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p21, p27 and p53 in estrogen and antiprogestin-induced tumor regression of experimental mouse mammary ductal carcinomas

Silvia Vanzulli[1], Alejo Efeyan[2], Fernando Benavides[3], Luisa A.Helguero[2], Giselle Peters[2], Jianjun Shen[3], Claudio J.Conti[3], Claudia Lanari[2] and Alfredo Molinolo[2,4]

[1]Academia Nacional de Medicina, Buenos Aires, Argentina, [2]Laboratory of Hormonal Carcinogenesis, Instituto de Biología y Medicina Experimental (IBYME-CONICET) Buenos Aires, Argentina and [3]Department of Carcinogenesis, The University of Texas M.D. Anderson Cancer Center, Science Park Research Division, Smithville, TX 78957, USA

[4]To whom correspondence should be addressed
Email: molinolo@dna.uba.ar

Metastatic mammary carcinoma tumor lines 59-2-HI and C7-2-HI originated in female BALB/c mice treated with medroxyprogesterone acetate and are maintained by syngeneic transplantation. Both lines express estrogen (ER) and progesterone receptors (PR) and regress completely after estradiol (E_2) or antiprogestin treatment. The BET tumor line, of similar origin and biological features, regresses only after E_2 treatment. To investigate possible differences between E_2- and antiprogestin-mediated effects we evaluated the morphological features, mitosis and apoptosis, and the differential expression of cell-cycle inhibitors associated with tumor regression. Treatments started when tumors reached 50–100 mm^2. After 24–96 h, tumors were excised and processed for morphological and immunohistochemical studies. Regression was associated with a significant and early decrease in the number of mitosis and with higher percentages of apoptotic cells. These phenomena were accompanied by an increase in p21 and p27 expression in the E_2 and antiprogestin-responsive lines treated with E_2, RU 38.486 or ZK 98.299 ($P < 0.05$). In BET tumors treated with E_2, p21 expression remained within basal levels and only p27 increased ($P < 0.05$). p53 was low in control 59-2-HI and C7-2-HI tumors and increased after treatment ($P < 0.05$) whereas BET untreated tumors already expressed high levels of p53 and MDM2. Although the immunohistochemical findings were compatible with alterations of p53, SSCP evaluation failed to disclose the presence of mutations, suggesting that the defective expression of p21 is related to an impaired p53 pathway. UV irradiation failed to increase p21 expression in BET, but was able to induce p53 and p21 in 59-2-HI and C7-2-HI tumors. The absence of an increased expression of p21 in E_2-regressing BET lesions suggests that this protein is not necessary for estrogen-induced regression, but may be essential for antiprogestin action. Our results also suggest that p53/MDM2 alterations may be one of the mechanisms responsible for selected hormone resistance in breast carcinomas.

Abbreviations: CDK, cyclin-dependent kinase; ER, estrogen receptors; E_2, 17-β-estradiol; H&E, hematoxylin–eosin; HPF, high power fields; MPA, medroxyprogesterone acetate; PR, progesterone receptors; RU, RU 38486 or mifepristone; SSCP, single-strand conformation polymorphism; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP biotin nick end labeling-technique; ZK, ZK 98299 or onapristone.

Introduction

Breast cancer is the most common malignant neoplasm throughout the occidental world and one of the leading causes of death in women, surpassed only by lung cancer (1,2). Epidemiological, clinical and experimental evidence indicate that female sex steroid hormones, and especially estrogens, are essential players in the induction of the disease, although the mechanism underlying their mitogenic effect has not been elucidated definitively. They may indirectly act through the activation of growth factor pathways and associated proteins (3), directly modulating the cell-cycle machinery (4) or by sequestering specific inhibitors (5).

More recent observations have demonstrated a physical association between estrogen receptors (ER) and progesterone receptor (PR) isoform B (6), p53 (7) or cyclin D1 (8) and have opened new insights into the mechanisms by which estrogens may modulate and be modulated by other mitogenic pathways.

More controversial is the role of progestins in breast cancer. Following the uterus model, progesterone has been regarded exclusively as a differentiating agent, as opposed to estrogens (9). There is, however, an increasing body of evidence indicating that it may play an important role in the induction and maintenance of the neoplastic phenotype in the mammary gland (10–18).

The standard therapy in hormone-dependent cancers, such as breast and prostate, is the hormonal ablation through surgical castration or by the use of synthetic antagonists (19). The use of the antiestrogen tamoxifen is still the treatment of choice in breast cancer expressing functional ER and PR but it has also been used in the past in patients with ER-negative tumors, suggesting the possible involvement of mechanisms other than the ER (20). Progestin antagonists have also been reported to exert some antitumor activity, a fact pointing towards alternative therapeutic modalities in the management of metastatic carcinoma (21).

Among the different mechanisms involved in the endocrine inhibition of tumor growth, an increase in the rate of cell death, apoptosis and/or decrease in the rate of cell proliferation, cytostasis, are especially important. Apoptosis is one of the essential mechanisms in the remodeling of hormone-dependent tissue (22). It is also involved in the involution of endocrine-dependent organs following withdrawal of trophic hormones, and there is evidence that it plays an important role in the growth of hormone-dependent tumors (23). The regulation of cell proliferation and death seems to proceed through a series of common pathways, the alteration of which have been associated with the development of neoplasms. It has been demonstrated that the inactivation of tumor suppressor genes such as p53 and RB is implicated in the development and or

progression of different tumors (24) and that the loss of function of a new family of cell-cycle regulators that act as cyclin-dependent kinase (CDK) inhibitors might also lead to tumor development. Based on sequence homology, these inhibitors fall into two families, KIP and INK. KIP family members include p21 (also known as WAF1, Cip1 or Sdi), $p27^{kip1}$ and $p57^{kip2}$. p21 binds to and universally inhibits the cyclin/CDK complexes, thereby preventing phosphorylation of the RB and thus inhibiting cell proliferation (25). Alterations in p53 function can result in loss of p21 expression and may be one of the mechanisms by which altered p53 influences tumor progression. However, it has been demonstrated that p21 expression may also be activated by p53-independent pathways (26). p27 is also a negative regulator implicated in G_1-phase arrest induced mainly by TGF-β1 and cell–cell contact (27).

