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Award Number DAMD17-94-J-4278

TITLE: Development of Ligand-Transformed Alpha-Fetoprotein for
Use Against Breast Cancer in Humans

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REPORT DATE: April 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

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1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE April 1999	3. REPORT TYPE AND DATES COVERED Final (1 Jul 94 - 31 Mar 99)	
4. TITLE AND SUBTITLE Development of Ligand-Transformed Alpha-Fetoprotein for Use Against Breast Cancer in Humans		5. FUNDING NUMBERS DAMD17-94-J-4278	
6. AUTHOR(S) James A. Bennett, Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Albany Medical College Albany, New York 12208		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
<div style="display: flex; justify-content: space-between;"> 11. SUPPLEMENTARY NOTES 19991214 070 </div>			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited		12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) The purpose of this study was to produce large quantities of the active form of alpha-fetoprotein (AFP) and assess its effectiveness in the control of estrogen-stimulated growth of experimental human breast cancers. Both recombinant and natural AFP were produced for this purpose. AFP stopped the estrogen-stimulated growth of MCF-7 and T47 human breast cancers and the MCF-10A human benign breast tumor grown as xenografts in immune-deficient mice. AFP did not interfere with the estrogen-independent growth of MDA-MB-231 and BT20 human breast cancer xenografts. Positivity for sex steroid hormone receptors was a marker of tumor sensitivity to the growth-inhibitory effects of AFP. Elevations in serum FSH and E ₂ were intermediate markers of AFP's biological activity. The histomorphometric profile of growth-inhibited tumors was one of cytostasis, not cytotoxicity, with cells accumulating in the G ₀ /G ₁ phase of the cell cycle. There was no evidence of toxicity associated with chronic treatment with AFP. The active site of this protein is an 8-amino-acid peptide located near the C-terminal part of the molecule. This peptide was synthesized in quantity and retained full biological activity. The peptide requires further study, since it is more clinically translatable as a pharmaceutical for the treatment of breast cancer than the full-length protein.			
14. SUBJECT TERMS Breast Cancer		15. NUMBER OF PAGES 34	
		16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

FOREWORD

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James A Bennett 4-29-99

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Final Report

INTRODUCTION

Subject: We have been studying the regulation of breast cancer growth by alpha-fetoprotein (AFP). AFP is a glycoprotein normally produced during gestation, initially by the fetal yolk sac and then by the fetal liver (1). It is a major protein constituent of the fetal plasma throughout gestation and has structural similarities to albumin (2). However, upon parturition, the gene for AFP is repressed, and its serum concentration diminishes to a negligible level. It is reexpressed during liver pathology such as hepatoma or cirrhosis. The restricted presence of this embryonic protein 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 and function of certain tissues such as liver (3), lymphocytes (4), placenta (5), ovaries (6), and uterus (7), and interact with certain ligands such as arachidonic acid (8), docosahexaenoic acid (8) and retinoic acid (8), all of which influence differentiation. Our own studies have shown that when either rodent or human AFP is incubated with a molar excess of estradiol, the protein undergoes a change in conformation (9). In this transformed state (tAFP), nanogram quantities of the material inhibit the growth of estrogen-stimulated tissues *in vivo*, including estrogen-stimulated breast cancers (10-13).

The physiological role of AFP, and especially tAFP, may be to act as a rudimentary servo mechanism that desensitizes endocrine tissues to the inappropriately high levels of steroid hormones that occur during gestation. This mechanism is fetoprotective, as the fetus develops in the presence of a large concentration of maternal and placental steroid hormones, and has receptors for these hormones, but it does not have the sophisticated control mechanisms of late fetal or adult life to regulate the production of and response to these hormones. A "side effect" of the proposed "servo mechanism" would occur when tAFP crossed the placenta into the maternal circulation where it would extinguish microscopic premalignant and/or cancerous foci in the breast that later on in life would be promoted to clinically detectable breast cancers. Such a "side effect" would explain the epidemiological data, which clearly show that the experience of full-term pregnancy decreases the lifetime risk of breast cancer (14).

Purpose: The purpose of our study is to produce large quantities of the active form of AFP and assess its effectiveness in the control of estrogen-stimulated growth of experimental human breast cancers.

The **specific aims** of our original grant proposal were:

1. Determine optimal conditions for producing the active form of AFP. Then, maximize the antitumor activity of AFP by manipulating its dose and schedule without introducing host toxicity in mice bearing human breast cancer xenografts.
2. Determine markers on tumors that predict tumor sensitivity to AFP.
3. Determine intermediate markers in the host which indicate that AFP is active *in vivo*.
4. Assess through histomorphometric studies the type of damage (lethal or non-lethal) done to the tumor by AFP.

These aims are specifically designed so that, upon their completion, the tools will be available for clinical trial of AFP for breast cancer.

Background: There are experiments of nature and laboratory experiments that point to AFP as a regulator of estrogen-stimulated growth of normal and malignant tissues. This has implications for AFP in the prevention and treatment of breast cancer, because almost all breast cancers start out as estrogen-receptor-positive and are stimulated in their growth by estrogen. By the time breast cancer is diagnosed, half of these breast cancers have further dedifferentiated to an estrogen-receptor-negative phenotype.

The evidence that supports the idea that AFP inhibits the response of tissues to estrogen is as follows. It is a well-known fact that hepatomas secrete AFP (15). In fact, serum AFP levels are used as a marker of tumor burden in this disease. What is less well known is that amenorrhea is one of the first symptoms of hepatoma in premenopausal women, and this symptom resolves following surgical removal of the tumor (16). Also, hyperestrogenemia and normal to elevated gonadotropins are present in hepatoma patients (17). Taken together, these data suggest that neither the uterus nor the hypothalamic-pituitary axis is responding to estrogen in hepatoma patients. It is our belief that elevated AFP levels could bring about this result. Data that support the contention that AFP can interfere with estrogen-dependent responses are as follows. Our own studies have shown that an isoform of AFP, upon exposure to estradiol, takes on a conformation that inhibits the estrogen-stimulated growth of normal mouse uterus (7). Soto et al. (18) have shown that AFP-containing serum from a hepatoma-bearing rat inhibits the estrogen-stimulated induction of progesterin receptor. These same investigators have shown that an AFP-secreting tumor induces the regression of an estrogen-dependent tumor (19). Epidemiological data suggest that AFP is the factor in pregnancy that confers on parous women their significant reduction in risk of breast cancer. As shown in Table 1a, AFP is elevated in maternal serum during pregnancy. Furthermore, there are factors in pregnancy, such as maternal race, weight, hypertension, consumption of alcohol, number of fetuses *in utero*, and neural tube defect in the fetus, where maternal serum AFP (MSAFP) is substantially altered from normal pregnancy levels. In studying the literature, we have found the consistent and striking correlation that in those pregnancy conditions associated with an elevated level of MSAFP, there was a significant reduction in the lifetime risk to the mother of acquiring breast cancer. Conversely, in pregnancy conditions characterized by low MSAFP (alcohol), subsequent breast cancer risk was elevated (Table 1a). We carried out epidemiologic studies analyzing retrospective data that extend and confirm the correlation between MSAFP levels and breast cancer risk (Table 1b). Recently Ekblom et al. (33) have published an epidemiological study which suggests that, at least in the case of hypertension during pregnancy, the reduction of breast cancer risk is also passed on to the fetus. He is in agreement with our speculation that it is AFP in the fetal and maternal circulation that protects the offspring as well as the mother against later development of breast cancer.

Table 1
Association of High Maternal Serum AFP with Decreased Breast Cancer Risk

Maternal Conditions			Maternal Serum AFP Concentration	Maternal Lifetime Breast Cancer Risk
1	2			
1a				
Pregnant	vs. Non-pregnant		1 > 2 (20)*	1 < 2(21)
Pregnant, black	vs. Pregnant, white		1 > 2 (22)	1 < 2(23)
Pregnant, lean	vs. Pregnant, obese		1 > 2 (22)	1 < 2(24)
Pregnant, consuming no alcohol	vs. Pregnant, consuming alcohol		1 > 2 (25)	1 < 2(26)
1b				
Pregnant, hypertensive	vs. Pregnant, normotensive		1 > 2 (27)	1 < 2(28)
Pregnant, with multiple fetuses	vs. Pregnant, with a single fetus		1 > 2 (29)	1 < 2(30)
Pregnant, fetus with neural tube defect	vs. Pregnant, fetus no neural tube defect		1 > 2 (31)	1 < 2(32)

*The numbers in the brackets are the reference sources for the data.

Recently, Richardson et al. (34) have reported measuring this association directly. She found that the concentration of AFP in cryogenically stored maternal sera was inversely correlated to the risk of breast cancer in these mothers 20 to 30 years after their pregnancies. As mentioned earlier in this report, we speculate that the AFP that crosses the placenta and enters the maternal circulation extinguishes microscopic premalignant foci that later on in life would be promoted into clinically detectable breast cancers. Our own work has shown that administration of natural mouse AFP (10), natural human AFP (11), or recombinant human AFP (13) can inhibit estrogen-stimulated growth of human MCF-7 breast cancer xenografts. The results of that work, made possible by the USAMRMC Breast Cancer Research Program, are described below.

