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Estrogen Causes Cell Death of Estrogen Receptor Stably Transfected Cells via Apoptosis

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We examined the effect of estrogen on the growth of estrogen receptor (ER) stably transfected cells (Rat1 + ER). 17-β-estradiol (E2, 10 nM) inhibited approximately 35–50% of Rat1 + ER growth after 3 d of treatment. The half-maximal growth inhibition occurred at 0.5–0.75 nM of E2 concentration and was saturated above 10 nM. This E2-induced antiproliferative effect was mediated through the ER since E2 did not cause any change in ER-negative parental Rat1 cells. Cells started to detach from plates and the adherent cells exhibited nuclear condensation. Apoptotic cell populations showed a 25% increase at 2 d of E2 treatment over controls that were quantified by fluorescence-activated cell sorter analysis. This indicates that E2 induced apoptosis in Rat1 + ER cells. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

Estrogen increases the growth of cells and tissues such as MCF-7 cells, breast, and uterus [1, 2]. The exact mechanism by which estrogen increases cell proliferation is not yet known. Many growth-related genes have been shown to be regulated by estrogen both directly and indirectly [3].

However, *in vivo* administration of estrogen to rats results in uterine or pituitary growth for only a short time [4]. DNA synthesis initially increases after estrogen is administered, but after 2–3 d of treatment, synthesis decreases to control or lower levels. Similarly, pituitary DNA synthesis decreases to control or lower levels after 3–7 d of estrogen treatment, with the exception of the Fischer 344 rat, in which continuous administration of estrogen results in pituitary tumors [5].

Previously, Rat1 + ER cells were constructed by stable transfection of human estrogen receptor (ER) cDNA (HEO) into Rat1 cells, which do not normally express ER [6]. HEO behaves normally except that it has a 10-fold lower affinity for the ligand and is unstable *in vitro* at 25°C as compared with the wild type ER due to a point mutation on glycine 400 [7]. Rat1 + ER cells stably express 20,000–50,000 ER/cell, which is equivalent to rat uterine ER levels [6]. The newly expressed ER is functional as determined by its specific affinity for estrogen and transcription activation of estrogen-responsive reporter plasmids [6]. Furthermore, two estrogen-responsive genes, the progesterone receptor and ER gene, were affected [6, 8, 9]. However, these estrogen responses were selective since two other endogenous estrogen-inducible genes, the prolactin and epidermal growth factor receptor genes, were not activated [6]. All this indicates that Rat1 + ER cells have acquired estrogen responsiveness.

We have studied the effects of estrogen on the growth of Rat1 + ER cells. Despite the fact that estrogen stimulates growth of breast cancer cells and many other tissues [9], the growth of Rat1 + ER cells is inhibited by 17-β-estradiol (E2) in a time-specific and dose-dependent manner. This paradoxical observation is consistent with reports by other investigators; that the growth of ER-stably transfected cells are either unaffected or inhibited by estrogen, or that certain cell lines are even killed by estrogen [3].

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Furthermore, Rat1 + ER cells start to detach from the plates at 1.5–2 d of E2 exposure. This appears to occur via an apoptotic pathway as determined by nuclear morphology, DNA laddering, and cell cycle analysis.

MATERIALS AND METHODS

Cell culture conditions

Cells were grown in phenol red-free, glucose (1000 mg/l) Dulbecco's modified Eagle's medium [DMEM (Sigma, St. Louis, MO) containing a 1× antibiotic/antimycotic mix (GIBCO, Gaithersburg, MD), 5 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid, and 0.37% sodium bicarbonate], supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT). Cells were grown at 37°C in a humidified atmosphere of 95% air/5% CO_2 and fed every 1–2 d.

