α-Fetoprotein Derived from a Human Hepatoma Prevents Growth of Estrogen-dependent Human Breast Cancer Xenografts

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ABSTRACT

α-Fetoprotein (AFP) is a transport protein that has growth-regulatory properties in many different tissues. It is known to interfere with responses stimulated by estrogen. The purpose of this study was to determine whether human AFP would inhibit the growth of human breast cancer. AFP was isolated from the culture supernatant of human hepatoma cells (HepG2) grown in serum-free medium and was purified by immunoaffinity chromatography. Human breast cancers were grown as xenografts under the kidney capsule of severe combined immunodeficient mice. The minimum inhibitory dose of AFP against estradiol (E₂)-stimulated growth of human MCF-7 breast cancer xenografts was 10 μg/mouse/day, and maximum inhibition (no growth) was achieved with 100 μg/mouse/day. Daily treatment was required to sustain inhibition. This 100-μg dose of AFP also inhibited xenograft growth of E₂-dependent T47 human breast carcinoma. Estrogen receptor-negative MDA MB 231 and BT20 human breast carcinoma xenografts were not inhibited by AFP (100 μg/mouse/day). Elevation in serum E₂ occurred during AFP treatment. AFP did not compete with agonists for the estrogen receptor. These laboratory results are consistent with the findings of a literature search, which consistently showed an association between elevated pregnancy levels of AFP and subsequent reduced risk for breast cancer later in life. We conclude that AFP can inhibit growth of estrogen-dependent breast cancer and warrants further development as an agent for the treatment and perhaps even the prevention of human breast cancer.

INTRODUCTION

AFP is a glycoprotein produced during gestation initially by the fetal yolk sac and then by the fetal liver and is a major protein constituent of the fetal plasma throughout gestation (1). It has 39% primary structure homology to albumin, and like albumin, it has a molecular weight of M₆ ~69,000 and is divided into a three-domain structure based on its disulfide bonding pattern (2, 3). However, unlike albumin, upon parturition the gene for AFP is repressed, which diminishes the serum concentration of AFP to a negligible level. The restriction of the physiological presence of this protein to embryonic life suggests a unique role for AFP in cell growth and differentiation, which are the hallmarks of embryonic life. Evidence for this role has been obtained in a variety of studies showing that AFP can regulate the growth or secretory activity of certain tissues such as liver (4), lymphocytes (5), placenta (6), ovaries (7), and uterus (8) and can interact with certain ligands such as arachidonic acid (9), docosahexanoic acid (9), and retinoic acid (9), all of which influence differentiation. However, a complete understanding of the physiological role of AFP is not yet available.

Previous studies have suggested a possible role for AFP in the regulation of breast cancer growth. Sonnenschein et al. (10) have shown that estrogen-dependent rat mammary tumors regressed in rats bearing hepatomas that were secreting milligram quantities of AFP into the serum. This serum also inhibited in a dose-dependent manner the estrogen-stimulated induction of progesterin receptor in cultures of rat pituitary tumor cells (11). Our own studies have shown that injecting mice with nanogram quantities of human cord sera AFP that had been preincubated with a molar excess of estrogen inhibited the estrogen-stimulated growth of the uterus (12) and of estrogen receptor-positive human breast cancer xenografts (13). In the absence of preincubation with E₂, this material was inactive when given in nanogram amounts (12, 13), suggesting that the molar excess of E₂ constituted a chemical environment that fixed more of the AFP molecules in their active form (14, 15). The data of Sonnenschein et al. taken together with our data suggested that a small proportion of the AFP molecules were already in their active form, and if we were to then raise the dose of AFP, we could achieve antiestrotrrophic activity with human AFP alone, abrogating the need for prior activation with ligand. To test this possibility, we needed a uniform source from which we could readily and reproducibly obtain milligram quantities of human AFP. Pooled human cord serum did not seem practical because of its heterogeneity and its large content of albumin relative to...
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Fig. 1 Effect of serum-free medium on the secretion of AFP by HepG2 cells. HepG2 cells were grown to confluence in serum-containing medium. After cells reached confluence, the medium was changed to a serum-free medium. Cells were then maintained in serum-free medium with a medium replenishment schedule of every 3–4 days. AFP concentration was measured in spent medium by enzyme immunoassay using the Abbott IMx automated immunofluorescence system as described previously (13).

