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Non-genomic Cell Growth Inhibition by Progesterone. Cell Cycle Retardation and Induction of Cell Death

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Abstract. *Background: Non-genomic mechanisms have been proposed to play a role in progesterone-dependent cell growth inhibition. Materials and Methods: The human cell line C-4I, derived from a squamous carcinoma of the uterine cervix, was progesterone receptor-negative. The culture medium contained 10% (v/v) fetal calf serum and the cells, growing in monolayer, were exposed to various progesterone concentrations. Flow cytometry and morphometry were employed to assess the effects. Results: Progesterone caused a concentration-dependent growth inhibition with an IC_{50} value of 2.06±0.46 μM (mean value±SEM, n=4). At 320 μM no viable and attached cells were left. Two mechanisms appeared to be responsible for the effect. Firstly, the cells accumulated in the G_1/G_0-phase indicating a cell cycle-specific arrest. Secondly, progesterone induced cell death with apoptosis and necrosis. Morphometric analysis showed that progesterone caused a marked reduction in the nuclear size, compatible with apoptosis. Conclusion: The present results show that progesterone exerts non-genomic effect(s) by reducing the input of and accelerating the exit of cells from the C-4I cell population.*

The growth altering properties of progesterone have been extensively reviewed (1-4). Earlier, the inhibition of proliferation was assumed to be mediated by progesterone receptors (5), being members of the type I family of nuclear hormone receptors and classically defined as ligand-activated transcription factors (6,7). This view was supported by a more recent study, which showed that progesterone and other synthetic progestins markedly inhibited DNA synthesis and cell growth in ER- and PgR-negative MDA-MB-231 cells transfected with PgR cDNA (8). However, more complex mechanisms have been identified, including crosstalk between progesterone receptors and other signal pathways (9,10). In addition, the action of progesterone involves signal pathways without binding to nuclear receptors. The non-genomic effects of progesterone have been comprehensively reviewed (11-14). Many of these effects are distinguished from the classical genomic effects by a rapid onset (15). In C-4I cells without detectable nuclear receptors (16,17), progesterone and other progestins caused a concentration-dependent growth inhibition (17,18). The fact that the anti-proliferative effect was not blocked by anti-progestins strengthened the idea of a non-genomic growth modulatory effect of progesterone (17). It has been reported that progesterone exerts it growth inhibitory effect by inducing apoptosis (19,20). The present study extended the previous observations to determine whether the non-genomic progesterone effect is cell cycle specific and involves apoptosis in C-4I cells.

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Materials and Methods

Cell culture. The cell line C-4I was derived from a squamous carcinoma of the uterine cervix (21) and obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% (v/v) fetal calf serum (Bio Whittaker, Walkersville, MD, USA), 0.1 g/l streptomycin (Sigma-Aldrich) and 0.06 g/l penicillin G (Sigma-Aldrich). The cells were seeded to obtain a final density of 4.0 – 5.0 x 10^4 cells /ml (if not otherwise stated). After 24 h, the medium and non-attached cells were aspirated. The cells were routinely grown in medium without phenol red since phenol red has been reported to have estrogen-like effects (22). However, separate experiments showed that the growth of C-4I cells was not influenced by the presence of phenol red (Figure 1); the doubling time in the presence and absence of phenol red was identical (51 - 53 h). The effect of progesterone (Sigma-Aldrich) on cell growth was determined within the range of 3.2 nM to 318 μM (1 ng/ml to 100 μg/ml) and given for the individual experimental set-

