Int. J. Cancer: **68,** 120–125 (1996)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

ANTAGONISM BETWEEN ESTRADIOL AND PROGESTIN ON BCL-2 EXPRESSION IN BREAST-CANCER CELLS

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Bcl-2 is a key protein involved in the control of apoptosis. Our previous studies on breast and endometrium indicated hormonal regulation of *bcl-2* in these tissues. In the present work we have analyzed Bcl-2 and Bax protein expressions in MCF-7 and T47-D, 2 hormone-dependent breast-cancer cell lines, by immunoblots. Estradiol markedly increased Bcl-2 protein content, both in short- and in long-term treatments of MCF-7 cells. Two types of anti-estrogens (4-hydroxytamoxifen and RU 58668) were able to reverse this effect. Also, a synthetic progestin (ORG 2058) was able to decrease the Bcl-2 level in T47-D cells. The level of Bax protein, however, was not affected in the same conditions of hormonal treatments. The level of Bcl-2 expression was 4.5-fold higher in MCF-7 than in MDA-MB 231 (an estradiol-independent cell line). From these results, we infer the existence of hormonal regulation of Bcl-2 expression and evoke a novel role for estradiol and progestin in the genesis of breast cancer.

The early stages of breast-cancer development depend on tumor initiators. Several lines of evidence emanating from *in vitro* studies of breast-cancer cell lines (Lippman *et al.,* 1986) and normal breast cells (Gompel *et al.,* 1986) highlight the proliferative effect of estrogens (E2). On the other hand, the role played by progesterone and progestins (P) is less clear. Data available from *in vitro* and *in vivo* experiments on the role of P are at variance with each other, leaving the controversy open (Going *et al.,* 1988; Gompel *et al.,* 1986).

Uncontrolled cell proliferation is an essential feature in tumorigenesis implying a degree of dysfunction of tissue homeostasis. Tissue homeostasis is the result of a fine balance between cell proliferation, differentiation and cell death. Spontaneous programmed cell death, termed apoptosis, is thought to play a key role in the development and growth regulation of normal and tumor tissues (Kerr *et al.,* 1972; Ellis *et al.,* 1991). Apoptosis can be induced in tissues and cell lines by withdrawal of essential growth factors and/or hormones. It has been described as exhibiting a cyclic variation in normal breast tissue (Ferguson and Anderson, 1981) as well as in breast-cancer cell lines, where it can be induced through estrogen deprivation (Kyprianou *et al.,* 1991). It is regulated in a complex manner, with several proteins intervening, of which Bcl-2 is of particular importance. *bcl-2* was first described as resulting from the t (14;18) translocation that occurs in a large number of human follicular lymphomas (Tsujimoto and Croce, 1986). The de-regulated expression of *bcl-2* and its association with B-cell malignancies suggest that *bcl-2* may be a proto-oncogene. It has been shown to prevent cells from going into apoptosis and to prolong cell-survival time without promoting cell proliferation. These observations have been made not only in hematopoietic tissues but also in neuronal cells, as well as in *bcl-2*-transfected fibroblasts (Garcia *et al.,* 1992; Hockenbery *et al.,* 1990; Vaux *et al.,* 1988). Moreover, *bcl-2* seems to cooperate with the c-myc product in promoting the proliferative activity of the latter (Vaux *et al.,* 1988). However transfection of *bcl-2* alone into breast epithelial cells does not confer tumorigenic capacity to the recipients (Lu *et al.,* 1995). Bcl-2 is a member of a larger family of proteins involved in the control of apoptosis. Among them, some promote apoptosis (Bax, Bcl-X_S) whereas others are anti-apoptotic (Bcl-2, Bcl-X_L). These proteins are capable, among themselves, of forming heterodimers and thereby affecting apoptosis. In instances of Bcl-2/Bax heterodimerization, the cellular content of Bax may remain unchanged whereas variation, if any, in Bcl-2 concentrations may affect the resultant action of the heterodimer on apoptosis (Oltvai *et al.,* 1993).

