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Award Number: DAMD17-03-1-0614

TITLE: Fibrin(ogen)-Mediated Extracellular Transport of Breast Cancer Cells by Macrophages: A New Idea Regarding Metastasis

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REPORT DATE: October 2004

TYPE OF REPORT: Final

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2004	Final (30 Sep 2003 - 29 Sep 2004)

4. TITLE AND SUBTITLE
Fibrin(ogen)-Mediated Extracellular Transport of Breast Cancer Cells by Macrophages: A New Idea Regarding Metastasis

5. FUNDING NUMBERS
DAMD17-03-1-0614

6. AUTHOR(S)

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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

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

We set out to test the hypothesis that fibrin tethers existing between macrophages and breast cancer cells facilitate the extracellular transport of tumor cells by macrophages. We successfully developed a model system to test our hypothesis, and used it to collect preliminary evidence in support of our theory. During the course of our research, we discovered that fibrin-coated droplets of olive oil — like fibrin-coated macrophages — bind avidly to fibrin-coated murine breast tumor cells *in vivo*. Because of its potential for immediate application to the treatment of breast and other cancers, we exploited our observation to develop a new vehicle by which to deliver lipophilic anti-cancer agents, e.g., docetaxel, to fibrin-coated tumors. Using the ascites form of the TA3/St murine mammary tumor, we showed that microscopic droplets of olive oil loaded with docetaxel and coated with fibrinogen prolong the survival of tumor-bearing mice by about 4 times with respect to survival conferred by the standard formulation of docetaxel, i.e., Taxotere. (The results of our studies have now been published in *Clin Cancer Res*, 10:7001-7010, 2004. A copy of that document is included with this report.) The survival benefit conferred by fibrinogen-coated droplets of docetaxel-loaded olive oil appears to be due to the thrombin-dependent retention of the drug-loaded droplets within the tumor microenvironment. With regard to the original hypothesis, we now propose that fibrinogen-coated particulate formulations of anti-cancer agents might be best suited to treating metastatic disease because macrophage-mediated trafficking of fibrinogen-coated, drug-loaded particles likely mimics macrophage-mediated trafficking of fibrinogen-coated tumor cells.

14. SUBJECT TERMS
Fibrinogen, Oil Droplets, Docetaxel, Drug Delivery, Breast Cancer

15. NUMBER OF PAGES
24

16. PRICE CODE

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

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Introduction

The morbid and mortal consequences of breast cancer — indeed, most solid cancers — are due, in large part, to metastasis. Much available evidence indicates cancer cells, fibrinogen and macrophages act cooperatively during metastasis. Heparin and other anticoagulants appear to disrupt that cooperation. No mechanistic understanding adequately explains either the cooperation or its disruption by anticoagulants. As for the involvement of fibrinogen, it has been proposed the protein: 1) envelops cancer cells, protecting them from immune surveillance, and/or 2) provides an intravascular matrix for trapping and anchoring cancer cells liberated from a parent tumor.

We set out to test the hypothesis that fibrin tethers existing between macrophages and breast cancer cells facilitate the extracellular transport of the tumor cells by the macrophages. Using model systems, we showed that macrophages can bind in fibrinogen-dependent fashion both to breast tumor cells (TA3/St murine mammary tumor cells) and to latex microspheres. We also showed that complexes formed between macrophages and either tumor cells or microspheres can move across a porous membrane. Heparin and hirudin prevent complex formation and macrophage-facilitated movement of fibrinogen-coated particles. During the course of our studies on macrophage-facilitated extracellular transport of particulate materials, we discovered that fibrinogen-coated droplets of olive oil — like fibrin(ogen)-coated macrophages — bind avidly to fibrin(ogen)-coated murine breast tumor cells. Because of its potential for immediate application to the treatment of breast and other cancers, we exploited our observation to develop a new vehicle by which to deliver docetaxel, a lipophilic anti-cancer agent, to fibrin(ogen)-coated tumors. We showed that microscopic droplets of olive oil loaded with docetaxel and coated with fibrinogen prolong the survival of mice bearing the fibrin(ogen)-rich ascites form of the TA3/St tumor by ~ 4 times with respect to survival conferred by the existing commercial formulation of docetaxel, i.e., Taxotere. The survival benefit appears to be due to thrombin-dependent retention of drug-loaded droplets within the tumor microenvironment. Based on our results and observations, we believe fibrinogen-coated particulate formulations of anti-cancer agents will prove ideally suited to treating metastatic disease because macrophage-mediated trafficking of fibrinogen-coated particles likely mimics macrophage-mediated trafficking of fibrinogen-coated tumor cells.

