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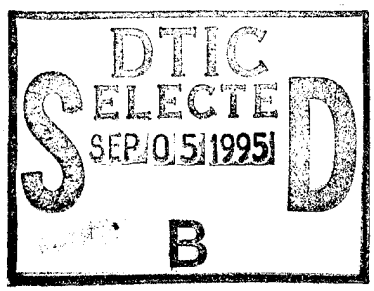
GRANT NO: DAMD17-94-J-4173

TITLE: Stromal Influences on Breast Cancer Progression

PRINCIPAL INVESTIGATOR(S): Sandra W. McLeskey, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057



REPORT DATE: July 14, 1995

TYPE OF REPORT: Annual

PREPARED FOR: Commander
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

Form Approved
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1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	7/14/95	Annual 15 Jun 94 - 14 Jun 95

4. TITLE AND SUBTITLE
Stromal Components of Breast Cancer Progression

5. FUNDING NUMBERS
DAMD17-94-J-4173

6. AUTHOR(S)
Sandra W. McLeskey, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20057

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Material Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Metastatic estrogen receptor positive breast carcinoma may be treatable with tamoxifen, an antiestrogen, but the tumor may subsequently become refractory to treatment, growing in the absence of estrogenic stimulation. We have developed a model of breast cancer progression by transfecting MCF-7 breast carcinoma cells, which are estrogen-dependent for growth in ovariectomized nude mice, with cDNAs for FGF-1 or FGF-4. The transfected cell lines are able to form tumors in ovariectomized nude mice. Since FGFs are angiogenic factors, this project investigates the importance of angiogenesis in the phenotypic transition of the transfected cells by looking for differences in the angiogenesis in tumors produced by the parental MCF-7 or the FGF-transfected cells. Our model is validated by a positive correlation of tumor microvessel density and tumor size. We have defined temporal and spatial events in the process of tumor-induced angiogenesis by identifing sprouting or proliferating endothelial cells. We have identified patterns of angiogenesis which are associated with regressing parental cell tumors and growing FGF-transfected cell tumors. We have begun to isolate endothelial cell populations from parental or FGF-transfected cell tumors to study FGF receptor gene expression. Future work will study gene expression in endothelial cells with a combination of *in vivo* and *in vitro* methods.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast cancer, MCF-7 cells, fibroblast growth factors, angiogenesis, endothelial cells	57
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unlimited	Unlimited	Unlimited	Unclassified

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INTRODUCTION

When initially diagnosed, human breast cancer may be estrogen receptor positive and amenable to hormonal therapy with the antiestrogen, tamoxifen. However, eventually such tumors may become refractory to tamoxifen treatment, progressing to an invasive, metastatic, phenotype that is essentially untreatable (1). We have developed a model tumor system that mimics some aspects of breast cancer progression to a tamoxifen-resistant, more metastatic state, by transfection of FGF-4 into estrogen-dependent MCF-7 cells. However, the dramatic change to a progressively growing, antiestrogen-resistant and metastatic phenotype produced by this transfection *in vivo* is not parallelled by a similar change in the *in vitro* phenotype of the transfected cells. They remain responsive to estrogen and inhibited by tamoxifen *in vitro* (2-5). Although growth requirements *in vitro* and *in vivo* may be somewhat different, this discrepancy between *in vitro* and *in vivo* behavior of these cells points to stromal factors as mediators of the tumorigenic, antiestrogen-resistant, metastatic phenotype in this system. Since FGFs are angiogenic growth factors, increased angiogenesis produced by the transfected cells may be responsible for this change in tumor phenotype. This project attempts to validate this system as a model of tumor angiogenesis and to seek to identify endothelial cell responses to FGF-4 which are important in mediating the tumorigenic, metastatic phenotype of these cells. This report covers work done in the first year of the project.

BODY

Three hypotheses were set forth in the original proposal. Each of these will be repeated in this report, followed by the results of work done to date. Originally, we had proposed to utilize FGF-4 transfected MCF-7 cells as our model. Since the proposal was written, FGF-1 transfected MCF-7 cells have become available (4). Since FGF-1 is more consistently expressed in human breast carcinoma (6) we have expanded our studies to utilize this cell line in parallel with the FGF-4 transfectants.

Hypothesis #1. We will validate the FGF-4 transfection of MCF-7 cells as a model of tumor angiogenesis by asking whether new blood vessel formation is positively correlated with tumor size and/or metastasis.

In the original proposal, immunohistochemistry with an antibody to von Willebrand factor was used to highlight blood vessels in tumors produced by FGF-4 transfected MCF-7 cells. Since that time, a rat monoclonal antibody to murine PECAM has become available (Pharmingen). PECAM is the mouse homolog of human CD31, an endothelial cell adhesion molecule which is considered very specific for endothelial cells (7). Consequently, we have switched our method of highlighting blood vessels in these mouse tumors to immunohistochemistry with this new PECAM antibody. We had proposed to assess microvessel density in tumors produced by FGF transfected or parental cells by counting the number of blood vessels highlighted with PECAM immunohistochemistry per 100X field and to correlate the blood vessel score with tumor size. The initial series of specimens selected for analysis were from two experiments in which mice bearing tumors produced by FGF-4 or FGF-1 transfected MCF-7 cells were treated with the antiestrogen tamoxifen, the angiogenesis inhibitor AGM-1470, or pentosan polysulfate, a drug which seems to

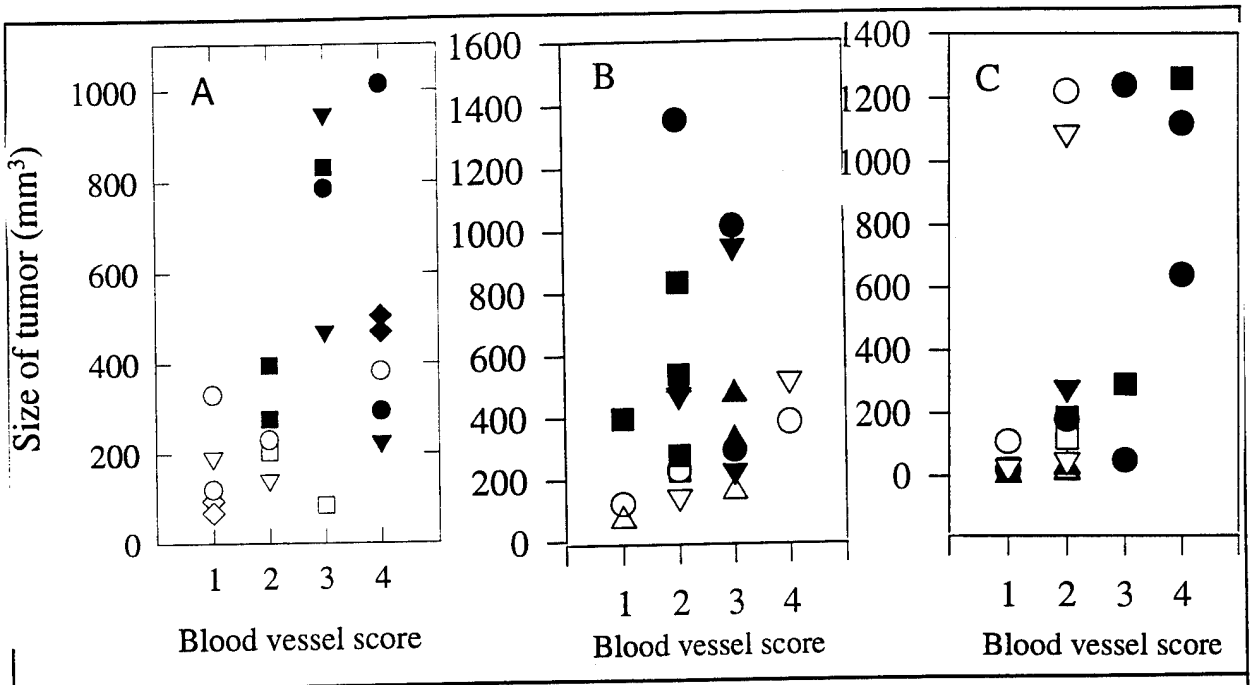

Figure 1. Semiquantitative scoring of microvessel density vs tumor size. Blood vessel scores of tumors produced by injection of FGF-4 transfected MCF-7 cells in relation to their size. Tumors were scored by a blinded observer by examination of sections stained with the rat monoclonal antibody to PECAM. Microvessel density was rated from 1 (few microvessels) to 4 (many microvessels). Solid symbols denote tamoxifen-treated animals. Circles represent control animals. Squares are AGM-1470-treated animals, diamonds are PPS-treated animals, and triangles represent animals treated with both PPS and AGM-1470.

abrogate the effects of FGFs, or combinations of these drugs. The experiment utilizing FGF-4 transfected MCF-7 cells was given as part of the preliminary data for the original proposal. The specimens were fixed in 10% buffered formalin for an undetermined time and embedded and sectioned by a commercial pathology laboratory. Because of variable results from staining of these specimens, presumably because of overfixation, we were not able to confidently count microvessels. Therefore, we gave each specimen a semiquantitative score, from 1 to 4, with 4 being the highest number of microvessels per 100X field, and 1 being the lowest. The experiment using FGF-4 transfected MCF-7 cells was evaluated twice, at two different times, with two different batches of antibody. In Figure 1, microvessel score is plotted against tumor size for these two experiments. Figures 1A and 1B represent two separate evaluations of one experiment using FGF-4 transfected MKL-4 cells. The results depicted in Figure 1C represent an evaluation of an experiment utilizing FGF-1 transfected α-10 cells. The data in Figure 1A and 1C shows a positive correlation of blood vessel score with tumor size. The data depicted in Figure 1B, although it came from tumor sections from the same tumors as used for Figure 1A, does not show a significant correlation between tumor size and blood vessel score.

Thus, we have obtained variable results in our efforts to correlate blood vessel density with tumor size, probably related to technical problems with the use of archival slides. However, our data do seem to support such a relationship. We now have developed a method for fixation and embedding of specimens which produces specimens which stain for PECAM immunoreactivity reliably. Future experiments to meet this goal will utilize this method, so this goal should be easily achievable.

Hypothesis #2. We will investigate the importance of an angiogenic growth factor in the spatial and temporal events of angiogenesis by asking the question: Does constitutive secretion of an angiogenic growth factor by tumor cells change the temporal and spatial pattern of angiogenesis as measured by endothelial cell proliferation or the presence of a marker for immature blood vessels?

We proposed to demonstrate proliferating endothelium in tumors produced by parental MCF-7 cells or FGF-4 - transfected cells by administering bromodeoxyuridine (BrdU) to mice previously injected with tumor cells, sacrificing the mice and subjecting the tumors to immunohistochemical detection of BrdU. Immature blood vessels were to be detected in serial tumor sections by *in situ* hybridization with *ets-1*, a transcription factor expressed only in immature blood vessels. This was to be done for very early time points after injection of cells and at subsequent time points until a small tumor has formed.

