A Proposed Unified Mechanism for the Reduction of Human Breast Cancer Risk by the Hormones of Pregnancy

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Abstract

Parity in women is associated with reduced lifetime risk of breast cancer, and hormones of pregnancy (estrogen E, progesterone P, human chorionic gonadotropin; hCG) are implicated. Parity also reduces mammary cancer risk in carcinogen-exposed rats, and administering pregnancy hormones to these animals is similarly effective. Because pregnancy hormones are also able to stimulate cancer growth, we proposed to resolve this dichotomy by determining whether administered pregnancy hormones elicit the cancer-inhibiting agent α-fetoprotein (AFP) from the liver, which would implicate AFP as a proximal effector of hormonal anticancer activity. Accordingly, we treated groups of nitrosomethylurea-exposed rats with saline, E₂, E₂ + P, E₃ + P, hCG, or allowed them to experience pregnancy, then monitored mammary cancer incidence and serum levels of AFP over time. Each hormone treatment reduced mammary cancer incidence and elevated serum AFP levels. To challenge human tissues, human HepG2 liver cells in culture were treated with the same hormonal agents. Each hormone regimen increased the levels of AFP in the culture medium. Medium containing AFP elicited by hCG inhibited the E₂-stimulated proliferation of cultured human MCF7 breast cancer cells, whereas hCG alone did not inhibit their growth. Furthermore, antibodies to AFP neutralized the growth-inhibiting effect of AFP-containing HepG2 medium. We conclude that in the treatment of carcinogen-exposed rats with the hormones of pregnancy, and by inference in women who have experienced pregnancy, that AFP is a proximal agent that inhibits mammary gland cancer.

An often-cited statistic notes that 180,000 women will be diagnosed with breast cancer each year in this country. It is also well known that parity has a profound effect on risk of acquiring breast cancer (1–3). Women who are parous when they are young have one-third to one-half the lifetime risk of acquiring breast cancer compared with nulliparous women (4). This observation has served as a provocative clue for researchers seeking modalties for the reduction of breast cancer risk. It has been reasoned that something in the serum of pregnant women must be an effector of the reduction in risk, and agents recognized as probable effectors are the steroidal estrogens (E₂ or E₃; refs. 5, 6), progesterone (P; refs. 5, 6), and human chorionic gonadotropin (hCG; refs. 7, 8). Additionally, α-fetoprotein (AFP) has been posited to be the risk-reducing agent (9, 10).

To study the parity-risk phenomenon, rat models using carcinogens such as N-methyl-N-nitrosourea (MNU; refs. 5, 11, 12) or dimethylbenzanthracene (DMBA; ref. 13) have proven useful. These agents are potent generators of mammary cancers; MNU especially generates estrogen receptor–positive (ER+) tumors (14), whereas DMBA generates both ER+ and ER− tumors. Carcinogen-exposed female rats that were subsequently mated and bore litters ultimately generated half as many tumors as those that remained nulliparous (15, 16). This is an obvious and striking parallel to the effect of parity on human breast cancer risk (4). Users of these rat models have hypothesized that administered pregnancy hormones elicit the cancer-inhibiting agent AFP (10). However, we have shown when AFP is the proximal effector, its presence in the serum of individuals within the diet, lifestyle, or race surrogates. We are not aware of any unifying hypothesis that would explain the same results obtained from these various surrogates.