AFP (12, 16), making purification of AFP difficult because of its 39% homology to albumin (2). The recombinant systems available to us also yielded insufficient quantities of human AFP. Tecce and Terrana (17) had reported that the human hepatoma cell line HepG2 secreted significant quantities of AFP into the culture supernatant when grown in serum-free medium and that AFP was the main protein secreted under these culture conditions. This seemed to be a reasonable source of AFP for the purpose of our study, which was to determine whether human AFP alone and in a dose-dependent manner could inhibit the growth of human breast cancers grown as xenografts in immunodeficient mice.

MATERIALS AND METHODS

Culture Conditions for HepG2 Cells. HepG2 cells were maintained and grown as a monolayer in αMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% serum (40% calf serum, 60% FCS), penicillin G (100 units/ml), and streptomycin (100 μg/ml). Cells were released from monolayer with 0.25% trypsin and 0.25% EDTA. Subculturing into additional flasks was carried out by fivefold dilution of cells in the above maintenance medium. Confluent flasks were switched to serum-free medium to up-regulate production of AFP as described previously (13).

Purification of AFP from Culture Supernatants of HepG2 Cells. HepG2 culture supernatants were pooled and concentrated using P-10 Centriprep concentrators (Amicon, Inc., Beverly, MA). Concentrate (10 ml) containing ~3 mg of AFP were loaded onto an 18 cm × 2.5 cm immunoaffinity column [rabbit anti-human AFP (DAKO, Carpinteria, CA) conjugated to cyanogen bromide-activated Sepharose 4B] in a loading buffer of 100 mM NaCl and 10 mM sodium phosphate (pH 7.4). The concentrate was incubated on the column at room temperature for 30 min. Non-AFP proteins were eluted with ~200 ml of loading buffer until no protein was detectable in the eluate by UV absorbance (280 nm). AFP was eluted with ~200 ml of 1.8 M MgCl2 and dialyzed immediately against an excess of 10 mM sodium phosphate buffer (pH 7.2). This material was washed and concentrated in a buffer composed of 100 mM sodium chloride and 10 mM sodium phosphate (pH 7.2).

Uterine Growth Bioassay. The immature mouse uterine growth bioassay is based on the finding that i.p. injection of 0.5 μg 17β-estradiol (E2) into 14- to 18-day-old female Swiss mice results in a 70% increase in uterine wet weight, with a corresponding parallel increase in mitotic index in 22 h (8). This provides a relatively quick in vivo bioassay for assessing agents that interfere with estrogen-stimulated growth. Immature female (15- to 18-day-old) Swiss mice (Taconic Farms, Germantown, NY) received i.p. injections of various quantities of AFP obtained from HepG2 cells, whereas control mice received injections of saline. One h after this first injection, test mice and positive-control mice received injections of 0.5 μg E2, and negative control mice received injections of 0.1 ml of saline. Twenty-two h after the second injection, the uteri were dissected, trimmed free of mesenteries, and immediately weighed. The uterine weights were normalized to mouse body weights (mg uterine weight/g of body weight) to compensate for differences in body weight among litters of the same age. Each experiment was performed with five to eight mice per group, and the mean normalized uterine weights ± SE for each group were calculated. The percentage of growth inhibition was calculated by the following relationships of normalized uterine wet weights:

\[
\frac{\text{positive control} - \text{test group}}{\text{positive control} - \text{negative control}} \times 100\%
\]