Hormones

All E2 treatments were done with 10% dextran/charcoal-stripped FBS [10] containing medium (DMEM + 10% ST-FBS). 10 nM of E2 was used to maximize the response unless otherwise noted. E2 was purchased from Sigma. ICI 182,780 (ICI) and monohydroxytamoxifen (MHT) were obtained from ZENECA Pharmaceuticals, Macclesfield, Cheshire, U.K. All estrogenic compounds were dissolved in ethanol and diluted 10^{4-5} with medium.

Cell growth assay

Rat1 and Rat1 + ER cells were plated onto 24-well plates at densities of 1000 cells per well in triplicate for a long-term growth response experiment and 10,000 cells per well in duplicate for a short-term growth response experiment and an E2 dose–response experiment. Before E2 induction, the cells were washed with Hank's buffered saline solution (HBSS; Sigma) and cultured in DMEM supplemented with DMEM + 10% ST-FBS for 1–2 d. The medium was changed each day while the cells were growing with or without ligand for the indicated time periods. The cell monolayers were washed with HBSS and stored at −20°C until the DNA assay was performed.

DNA assay

0.5 ml of hypotonic 0.1× calcium- and magnesium-free HBSS was added to each well and the cells were lysed by sonication with a Kontes ultrasonic cell disrupter (Kontes; Vineland, NJ). To determine the DNA concentrations in the cell lysates, 100 μl of each sample was incubated in the dark for at least 1 h with 1.5 ml of PES (2 M NaCl, 50 mM sodium phosphate, and 1 mM EDTA, pH 7.4) and 1 μg/ml Hoechst 33258 dye [11]. The fluorescence of each sample was measured with a SLM-Aminco Fluoro-Colorimeter (SLM Instrument, American Instrument, Urbana, IL). A linear standard curve was generated using sheared calf thymus DNA. Total DNA content was used as a measure of cell number. Each data point represents an average of duplicate or triplicate points.

Nuclei staining

Cells were grown in 100 mm dishes and treated with estrogen for 1.5–2 d. Adherent cells were fixed with 4% paraformaldehyde for 2 min and stained with 10 μg/ml Hoechst 33258 for 5 min at room temperature in the dark. Cells were viewed under an Nikon Optiphot-2 fluorescence microscope (Nikon, Tokyo, Japan) using a 4′,6′-diamidino-2-phenylindole (DAPI) filter.

Flow cytometric analysis of apoptosis

Both adherent and floating cells were pooled and washed with PBS. Cells were fixed with 75% ethanol and kept at 4°C for up to 7 d until measured. Before the analysis, cells were washed again in phosphate citric acid buffer (0.19 M sodium phosphate, 0.4 mM citric acid, final pH 7.8), suspended in 1 ml cold propidium iodide solution (1 mg/ml RNaseA, 50 μg/ml propidium iodide, 0.2% NP-40, in PBS), and incubated on ice for at least 30 min in the dark. Fluorescence-activated cell sorter (FACS) analysis was performed on a FACScan (Becton Dickinson, Sparks, MD) flow cytometer. The percentage of cells in each phase of the cell cycle was analyzed by PC-LYSYS software (Becton Dickinson).

RESULTS

Time-course of cell growth

The growth of Rat1 + ER cells was inhibited in the presence of 10 nM E2. These effects were apparent in this experiment beginning on the third day of treatment (Fig. 1(B)) and continued for 8 d (Fig. 1(A)); the final time point of the experiment. Figure 1(A) and (B) are shown separately, because the concentration of DNA in the samples before day 4 in Fig. 1(A) was below the sensitivity of the fluorometric assay [11]. The total amount of DNA in the Rat1 + ER cells grown in 100 nM MHT was similar to that in 10 nM E2. MHT, which is a partial antagonist, behaved like an E2 agonist in this system.