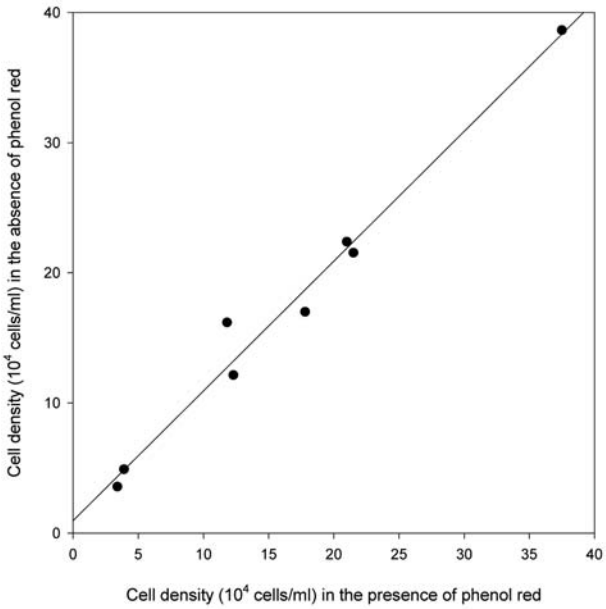

Figure 1. *Cell densities in the absence and presence of phenol red. C-4I cells were grown in RPMI1640 with 10 % fetal calf serum with and without phenol red for a period of 240 h. The medium was renewed every 24 h. The correlation between cell densities obtained in the absence and presence of phenol red could be described by first order regression line, y=a x + b, where a=1.0, b=0.94 and r=0.99.*

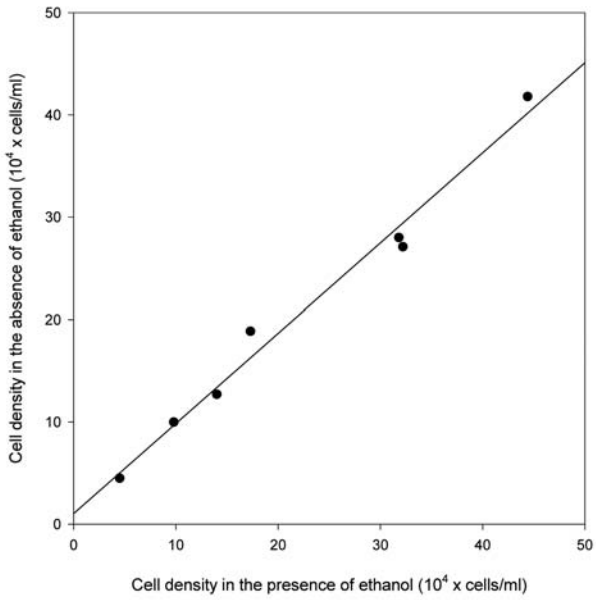

Figure 2. *Cell densities in the absence and presence of ethanol. C-4I cells were grown in RPMI1640 with 10 % fetal calf serum with and without 0.2 % (v/v) ethanol for a period of 168 h. The medium was renewed every 24 h. The correlation between cell densities obtained in the absence and presence of ethanol could be described by the first order regression line y=a x + b, where a=0.88, b=1.04 and r=0.99.*

up. Twenty-four h after seeding, the culture medium was aspirated and renewed with or without various concentrations of progesterone. Progesterone was dissolved in 96% (v/v) ethanol before addition to the medium. The ethanol concentration in the medium was equal irrespective of progesterone concentrations and never exceeded 0.2% (v/v). The cell culture series without progesterone contained equivalent ethanol concentrations. Separate experiments showed that cell growth was not influenced by the presence of 0.2% (v/v) ethanol (Figure 2). The doubling time in the presence and absence of ethanol was similar: 46 - 49 h. The cells were detached by trypsin and counted in a cytometer. Viability was determined by the trypan blue exclusion test.