We have developed a model of hormonal carcinogenesis in female virgin BALB/c mice, in which the administration of high doses of medroxyprogesterone acetate (MPA) leads to the development of ER(+), PR(+) ductal metastatic mammary carcinomas (16,17). Several of these tumors have been maintained through syngeneic passages in MPA-treated animals, to preserve their original progestin-dependent growth behavior. From these *in vivo* lines we have obtained progestin-independent lines that are able to grow in untreated or ovariectomized animals. All progestin-dependent and independent tumor lines express ER and PR (13). In *in vitro* primary cultures, we showed the direct proliferative effect of MPA, which is mediated by PR (28). Antiprogestins and PR antisense oligodeoxynucleotides significantly inhibit tumor growth, demonstrating that PR are involved in cell growth, even in absence of a specific ligand (14,29). We have also demonstrated that estrogens may induce complete regression in *in vivo* progestin-dependent and independent tumor lines (30).

In this paper, we have explored the involvement of cell-cycle regulators in hormone-induced tumor regression. For this purpose, we have used three of our progestin-independent tumor lines, BET, which regresses completely after estrogen treatment but is antiprogestin resistant, and the 59-2-HI and C7-2-HI tumor lines which regress with either treatments. We have analyzed the histological features, regarding the involvement of apoptosis and mitosis in this phenomenon, and the differential expression of p53, p21, p27 and MDM2. We demonstrate a high expression of wild-type (wt) p53 and MDM2 associated with non-inducible p21 in the antiprogestin resistant line, which may explain the differential response of these tumors to estrogens and antiprogestins.

Materials and methods

Tumors

The progestin-independent tumor lines 59-2-HI, C7-2-HI and BET were selected for this study. They are mammary ductal carcinomas induced by MPA in female BALB/c mice that express PR and ER, and are maintained by syngeneic transplants in untreated female mice. 59-2-HI regress with both estrogen and antiprogestin treatments (29), while BET regress only with estrogen treatment (31). In selected experiments an estrogen/antiprogestin resistant tumor line, 59-HI, was also evaluated.

Hormones and antihormones: treatments

17-β-Estradiol (E_2) (Sigma Co., St Louis, MI) 5 mg silastic pellets were implanted subcutaneously (s.c.) as described previously (30).

ZK 98299 or onapristone (ZK) was kindly provided by Schering AG (Berlin, Germany). This is a Type 1 antiprogestin that forms stable complexes with the PR that are unable to bind to DNA (32). RU 38486 or mifepristone (RU) was a gift from Roussel Uclaf (Romainville, France). Mifepristone is a Type II antiprogestin forming, with PR, complexes that bind DNA, but are unable to induce transcription. Both are very efficient antiprogestins; RU486 also has antiglucocorticoid effects (18). Both antihormones were administered s.c. in daily doses of 10 and 6.5 mg/kg body wt in saline, respectively, in the contralateral flank of the tumors.

Experimental design

The tumors were transplanted s.c. by trocar (1–2 mm^2) in the right inguinal flank of 2-month-old virgin female BALB/c mice. Tumor size was measured every day during the first week, and every 2–3 days thereafter, with a Vernier caliper (length and width). The treatments were started when the tumors reached a size of ~50–100 mm^2. Mice bearing 59-2-HI tumors were treated with E_2, ZK or RU (16–19/group); six animals were inoculated daily with saline solution and remained as controls. Twenty mice bearing BET tumors were treated only with E_2 and four animals remained as controls. Three or four animals per group were killed daily between 24 and 96 h post-treatment. Both experiments were repeated twice at 24 and 48 h. Mice transplanted with C7-2-HI were treated only for 48 h. Mice bearing 59-HI were treated with E_2, ZK or RU for control studies and three animals per group were killed 48 h post-treatment.

UV-B radiation. Mice bearing 59-2-HI ($n = 3$), C7-2-HI ($n = 3$) or BET ($n = 3$) tumors of ~100 mm^2 were subjected to 100 mJ/cm^2 during 1 min under anesthesia, during 4 consecutive days, using a National Biological Corporation Panosol II-4 lamps SSX24T12/UV-B-HO. Four days after the last irradiation set, the animals were killed together with control groups ($n = 2$/tumor line). Tumor samples were dissected out together with the skin and processed for histological studies.

Morphological studies

Tumor tissues were fixed in 10% buffered formalin and embedded in paraffin for histological studies. The morphological features of both tumor parenchyma (histological type, differentiation and growth pattern) and stroma (blood vessels, cellular infiltrates, fibrosis and inflammation) were evaluated in hematoxylin–eosin (H&E) stained slides. Mitosis and apoptosis were counted in 5 μ H&E stained sections by direct evaluation of at least 10 high power fields (HPF) or 500 tumor cells. The number of mitosis is expressed as (total number of cells in mitosis×HPF/total number of cells×HPF) ×100. Apoptosis was evaluated by identifying cell shrinkage with condensation, peripheral clumping or fragmentation of nuclear chromatin and by the presence of apoptotic bodies (33). The number of apoptosis is expressed as (total number of cells in apoptosis×HPF/total number of cells×HPF) ×100. Cell proliferation was evaluated by bromodeoxyuridine labeling (Cell proliferation kit, Amersham, UK) and apoptosis by the modified TUNEL [terminal deoxynucleotidyl transferase (TdT)-mediated dUTP biotin nick end labeling-technique] (Apoptag Plus; Oncor, Gaithersburg, MD) (34). Both assays were performed on selected samples according to instructions of the manufacturer. Positively stained nuclei were evaluated as described for mitosis and apoptosis.