A compelling clue for a unifying hypothesis comes from studies which have linked AFP to parity and breast cancer growth. Several epidemiologic studies (reviewed in ref. 19) have shown that pregnancy conditions in which AFP is increased above levels normally found in pregnancy are...
conditions which are associated with a reduction in breast cancer risk below that found with normal singleton pregnancy. Moreover, Richardson et al. (20, 21) have reported that parity-associated protection from breast cancer is directly proportional to a woman’s blood level of AFP during her pregnancy. Consistent with this line of thinking are laboratory studies which have shown that mammary cancer growth is inhibited by the direct addition of AFP to mammary cancer cells growing in culture or in rodents (19, 22–26). Noting that the concentrations of test substances such as E3, E2, P, or hCG administered in published prevention trials were quite high, we hypothesize that these test substances may be effective cancer preventers by virtue of their ability to elicit AFP secretion from the nonpregnant rat liver, rather than by having a direct effect on the mammary gland. Furthermore, we hypothesize that AFP, through its direct inhibition of breast cancer growth, is a key proximal agent by which the reduction in breast cancer risk is obtained. We tested this hypothesis by repeating the hormone treatments (E2, E3, P, and hCG) that were reported to be effective at reducing rat mammary cancer, in the same experimental configuration used by the previous researchers, and measured cancer incidence as well as serum AFP levels in these carcinogen-exposed, hormone-treated animals. Furthermore, as a surrogate for human liver, we examined the ability of these hormones to elicit AFP from human liver cells in culture. We report here that these hormone treatments do, in fact, lead to the production of AFP, that hormone-elicited AFP directly inhibits the growth of human breast cancer cells in culture, and that antibody to AFP specifically blocks the anti-breast cancer activity of hormone-elicited AFP. A preliminary report of this work has been published (27).

Materials and Methods

Animals

Female Sprague-Dawley rats were obtained from Taconic Farms at 34 d of age and were placed immediately on a controlled diet (Agway Pro-Lab 2000; Agway Corporation), allowed free access to food and water, and maintained on a 12-h light-dark cycle at a constant temperature (22°C) for the duration of the study. All procedures involving animals were reviewed and approved by the Albany Medical College Institutional Animal Care and Use Committee.

Carcinogen exposure

There were 30 rats in each experimental group (unless otherwise specified) to assure a 95% probability of detecting a difference between groups (ratios) of 40%, which was the difference seen for pregnancy (5, 15). MNU was obtained from the National Cancer Institute carcinogen repository (MRI, Inc.) and was dissolved in sterile physiologic saline, (1% w/v), buffered to pH 5.0 with 3% acetic acid. At 50 d of age, rats were randomized and a single injection of MNU, 50 mg/kg body weight, was administered into the jugular vein. Treatment with hormones occurred during the time period beginning 10 to 21 d after MNU exposure and continued so that the same start date and treatment durations as were used by the earlier workers were reproduced in the experiments reported herein. For tumor detection and monitoring tumor growth, animals were palpated twice weekly, starting at day 30 (after MNU exposure) and continuing for at least 102 d. Tumor incidence is reported as the number (or percentage) of rats in a group that experienced at least one tumor, whereas multiplicity is the average number of tumors per rat. The dimensions of each tumor were determined by noninvasive measurement with a caliper, and tumor volume was estimated as an ellipsoid of revolution \( V = \frac{\pi}{6} \, d^2 \, D \), where \( d \) and \( D \) are the respective short and long diameters. For each group, mean tumor volume was calculated.

Hormone treatments

Each hormone treatment followed the doses and schedules specified in the publications being replicated, and therefore, differ from one another in modest ways. In every case, we used MNU, although in some of the previous investigations, DMBA was used (7, 8, 13, 17, 18). E2 + P. Following the procedures of Grubbs et al. (5), who used MNU as the carcinogen, 10 d after carcinogen administration, 30 female rats received 20 \( \mu \)g of E2 (Sigma Aldrich) plus 4 mg of P dissolved in sesame oil, daily, by s.c. injection (0.2 mL), for 40 d. E3 + P. Following the procedures of Rajkumar et al. (6), who used MNU, 13 d after carcinogen administration, 30 female rats received two individual subcutaneous Silastic capsules (0.078 in. ID \( \times 0.125 \) in. OD, 2 cm long; Dow Corning) one packed with 30 mg of E3 and another packed with 30 mg of P (Sigma Aldrich). Each Silastic capsule was implanted s.c. dorsally, on either side of the upper spine while animals were under isoflurane anesthesia. Implants were left in place for 21 d to mimic pregnancy, and removed after 21 d. hCG. Similar to the procedures of Russo et al. (8) who used DMBA, 21 d after MNU administration, 30 female rats received an s.c. injection of 100 IU of hCG (Sigma Aldrich) reconstituted with deionized water (pH 7.2), daily, for 60 d.