This goal has been addressed by injecting parental or FGF-4 - transfected cells into the mammary fat pads of ovariectomized nude mice at two sites, and sacrificing the mice at 3-5 day intervals from the first day following tumor cell injection to the 40th day. Eight hours before sacrifice, the mice are injected intraperitoneally with 1 mg BrdU in 0.1 ml PBS. At the last time points, the parental cell tumors are undergoing regression and are visible only by because the tumor cells express the bacterial *lacZ* gene and can be visualized when the tumor sites are harvested and placed in the chromogenic substrate, X-gal. We have developed our own system for X-gal staining, fixation, embedding, and sectioning, which gives reliable PECAM immunoreactivity in endothelial cells in these sections. As above, blood vessels are highlighted with immunohistochemistry utilizing the rat monoclonal PECAM antibody. Cells which have incoroporated BrdU are revealed with immunohistochemistry utilizing a rat monoclonal antibody for BrdU (Accurate Antibodies). Because of the difficulty in correlating individual cells between two serial sections of the tumor, a double staining technique has been developed in which the sections are first stained for PECAM utilizing a biotin-avidin complex system coupled to peroxidase. A brown substrate for peroxidase, diaminobenzidine, is used to disclose the PECAM-positive endothelial cells. Following that staining procedure, the sections are stained for BrdU utilizing a second biotin-avidin complex system coupled to alkaline phosphatase. A red substrate for alkaline phosphatase, Vector red, is used to disclose the BrdU labeled cells. This technique gives reliable brown-stained blood vessels and cherry-red nuclear staining for BrdU. We include a piece of skin in all sections which gives us skin blood vessels as positive controls. Because the same secondary antibody and a biotin-avidin complex system is used for both reactions, we occasionally get cross-reactivity in which the PECAM-stained cells are reddish-brown. Since the PECAM staining is localized to membrane and the BrdU staining is nuclear, this does not really present a problem in differentiating PECAM-positive cells from BrdU

Figure 2. FGF-transfected MCF-7 cells produce tumors with more cellular stroma than parental cells. Hematoxylin and eosin staining of tumors produced by injection of (A) parental MCF-7 (ML-20) or (B) FGF-4 transfected cells (MKL-F) harvested 5 days following injection. The stromal reaction produced by MKL-F cells as depicted in B is more cellular than that produced by ML-20 cells as depicted in A. (Both MKL-F and ML-20 cells express ß-galactosidase. The blue-staining tumor cells in A were stained with X-gal prior to sectioning.)

Figure 3. Difference between neovascularization produced by parental and FGF-transfected cells. Brown -staining PECAM immunohistochemistry is seen in a tumor produced by parental cells (A) or by FGF-4 transfected MCF-7 cells (B) 10 days after injection. Cherry-red nuclear staining indicates immunoreactivity to BrdU.

Figure 4. Endothelial cell double-stained for PECAM and BrdU immunoreactivity. Brown cytoplasmic staining is PECAM immunoreactivity, cherry-red nuclear staining is BrdU immunoreactivity.

positive cells. However, in a particular case, if there were a question of interpretation because of cross-reactivity, we could stain a serial section with only one of the antibodies and easily resolve the question.

Although these initial studies were intended to identify endothelial cells in nascent tumors so that we could study their gene expression, several interesting findings have resulted. First, tumors produced by the FGF-transfected cells have a more cellular stroma than those produced by the parental cells. This is not surprising, since FGFs are known mitogens for both fibroblasts and endothelial cells. Figure 2 shows a comparison between the two tumor types 3 days after injection. Second, beginning at about 5 days after tumor cell injection, new microvessels begin to appear in the tumors. New blood vessels are evident in tumors produced by FGF transfectants, but parental cells also seem to be able to stimulate neovascularization. However, the morphology of the new blood vessels is somewhat different in tumors produced by FGF-transfected cells when compared with parental cells. Figure 3 shows representative sections of PECAM stained tumors. In this figure, we see that the parental cells stimulate neovascularization that seems to be mostly on the periphery of the tumor and composed of large capillaries filled with blood cells. In contrast, the tumor produced by FGF-transfected cells seems to contain smaller vessels, many of which are inside the tumor, and do not contain visible blood cells. Our pathology consultant is in the process of analyzing these sections in detail.

Double PECAM - BrdU staining of sections of tumors produced in this experiment reveals many endothelial cells which are positive for BrdU incorporation. Figure 4 shows an examples of such a cell. Our pathology consultant is in the process of quantifying and localizing BrdU positive endothelial cells from these specimens. Image analysis may be used to perform this task.

To disclose immature endothelial cells, we proposed to do *in situ* hybridization with a probe for *ets-1*, a transcription factor preferentially expressed in immature endothelial cells. We have obtained a riboprobe vector from Dr. Dennis Watson at Medical College of South Carolina which has been used for *in situ* hybridization for *ets-1* (8). However, in the meantime, an antibody for

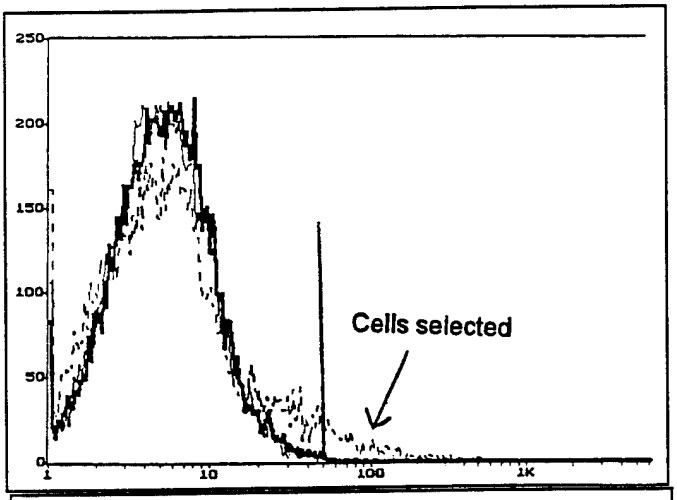

Figure 5. Isolation of tumor-derived endothelial cells. FACS sort of PECAM-expressing cells obtained by dispersion of a tumor produced by injection of FGF-4 transfected MCF-7 cells. —— No primary antibody; ▬▬ PECAM antibody but no secondary antibody; ---- PECAM primary antibody and fluorescein-coupled secondary antibody. Cells were selected which were brighter than 50 on the scale of relative fluorescence.

ETS-1 became available (Santa Cruz) which purportedly can be used in immunohistochemistry. Since demonstration of protein expression is a more stringent way of demonstrating expression of a particular gene, we have attempted to utilize this antibody for immunohistochemistry in our sections. However, despite a number of attempts utilizing a variety of conditions, we have been unable to obtain specific nuclear staining with this antibody. We plan to abandon our efforts to do immunohistochemistry with this antibody and continue with our plans to do *in situ* hybridization using the riboprobe vector which is known to work.

In summary, this goal is well underway and has revealed some interesting morphologic differences between parental and transfected cells. Once the pathologic analysis of the sections is complete, we will analyze the data and prepare to publish these findings.

Hypothesis #3. We will test the hypothesis that interactions between tumor and stroma produce changes in gene expression and growth responses within the tumor.

We proposed to study FGF receptor gene expression in endothelial cells in two ways. First, we proposed to utilize *in situ* hybridization with probes for specific FGF receptors to study endothelial cell gene expression *in vivo*. Second, we proposed to isolate endothelial cells from tumors and study endothelial cell FGF receptor gene expression by RT-PCR. Growth responses of isolated endothelial cells were to be studied in an *in vitro* growth assay utlizing a method which measures invasion of a matrix by endothelial cells previously differentiated into tubular structures.

For this goal in this year, we have concentrated on trying to isolate endothelial cells from tumors. In the original proposal, we put forth a method employing size and density fractionation. However, since then, the rat monoclonal antibody to murine PECAM mentioned in above has become available. Consequently, we attempted to isolate endothelial cells with fluorescence activated cell sorting, using this antibody and a secondary antirat antibody coupled to fluorescein isothiocyanate. Figure 5 shows histograms for one such sort in which we obtained cells positive for LDL uptake (Figure 6). Although more tests are necessary before we can say that the cells we obtained by this method are definitely endothelial cells, we feel very encouraged by this finding. Unfortunately, we obtained very few cells from this procedure, possibly as a result of overzealous digestion of the tumor in an attempt to obtain a single-cell suspension. Our plans for this goal are

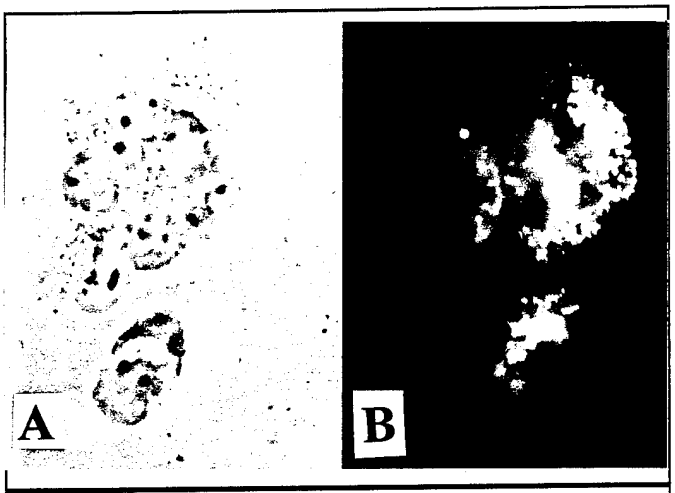

Figure 6. (A) Phase-contrast micrograph of cells obtained by PECAM-FACS (400X). (B) Fluorescence micrograph of the same cells treated with acetylated LDL coupled to 1,1'-dioctadecyl-1-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Biomedical Technologies, Inc.). Mouse fibroblasts treated in the same way exhibited no fluorescence.

to optomize digestion of the tumor so as to preserve the PECAM epitope on the endothelial cells as well as cell viability. In addition, there is a newly published procedure (9) in which the PECAM antibody is immobilized on magnetic beads, a cell suspension is poured over it so that endothelial cells are retained. Cells isolated in this way stably express endothelial cell markers over several passages. We will be attempting this procedure to isolate tumor-derived endothelial cells in the near future.

CONCLUSION

We have made significant progress in each section of this proposal, particularly in hypotheses #1 and #2. The only person who received pay from this grant was Sandra McLeskey, PhD. There have been no publications to date. Portions of the work were presented as a poster at the recent Gordon Conference on wound healing, July 2-7, in New London, NH. The results of our early studies will be presented as a manuscript to be prepared in late 1995. We also plan a presentation at the annual meeting of the American Association of Cancer Research in Washington, DC, in May, 1996.