Distribution of C-4I cells within cell cycle. The cell cycle effects of progesterone were determined by flow cytometry using a FACScan Analyzer (Becton Dickinson, San Jose, CA, USA) with argon laser excitation at 488 nm. Red fluorescence (FL2; DNA) was detected through a 563-607 nm bandpass filter. Viable cells were prepared according to Brons (23). The detached cells were kept at 2 - 4˚C and mixed with the aspirated culture medium. The suspension was centrifuged for 5 min at 200 x g. The cell pellet was resuspended in 250 µl PBS (pH 7.0) with 1% (v/v) fetal calf serum at 2 - 4˚C and a volume of 125 µl of trisodium citrate containing 100 µg/ml ribonuclease A (Sigma-Aldrich) and 20 µg/ml propidium iodide (Molecular Probes, Eugene, OR, USA). Triton X-100 (Sigma-Aldrich) was added to achieve a final concentration of 0.1% (v/v). The samples were filtered before flow cytometry. The carrier in the flow cell was isotonic saline. The cell cycle distribution was determined by the WinCycle software (Phoenix Flow Systems, San Diego, CA, USA).

Apoptotic, necrotic and viable cells. Apoptotic and necrotic cells were distinguished by the Annexin V-FITC method. This utilizes a well established system to differentiate between necrotic and apoptotic cells. In this system, the anticoagulant Annexin V is used in the presence of calcium to bind phosphatidyl serine, which is normally a cytoplasmic protein, but translocated to the cell surface in the early stages of apoptosis. Because Annexin V is conjugated to fluorescein isothiocyanate (FITC), it can be detected using flow cytometry. The membranes of necrotic cells are permeable and will also bind Annexin V-FITC, but propidium iodide is used to distinguish between viable, early apoptotic and necrotic or late apoptotic cells. Propidium iodide is excluded from viable (FITC- negative) and early apoptotic (FITC-positive) cells. Late apoptotic cells will stain with both FITC and propidium iodide due to the final necrotic-like disintegration of the cells.

C-4I cells (ATTC) were detached by trypsination and mixed with the cells released to the medium before trypsination, washed with PBS (pH 7.0) and in Hepes buffer (pH 7.4). The cells were resuspended in 50 µl Hepes buffer with 5 µg propidium iodide (Molecular Probes, Eugene, OR, USA) and 0.125 µg Annexin V-FITC (R & D Systems, MN, USA) and kept at room temperature for 20 min before addition of 500 µl Hepes buffer. The flow cytometric set-up comprised a FACScan Analyzer (Becton Dickinson) with argon laser excitation at 488 nm, combining green fluorescence (FL1; F-Annexin V) with 515-545 nm band pass filter and red fluorescence (FL2; DNA and RNA) by 563-607 nm band pass filter. The carrier in the flow cell was isotonic saline. All samples were analyzed within 30 min.

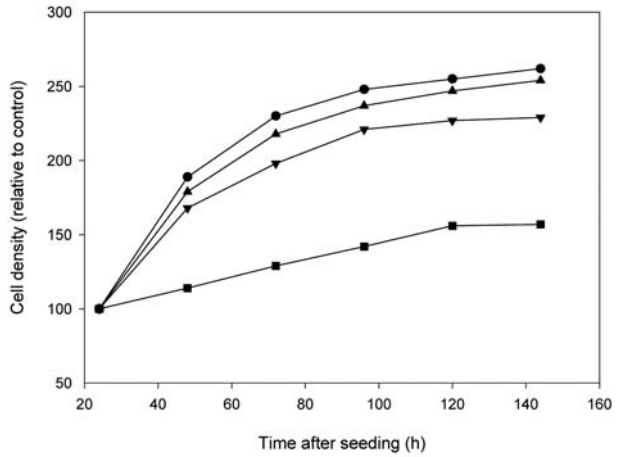

Figure 3. *Time-course of growth and effects of progesterone. C-4I cells were grown in RPMI1640 with 10 % fetal calf serum without phenol red. The medium without (●) and with progesterone (3.2 nM (▲), 0.32 μM (▼) and 32 μM(■)) was renewed every 24 h. At 144 h after seeding, the cell densities in the control series were 262±10% of the 24 h values (mean±SEM, n=3). The values in the presence of 3.2 nM, 0.32 μM and 32 μM progesterone were 254±40%, 229±48% and 157±27% of the 24 h values (mean±SEM, n=3), respectively.*