BODY

Initially, our source of natural human AFP was pooled cord sera, and our recombinant human AFP was obtained from our collaborators at McGill University. Both of these isolates were purified to a single band and compared to one another for antiestrotrophic activity in our screening bioassay of estradiol (E₂)-stimulated growth of immature mouse uterus. Preincubation of both natural and recombinant AFP with a molar excess of E₂ resulted in a conformational change in the molecule as measured by difference spectroscopy. As shown in Table 2, nanogram doses of the E₂-transformed AFPs produced significant inhibition of E₂-stimulated growth of immature mouse uterus. Antibody to human AFP completely eliminated the growth-inhibitory

Table 2
**Inhibition of Estradiol-stimulated Mouse Uterine Growth
 by Transformed Natural and Recombinant Human AFP^a**

Group Number	Injectant 1	1hr →	Injectant 2	Mean Uterine ^e Weight ± S.E. (mg/g body wt)	Growth ^f Inhibition %
I	Saline		Saline	0.98 ± 0.04	
II	Saline		E ₂ ^d	1.61 ± 0.06	
III	E ₂		E ₂	1.64 ± 0.06	
IV	nAFP/Sal ^b		E ₂	1.60 ± 0.05	2
V	nAFP/E ₂ ^c		E ₂	1.43 ± 0.04 ^g	32
VI	rAFP/Sal ^b		E ₂	1.67 ± 0.07	0
VII	rAFP/E ₂ ^c		E ₂	1.45 ± 0.03 ^g	29
VIII	nAFP/E ₂ + Anti-AFP		E ₂	1.61 ± 0.05	5
IX	rAFP/E ₂ + Anti-AFP		E ₂	1.66 ± 0.07	0

^a Natural (n) AFP is a natural preparation of AFP purified from pooled human cord sera. Recombinant (r) AFP is the recombinant preparation of AFP produced in an *E. coli* expression system.

^b 5 µg/ml AFP was added to an equal volume of saline and incubated at room temperature for 1 hr. 0.1 ml of this incubation mixture was injected i.p. into each mouse.

^c 5 µg/ml AFP was added to an equal volume of 10 µg/ml E₂ and incubated at room temperature for 1 hr. 0.1 ml of this incubation mixture was injected i.p. into each mouse.

^d 0.5 µg E₂ in 0.1 ml was injected i.p. to stimulate uterine growth.

^e Twenty-two hours following administration of injectant 2, uteri were excised and uterine wet weights were determined. There was a minimum of 5 replicate mice per group.

^f Percent growth inhibition was calculated as the reduction in E₂-stimulated uterine growth divided by the full E₂-stimulated uterine growth multiplied by 100%. For example, the growth inhibition in group V was calculated by relating the uterine weights as follows:

$$\frac{\text{III} - \text{V}}{\text{III} - \text{I}} \times 100\%. \text{ For group IV it would be } \frac{\text{II} - \text{IV}}{\text{II} - \text{I}} \times 100\%.$$

^g Significant inhibition, *p* < 0.05; Wilcoxon Sum of Ranks Test.

activity in both natural and recombinant AFP. Also, without prior exposure to E₂, these AFP preparations were not inhibitory at the nanogram doses tested. The dose response curves of E₂-activated natural and recombinant AFP were also similar (Fig. 1). We had previously shown that ligands other than E₂ can transform natural AFP to its antiestrotrophic form, and the common bond of these ligands was that they all had receptors in the steroid/thyroid hormone receptor superfamily (35). This concept was tested for recombinant human AFP and appeared to hold true. As shown in Table 3, estradiol, 13-cis-retinoic acid and cholecalciferol (vitamin D₃) all converted recombinant AFP to an antiestrotrophic form, whereas arachidonic acid and ascorbic

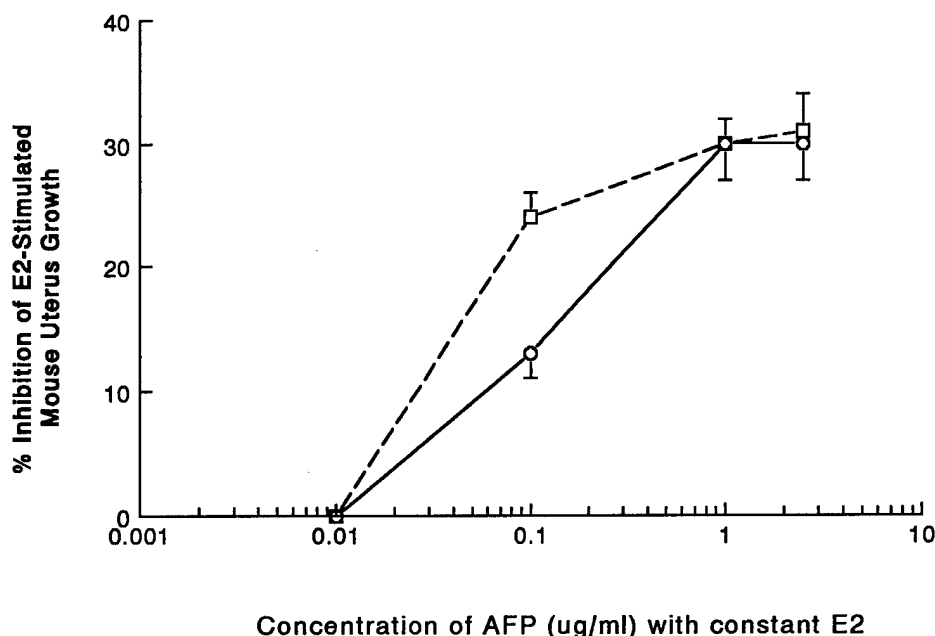


Fig. 1. Similarity of dose-response curves between natural (—○—) and recombinant (—□—) transformed human AFP. Various concentrations of AFP were incubated with 5 $\mu\text{g/ml}$ E_2 for 1 hr at room temperature to induce transformation of AFP. Each incubation mixture was injected i.p. in a volume of 0.1 ml into 5 replicate mice. Mice were then challenged with E_2 and uterine wet weights were measured as described in Table 1. Inhibition of uterine growth was determined by comparing uterine weights in the AFP-treated groups to those in groups injected with saline (unstimulated) and those injected with E_2 only (fully stimulated) as described in Table 1. The mean percent growth inhibitions \pm the standard errors were calculated for each dose of AFP.

Table 3
Inhibition of Estradiol-stimulated Mouse Uterine Growth by Recombinant Human AFP Transformed by Estrogen, by Vitamin A or by Vitamin D

TREATMENT 1 HR PRIOR TO INJECTION OF E_2 ^a	GROWTH INHIBITION %
rAFP/Sal	2 \pm 3
rAFP/ E_2	35 \pm 2
rAFP/13cRA ^b	32 \pm 4
rAFP/AA ^c	7 \pm 1
rAFP/ D_3 ^d	29 \pm 4
rAFP/C ^e	1 \pm 1

^a The bioassay was carried out as described in the legend to Table 1 using the same AFP-to-ligand concentrations as described in that legend.

^b 13-cis-retinoic acid or vitamin A

^c arachidonic acid

^d cholecalciferol or vitamin D_3

^e ascorbic acid or vitamin C

acid were inactive in this regard. We compared natural to recombinant human AFP for inhibition of human breast cancer xenograft growth. As shown in Fig. 2, E₂-activated natural and recombinant AFP were similar in their ability to inhibit estrogen-dependent growth of the human MCF-7 breast cancer xenograft. The level of inhibition achieved was similar to that obtained with tamoxifen. Daily treatment was required to maintain the prevention of tumor growth. Both AFP and tamoxifen produced a cytostatic effect, as evidenced by the tumor regrowth upon cessation of treatment, and this cytostasis was confirmed morphologically by microscopic examination of H & E-stained, growth-inhibited tumors. As shown in Figure 3, both recombinant and natural E₂-activated AFP did not interfere with the estrogen-independent growth of MDA-MB-231 human breast cancer xenografts. A detailed report of our comparison of natural AFP to recombinant AFP has been published (36).

The activation step seemed like it would be problematic to translate clinically, especially if the activating ligand were E₂. We hypothesized that a small portion of AFP molecules were already in their active form and that if we raised the dose of AFP, we could achieve antiestrotrophic activity without activating ligands. As shown in Fig. 4, this indeed was the case. The antiestrotrophic activity obtained with 100 µg of AFP not pretreated with E₂ was similar to that obtained with 100 ng of AFP that was pretreated with E₂. This was true for natural as well as recombinant AFP. Since we had seen no evidence of toxicity with AFP, and since it is known that the human fetal liver produces milligram quantities of AFP (1), it seemed reasonable to proceed with higher doses of AFP not pretreated with ligand. Unfortunately, it was around this time in the investigation that our collaborators at McGill ran into production problems with recombinant AFP and were not able to supply us with the quantities of recombinant AFP required for this approach. Moreover, cord sera was not a practical source for isolating large quantities of pure AFP. To solve these problems, we embarked on producing our own recombinant AFP and finding a system that produced large quantities of natural human AFP. The latter problem was more readily solved. We found in the literature that a human hepatoma (HepG-2) cell culture line, when grown in serum-free medium, secreted microgram quantities of relatively pure AFP into the culture supernatant (37). We obtained this cell line, validated its ability to grow in serum-free medium and secrete AFP, and demonstrated that the secreted AFP had antiestrotrophic activity. We published these findings, along with the anti-breast cancer activities of this AFP (38). We essentially finished the aims of this grant using this AFP, and those results are described below. At the end of this report, our experience of producing recombinant AFP, as well as truncated forms of AFP, will be described.