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APPENDIX

Br. J. Cancer

EFFECTS OF AGM-1470 AND PENTOSAN POLYSULFATE ON TUMORIGENICITY AND METASTASIS OF FGF-TRANSFECTED MCF-7 CELLS

Sandra W. McLeskey, Lurong Zhang, Samir Kharbanda, Yiliang Liu, Bruce J. Trock, Marco M.Gottardis, Marc E. Lippman, and Francis G. Kern

Lombardi Cancer Center (S.W.M., L.Z., S.K., Y.L., B.J.T., M.E.L., F.G.K.), Department of Biochemistry (F.G.K., L.Z.), Department of Pharmacology (S.W.M., M.E.L.), Department of Medicine (B.J.T., M.E.L.), School of Nursing (S.W.M.), Georgetown University Medical Center, Washington, D.C. 20007, and Ligand Pharmaceuticals, Inc. (M.M.G.), La Jolla, CA 92037

SUMMARY: We have previously transfected MCF-7 breast carcinoma cells, which are estrogen-dependent for growth in ovariectomized nude mice, with one of two angiogenic growth factors, FGF-1 or FGF-4. These transfections have resulted in cell lines which are tumorigenic and metastatic in untreated or tamoxifen-treated ovariectomized nude mice. We assessed the effects of AGM-1470, an antiangiogenic agent, and pentosan polysulfate (PPS), an agent which binds FGFs, on tumor growth and metastasis produced by FGF-1 or FGF-4 transfected MCF-7 cells. Otherwise untreated or tamoxifen-treated ovariectomized mice were injected with FGF-transfected cells, treated with AGM-1470 or PPS, and tumor growth and metastasis analyzed. Sensitivity of FGF-transfected and parental MCF-7 cells to AGM-1470 or PPS was also determined *in vitro*. Both AGM-1470 and PPS inhibited tumor growth in otherwise untreated or tamoxifen-treated mice injected with either FGF-1 or FGF-4 transfected MCF-7 cells. This

effect was more reliably seen in tamoxifen-treated animals. AGM-1470 was about 10^5 times less potent in inhibiting the anchorage-dependent growth of parental MCF-7 or FGF-transfected MCF-7 cells than in inhibiting the growth of human umbilical vein endothelial cells. PPS did not affect the *in vitro* growth of the transfectants or parental cells. Thus, the growth-inhibitory effect on tumors was in excess of the effect of either drug on the same cells in tissue culture, implying that stromal elements, possibly influenced by paracrine signalling, are important determinants of the effects of these drugs. Neither drug had a significant effect on metastasis beyond what would be expected from their effect on tumor size. AGM-1470 or PPS may be helpful in cases of breast carcinoma in which angiogenesis is due to expression of FGFs by the tumor cells and may be more effective when combined with tamoxifen. (286 words)

RUNNING TITLE: FGF-transfected tumors growth-inhibited by AGM-1470 and PPS

KEY WORDS: AGM-1470, Angiogenesis, FGF, Pentosan polysulfate, MCF-7 cells, breast cancer

INTRODUCTION

The acquisition of the ability to promote neovascularization has been described as a seminal event in tumorigenicity, enabling uncontrolled growth, invasion, and metastasis of a previously indolent lesion (Folkman *et al.*, 1989; Folkman & Shing, 1992; Weinstat-Saslow & Steeg, 1994). We have previously transfected MCF-7 breast carcinoma cells with expression vectors for one of two angiogenic growth factors, FGF-4 (McLeskey *et al.*, 1993) or FGF-1 (Zhang *et al.*, 1994). These transfections have produced cell lines that are tumorigenic and metastatic in ovariectomized and tamoxifen-treated athymic nude mice. This behavior is in direct contrast to parental MCF-7 cells, which require estrogen supplementation for formation of small, poorly metastatic tumors in ovariectomized nude mice (Soule & McGrath, 1980; Osborne, Hobbs & Clark, 1985). The change in *in vivo* phenotype produced by these transfections may mimic the transition that occurs in estrogen receptor positive human breast carcinomas which are initially responsive to antiestrogen therapy. After prolonged antiestrogen therapy, such carcinomas may become refractory to the antiestrogen, and acquire a more invasive and metastatic phenotype, ultimately leading to the death of the patient.

Although the acquisition of angiogenic ability may be multifactorial in a given tumor and may involve different mechanisms in different tumors, there is evidence that FGFs or FGF receptors may be involved in some transitions of tumors to an angiogenic phenotype. Expression of FGFs has been associated with a switch to an angiogenic phenotype in fibrosarcomas (Kandel *et al.*, 1991), and is prominent in melanomas (Halaban, 1993) and in brain tumors which are very vascular (Brem *et al.*, 1992). Several investigators have shown specific FGF receptors to be

newly expressed at a time of phenotypic transition of tumors from indolent to aggressive or metastatic (Yan *et al.*, 1993; Yamaguchi *et al.*, 1994; Luqmani *et al.*, 1995). We have found FGF-1 or FGF-2 mRNA to be expressed in many breast carcinoma specimens (Ding *et al.*, 1992) and FGF ligands to be preferentially expressed by estrogen receptor negative breast carcinoma cell lines (Flamm *et al.*, 1989). Amplified and/or overexpressed FGF receptors have been identified in breast carcinoma specimens, implying that FGF signalling contributes to the phenotype of these tumors (Adnane *et al.*, 1991).

Since the transfection of FGFs into MCF-7 cells, an estrogen-dependent, poorly tumorigenic, and relatively nonmetastatic breast carcinoma cell line, has produced cell lines which cause aggressive, metastasizing tumors in the absence of estrogenic growth stimulation, it seemed important to test the hypothesis that this phenotypic change is the result of increased angiogenesis. Therefore, we have treated ovariectomized mice injected with either FGF-1 or FGF-4 transfected MCF-7 cells with AGM-1470 (a.k.a. TNP 470), a drug purported to be antiangiogenic (Ingber *et al.*, 1990; Kusaka *et al.*, 1991; Yamamoto, Sudo & Fujita, 1994; O'Reilly, Brem & Folkman, 1995), or pentosan polysulfate (PPS), a drug which binds FGFs, as well as other heparin-binding growth factors (Belford, Hendry & Parish, 1993), and which under some circumstances inhibits the effects of FGFs (Wellstein *et al.*, 1991; Zugmaier, Lippman & Wellstein, 1992). We now report that both agents were growth-inhibitory to tumors produced by FGF-transfected cells in both ovariectomized and tamoxifen-treated mice. These effects were in excess of the *in vitro* effects of these agents on the transfected and parental cells. In spite of the negative effect of each drug on tumor growth and contrary to published reports of an inhibitory

effect of AGM-1470 on metastasis in other systems (Yanase *et al.*, 1993; Yamaoka *et al.*, 1993; Brem *et al.*, 1993; Mori *et al.*, 1995; Kato *et al.*, 1994), neither drug had a detectable inhibitory effect on metastasis in this system beyond that which could be predicted by the decrease in tumor size produced by the drug.

METHODS

Cell lines. MKL-4 cells are MCF-7 cells sequentially transfected with expression vectors for FGF-4 and *lacZ* as described (McLeskey *et al.*, 1993; Kurebayashi *et al.*, 1993). α-21 and α-10 cells are clonal G-418 resistant cell lines isolated from a transfection of ML-20 cells [MCF-7 cells first transfected with an expression vector for *lacZ* (Kurebayashi *et al.*, 1993)] with an expression vector encoding amino acids 21-154 of FGF-1(Burgess *et al.*, 1986; Burgess & Maciag, 1989), and further characterized as producing high levels of the transfected protein and forming tumors in nude mice (Zhang *et al.*, 1994). MCF-7 cells were approximately passage 60.

Drugs. Pentosan polysulfate (PPS) was obtained from beneChemie GmbH, München, FRG. AGM-1740 (a.k.a. TNP 470) was kindly supplied by Katsuichi Sudo, Takeda Chemical Industries, Osaka, Japan. Tamoxifen pellets were obtained from Innovative Research, Inc., Toledo, OH.

Cell culture and injection of mice. Cells were maintained in improved minimal essential medium (IMEM) supplemented with 5% fetal bovine serum (FBS) in a 5% CO_2, 37° incubator. The day of injection, cells were scraped into their normal growth medium and viable cells were counted using trypan blue exclusion. Ten million viable cells were injected into the upper right mammary fat pad of each mouse in an injection volume of 0.15 ml. This number of injected cells was used to produce 100% tumor take (McLeskey *et al.*, 1993) and is consistent with the numbers of cells injected by others (Haran *et al.*, 1994). Mice were randomized into groups of 5 and sustained-release tamoxifen pellets (Innovative Research, Toledo, OH), were

implanted in the interscapular area as described (McLeskey *et al.*, 1993) for half of the groups. Drug treatments were begun the following day. PPS was injected intraperitoneally at a dose of 5 mg/kg in 0.1 ml phosphate-buffered saline (PBS) 6 days/week. AGM-1470 was injected subcutaneously at a dose of 30 mg/kg in 0.1 ml 30% ethanol in PBS three times per week. The control group received subcutaneous injections of 30% ethanol in PBS. Tumors were measured in three dimensions twice weekly with calipers. Tumor volume was calculated as the product of the largest dimension, the orthogonal measurement, and tumor thickness. For some experiments, dissected tumors were weighed at the time of sacrifice.

Statistical analysis of tumor growth. Only mice which survived until the end of the experiment were included in the statistical analysis. (Three mice expired of unknown causes, not related to tumor burden, during the course of the four experiments.) Mean tumor volume for each treatment group was obtained using the calculated volumes of each tumor, with zeros being used as the volume when no tumor arose in an animal.

Because the data included tumor volume measurements at multiple time points for each animal, and variances differed between treatment groups and over time, repeated measures analysis of variance (RMANOVA) was used to analyse the data (Heitjan, Manni & Santen, 1993). This analysis is considerably more powerful than analyses which compare tumor growth at a single time point. Lack of normality was an inconsistent finding and logarithmic transformations did not improve model fit, so the untransformed data were used for all analyses described here.

The effects of PPS and AGM-1470 were evaluated, singly and in combination, in both

untreated and tamoxifen-treated mice. A 2^3 factorial design was used, resulting in 8 treatment groups, with tamoxifen and each study drug occurring in four of the treatment groups: 1. untreated, 2. AGM-1470 alone, 3. PPS alone, 4. AGM-1470 + PPS, 5. Tamoxifen alone, 6. Tamoxifen + AGM-1470, 7. Tamoxifen + PPS, 8. Tamoxifen + AGM-1470 + PPS. The analyses were conducted in two ways. The first analysis examined the impact of each drug on tumor volume at all time points, and evaluated the evidence for interactions (synergism or antagonism) between drugs. For each drug, the analysis evaluated whether treatment groups including the drug had smaller mean tumor volumes than treatment groups without the drug, and also whether the magnitude of a drug's effect varied according to the presence or absence of the other drugs. The second analysis considered the 8 groups above as distinct treatment groups and compared mean tumor volume between pairs of treatment groups at successive time points. Because it evaluates differences between pairs of treatment groups, each of which uses only five mice, this second analysis has somewhat lower statistical power than the previous analysis. Mathematically, these two analyses are equivalent, but they provide different perspectives of the data.