Human Breast Cancer Xenografts. The MCF-7 and MDA-MB-231 human breast cancer cell lines were obtained from ATCC, Rockville, MD and were grown in DMEM sup-
Table 1  Inhibition of E2-stimulated mouse uterine growth by AFP derived from HepG2 cells

<table>
<thead>
<tr>
<th>Test material^</th>
<th>Dose^</th>
<th>% inhibition of E2-stimulated growth of mouse uterus^</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude supernatant</td>
<td>100 µg AFP</td>
<td>45 ± 4</td>
</tr>
<tr>
<td>Fraction of crude supernatant non-adherent to anti-AFP column</td>
<td>100 µg Protein</td>
<td>0</td>
</tr>
<tr>
<td>Fraction of crude supernatant adherent to anti-AFP column</td>
<td>100 µg AFP</td>
<td>45 ± 3</td>
</tr>
</tbody>
</table>

^ Three-day supernatant from HepG2 cells that had been grown in serum-free medium for 10 days.
^ One hundred µg of material were injected i.p. in a volume of 0.5 ml 1 h before challenge of mice with an i.p. dose of 0.5 µg of E2 in a volume of 0.2 ml. Negative-control mice received two injections of saline. Positive-control mice received one injection of saline followed 1 h later by an injection of 0.5 µg of E2.
^ Mice were sacrificed 22 h after injection of E2; uteri were excised and weighed immediately. Uterine wet weight (mg) was normalized to mouse body weight (g). Percent inhibition was calculated by dividing the mean E2-induced increase in uterine weight in the presence of test material by the mean E2-induced increase in uterine weight in the absence of test material. There were five to eight mice per treatment group.

Fig. 3  Antiuterotrophic activity of AFP. Various doses of HepG2-derived crude (●) and purified (○) AFP were injected i.p. into immature female Swiss mice. One h later, 0.5 µg of E2 were injected i.p. into these mice. Twenty-two h later, uteri were dissected and weighed. The percentages of growth inhibition calculated for each dose of AFP are presented as mean values; SE (bars). There were five to eight replicate mice per treatment group.

supplemented with 5% FCS, 1% nonessential amino acids, 10 ng/ml insulin, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. The BT20 human breast cancer cell line (ATCC) was grown in EMEM supplemented with 10% FCS, 1% nonessential amino acids, and penicillin/streptomycin as above. The T47D human breast cancer cell line (ATCC) was grown in EMEM supplemented with 10% FCS, 1% nonessential amino acids, 5 mM L-glutamine, 100 units/ml insulin, L-glutamine, and penicillin/streptomycin as described above. Confluent cells growing in culture were released from monolayer by trypsinization, diluted into single-cell suspension, and then solidified by centrifugation into a pellet, which was subsequently exposed to 10 µl of fibrinogen (50 mg/ml) and 10 µl of thrombin (50 units/ml) for 30 min at 37°C. Fibrin clots containing tumor were cut into pieces ~1.5 mm in diameter. Each piece of tumor was implanted under the kidney capsule of an immunodeficient ICR-SCID mouse (Taconic Farms) that weighed ~25 g, as described previously (13, 18). Estrogen supplementation of mice was required for the growth of MCF-7 and T47 tumors. Supplementation was accomplished by s.c. implantation of a Silastic tubing capsule containing solid E2 (2 mm in length) inserted on the day of tumor implantation. Tumor growth was then monitored during survival laparotomy at 10-day intervals by measurement of the diameters of the short and long axes of each tumor, using a dissecting microscope equipped with an ocular micrometer (13). Tumor volumes were calculated using the formula π/6(D)²H, assuming the tumor shape to be an ellipsoid of revolution around its long axis (D).

Quantitation of AFP and Estrogen. The Abbott IMx automated immunofluorescence system was used to quantitate AFP. In this system the analyte initially contacts and becomes bound to microparticles that are coated with monoclonal antibodies to human AFP. Subsequently the particle-bound AFP is exposed to polyclonal anti-human AFP conjugated to alkaline phosphatase. After washing to remove excess material, substrate for the alkaline phosphatase (4-methylumbelliferyl phosphate) is added, and the fluorescent product is measured in the optical system of the instrument.