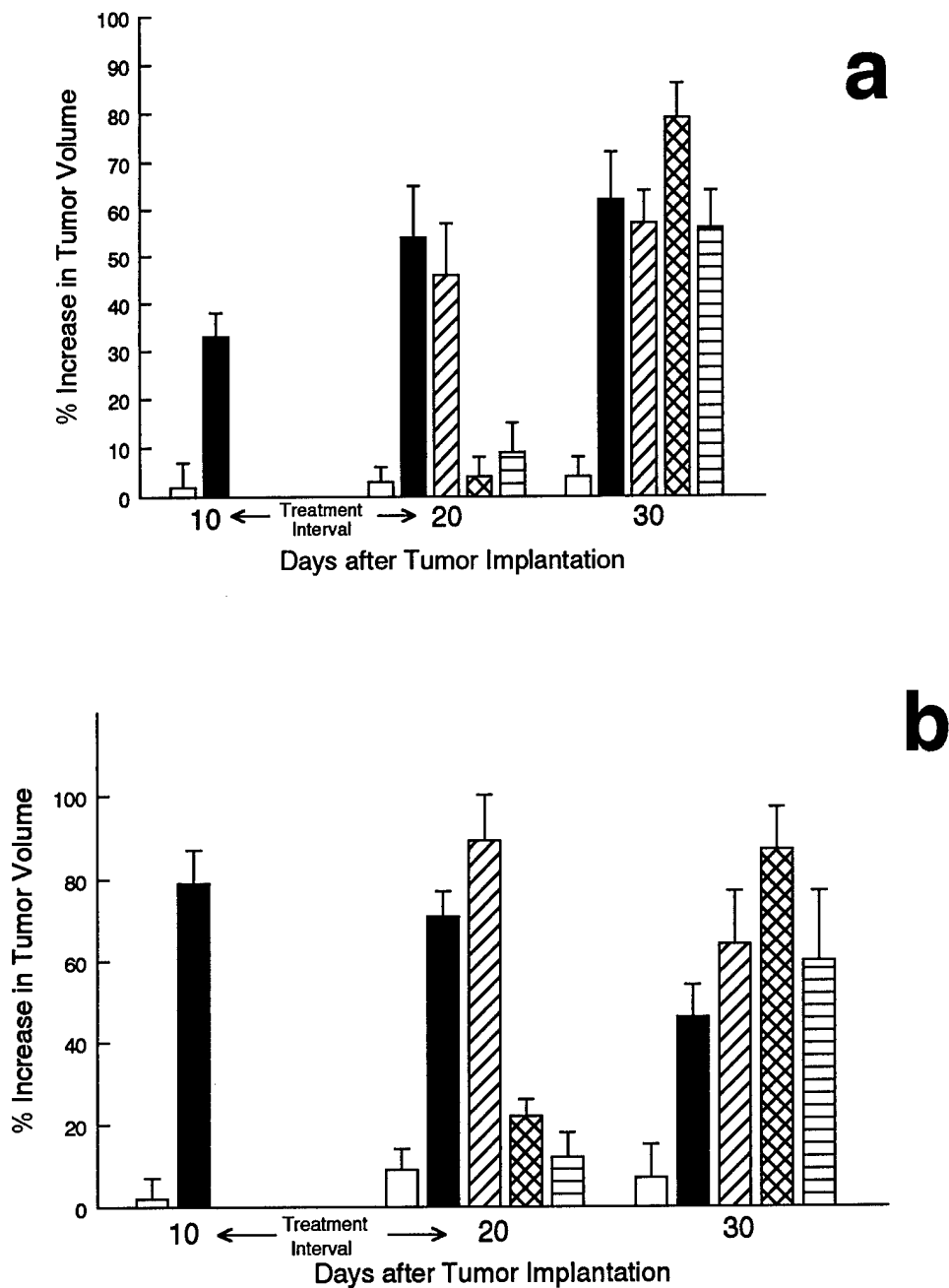
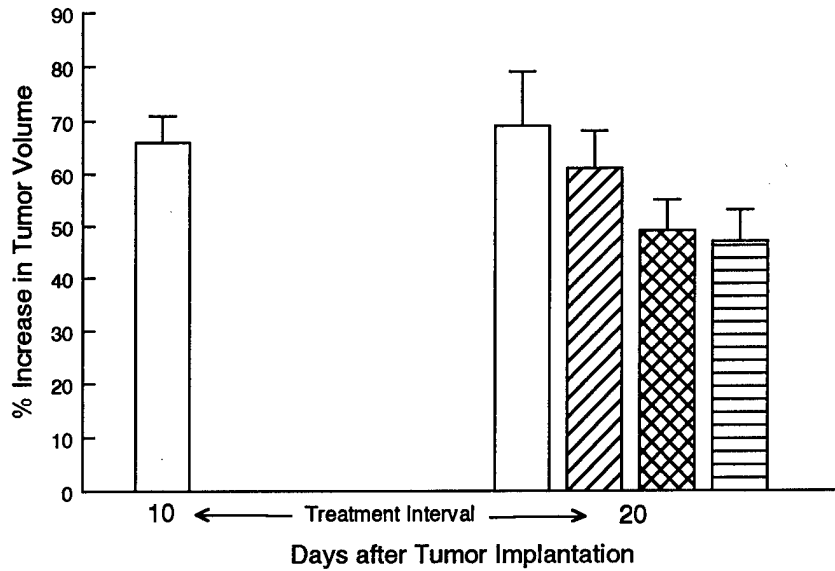


Fig. 2. Effect of natural (a) and recombinant (b) transformed human AFP on the growth of estrogen-dependent human MCF-7 breast cancer xenografts. Open bars represent non-estrogenized mice. All other mice were estrogenized (filled bars) by s.c. placement of Silastic E₂ implants at the time of tumor implantation. Mice received daily i.p. injections from day 10 through day 20. Injectants contained 0.25 µg transformed AFP (cross-hatched), 0.25 µg untransformed AFP (diagonal lines), or 50 µg tamoxifen (horizontal lines). The change in tumor size between day 10 and day 20 in the groups treated with transformed natural AFP, transformed recombinant AFP or tamoxifen is significantly different from the change in tumor size in the group treated with untransformed natural or recombinant AFP during the same interval, $p < 0.01$, Wilcoxon Mann-Whitney test. $n = 8-10$ mice per group. Means \pm standard errors are shown.

a



b

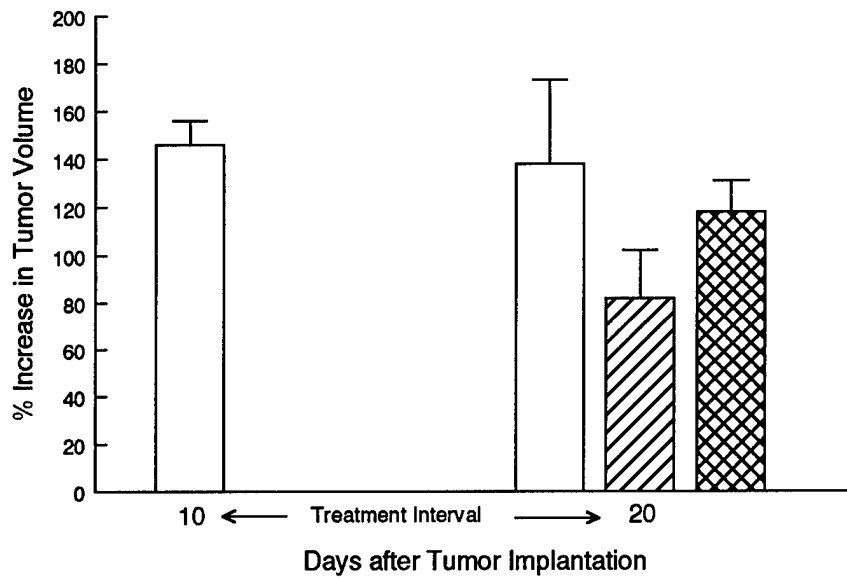
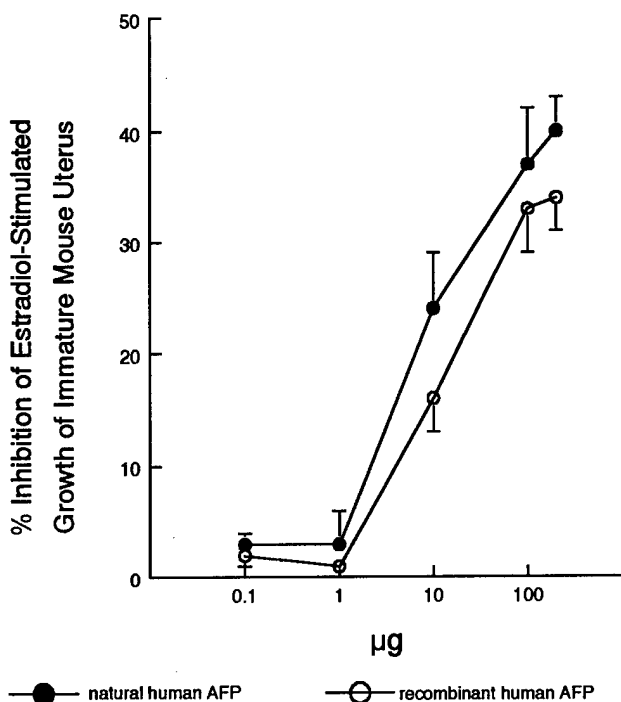


Fig. 3. Effect of natural (a) and recombinant (b) transformed human AFP on the growth of estrogen-independent human MDA-MB-231 breast cancer xenografts. Mice received daily i.p. injections from day 10 through day 20. Injectants contained 0.25 µg transformed AFP (cross-hatched), 0.25 µg untransformed AFP (diagonal lines) or 50 µg tamoxifen (horizontal lines). The unfilled (white) bars represent uninjected controls. n = 8-10 mice per group. Means ± standard errors are shown.

Fig. 4. Inhibition of Estrogen-Stimulated Growth of Immature Mouse Uterus by Higher Doses of Natural or Recombinant Human AFP Not Pre-exposed to Activating Ligands



1. Activity of human AFP (HepG-2-derived) against human cancer xenografts.

Several human breast and non-breast cancer cell lines were grown as a monolayer in culture. To prepare these cell lines for growth as xenografts, confluent monolayers were trypsinized, pelleted by centrifugation, and solidified by exposure to 15 µl of fibrinogen (50 mg/ml) and 10 µl of thrombin (10 units/ml) for 30 minutes at 37°C. Fibrin clots were then cut into pieces approximately 1.5 mm in diameter, and each piece was implanted under the kidney capsule of severe combined immunodeficient (SCID) mice (36, 38). For estrogen-dependent tumors, mice were supplemented with a Silastic tubing implant containing estradiol (Si/E₂), which was placed subcutaneously (36, 38). AFP was injected i.p. once a day for the times indicated in the figures. There were 5 to 10 replicate mice per treatment group.