Immunohistochemistry

Immunohistochemical detection of p21, p27, p53, MDM2, cytokeratins and CD18 was performed on formalin-fixed, paraffin-embedded tissues, using the ABC technique (35). Briefly, paraffin sections were de-waxed in xylene and rehydrated through graded ethanols. Endogenous peroxidase activity was inhibited using 3% H_2O_2 in distilled water. Sections were blocked in 2% normal goat serum and then incubated overnight at room temperature with the specific antibodies. Microwave antigen retrieval (p53) or enzyme digestion (cytokeratins) were performed before immunostaining. The antibodies and dilutions used are listed in Table I. After incubation with the primary antibody the slides were washed three times with phosphate buffered saline (PBS) and incubated with a biotin-conjugated secondary antibody (1:150 in PBS, 3% normal goat serum, Vector Laboratories, Burlingame, CA) for 30 min at room temperature. After washing three times with PBS, the slides were incubated with the ABC complex, prepared according to the instructions of the manufacturer, for 30 min at room temperature. The slides were then washed with PBS and developed with 3-,3′-diaminobenzidine, 0.30 mg % in PBS with H_2O_2 to a final concentration of 0.5%, under microscopic control. As positive controls, we used mouse-irradiated skin (p21), a human colon adenocarcinoma (p53), normal epidermis (cytokeratins) and a non-specific granuloma for CD18. Primary or secondary antibodies were omitted in the negative controls. The immunostained nuclei were quantified in each case. All counting was performed under a standard light microscope in 1000× field evaluating number of mitosis or apoptosis or positive nuclei/total number of cells. Ten fields or at least 500 cells were counted on each section and the results are expressed as mean ± standard deviation.

Analysis of p53 mutations

Three samples from each of the three murine ductal mammary carcinomas studied, 59-2-HI, C7-2-HI and BET, were evaluated for p53 mutations.

Table I. Antibodies used

Antibody	Source	Clone	Type	Dilution
p21	Santa Cruz Biotechnology, Santa Cruz, CA	C-19	Polyclonal	1:50
p27	Santa Cruz Biotechnology, Santa Cruz, CA	C-19	Polyclonal	1:50
Cytokeratin	Dako, Carpinteria, CA	Z0622	Polyclonal	1:150
p53	Biogenex Laboratory, San Ram′n, CA	CM-1	Polyclonal	1:30
CD18	Kindly provided by Dr Mirta Giordano, National Academy of Medicine, Buenos Aires, Argentina	M1-70	Monoclonal	1:50
MDM2	Santa Cruz Biotechnology, Santa Cruz, CA	SMP-14	Polyclonal	1:50
ER	Santa Cruz Biotechnology, Santa Cruz, CA	MC-20	Polyclonal	1:100
PR	Santa Cruz Biotechnology, Santa Cruz, CA	C-20	Polyclonal	1:100

p53 gene mutation analysis was conducted by single-strand conformation polymorphism (SSCP) followed by DNA sequencing of unique SSCP bands. Genomic DNA isolated from BALB/c mice was used as control template to amplify regions containing *Tp53* exons 5–8.

SSCP analysis. The SSCP protocol used by Shen *et al.* (36) was followed with minor modifications. Briefly, DNA fragments were labeled by the addition of 0.25 µl of [^{33}P]dCTP (NEN, Boston, MA) into each 25 µl of PCR reaction. Two microliters of each PCR-amplified sample were taken out and added into 8 µl of stop solution (95% formamide, 10 mM NaOH, 0.25% bromophenol blue and 0.25% xylene cyanol). The sample was denatured for 5 min at 95°C in a heating block and quenched immediately on ice. Five microliters of each sample were loaded onto two types of gels: 0.5× MDE gel (BMA, Rockland, ME) containing 5% (v/v) glycerol and 0.5× MDE gel without glycerol. The gels were electrophoresed at 6–8 W at room temperature for 18 h. The gels were dried on 3 MM Whatman paper and exposed to Kodak Biomax MR films.

Statistical studies

Differences between groups in the number of mitosis, apoptosis or stained nuclei (immunohistochemistry) were evaluated using ANOVA followed by the Tukey *t*-test to compare differences between experimental and control groups.

Results

Hormone response and hormone receptor expression in 59-2-HI, C7-2-HI and BET

Complete tumor regressions were observed with RU, ZK and E_2 in 59-2-HI and C7-2-HI tumors, but only with E_2 in BET tumors, as described previously (31) (Figure 1). Tumor size variations during the evaluation period are shown in the insets; differences between untreated and treated animals were already evident during this period. ER and PR were evaluated by immunocytochemistry, western blot and binding techniques. PR and ER were detected as positively stained nuclei with immunocytochemistry (Figure 2) and both PR_A and PR_B isoforms were present in all tumors as determined by western blots (results not shown; Helguero,L., Lamb,C., Lannri,C. and Molinola,A.). The finding of cytoplasmic/membrane staining for PR in some tumors is still unclear and it has been reported previously (37). Both receptors, ER and PR, were able to bind the hormone [BET: ER 42 ± 34 fmol/mg prot, PR 153±113 fmol/mg prot (n = 4); 59-2-HI: ER 113 ± 12 fmol/mg prot, PR 168 ± 20 fmol/mg prot (n = 4); C7-2-HI: ER 101±42 fmol/mg, PR 223 ± 32 fmol/mg prot, (n = 4)]. MPA was able to down regulate PR, which suggests a functional receptor (not shown). Our data indicate that there are no evident differences at hormone receptor levels, which may justify the differences in hormone response.

Morphological studies

Controls. 59-2-HI (Figure 3A) and C7-2-HI (not shown) tumors are moderately differentiated ductal carcinomas. BET tumors displayed the features of poorly differentiated ductal carcinomas (Figure 3B). All tumors exhibited high mitotic activity, low levels of apoptosis and foci of necrosis.

