ABSTRACT

Proper function of the p53 tumor suppressor gene is critical for inhibiting tumor development in a broad spectrum of tissues. Although the mammary gland is highly susceptible to tumor formation, the functional status of p53 in the normal tissue had not been investigated. Therefore, expression, localization, and activity of p53 were examined in normal mammary tissues. High levels of p53 protein were found expressed in the cytoplasm of the ductal epithelium of the quiescent mammary gland. Ionizing radiation failed to recruit p53 to the nucleus, and p53-dependent response were minimal. However, transient hormonal stimulation resulted in nuclear accumulation of p53, an induction of p21/WAF1, and a 5-fold increase in apoptosis after ionizing radiation. Therefore, the functional state of wild-type p53 in the mammary epithelium can be regulated by hormonal stimuli.

INTRODUCTION

Heightened susceptibility of the breast to tumor development has been associated with early menarche, nulliparity, exposures to ionizing radiation, and family history (1, 2), but the underlying molecular mechanisms are poorly understood. Heightened susceptibility during critical periods of breast development has been associated with a subpopulation of ductal epithelial cells that spends the least time in G1 and exhibits a high proliferative index (3–6). These cells were unable to repair damaged DNA on genotoxic insult (4–6), which indicates that DNA repair may be compromised and responsible for the increased incidence of breast cancer.

Hormones are known to regulate the critical periods of breast development and susceptibility of the breast to tumorigenesis (5, 7). Treatment with estrogen and progesterone stimulated lobular-alveolar development and susceptibility of the breast to tumorigenesis (5, 7). Hormones are known to regulate the critical periods of breast development and susceptibility of the breast to tumorigenesis (5, 7). Treatment with estrogen and progesterone stimulated lobular-alveolar development and was shown to reduce the incidence of mammary tumor formation in rats (8), whereas the protective effects of placental hormones have been associated with the activation of apoptosis (9).

The p53 tumor suppressor gene is also important in the development of breast cancer because it is commonly mutated in tumors (10). In normal cells, DNA damage leads to an accumulation of p53 protein in the nucleus, in which p53 acts as a transcriptional activator for a group of genes involved in cell cycle arrest, DNA repair, or apoptosis (11–14). It is in this manner that p53 serves to maintain the integrity of the genome. Germ line mutations in p53 have been shown to segregate with the Li-Fraumeni cancer susceptibility syndrome (15), which confers on women an increased risk of developing breast cancer compared with the general population (15–17). In addition, mutations in p53 have been detected in over 40% of spontaneous breast carcinomas (18, 19). These observations implicate p53 not only in the pathogenesis of breast cancer but in the susceptibility to it as well.

Therefore, in an attempt to understand the molecular mechanism responsible for the susceptibility of the mammary gland to tumorigenesis, the expression and functional status of p53 were examined in the normal mammary tissue after hormonal treatment in vivo. High levels of p53 protein were detected in the ductal epithelium of the mature mammary gland and were localized within the cytoplasm. In addition, p53 remained localized to the cytoplasm after radiation-induced DNA damage. However, treatment of mice with placental hormones resulted in the nuclear accumulation and activation of p53 in response to DNA damage. These results demonstrate that hormones can alter the functional status of p53 in the mammary gland.

MATERIALS AND METHODS

Mice and Tissues. Fourth inguinal mammary gland and adjacent skin were isolated from 9 week BALB/c and BALB/c-p53 knockout female mice (20) before (n = 6) or after 5 Gy whole-body γ-radiation (1 h postirradiation, n = 2; 6 h postirradiation, n = 4). Radiation was supplied by a 137Cs irradiator. Separate groups of 9-week BALB/c and BALB/c-p53 knockout female mice were given 5 IU of PMSG3 isolated from pregnant mares’ serum (Sigma, St. Louis, MO) followed by 5 IU hCG from human urine of pregnancy (Sigma) 48 h later. Animals were then subjected to 5 Gy whole body γ-radiation 6 h after injection with hCG and killed at 1 or 6 h postirradiation. Two animals were evaluated at each time point corresponding to the unirradiated time point and the 1- and 6-h postirradiation time points. Vaginal smears were taken at the time of sacrifice to evaluate the phase of the estrous cycle. Normal human breast tissue was obtained from reduction mammoplasties at Baystate Medical Center (n = 4). All of the tissues were fixed in 10% phosphate buffered formalin, rinsed in 70% ethanol, and embedded in paraffin.