ICI, a pure antagonist that acts as a competitive inhibitor of estrogen, allowed normal growth in the Rat1 + ER cell line. This suggests that the basal level of growth was not influenced by the low endogenous estrogen from 10% dextran/charcoal-stripped FBS containing medium (DMEM + 10% ST-FBS). This is at least partially due to the fact that the point mutation in the HEO lowered the affinity for estrogenic

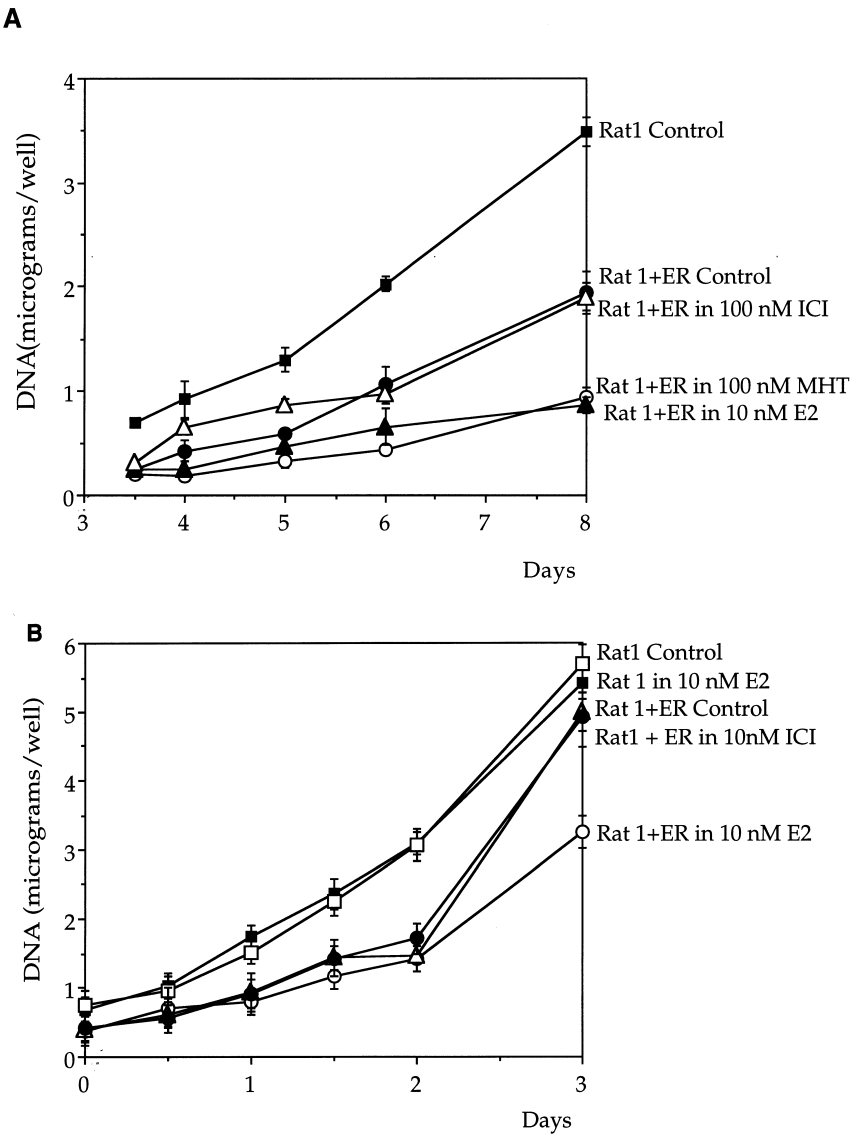

Fig. 1. Estrogenic hormone effects on Rat1 and Rat1 + ER cell growth. Rat1 and Rat1 + ER cells were plated onto 24 well plates at densities of 1000 cells per well in triplicate for the long-term growth response experiment and 10,000 cells per well in duplicate for the short-term growth response experiment. Before hormone treatment, cells were cultured in DMEM + 10% ST-FBS for 24–48 h. The medium was changed each day while the cells were growing with or without ligands, for the indicated time periods. The cell monolayers were washed with HBSS and stored at −20°C until the DNA assay was performed. Total DNA was then measured from the cell lysates fluorometrically using Hoechst 33258 dye [11]. Total DNA content was regarded as a measure of cell number. Each data point represents an average of duplicate or triplicate points. (A) Long-term growth response assay: cells were grown under the ligands up to 8 d. Total DNA per well from 3.5–8 d is shown. – ■ –, Rat1 control; – ● –, Rat1 + ER control; – △ –, Rat1 + ER in 100 nM ICI; – ▲ –, Rat1 + ER in 100 nM MHT; – ◦ –, Rat1 + ER in 10 nM E2. (B) Short-term growth response assay: total DNA per well from 0-3 d from each sample is shown. – ■ –, Rat1 control; – □ –, Rat1 in 10 nM in E2; – ● –, Rat1 + ER control; – △ –, Rat1 + ER in 100 nM in ICI; – ◦ –, Rat1 + ER in 10 nM E2.

ligands as compared with the wild type ER, such that the HEO was unaffected by the low levels of estrogen in the medium [7, 12].

