p53 Function Is Required for Hormone-Mediated Protection of Mouse Mammary Tumorigenesis

Daniel Medina and Frances S. Kittrell

Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030

Abstract

Limited hormonal stimulation of the mammary gland during a critical window in postpubertal development imparts a long-lasting protective effect against breast cancer in humans and in rodent models. The hormonal stimulation can be achieved by full-term pregnancy or low doses of estradiol-17β and progesterone administered for 21 days. The mechanism(s) behind this effect of hormones is not understood at the molecular level. The experiments reported here demonstrate that the absence of p53 tumor suppressor gene function abrogates the protective effect of hormones against carcinogen-induced mammary carcinogenesis in BALB/c mice. This is the first identification of a specific gene product that mediates the protective effect of hormones. Additionally, the experiments highlight the usefulness of transgenic mouse models in the testing of hypotheses derived from the classic rat mammary models.

Introduction

Breast cancer remains the major cancer among women in the United States in terms of noncutaneous cancer incidence and is the second leading cause of cancer deaths (1). Among the many risk factors for breast cancer, reproductive history, genetic background, and age are the strongest and most consistent (2, 3). However, the strongest protective factor is also related to reproductive history, i.e., early age at first pregnancy (≤20 years of age), which confers a 50% reduction in lifetime risk compared with the lifetime risk of breast cancer in nulliparous women. The protective effect of early first pregnancy has been demonstrated repeatedly in numerous epidemiological studies and provides a physiologically operative model to achieve practical and affordable prevention of breast cancer in humans (2, 4, 5).

The protective effect of early pregnancy against chemical carcinogen-induced mammary tumorigenesis has been demonstrated in both rat (6–15) and mouse models (16). The effect of pregnancy is mimicked by treatment with estrogen and progesterone (6, 15) and treatment with human chorionic gonadotropin (8). Doses of estrogen and progesterone that produce circulating levels of these hormones equivalent to that found in midpregnancy replicate exactly the effects of pregnancy on the protection against carcinogen-induced mammary cancer (6, 15). The molecular mechanisms that underlie the basis for the protective effect have not been elucidated, although recent experiments have provided several candidate genes. Two different groups have used gene expression profiling methods to identify genes that are differentially expressed in the involuted gland compared with the AMV3 gland, several genes involved in chromatin remodeling; i.e., GB7 and RbAp46, were novel. These authors suggested the hypothesis that “hormones up-regulated epigenetic factors responsible for persistent changes in gene expression may be related to the determination of cell fate.” In a second study, D’Cruz et al. (18) used cDNA microarray technology to identify genes differentially expressed in the involuted gland. This tour de force study examined both rat and mouse models and demonstrated that several classes of genes, especially growth factors such as amphiregulin, pleiotrophin, insulin growth factor 1, and tumor growth factor β3 were correlated with hormone-induced protection. These authors concluded that parity (i.e., hormones) produced a persistent increase in differentiated gene products. Interestingly, there was also a persistent change in the populations of hematopoietic cells populating the mammary gland.

In studies using the Wistar-Furth rat and BALB/c mouse models, Sivaraman et al. (12, 19) demonstrated that hormone-induced protection is manifested at the cellular level as a block in carcinogen-induced proliferation shortly after carcinogen treatment. An examination of several cell cycle-related genes demonstrated that cellular p53 protein content and nuclear localization were increased in the involuted gland compared with the AMV gland before and after carcinogen treatment. Furthermore, p21Cip1 protein levels were also increased in the involuted gland. These results suggested the general hypothesis that p53 is one of several genes up-regulated in a persistent fashion by hormonal stimulation of the virgin gland and that p53 functions as a cell cycle block in response to carcinogen-induced DNA damage. Several recent reports have demonstrated that p53 protein function is activated as a consequence of carcinogen exposure (20, 21).

Several mouse strains [C3H and (DBA/2fxC57BL)F1] have been shown to be suitable models for hormone-induced refractoriness to chemical carcinogenesis (16). Additionally, mouse strains C57BL/6, FVB, 129SvEv, and BALB/c demonstrate the same persistent changes in parity-induced gene expression as the Sprague-Dawley and Lewis rats (18). One missing piece of information is the demonstration that hormone-induced protection of carcinogen-induced mammary tumorigenesis is operative in the BALB/c mouse. The availability of the BALB/c p53-null mammary epithelium model provides a means to test the functional role of p53 in hormone-induced protection. This model has been extensively characterized for its hormonal responsiveness, developmental properties, genetic alterations, and tumorigenesis (22, 23). The absence of p53 function increases risk for tumorigenesis but does not alter either hormonal responsiveness or normal development (23, 24). The results presented here address two questions: (a) Is the BALB/c mouse a suitable model to study hormone-induced protection? (b) Does absence of p53 function abrogate hormone-induced protection?
Materials and Methods

Experiments. All mice were bred and maintained in a conventional mouse facility at Baylor College of Medicine with food and water provided ad libitum and the room temperature set at 70°F. The animal facility is AALAC accredited.