As shown in Figures 5 and 6 (same experiment), the MCF-7 breast cancer remained dependent on estrogen for its growth. Significant growth inhibition was obtained with 10 µg of AFP and complete growth inhibition was achieved with 100 µg of AFP. A dose of 100 µg of AFP also stopped the growth of MCF-7 tumors which had been allowed to seed and grow for 10 days prior to treatment (Fig. 7). Upon cessation of AFP treatment, tumor growth resumed (Fig. 7). This pattern of growth regulation was similar to that found with tamoxifen in this model (Fig. 7b). Albumin, given at a dose of 250 µg per mouse per day, did not inhibit the growth of MCF-7 breast cancer xenografts (Fig. 7b).

Fig. 5 MCF-7

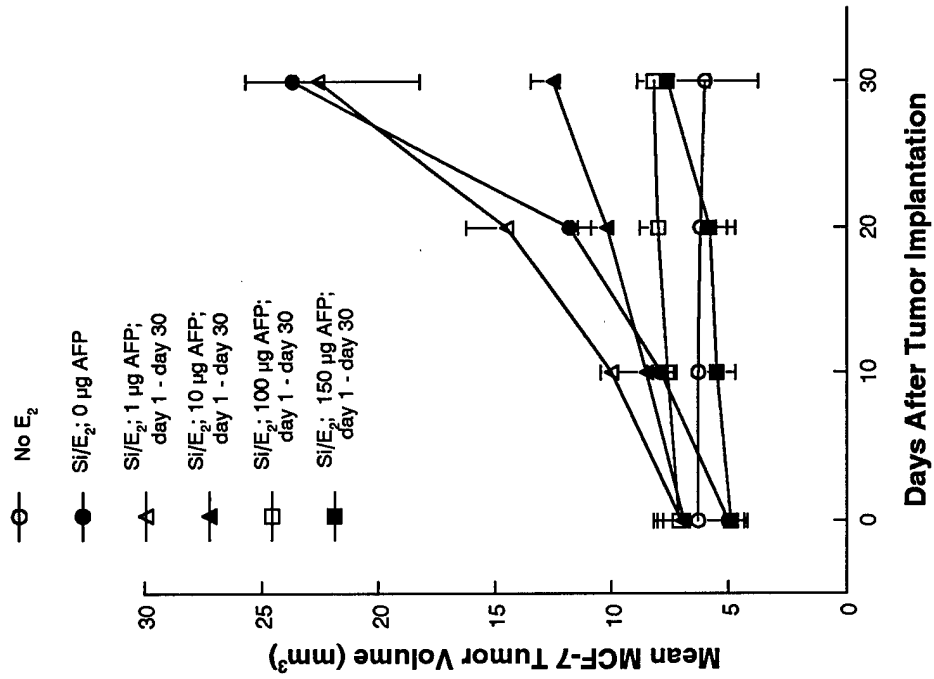


Fig. 6 MCF-7

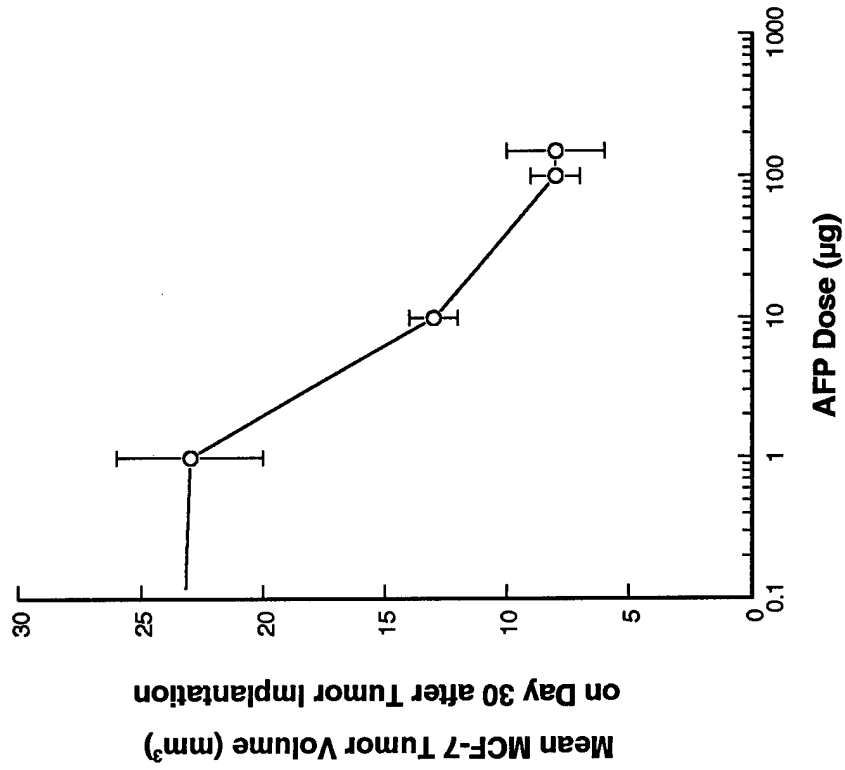
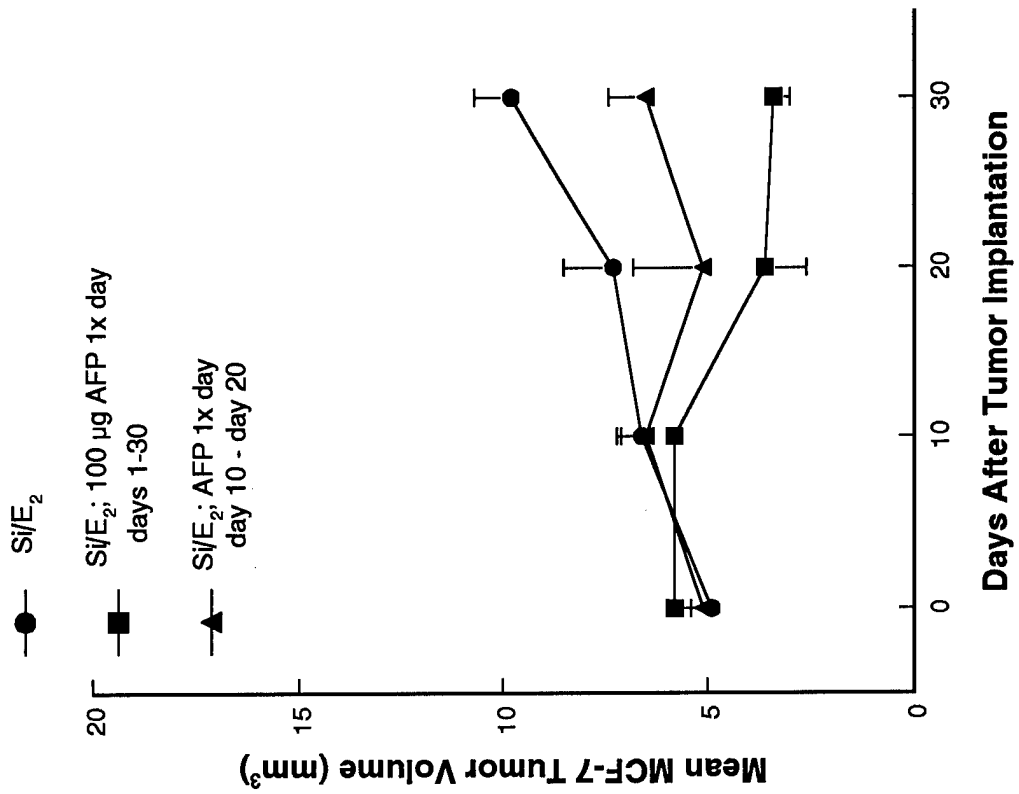


Fig. 7

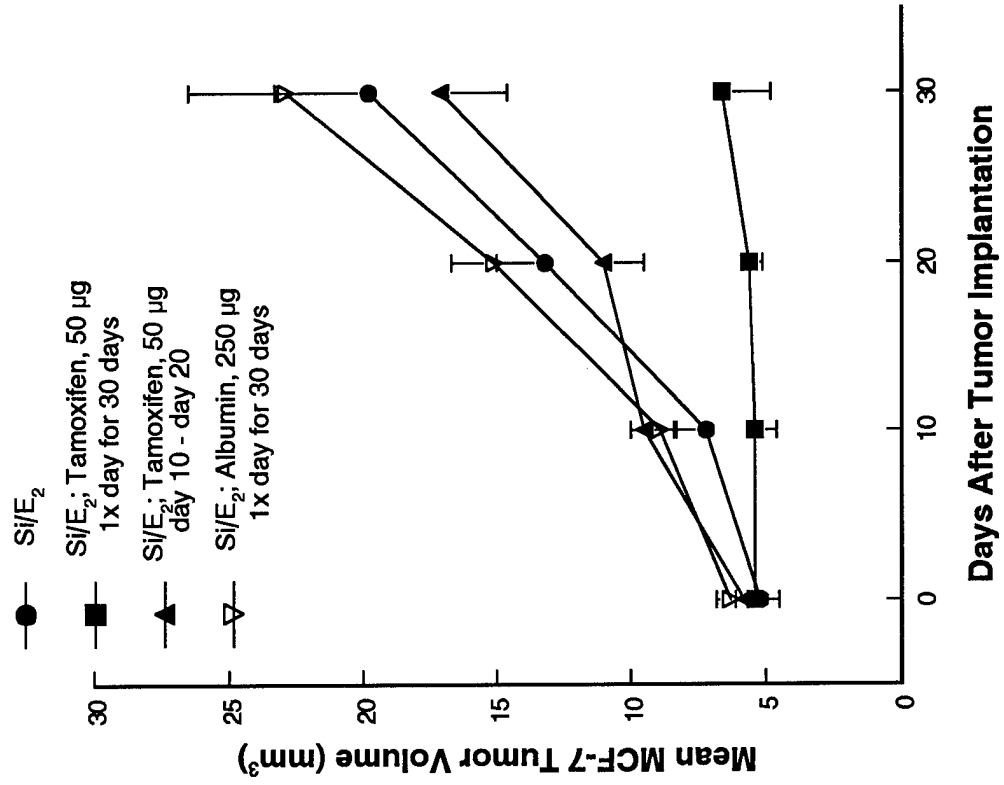
MCF-7

a



b

MCF-7



The T-47 D human breast cancer was dependent on estrogen for growth in our model (Fig. 8). AFP given daily completely stopped the growth of this tumor (Fig. 8). AFP also stopped the growth of T-47 D tumors which had been allowed to seed and grow for 10 days prior to treatment (Fig. 8). Again, upon cessation of AFP treatment, tumor growth resumed.