Estradiol was quantitated in a competitive RIA using tubes coated with rabbit antibody to estradiol (Coat-A-Count, Diagnostic Products Corp., Los Angeles, CA). 125I-labeled estradiol, which was provided in the assay kit, was used to compete with estradiol in the sample for antibody sites on the coated tubes. Samples (100 µl) and 125I-E2 (1.0 ml) were added to a tube, and the tubes were incubated for 3 h at room temperature. The tubes were thoroughly decanted and then counted in a gamma counter. Final counts in the tubes were inversely related to the amounts of E2 present in the samples. A predetermined standard curve generated with standards provided in the assay kit was used to relate counts to concentration.

Assessment of Estrogen Receptor Antagonism. Commercially obtained rabbit uterus (Pel-Freez Biologicals, Rogers, AR) was used as a source of estrogen receptor. Uteri were pulverized in a stainless steel impact mortar under liquid nitrogen and homogenized (20% w/v) in assay buffer [10 mM Tris
edges in the larger flasks when these cells were being maimed because confluent monolayers tended to peel away from the culture flasks. For bulk production of AFP, multiple 25-cm² flasks were preferable to fewer flasks with larger surface areas which was adjusted with assay buffer to 2.5 mg protein/ml. All incubations were carried out in triplicate, each containing 100 μl of cytosol, 20 μl of 10 μM 6,7-[3H]estradiol (50 Ci/mmol; DuPont NEN Research Products, Boston, MA), and 80 μl of putative antagonist in assay buffer. Total count tubes received 20 μl of [3H]estradiol and 180 μl of assay buffer. After incubation overnight at 4°C, all the total count tubes received 300 μl of dextran-coated charcoal suspension; tubes were agitated for 15 min and then centrifuged (1,000 g) for 15 min. Supernatants were decanted into counting vials, scintillant was added, and protein-bound tritium was determined by liquid scintillation counting.

RESULTS

HepG2 cells were grown to confluence in 25-cm² tissue culture flasks. For bulk production of AFP, multiple 25-cm² flasks were preferable to fewer flasks with larger surface areas because confluent monolayers tended to peel away from the edges in the larger flasks when these cells were being maintained in serum-free medium. As shown in Fig. 1, when HepG2 cells were switched to serum-free medium, AFP production increased by approximately fivefold. Levels plateaued after 7 to 10 days in serum-free medium and were then sustained for as long as 100 days. Gel electrophoresis indicated that there were multiple proteins in addition to AFP present in the culture supernatant (Fig. 2). The dense protein band migrating at a molecular weight of M₅₄, ~69,000 (Fig. 2), consistent with the molecular weight of AFP, was subsequently confirmed to be AFP by Western blot (data not shown). AFP in this protein mixture was purified to a single band, using immunoaffinity chromatography (Fig. 2); the presence of AFP in that band was again verified by Western blot (data not shown).

Protein in the culture supernatant was concentrated by centrifugal force-augmented hydrostatic pressure using Centriprep concentrators with M₅₄ 10,000 cutoff (Amicon, Beverly, MA). Injection of concentrate containing 100 μg of AFP into immature female mice significantly inhibited their uterotrophic response to E₂ (Table 1). This procedure was used as a screening assay for antiestrogic activity in our preparations because of its rapid turnaround time and cost-effectiveness. Optimal activity was found in supernatants from cells that had been in serum-free medium for 7 to 21 days. After immunoaffinity purification, the chromatographic fraction devoid of AFP had no antiusretropic activity, whereas the immunoaffinity-purified AFP-containing fraction accounted for all of the activity in the crude supernatant (Table 1). Dose-response studies indicated that the antiusretropic activity plateaued at 50 μg of AFP and titrated out between 1 and 10 μg AFP/mouse (Fig. 3).