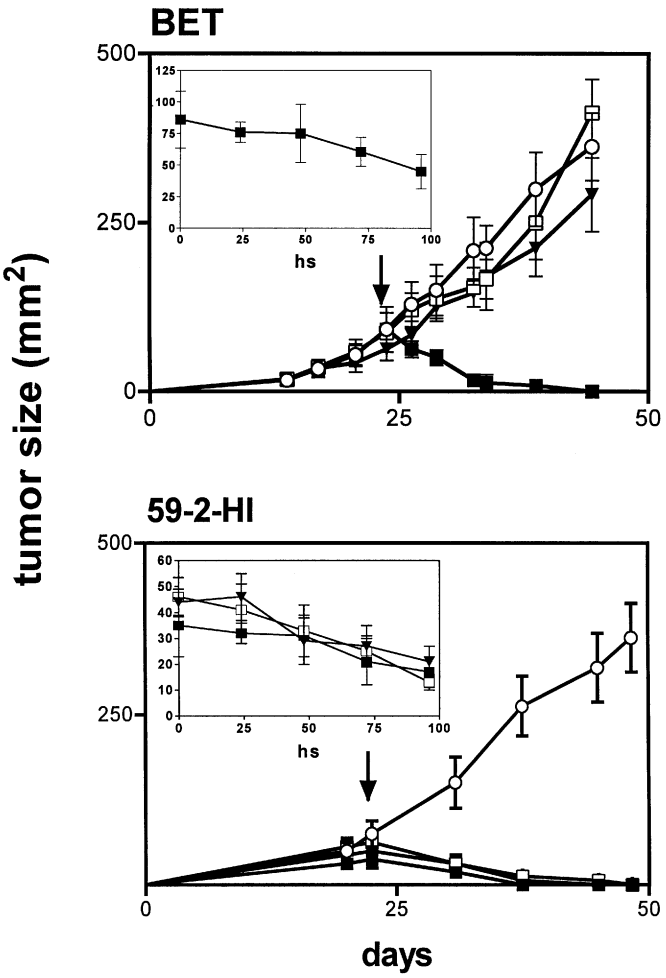

Fig. 1. Effect of antiprogestins and E_2 treatment on tumor growth of two tumor lines: BET and 59-2-HI. The arrow indicates the initiation of treatments. (Inset) Changes in tumor sizes registered during the 96 h period in which tumors were evaluated. E_2 (filled square) was supplied as one silastic pellet of 5 mg implanted s.c. in the back of the animal, RU 6.5 mg/kg (square) or ZK 10 mg/kg (filled inverted triangle) were administered daily s.c. in the left inguinal flank; control animals (circle) received saline solution.

Tumor regression. No histological differences were observed between 59-2-HI or C7-2-HI tumors regressing with RU, ZK or E_2 or in BET tumors regressing with E_2. Twenty-four hours after the beginning of the treatments the predominant picture was the presence of solid epithelial sheets of cohesive cells

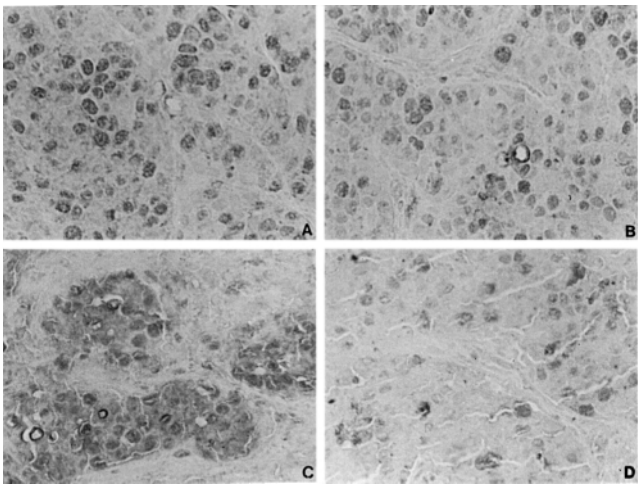

Fig. 2. (**A**) PR staining in 59-2-HI: almost all neoplastic cells show nuclear staining. (**B**) ER staining in 59-2-HI: moderate to strong positive nuclear staining for ER is present in most malignant cells. (**C**) PR staining in BET: malignant cells show nuclear staining for PR; cytoplasmic staining is also evident. (**D**) Variable staining for ER in this BET tumor. Strong to mild immunoreactive nuclei are seen. All pictures are 400×.

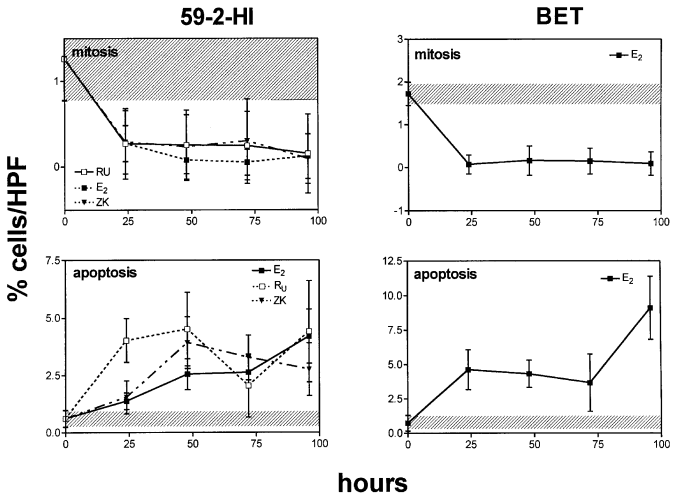

Fig. 4. Number of cells in mitosis and number of apoptotic cells expressed as percentage of cells per HPF in 59-2-HI and BET tumor lines treated with antiprogestins and/or E_2. A significant decrease of the number of mitosis ($P < 0.001$) was observed in both tumors with all treatments. In 59-2-HI, a significant increase of the number of apoptotic cells ($P < 0.001$) was observed in RU-treated tumors after 24, 48 and 96 h and in ZK- or E_2-treated mice at 48, 72 and 96 h. The increase in apoptosis in BET tumors treated with E_2 was statistically significant ($P < 0.01$) at 24, 48, 72 and 96 h after treatment initiation. Data represent the media ± SD of 45 fields (15/sample).