Pregnancy. Following the procedures of Grubbs et al. (15), 10 d following carcinogen administration, 30 female rats were introduced to males (three females per male). Females stayed with males for 7 d, after which they were removed and separated into individual cages. Between 21 and 23 d later, 19 females bore litters and were allowed to nurse-feed for 15 d. Females that did not become pregnant were excluded from the study.

Controls. The no-treatment group consisted of 30 female rats exposed to MNU without subsequent treatment, and experienced the maximal number of tumors over the course of the ensuing 4 mo. A group of animals that was not exposed to MNU developed no tumors during the course of the study.

Blood samples

Blood was drawn from animals in each of the six groups of rats at four time points: bleed 1 was prior to the first dose of treatment (for pregnancy, on the 4th day females were housed with males), bleed 2 occurred at the midpoint of the treatment regimen, whereas bleed 3 was on the last day of the treatment regimen. Bleed 4 occurred 7 d following the termination of the treatment regimen, or, for the pregnant group, 7 d postpartum. These bleeding times are indicated in Fig. 1. Control animals were bled 30, 40, and 50 d after MNU administration. While rats were anesthetized under 3% isoflurane, 1 mL blood samples were drawn from the tail vein with a 25-gauge needle and transferred to glass centrifuge tubes and allowed to clot at room temperature. Serum drawn from each sample was spun thrice (1,500 rpm, for 10 min) after which supernatant was collected and stored at –80°C until used for AFP measurement. No animal had more than one blood draw.

Measurement of hormone-elicited rat AFP in rat serum

Detection of AFP in rat sera was done by Western blot analysis using a standard curve based on AFP found in rat amniotic fluid. Amniotic fluid was drawn from the amniotic sacs of 15-d pregnant Sprague-Dawley rats, centrifuged thrice at 2,500 rpm for 5 min to clarify, and then diluted to various protein concentrations with PBS.
and subjected to electrophoresis on a 10% Tris-HCl agarose gel. Amniotic fluid AFP was detected as described below.

Preparation of the serum samples included a 1:60 dilution with distilled water, and then resuspension in electrophoresis sample preparation buffer. Samples were boiled for 6 min. A Bradford assay was used to determine the protein concentration of each sample (including amniotic fluid). Each sample (20 μL) was applied to a 10% polyacrylamide gel. Following electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane using a semidry transfer cell. Membranes were blocked with 5% milk in 0.1% Tween TBS for 1 h at room temperature, then incubated overnight at 4°C in goat polyclonal anti-rat AFP conjugated to horseradish peroxidase (Santa Cruz Biotechnology). The polyvinylidene difluoride membrane was then washed four times in Tween TBS at room temperature and incubated for 5 min in Western Lightning reagent (Perkin-Elmer), a 1:1 mixture of luminal and oxidizing agent (enhanced chemiluminescence reagent). Film was exposed to polyvinylidene difluoride membranes and developed to reveal bands representing AFP. Quantification of AFP in rat sera was through densitometry measurements using ImageQuant Densitometry software. Densitometry measurements for various samples were compared with that of amniotic fluid, which is reported to be 1 mg/mL immediately prior to parturition (28).

**Measurement of hormone-elicted human AFP in HepG2 supernatants**

HepG2 cells (10^5 cells/well) were maintained and grown as a monolayer in αMEM (Life Technologies, Inc.) supplemented with 5% serum (40% bovine calf serum, 60% fetal bovine serum), penicillin G (100 units/mL), and streptomycin (100 μg/mL) in 24-well culture plates in triplicate. Cells were changed to serum-free medium when confluent and medium was changed every 3 d (19). For treatments with hormones, E3, E2 + P, or P (Sigma) was dissolved in 95% ethanol, diluted in buffer and brought to 10^{-8} mol/L in the cell culture dish; final ethanol concentration in each well was 0.095%.