Because the tumor volume measurements for tumors produced by FGF-1 transfectants included the volume produced by the "bloody sac" (Zhang *et al.*, 1994) surrounding the tumors for some tumors, especially at early time points, we weighed the harvested tumors from two of the experiments utilizing these FGF-1 transfectants. These data were not normally distributed, even upon logarithmic transformation. Consequently, a one-way analysis of variance (ANOVA) was used on the ranks of the mean tumor weights for each treatment group. This nonparametric

test of significance at one time point does not have the power of the RMANOVA conducted over multiple time points on the tumor volume data described above, and thus may fail to detect a difference between treatment groups when one in fact exists (Type I error).

***In vitro* growth curves.** Ten thousand MCF-7 and FGF-transfected MCF-7 cells/well were plated in IMEM with 5% FBS in 24 well plates and allowed to attach overnight. Treatments as indicated in a final volume of 1 ml were added on the following day (day 0). Untreated wells received ethanol vehicle of AGM-1470 (0.1%). Cells were harvested with 0.1 mM EDTA in PBS on appropriate days and counted using a Coulter automated cell counter. Human umbilical vein endothelial cells (HUVEC) were plated in 24 well plates at 10,000 cells/well using their normal growth medium (IMEM supplemented with 10% FBS, 10 ng/ml FGF-1, and 10 µg/ml heparin). Drug treatments were added the day following plating. Cells were harvested as above and counted using a hemocytometer.

Detection and rating of metastases. Metastases in proximal axillary and distal axillary and inguinal lymph nodes and selected whole organs (brain, kidneys, liver, spleen, lungs, and heart) were harvested, fixed in 2% formaldehyde, 0.2% glyceraldehyde for 2-3 hours, and subjected to staining using X-gal (5–bromo–4–chloro–d–indoyl–ß–D– galactopyranoside) (1 mg/ml X-gal in 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM magnesium chloride in PBS) overnight at 4°. Organs were examined under a dissecting microscope and rated for the presence of blue-staining metastases as described (Kurebayashi *et al.*, 1993). Metastases were rated according to the following rating system: 0 - No visible blue spots. 1+ - Few diffuse blue spots (less than about 5) or one microscopic focus of a few blue spots. 2+ -

Diffuse blue spots (about 5-15) or several foci of a few blue spots. 3+ - Many diffuse blue spots (about 15-50) or a barely visible macroscopic focus of blue. 4+ - Very many blue spots (more than about 50) or a large macroscopic focus of blue (Kurebayashi *et al.*, 1993).

We used analysis of covariance to evaluate the drug effects on the metastasis score for proximal, distant, lung, and total metastases (the latter was the sum of scores for the former) while adjusting for the effects of tumor volume at the final time point. In addition, we converted the score to a binary variable for metastases present (yes vs. no), or low vs. high metastases score (0,1 vs. 2,3,4). Logistic regression was used to evaluate this binary outcome, again, adjusting for the effect of final tumor volume. Because of the small number of animals per treatment group, we included only the effects of single drugs vs. control in the models (*e.g.*, main effect).

RESULTS

Tumors produced by FGF-1 - or FGF-4 - transfected MCF-7 cells in nude mice are growth-inhibited by treatment with pentosan polysulfate or AGM-1470. We treated ovariectomized mice injected with FGF-transfected MCF-7 cells with pentosan polysulfate (PPS), an agent which is capable of abrogating the effects of FGFs and other heparin-binding growth factors *in vitro* and *in vivo* (Wellstein *et al.*, 1991; Zugmaier, Lippman & Wellstein, 1992). This agent presumably acts by binding to FGFs (Belford, Hendry & Parish, 1993), preventing them from reaching their receptors on tumor or stromal cells. PPS would therefore be expected to abrogate both the autocrine and paracrine effects of the transfected FGFs, reverting the cells back toward their parental phenotype. Since FGFs are known angiogenic factors, we also examined the angiogenic component of the tumorigenic phenotype of the transfectants by treating ovariectomized mice injected with FGF transfected cells with AGM-1470, an agent with preferential toxicity for endothelial cells (Kusaka *et al.*, 1994) which is an inhibitor of angiogenesis in many assays (Ingber *et al.*, 1990; Kusaka *et al.*, 1991; Yamamoto, Sudo & Fujita, 1994). Since we have previously shown that tamoxifen treatment of mice injected with FGF-4 - transfected MCF-7 cells stimulates tumor growth (McLeskey *et al.*, 1993), we also tested the effects of PPS or AGM-1470 on tumor growth of FGF-transfectants in tamoxifen-treated animals. Our rationale was that abrogation of the effect (either angiogenesis alone with AGM-1470 or all autocrine and paracrine effects with PPS) responsible for the change in phenotype of the transfectants would return them to their parental phenotype of being growth-inhibited by

tamoxifen treatment.

The FGF-1 transfected MCF-7 cell lines we chose to use in these experiments are transfected with an expression vector encoding FGF-$1_{21\text{-}154}$, a biologically active form of FGF-1 that lacks the first 21 amino acids (Burgess *et al.*, 1986; Burgess & Maciag, 1989; Forough *et al.*, 1993). Although this species lacks a signal peptide sequence, FGF-1 is present in media conditioned by the transfectants. These cells are tumorigenic and obviously have an effect on stromal cells, as evidenced by the sac filled with bloody fluid surrounding the tumor in many animals (Zhang *et al.*, 1994).

Tumor growth curves from four experiments, one with FGF-4 transfected cells and three with FGF-1 transfected cells, are depicted in Figure 1, A-D. We first analyzed the drug effect for each drug at all time points and in all treatment groups for each experiment (Table I), using tumor volume as the dependent variable. We also compared pairs of treatment groups for the effect of an added drug on tumor volume. This pairwise comparison was carried out for all time points for each treatment pair for each experiment. The results of the pairwise comparison for the final time point of each experiment are presented in Table II. P-values for all the time points for each comparison are similar to those given in Table II.

The overall effect of PPS was to reduce tumor volume in mice transfected with each of the cell lines (Figure 1, A-D). Table I shows that this effect of PPS was statistically significant in three experiments (Figure 1, A, B, and D) out of four. In the fourth experiment (Figure 1C), the overall effect of PPS was marginally significant (p=.079). In the pairwise comparison, the effect of PPS was most pronounced among tamoxifen-treated animals. These pairwise comparisons

show that reductions in tumor volume due to PPS were larger and of greater statistical significance among tamoxifen-treated mice than in otherwise untreated mice (Table II).

In contrast, although AGM-1470 decreased mean tumor volume among each of the transfectants (Figure 1, A-D), the overall effects were statistically significant only in one experiment involving FGF-1 transfectants (Figure 1B), although the result for the FGF-4 transfectants (Figure 1A) was near the conventional significance level of $p<.05$ ($p=.064$, Table I). For each transfectant, the magnitude of the reduction is smaller than that obtained with PPS. As with PPS, pairwise comparison of AGM-1470 treatment groups showed the effect of AGM-1470 was greater among tamoxifen-treated animals, with 3 out of 4 experiments showing statistically significant reduction in mean tumor volume with AGM-1470 treatment, compared to none of the comparisons among untreated groups (Table II).

Since PPS presumably reduces FGF-mediated effects in a dose-dependent manner, we could therefore hypothesize that at some dose, PPS would abrogate the effects of FGFs completely, returning the transfectants to their parental phenotype of being growth-inhibited by tamoxifen. At the dose used in these experiments, animals treated with PPS and tamoxifen and injected with FGF-4 transfected cells had a larger mean tumor volume than animals treated with PPS alone (Figure 1A). However, the stimulatory effect of tamoxifen was not as large as that observed in otherwise untreated animals. In contrast, animals injected with the FGF-1 transfected cells had very small or no tumors in the PPS-treated group, and the addition of tamoxifen did not increase tumor growth or incidence (Figure 1, B-D). Therefore, for the FGF-1 transfectants, the stimulatory effect of tamoxifen was not evident in PPS-treated animals. In fact,

in one experiment involving FGF-1 transfectants, PPS was significantly antagonistic to the stimulatory effect of tamoxifen (p=.006) (Fig. 1B). Giving a combination of AGM and PPS did not increase the growth inhibitory effect of PPS alone in either otherwise untreated or tamoxifen-treated animals in any cell lines tested (data not shown).

Since PPS inhibited tumor growth to a greater degree than AGM-1470, and since AGM-1470 is thought to be an angiogenesis inhibitor, these data suggest that the increase in tumorigenicity observed in FGF-transfected MCF-7 cells when compared with parental MCF-7 cells is not solely due to FGF-mediated angiogenesis. However, it is also possible that at the dose used, AGM-1470 did not inhibit angiogenesis completely. In fact, several of the AGM-1470-treated animals from the three experiments with FGF-1 transfectants had sacs filled with bloody fluid surrounding their tumors. Only one of the PPS-treated animals from three experiments injected with FGF-1 transfectants had a sac filled with bloody fluid surrounding its tumor. Although we do not know the origin of this sac and have no indication that it represents angiogenesis, its presence could be interpreted as evidence that FGF-1 mediated effects on stromal tissue was more completely inhibited in these animals by PPS treatment. The sac in this one animal arose quite late in the experiment, and the sudden rise in volume of the tumor lesion due to the sac formation in this one animal is responsible for the sudden increase in mean lesion volume of the PPS treatment group depicted in Figure 1C. When the animals were sacrificed, the tumor inside this sac was found to be quite small (Fig. 1C, inset).

The presence of the "bloody sac" in some animals but not others confounds the measurement of tumor volume, since it is possible that the volume of the bloody sac surrounding the

tumor is larger than the tumor inside, as exemplified above. For that reason, at the time of tumor harvest in two experiments, we weighed tumors produced by FGF-1 transfected cells. These data are graphically depicted in the insets for Fig. 1, C and D. Since these data were not normally distributed even upon logarithmic transformation, a one-way ANOVA on the ranks of the mean tumor weights for each treatment group was used to test for significant differences between treatment groups. As mentioned, the use of this nonparametric test at one time point does not have the power of the RMANOVA used to analyze the repeated measurements of mean tumor volume above (Heitjan, Manni & Santen, 1993). In this analysis of effects of drug treatment on tumor weight, there were no significant differences between treatment groups in the experiment depicted in 1C. For the experiment depicted in 1D, only tamoxifen produced an overall significant effect on tumor weight ($p=0.032$). Pairwise comparisons of treatment groups in this experiment showed that the addition of PPS to tamoxifen treatment produced a significantly lower mean tumor weight when compared with tamoxifen alone ($p=0.008$). For the experiment depicted in Figure 1C, ANOVA done on the ranks of the mean tumor weights for each treatment group showed no significant drug effect ($p=0.277$). Therefore, no further comparison of treatment groups was appropriate. Thus, although the statistical analysis of data produced by tumor weight measurements was not as powerful as the RMANOVA, it did confirm the significant overall effect of tamoxifen detected by the RMANOVA, as well as the significance of the pairwise comparison of tamoxifen alone vs tamoxifen plus PPS, in the experiment depicted in Figure 1D. Analysis of tumor weight measurements failed to detect an overall effect of PPS which was detected by the RMANOVA, probably because of the wide variability in tumor

weights (insets of Fig. 1C and 1D) and lack of power of the nonparametric test.