In earlier works we have investigated the expression of Bcl-2 in hormone-dependent tissues, namely, normal human breast and endometrium. (Gompel *et al.,* 1994; Sabourin *et al.,* 1994). We then observed a cyclic variation in the expression of Bcl-2 in breast epithelial cells and the glandular endometrium; the maximal expression being at the end of the follicular phase and a clear decrease seen during the luteal phase, highly suggestive of an hormonal regulation of Bcl-2 expression. Up-regulation by E2 and down-regulation by P of Bcl-2 and their consequences on apoptosis may represent a novel mechanism which contributes to breast carcinogenesis. In order to verify this assumption we have studied the level of expression of Bcl-2 in hormone-dependent breast-cancer cell lines after treatment by E2, anti-estrogens and P. The levels of expression of Bcl-2 both in hormone-dependent and in hormone-independent cell lines were also compared. The results of our present work confirm and consolidate the fact that Bcl-2 expression is indeed stimulated by E2 and inhibited by P.

MATERIAL AND METHOD

Materials

Thymidine and 17β estradiol (E2) were purchased from Sigma (St. Quentin-Fallavier, France). 4-hydroxytamoxifen (OH-TAM), RU 58668 (RU) and RU 38486 (RU 486) were provided by Dr. Van De Velde (Roussel-Uclaf, Romainville, France) and ORG 2058 (ORG) by Dr. Schott (Organon, Oss, The Netherlands). Reagents for cell cultures were from GIBCO, (Cergy-Pontoise, France). MCF-7 cell lines were purchased from the ATCC (Rockville, MD). T47-D and MDA-MB231 cell lines were provided by Dr. F. Kuttenn (Hôpital Necker, Paris, France) and by Dr. C. Mercier-Bodard (Inserm, Kremlin-Bicêtre, France) and originally came from Dr. K. Horwitz (Denver, CO).

Cell culture and synchronization

Cell cultures were done in a 5%-CO_2-enriched atmosphere at 37°C. MCF-7 and MDA-MB 231 were cultured in DMEM without phenol-red but containing 2 mM glutamine, antibiotics and 5% FCS. T47-D were cultured in RPMI without phenol-red but containing 2 mM glutamine and antibiotics.Synchronization of MCF-7 and T47-D cells were conducted in their corresponding medium containing 5%-steroid-stripped FCS (DCC-FCS) (using dextran-coated charcoal). Thymidine (2 mM final concentration) was applied during 2 pulses of 16 hr separated by a 12-hr wash in DCC-FCS medium (Wiling *et al.,* 1988).

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Received: February 12, 1996 and in revised form June 4, 1996.

Hormone treatments

The effects of E2 and anti-estrogens were studied mainly in MCF-7 cells, known constitutively to express high levels of estradiol receptors (ER) (Brooks *et al.*, 1973).

Hormones were added to the corresponding DCC-FCS medium with an ethanol concentration less than 0.1%. The control medium (C) also contained the same ethanol concentration. MCF-7 cells were either treated with E2 (10^{-8}M) alone or in association with one of the 2 anti-estrogens; a pure steroidal anti-estrogen, RU, or a triphenylethylene anti-estrogen, OH-TAM, added both at a final concentration of 10^{-7}M. The effect of E2 treatment was monitored at time-points: 2, 4, 8, 10–12, 24 and 48 hr or 4 and 7 days. The effects of the anti-estrogens were monitored at 2, 4, 8, 24 and 48 hr. The dose-dependent effect of E2 was studied after 4 hr treatment using concentrations ranging from 10^{-10}M to 10^{-7}M. The T47-D cells were also treated with E2 (10^{-8}M) for times ranging from 2 hr to 5 days.

The effects of the synthetic pregnane progestin, ORG 2058, were studied mainly in T47-D cells, which are highly progesterone-dependent and poor responders to E2 (Horwitz *et al.*, 1982). The T47-D cells were treated following synchronization with ORG (10^{-7}M), either alone or in addition with E2 (10^{-8}M) for durations of 2, 4, 8, 10–12 hr, 24, 48 hr or 5 days.