The MCF-10A benign breast adenoma grew in an estrogen-dependent manner and was inhibited in its growth by daily treatment with AFP (Fig. 9).

The MDA-MB-231 and BT-20 human breast cancers grew in an estrogen-independent manner in our xenograft model. Neither of these tumors were inhibited by either AFP or Tamoxifen (Figs. 10 and 11) when these agents were given at the dose and schedule which inhibited the estrogen-dependent human breast cancers described above.

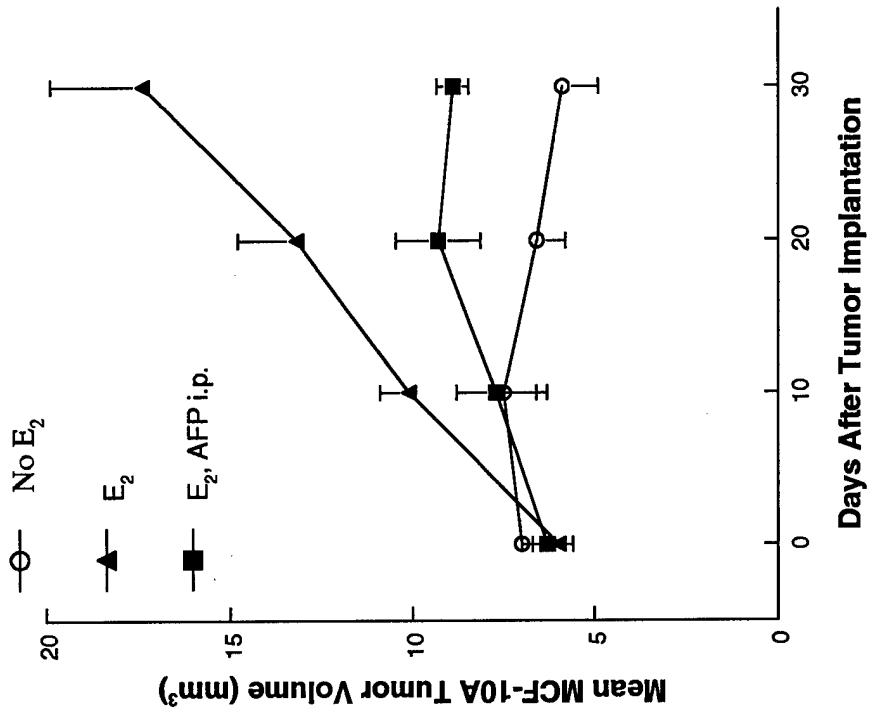
We have also investigated the sensitivity of non-breast cancers to AFP. The LNCaP human prostate cancer was dependent on androgen supplementation (testosterone propionate, 0.4 mg/mouse s.c. every other day) for its growth (Fig. 12). This tumor was completely stopped in its growth by daily administration of 100 μ g of AFP. In contrast, the DU 145 human prostate cancer did not require androgen supplementation for growth and was not inhibited by AFP (Fig. 13).

The OVCAR-3 human ovarian cancer did not require hormone supplementation for growth and grew in a cyst-like pattern. Growth was slightly retarded in the presence of AFP, but the differences were not significant (Fig. 14). Similarly, the MFE-296 human endometrial cancer did not require hormone supplementation for growth and was not inhibited by daily treatment with AFP (Fig. 15).

The histopathology of tumors growth-inhibited by AFP continued to be a profile of cytostasis with an accumulation of cells in G_0G_1 and no evidence of necrosis. Ploidy was quantitated using a CAS-200 Image Analyzer on sections of ten MCF-7 xenografts stained with Feulgen's reagent. Cells in the S phase of the cell cycle dropped on average from 38% to 10% as a result of AFP treatment. Correspondingly, cells in the G_0/G_1 phase increased from 49% to 76% in the AFP group.

