Estrogen or antiprogestin treatment induces complete regression of pulmonary and axillary metastases in an experimental model of breast cancer progression

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In this paper we demonstrate, using the C7-2-HI metastatic transplantable ductal mammary tumor, that endocrine therapy can induce complete regression of spontaneous lymph node and lung metastases in a mouse model of breast cancer progression. This tumor expresses high levels of estrogen and progesterone receptors and shows a high incidence of early axillary lymph nodes and lung metastases; using this model we had previously shown complete tumor regression of subcutaneous implants. Interestingly, although the metastases showed a more differentiated histology as compared with the primary growth, they underwent complete regression when treated with estrogens or antiprogestins. This phenomenon was associated with sustained cytostasis and apoptosis accompanied by increases in p21 and p27 expression and early tissue remodeling. These results highlight the essential role of PR in regulating cell proliferation in this model as well as its possible use as therapeutic target.

Introduction

Adjuvant therapy is defined as the treatment given in addition to the primary therapy to destroy any cancer cells that may have spread, even if they cannot be detected by radiology or laboratory tests; several studies have shown that adjuvant therapy for breast cancer may increase the chance of long-term survival by preventing a recurrence, http://cis.nci.nih.gov/fact/7_20.htm (1). Endocrine treatment such as tamoxifen (TAM) (2,3), and more recently first-generation aromatase inhibitors (4), are the adjuvant therapy of choice in operable breast cancer as well as in metastatic estrogen receptor alpha (ER) positive carcinomas (5). Interestingly, although the antiestrogen TAM has been used for decades with excellent results, post-menopausal women with breast cancer have also been successfully treated with estrogens, such as diethylstilbestrol or ethinylestradiol (6–8). The exact mechanism by which TAM inhibits tumor growth is still not completely understood (9–12), although it is currently accepted that, as a selective estrogen receptor modulator or SERM, it behaves as an anti-estrogen within the physiological context of the mammary gland. The pathways through which estrogens can inhibit the growth of a hormone-dependent breast cancer are also poorly understood. Experimental support for a growth-inhibitory action has been given by the facts that estrogen treatment induced complete regression in the T61 human xenograft model (13) and also in a T47D variant with overexpression of protein kinaseC alpha or PKC alpha (14). Even more controversial is the fact that antiprogestins may induce tumor regression in the same models in which antiestrogens inhibit tumor growth (15,16). The role of progesterone in breast cancer development has not been as thoroughly researched as the one of estrogen, although many experimental and epidemiological data available point toward a relevant one. The possible relevance of progestins has been highlighted by the results of the Women’s Health Initiative (17) and the Million Women Study (18) that specifically link the use of progestins with human breast cancer.

In a previous publication we sought to evaluate the beneficial effects of estrogen and antiprogestins in mammary cancer, using a hormone-responsive model of breast cancer progression; we demonstrated that in BALB/c mice, mammary ductal carcinomas regress completely when treated with 17-β-estradiol (E2) or antiprogestins (19–21) and partially when treated with TAM (22). This phenomenon is mediated by an important cytostatic effect accompanied by an increase in apoptosis (21). As the carcinomas in this model will eventually develop lymph node and lung metastases, we decided to extend our previous investigations to study the effects of endocrine treatment in advanced disease stages. This is a non-genetically manipulated model, especially suited to evaluate tumor progression (23–25) as the tumors evolve from a hormone-sensitive to a hormone-resistant state, a transition mimicking the acquisition of hormone resistance in human breast cancer. This transition is accompanied by distinctive changes in progesterone receptor (PR) expression; tumors which have acquired hormone independence but are still responsive to endocrine treatment express high levels of steroid receptors while unresponsive tumors show a different pattern of PR expression (26). Most of the experiments regarding hormone or antihormone action have been carried out using 3 or 4 human breast cancer cell lines out of more than 30 available, and in their respective xenografts in immunosuppressed mice (27–29) or in the rat methyl nitrosourea (MNU) (30) or in the dimethylbenz[a]anthracene (DMBA) models (31). As progression models, these tumors have the
disadvantage of not giving rise to metastases, a fact that has been partially addressed, with the models generated in transgenic mice, but unfortunately most of them do not express ER and PR.