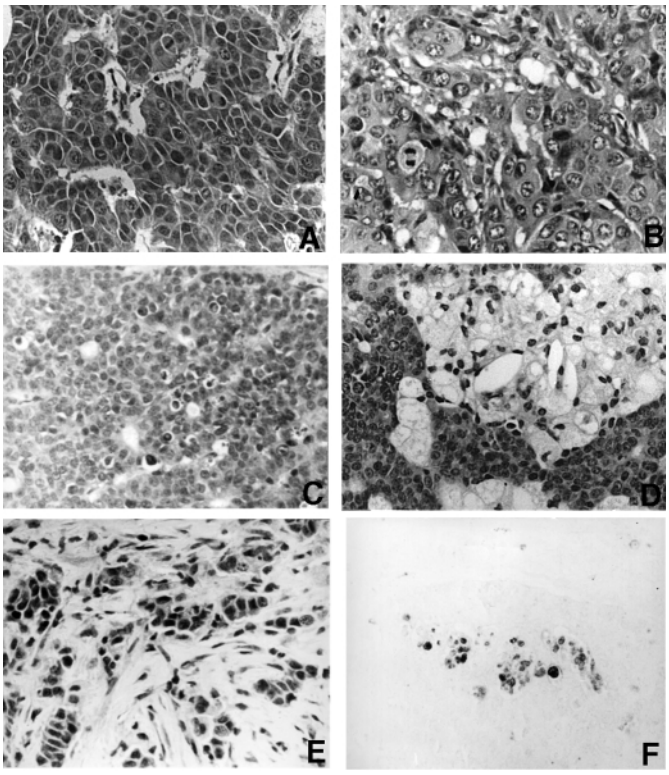

Fig. 3. (**A**) Control 59-2-HI tumor showing solid sheets of cancer cells with occasional glandular differentiation (arrow). Several mitotic figures are evident (arrowhead) (H&E, 400×). (**B**) Control BET tumor disclosing nests of tumor cells, separated by thin strands of stroma (H&E, 400×). Mitotic images (arrow) indicating cellular proliferation and a low rate of apoptosis were the hallmark of untreated tumors. (**C**) Several isolated apoptotic cells (arrow) in a treated 59-2-HI carcinoma (200×). (**D**) Groups of vacuolated cells with abundant foamy cytoplasms and central round or ovoid nuclei (arrow) (H&E, 400×). Immunocytochemical identification of CD18 confirmed their histocytic origin (not shown). (**E**) Regressing BET tumor showing abundant fibroblastic stroma and a few strands of remaining neoplastic cells (arrow) (H&E, 400×). Note the absence of mitotic figures. (**F**) Apoptotic bodies identified with TUNEL technique (200×).

with scanty intratumoral stroma. A few number of mitosis and numerous apoptotic images were observed. At 48 h post-treatment, a higher number of either isolated or clustered apoptotic cells (Figure 3C), and variable amount of necrosis was observed in all tumors. Seventy-two hours post-treatment, small nests separated by increased stroma replaced the sheets. Groups of epithelial-like cells with vacuolated cytoplasms were observed intermingled with polygonal cells showing clear nuclei and foamy cytoplasms (Figure 3D). Polygonal foamy cells were positive for CD18 and negative for cytokeratin (not shown) indicating their histiocytic origin. Ninety-six hours post-treatment, the tumor parenchyma was reduced to lines or clumps of a few cohesive neoplastic cells with an evident increase in the intratumoral stroma (Figure 3E). Initially, there was a fibroblastic proliferation mimicking single neoplastic cells, together with some eosinophils and lymphocytes. At 7 days of treatment, the stromal changes were characterized by progressive fibrosis with mild lymphocytic infiltrates and occasional foci of calcification. No evident differences were observed in vascularization between untreated and treated tumors, regardless of the treatment type. A high correlation was observed between the identification using TUNEL technique and the morphological evaluation of apoptosis in H&E-stained slides. A cluster of apoptotic cells stained with TUNEL is shown in Figure 3F.

Mitosis and apoptosis counting

The number of mitosis decreased significantly ($P < 0.001$) at 24 h, and remained constant up to the end of the experiment. The pattern of inhibition was similar with all treatments in 59-2-HI (Figure 4) and C7-2-HI (not shown) and with E_2 in BET (Figure 4). These results were also confirmed in selected samples using bromodeoxyuridine labeling (not shown).

Apoptosis was quantified by evaluation of H&E-stained slides. Low levels were detected in controls, and significant increases ($P < 0.001$) were observed after treatment with RU or ZK (59-2-HI or C7-2-HI), with the highest values at 48 h.

Similar increments were observed in E_2-treated mice with the highest values at 96 h (Figure 4). A decrease in the fraction of cells in apoptosis was also observed at 72 h, but the percentage increased to previous values after 96 h. These results were confirmed using the TUNEL technique (i.e. BET, control: TUNEL 0.87 ± 0.66, H&E 1.03 ± 0.93; 48 h E_2: TUNEL 9.47 ± 3.15, H&E 8.48 ± 2.43). A consistent correlation was observed when comparing the results obtained with TUNEL and evaluating H&E-stained slides in all cases. No change in the number of mitotic and apoptotic cells was detected in the unresponsive 59-HI-treated tumors (not shown).

p21, p27, p53 and MDM2 expression

Using immunocytochemistry, all cell-cycle proteins gave nuclear specific staining. Occasional positive staining was found in the cytoplasm, attributable mostly to background staining, as demonstrated in the negative controls (not shown). The expression of cell-cycle inhibitory proteins was studied at different times in 59-2-HI and BET tumor lines and after 48 h of treatment in C7-2-HI tumor line. In 59-2-HI tumor line, the expression of p21 and p27 was significantly increased ($P < 0.001$) at 24 h post-treatment. This increase was sustained only in tumors treated with E_2 and ZK (Figure 5) reaching its peak at 48 h. In RU-treated mice, p21 and p27 expression decreased significantly after 24 h, over control values for p21 and to control values for p27 (Figure 5), reaching their lowest levels at 72 h. With all treatments, there was a parallelism between p21 and p27 expression. In BET tumors, only an increase in p27 expression was detected with E_2 treatment; no differences were detected for p21 as compared with untreated tumors (Figures 6 and 7). The basal p21 levels in control BET tumors were even lower than the levels found in untreated 59-2-HI ($P < 0.05$, Figure 7). To rule out the possibility that, in BET tumor, a change in p21 expression may have occurred before the first 24 h after E_2 treatment, two mice were treated for only 6 or 12 h and similar results were obtained (not shown). p21 and p27 were also significantly increased in C7-2-HI tumors after 48 h of E_2 or antiprogestin treatment (Figure 7).