The Rat1 cells grew faster, based on total amount of DNA, than the Rat1 + ER cells (Fig. 1(A) and (B)). The cells were counted once and equal volumes of the samples were seeded to avoid a counting error. These results were observed consistently in several experiments. It may be that transformation caused the Rat1 + ER cells to grow at a slower rate than the Rat1 cells or that unoccupied ER may have some other effect on Rat1 + ER growth.

Short-term growth was assayed to determine when the decrease in total DNA was first evident after E2 treatment. The decrease in the number of Rat1 + ER cells treated with E2 compared with no treatment was significant at 3 d of growth (Fig. 1(B)). This experiment also showed that the growth of Rat1 cells

was not affected by the presence of E2, which confirmed that Rat1 cells are ER-negative.

Dose response of E2 on the growth of Rat1 + ER cells

A dose–response experiment was performed to determine if the decrease in DNA is correlated with ER occupancy. Rat1 + ER cells were treated with increasing concentrations of E2 for 3 d and the medium was replenished each day during the treatment period. The total DNA from adherent cells decreased as the dose of E2 increased from 0.01–10 nM (Fig. 2). In this experiment, the half-maximal decrease in total DNA occurred between 0.5 and 0.75 nM E2 concentration. This agrees with the reported equilibrium dissociation constant (K_d) of approximately 1 nM for [7]. The cell growth response was saturated above 10–50 nM. Therefore, the decrease in cell number was dependent on receptor occupancy.

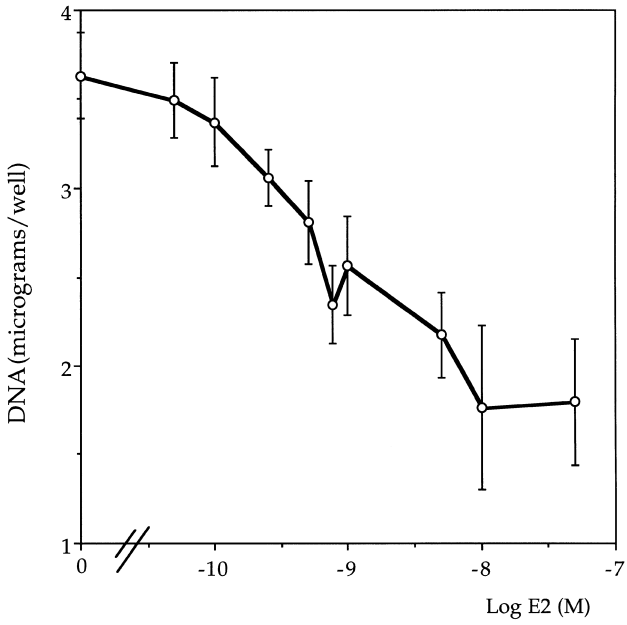

Fig. 2. Dose–response of E2 on the growth of Rat1 + ER cells. Rat1 + ER cells were plated onto 24-well plates at densities of 10,000 cells per well in duplicate. Before hormone induction, cells were cultured in DMEM + 10% ST-FBS for 24–48 h. The medium was changed each day while the cells were growing, with increasing concentrations of E2 for 3 d. Measurement were performed as in Fig. 1. Each data point represents an average of duplicate points.