Body (Follows the approved Statement of Work)

Task 1. Characterize and document the fibrinogen (fgn)-dependent extracellular transport of breast cancer cells by macrophages *in vitro*

A. Optimize the chemotaxis system used to monitor the transport process.

We developed a model system to test whether macrophages (Mϕ's) are able to transport particles and cancer cells in fgn-dependent extracellular fashion. In preliminary studies, we worked with fgn-coated poly(styrene-divinylbenzene) microspheres (MS's). MS's were incubated with stimulated Mϕ's (either murine peritoneal Mϕ's, or THP-1 cells as surrogates of human Mϕ's) so as to form MS - Mϕ complexes. MS - Mϕ complexes were applied to the upper chambers of a 96-position chemotaxis apparatus fitted with a porous membrane. If the chemoattractant N-formyl-Met-Leu-Phe (fMLP) was added to the lower chambers of the apparatus, 11 ± 2% (peritoneal Mϕ's) or 9 ± 3% (THP-1 cells) of the complexes — and only complexes, i.e., no individual MS's and no individual Mϕ's — was recovered from the lower chambers after 12 h (n = 50, i.e., 5 attempts x 10 repetitions/attempt), **Fig. 1A**. In the absence of fMLP, ≤ 0.3% of complexes was recovered from the lower chambers. Plasmin treatment of MS - Mϕ complexes recovered from the lower chambers liberated all of the MS's from their viable (>98%) "cellmates," **Fig. 1B**. If plasmin was included in the upper chambers from the start, the number of Mϕ's reaching the lower chambers was not different, but there were no MS's in those chambers. We take these results to mean Mϕ's can transport fgn-coated particles in extracellular fashion.

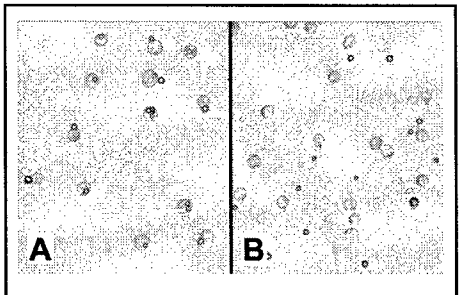

Figure 1. *A, MS - Mϕ complexes.* ***B****, Treatment of MS - Mϕ complexes with plasmin, 1.0 U/mL, yields individual MS's and intact, viable, individual Mϕ's.*

We substituted cancer cells for MS's in the chemotaxis assay. As cancer cells, we used TA3/St mammary adenocarcinoma cells that had been propagated as ascites tumors in A/J mice. The use of TA3/St cells was driven by 3 considerations: 1) the ascites fluid that baths the cells is fgn-rich,[1] 2) a large number of viable cancer cells can be recovered from the abdominal cavities of tumor-bearing mice, and 3) fgn coats the cancer cells *in vivo*, **Fig. 2**. After fluorescently labeling the cancer cells, they were co-incubated with stimulated THP-1 cells in the upper chambers of the chemotaxis apparatus. We then monitored the appearance after 12 h of fluorescence in the corresponding fMLP-spiked lower chambers. The results of our preliminary studies were gratifying. If THP-1 cells were included with the TA3/St cells in the upper chambers, 8 ± 3% (n = 50, i.e., 5 mice x 10 positions/mouse) of the fluorescent cancer cells was recovered from the lower chambers of the chemotaxis apparatus. In the absence of THP-1 cells, however, < 1% (n = 50) of the fluorescent cancer cells was recovered from the lower