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**Fig. 1.** Breast cancer incidence in hormone-treated, carcinogen-exposed rats. For each treatment regimen, 30 female Sprague-Dawley rats received MNU (50 mg/kg) at the age of 50 d. Black bars, times and durations of hormone treatment (or pregnancy), as described in detail in Materials and Methods. See Table 1 for significance of the differences in incidence.

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hCG (Sigma; 10,000 IU/vial) was dissolved in sterile deionized water and used at a final concentration of 10^{-5} mol/L. Control cells were grown in serum-free medium alone (corrected for ethanol concentration where appropriate). Hormone-treated cells in serum-free medium were administered hormones daily for 21 d. Medium containing AFP was removed every 3 d and replaced with fresh serum-free medium and hormones. The collected media containing secreted AFP was stored at −20°C to prevent AFP degradation, and subsequently assessed for human AFP using a Beckman-Coulter Access in the Clinical Chemistry Laboratories of the Albany Medical Center Hospital.

**Addition of AFP-containing HepG2 supernatant to MCF-7 cells**

To obtain sufficient quantities of human AFP for the treatment of MCF-7 cells, HepG2 cells were grown in multiple T25 flasks at 2 × 10^6 cells per flask in 4 mL of DMEM containing 1.5% antibiotic-antimycotic, and 10 ng/mL bovine insulin). For the cell proliferation data, Dunnett’s test was used. In either test, P < 0.05 was considered significant.

**Statistics**

The level of cancer chemopreventive activity provided by each hormone treatment was analyzed by comparing the incidence (number of rats bearing tumors) in the treatment group with that of the MNU-only control using Fisher’s exact test. For cell proliferation data, Dunnett’s test was used. In either test, P < 0.05 was considered significant.

**Results**

Five groups of carcinogen-exposed rats were treated with different hormonal regimens, whereas a sixth group received no additional treatments. The outcomes in terms of mammary cancer appearance are shown in Fig. 1A-E. In each panel, the upper curve shows cancer appearance in the positive control rats (animals that received only carcinogen), whereas the lower curve represents cancer appearance in animals treated with either E2 + P (A), E3 + P (B), E3 alone (C), hCG (D), or pregnancy (E). In each case, the reduction of mammary cancer incidence was similar to that reported by earlier workers (5–8). Table 1 indicates that the decreased incidence is statistically significant (compared with controls) for each treatment.

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<th>Table 1. Treatment doses, schedules, and tumor incidence in MNU-treated Sprague-Dawley female rats</th>
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*For difference from MNU control.

†Sum of all tumor volumes in a group/total number of animals in a group.

‡Normal gestation period in rats.

§Normalized for group size of 30 animals.
that incidence reduction in this study is comparable to that in the studies being replicated, that multiplicity was decreased by each treatment, and that tumor volume was decreased by each of these treatments.

Serum from animals in the prevention study was harvested immediately prior to regimen initiation (bleed 1), at the midpoint of each regimen (bleed 2), and again (from different animals) at the end of the treatment regimen (bleed 3). Additionally, animals were bled 1 week after the end of the treatment regimen (bleed 4). Rat AFP levels in these serum samples were estimated using Western blot analysis based on a standard curve using rat amniotic fluid AFP (Fig. 2A). A typical gel showing AFP bands in bleed 2 sera from the different treatment regimens is shown in Fig. 2B. Each of the regimens resulted in an increase in serum AFP levels during treatment. The extent of AFP increase varied among treatment regimens. The kinetics of increase and the intragroup and intergroup variability were thoroughly evaluated by measuring AFP band mean densitometry among five replicate animals in a group treated with steroid hormones (E3 + P) and in the group treated with hCG (Fig. 2C). These two groups were chosen because they showed a very consistent decrease in mammary cancer incidence over time (Fig. 1B and D). As shown in Fig. 2C, prolonged treatment with hCG resulted in a significant increase in
serum AFP levels during treatment and levels were still increased 1 week following treatment cessation. In the group treated with E$_3$ + P$_1$, serum AFP levels were significantly increased by midtreatment (bleed 2) but returned to pretreatment levels by the end of treatment and remained there 1 week posttreatment.