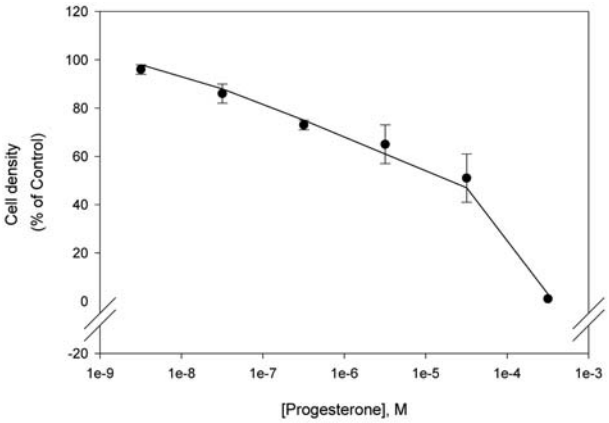

Figure 4. *Effects of progesterone concentrations on growth. C-4I cells were grown in RPMI1640 with 10 % FCS without phenol red. After 24 h, the non-attached cells and medium was aspirated and fresh medium with and without progesterone (3.2 nM – 320 μM) was renewed every 24 h and the cells were harvested 168 h after seeding. The cell density in the absence of progesterone was 10.2±1.0 x 10^4 (mean±SEM, n=4) cells/ml 168 h after seeding.*

Morphometry. The morphology of the C-4I cells was studied by image analysis. The cells were grown in Petri dishes containing micro slides. The medium with and without 79.8 μM progesterone was changed every day. After 6 days, the micro slides were routinely processed, fixed in buffered formaldehyde and stained with hematoxylin and eosin. The nuclear morphometric measurements were performed with a 100 x (numerical aperture 1.30) oil immersion objective, using automated scanning stage controlled by an image analysis system (QPRODIT, Leica, Cambrigde, UK).

Progesterone receptors. The C-4I cells were grown in Petri dishes containing micro slides and the medium, with and without 79.8 μM progesterone, was renewed daily. The micro slides were transferred to dry ice 144 h after seeding. Immunohistochemistry with monoclonal antibodies against human progesterone receptors (Novocastra Laboratories Ltd, Newcastle, UK) was used to identify receptors. The receptor status was assessed with light microscopy and the average number of positive cells per 100 counted cells in 10 different fields in each slide (magnification: 40 x) was determined. Proliferative breast carcinoma was employed as a positive control for progesterone receptors.

Table I. *Time course and non-genomic effect of progesterone on cell cycle distribution. C-4I cells were grown in RPMI1640 with 10 % fetal calf serum without phenol red. After 24 h, the non-attached cells and medium were aspirated and fresh medium with and without progesterone (31.8 μM) was renewed every 24 h and the cells were harvested 48, 72 and 96 h after seeding. The cells were prepared and flow cytometry performed as described in Materials and Methods. The FL2 histograms were analyzed by WinCycle software. The results are presented as mean value±SEM (n=3).*

Time after seeding	24 h	48 h	72 h	96 h
% of cells in G_0/G_1				
Control	48.9±0.4	54.3±0.8	55.6±0.2	59.6±1.2
Progesterone (31.8 μM)	49.9±0.3	58.2±1.1	60.4±0.7	65.1±0.
% of cells in S				
Control	37.8±0.4	32.1±0.8	33.1±0.4	30.3±1.9
Progesterone (31.8 μM)	37.4±0.1	29.7±0.7	29.8±0.8	25.9±0.8
% of cells in G_2/M				
Control	13.2±0.4	13.5±0.4	11.4±0.6	10.1±0.8
Progesterone (31.8 μM)	12.8±0.4	12.1±0.7	9.8±0.6	9.0±0.6

Results

Progesterone receptors. Previous studies have reported that C-4I cells were devoid of progesterone receptors (16,17). The present study also confirmed the absence of progesterone receptors in C-4I cells (data not shown).