The dose-dependent effect of ORG was studied after 4 hr treatment, using concentrations ranging from 10^{-10}M to 10^{-7}M, either alone or combined with E2 at 10^{-8}M. RU 486 (10^{-7}M), a steroidal anti-progestin, was added alone or combined with ORG 2058 (10^{-7}M) for durations of 2, 4, 8, 24 or 48 hr.

Western blotting

Western blots were performed according to Sambrook *et al.* (1989) and adapted as follows. After lysis of the cells in 2% SDS, 100 mM DTT, the protein extracts were quantitated using the method of Lowry. Proteins (30 to 60 μg) in loading buffer (2% SDS, 100 mM DTT, v/v) were separated under denaturing conditions by 12.5% SDS-PAGE and electro-transferred to PVDF membrane (Millipore, St. Quentin-en-Yvelines, France). Non-specific binding sites were blocked with 10% skimmed milk in TBS buffer (50 mM Tris, pH 7.5, 150 mM NaCl) containing 0.05% Tween-20. The Bcl-2 protein was detected by applying successively, in TBS supplemented with 0.1% Tween-20 and 1% skimmed milk, a mouse monoclonal anti-Bcl-2 antibody (Dako, Paris, France) 1:5000 overnight, 1:1000 biotinylated goat anti-mouse antibody (Dako), 1:50 streptavidin/biotinylated horse-radish-peroxidase complex (HRP) (Dako), and revealed using an ECL chemiluminescence system (Amersham, Les Ulis, France). Each of these steps was separated by 5 × 8-min washes in TBS containing 0.2% Tween-20. The Bax protein was detected using a rabbit polyclonal anti-Bax antibody (Santa Cruz Biotechnology, Santa Cruz, CA) 1:8000 overnight, 1:2500 biotinylated goat anti-rabbit antibody (Dako) and 1:50 streptavidin/biotinylated HRP complex (Dako), and revealed using ECL. Actin was detected using a mouse monoclonal antibody (Amersham) 1/1000, incubated for 2 hr.

Relative amounts of Bcl-2 protein were quantitated using a CD camera (ISS, Pessac, France). The level of Bcl-2 protein was normalized with the respect to actin. So as to avoid the inter-assay variations, the values obtained were also normalized with the value measured for t_0 cells in each experiment.

RESULTS

Bcl-2 protein

The data obtained from the Western blots and microdensitometric measurements reveal that the level of Bcl-2 expression was 4.5-fold higher in the hormone-dependent cell lines MCF-7 and T47-D than in the estradiol-independent cell line MDA-MB 231 (Fig. 1).

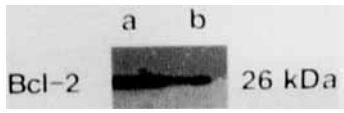

FIGURE 1 – Western-blot detection of Bcl-2-protein levels in MCF-7 and MDA-MB 231. The cell lines were sub-cultured in 75-cm² flasks containing 5% FCS-DMEM without phenol red, during 7 days; 50 μg of total protein were loaded per well for the SDS-PAGE. *(a)* MCF-7, *(b)* MDA MB 231.

The level of Bcl-2 protein was always higher following synchronization (t_0 cells) than in control cells (C) cultured for 2 hr (Fig. 2*b*).

The E2-treated MCF-7 cells (E2) revealed an increase in Bcl-2 protein content as compared with the control cells (C); a rapid increase (2-fold) as early as 2 hr was observed following E2 treatment and was sustained for 8 to 10 h, with a maximum (2.6-fold) situated at around 4 hr (Figs. 2, 3). Thereafter, and till 24 hr, the levels remained unaltered and comparable between E2-treated and control cells (Fig. 2*a*). Furthermore, a second phase in increase (3- to 4-fold) of Bcl-2 expression was also observed from day 2 to day 7 (Figs. 2, 3). The 2 anti-estrogens, RU (Fig. 2*b*) and OH-TAM (not shown) reversed the effects of E2. However, when added alone, OH-TAM and RU had no notable effect on Bcl-2 content (Fig. 2*b*). The dose-dependent effect of E2 on MCF-7 cells was studied after 4 hr of treatment. No variation in Bcl-2-protein levels was observed when E2 concentrations varied between 10^{-10} M and 10^{-7} M (data not shown).