In this model we demonstrate that endocrine therapy can induce regression of tumor metastases, a process associated with cytostasis, apoptosis and expression of Cdk inhibitors.

Materials and methods

Experimental model

Animals. Two-month-old virgin female BALB/c mice (Instituto de Biología y Medicina Experimental Animal Facility) were used. The animals were housed in groups of four per cage in an air-conditioned room at 20 ± 2°C under a 12 h light/dark cycle and access to food and tap water ad libitum. Animal care and manipulation were in agreement with institutional guidelines and the Guide for the Care and Use of Laboratory Animals (32).

Tumor. C7-2-HI is a metastatic transplantable ductal mammary tumor induced by the continuous administration of medroxyprogesterone acetate (MPA) to a BALB/c female mouse (23), maintained by serial subcutaneous (s.c.) transplantations into syngeneic female mice. It is a progestin-independent tumor that expresses high levels of ER and PR and shows a high incidence of early (40-60 days) spontaneous metastasis in homolateral axillary nodes and lung (33). The primary implants regressed completely after E2 or antiprogestin treatment (21).

Treatments. Silastic pellets containing 5 mg of 17-β-estradiol (Sigma, St Louis, MI) or 6.5 mg of the antiprogestin RU 38486 (RU) (mifepristone; Sigma) were subcutaneously implanted as described previously (19-21).

Experimental design

Tumor samples were transplanted subcutaneously by a trocar into the right inguinal flanks of mice and the growth evaluated three times a week (length and width) with a Vernier caliper. Axillary node metastases were detectable by palpation ~45 days post-transplantation; at this time the size of the primary tumors was ~150 mm³. Several animals were euthanized 60 days after tumor transplantation and the lungs evaluated histologically for metastases. After positive confirmation the treatments were started in the rest of the animals (Figure 1).

The mice were separated at random and sham operated or treated with E2 (n = 24) or with RU (n = 22). From each group 3 or 4 animals were killed every day between days 1 and 4 and subsequently, on days 7, 15 and 25 post-treatment as shown in Figure 1. Control animals were euthanized on day 60, when treatments were initiated (Control 0, n = 4) and at the end of the experiment, on day 25 after treatment and on day 85 after tumor inoculum (Control 25 days, n = 3-4). Lymph nodes and lungs were fixed in 10% buffered formalin, embedded in paraffin and stained with Hematoxylin-Eosin (H&E) for histological diagnosis. Additional sections of 5 μm thickness were prepared for immunohistochemical studies. The number and size of pulmonary metastases were evaluated on histological sections.

Morphological studies. The morphology of tumor parenchyma (growth pattern, differentiation) and stroma, as well as mitosis and apoptosis were evaluated on H&E stained sections. The latter were counted in 10 and 15 high power fields (hpf), of each section using a 1000× magnification, and expressed as a mean ± standard deviation of the percentage of the ratios between the total number of events (mitosis or apoptosis) and the total cell number per hpf. Mitotic figures were identified morphologically by the condensed ‘hairy’ aspect of the chromosomes. Morphological identification of apoptosis was performed according to criteria previously reported (21); selected samples were also reacted using the deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method according to the manufacturer’s instructions (Boehringer Mannheim, UK) (21). The number of lung metastases was evaluated in two non-consecutive histological sections using 10× objective. Only metastases with sizes >0.5 mm in diameter were included to evaluate the size decrease of lung metastases after treatment.