Morphological and biochemical evidence for apoptosis

Interestingly, we observed that cells detach from the plates at 1.5–2 d of E2 treatment. The phenomenon was further explored and examined. The floating Rat1 + ER cells were shrunken and excluded trypan blue (data not shown), suggesting that at least these cells were not necrotic. Initially, cells were treated with estrogen for 1.5–2 d and stained with Hoechst dye. Hoechst dye traverses through intact plasma membrane such that the nuclei of apoptotic and live cells can be stained. Under a fluorescence microscope, condensed nuclei were detected with E2-treated adherent cells whereas very few condensed nuclei were found in untreated cells (Fig. 3).

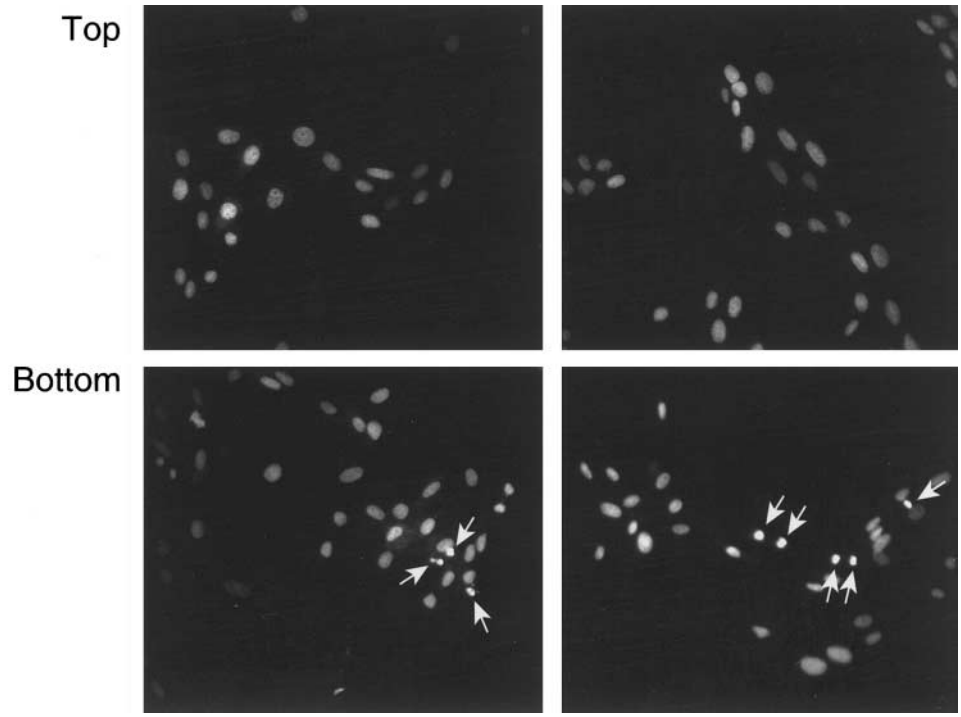

Fig. 3. Morphology of Rat1 + ER cells. Cells were grown and treated with E2 (10 nM) for 1.5–2 d. The medium was removed and adherent cells were fixed with 4% paraformaldehyde and stained with 10 μg/ml Hoechst 33258 solution in PBS for 5 min at room temperature in the dark. Stain solutions were washed out and observed using a fluorescence microscope under a DAPI filter. (Top) Nuclei from untreated Rat1 + ER cells. (Bottom) Nuclei from E2-treated Rat1 + ER cells. The arrows indicate condensed nuclei.

Flow cytometric quantification of apoptosis

Flow cytometric measurement was used to quantify the extent of apoptosis in the total cell population, combining both adherent and floating cells. A hypodiploid DNA peak under FACS analysis corresponds to apoptotic nuclei with low DNA content and has been considered to be a marker of apoptosis [13]. This analysis has the additional advantage in that the distribution of normal cell cycle phases can be quantified as well.