MCF-10A

Fig. 9



T47

Fig. 8

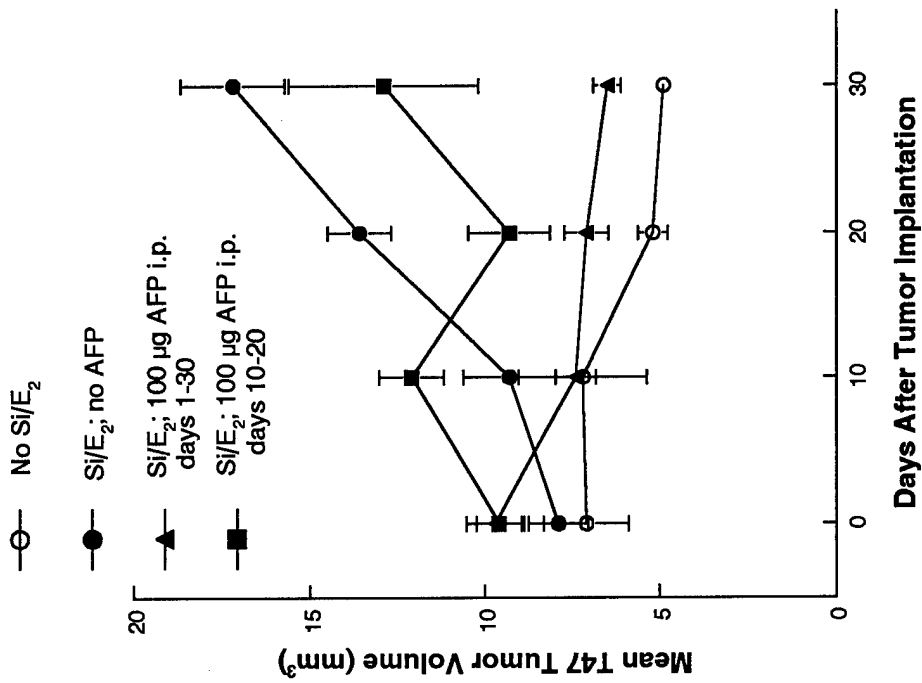


Fig. 11 **BT20**

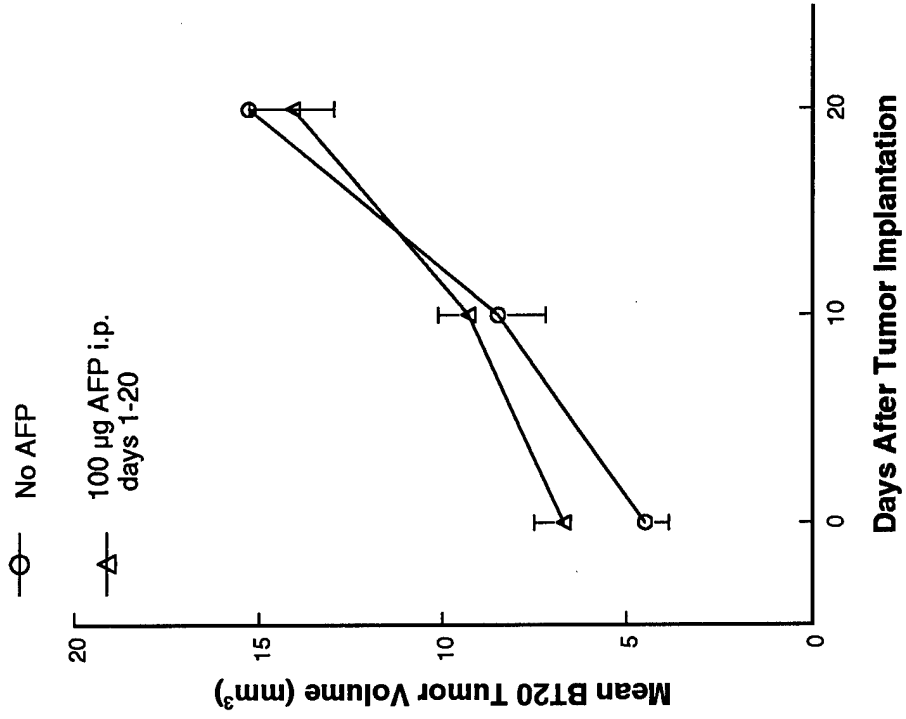


Fig. 10 **MDA-MB-231**

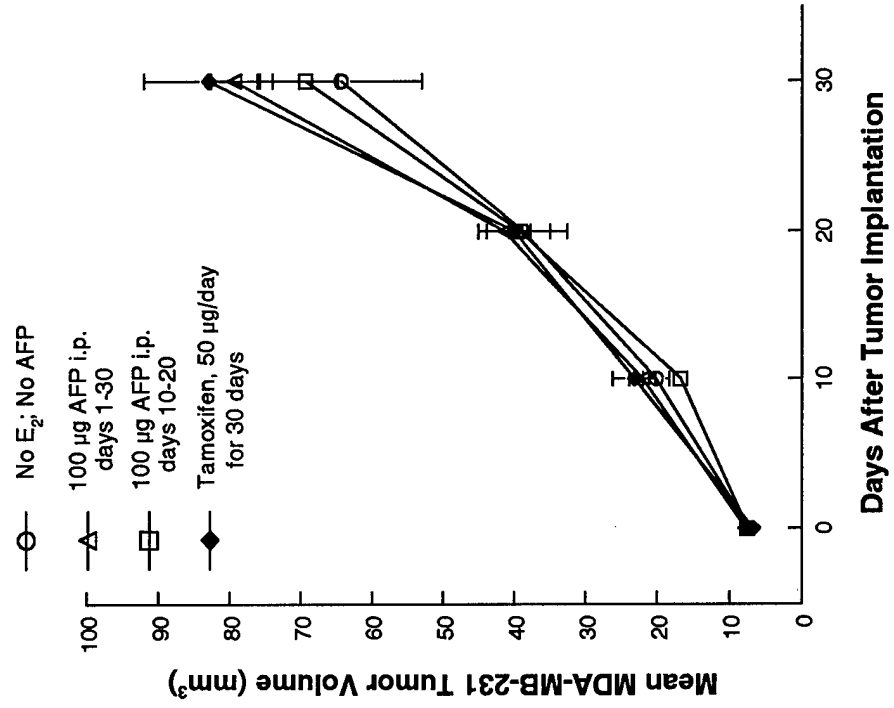


Fig. 13 **DU 145**

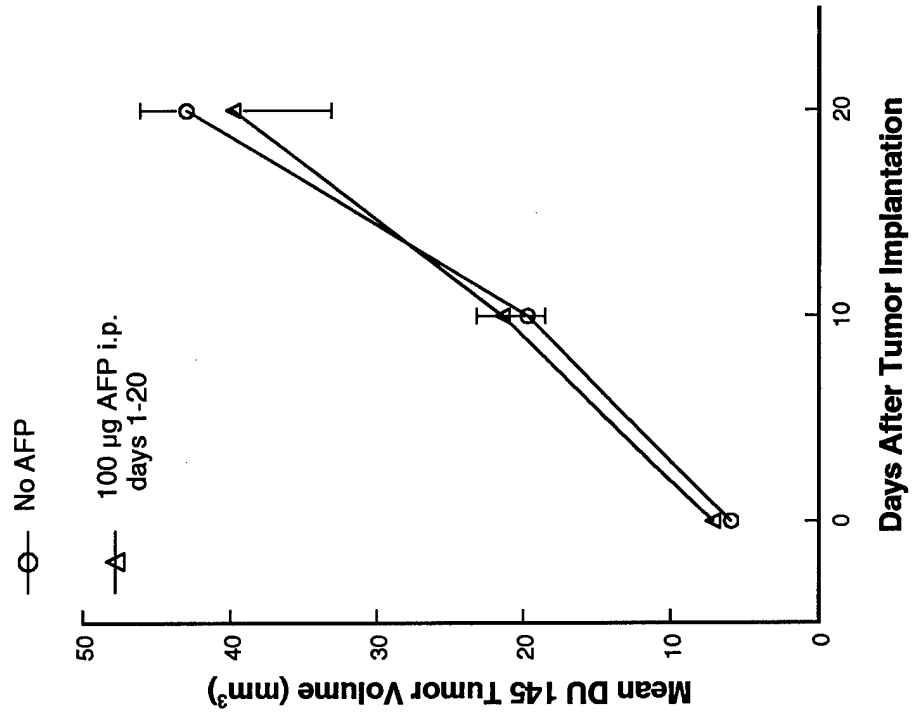


Fig. 12 **LNCaP**

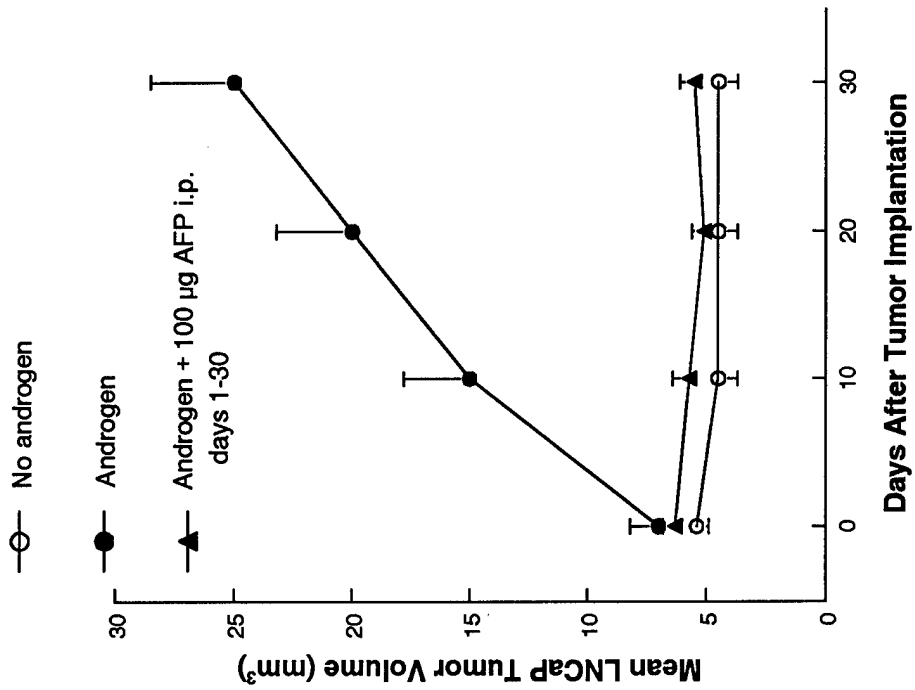


Fig. 15
MFE-296

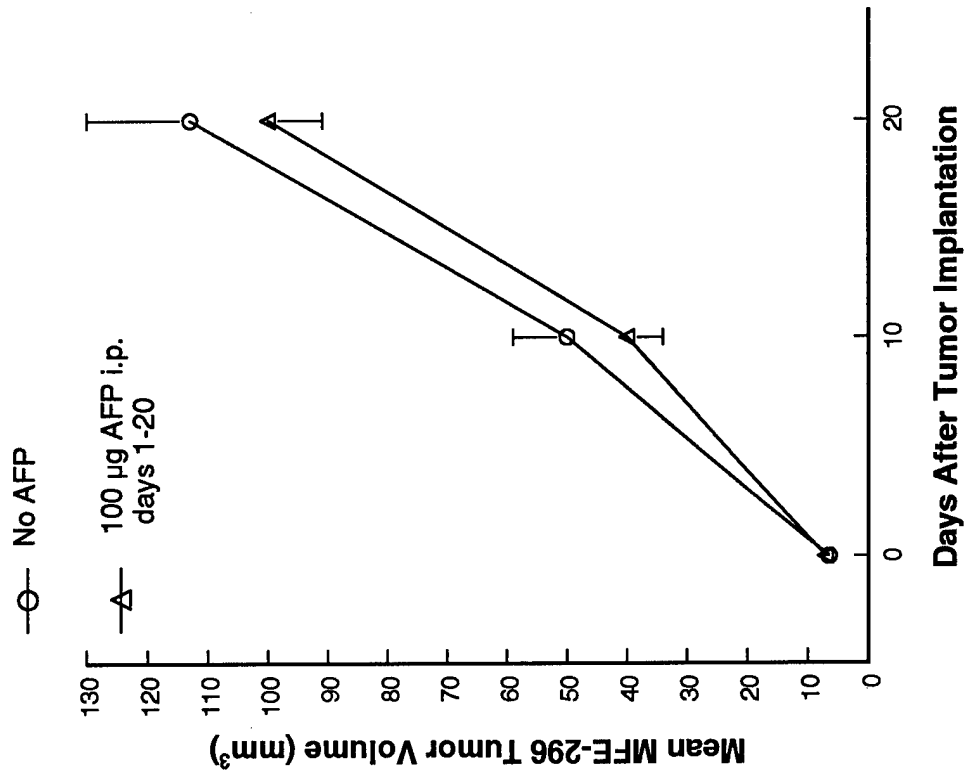
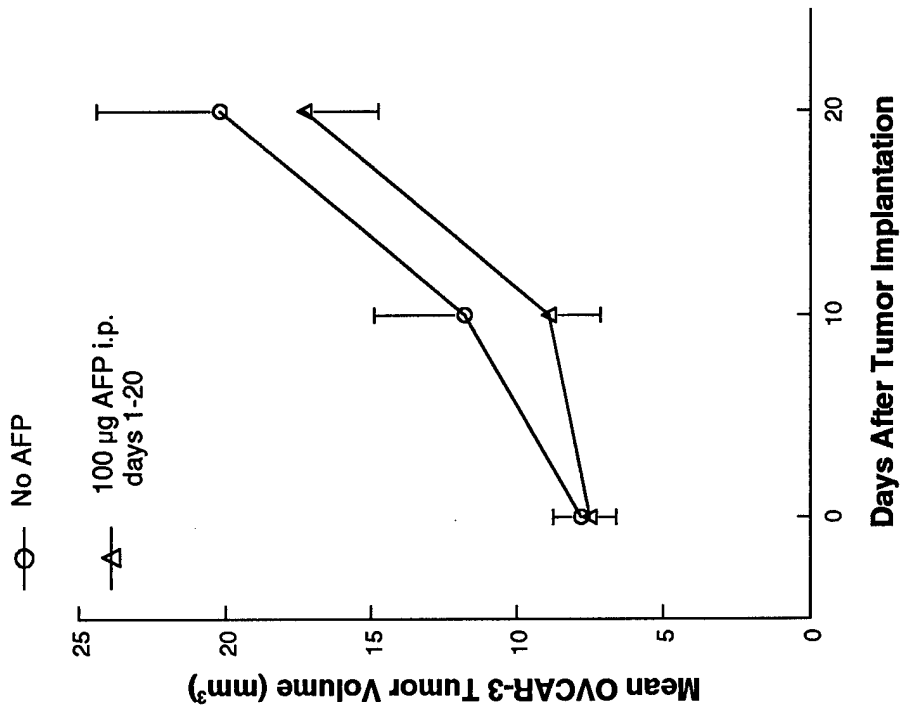


Fig. 14
OVCAR-3



2. Markers on Tumors Which Predict Tumor Sensitivity to AFP

a. Sex steroid hormone receptors

Cells were grown in bulk culture, harvested by trypsinization, pelleted, resuspended in buffer and stored at -70°C until used. All three receptor determinations (estrogen receptor [ER], progesterone receptor [PR] and androgen receptor [AR]) were done at one time, and required 600 mg of each cell line to yield sufficient cytosol volume and protein concentration. Aliquots of cells were thawed, homogenized and centrifuged at 60,000 rpm for one hour at 4°C . Supernatant (cytosol) protein concentration was determined using BioRad (Bradford) protein assay, and adjusted to between 2.0-2.5 mg/ml in a final volume of 6.0 ml.