When the effect of E2 on Bcl-2 expression was assayed in T47-D cells, we found it to be weaker than that observed for MCF-7 cells. The Bcl-2 expression reached only 1.5- to 2-fold increase during the treatment regimen (Fig. 4). This increase was observed constantly only after 24 hr and lasted until 5 days (Fig. 4).

The effects of the synthetic progestin, ORG, was studied in T47-D cells. ORG decreased the expression of Bcl-2 progressively and as early as 2 hr after treatment. Bcl-2 level at 48 hr of progestin treatment decreased to a minimum (Figs. 4, 5). It was as low as 20% of the value obtained for control cells (Fig. 5). This decrease was maintained during the following days at a mean level of 30% as compared to the controls (Fig. 5).

The addition of E2 to ORG-treated cells did not alter the observed decrease and had even a synergetic effect (Figs. 4, 6) at the lower progestin concentrations used (see below). The progestin effect on Bcl-2 expression was reversed on addition of the anti-progestin RU 486 at a concentration of 10^{-7} M (Fig. 4*d*). The anti-progestin alone had no detectable agonistic effect on Bcl-2 expression in these experimental conditions (Fig. 4*d*). The dose-dependent effect of ORG 2058 after 4 hr treatment was also studied in T47-D cells. A concentration of 10^{-7} M was the most effective one with a progressive inhibition of Bcl-2 expression noticeable from 10^{-10} M up to 10^{-7} M, in the presence or in the absence of E2 (Fig. 6). The inhibition was more effective when E2 was present together with ORG (except at 10^{-7} M). The addition of ORG 2058 to MCF-7 cells, however, did not affect the Bcl-2-protein level. In the presence of E2, ORG did not modify the stimulatory effect of the former (not shown).

Bax protein

The level of Bax protein did not vary under the different conditions of hormonal treatment. In MCF-7, E2 treatment at short- and long-term did not affect the level of Bax as assayed in the same cellular extracts as those for Bcl-2 (Fig. 2*a*). In T47-D cells, the P treatment did not alter the level of expression of Bax (Fig. 4*c*), whereas at corresponding time-points it lowered Bcl-2 levels. The level of Bax protein remained unaltered even in synchronized cells (Fig. 2*a*).