We observed that E2 caused about a 25% increase in apoptotic cells from a total population of cells treated for 2 d with E2 as compared with the untreated groups (Fig. 4). Not all the floating cells exhibited a single sub-G1 peak due to the loss of DNA. This probably is because S phase or G2 phase cells that contained more than the diploid content of DNA and were also undergoing apoptosis did not appear in the sub-G1 peak. Floating cells collected on the second day of E2 treatment also showed a similar sub-G1 peak, although the histograms of the peaks were different between the combined sample and the floating sample. Adherent cells that were initiating apop-

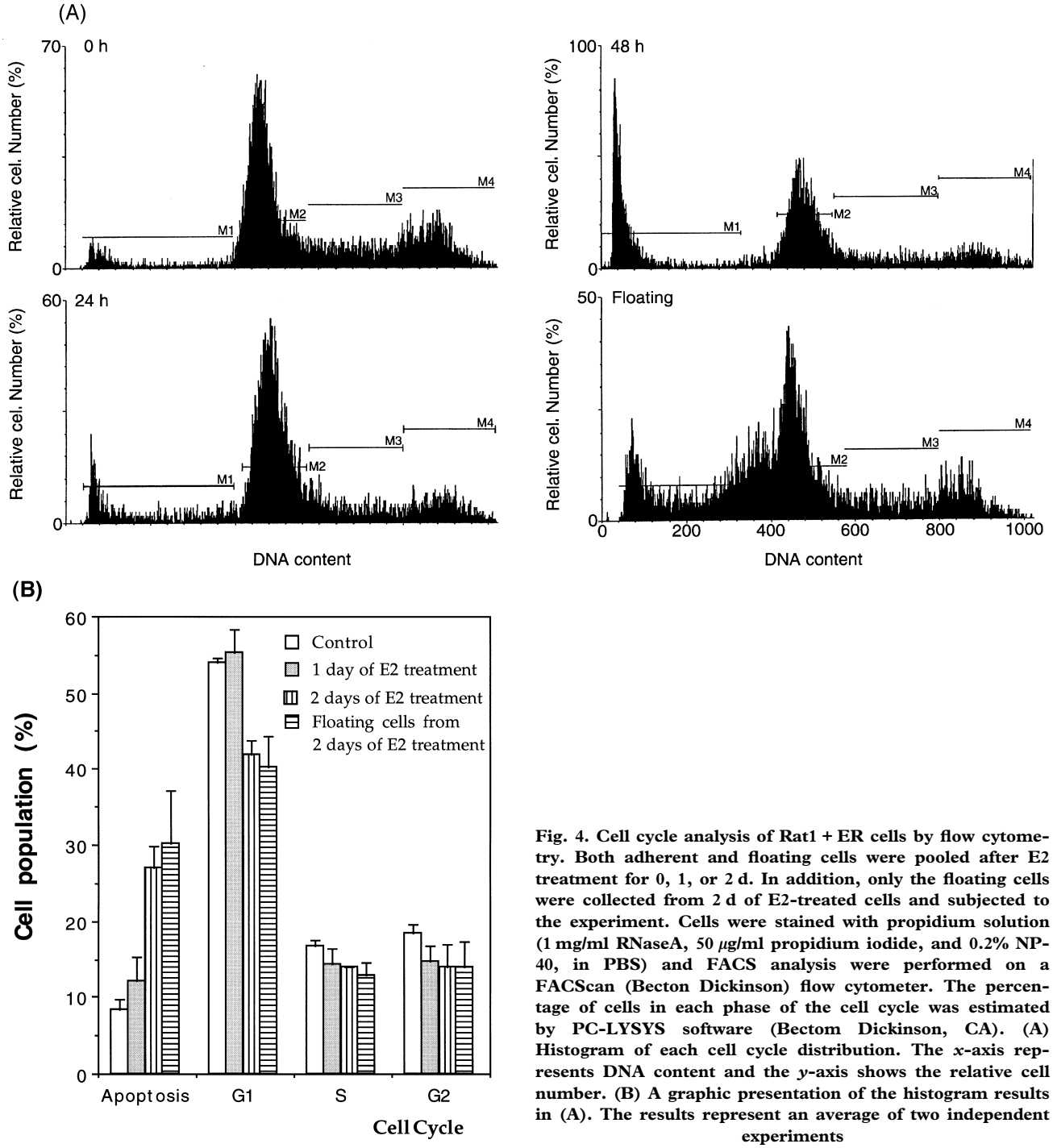

Fig. 4. Cell cycle analysis of Rat1 + ER cells by flow cytometry. Both adherent and floating cells were pooled after E2 treatment for 0, 1, or 2 d. In addition, only the floating cells were collected from 2 d of E2-treated cells and subjected to the experiment. Cells were stained with propidium solution (1 mg/ml RNaseA, 50 μg/ml propidium iodide, and 0.2% NP-40, in PBS) and FACS analysis were performed on a FACScan (Becton Dickinson) flow cytometer. The percentage of cells in each phase of the cell cycle was estimated by PC-LYSYS software (Bectom Dickinson, CA). (A) Histogram of each cell cycle distribution. The *x*-axis represents DNA content and the *y*-axis shows the relative cell number. (B) A graphic presentation of the histogram results in (A). The results represent an average of two independent experiments

tosis may have contributed to the sub G1-peak in the total cell population.

Overall these results indicate that apoptotic cell death caused an approximate 35% decrease in cell numbers observed at 3 d of E2 treatment (Fig. 1(B)).

DISCUSSION

Estrogen is a mitogen in many estrogen-responsive cells and tissues [14]. Upon estrogen withdrawal, some cells undergo apoptosis, such as the cyclical regression of the uterine epithelium [15]. In contrast to these observations, estrogen treatment did not induce growth but killed a portion of Rat1 + ER cells. Interestingly, in all ER-stably transfected cell lines