Time course of cell growth in presence and absence of progesterone. Twenty-four hours after seeding the medium was renewed and various progesterone concentrations were added. This procedure was repeated with 24-h intervals and

Table II. *Effect of increasing progesterone concentrations on cell cycle distribution. C-4I cells were grown in RPMI1640 with 10 % fetal calf serum without phenol red. To obtain a sufficient number of cells for the flow cytometry, a starting density of 9 x 10^4 cells/ml was achieved in 25-cm^2 culture flasks with 15 ml medium. After 24 h, the non-attached cells and medium were aspirated and fresh medium with and without progesterone was renewed every 24 h. The cells were harvested 96 h after seeding. The cells were prepared and flow cytometry performed as described in Materials and Methods. The FL2 histograms were analyzed by WinCycle software. In parallel, attached cells were counted. The results are presented as mean value±SEM (n=3).*

Progesterone (μM)	G_0/G_1 (%)	S (%)	G_2/M (%)	Cell density (10^4 x cells /ml)
0	52.2±1.0	38.3±0.2	8.2±0.4	47.5±3.4
31.8	64.4±0.6	28.0±0.6	7.7±0.1	19.5±1.2
79.5	74.3±1.1	19.6±1.0	6.1±0.2	15.4±1.0
159	69.5±0.5	23.4±0.3	7.1±0.6	7.5± 1.7
318	n.d.	n.d	n.d.	0

n.d.: Not determined due to low cell number

Table III: Effect of progesterone concentrations on cell death. C-4I cells were grown in RPMI1640 with 10 % fetal calf serum without phenol red. After 24 h, the non-attached cells and medium were aspirated and fresh medium with and without progesterone was renewed every 24 h. C-4I cells floating in the medium and attached cells were harvested 96 h after seeding. Apoptotic and necrotic cells were distinguished by flow cytometry using propidium iodide and Annexin V-FITC. The fractions of viable and dead cells are presented as % of total cell number (mean value±SEM, n=3). The estimated ratios between fractions of apoptotic and necrotic cells are also presented.

Progesterone (μM)	% Viable cells	% Dead cells	Apoptotic/ Necrotic
0	84.5±0.8	15.5±0.8	3.0
31.8	86.1±0.9	13.9±0.9	2.3
79.5	80.6±1.5	19.4±1.5	1.9
159.0	69.1±0.1	30.9±0.1	1.1
238.5	45.1±2.7	54.9±2.7	0.8

the total exposure period was 120 h. Figure 3 shows cell proliferation during the culture period, with and without progesterone up to 144 h after seeding. There was no effect at the lowest concentration (3.2 nM) compared to the control situation and a minor effect of 0.32 μM progesterone. The highest concentration (32 μM) caused a clear reduction in cell growth with approximately 40% reduction after 120-h exposure.

Progesterone concentrations and C-4I cell growth. The C-4I cells were exposed to various progesterone concentrations for 144 h. Figure 4 shows that progesterone inhibited the cell growth in a concentration-dependent manner. A concentration of 320 μM progesterone resulted in no viable and attached cells in the culture flasks. The IC_{50}-value, calculated according to Chou (24), was 2.06±0.46 μM (mean value±SEM, n=4).

Time-dependent and progesterone effects in the cell cycle. Flow cytometry was employed to determine whether the non-genomic effect of progesterone was cell cycle-specific or not. Table I shows the time course of cell cycle fractions with and without 31.8 μM progesterone. The fraction of cells in G_0/G_1 increased during the culture period and the presence of progesterone caused an additional increase. In the G_2/M-phase the fraction tended to decrease, but with no marked difference between the control and progesterone series. In the S-phase the fraction showed a time-dependent decrease which was markedly greater in the presence of progesterone. Table II shows the effect on C-4I cell cycle fractions after 72-h incubation in the presence or absence of various concentrations of progesterone. Small changes were seen in the G_2/M-phase fraction. In G_0/G_1 the lowest and the intermediate progesterone concentration showed a clear increase, whereas the highest concentration gave no further increase. In the S-phase the fraction showed a mirror image of the G_0/G_1-phase. In parallel, the concentration-dependent reduction in cell density was evident (Table II).