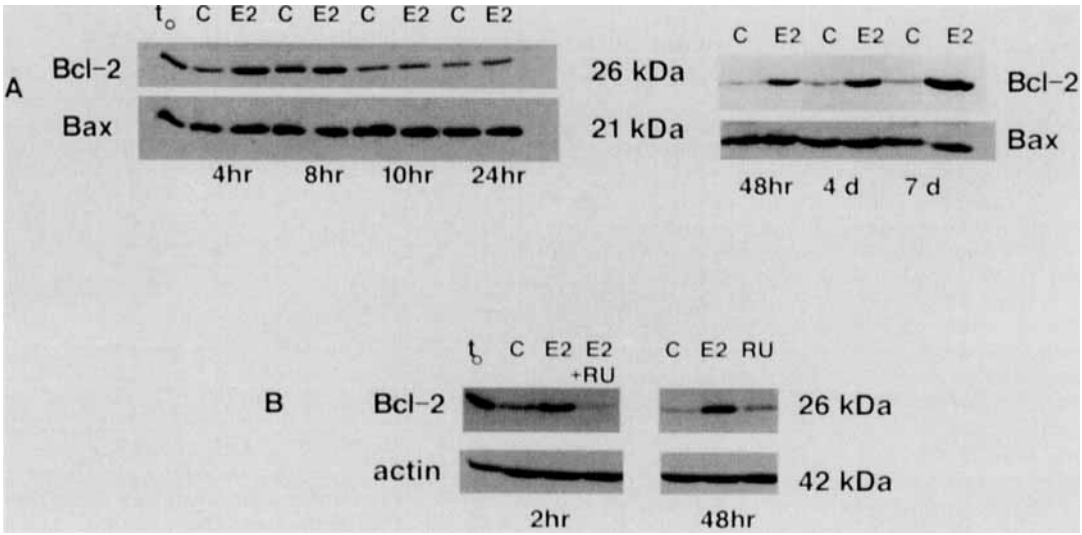

FIGURE 2 – Western-blot detection of Bcl-2 and Bax after E2 and anti-estrogen treatments of MCF-7 cells. MCF-7 cells were sub-cultured in 5% FCS-DMEM for 7 days in 75-cm^2 flasks. A double thymidine blockade (final concentration 2 mM) in DCC-FCS medium was applied in order to synchronize the cells. Bcl-2-, Bax- and actin-protein levels were revealed by Western-blot analysis of 30 µg of total protein loaded per well. *(a)* After E2 (10^{-8} M) treatment in 5% DCC-FCS-DMEM without phenol red, for periods ranging from 4 hr to 7 days (7 d). E2 was added in $<0.1\%$ ethanol to the cells immediately following synchronization, and the same concentration of ethanol was added to control (C) cells. *(b)* The steroidal anti-estrogen, RU (10^{-7} M) was added alone or with E2 (10^{-8} M) from 2 to 48 hr.

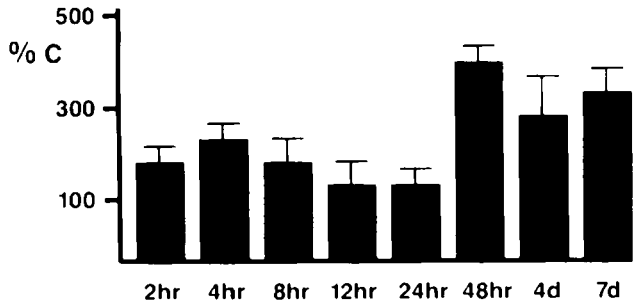

FIGURE 3 – Bcl-2-protein levels in E2(10^{-8}M)-treated MCF-7 cells as a function of the duration of treatment. Bcl-2-protein levels were quantitated from Western blots (see "Material and Methods") using a CD camera. After scanning, results were normalized to the values obtained for actin and cells immediately following synchronization within the same experiment and designated t_0. Each histogram corresponds to the mean of at least 4 experiments, each in duplicate. Results are expressed as percent of the control at each time point. Bars on histograms show standard deviation.

DISCUSSION

The protein Bcl-2, initially identified in hematopoietic tissues, was more recently shown to be ubiquitous. Its main function seems to be to over-ride apoptosis. Data from transfection and transgenic mice experiments have shown that Bcl-2 prolongs cell survival without affecting proliferation and is able to prevent apoptosis induced by growth factors deprivation (Garcia *et al.*, 1992; Vaux *et al.*, 1988). Over-expression of Bcl-2 was recently shown to inhibit cell death at confluence or in conditions of serum deprivation in breast cells without inducing a tumorigenic phenotype (Lu *et al.*, 1995). Bcl-2 is a member of a larger family of proteins involved in the regulation of apoptosis. Among them, Bax plays an important role. It is able to heterodimerize with Bcl-2 and alter the latter's anti-apoptotic action (Oltvai *et al.*, 1993; Sato *et al.*, 1994). It is therefore important to estimate simultaneously the level of