AGM-1470 and PPS have little effect on FGF transfectants or parental MCF-7 cells in tissue culture. Growth requirements may differ substantially between *in vitro* and *in vivo* environments, since many tumor cells are immortal in tissue culture but are not tumorigenic in animals. However, we felt that it was important to test the effects of AGM-1470 and PPS on the FGF transfectants in tissue culture in order to establish the presence of any directly toxic effects of either drug on the transfected cells.

In anchorage-dependent growth assays, AGM-1470 has been shown to have a cytostatic effect on endothelial cells with an EC_{50} of about 10 pg/ml (Kusaka *et al.*, 1994). The batch of AGM-1470 used in these *in vivo* experiments was tested on human umbilical vein endothelial cells (HUVEC) and found to inhibit their growth with approximately the same potency as has been published (Kusaka *et al.*, 1994) (data not shown). In anchorage-dependent growth assays using FGF-transfected or parental MCF-7 cells, AGM-1470 inhibited growth with an EC_{50} of approximately 10-30 μg/ml (Figure 2, A-D). PPS from the same batch as was used in *in vivo* experiments at maximal concentrations of 100 μg/ml had no effect on FGF-transfected, parental MCF-7, or HUVEC growth (data not shown). Thus, the inhibitory effect of AGM-1470 or PPS on tumorigenicity *in vivo* is probably not simply due to a nonspecific toxic effect on the growth of tumor cells and more likely involves one or more tumor or stromal cell parameter(s) important for *in vivo* growth.

AGM-1470 or PPS treatment does not affect metastasis of FGF-transfected MCF-7 cells. As described (McLeskey *et al.*, 1993; Kurebayashi *et al.*, 1993; Zhang *et al.*, 1994), FGF-

transfected MCF-7 cells are reliably metastatic, primarily to proximal and distal lymph nodes and lungs. In one investigation, the incidence of metastases in FGF-4 transfected cells was correlated with size of the tumor, with tumors greater than 100 mm^3 having 100% incidence of metastasis to the proximal lymph node. These metastases are detected by X-gal staining for ß-galactosidase activity of the *lacZ* transfected cells. Thus, microscopic metastases can be detected as well as macroscopic (Kurebayashi *et al.*, 1993). Since angiogenesis has been thought to be an important determinant of metastasis (Weinstat-Saslow & Steeg, 1994), it is possible that the increased incidence of metastasis observed with FGF-transfected MCF-7 cells is due to the increased angiogenesis in the primary tumor or metastatic focus produced by the transfected FGF. To test the hypothesis that decreasing the angiogenic or other effects of the transfected FGF would decrease the incidence of metastasis, we examined proximal axillary and distal axillary and inguinal lymph nodes, and selected organs (lungs, liver, brain, kidneys, spleen, and heart) using X-gal detection to disclose the presence of blue-staining cancer cells expressing ß-galactosidase. Because the incidence of metastasis in FGF-transfected cells had previously been correlated with tumor size (Kurebayashi *et al.*, 1993), we wanted to know if tumors large enough that they would be expected to metastasize failed to do so, or if tumors so small that they would not be expected to metastasize, produced metastasis. To visualize the results of this analysis, we used a rating scale for the extent of metastasis in a given organ (Kurebayashi *et al.*, 1993) and plotted tumor volume at the end of the experiment with relation to the extent of metastasis (Figure 3, A-F). Data from the experiments depicted in Figure 1A and Figure 1B from proximal and distal lymph node metastases and pulmonary micrometastases are presented, since these were sites most

reliably involved. Because of lower than expected rate of metastases in control groups in the experiments depicted in Figure 1C and Figure 1D. data from these experiments was not analyzed. The analyses were conducted separately for each cell line. None of the drug treatment groups were shown to have a significant effect on metastases after adjusting for tumor volume, in either analysis of covariance or logistic regression models (data not shown). We also compared extent of metastases between the drug groups without adjusting for tumor volume to rule out the possibility that the correlation between volume and drug was distorting the drug effect in the models. These analyses also showed no significant drug effect on metastases (data not shown).

False negative error in detecting metastasis is very likely, since the X-gal stain only penetrates the organ a few millimeters and internal metastases remain undetected. Another source of false negative error for the FGF-4 transfectants (Figure 1A) is that only about 30% of these cells were blue-staining *in vitro* before injection (McLeskey *et al.*, 1995). False positive error in metastasis detection is not as likely, as reaction conditions minimize the ability of endogenous ß-gal activity to produce blue color, and visual inspection of the metastases under magnification leads to rejection of nonspecific blue staining. Thus, we feel that the presence of metastases in AGM-1470 or PPS treated animals is an indication that these drug treatments as administered in this study did not have a significant inhibitory effect on metastasis.

DISCUSSION

We have demonstrated a growth-inhibitory effect of AGM-1470 and PPS on tumors produced by FGF-transfected MCF-7 cells in ovariectomized and tamoxifen-treated nude mice. These effects were present in four separate experiments utilizing three different FGF-transfected cell lines. Although the statistical significance of the drug effects was not uniform over all four experiments, it seems clear that PPS is growth-inhibitory for these tumors in most circumstances and AGM-1470 is growth-inhibitory for these tumors at least under conditions of tamoxifen treatment (Tables I and II). Neither agent was able to abrogate tumor growth completely in any experiment with the exception of the combination of PPS and tamoxifen in one experiment involving an FGF-1 transfected cell line (Figure 1B). Since we only used one dose of each agent, it might be argued that the dose used was insufficient to completely abrogate the effects of the transfected FGF.

Although PPS is believed to act by binding to FGFs, it also binds many other heparin-binding growth factors (Zugmaier, Lippman & Wellstein, 1992). Moreover, PPS had no effect on *in vitro* growth of the transfectants or the parental cells. Thus, the effects of PPS in our experiments may be due to effects of PPS on heparin-binding growth factors which may be produced by the transfected or parental cells and which may have paracrine effects on stromal cells. However, since the transfected FGF is the factor responsible for the increased the tumorigenicity of these cells (McLeskey *et al.*, 1993; Zhang *et al.*, 1994), we must conclude that the activity of PPS on the transfected FGF is at least one of the factors responsible for the reduced tumor growth in PPS-treated animals. When used in tamoxifen-treated animals, the

inhibitory effect of PPS on tumor growth was more often significant over the four experiments (Table II). These data are evidence for the activity of the transfected FGF in promoting the tamoxifen stimulation of tumor growth in these transfectants, but also suggest that tamoxifen may be influencing some other factor which is stimulatory for tumor growth in this model and which is also affected by PPS.

We felt that the FGF-1 transfected MCF-7 cells, in particular, might be an ideal cell line in which to test the effects of an antiangiogenic drug such as AGM-1470. These cells form a sac filled with bloody fluid around the tumor which initially is much larger than the tumor itself (Zhang *et al.*, 1994). If this sac is the product of excessive angiogenesis, giving an antiangiogenic drug might inhibit its formation. Neither AGM-1470 nor PPS completely prevented the formation of the sac, although its appearance was limited to only one animal and much delayed in PPS-treated animals (Figure 1C).

We have shown the FGF-transfected and parental MCF-7 cells to be much less sensitive to the *in vitro* growth-inhibitory effects of AGM-1470 cells than endothelial cells (Figure 2). In addition, the potency of our batch of AGM-1470 in inhibiting *in vitro* endothelial cell growth agrees with published reports (Kusaka *et al.*, 1994) (data not shown). When pharmacologic doses of AGM-1470 are administered to rats, plasma concentrations are below 1 μg/ml except for very short periods after subcutaneous or bolus intravenous injection (personal communication, K. Sudo). Since this is below the concentration required for growth inhibition of the tumor cells *in vitro*, it is tempting to ascribe the *in vivo* growth-inhibition by AGM-1470 of tumors produced by FGF-transfected cells to its preferential toxicity for endothelial cells and resultant

inhibition of angiogenesis. However, the drug apparently is taken up into many types of cells, and neither the active species nor the site of action for AGM-1470 has been determined, making it difficult to know the concentration of the drug at its site of action. Therefore, although specific inhibition of angiogenesis may indeed be the mechanism whereby AGM-1470 inhibits tumor growth, its general toxicity for tumor or other cells cannot be excluded as a mediator of the inhibition of tumor growth observed in this study.

AGM-1470 significantly inhibited tumor growth more frequently in tamoxifen-treated animals than in otherwise untreated ones (Table II). If AGM-1470 is indeed an antiangiogenic drug, then the question is raised as to whether the effects of tamoxifen in stimulating tumor growth are due to a stimulation of angiogenesis which is additive to that of the transfected FGF. This would be an heretofore undescribed effect of tamoxifen and in fact, there are some reports of antiangiogenic effects of tamoxifen (Gagliardi & Collins, 1993; Haran *et al.*, 1994). If such an proangiogenic effect of tamoxifen exists, it could be due to a direct effect of tamoxifen upon endothelial cells, an effect of tamoxifen in increasing FGF production by the tumor cells or an effect of tamoxifen in increasing production of another angiogenic growth factor which can act in synergy with the FGF. We do not find estrogen or tamoxifen to affect expression of the transfected FGF-4 in MKL-4 cells (Miller *et al.*, 1994). TGF-ß has been shown to have a synergistic effect with FGF-2 in an *in vitro* assay of angiogenesis (Gajdusek, Luo & Mayberg, 1993). Although TGF-ß has been shown to inhibit the growth of both breast carcinoma and endothelial cells *in vitro* (Knabbe *et al.*, 1987; Barnard, Lyons & Moses, 1990; RayChaudhury & D'Amore, 1993), its effects *in vivo* are unclear (Welch, Fabra & Nakajima, 1990; Walker &

Dearing, 1992; Arteaga *et al.*, 1993; Dalal, Keown & Greenberg, 1993). Thus, tamoxifen-induced TGF-ß expression (Knabbe *et al.*, 1987; Butta *et al.*, 1992) in the tumor could be synergistic with the transfected FGF in stimulating angiogenesis *in vivo*. If so, we might expect that abrogating angiogenesis with AGM-1470 or abrogating the effect of heparin-binding growth factors (both the FGF and the TGF-ß) with PPS would inhibit growth of tumors produced by FGF transfectants in tamoxifen-treated animals more significantly than in otherwise untreated animals. Experiments are planned to investigate this possibility