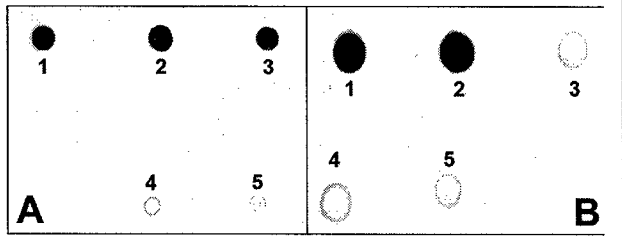

Figure 2. ***A, Fgn immunoblot:*** *1, murine fgn; 2, cell-free ascites from TA3/St tumor-bearing mouse; 3, 1 x 10^4 washed (x10) TA3/St tumor cells from same ascites; 4, medium from final wash of ascites tumor cells; 5, 1 x 10^4 TA3/St tumor cells exposed to AP-conjugated 2° antibody alone.* ***B, Murine serum albumin immunoblot:*** *1, murine serum albumin; 2, cell-free ascites from TA3/St tumor-bearing mouse; 3, 1 x 10^4 washed (x10) TA3/St tumor cells from same ascites; 4, medium from final wash of ascites tumor cells; 5, 1 x 10^4 TA3/St tumor cells exposed to AP-conjugated 2° antibody alone.*

chambers. Microscopic examination of fluorescent TA3/St cells in the lower chambers revealed each was complexed with at least one THP-1 cell, **Fig. 3**. Just as plasmin dissociated MS - Mϕ complexes, so, too, did that enzyme dissociate complexes of TA3/St cells and THP-1 cells.

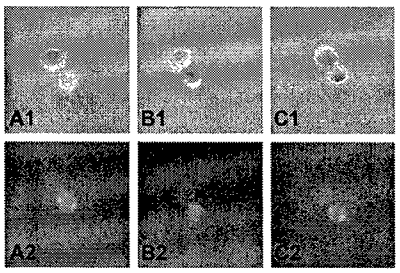

Figure 3. *TA3/St cell – THP-1 cell duplexes recovered from the lower chamber of a chemotaxis apparatus. See text for details. Upper panels, 1, are duplexes as seen using phase contrast microscopy. The larger cell in each duplex is a Mϕ-like THP-1 cell. Lower panels, 2, are the same duplexes as seen using fluorescence microscopy. The fluorescent cell in each duplex is a TA3/St breast cancer cell.*

B. Establish the fgn dependency of the transport process.

To test more directly the involvement of fgn in the extracellular transport process, we used B16F10 melanoma cells isolated from the abdominal cavities of either wild type (WT) C57BL/6 mice or fgn (−/−) mice of C57BL/6 background. We chose B16 cells because in WT C57BL/6 mice the melanoma cells become coated with fgn.[2,3] Whereas co-incubation (12 h) of THP-1 cells with fluorescent B16F10 cells from WT mice resulted in the translocation of 13 ± 4% (n = 30, i.e., 3 mice x 10 repetitions/mouse) of the fluorescent cells, co-incubation of THP-1 cells with fluorescent B16F10 cells from fgn (−/−) mice resulted in the translocation of 1 ± 0.5% (n = 30) of the fluorescent cells. Once again, no B16F10 cells were translocated in the absence of THP-1 cells, and plasmin dissociated complexes formed between THP-1 cells and B16F10 cells from the WT mice.

C. Establish the role of macrophage stimulation in the transport process.

We have found that macrophages must be activated before they will bind to fibrin(ogen)-coated microspheres or tumor cells *in vitro*. Formerly, we used phorbol 12-myristate 13-acetate (PMA) at a final concentration of 20 nM as activator. Since the submission of this proposal, we explored the use of 1α,25-dihydroxy-vitamin D_3 as activator of both THP-1 cells and mouse resident peritoneal Mϕ's. At a final concentration of ~ 12 nM, the vitamin yielded results that were about the same as those obtained when using PMA as activator.

D. Develop time-lapse cinematographic evidence of the transport process.

We did not have enough time and personnel to address this specific aim of ***Task 1***.