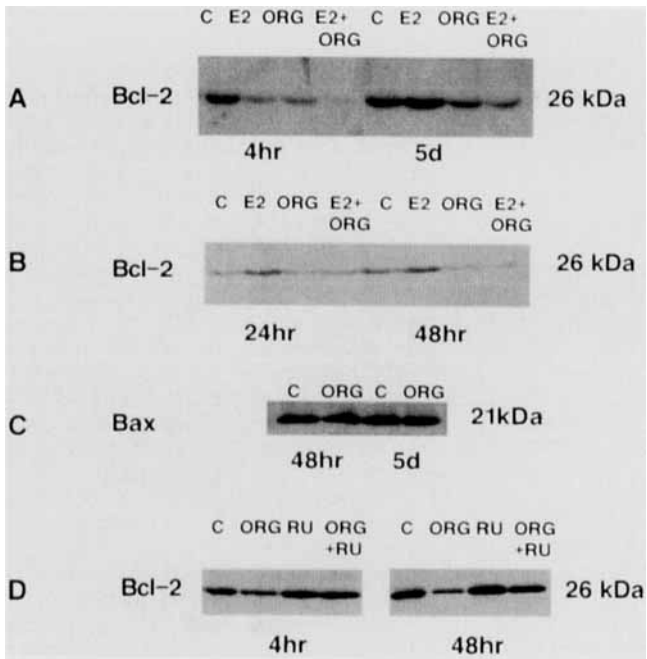

FIGURE 4 – Western-blot detection of Bcl-2 and Bax in T47-D cells in control cells, cells treated with E2 and/or a synthetic progestin, ORG 2058 (ORG), after loading 50–60 µg of total proteins per slot. T47-D were sub-cultured in 75-cm^2 flasks in RPMI with 5% FCS and without phenol red for 7 days. After a double thymidine blockade (2 mM final concentration) in 5% DCC-FCS-RPMI, the effects of the hormones were studied and compared with control cells (C). *(a)* Bcl-2 protein level in T47-D cells at 4 hr and 5 days (5 d) of steroidal treatments: E2 (10^{-8} M), ORG (10^{-7} M) or both products. *(b)* Bcl-2-protein level in T47-D cells at 24 and 48 hr of the same steroidal treatments as in Figure 2*a*. *(c)* Bax protein levels at 48 hr and 5 days (5 d) of progestin treatment. *(d)* Bcl-2-protein levels in the presence of ORG (10^{-7} M), the antiprogestin, RU 486 (RU, 10^{-7} M) or ORG + RU 486 (10^{-7} M) at 4 hr and 5 days (5 d).

expression of the 2 proteins so as to discern the overall effect of steroid hormones in cell survival and cell death.

The present work demonstrates progestin-dependent regulation of Bcl-2 in breast-cancer cells and provides evidence of variation in Bcl-2-protein content after E2 and anti-E2 treatments. We have observed a discernable induction of Bcl-2 expression in cells subjected to E2 treatment, which was reversible after anti-estrogen exposure, suggesting a specific E2-dependent effect that may involve an E2-receptor-mediated mechanism. No significant agonistic effect of the anti-estrogens was observed. E2 appeared to trigger Bcl-2-protein induction as early as 2 hr after treatment of the cells, and the induction was sustained for 8 to 10 hr. A second phase of induction was observed after 24 hr and lasted through the following few days. The induction of Bcl-2 expression, induced on addition of E2, suggests that a series of different mechanisms may intervene in this process. It could result from transcriptional activation and/or stabilization of otherwise labile Bcl-2 transcripts, or increase in translational efficiency, and/or increase of the protein half-life. Wang and Phang (1995) have provided evidence of a time-dependent increase of Bcl-2 mRNA in MCF-7 cells subjected to E2 treatment. The authors described the increase in Bcl-2 mRNA, however after only 6 hr of E2 treatment, a maximum being reached at 48 hr. In their report, time-points earlier than 6 hr and later than 48 hr were not checked. We have analyzed the expression of Bcl-2 as early as 2 hr and up to 7 days post-E2 treatment, to provide a more complete picture of E2 action. On the other hand, Teixeira *et al.* (1995), investigating the effect of E2 on Bcl-2 expression, both at the protein and at mRNA levels, have also reported an increase in Bcl-2 mRNA after 8 hr and the protein after 24 hr. We detected the increase in expression of Bcl-2 at a much earlier time-point, and this may perhaps be due to the fact that we used synchronized cells. This could be linked to the cell-cycle-dependent expression of Bcl-2 (Lu *et al.*, 1994).

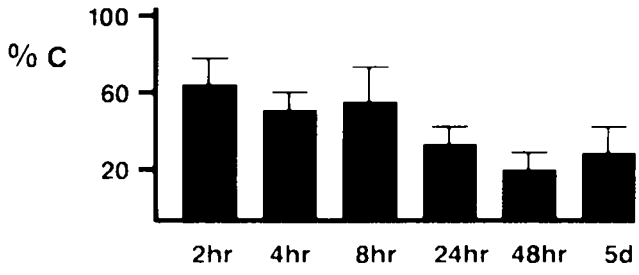

FIGURE 5 – Bcl-2-protein levels in ORG(10^{-7} M)-treated T47-D cells as a function of the duration of treatment quantitated and expressed as in Figure 3.