Experiment 1 tested the suitability of the BALB/c mouse as a model to study hormone-induced protection. The virgin BALB/c mouse at 8–12 weeks of age is modestly responsive to DMBA-induced mammary tumorigenesis and achieves a 60% tumor incidence by 10 months after treatment (22). In contrast, the virgin FVB mouse is minimally responsive to DMBA (22). In group 1, mice received s.c. implants of a silastic tube containing 50 µg of estradiol-17β and 20 µg of progesterone at 28 days of age. The tube remained in place for 13 days before removal. The mammary glands were allowed to regress for 15 days, and DMBA treatment was initiated at 56 days of age (1 mg/week for 6 consecutive weeks). In group 2, mice were treated identically except that a silastic tube containing cellulose but no hormones was implanted s.c. At the time of the first DMBA treatment, two mice from each group were terminated for whole-mount analysis of the mammary glands. The mice were evaluated weekly for tumor formation. Mice with mammary tumors were terminated when the tumors were 5–10 mm in largest diameter, and the tumors were fixed in 10% neutral-buffered formalin for subsequent histological sections. The mice were followed for 37 weeks after removal of the silastic tubing (i.e., 43 weeks of age).

In experiment 2, samples of mammary duct were isolated from 7–8-week-old p53-null BALB/c mice and transplanted into the cleared mammary fat pads of 3-week-old wild-type BALB/c mice (23). The transplanted duct samples grew and filled the fat pads in 6–8 weeks. In each group, two mammary fat pads were processed as whole mounts at 8 weeks to examine the growth and morphology of the outgrowth. The mice were mated at 6 weeks of age, underwent a single pregnancy, and nursed their pups for 7 days; the mammary glands were allowed to involute for 21 days before the mice received 1 mg of DMBA/week for 4 consecutive weeks. The mice were palpated weekly for mammary tumors for 45 weeks after transplantation (48-week-old mice). Mammary tumors were fixed in 10% neutral-buffered formalin for histological sections. Samples of mammary duct from p53 wild-type BALB/c mice were not transplanted as contralateral controls because previous experiments had demonstrated that the frequency of DMBA-induced transformation in single outgrowths of normal gland was very low and would not have provided a baseline to detect a protective effect of hormones (22).

Statistics. The tumor incidences were analyzed by Fisher’s exact test.

Results

Whole-mount preparations of the 8-week-old, hormone-stimulated, involuted mammary gland demonstrated that the gland had undergone complete involution within the 15 days after removal of estrogen and progesterone. The results of DMBA-induced mammary tumorigenesis in hormone-treated and AMV BALB/c mice are shown in Fig. 1. Mammary tumor incidence was reduced from 63% (19 of 30) to 17% (5 of 30; P < 0.05) in mice that received the short-term hormone treatment before DMBA exposure. These results are similar to the results obtained from the original experiments that demonstrated hormone-mediated protection against DMBA-induced mammary cancer in mice (16) and demonstrate that the BALB/c mouse is a suitable animal model for these types of experiments.

The results of the effect of pregnancy on DMBA-induced tumorigenesis in p53-null mammary epithelium are shown in Fig. 2. DMBA induced a high incidence of mammary tumors in the p53-null mammary epithelium (65%; 13 of 20) compared with the untreated p53 null mammary cells (27%; 6 of 22; P < 0.05). A single pregnancy did not significantly affect mammary tumorigenesis in untreated p53-null mammary cells (15%; 3 of 22; P > 0.05). Importantly, unlike the case with p53 wild-type mammary epithelium, the short-term hormonal stimulation did not significantly reduce the tumorigenic response of the p53-null epithelium to DMBA (60%; 12 of 20; P > 0.05). The mammary tumors had a similar histopathology in both groups and included both squamous adenocarcinomas and more anaplastic adenocarcinomas. Thus, the absence of p53 function abrogates a significant portion of the protective effect exerted by short-term hormonal stimulation against a chemical carcinogen-induced tumorigenic stimulus.