These results led us to study the expression of p53 and MDM2. A very high expression of p53 (Figures 7 and 8) and MDM2 proteins was detected in treated and untreated BET tumors (not shown). In 59-2-HI and C7-2-HI untreated tumors, p53 expression was lower as compared with BET ($P < 0.05$) and it significantly increased after E_2 and antiprogestin treatment, suggesting that p21 may be p53-dependent. Under the same experimental conditions, no differences in p21 and p27 expression were observed in the 59-HI-unresponsive tumor line.

As it has been reported that a high nuclear expression of p53 by immunohistochemistry is strongly associated with a mutated protein (38), we investigated the occurrence of mutations in the p53 gene by SSCP in these tumor lines. No mutations were found when analyzing exons 5–8 in any of the tumors studied (not shown). The fact that wt p53 and MDM2 levels were high in BET tumors suggests that this pathway be altered in this tumor line. To test this hypothesis we evaluated the expression of these proteins using an experimental setting in which hormones were not involved.

It is already established that UV-B exposure induces an increase in p53 and p21 expression. To further corroborate that p21 was non-inducible in BET tumor line, BET-, 59-2-HI- and C7-2-HI-tumor bearing mice were exposed to UV-B and the expression of p53 and p21 was evaluated in tumor cells close to the epidermis. Both p53 and p21 expression was increased in 59-2-HI or C7-2-HI tumors but only p53 was highly expressed in BET tumors (Figure 9).

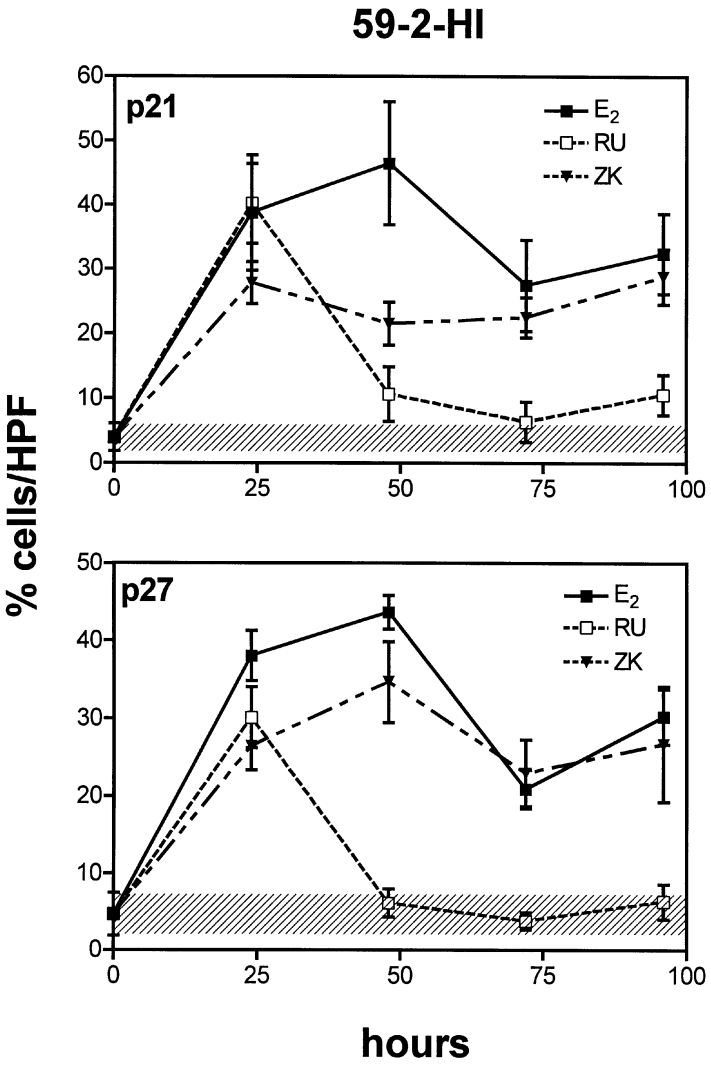

Fig. 5. p21 and p27 expression (% stained nuclei/HPF) in 59-2-HI-treated tumor. A significant increase ($P < 0.001$) of p21 and p27 protein was observed at 24, 48, 72 and 96 h after treatment with E_2 and ZK. The increase in p21 expression was significant ($P < 0.001$) 24 and 96 h after treatment with RU; p27 increase became significant ($P < 0.001$) only 24 h after treatment was initiated. Data represent the media ± SD of 45 fields (15/sample).

Discussion

Our results demonstrate that an intact p53/p21 pathway is not necessary for estrogen-induced tumor regression and suggest that p21 may be essential in mediating antiprogestin-induced regression. The increase in p21 and p27 is associated with estrogen and antiprogestin-induced cytostasis and apoptosis in two estrogen and antiprogestin-responsive tumor lines. Only p27 was increased in response to E_2 treatment in a tumor line sensitive only to E_2. All tumors expressed similar levels of ER and PR; the morphological and biological features of tumor regression were also similar in all.

The lack of regulation of p21 by E_2 in BET led us to evaluate the expression of p53 and MDM2; high cellular expression levels of both proteins were observed. The presence

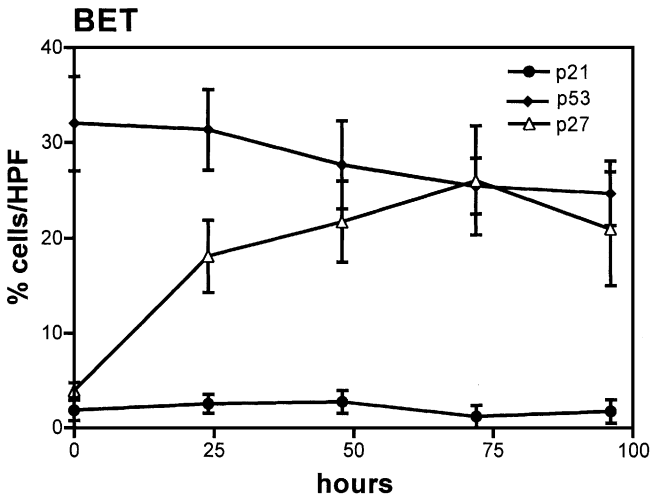

Fig. 6. p21 and p27 and p53 expression (% stained nuclei/HPF) in BET tumor treated with E_2. A significant increase ($P < 0.001$) of p27 protein was observed at 24, 48, 72 and 96 h after treatment was started. A slight decrease in p53 ($P < 0.05$) was observed 72 and 96 h after treatment.