To evaluate the effect of hormones on human liver cells, HepG2 cells were incubated with either E$_3$, E$_2$ + P, hCG, or P alone, and human AFP released into the cell culture medium was quantified. Figure 3 indicates that treatment with E$_3$, E$_2$ + P, or hCG led to sustained elevated levels of human AFP in the medium, whereas treatment with P alone did not stimulate AFP production. Levels were elevated as early as day 3 after hormone addition, reached a maximum by 6 days, and remained elevated throughout the 21 days of the experiment.

To assess whether human AFP elicited from HepG2 cells could inhibit breast cancer cell growth in culture, supernatants from HepG2 cells that had been treated with hCG were added to cultures of MCF-7 human breast cancer cells growing in culture. As shown in Fig. 4, the AFP-containing supernatant significantly inhibited the estrogen-stimulated growth of these human breast cancer cells. The basal growth of these cells (cells in the absence of E$_2$) was not inhibited by this supernatant. A key finding is that treatment of the HepG2 supernatant with antibody to AFP neutralized the growth-inhibitory activity of the supernatants, whereas treatment of these supernatants with isotype control did not neutralize their growth-inhibitory activity (Fig. 4). Treatment of MCF-7 cells with $10^{-7}$ mol/L of hCG alone (which was the concentration of hCG used to elicit AFP into the HepG2 supernatants) did not inhibit constitutive growth of MCF-7 cells or the E$_2$-induced proliferation of these cells (Fig. 4). Interestingly, addition of HepG2 supernatants treated with anti-AFP (Fig. 4), or the addition of anti-AFP alone (data not shown) to MCF-7 cells actually stimulated the E$_2$-induced growth of these cells.

Discussion

Pregnancy reduces breast cancer risk in women (4) and in rats (15). These observations led several independent investigators (5–8) to propose and then show that administering the hormones of pregnancy (E$_3$, E$_2$, or hCG) leads to reduced breast cancer incidence in rats exposed to carcinogens. We have confirmed this observation and show here that the hormones of pregnancy alone are sufficient to induce elevated AFP levels in nonpregnant adult rat serum and in cultures of human liver cells. We note here the well-established finding that pregnancy leads to elevated serum levels of AFP in women (30) and in rats (28), and that this leads to reduced breast cancer incidence (9, 15, 20). AFP inhibits cancer growth directly, whereas the hormones of pregnancy do not. Taking all of these data together, it is logical to conclude that AFP is a key mediator through which the pregnancy-induced, or hormone-induced, reduction in breast cancer is realized. Capturing this oncostatic property in analogues of AFP could lead to a rational process for the treatment and prevention of breast cancer. These concepts are discussed below.

We show that treatment of nonpregnant, carcinogen-exposed rats with the hormones of pregnancy leads to a reduction in mammary cancer incidence compared with animals exposed only to carcinogen. Our experimental design called for repetition of the prevention studies done earlier (5–8), with the only significant difference being that some groups (7, 8, 18) have used DMBA, whereas we and others used MNU as the carcinogen in all cases. The incidence of mammary cancers (number of rats that had one or more tumors), the multiplicity of tumors (number of tumors per rat), and tumor burden (assessed as volume), were lower in the hormone-treated groups than in the carcinogen-only group and were very similar, in each instance, to that reported in the earlier studies (Table 1). It is difficult to compare latency (time to appearance of tumors after treatment with carcinogen) across publications because DMBA results in a different latency period than does MNU, but in our study, latency was prolonged for each treatment group except pregnancy.