We have also shown that, following progestin treatment, there was a progressive decrease in the expression of Bcl-2 in T47-D cells. This decrease was noticeable as early as 2 hr after-treatment. After 5 days of treatment, the Bcl-2-protein content declined to a barely detectable level. This decrease was not affected by the presence of E2 added along with P at 10^{-7} M. This concentration (10^{-7} M or 30 ng/ml) corresponds to the plasma level of progesterone in the luteal phase (> 10 ng/ml) (Abraham *et al.*, 1974). The anti-progestin, RU 486, reversed the action by P which is consistent with the receptor (PR)-mediated effect of the molecule. The inhibitory action of P on Bcl-2 expression can be the sum of a specific progestin effect as well as an anti-estrogen action of the progestin. The early decrease in Bcl-2-protein level, may reflect a reduction in bcl-2 transcripts and the action of P at the gene level. The failure of P to have an effect on MCF-7 could result from the use of culture medium lacking phenol red and the absence of E2 priming. These cells contain only very low levels of PR in the absence of E2 stimulation (Nardulli *et al.*, 1988). Similarly, the relatively weaker effect of E2 on Bcl-2 expression in T47-D

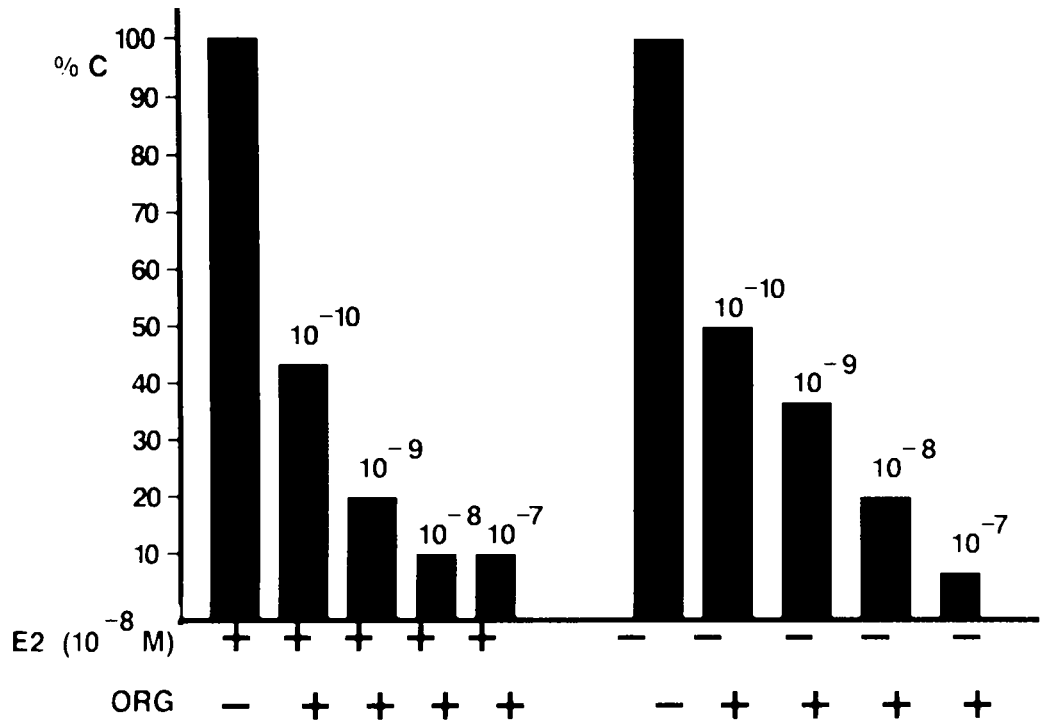

FIGURE 6 – Dose-dependent effect of ORG 2058 in T47-D cells on Bcl-2 expression. T47-D cells were treated by ORG 2058 at a concentration from 10^{-10} M to 10^{-7} M with or without E2 (10^{-8} M) for 4 hr after synchronization. Bcl-2-protein levels were quantitated using Western blots and a camera (see "Material and Methods"). Results are expressed as percent of Bcl-2 values either in E2 (10^{-8} M)-treated cells or in control (C) cells. In these experiments, the inhibition at 10^{-7} M after 4 hr was maximal.