In an additional series of experiments, the cell cycle effect of progesterone (31.8 μM) was tested without daily renewal of medium and addition of progesterone. After 24 h, the G1-fractions were 56.3 and 61.3% in the control and progesterone series, respectively. The corresponding values after 72 h were 64.7 and 74.3%. The S-phase showed no change in the control series (29.6 to 28.3%), whereas the presence of hormone caused a reduction from 27.3 to 19.6%. The G_2/M-fraction decreased from 14.0 to 8.2% in the control and from 11.4 to 6.1% in the presence of progesterone.

Effect of progesterone on apoptosis and necrosis. After repeated exposure to progesterone for 72 h, the density of viable and attached C-4I cells was concentration-dependently reduced (Table III). In the absence of progesterone approximately 12 and 4% of the cells were apoptotic and necrotic, respectively. The cytometric analysis showed a mixed pattern, but apoptosis appeared to increase continuously from 31.8 μM, whereas necrosis showed an apparent steep concentration response curve from 159 μM.

Progesterone effects on cell morphology. Light microscopy gave the impression of a smaller nuclear size and the loss of cytoplasm in the presence of 79.5 μM progesterone (results

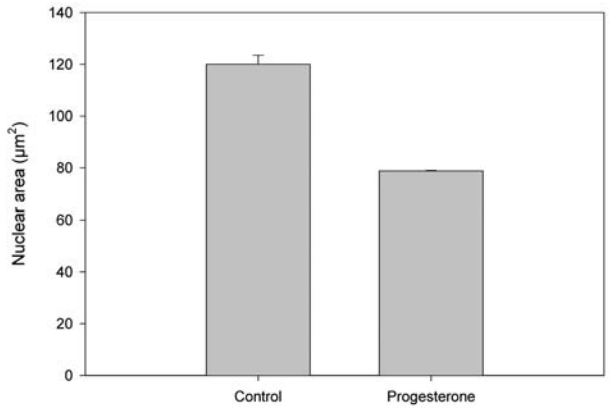

Figure 5. *The effect of progesterone on nuclear size. C-4I cells were grown in RPMI1640 with 10% fetal calf serum without phenol red in Petri dishes containing micro slides. After 24 h, the non-attached cells and medium was aspirated and fresh medium with and without 79.5 μM progesterone was renewed every 24 h. The culture was terminated 96 h after seeding. The cells on the micro slides were prepared as described in Materials and Methods. The results are presented nuclear area (mean value±SEM, n=3).*

not shown). In the control series there was a high frequency of mitosis, whereas no or low mitotic activity was observed in the hormone-treated cells. Morphometry was employed to quantify changes of the cell nucleus. Image analysis showed a significant reduction in nuclear size in the presence of 79.5 μM progesterone (Figure 5). The nuclear area, nuclear perimeter, and longest and shortest nuclear axes were reduced by 34.2±1.7%, 18.1±0.8%, 21.2±1.4% and 16.9±0.8%, respectively.