Six-point Scatchard plot analyses were performed for each of the receptors at the following concentrations of radioligand: 0.1, 0.5, 1.0, 2.0, 4.0 and 8.0 nM with 12.5 μM radioinert steroid as inhibitor. ER was determined using ^3H estradiol (E_2) and unlabeled E_2 ; PR with ^3H progesterone and 6α -methyl- 17α -hydroxy progesterone acetate (MPA); and AR with ^3H dihydrotestosterone (DHT) and DHT. Samples were assayed in duplicate at 4°C .

Tubes used to assess total counts contained only labeled steroid in buffer. Tubes used to assess total binding contained 100 μl cytosol and labeled steroid. Tubes used to assess non-specific binding contained the same as specific-bound tubes, but with the addition of a molar excess of unlabeled steroid as inhibitor. Specific binding was calculated as the difference between total binding and non-specific binding.

Controls were performed for every assay using rabbit kidney cytosol as negative control for all three receptors, rabbit uterus cytosol as positive control for ER and PR, and castrated rat prostate cytosol as positive control for AR.

After overnight incubation all tubes except total counts were stripped of unbound steroid with charcoal. Following centrifugation at 1500 rpm at 4°C , supernatants containing bound steroid were decanted into scintillation vials for counting.

The sex steroid receptor values for the various tumor cell lines are shown in Table 4. Those tumors that were dependent on estrogen or androgen for growth were positive for those receptors. Tumors that did not require estrogen or androgen for growth were negative for those receptors. We had difficulty growing the ZR-75-1 and Hs578T breast cancers as xenografts.

Table 4

Tumor Line	ER	PR (fmol/mg cytosol protein)	AR
MCF-7	17	96	89
T-47 D	16	151	30
ZR-75-1	0	233	34
MDA-BM-231	0	22	0
BT-20	0	14	0
Hs 578 T	0	0	0
LNCaP	0	0	560
DU 145	0	0	0
OVCAR-3	65	0	19
MFE-296	0	0	37

An assay for AFP receptor was developed by our group. The published procedure using radioiodinated AFP utilizes prohibitively large amounts of cold AFP in incubations to block receptors so that non-specific binding of the radioiodinated material can be evaluated (39). Therefore, a technique that did not require such a step was needed. We took advantage of the exquisite sensitivity of the Abbott IM χ immunoquantitation of AFP (0.2 ng/ml) and the published report that AFP is dissociated from its receptor in 0.4 M KCl (40). Replicate tubes containing 2.5×10^6 cells in 0.2 ml serum-free medium were incubated with varying concentrations of AFP for 3 hours at 4°C. Cells were washed four times by centrifugation and resuspension in serum-free medium. After the final washing there was no detectable AFP in the supernatant (i.e., less than 0.2 ng/ml, indicating virtually complete removal of AFP from lower affinity non-specific sites). Sodium azide (20 nM, 5 min, 4°C) was then added to prevent receptor-ligand complexes on the cell membranes from internalizing when cells were subsequently warmed. KCl (0.4 M final concentration) was then added and incubated for one hour at 37°C to dissociate AFP-AFP receptor complexes. Cells were then centrifuged at 2,000 rpm for 10 minutes, supernatant was removed and the KCl-liberated AFP content of the supernatant was determined. By Scatchard plot analysis of AFP bound at different incubation concentrations, the number of specific binding sites per cell and their binding affinities were determined. Binding of AFP increased with increasing concentration and plateaued at 30 ng/ml. Bound AFP was approximately 0.1% of total AFP added to the cells. Therefore, concentration of free AFP was assumed to be equal to that of total AFP.

All of the tumors studied thus far contained high-affinity binding sites for AFP. The number of binding sites per cell was found to be consistent with cells that have high-affinity receptors for ligand. Table 5 shows the results of the binding affinity and number of receptors per cell for four tumor cell lines tested to date.

Table 5
AFP Receptor Binding Study Results

Tumor (P: prostate, B: breast)	MDA B / ER-	MCF-7 B / ER+	T47 B / ER+	LNCaP P / AR+
AFP Binding Kd	6×10^{-8}	3.8×10^{-8}	1.8×10^{-10}	3.9×10^{-9}
Receptors - #/cell	140,000	43,800	30,200	31,000

The highest affinity was found for the T47 tumor and the MDA-MB-231 cell line had the greatest number of receptors per cell. There appeared to be a wide range of receptor affinities for AFP (nearly 2 orders of magnitude). Neither the number of receptors nor the affinity of AFP for those receptors seemed to correlate with tumor sensitivity to growth inhibition by AFP. Therefore, we did not pursue the marker any further in this study. The above results are consistent with findings of another AFP study in which we are collaborators. In that study, AFP was radiolabeled with Technetium-99m and was tested as a radiopharmaceutical for the detection of breast cancer through scintigraphic imaging. In that study, all of the tumors described in Table 5 imaged equally well with AFP when they were growing subcutaneously as xenografts in SCID mice. In fact, Tc-99m-AFP yielded significantly higher tumor-to-background ratios when compared to Tc-99m Sestamibi, which is currently in clinical trial for this purpose. A detailed report of this imaging study was submitted for publication to the *Journal of Nuclear Medicine* in January, 1999.

3. Intermediate Markers in the Host Which Indicate That AFP is Active *in Vivo*

a. Gonadotropin Assay

Radioimmunoassay Reagent Preparation. LH and FSH levels were estimated by double antibody RIA using reagents from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). LH and FSH preparations (NIDDK-rLH-I-9 and NIDDK-rFSH-I-8) were iodinated by the chloramine-T method (41). Briefly, LH or FSH iodination preparations were thawed, and one millicurie of ^{125}I was added to the tube and mixed. Chloramine-T was added (25 μl of a 1 mg/ml solution), and this solution was mixed and allowed to react for 2 minutes at room temperature. Sodium metabisulfite (50 μl of 2.4 mg/ml in 0.05 M PBS) was then added to quench the reaction. In order to separate labeled protein from free ^{125}I , this solution was gently layered on top of a gel surface (G-75 Sephadex column) and allowed to run into the column. The column was then continuously washed with 1% BSA in PBS until 15-20 1-ml fractions were collected. Aliquots of 5 μl were counted. The first radioactive peak was selected as labeled hormone, and the second peak was discarded as free ^{125}I .

The fractions making up the peak of iodinated hormone were frozen, and the one with the greatest count was utilized in the radioimmunoassay. The "cold standards" (NIDDK-rLH-RP-3 and NIDDK-rFSH-RP-2) were reconstituted with 1 ml of distilled water to yield a solution of 5 $\mu\text{g/ml}$ in 1% BSA. The reference protein was aliquoted at 25- μl volumes and frozen for later use in assays. The first antibodies for LH and FSH (NIDDK-anti-rLH-S11 and NIDDK-anti-rFSH-S-11) were reconstituted in 1 ml of distilled water, aliquoted and stored frozen. Final dilutions of all NIDDK reagents were conducted just prior to use in assays.

Radioimmunoassay Procedure. All NIDDK reactants were added to RIA tubes in one sitting, at refrigerator temperature, in the sequence: a) buffer, b) "cold standard" or unknown, c) radioiodinated hormone (NIDDK-rLH-I-9 or NIDDK-rFSH-I-8), d) antiserum (1:750,000 dilution for anti-LH and 1:125,000 dilution for anti-FSH). The reactants were then incubated at room temperature for 24 hours. The "second" antibody (goat anti-rabbit IgG, Sigma # R-5506) was added after this 24-hour incubation and allowed to incubate for another 24 hours. The following morning 0.5 ml of 25% polyethylene glycol was added to each tube, then tubes were vortexed and allowed to incubate for 15 minutes. All tubes were centrifuged at 1000 x g. The supernatants were aspirated and the pellets counted in a Beckman Crystal Plus gamma counter. Specific binding was obtained by subtracting non-specific binding (tubes pipetted with tracer, buffer, and second antibody, omitting first antibody) from total binding. Standards and unknowns were calculated as a percent of specific binding based on their competitive reduction of that binding. Unknowns were assayed in duplicate and calculated from a weighted logit linear regression treatment of the standard curve. Standard curves were prepared in triplicate and covered a range of 7.8-500 ng/tube. Unknowns were assayed in duplicate and diluted so as to be read near the middle of the standard curve. This allowed the use of the more linear portion of the curve (25-250 ng/tube). The ED_{50} for LH was approximately 123 ng/tube, and the ED_{50} for FSH was approximately 115 ng/tube. Specific binding was calculated to be 30% and 45% for FSH and LH, respectively. The coefficient of variance between replicates did not exceed 15%.

Trial-and-Error Component. Difficulties were encountered in establishing workable standard curves. Iodinations were conducted at the Albany Medical Center core facility. Initial iodinations of gonadotropins resulted in low specific binding of FSH to its antibody (14%) and no binding of LH to its antibody. It was felt that the low and absent binding was due to harsh iodination conditions that possibly destroyed or significantly altered the tertiary structure of the gonadotropin molecules. Both gonadotropins had extremely high specific activities. New strategies were developed to reduce the specific activity of the gonadotropins and to increase the specific binding of gonadotropin to its respective antibody. It was decided that iodinations would be carried out utilizing iodogen compound, which would result in milder conditions that would conserve the integrity of the molecules. This procedure was conducted with both gonadotropins on two additional occasions. Lower specific activities were achieved; however, specific binding ranged between 2%-10%.

At this time we began to suspect that our problem was with the first antibody concentrations. In order to test this hypothesis, binding curves utilizing ten different levels of first antibody were set up, and specific binding was examined utilizing the hormone iodinated with iodogen. These curves also yielded low specific binding. We then obtained an anti-ovine LH antibody that had successfully been utilized in a rat LH assay set up at the New York State Department of Health. Again, ten LH binding curves were assayed in an attempt to discover a titre that would yield between 30%-40% specific binding. This attempt was also unsuccessful.