cells is consistent with their low responsiveness to E2 (Horwitz *et al.*, 1982). It is also interesting to mention that we have constantly observed that the Bcl-2-protein content dropped considerably as early as 2 hr of removing the thymidine blockade and without any E2 treatment (Fig. 2*b*). This may suggest the prevalence of a short half-life for the Bcl-2 protein in these cells. This assumption is reinforced by the fact that P was able to "down-regulate" Bcl-2 after only 2 to 4 hr of treatment (Fig. 5).

The highly complex structure of the normal *bcl-2* gene (Seto *et al.*, 1988; Tsujimoto and Croce, 1986) indirectly suggests that several levels of control and a host of regulatory factors may be involved in its expression. The 5′ region of the *bcl-2* gene contains several distinct transcription initiation sites, as reported for certain oncogenes (c-*abl*, c-*myb*), and the first exon of *bcl-2* is untranslated, as in c-*myc*. Interestingly, the expressions of c-*myb*, c-*fos* and c-*myc* have also been reported to be regulated by E2 in breast-cancer cell lines (Dubik *et al.*, 1987) and in normal epithelial breast cells (c-*myc*) (Leygue *et al.*, 1995). The existence of a negative regulatory element situated between the 2 major *bcl-2* promoters has also been observed in addition to a *p53*-negative regulatory element (Haldar *et al.*, 1994). Though considerable interest has been generated regarding the role of *bcl-2*, little information has been forthcoming, in the last few years, concerning the regulation of expression of this gene. Mitogens are known to induce Bcl-2 expression, noticeable at the transcriptional level in normal lymphoblasts, whereas in lymphoid follicules (Chleq-Deschamp *et al.*, 1993; Reed *et al.*, 1987) regulation at the translational level has been proposed. Studies of mRNA as well as protein levels suggested, however, the existence of post-transcriptional regulation (Reed *et al.*, 1987).

Our results show a stimulatory effect of E2 and a negative or inhibitory effect of progestin on Bcl-2 expression. These data are in agreement with findings reported for normal breast tissues and the endometrium (Gompel *et al.*, 1994; Sabourin *et al.*, 1994). Other studies have indicated a strong correlation between the presence of Bcl-2 and estrogen receptor in breast-cancer biopsies (Leek *et al.*, 1994). The variation in the levels of Bcl-2 expression that we have observed in different breast-cancer cell lines can also be considered in the light of their hormone-dependency. The level of Bcl-2 expressed was higher in E2-dependent cells than in E2-independent cells, which reinforces the notion of hormone-dependent control of Bcl-2 expression.

Other reports on Bcl-2 and Bax expression in MCF-7 cells under E2 treatment have, as in the present work, failed to detect any significant variation in Bax expression. (Teixeira *et al.*, 1995; Wang and Phang, 1995). The absence of any variation in Bax-protein levels agrees well with the reported anti-apoptotic effect of E2 on MCF-7 cells (Kyprianou *et al.*, 1991). The low level of Bcl-2, together with a stable level of Bax protein, argue in favour of a pro-apoptotic effect of P.

Our data indicate that E2 could be a key molecule in the regulation of the *bcl-2* gene in breast-cancer cells, and P could have a counterbalancing effect on E2-mediated tumor promotion and/or apoptosis.

ACKNOWLEDGEMENTS

We are grateful to the French Ministry of Foreign Affairs IFCPAR/CEFIPRA (Project 404-2) and ARC for support and encouragement. We thank Dr. F. Marano for providing *in vitro* culture facilities. The assistance of Ms. S. Zyzemski in preparing this manuscript is gratefully acknowledged.

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