Discussion

In humans progesterone appears to be a major stimulus for breast epithelial cell proliferation during the menstrual cycle and in pregnancy, in contrast to the marked inhibition of estrogen-induced proliferation and induction of differentiation by progesterone in uterine tissues (1). The uterine cervix is not regarded as being progesterone-sensitive. However, in the present study we showed that progesterone caused growth inhibition of a cell line derived from a squamous carcinoma of the uterine cervix (21), confirming our previous observations (17,18). The fact that these cells are devoid of progesterone receptors, as shown in the present and earlier studies (16,17), suggests that progesterone exerts its effect through non-genomic mechanism(s). In a recent study, the progesterone-dependent growth inhibition was characterized by an IC_{25}-value of 7.7 nM (17). Reanalysis of these data (17)gave an IC_{50} of 5.9±3.7 μM. In close correspondence, we found an IC_{50} value of 2.1±0.5 μM in the present study. This value is similar to the IC_{50} value (4 μM) of the progesterone membrane binding site in amphibian oocytes (25) and EC_{50}-values of 0.5 to 5 μM for the progesterone-induced increase in intracellular calcium in smooth vascular muscles (26). The concentration-dependent growth inhibition suggests the existence of specific binding sites for progesterone. Such sites may be involved in opening of plasma membrane calcium channels (27-30), activation of protein kinase C (31,32), stimulation of protein tyrosine phosphorylation (33-36) or inhibition of various membrane transport system such as P-glycoprotein (37-40), multidrug-resistant proteins (41) and the transporter responsible for cellular cGMP efflux (42). The progesterone effect on cGMP efflux from intact C-4I cells appears to be composite since prolonged incubation (96 h) caused retention of intracellular cGMP (17), whereas a relatively short incubation time (0.5 -1 h) caused increased distribution to the extracellular compartment (43). A similar paradox is also observed for human erythrocytes, where progesterone causes increased efflux from intact cells (44), but inhibits cGMP uptake into vesicles (42). One or more of the reported non-genomic effects, including an increase in intracellular calcium levels, activation of protein kinase C, tyrosine phopshorylation or changes in intracellular levels of cyclic nucleotides, may be responsible for the anti-proliferative effect of progesterone in C-4I cells.

We observed a concentration-dependent accumulation of C-4I cells in the G_1/G_0-phase of the cell cycle. This reduces the cell number entering the growing population of C-4I cells and can, in part, explain the growth inhibition by progesterone. Two distinct, opposing effects of progestins on cell cycle progression can be observed within one cell type (T-47D human breast cancer cells), emphasizing the complexity of the effects of progestins on cell proliferation, comprising an initial transient acceleration through the G1-phase and a subsequent increase in the S-phase fraction, followed by cell cycle arrest and growth inhibition accompanied by a decrease in the S-phase fraction (45). It is possible that the effects are mediated by distinct mechanisms that manifest themselves to different degrees in different cell types, leading to the predominance of either a stimulatory or inhibitory growth response. Since progesterone does not stimulate growth in C-4I cells and these cells are receptor-negative, it is possible that growth stimulation depends on the activation of progesterone receptors. However, this idea is in disagreement with the observation that progesterone and other synthetic progestins markedly inhibited DNA synthesis and cell growth when ER- and PgR-negative MDA-MB-231 cells were transfected with PgR cDNA (8).

Apoptosis or programmed cell death (PCD) is recognized as an essential feature in the balance between cell death and cell proliferation, in normal as well as in neoplastic tissues

(46-48). It has been reported that progesterone exerts it growth inhibitory effect by inducing apoptosis (19,20). In agreement, we found that progesterone caused a reduction of the growing cell population by induction of cell death in a concentration-dependent manner. The observation that progesterone caused a clear reduction in the nuclear area of the C-4I cells is also compatible with the well-known features of apoptosis, including chromatin condensation and cell shrinkage.

Progestins have an established role in the therapy of endometrial cancers (49) and high local gestagen concentration has proved its effectiveness in treatment of endometrial hyperplasia, an initial stage of potential development of endometrial cancer (50). The present study suggests that high concentrations of progesterone and possibly other gestagens have two distinct, synergistic effects on cell growth, namely retardation of the cell cycle and induction of cell death. The present work presents evidence of the non-genomic nature of the mechanism(s) of C-4I cell growth inhibition by progesterone.

Acknowledgements

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