Northern Blot Hybridization. Tissues were homogenized and total RNA was isolated and subjected to Northern blot analysis as described previously (20). Briefly, 10 μg of total RNA was separated on a 1.2% agarose-formaldehyde gel, blotted onto a nylon membrane (Zetabind; CUNO, Meriden, CT), and hybridized sequentially with 32P-labeled p53, p21/WAF1, and 18S cDNA probes.

Immunohistochemistry. Four-μm sections were deparaffinized and subjected to antigen retrieval (20). Sections were incubated overnight at 4°C with rabbit polyclonal CM5 antisera (1:200; Novacstra, Newcastle upon Tyne, United Kingdom), CM1 (1:200; Novacstra) or Ab-5 (1:50; Oncogene Research, Cambridge, MA) specific for murine p53, human p53, and murine p21/WAF1, respectively. Immunocomplexes were visualized by the ABC method (Vector Laboratories, Burlingame, CA). Sections were counterstained with methyl green.

In Situ End-Labeling Method. Paraffin-embedded sections were deparaffinized, treated with methanol and hydrogen peroxide to quench endogenous peroxidases, and subjected to TUNEL, using FragEL DNA Fragmentation kit (Oncogene Research). Sections were counterstained with methyl green and analyzed for apoptosis by counting a total of 2000–8000 cells per tissue (2–6 animals/treatment; 3–5 fields/animal). Differences in TUNEL-positive cells among treatments were compared using a 99.95% confidence interval test of 2 proportions.

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The abbreviations used are: PMSG, pregnant mare serum gonadotropin; hCG, human chorionic gonadotropin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end-labeling.
RESULTS

Localization and Expression of p53 during Normal Mammary Gland Development. Expression of p53 mRNA was examined by Northern blot along with p21^WAF1, a gene that is transcriptionally activated by p53 (Fig. 1A). High levels of p53 mRNA were detected in the resting mammary glands of mature nulliparous mice (N), declined during lactation (L5), then were induced rapidly in tissues undergoing intense apoptosis during involution (W2, W5). Levels remained elevated into late involution (W10). Despite similar levels of p53 mRNA in nulliparous and postweaning tissues (N versus W2), p21^WAF1 was induced only during the early postweaning period (W2–W5) when nuclear p53 protein was detected in association with apoptosis (20). Similarly, basal levels of other p53 target genes (mdm2 and bax) were detected in nulliparous mammary tissue despite high levels of p53 mRNA (data not shown). High levels of p53 protein were also detected in nulliparous mammary glands by immunoprecipitation-immunoblot analysis (data not shown). This suggested that the differences in the transcriptional activity of p53 in the nulliparous versus weaning-2-day tissues were attributable to differences in the activity of p53 protein, not differences in abundance.

It has been shown in a variety of cell lines and tumors that cytoplasmic sequestration of p53 impairs the ability of p53 to transactivate downstream target genes. Therefore, the subcellular localization of p53 protein was examined by immunohistochemistry in the murine mammary gland and in human breast tissue. The p53 protein was localized within the cytoplasm in a perinuclear pattern throughout the ductal epithelium of the nulliparous murine gland (Fig. 1B) and in 50% of normal human breast tissues (Fig. 1C). The cytoplasmic localization was not attributable to cross-reactivity because mammary glands from age-matched BALB/c-p53 knockout mice were devoid of immunostaining (Fig. 1D). This suggested that the high levels of wild-type p53 protein in the resting nulliparous mammary gland are inactive because of cytoplasmic sequestration and that the mechanism is conserved among humans and mice.

Localization and Activity of p53 in Response to DNA Damage. To determine whether the cytoplasmic p53 in the mammary epithelium could be activated, whole-body γ-radiation was administered to nulliparous female mice to induce p53-dependent responses (Fig. 2). Transcriptional activation of target genes by p53 would require its translocation to, and accumulation in, the nucleus after DNA damage.
However, at 1 h after irradiation, p53 remained in the cytoplasm with no reactivity detected in the nuclei of the mammary epithelium (Fig. 2A). By 6 h after irradiation, faint nuclear staining was detected in a few cells (<1%) of the ductal epithelium, but the majority of p53 protein remained sequestered within the cytoplasm (Fig. 2B). In contrast, the epithelium of the follicular hair bulges in the skin, and a substantial proportion of lymphocytes (Fig. 2, C and D) stained strongly for nuclear p53 at 1 and 6 h postirradiation. Mammary tissue and skin and the intramammary lymph node of irradiated knockout mice were negative for p53 immunostaining (data not shown).