Immunohistochemistry. Sections of formalin-fixed, paraffin-embedded tissue were reacted with the different antibodies using the avidin-biotin-peroxidase complex technique (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). In brief, endogenous peroxidase activity was inhibited using 3% H₂O₂ in distilled water. Blocking solution (2% normal goat serum) was used before the specific antibody. Polyclonal antibodies to p21 (Oncogene Research Products, Boston, MA), p27 (C-19, Santa Cruz Biotechnology, Santa Cruz, CA), ER (MC-20, Santa Cruz Biotechnology) and PR (C-20, Santa Cruz Biotechnology) were used at 1:50 dilution and incubated overnight at room temperature before the secondary antibody. Sections were reacted with the different antibodies using the avidin-biotin-peroxidase complex technique (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). In brief, endogenous peroxidase activity was inhibited using 3% H₂O₂ in distilled water. Blocking solution (2% normal goat serum) was used before the specific antibody. Polyclonal antibodies to p21 (Oncogene Research Products, Boston, MA), p27 (C-19, Santa Cruz Biotechnology, Santa Cruz, CA), ER (MC-20, Santa Cruz Biotechnology) and PR (C-20, Santa Cruz Biotechnology) were used at 1:50 dilution and incubated overnight at room temperature before the secondary antibody.
temperature. Microwave antigen retrieval (4 cycles of 5 min each in 0.1 M citrate buffer) using 750W Philips M902 microwave oven was used before p21 stain. The reaction was developed with 3, 3'-diaminobenzidine, 0.30 mg % in PBS with H2O2 to a final concentration of 0.5%, under microscopic control. Specimens were lightly counterstained with hematoxylin 10%, dehydrated and mounted. Quantification was performed as described above in Morphological studies.

Statistical analysis
Differences between groups in the number of mitosis, apoptosis or stained nuclei were evaluated using ANOVA followed by the Tukey t-test to compare differences between experimental and control groups. Mann-Whitney non-parametric test was used to compare the number of metastases.

Results
BALB/c mice bearing s.c. C7-2-HI tumors with axillary node metastasis were treated with E2 or RU. Growth differences were evident enough to be detected as early as treatment day 2. The decrease in tumor size was more remarkable in E2-treated mice than in RU-treated mice, since in the latter it took more time to induce complete tumor regressions (Figure 2).

To evaluate the effects of both agents on lung metastasis, randomly selected animals were euthanized at different times, according to the experimental design shown in Figure 1. Four out of six animals euthanized on day 25, showed complete metastases regression; only in one animal a metastasis with a size >0.5 mm was observed. The progressive decrease as well as the total number and the sizes observed in both groups are shown in Figure 3. The differences in both parameters were statistically significant ($P < 0.05$, Mann-Whitney test).

A similar trend was observed with RU and, as described for axillary node metastasis, this treatment was less efficient than E2, since after 25 days of RU treatment regression was not complete in three out of four animals (data not shown).

Morphological features
C7-2-HI tumors growing subcutaneously were classified as ductal semi-differentiated carcinomas; node metastases showed a different pattern of growth, as they displayed the features of well-differentiated ductal carcinomas regardless of the size (Figure 4A and K). Lung metastases on the other hand, showed a poorly differentiated pattern forming nests of highly cohesive cells when they were small (when the treatment was...
initiated (Figure 4B) and a well-differentiated pattern at larger sizes (25 days later) (Figure 4L).

A progressive decrease in the number of tumor cells associated with an increase in stroma was observed in E2- or RU-treated lymph node metastases (Figure 4C, E, G and I). After 4 days of treatment, the stroma consisted mainly of abundant extracellular matrix and fibroblasts; foci of calcification (Figure 4E, arrow) and sclerohyalinosis (Figure 4I, arrow) were also evident after 25 days of treatment. Inflammatory cells, such as granulocytes or lymphocytes, were not a conspicuous part of the stroma reaction. An extensive central necrosis was observed in node metastases of control mice, a phenomenon probably related to the size of the tumors, while only foci of necrosis were present in regressing node metastases.

As already mentioned, both treatments induced regression of pulmonary metastases, but E2 treatment was much more effective than RU treatment. Few and isolated neoplastic cells were observed in lungs after 4–7 days in E2-treated mice (Figure 4D). Lung metastasis treated with RU began to display glandular formation on day 4 post-treatment (Figure 4H, arrow) and on day 25 a few, small glands separated by increased fibrous tissue and lymphocytes were observed (Figure 4J). Necrosis was not observed in either treated or untreated lung metastases.