Task 2. **Determine whether compounds that either prevent fibrin formation, mask adsorbed fibrinogen, or block macrophage fibrinogen receptors interfere with the extracellular transport of breast cancer cells by macrophages *in vitro*.**

A. Delineate the effect of heparin and pentosan polysulfate on the transport process.

Heparin and pentosan polysulfate are polyanions that bind rather specifically to *adsorbed* vis-à-vis *solution phase* fgn.[4,5] As a consequence, they prevent interactions of bound fgn with proteins on other surfaces. Importantly, whereas in the absence of the polyanions THP-1 cells adhere to, and form rosettes with, fgn-coated beads, **Fig. 3A**, in their presence, even activated THP-1 cells do not adhere to such beads, **Fig. 3B**. By preventing contact between the cells and the

beads, the polyanions effectively prevent interaction of bound fgn with fgn receptors on the cells. We hypothesize these polyanions will prove to be "anti-adhesive" agents, and are now testing that hypothesis.

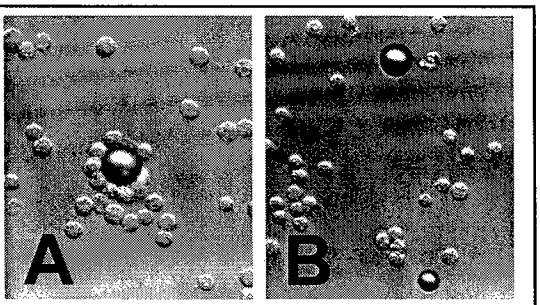

Figure 3. *A, Activated THP-1 cells form rosettes with fgn-coated beads. B, In the presence of heparin or related polyanions, activated THP-1 cells do not form rosettes with fgn-coated beads.*

B. Delineate the effect of hirudin on the transport process.

During the course of our investigations on the effect of hirudin on the extracellular transport of fibrinogen-coated tumor cells and particles by macrophages, we discovered that even fibrinogen-coated droplets of olive oil adher to fibrin(ogen)-coated breast tumor cells. That adherence is prevented by hirudin. We had a hunch fibrinogen-coated oil droplets might be ideally suited to delivering a lipophilic anti-cancer agent, e.g., docetaxel, to breast tumors. Because we believed fibrinogen-coated oil droplets might have immediate applicability to the treatment of breast and other cancers, we spent much of the year testing our hunch. Those experiments culminated in the recent publication of an article in Clinical Cancer Research (Einhaus CM, *et al.* 2004. Fibrinogen-coated droplets of olive oil for the targeted delivery of docetaxel to a fibrin(ogen-rich ascites form of a murine mammary tumor. *Clin Cancer Res* 10:7001-7010). The article is included as an appendix to this report.

C. Delineate the effects of plasmin and tPA on the transport process.

See ***Task 1***, **Specific Aim A.**

D. Delineate the effect of free fibrin D- and E-domains on the transport process.

We did not have enough time and personnel to address this specific aim of ***Task 2***.

E. Delineate the effect of anti-fibrinogen antibodies on the transport process.

We did not have enough time and personnel to address this specific aim of ***Task 2***.

F. Delineate the effect of anti-fibrinogen receptor antibodies on the transport process.

We did not have enough time and personnel to address this specific aim of ***Task 2***.

Key Research Accomplishments

1. A model system was developed with which to study the ability of macrophages to transport either tumor cells or particles in fibrinogen-dependent extracellular fashion. (Manuscript in preparation.)

2. Fibrinogen-coated oil droplets were developed as vehicles to deliver lipophilic anti-cancer agents to fibrin(ogen)-rich tumors. (Manuscript published. See Appendix.)

Reportable Outcomes

1. Einhaus CM, Retzinger AC, Perrotta AO, Dentler MD, Jakate AS, Desai PB, **Retzinger GS**. 2004. Fibrinogen-coated droplets of olive oil for delivery of docetaxel to a fibrin(ogen)-rich ascites form of a murine mammary tumor. *Clin Cancer Res* 10:7001-7010.