It may be that the pharmacologic levels and the chronic administration of hormones used in this study, as well as in other studies (5–8), resulted in a generalized liver distress that increased the levels of AFP. Drug-induced liver distress that has increased the level of AFP has been reported (31). Drug-induced liver distress that has reduced the incidence of mammary cancer has also been reported (32–34). Unfortunately, AFP levels were not measured in these later studies.

It may be questioned whether the cancer prevention experiments in rats constitute a rational extension of the phenomenon occurring in human breast cancer. Because there were no experimental means to elicit AFP from human liver,
we undertook a more practical alternative, specifically, a challenge of cultured HepG2 human liver cells with these same hormones. With challenges of E3, E2 + P, or hCG, the level of human AFP found in culture medium was sustained at significantly elevated levels over the basal level secreted by untreated cells. A significant negative control observation was that P alone did not elicit AFP from human liver cells. It is also known that P alone fails to protect rats against carcinogen-induced mammary cancer (35).

To assess whether hormone-elicited AFP is sufficient to attenuate cancer, we used the culture medium of hCG-stimulated HepG2 cells to influence the proliferation and response of MCF-7 human breast cancer cells. We avoided the use of steroid stimulation so as to preclude any trophic effects of steroids on the MCF-7 cells. In other work (36–39), as well as in this report, we have shown that E2 stimulates the growth of MCF-7 and T47D cells; it certainly does not inhibit proliferation. As shown clearly in Fig. 4, the culture medium of hCG-stimulated HepG2 cells is sufficient to inhibit E2-induced proliferation of MCF-7 cancer cells. When the HepG2 medium was stripped of human AFP, the medium no longer inhibited the growth of the cancer cells, and in fact, led to an overgrowth of cells, suggesting that AFP is a proximal agent of cancer inhibition and that its removal might lead to accentuated breast cancer growth. AFP operates via a membrane-associated AFP receptor which is expressed by MCF-7 cells and by other malignancies (40). Interestingly, MCF-7 cells also produce AFP (41), the ligand for that receptor, suggesting that these cells might be experiencing some restriction in growth through AFP-mediated autocrine inhibition of estrogen responsiveness. AFP removal by anti-AFP may have released the cells from this restriction which may have led to the accentuated tumor growth seen under the conditions of anti-AFP treatment of MCF-7 cells. hCG alone did not inhibit E2-induced proliferation of these cells, indicating that hCG is not the proximal agent of cancer inhibition. Neither did it stimulate proliferation.

The ability of AFP in the culture media of HepG2 cells to inhibit proliferation of MCF-7 cells is consistent with earlier reports using different systems. Sonnenschein et al. (22) elegantly showed the ability of transplanted AFP-secreting hepatomas to inhibit the growth of mammary cancers growing in rats. Using a heterologous system, we showed that human AFP could stop the growth of human breast cancers growing as xenografts in immune-deficient mice (19), and in a homologous system, that rodent AFP could stop the growth of MTW9A mammary cancers in rats (42).

Based on these data and epidemiologic data described below, we postulate that AFP is a key pregnancy-associated molecule that leads to a reduction of breast cancer incidence later in life. In 1989, Jacobson and Janerich (10) proposed that AFP in the serum of pregnant women may be the mediator of the reduction in lifetime breast cancer risk that is associated with early term pregnancy. The pregnancy-associated epidemiologic data became particularly compelling in suggesting a role for AFP in reducing breast cancer risk when it was found that a twin pregnancy, in which AFP levels are almost twice those in singleton pregnancies, afforded greater breast cancer risk reduction when compared with that obtained after a singleton pregnancy (9). Then, Richardson et al. (20) also reported a direct correlation within a single cohort of women, between the level of AFP during pregnancy and subsequent reduction in breast cancer risk, further linking AFP to risk reduction. Jacobson et al. (10) further hypothesized that during pregnancy, the transient relaxation of immune competence that is believed to play a role in averting fetal rejection permits some subclinical estrogen-responsive tumors to progress into active growth, a state in which estrogen is necessary to sustain tumor viability. The newly emerging tumors immediately encounter an environment (high concentration of AFP) that blocks their responsiveness to...
References

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