examined to date, cells were either unaffected in growth, underwent growth inhibition, or were killed by estrogen treatment [3]. The reason for this paradoxical estrogen-induced growth response is not known [9]. Evidence has been accumulated from 20 examples, and the results are summarized in detail in a review by Levenson and Jordan [3].

Although the estrogen-induced death of cells in ER transfected cell lines is well documented, in most cases the mechanism of cell death has not been demonstrated. In this report, we have shown that cell death was not due to random poisoning of cells but was due to the activation of programmed cell death or apoptosis. The mechanism by which estrogen triggers an apoptotic pathway in Rat1 + ER cells is not understood. It may be that estrogen directly activates apoptotic genes. Several growth-related genes that are regulated by estrogen have been shown to be implicated in apoptosis [16]. Some of these genes were also regulated in ER-stably transfected cell lines. In ER transfected HeLa cells, it was reported that estrogen down-regulated c-myc expression and inhibited growth [17]. In other studies, estrogen activated lysosomal protease cathepsin D, which plays a part in apoptosis and cell invasion, in ER-stably transfected HeLa cells [18].

The growth of Rat1 + ER cells was examined in the presence of estrogen and the results showed that E2 decreased cell number in a time-specific and dose-dependent manner. Apoptosis must be occurring through the ER because the phenomenon did not occur in the parental Rat1 cells, which are ER-negative. Furthermore, the partial agonist MHT also induced an apoptotic response. In previous studies of Rat 1 + ER cells, the estrogen antagonist ICI 164,384 blocked all estrogen responses and MHT had partial agonist effects similar to those observed here. It seems likely that other cell lines stably transfected with ER, in which estrogens induce cell death, are also undergoing cell apoptosis. Whether this phenomenon has other applications remains to be determined but presents interesting possibilities.

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