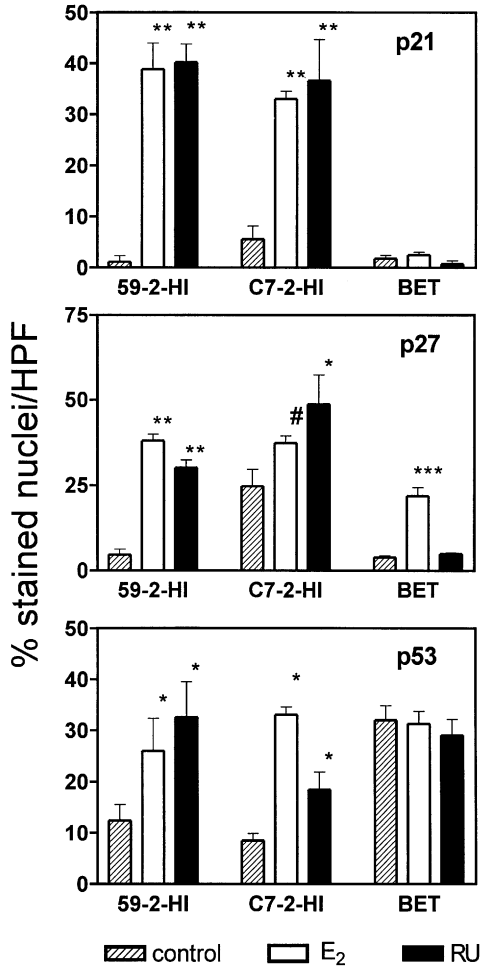

Fig. 7. p21, p27 and p53 (% stained nuclei/HPF) expression in 59-2-HI (24 h), C7-2-HI (48 h) and BET (48 h) tumors treated with E_2 or RU. Bars represent the media ± SE of three samples. Ten to fifteen fields were counted in each sample. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ between experimental and control groups; $^{\#}P < 0.05$ in each individual experiment. Data from 24 h from 59-2-HI were selected for this representation because the highest values for RU were obtained.

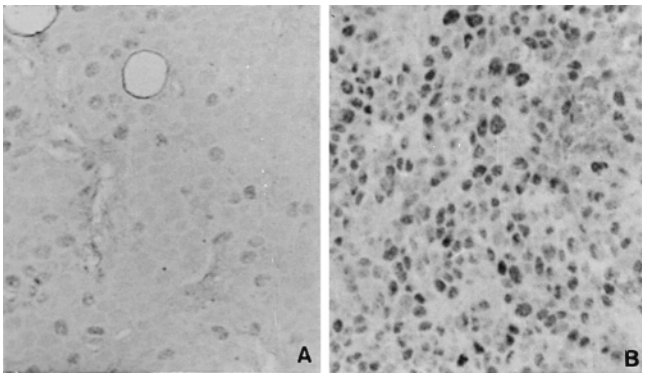

Fig. 8. p53 expression in untreated 59-02-HI and BET tumors. (**A**) 59-2-HI: only a few carcinomatous cells show mild to moderate nuclear staining (400×). (**B**) BET: a high percentage of malignant epithelial cells disclose strong to mild positive nuclear staining (200×).

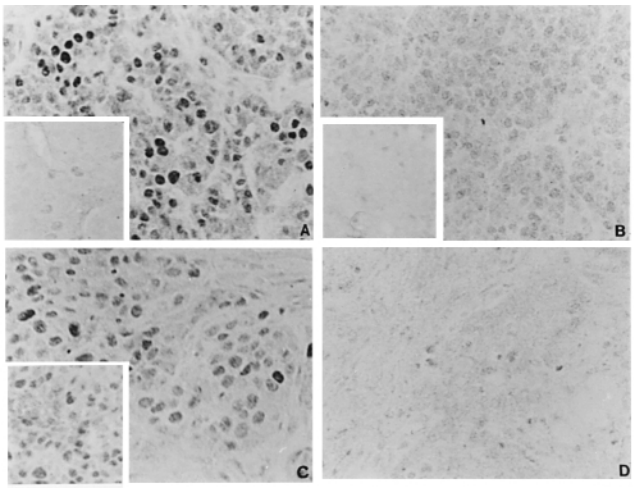

Fig. 9. Immunostaining of p21 and p53 after UV-B exposure. (**A**) 59-2-HI, p53: ~50% of the cells in this lesion disclose strong nuclear staining. Stromal cells are negative. (**B**) 59-2-HI, p21: most malignant cells show positive nuclear staining for p21. (**C**) BET, p53: close to 100% of the cells shows fine granular nuclear staining. (**D**) BET, p21: only a few isolated cells are positive. All figures are 400×. (Insets) Immunostaining in untreated control animals.

of an altered p53/p21 pathway in this tumor line was also confirmed by the fact that these proteins remained unchanged after UV-B radiation, under experimental conditions in which both p21 and p53 were increased in the sensitive lines. An SSCP analysis failed to reveal p53 mutations, although we cannot rule out the presence of mutations not identified by this technique.

A significant degree of necrosis was always present in our tumors. A catastrophic event such as necrosis plays an important role in tumor shrinkage, as measured by tumor volume. This event may also be directly related to apoptosis, as the death of a few strategically located cells may induce tissue collapse that may in turn trigger vascular occlusion and subsequent hypoxia, leading to necrosis and more apoptosis. In hormone/antihormone-induced regression, non-neoplastic stromal cells, such as those supporting the vascular structures, may also be the targets for hormone action (39,40). Although both parenchymal and stromal events account for final tumor volume, specific tumor growth in carcinomas is regarded as a

positive balance between malignant epithelial cell division and cell death. At a constant apoptosis rate, an increase in cytostasis, associated or not with cell differentiation, may, by itself account for tumor regression. Tumor inhibition, however, may also mean tumor dormancy, a process in which a significant portion of malignant cells enters G_0, thus avoiding the deleterious effects of a therapeutic agent. Hence, the importance of using models in which complete tumor regression, rather than tumor inhibition, constitute the experimental end-point. Although we did not address angiogenesis specifically in our experiments, no evident differences in tumor vessels were observed between control and treated groups. We cannot however rule out subtle changes that may become evident under specifically designed experiments, that may contribute to the alterations observed.