The human MCF-7 breast cancer was grown as a xenograft under the kidney capsule of SCID mice. As shown in Fig. 4, the tumor was dependent for growth on exogenous estrogen supplied via a Silastic capsule, placed s.c., that contained solid E₂, which yielded a steady-state plasma E₂ level of ~100 pg/ml. Daily injections of AFP inhibited the growth of this tumor in a dose-dependent manner (Fig. 4). Over a 30-day period, AFP at 10 μg/mouse/day retarded the growth of the MC-7 breast cancer and 100 μg/mouse/day completely stopped the growth of MC-7.
MCF-7

Fig. 5 Effect of HepG2-derived AFP on the growth of estrogen-dependent and estrogen-independent human breast cancer xenografts. Open symbols represent groups that were not estrogenized; closed symbols represent groups that were estrogenized. AFP-treated mice (▲, △) received daily i.p. injections of 100 μg of AFP. Tamoxifen-treated mice (■, □) received daily i.p. injections of 50 μg of Tamoxifen. *, significantly different from E2-alone group: P < 0.05, Wilcoxon rank-sum test.

(Figs. 4 and 5). The growth prevention achieved with AFP at 100 μg/mouse/day was similar to that obtained with Tamoxifen at 50 μg/mouse/day (Fig. 5). Both of these agents produced a cytostatic effect as evidenced by the resumption of growth after treatment was stopped and a histological profile showing no evidence of necrosis, but rather a significant reduction in mitotic figures and an increase in cells with G0/G1 ploidy in growth-inhibited tumors. This dose and schedule of AFP also inhibited the estrogen-dependent growth of the T47 human breast cancer (Fig. 5). Xenografts of two human tumors that grew independent of estrogen (MDA MB 231 and BT20) were not inhibited by daily treatment with 100 μg of AFP (Fig. 5). Blood samples taken at the end of 30 days of AFP treatment contained higher levels of E2 than blood samples from animals not given AFP (Table 2), suggesting that AFP was interfering with another E2 response, the feedback at the hypothalamic-pituitary axis, which normally would decrease the output of follicle-stimulating hormone in the presence of elevated serum E2 levels and prevent such an increase in E2. The mechanism by which AFP interferes with E2 responses is not clear at this time. It is known that human AFP does not have a high affinity binding site for E2 (19); therefore, sequestering of E2 does not seem to be a likely mechanism. In our own studies, we examined whether AFP interferes with the ability of E2 to bind to the estrogen receptor. As shown in Fig. 6, AFP does not have the characteristics of a typical estrogen receptor antagonist.

DISCUSSION

The results of this study demonstrate that human AFP can interfere with estrotrophic responses, including the estrogen-stimulated growth of human breast cancer xenografts. The estrogen receptor-positive MCF-7 and T47 human breast cancers did not grow in the presence of AFP. In contrast, the estrogen
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Table 2  Effect of AFP on serum estradiol levels

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Serum E$_2$ concentration* (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>56 ± 7</td>
</tr>
<tr>
<td>E$_2$</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>E$_2$-AFP</td>
<td>187 ± 8</td>
</tr>
</tbody>
</table>

* Treatment groups were taken from those shown in Fig. 5.