To further assess the functional status of p53, transcriptional targets and biological responses to ionizing radiation were evaluated. Induction of p21/WAF1 protein in tissues from nulliparous female mice was used as a cellular reporter for transactivation by p53. At 1 h postirradiation, no p21/WAF1 protein was detected in the epithelium of the mammary gland (Fig. 2E). By 6 h after radiation treatment, less than 1% of the cells expressed nuclear p21/WAF1 protein in the ductal epithelium (Fig. 2F). Nuclear p21/WAF1 protein could be detected in some of the nuclei of the stromal fibroblasts and adipocytes surrounding the ductal epithelium. In contrast, the follicular bulbs and the intramammary lymph node stained strongly for nuclear p21/WAF1 protein at 1 and 6 h after radiation treatment (Fig. 2, G and H). The mammary gland, skin, and lymph node of BALB/c-p53 knockout mice were negative for p21/WAF1 protein induction at 1 and 6 h postirradiation, confirming the response as p53-dependent (data not shown).

Levels of apoptosis were quantitated to determine whether biological responses to p53 were impaired as well (Fig. 3A). In the unirradiated mammary gland, 2.38% of mammary epithelial cells were TUNEL-positive. Similarly, 0.07% of the follicular hair bulges in skin and 1.94% of the intramammary lymphocytes were TUNEL-positive. Apoptosis was increased greater than 10-fold in the follicular bulges of the skin and the intramammary lymph node after γ-radiation (P < 0.05) compared with a 1.5-fold induction of TUNEL-positive cells in the mammary epithelium after γ-radiation. Although the increase in apoptosis in the mammary epithelium was modest compared with that observed for the other tissues, these increases were statistically significant (P < 0.05). These responses were p53-dependent because there was no induction above basal apoptotic levels in the tissues from BALB/c-p53 knockout mice after irradiation. Thus, despite the expression of high levels of p53 protein in the mammary epithelium of nulliparous BALB/c mice, p53-dependent responses were impaired after genotoxic insult.

Hormones Induce p53 Activity in the Mammary Gland. PMSG and hCG were administered to induce a superovulatory state, in which exogenous gonadotropins and endogenous steroidal and peptide hormones are present, to determine whether this hormonal milieu had an effect on the activity of p53. Short-term responses (6–12 h post-hCG) were evaluated to distinguish the direct effects of hormonal environment on p53 activity from indirect effects occurring later as a consequence of hormone-induced differentiation. No morphological signs of differentiation were evident at 12 h post-hCG treatment. Hormone treatment alone did not alter the localization of p53 in the mammary epithelium, with protein localized within the cytoplasm of the ductal epithelium (Fig. 4A). Therefore, mice were challenged with ionizing...
radiation 6 h post-hCG treatment, then analyzed 1 and 6 h later. Nuclear p53 was detected in the ductal epithelium of the mammary gland 1 h after irradiation (Fig. 4B). At 6 h postirradiation, p53 remained localized to the nucleus of the epithelial cells, with diffuse cytoplasmic p53 also evident (Fig. 4C). However, there was a difference in the cell types that expressed nuclear p53 at 1 h versus 6 h postirradiation. At 1 h, both luminal and basal epithelial cells within the ducts were positive for nuclear p53; yet, by 6 h postirradiation, staining was more prevalent in the basal cells.

Induction of p21/WAF1 protein was also evident within ductal structures of the mammary epithelium and in the stroma of PMSG/hCG-treated mice at one h postirradiation (Fig. 4D). By six h postirradiation, intense nuclear staining of p21 protein was detected in the majority of the ductal epithelial cells (>60%) as well as in the surrounding stroma (Fig. 4E). Similar to the pattern of p53 immunostaining, there was a shift from luminal and basal cell expression of p21/WAF1 at 1 h to a predominance of basal cell expression at 6 h. The absence of p21/WAF1 in mammary glands of BALB/c-p53 knockout mice postirradiation confirmed that the response was p53-dependent (Fig. 4F).