Discussion

The results of these experiments support the hypothesis that p53 function is an important downstream component for the hormone-induced protective effect against chemical carcinogen-induced mammary tumorigenesis. We propose that p53 exerts a G1 cell cycle block, perhaps through up-regulation of the cyclin kinase inhibitor, p21Cip1. Earlier results demonstrated that p21 is also up-regulated by hormone treatment (19). Several important questions are of interest concerning the mechanism of hormone-induced activation of p53. Earlier experiments suggest that activation is at a posttranslational level because p53 mRNA levels were not increased by hormone stimulation. It will be important to determine what posttranslational modification (i.e., site of phosphorylation and/or acetylation) is induced by hormones, the kinase or molecules involved in posttranslational modification,
and the exact mechanism by which estrogen and/or progesterone regulate the molecules involved in posttranslational modification. Equally important, it will be important to determine the exact role of those molecules (e.g., RbAp46, G-B7) in inducing persistent changes in gene expression as a consequence of hormone stimulation. These results, along with other recent reports (17, 18, 25), begin to provide a mechanistic basis for the well-established phenomenon of hormone-induced protection.

Another issue raised by these experiments is the short window of susceptibility of the BALB/c mammary gland compared with the larger window of susceptibility seen in rat strains. The BALB/c mouse is more susceptible to DMBA-induced tumorigenesis than is the C57BL/6 mouse or the FVB mouse (22), two strains often used to make transgenic animals or backcross genetically altered mice. However, there are limitations to the window of susceptibility to DMBA-induced tumorigenesis. Initially we tried to use a single pregnancy followed by complete involution before DMBA treatment; however, the mice were 13–15-weeks-old at first DMBA treatment. The 100-day-old virgin rat mammary gland is susceptible to DMBA or nitrosomethylurea-induced carcinogenesis; however, very few mammary tumors developed in the DMBA-treated 90–100-day-old virgin BALB/c mammary glands, which made age-matched controls for the parous mice impossible. We also tried a single pituitary isograft for 3 weeks starting at 4 weeks of age, followed by 4 weeks of involution, but again only a modest incidence of mammary tumors (25%) developed in the DMBA-treated 80-day-old AMV controls. Interestingly, the DMBA-treated, hormone-stimulated mice developed a 30% incidence of mammary tumors.

The absence of any kind of protection by the short-term exposure of a pituitary isograft, which increases primarily prolactin and progesterone hormones, suggests that estrogen is an important hormone for the protective effect. This idea is supported by the result that perphenazine, a drug that increases prolactin secretion from the pituitary gland, is not protective in a similar but slightly different model of hormone-induced protection (15). Estrogen alone is not sufficient for protection in a pretreatment model; earlier results have demonstrated that both estrogen and progesterone are important for the protective effect in the rat model (6, 12).

The basis for the marked differences in susceptibility between the rat and mouse mammary gland is not understood at the cellular or molecular levels. The steady-state proliferation activity is not significantly different in the 100-day-old glands, being low in both species. The morphological state of the involuted gland is not predictive of susceptibility. For example, the Sprague-Dawley mammary contains more tertiary branching and smaller alveolar buds than the BALB/c gland; however, the involuted gland of the Lewis rat shows less tertiary branching than that of BALB/c mice (14). The hormonal responsiveness of the virgin rat gland is different from that of the virgin BALB/c mouse gland (26), but how this is related to increased susceptibility to carcinogen-induced initiation is not known. Until we understand the molecular basis of carcinogen-induced initiation, it will be difficult to unravel the factors that determine the short window of susceptibility in BALB/c mice.

One important function of p53 is to respond to DNA damage by exerting a cell cycle checkpoint, allowing time for DNA repair or to initiate the apoptotic program. An important issue is whether alterations in other genes involved in the cellular response to DNA damage alter breast cancer risk. Individuals with germ-line mutations of \textit{BRCA-1} have an increased risk for breast cancer. It has been reported that pregnancy at an early age affords no protection in these patients (27). Li-Fraumeni patients are heterozygous for the \textit{p53} gene. The protective effect of early pregnancy has not been reported in these patients, primarily because of the low incidence of patients. The p53 BALB/c model would be a good model to test the protective effect of pregnancy as the tumor distribution (both mammary and other cell types) mimics that found in Li-Fraumeni patients (28).

In summary, these experiments report the first results where a candidate molecule has been directly tested for its role in hormone-induced protection. The results demonstrate that the absence of p53 function abrogates the protective effect of hormones against chemical carcinogen-induced mammary cancer. Furthermore, the experiments emphasize the feasibility of using transgenic mouse models to test and understand the role of candidate genes that were identified originally from results using classic rat mammary models.
References

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