In our studies, we found that human AFP inhibited the estrogen-stimulated growth of both normal mouse uterus and of human breast cancer xenografts. This effect of AFP was dose-dependent and reached its maximum effect at 100 µg/mouse/day. That dose resulted in a blood AFP level which was well below the fetal physiological range of AFP, since normal fetal blood levels of AFP are ~1–3 mg/ml (1). This may very well explain why no evidence of toxicity, such as weight loss or reduction in activity, was seen in mice that received this dosing regimen of AFP. It also suggests that AFP would be well tolerated if it was applied as a therapeutic agent in humans. The mechanism of the antiestrogenic effect of AFP is not clear at this time. Sequestering of estrogen by AFP is unlikely because human AFP does not have a high-affinity binding site for estrogen (19). In fact, the AFP found in most species other than rodents (22) does not have a high-affinity binding site for estrogen (23). In our own studies, we found that human AFP did not compete with estrogen for binding to the estrogen receptor (Fig. 6). Thus it would seem that there is a point downstream from the binding of estrogen to its receptor where AFP, or a signal emanating from AFP associating with its receptor, interferes with a signal coming from the receptor-bound estrogen. Additional study of this area is ongoing. The fact that AFP does not behave as a classical estrogen receptor antagonist may offer advantages in that it may be additive or synergistic in combination with classical estrogen receptor antagonists, or it may be effective against estrogen receptor-positive tumors that have become resistant to the classical estrogen receptor antagonists. However, it does not appear that AFP will impact on the estrogen receptor-negative breast cancers, which are some of the most difficult breast cancers to treat with chemotherapy.

Several reports suggest that AFP interferes with estrogen-induced responses in normal and malignant tissues in humans. For example, premenopausal women with AFP-secreting hepatomas experience amenorrhea (24, 25) and elevations in serum E$_2$ (26). After surgical removal of the hepatoma, normal monthly menstruation resumes and serum E$_2$ returns to normal levels. Elevations of E$_2$ in women with AFP-secreting hepatomas are in agreement with our results and can be explained by AFP-induced interference with the E$_2$ feedback response at the hypothalamic-pituitary axis, which would normally reduce follicle-stimulating hormone secretion and maintain a steadier blood level of E$_2$. With regard to the effects of AFP on human malignancies, the growth of most human breast cancers, at least in their early stages, is stimulated by estrogen, and interference with that response has been a therapeutic strategy in the management of this disease. That AFP may be playing a role in the control of this disease is supported by several reports in the literature, which, when taken together, suggest that individuals who experience elevations in AFP are subsequently at reduced risk for breast cancer. These reports are summarized in Table 3. For example, women who have experienced full-term pregnancy have had elevations of AFP in their serum (27), and subsequently they have been found to be at reduced risk of breast cancer when compared with women who have never experienced pregnancy (28). Furthermore, there are factors in pregnancy, such as maternal race, weight, hypertension, number of fetuses in utero, and neural tube defect in the fetus, where maternal serum (MS) AFP is substantially elevated above nor-

Table 3 Association of pregnancy AFP levels with subsequent risk of breast cancer

<table>
<thead>
<tr>
<th>Maternal condition</th>
<th>Maternal serum AFP concentration</th>
<th>Maternal lifetime breast cancer risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnant</td>
<td>1 &gt; 2 (27)*</td>
<td>1 &gt; 2 (28)*</td>
</tr>
<tr>
<td>Pregnant, black</td>
<td>1 &gt; 2 (29)</td>
<td>1 &gt; 2 (30)</td>
</tr>
<tr>
<td>Pregnant, lean</td>
<td>1 &gt; 2 (29)</td>
<td>1 &gt; 2 (31)</td>
</tr>
<tr>
<td>Pregnant, hypertensive</td>
<td>1 &gt; 2 (32)</td>
<td>1 &gt; 2 (33)</td>
</tr>
<tr>
<td>Pregnant with multiple fetuses</td>
<td>1 &gt; 2 (34)</td>
<td>1 &gt; 2 (35)</td>
</tr>
<tr>
<td>Pregnant, fetus with neural tube defect</td>
<td>1 &gt; 2 (36)</td>
<td>1 &gt; 2 (37)</td>
</tr>
<tr>
<td>Pregnant, consuming no alcohol</td>
<td>1 &gt; 2 (38)</td>
<td>1 &gt; 2 (39)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses are the reference sources for the data.

REFERENCES
Alpha-fetoprotein derived from a human hepatoma prevents growth of estrogen-dependent human breast cancer xenografts.


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