Foamy macrophages were observed in all regressing metastases after day 3 post-treatment (Figure 4G, arrow). Similar findings were reported previously in regressing tumors growing in s.c. tissue (21).

In lungs, where there is an increase of tumor differentiation with time, RU treatment induces an early differentiation that together with other phenomena will induce tumor regression. Differentiation alone is not a marker of quiescence in these tumors since untreated metastases show this differentiated phenotype.

Mitosis and apoptosis

Tumor metastasis regression was associated with a conspicuous cytostatic effect and an increase in apoptosis. The growth kinetics of both phenomena was similar in both organs for the same treatment (Figure 5). In E2-treated animals lung metastases were studied only during the first 96 h, because at later times, owing to tumor regression, the number of remaining neoplastic cells was too small for meaningful analysis. In the case of RU treatment it took more time to duplicate the apoptotic index of controls in both axillary and lung metastases; these results were in agreement with those observed in the growth curves when comparing E2 with RU treatment. The same happened with the mitotic index in which a 50% decrease was already observed at 24 h in E2-treated mice ($P < 0.05$)

Fig. 5. Mitotic cells (A) and apoptotic cells (B) were counted in 10 or 15 hpf, respectively, of each section using a 1000× magnification, and expressed as a mean ± standard deviation of the percentage of the ratios between the total number of events (mitosis or apoptosis) and the total cell number per hpf. Mitotic figures were identified morphologically by the condensed ‘hairy’ aspect of the chromosomes and morphological identification of apoptosis was performed according to criteria previously reported (21). A significant decrease in mitosis was observed since the first day in E2-treated metastasis ($P < 0.05$) and after 3 days in RU-treated metastasis ($P < 0.05$) and a significant increase in apoptosis was observed from day 2 in E2- and RU-treated metastases ($P < 0.05$).
whereas it took >72 h to reach similar values in RU-treated mice (P < 0.05, Figure 5A–D).

p21 and p27 expression

In this study, we evaluated the kinetics of p21 and p27 expressions in axillary and lung metastases treated with E₂ or RU (Figure 6). The p21 levels were significantly increased and reached their peak after 24 h in axillary and lung metastases of E₂-treated mice (P < 0.05). In lymph node metastasis, p27 followed a similar trend, although the increase became statistically significant on day 3 and was not as even as p21. Moreover, an unexplained decrease was observed on day 15.

Fig. 6. p21 and p27 expressions (expressed as the ratio between the percentage of stained cells/hpf from experimental groups and the control group on day 0) in regressing lymph node and lung metastases of E₂(A) and RU-treated mice (B). P21 significantly increased (P < 0.01) after 1 day of E₂ treatment in both lymph node and lung metastases while the increase in p27 was significant after 3 days (P < 0.05). In RU-treated mice the rise in p21 and p27 was significant after the third day of treatment (P < 0.05).
In lung metastasis, a statistically significant decrease in p27 expression was observed after the first day of treatment ($P < 0.05$) and the increase in p27 expression was observed on day 3 ($P < 0.001$). In RU-treated mice, p21 and p27 expressions in both axillary nodes and lungs followed a similar trend. As has been described previously for mitotic and apoptotic indices, the increase in p21 and p27 expressions was statistically significant ($P < 0.05$) after the third day of treatment. Representative immunostaining of p21 and p27 expressions in lymph node and lung metastases are shown in Figures 7 and 8, respectively. An early stage of an E2-treated tumor and a late stage of an RU-treated tumor were selected. A high increase in

Fig. 7. Immunostaining of p21, p27, ER and PR in lymph node metastasis of C7-2-HI tumor treated or untreated with E2 or RU. The experimental procedures as well as the antibodies used are described in Materials and methods.

Fig. 8. Immunostaining of p21, p27, ER and PR in lung metastasis of C7-2-HI tumor treated or untreated with E2 or RU. The experimental procedures and the antibodies used are described in Materials and methods.
nuclear staining can be observed in all E2- or RU-treated tumors as compared with control tumors.