The failure of both drugs to prevent metastasis in spite of their inhibitory effects on tumor growth is surprising in light of the previous correlation of the number of metastatic foci of FGF-4 transfectants with tumor size (Kurebayashi *et al.*, 1993) and in light of previous reports that AGM-1470 decreased metastasis (Yanase *et al.*, 1993; Yamaoka *et al.*, 1993; Brem *et al.*, 1993; Kato *et al.*, 1994; Mori *et al.*, 1995). We do not believe the metastases in our system are produced by seeding of distant organs at the time of tumor cell injection. The evidence to support this belief is the previously mentioned correlation of extent of metastasis with tumor size after injection of equal numbers of cells (Kurebayashi *et al.*, 1993) and the fact that in the past, we have been unable to detect blue-staining cells in the animals' distant organs between 2 and 10 days after tumor cell injection (data not shown). However, our failure to find an effect of drug on metastases must be interpreted with caution due to the small sample size. With only 5 animals per drug group and the need to incorporate three dummy variables into the models to parameterize the drug effects, the power to detect differences between groups was low. The discrepancy between our findings and those of others may also be due to experimental design,

since metastasis may also be studied by injecting tumor cells into the venous circulation (Yamaoka *et al.*, 1993; Mori *et al.*, 1995; Kato *et al.*, 1994) or by excising primary tumors from untreated animals and then beginning treatment during the period of presumed metastatic growth (Yamaoka *et al.*, 1993). In addition, other investigators have not taken the size of the primary tumor into consideration when evaluating the incidence of metastasis (Yanase *et al.*, 1993; Yamaoka *et al.*, 1993; Brem *et al.*, 1993). Because we have previously shown the correlation of tumor size with the number of metastatic foci, it would seem likely that decreasing tumor size by any means should decrease the likelihood of metastasis. It may be that a different dose-response relationship for these drugs applies to parameters affecting metastasis than applies to parameters affecting tumor growth in our system. This situation could pertain if the drugs were to act on each parameter through different mechanisms. Since we have only limited information on the mechanism of action of PPS and essentially no information on the mechanism of action of AGM-1470, it is difficult to comment on this possibility.

In conclusion, we have shown a growth-inhibitory effect of PPS and AGM-1470 on tumors produced by FGF transfected MCF-7 cells. These inhibitory effects confirm the importance of the transfected FGF for the tumorigenic phenotype of the transfectants and also suggest that increased angiogenesis is an important factor in this phenotype. Since FGF-1 has been shown to be expressed in human breast carcinomas (Ding *et al.*, 1992; Zhang *et al.*, 1994), it is possible that such therapeutic modalities might become important in the treatment of cases of human cancer where FGF or other heparin binding angiogenic growth factor production is a determinant of tumor growth. Because the effect of the drugs was more pronounced in

tamoxifen-treated animals, the use of these agents in combination with tamoxifen or in women whose cancer has become refractory to tamoxifen might offer additional benefit.

ACKNOWLEDGEMENTS

The authors would like to thank K. Sudo of Taneka Chemical Industries, Inc. for supplying the AGM-1470. A. Wright assisted with the animal experiments. D. El-Ashry thoughtfully critiqued this manuscript. This research was supported by NIH grants CA50376 and CA53185. L. Zhang is a Susan Komen Foundation post-doctoral fellow. Animal protocols for this work were approved by the Georgetown University Animal Care and Use Committee.

Table I: Comparison of drug effects over all treatments and time points. Statistical significance (p values) is given for overall effects of individual drugs on mean tumor volume in tumors produced by FGF-1 or FGF-4 transfected MCF-7 cells.

DRUG	FGF-TRANSFECTED CELLS INJECTED			
	FGF-4 (Fig.1A)	FGF-1 (Fig. 1B)	FGF-1 (Fig. 1C)	FGF-1 (Fig.1D)
TAM	.0007	.385	.595	.013
PPS	.014	.0007	.079	.0003
AGM	.064	.041	.505	.174

Table II: Pairwise comparisons between treatment groups. This is an example of the comparison between pairs of treatment groups showing the effect of PPS and AGM-1470 on mean tumor volume produced by injection of FGF transfected MCF-7 cells in otherwise untreated or tamoxifen treated mice, using the final time point of each experiment. *Mean tumor volumes in mm^3 ± standard error of the mean. Comparisons of other time points yielded similar p-values.

TREATMENT GROUPS COMPARED	FGF-TRANSFECTED CELLS INJECTED			
	FGF-4 (Fig.1A)	FGF-1 (Fig.1B)	FGF-1 (Fig.1C)	FGF-1 (Fig. 1D)
UNTREATED	370±113.7*	494±274.4	1533±805.0	1137±1006.5
PPS	117±14.9	24±14.6	642±623.6	248±152.6
p-value	.101	.034	.315	.390
UNTREATED	370±113.7	494±274.4	1533±805.0	1137±1006.5
AGM-1470	137±35.1	332±71.6	342±163.1	819±727.8
p-value	.146	.451	.182	.757
TAMOXIFEN ALONE	829±175.1	914±325.7	1365±821.6	4068±926.9
TAMOXIFEN + PPS	303±91.6	0±0.0	83±75.5	482±432.3
p-value	.002	.001	.151	.002
TAMOXIFEN ALONE	829±175.1	914±325.7	1365±821.6	4068±926.9
TAMOXIFEN+AGM-1470	421±128.8	157±75.0	792±597.8	2001±1119.9
p-value	.014	.003	.515	.052

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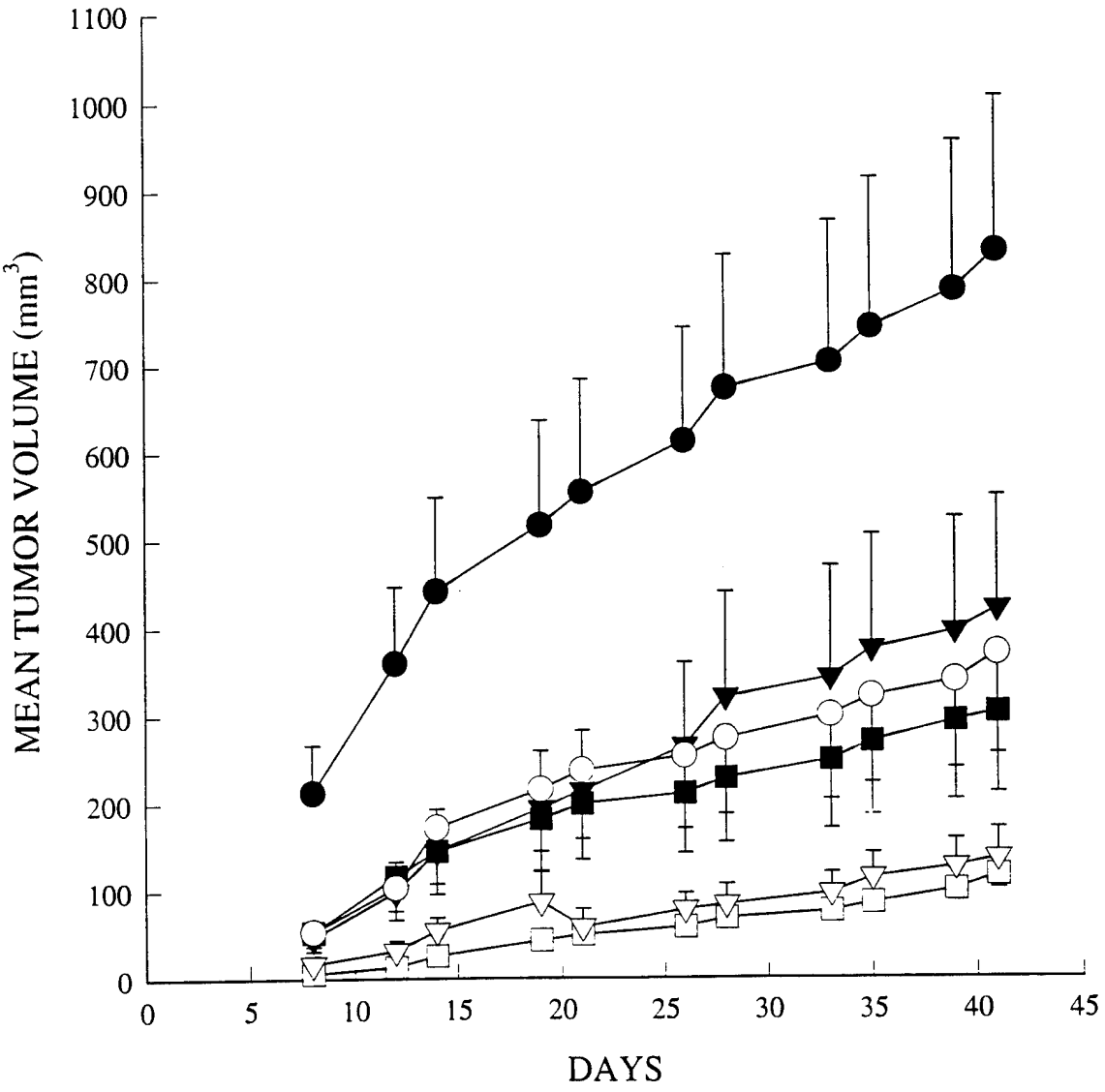
FIGURE LEGENDS

FIGURE 1. Effects of AGM-1470 and PPS on growth of tumors produced by FGF-transfected MCF-7 cells. Ovariectomized nude mice were injected as described with 10 million cells of the indicated cell line. Randomized groups of 5 mice each were treated with indicated drugs as described. ○-vehicle (30% ETOH in PBS), ●-tamoxifen, □-PPS, ■-PPS + tamoxifen, ▽-AGM-1470, ▼-AGM-1470 + tamoxifen. Error bars represent one standard error of the mean. **A.** Growth of tumors produced by FGF-4 transfected MCF-7 cells (MKL-4 cells (Kurebayashi *et al.*, 1993)). **B-D.** Three experiments depicting the growth of tumor lesions (sacs filled with bloody fluid containing the solid tumor or solid tumors without a sac) produced by two different clonal cell lines of FGF-1 transfected MCF-7 cells. **B.** Clonal cell line α-21. **C.** A second experiment with clonal cell line α-21. Inset: Postmortem tumor volumes from measurements of the dissected tumors after sacrifice. These measurements show that the sudden increase in volume of the lesions in PPS treated animals at day 50 represented growth of the sac filled with bloody fluid, not the tumors themselves. Symbols represent individual tumor volumes. Bars represent mean volumes. Error bars represent one standard error of the mean. T, tamoxifen; A, AGM-1470; P, PPS. **D.** Clonal cell line α-10. Inset depicts postmortem tumor weights as in 1C.

