISCUSSION

In cell types genetically programmed to divide, the expansion of a tissue mass is determined by the rate of cell division and the rate of cell death. Tumorogenesis

may be viewed therefore, as either an increase in the rate of cell division with no increase in cell death, or no change in the rate of cell division but a decrease in the rate of death. Evidence has now been presented for the latter for a number of cell types in response to tumour promotors [40]. Until relatively recently, the majority of studies conducted on new anti-endocrine agents have focussed on their anti-mitogenic potential. This situation is now undergoing rapid revision due to the growing awareness that a cellular pathway involved in increasing the rate of cell division can also be involved in cell survival [41]. The observations described in this report amply support this concept.

Therefore using pituitary GH_3 cells, for which we and others have previously shown the ER to be a mediator of growth [26, 42], the current study demonstrates that blockade of the ER by the pure antiestrogen, ZM 182780 induces widespread death of these cells, 3–5 days after first exposure. For our previous studies we used cells adapted to grow in a low serum, steroid free medium. Growth of these cells was still apparent in the absence of serum and this could be increased by the cytokines, insulin-like growth factor-1 (IGF-1) [26, 27] and interleukin-2 (IL-2) [28] and these growth effects were blocked by pure antiestrogens. Antiestrogen induced cell death was never observed in any of these experiments nor were these cells shown to be growth responsive to estrogens. By contrast, cell death reported here was observed when the pure antiestrogen ZM 182780 was added to serum and steroid replete cells and death in the presence of antiestrogen was further enhanced by the acute removal of steroids. The only other published *in vitro* observations similar to those of the current study, using concentrations of an antiestrogen appropriate for transcriptional blockade of the ER only, come from a study on the MCF-7 breast cancer cell line [35]. These authors reported that clear death could be induced 3–4 days after exposure to the antiestrogen, 4-OHT. Their observations, as well as those reported here, suggested that the process of death was closer to that reported as apoptosis than it was to necrosis [37].

More usually an apoptotic process is associated with rapid death following exposure to a particular agent [37, 38, 43]. However, the observations on the time course of antiestrogen-induced death presented here are not necessarily in conflict with this, if one considers the potential mechanism for the initiation of the death process. From the decrease in cell size on antiestrogen treatment, which from previous serum free studies [25–28] was not a strict indicator that death would follow, a decrease in synthetic capacity was indicated. It follows from this, that as opposed to control by the ER of a component or components of the processes of cell management, such as metabolism or respiration, a more specific pathway is modified by the antiestrogen. In this respect the conditioned medium experiment of Fig. 8 strongly suggests that in addition to maintaining a growth stimulus, the transcriptionally activated ER is responsible for the production of autocrine/paracrine survival factors. Therefore on first exposure of cells to the pure antiestrogen, transcription of ER target genes should be greatly reduced. However, the decline in the proteins/peptides coded would depend on mRNA half-life and that of the protein or peptides coded. This would therefore allow significant growth before such growth/survival mediators were exhausted, leading to growth arrest and cell death. For many experiments, including the one presented in Fig. 2(a), it was clear that cells were still able to divide, albeit at a greatly reduced rate as compared to controls, upto the time of obvious cell death on day 4.

Although stressed earlier in the results section of this paper, it is important to reiterate here that the experiment of Fig. 8 was performed under steroid-free culture conditions. As the experiment of Fig. 7 demonstrates, the requirement for estrogen to maintain survival of these cells is circumvented by seeding these cells at high density. From our transfection data presented here and shown in an earlier report from this laboratory [28], it is clear that the ER within GH_3 cells is made transcriptionally active by signal transduction pathways activated by autocrine or paracrine acting peptide factors. Therefore the observations from the experiment of Fig. 8(b), showing that conditioned medium from high density cells treated with antiestrogen, fails to protect low density cells from death, allows for the conclusion that it is the peptide factor activated ER that is responsible for the survival of these cells. Indeed, it may be that peptide factors that induce the activation of the ER, also act as survival factors thus giving a strong re-inforcing survival feedback loop. This proposal may have significant implications for tumour development, since by this means, tumour expansion that is initially estrogen dependent, will gradually progress to a state of total estrogen independence.

From studies on fibroblast cell lines it has been proposed that growth and death are linked and that death by apoptosis occurs when growth pathways are switched on in the absence of survival pathways [44]. Although not presented here, in a number of experiments, death of GH_3 cells after acute steroid depletion, failed to occur in the presence of 10 or 100 nM ZM 182780. Instead complete growth arrest was observed. These observations were consistent with our original studies under serum free conditions and suggested that the degree of growth escape in the presence of antiestrogen observed in Fig. 2 and in all experiments where obvious death occurred after 4 days of treatment, was necessary for the initiation of the death process. Growth in the presence of antiestrogen may therefore have been due to an incomplete blockade of the ER or be due to the activation of growth pathways that were independent of the ER.

Under serum-free conditions it has been observed that a number of non-steroidal mitogens for GH_3 cells

decrease the effectiveness of pure antiestrogens to block growth [Newton C. J., unpublished observation]. This may be attributable to the strong activation of ER independent pathways or it could be due to the activation of signal transduction pathways that directly modify the transcriptional activity of the ER [45]. In this respect, it has been shown that depending on the cell and promotor context, strong activation of the cAMP response pathway converts the 'pure antiestrogen', ICI 164384, into an estrogen [46]. Potentially therefore the ER may retain a sufficient degree of transcriptional activity in the presence of antiestrogen to maintain expression of growth related genes but not of genes required for cell survival. At present it cannot be ascertained why for some experiments pure antiestrogens were able to completely block growth. However, over a 3 year period of cultivation within this laboratory, changes in basal growth rate and morphology of GH_3 cells have been observed. These changes may be indicative of subtle alterations in intracellular pathways, some of which could directly effect the ER. In turn, these changes might reduce the efficiency with which antiestrogens block the transcriptional ability of the ER.

Further evidence to support an idea that death of GH_3 cells might be induced by an imbalance between ER controlled growth pathways and ER induced survival pathways, comes from a series of more recent, unpublished studies in this laboratory where growth arrest, without cell death, was achieved with 10 nM ZM 182780. Here it was noted that marked cell death was observed with a concentration of ZM 182780 capable of giving partial ER blockade. In the absence of estrogens, the concentrations of pure antiestrogen needed was between 0.1 and 1 nM. Here marked growth was observed but, around day 5 of treatment, rapid cell death occurred over a subsequent 24 to 48 h period affecting as much as 90% of the cell population. No obvious cell death was noted in the control wells, where cell density was higher, indicating that this was not due to medium depletion. These findings might go part way to explain the observation from studies on aromatase inhibitors, where partial estrogen deprivation was shown to be sufficient for the regression of DMBA induced tumours in rats without having any obvious effect on the uterus [47]. Based on a theory of an imbalance between cellular growth and survival signals as suggested here and previously [48] these data support the idea that death is occurring by apoptosis. At present we are continuing these studies on suboptimal receptor blockade, by looking at the effect of a range of partial ER antagonists, included amongst which is 4-OHT.

As yet the precise intracellular mechanisms controlling growth and survival of the pituitary GH_3 cell line are are not yet elucidated but it would appear that the ER has a major role to play in this respect. Studies are currently in progress in this laboratory that show that low molecular weight autocrine/paracrine factors are involved in GH_3 cell survival. The elucidation of the nature of these factors and the molecular events they facilitate to prevent antiestrogen induced cell death may help in the wider understanding of the mechanisms by which the ER is involved in maintaining cellular homeostasis and also as to the way antiestrogen resistance develops in therapeutic target tissues such as the breast [6].

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