At this time we decided to go back to our original plan for iodination and obtain all new reagents from the NIDDK. Iodinations of the NIDDK-provided antibody were conducted utilizing lower concentrations of chloramine-T (approximately 10 $\mu\text{g}/1 \mu\text{g}$ of antigen). Binding curves were set up using four different dilutions in an attempt to establish the appropriate antibody titre. Finally, adequate specific binding was obtained using the NIDDK antibodies at a slightly lower final tube dilution and with antigen iodinated with the chloramine-T concentrations suggested by the NIDDK. Specific binding for LH was 45% and 30% for FSH. We have applied this assay to assess gonadotropin levels in serum samples from tumor-xenograft-bearing mice receiving AFP.

Estradiol was quantitated in a competitive RIA using tubes coated with rabbit antibody to estradiol (Coat-A-Count, Diagnostic Products Corp., Los Angeles, CA). ^{125}I -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 ^{125}I -E₂ (1.0 ml) were added to a tube, and the tubes were incubated for 3 hours 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.

Female SCID mice bearing MCF-7 breast cancer xenografts were treated daily for 30 days with 100 μg of AFP. As previously shown in Figure 1, this treatment completely prevented tumor growth. Blood samples were taken from these mice at the end of the 30-day treatment interval. As shown in Table 6,

Table 6

Treatment	FSH (ng/ml)	LH (ng/ml)	E ₂ (pg/ml)
No E ₂ ; No AFP	127 ± 34	214 ± 88	56 ±
Si/E ₂ ; No AFP	89 ± 25	210 ± 76	100 ± 5
Si/E ₂ ; AFP 100 µg mouse/day	119 ± 22	203 ± 69	187 ± 8

supplementation of mice with Silastic estradiol (Si/E₂) implants increased the serum level of E₂ and decreased the level of FSH. This is consistent with an E₂ feedback mechanism on the hypothalamic pituitary axis down-regulating the output of FSH. AFP treatment prevented this decrease in FSH and further increased serum E₂ levels. This is consistent with an inhibition by AFP of the estrogen-dependent feedback response on FSH secretion at the hypothalamic pituitary axis. There seemed to be no effect of either E₂ supplementation or AFP treatment on serum LH levels. We want to repeat these studies in male mice. Also, we want to look at changes in gonadotropin and androgen levels in male mice growing androgen-dependent LNCaP prostate cancer xenografts. At the time of this writing, it would appear that increases in serum gonadotropin and estrogen levels are intermediate markers which indicate that AFP is having its intended inhibitory effect on estrogen-dependent responses *in vivo*.

b. Breast-cancer-associated antigen assay

In our original proposal we postulated that a decrease in serum levels of tumor-associated antigen would be an intermediate marker indicating that AFP is having a direct inhibitory effect on the tumor. We reasoned that if AFP were stopping tumor growth, it would be slowing down the metabolic activity of the tumor, which should translate into less shedding of tumor-associated antigen. In our original proposal we indicated that we would measure serum levels of CA 15-3 in tumor-xenograft-bearing mice, since this tumor-associated antigen has been found to be elevated in serum of patients with breast cancer (42). However, upon further study of this issue, we have learned that CA 27.29 is more sensitive than CA 15-3 for detecting tumor presence (43). CA 27.29 is measured by a competitive inhibition radioimmuno-assay using the Truquant BR RIA kit purchased from Biomira Inc. (Canada). This is a solid phase RIA in which polystyrene tubes coated with CA 27.29 antigen are incubated with standards, normal serum controls or serum samples from tumor-bearing mice, then with ¹²⁵I-labeled BR 27.29 monoclonal antibody for 3 hours at room temperature. Tubes are washed twice with distilled water, and bound radioactivity in the tubes is measured in the gamma counter. Several serum samples were obtained from tumor xenograft-bearing mice at various stages of tumor development. In all cases we were not able to detect CA27.29 in these samples.

Continued Development of Truncated Forms of AFP

AFP is a glycoprotein consisting of 590 amino acids. Based on its structural homology to albumin, it has been divided into three domains (I, II, III) with each domain having three subdomains (A, B, C) (Fig. 16) (44). Through our collaboration with others and through our own work, we have determined that the active site of AFP is in a small portion of Domain III B of the molecule. This was an arduous, stepwise task. Our collaborators from Japan provided us

with Domain I of the molecule, and it was inactive in our estrogen-dependent growth assays. Our collaborators in Montreal provided us with Domain II-III of the molecule, and it was active in our estrogen-dependent growth assays. We produced Domain III in a baculovirus system and found that it was active in our estrogen-dependent growth assays. Our experience with Domain III has recently been published (45). We then produced a portion of Domain III (Domain III AB, approximately 100 amino acids) in a baculovirus expression system and found that it was active in our estrogen-dependent growth assays. All of this work localized the active site to the Domain III AB section of the AFP molecule. However, a problem with all of these recombinant molecules is their low yield, approximately 100 µg of protein per 3 x 10⁸ SF-9 cells. While we were in the process of making Subdomains III A and III B, one of our extramural collaborators, Dr. Mizejewski, reported on an active 34-mer peptide (amino acids 447-480) in Domain III B of AFP which inhibited estrogen-stimulated mouse uterine growth (Fig. 16) (46). We have obtained this peptide from Dr. Mizejewski and have tested it in our estrogen-dependent uterine growth assay. We have had variable results with this peptide. In our first experiment there was 37% growth inhibition, which compared favorably with Hep G-2 derived AFP (44% inhibition). In the second experiment there was only 18% growth inhibition, which was not significant in our statistical analysis (Table 7). One of the problems with the peptide is that it tends to aggregate in

Table 7
Antiestrotrophic Activity of AFP-derived Peptides

Test Agent	% INHIBITION OF E ₂ STIMULATED GROWTH OF IMMATURE MOUSE UTERUS	
	No urea	Urea
Mizejewski synthesis of peptide 447 (fresh)	39 *	N.D.
Mizejewski, peptide 447 (stored for 1 year)	18	41 *
Bennett synthesis of peptide 447 (fresh)	22	30 *
Bennett, peptide 447-1, substitution of alanines for cysteines (fresh)	15	29 *
Bennett, peptide 447-2, substitution of aspartates for cysteines 455 & 468 and aspartate for isoleucine 463 (fresh)	28 *	44 *
Mizejewski, scrambled peptide 447 (fresh)	0	2
Mizejewski, analogous albumin peptide (amino acids 440-473)	2	3
Bennett (447-456, Ala for Cys 455; fresh)	3	0
Bennett (457-466; fresh)	1	4
Bennett (467-480; Ala for Cys 468; fresh)	33 *	42 *
Bennett (472-479, fresh)	36 *	43 *
Bennett (472-478, fresh)	9	13

Bioassay was carried out as described in Table 2. Growth inhibition obtained with the optimal dose of peptide, 1,000 ng, is reported. Vehicle alone with or without urea (final concentration of urea in injectant was 100 mM) was not inhibitory. This urea concentration is well below the renal physiological level of mammalian systems.

* Significant inhibition ($p < 0.05$) compared to estrogen-stimulated mice not pretreated with peptide. Wilcoxon Sum of Ranks Test. There were 5 replicate mice per group.

solution, which inactivates its function. These microaggregates have been detected by using molecular sieving columns and by mass spectroscopy. Aggregation increases proportional to the concentration and time of peptide in solution. We are currently working with Dr. Mizejewski, trying to resolve this problem. Dr. Mizejewski has synthesized and tested another peptide (amino acids 511-560) in Domain III C of AFP, and we have synthesized and tested other peptides (amino acids 434-444 and 428-444) in Domain III A of AFP, and none of these peptides has demonstrated activity by inhibiting estrogen-stimulated growth of immature mouse uterus. The two very significant advantages of using a peptide such as AFP 447-480 are that it does not require incubation with lipophilic substances to convert it to its active form and it can be produced in large quantity with substantially less effort required of recombinant systems.

Recently, we have synthesized more hydrophilic forms of peptide 447 as well as truncated forms of this peptide. Aggregation during storage has remained a problem for maintaining activity. Incubation of stored peptides in 1 M urea for one hour has dissociated the aggregates and restored activity (Table 7). Interestingly, an 8-mer, peptide 472-479, has full activity, appears to be the smallest amino acid sequence that maintains activity, and has retained its activity up to three months in storage (Table 7, Fig. 16). Even though there are other modifications (cyclization, amino acid substitution, peptidomimetic, ...) that could still be made on this peptide, at the present time, this 8-mer is our lead compound, and we are trying to secure funding that would support the testing of this agent against human breast cancer xenografts as well as explore its mechanism of action.

CONCLUSIONS AND FUTURE WORK

Conclusions

1. The site of AFP that is active in blocking estrogen-dependent growth is localized in the third domain of the molecule and appears to be contained in an 8-mer amino acid sequence found in domain III B. This peptide needs to be tested against breast cancer xenografts.
2. Increasing the dose of full-length AFP (10 μ g or above) exempts the protein from the ligand-induced activation requirement and simplifies its use for therapeutics. At this higher dose, full-length natural human AFP stopped the growth of estrogen-dependent breast cancers and androgen-dependent prostate cancer. It did not affect the growth of estrogen-independent breast, ovarian and endometrial cancer and androgen-independent prostate cancer.
3. Positivity for sex steroid hormone receptors appears to be a marker of tumor sensitivity to the growth-inhibitory effects of AFP.
4. Elevations in serum FSH and E_2 appear to be intermediate markers of AFP's ability to interfere with estrogen-dependent responses *in vivo*.
5. The growth-inhibitory effects of AFP are reflected in a histomorphometric profile of cytostasis whereby cells pile up in the G_0G_1 phase of the cell cycle and cell renewal is repressed.

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