Figure 2. AGM-1470 and PPS have low potency for growth inhibition of parental or FGF transfected MCF-7 cells *in vitro*. Ten thousand cells per well were plated in IMEM plus 5% FBS in 24-well plates and allowed to attach overnight. Medium was changed to IMEM plus 5% FBS with indicated treatments on day 0. ○ - 0.1% ethanol, - 0.3 μg/ml AGM-1470, ▽ - 1 μg/ml AGM-1470, △- 3 μg/ml AGM-1470, □ - 10 μg/ml AGM-1470, ● - 30 μ/ml AGM-1470. **A.** FGF-1 transfected

cell line, α-10. **B.** FGF-1 transfected cell line, α-21. **C.** FGF-4 transfected cell line, MKL-4. **D.** Parental MCF-7 cells.

Figure 3. PPS and AGM-1470 do not prevent metastasis of FGF-transfected MCF-7 cells. Lymph nodes and lungs from animals injected with FGF-transfected MCF-7 cells were treated with X-gal to reveal blue-staining tumor cells. The extent of metastasis in each organ was rated on a scale of 0 (absent) to 4 (extensive) as described (Kurebayashi *et al.*, 1993). ○-vehicle (30% ETOH in PBS), ●-tamoxifen, □-PPS, ■-PPS + tamoxifen, ▽-AGM-1470, ▼-AGM-1470 + tamoxifen. **A-C** MKL-4 cells (FGF-4 transfectants). **D-F** α-21 cells (FGF-1 transfectants).**A, D** - proximal lymph nodes, **B, E** - distal lymph nodes, **C, F** - lungs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800
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10
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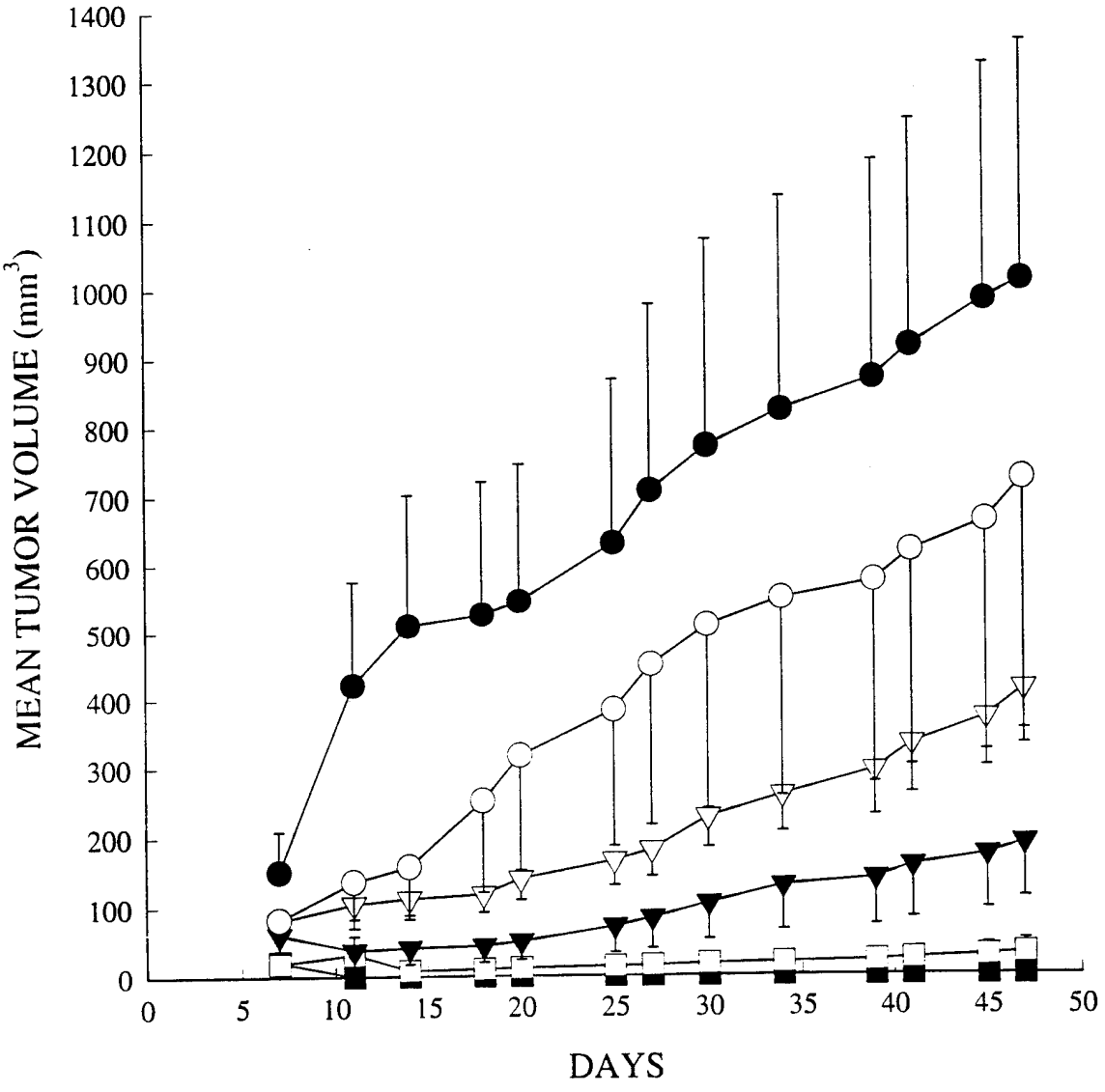
20
25
30
35
40
45
DAYS

Figure 1A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800
700
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45
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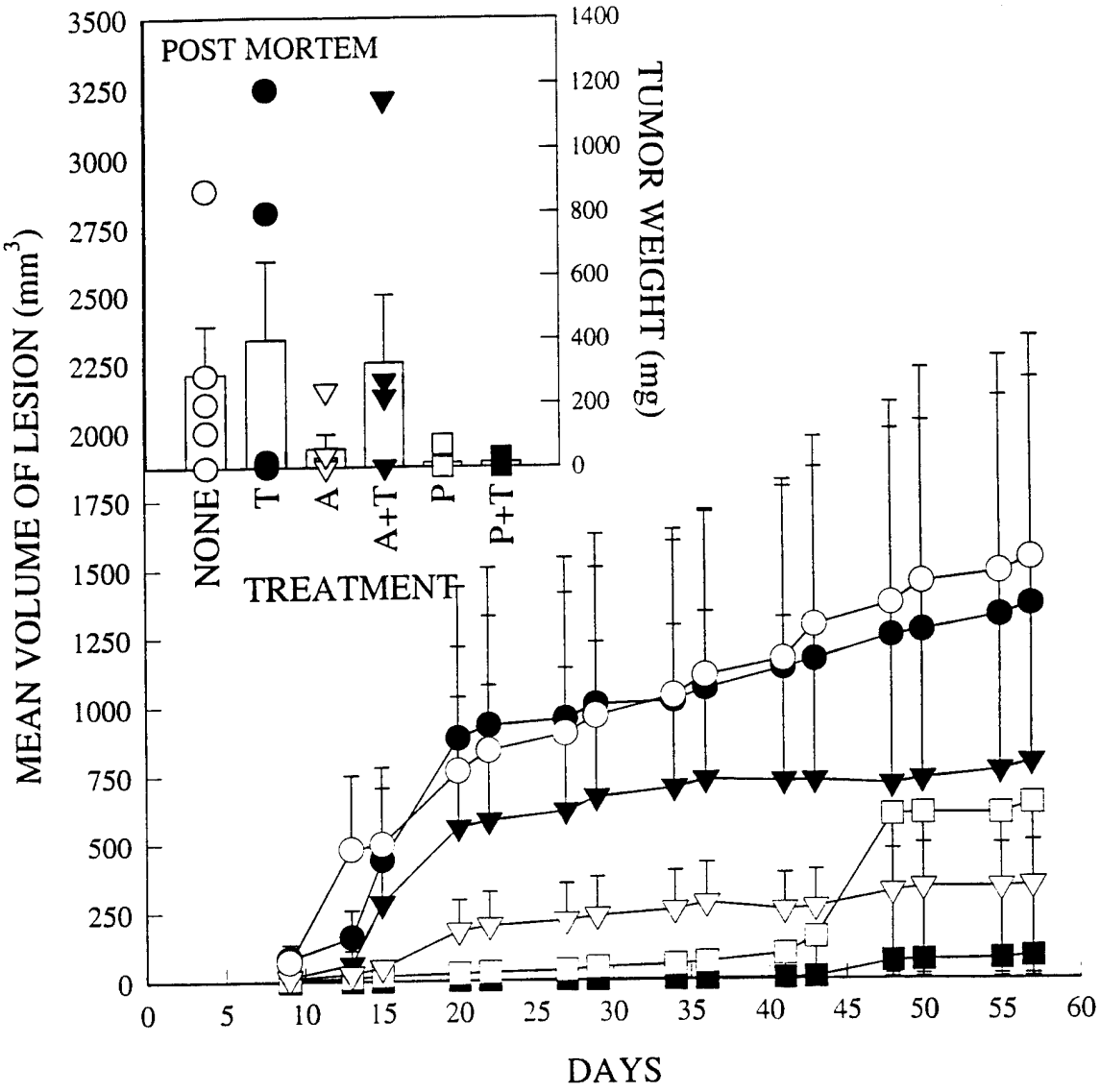
DAYS