We have used a very reliable experimental tumor model (16) in which, unlike most, complete tumor regressions are observed (30). The histological picture of regression, characterized by a decrease in cell division and increased apoptosis rates, was the same under antiprogestin and estrogen treatments. In our model, unlike the classical description in which isolated cells enter apoptosis, numerous cells underwent apoptosis simultaneously generating apoptotic areas. This phenomenon may point to clones or especially sensitive groups of cells or to uneven tissue distribution of the hormone. Inflammatory or reactive cell infiltration was not a relevant histological feature, except for foamy macrophages. We did not observe morphological changes suggestive of changes in cell differentiation in this model of tumor regression, probably because, on one hand, the time frame in which this phenomenon is occurring, hours and days, precludes the induction of genes responsible for switching on cell differentiation and, on the other hand, this set of genes may not be the primary target for E_2 and antiprogestins. In other models of hormone-induced tumor regression (23,32,41–43) cytostasis and terminal cell death, associated with cell differentiation and arrest in G_0/G_1, were the predominant regression mechanisms. Cytostasis is predominant over cell death in tumor models in which only partial regressions or growth arrests are observed, such as the antiestrogen treatment in rats bearing MNU-induced mammary carcinomas (44), or in an *in vivo* xenograft model of prostate cancer after androgen ablation (45,46). Increased levels of apoptosis, on the other hand, were described in human mammary tumor biopsies that had shown a positive response to steroid and non-steroid antiestrogens (47).

The characterization of the expression of cell-cycle proteins associated with apoptosis and/or cytostasis and tumor regression has been essential to understand the mechanisms related to tumor growth control. Apoptosis has been regarded as an early event when mediated by p53 (48) and, when mediated by the p27 pathway, it has been reported to be evident only 72 h after the increase in the protein levels (49). Coincidentally, in the BET tumor line, in which no increase in p21 was detected, apoptosis was also a late event. p21 has been involved in cell-cycle arrest in both p53-dependent or independent mechanisms, and it has been related mainly with cytostasis and cell differentiation rather than apoptosis (50). There are, however, several reports indicating that, under certain conditions p21 may be necessary for cell proliferation and that PR may increase p21 expression directly by an interaction with co-activators Sp1 and CBP/p300 (51). In our experimental setting it seems more probable that p21 regulation by PR is p53-dependent, as both proteins were simultaneously increased in treated tumors. The role of p27 is also puzzling as, although it has been associated with cytostasis, it has also been implicated in apoptosis (27,49). That both proteins play crucial roles in cytostasis has been assessed further in experiments in which p21 or p27 antisense oligonucleotides counteract the inhibitory effects of antiestrogens in MCF-7 cells (52).

Interestingly, it has been reported recently that overexpression of p21 can induce the expression of estrogen-binding proteins thus mimicking estrogen action in receptor-negative cells (53). It may be then possible that the antiprogestin-induced increase in p21 expression can activate the ER pathway. When blocking PR with antiprogestins, the consequent increase in p21, together with the increase in p27 expression, may trigger the cascade of signals leading to cytostasis and apoptosis. E_2 can induce regression regardless of p21 activation in BET, suggesting that both signals converge downstream; p27 may be necessary but not sufficient for these effects. In C7-HI, a similar tumor line, which regresses partially with antiprogestins, p21 expression was transiently increased in antiprogestin-treated tumors. No changes in G_1 cyclins were detected whereas a significant decrease in cyclin A was evident even 14 days after treatment (54). It is possible then that high levels of p21 and p27 may neutralize high levels of G_1 cyclin/CDK complexes. The low levels of cyclin A may be either the consequence of an antiproliferative state or may be responsible for maintaining this state when p21 and p27 levels become normalized. That estrogens may promote cell-cycle progression at multiple sites has been reviewed recently by Foster *et al.* (55). They have demonstrated that, in addition to regulating cyclin D-CDK4 function, estrogens might independently regulate p21 and p27 expression in MCF-7 cells.

As a result of its high turnover, p53 is usually undetectable by immunohistochemistry, and when present, it has been regarded as evidence of mutations (56,57) or as non-mutated protein stabilized by different factors (58,59). High levels of p53 were detected in BET-treated tumors as well as in untreated controls. In 59-2-HI tumors, p53 nuclear immunoreactivity was high only in treated animals, suggesting that the concomitant increase in p21 is mediated by p53. As stated, SSCP analysis failed to reveal the presence of mutations in exons 5–8, which may point to a deregulated p53 function in this tumor. MDM2 is a p53-induced protein that regulates p53 expression by different mechanisms including ubiquitination, direct binding to the p53 transactivation domain and also by increasing its traffic to the cytoplasm (60). MDM2 is overexpressed in BET tumor and may be playing a role in a non-functional p53/p21 pathway, directly inhibiting the p53 transactivation domain (61). Others have also observed co-expression of high levels of MDM2 and non-functional p53 (61,62).

In conclusion, in this paper we demonstrate that an intact p21 pathway is not necessary for estrogens to induce tumor regression in responsive mouse mammary carcinomas. Our data also suggest an essential role of p21 in antiprogestin-induced regression, and point to possible mechanisms of hormone resistance. We also provide data regarding the morphological aspects and the role of apoptosis, mitosis and cell-cycle inhibitors in both estrogen and antiprogestin-induced tumor regression in a well-characterized mouse mammary tumor model. Our findings are especially interesting because they emerge from a fully characterized *in vivo* model in which the natural evolution of hormone-treated tumors ends in complete clinical regression.

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