Levels of apoptosis were also measured in hormone-treated animals (Fig. 5A). Basal levels of apoptosis in mammary gland, follicular hair bulges of the skin, and intramammary lymph node 6 h after irradiation in wild-type mice. Basal levels of apoptosis were detected in tissues from p53-null mice. Apoptosis was not quantitated in the lymph nodes of irradiated p53-null mice in this experiment but were shown to be basal in previous work (*), a statistically significant difference compared with the unirradiated tissue (P < 0.05). B, histology of representative TUNEL-positive cells within the mammary duct of an irradiated BALB/c nulliparous female (i), follicular bulbs of the skin (ii), and the intramammary lymph node (iii).

DISCUSSION

In this study, cytoplasmic localization of wild-type p53 was observed in both the normal mouse mammary epithelium and the human breast epithelium (Fig. 1). Cytoplasmic localization of wild-type p53 has been reported in breast tumors as well as other neoplasms and embryonic stem cells (21–24). Particular domains of the p53 protein have been demonstrated to be critical for its localization and retention. The tetramerization domain has been shown to be necessary for nuclear import and retention, whereas, residues Arg-306 and Lys-305, in addition to the cytoplasmic sequestration domain, have been reported responsible for cytoplasmic sequestration (25–27). These results suggest that the conformation of p53 is important for its localization and ability to translocate to the nucleus.
protein has been detected with the PAb240 monoclonal antibody, which recognizes a denaturation-sensitive epitope (28, 29). Therefore, conformational shift is one mechanism by which p53 may be sequestered in the cytoplasm of mammary epithelial cells. This may involve either exposing the cytoplasmic sequestration domain or concealing a domain critical for import and retention in the nucleus. Cytoplasmic localization of p53 may also be a consequence of conformation-independent mechanisms involving excessive export or tethering of p53 by cytoplasmic proteins such as P53BP2 or hsp84 (30–32).

Although the precise mechanism responsible for cytoplasmic sequestration and impaired function of p53 in the quiescent mammary epithelium remains to be established, we show that it is reversible by exposure to hormones.

**p53 Is Functionally Compromised in the Mammary Epithelium.** The p53 responses to ionizing radiation have been well documented in vivo. Under normal conditions, p53 levels are below the level of detection by immunohistochemistry and require very sensitive methods to detect protein (33). Responses to γ-radiation have been shown to vary widely among tissues and cell types. Some tissues such as skin, intestinal epithelium, and lymphoid tissues displayed an increase in protein accumulation, increased p53 transcriptional activity, and induction of apoptosis, whereas other tissue types such as liver, skeletal muscle, and brain showed no response (33–35). Previous studies had characterized the apoptotic response to ionizing radiation in preneoplastic lesions (36, 37), but p53 responses in the normal mammary gland had not been fully characterized.

In the present study, we demonstrated that p53-dependent responses to γ-radiation in the mammary epithelium differed from skin and lymphocytes (Fig. 2). In the mammary epithelium, p53 was localized to the cytoplasm of the ductal epithelium, with little induction of p21/WAF1 or apoptosis after γ-radiation (Fig. 2 & 3). Previous studies have reported a spontaneous apoptotic index of less than 1% in nulliparous BALB/c mice, with an increase to ~3% after γ-irradiation (36), which was similar to the results observed in Fig. 3. Although modest, induction of apoptosis in response to DNA damage in the nulliparous mammary epithelium was observed in both studies.

**Activation of p53 by Placental Hormones.** Epidemiological studies have established that pregnancy exerts a prophylactic effect against breast cancer (7). The mechanism by which pregnancy exerts this protective effect is not clear; however, ovarian and placental hormones have been shown to mimic these effects by causing differentiation of the mammary epithelium similar to a pregnant state (8, 38–40). Apoptotic responses play a pivotal role in rendering the gland resistant to tumors because apoptotic responses to γ-radiation were greater in pregnant mammary glands compared with the nulliparous gland (36). Treatment with placental hormones also rendered the mammary gland resistant to tumor development and increased apoptotic responses after carcinogen-induced DNA damage (9). Therefore, hormonal status affects susceptibility to tumor development and apoptotic responses.