Figure 1B


POST MORTEM
MEAN VOLUME OF LESION (mm3)
TUMOR WEIGHT (mg)
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A
A+T
P
P+T
TREATMENT
DAYS
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500
750
1000
1250
1500
1750
2000
2250
2500
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3000
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0
200
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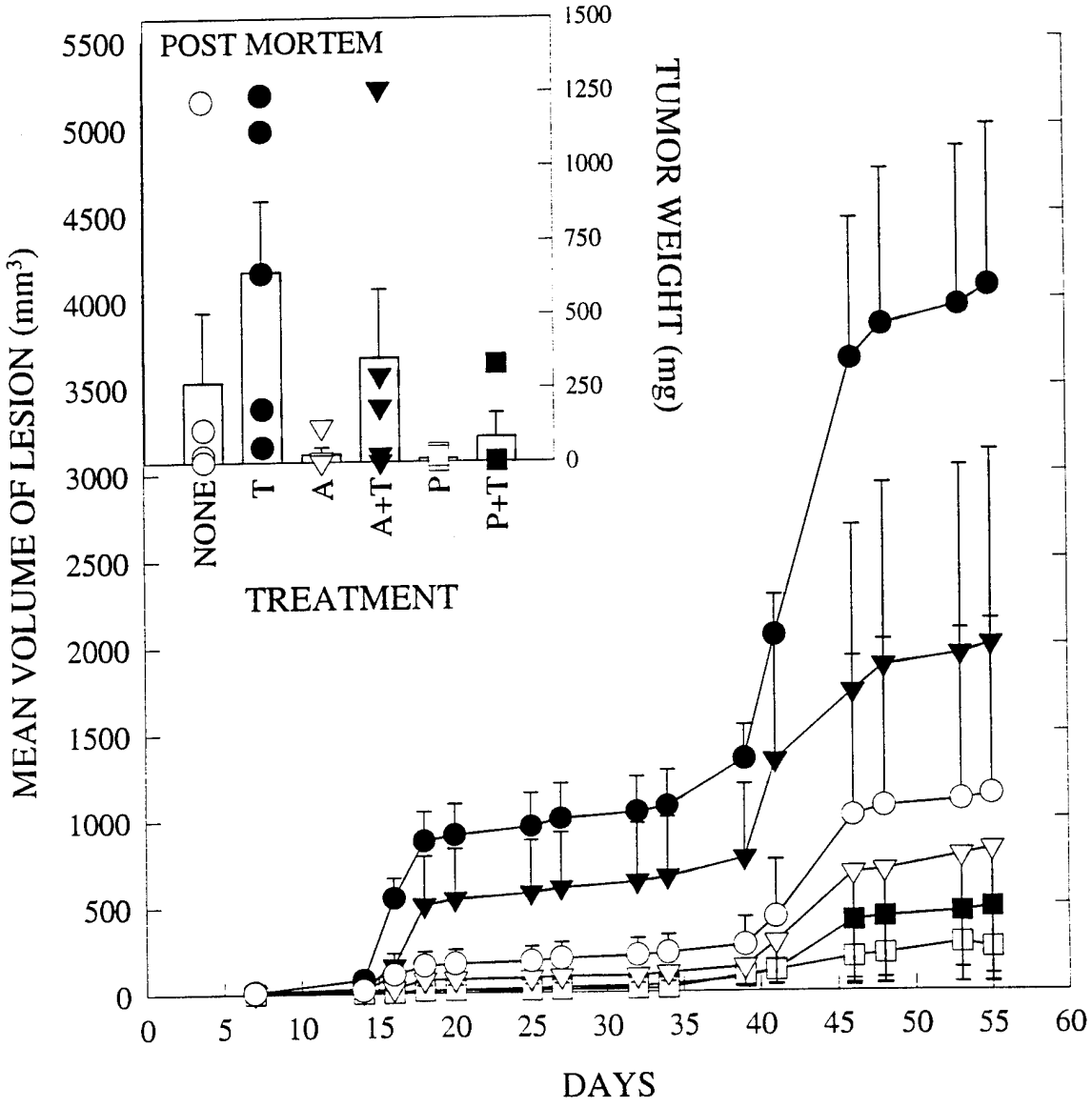
Figure 1C


POST MORTEM
MEAN VOLUME OF LESION (mm³)
TUMOR WEIGHT (mg)
TREATMENT
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A+T
P
P+T
DAYS
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4500
4000
3500
3000
2500
2000
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1000
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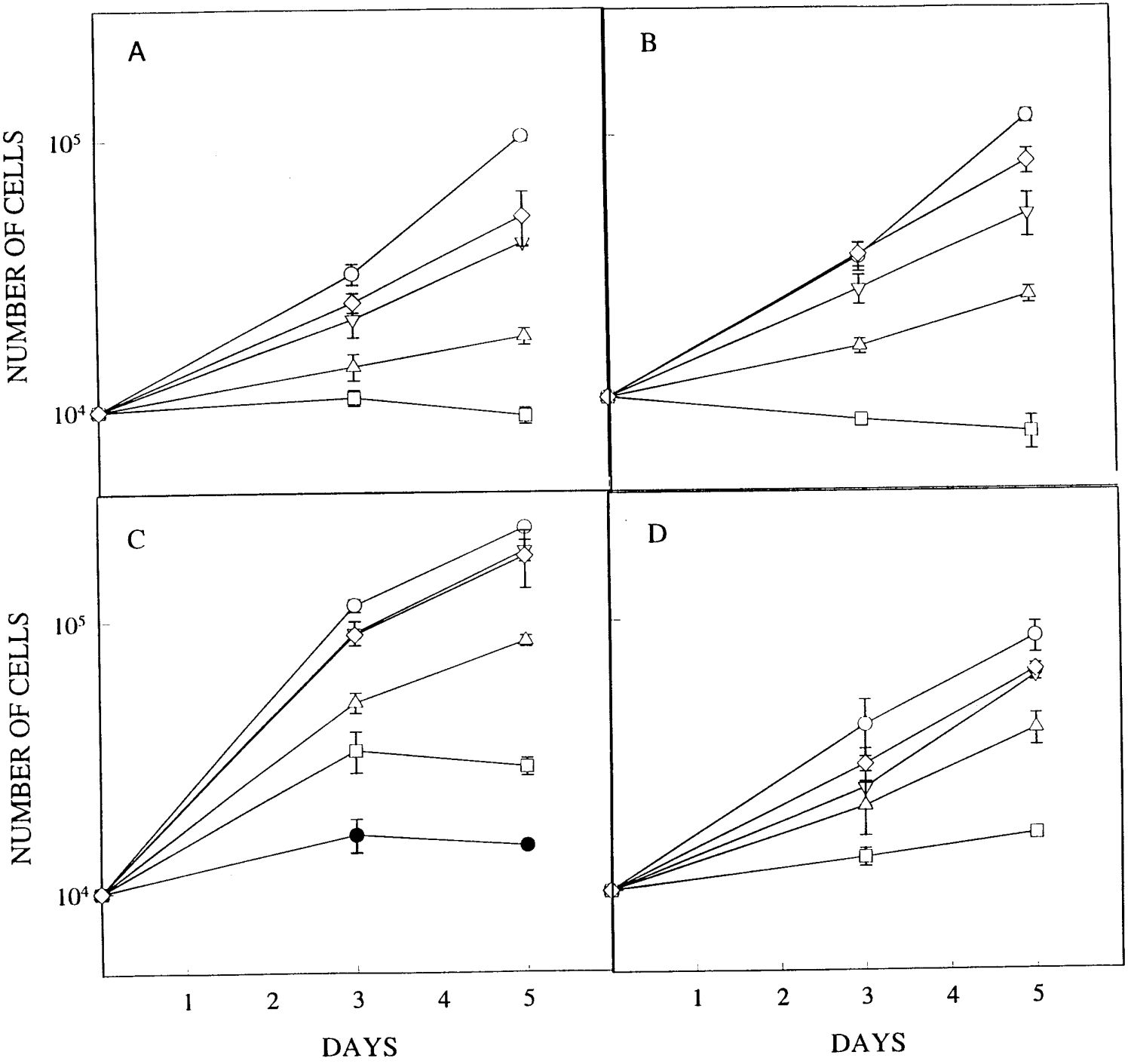
Figure 1D


A
B
C
D
NUMBER OF CELLS
NUMBER OF CELLS
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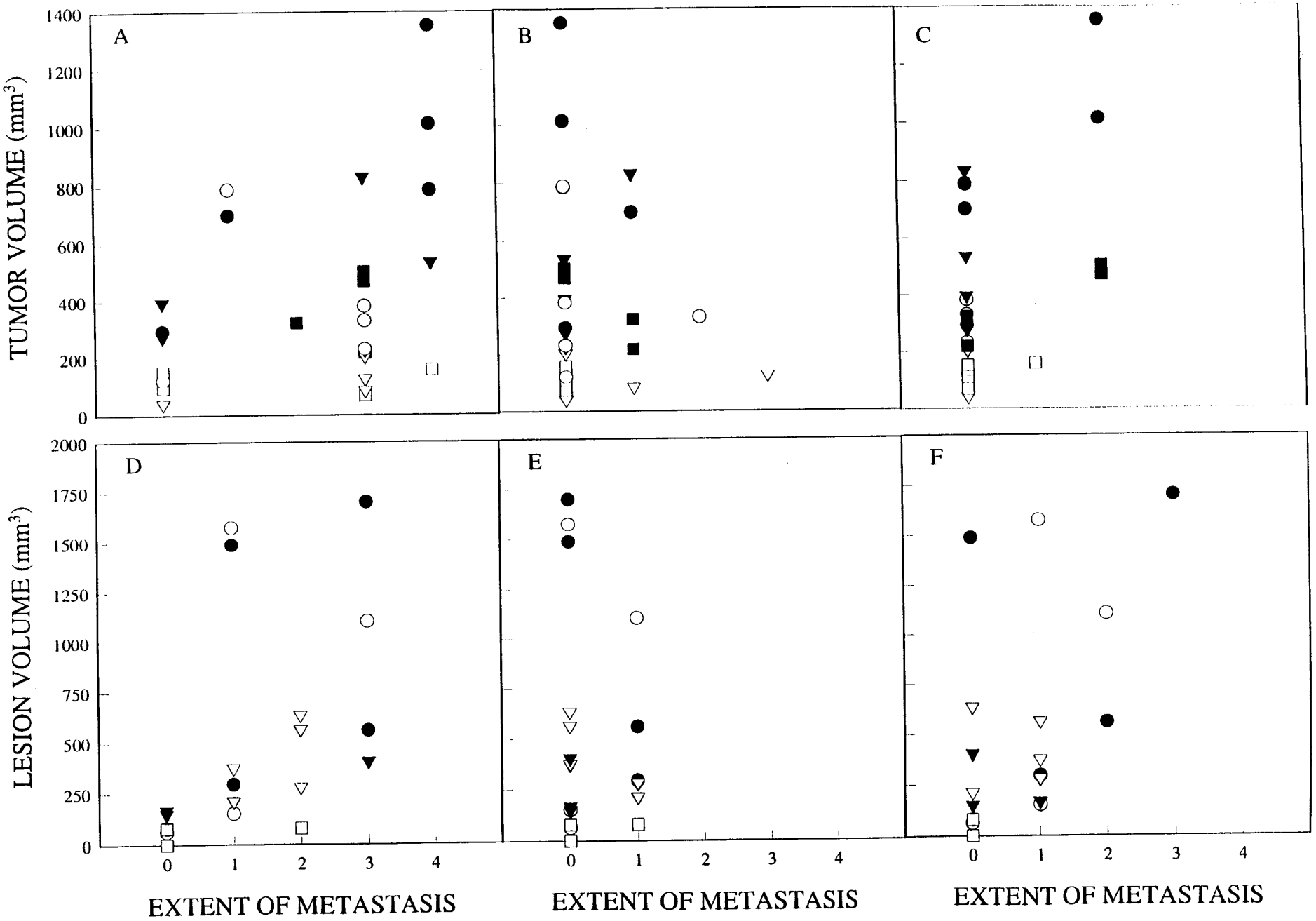
1
2
3
4
5
DAYS
DAYS

Figure 2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B
C
D
E
F
TUMOR VOLUME (mm3)
LESION VOLUME (mm3)
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1200
1000
800
600
400
200
0
2000
1750
1500
1250
1000
750
500
250
0
0
1
2
3
4
EXTENT OF METASTASIS
EXTENT OF METASTASIS
EXTENT OF METASTASIS

Figure 3