**ER and PR expressions**

The expression of ER and PR was significantly decreased in E2- or RU-treated lymph node metastases as well as in RU-treated lung metastases. This inhibition was already observed 24 h after treatment (Figure 9). In lung metastases of E2-treated mice this effect was not observed during the first 48 h although reduction in primary tumor sizes was already evident. The percentages of ER stained cells were very similar to those of PR regardless of the treatment or site of the metastases. Nuclear PR and ER staining in treated and untreated metastases are shown in Figures 7 and 8.

**Discussion**

In this paper we have explored the effects of hormone therapy in breast cancer metastases using a model of tumor progression in which experimental mammary ductal carcinomas, expressing ER and PR, metastasize to regional lymph nodes and lungs (33). As we have previously shown (21) the C7-2-HI tumor model is very suitable to evaluate hormone response, and in this context, we wanted to extend to the metastatic process our previous observations that primary tumors regressed with endocrine treatment. It is worthwhile pointing out that this is not a genetically engineered model, and not many are available to study all steps of tumor progression. Our results show that estrogen and antiprogestin treatment effectively induced regression of both axillary lymph node and lung metastases. This regression proceeded through cytostasis and increase in apoptosis with high levels of p21 and p27 expressions, as in primary tumors (21). In this model PR is an essential proliferative pathway; antiprogestin treatment (20) or treatment with PR antisense oligonucleotides (C.A. Lamb, L.A. Helguero, S. Giulianelli, R. Soldati, S.I. Vanzulli, A. Molinolo and C. Lanari, manuscript submitted) inhibit tumor growth. Even in tumors unresponsive to progestins, the proliferative effects of growth factors such as FGFs proceed through a cross-talk with the PR (34). Estrogens are also very effective in inhibiting tumor growth, although their mechanisms of action are still under investigation. We have found no histological differences between RU- and E2-treated tumors, and the same is true for p21 and p27 expressions. Both RU and estrogens induced an inhibition in receptor expression in regressing tumors. Although the possibility arises that tumor regression may be associated with a decrease in steroid receptor expression, the data obtained in E2-treated lung metastases, where an early decrease in tumor size was observed even in the absence of diminished levels of steroid receptor expression, suggests that the downregulation in receptors may be the consequence, rather than the cause of this phenomenon. An interesting finding was the fact that lymph node and lung metastases were histologically more differentiated, as compared with the primary implants. This higher degree of differentiation, however, did not correlate with any difference in hormone response. Experiments in which tumor cells were directly inoculated in lymphoid tissue and experiments in which tumor metastases were subcutaneously transplanted (data not shown), suggest that it is the organ in which the tumor is growing which confers the differentiated phenotype.

To our knowledge only a few reports are available exploring the regression of metastases in breast cancer. Among these, are the studies in the *neu* (35) and in the *wnt* 1 conditional transgenic mice (36) and those reported by Connolly et al. (37), in which the inhibition of cyclooxygenase reduced tumor growth and lung metastasis, although in this case only micro-metastases were present at the time of treatment initiation.
Complete tumor regression was not achieved in these experiments, unlike what was reported in the *neu* conditional transgenic study (35), and the percentage of proliferating cells was similar in treated and untreated tumors. This lack of complete regression may be related to the failure of the model to induce sustained tumor cell apoptosis. In both the *neu* conditional transgenic model and in ours, the inhibition of the expression of a critical regulatory pathway i.e. *neu* and PR, is enough to induce complete regressions, demonstrating a hierarchical role for these pathways in the maintenance of the neoplastic phenotype. Interestingly, *neu* is also overexpressed in some of the tumors of our model (38) that also regress with estrogens and anti-progestins (C. Lanari, unpublished data).

Our data as well as that of others point toward the importance of hierarchical targets, the blockage of which would lead to sustained cytostasis and apoptosis. Triggering both phenomena will lead to tumor regression. Increases in p21 and p27 expressions are also behaving as markers of tumor regression at early stages. An increase in the expression level of these proteins after treatment initiation may result in a predictable assay to evaluate treatment responsiveness.

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