On the basis of these associations, placental hormones were used to modify p53-dependent responses to DNA damage. Irradiation of mice...
6 h after hCG treatment allowed us to evaluate the immediate effects of the hormones rather than the secondary effects attributable to differentiation, which occur later and would complicate the interpretation. Mice treated with PMSG and hCG showed nuclear localization of p53 protein and p21/WAF1 at 1 h postirradiation (Fig. 4) followed by a 5-fold induction of apoptotic responses at 6 h (Fig. 5). Although γ-radiation-induced apoptosis in the intramammary lymph nodes was not affected by hormonal treatment, apoptotic responses were increased in the follicular hair bulbs. Therefore, the effects of hormonal treatment on the activity of p53 may not be limited to the mammary epithelium. These data suggest that placental hormones promoted nuclear accumulation of p53 protein within the mammary epithelium, which mediated p53-dependent induction of p21/WAF1 and apoptosis after DNA damage, whereas responses to DNA damage were minimal in untreated mice (Fig. 3). Furthermore, the effects were rapid and preceded the differentiation of the mammary epithelium.

Treatment with PMSG and hCG is used to induce superovulation and is known to cause complex hormonal changes affecting ovarian and pituitary feedback mechanisms (41). As a result, the effects on p53 function may be attributable to the combined actions of gonadotropins, steroids, and peptide hormones. Vaginal smears from PMSG/hCG-treated mice revealed cornified epithelium indicative of estrus phase (41). Therefore, high levels of endogenous estrogen were present in the mice at the time of irradiation. Estrogen may cause the activation of p53 function in the mammary epithelium inasmuch as it has been shown to increase the levels of p53 protein in estrogen receptor-positive mammary epithelial cell lines (42) and activate the P1 promoter in the p53 gene (43). However, direct effects of the exogenous hCG cannot be ruled out because receptors for hCG have been found on rat and human mammary epithelial cells (44, 45), and hCG may not be cleared within the 6–12 h period postinjection analyzed in these experiments. Furthermore, p53-dependent activation of apoptosis and growth inhibition by hCG have been observed in both human breast epithelial cells and rat mammary epithelial cells (9, 38, 39, 46). The exact hormone(s) necessary for the altered p53 response in the mammary gland remains unclear. Nevertheless, the lack of p53 activity in the nulliparous mammary epithelium could be reversed by hormonal stimulation.

Fig. 5. A, the apoptotic response in hormone-treated mice 6 h after treatment with ionizing radiation. Apoptotic index was measured by TUNEL in the mammary gland, intramammary lymph node, and follicular hair bulges of the skin after treatment with hormones (PMSG/hCG) or with hormones and radiation (PMSG/hCG + IR). *, a statistically significant difference compared with the unirradiated tissue. B, histology of TUNEL-positive cells within the mammary duct (i), follicular bulbs of the skin (ii), and the intramammary lymph node (iii) taken from wild-type BALB/c female mice 6 h postirradiation.

The relationship between hormonal stimuli and the pattern of p53 expression may, in fact, be quite complex as suggested by the absence of cytoplasmic p53 in 50% of normal breast samples. This may be attributable to the phase of the menstrual cycle or the hormonal status of the individuals at the time of surgery. The use of oral contraceptives, hormone replacement therapy, or the menopausal state of a woman could also account for more variable expression and localization of p53 in human breast tissues.

Hormonal Control of p53 Function and Chemoprevention. Overall levels of p53 in the mammary epithelium may influence susceptibility to tumor formation. Reduction in levels of p53 was shown to be sufficient to enhance tumor formation in the thymus of mice heterozygous for p53 (47). Complete loss of p53 in the mammary epithelium rendered the mammary epithelium extremely sensitive to tumor formation (48). Therefore, the compromised function of p53 observed in normal resting mammary epithelium in nulliparous mice may contribute to the heightened susceptibility of the gland to tumorigenesis. Russo et al. (4) demonstrated that the mammary epithelium was unable to repair its DNA after carcinogenic insult. Therefore, compromised function of p53 provides a molecular mechanism...
that may be responsible for impaired DNA repair and apoptosis in the nulliparous mammary epithelium leading to heightened susceptibility to mammary tumors.

Susceptibility of the mammary epithelium is also influenced by endocrine status. Likewise, p53 function was shown to be subject to hormonal regulation. Treatment with PMSG and hCG activated p53 function, which could render the mammary epithelium less susceptible to DNA damage. Clearly, identification of the hormone(s) responsible for the functional activation of p53 will be necessary to fully understand the mechanism of p53 regulation in the mammary epithelium. Hormonal therapies designed to activate p53 may provide an effective prophylactic therapy as well as a means to increase the efficacy of chemotherapy by diminishing DNA damage in surrounding normal epithelium while increasing the responsiveness of breast tumors with wild-type p53.

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