2. **Retzinger G.S.**, Recipient and Principal Investigator, "Fibrinogen-Facilitated Extracellular Transport of Tumor Cells by Macrophages," Johnson & Johnson Focused Giving Award, $92,000 (annual), February 1, 2004 – January 31, 2007.

Conclusions

1. Fibrinogen-coated microparticles, including oil droplets and latex microspheres, can form complexes with either fibrin(ogen)-coated macrophages or fibrin(ogen)-coated tumor cells.

2. Heparin and hirudin prevent formation of such complexes.

3. Macrophage – particle/tumor cell complexes can move across a porous membrane in response to a chemoattractant.

4. Fibrinogen-coated oil droplets are an effective means by which to deliver lipophilic anticancer agents to a fibrin(ogen)-rich ascites form of a murine mammary tumor.

5. **Significantly more time and resources are required to move forward in a meaningful way with this research.**

So What?

1. The demonstration that macrophages can transport tumor cells in fibrinogen-dependent extracellular fashion opens up a whole new way of thinking about metastasis and its treatment. Perhaps anticoagulants will prove useful as anti-adhesive agents for the treatment of metastatic disease.

2. Fibrinogen-coated particulate formulations of various medicinals might have relevance to the treatment of a host of pathologic lesions where fibrin(ogen) deposition has been shown to occur, e.g., abscesses, malignant tumors, atherosclerotic plaques, granulomas, etc.

3. If both fibrinogen-coated particulate materials and fibrinogen-coated tumor cells are trafficked by macrophages in similar fashion, then fibrinogen-coated particulate formulations of anti-cancer agents might be ideally suited to treating metastatic disease.

4. Perhaps platelets, like fibrinogen-coated oil droplets, will prove useful for drug delivery (to tumors and other fibrin(ogen)-rich inflammatory lesions).

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Personnel Receiving Pay from this Research Effort

Principal Investigator

Gregory S. Retzinger, MD, PhD (9.03 – 10.04)

Research Assistants

Michael Dentler (10.03 – 7.04)

Michael Walker (9.04 – 10.04)

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Appendix

Einhaus CM, Retzinger AC, Perrotta AO, Dentler MD, Jakate AS, Desai PB, **Retzinger GS**. 2004. Fibrinogen-coated droplets of olive oil for delivery of docetaxel to a fibrin(ogen)-rich ascites form of a murine mammary tumor. *Clin Cancer Res* 10:7001-7010.

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Fibrinogen-Coated Droplets of Olive Oil for Delivery of Docetaxel to a Fibrin(ogen)-Rich Ascites Form of a Murine Mammary Tumor

Charity M. Einhaus,[1] Andrew C. Retzinger,[2] Andre O. Perrotta,[3] Michael D. Dentler,[4] Abhijeet S. Jakate,[5] Pankaj B. Desai,[6] and Gregory S. Retzinger[7]

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ABSTRACT

Micronized droplets of olive oil loaded with docetaxel and coated with functional fibrinogen were administered intraperitoneally to mice bearing the fibrin(ogen)-rich ascites form of the TA3/St mammary tumor. When compared with docetaxel administered intraperitoneally as its commercial formulation (*i.e.*, Taxotere) docetaxel-loaded oil droplets coated with murine fibrinogen prolonged the median survival time of tumor-bearing mice from 14.5 to 29.5 days. Drug-free oil droplets provided no therapeutic benefit. Significantly more docetaxel was associated with tumor cells 24 and 48 hours after administration of the drug in fibrinogen-coated oil droplets than after its administration as Taxotere. Consistent with a role for thrombin in the retention of fibrinogen-coated oil droplets within the tumor microenvironment, hirudin significantly reduced the association of tumor cells with docetaxel delivered in fibrinogen-coated oil droplets and, at the same time, reduced the therapeutic efficacy of the droplets to that of Taxotere. Importantly, fibrinogen-coated oil droplets formed rosettes with tumor cells *in vivo*, a process prevented by hirudin. Although mice treated with oil droplets developed antifibrinogen antibodies, those antibodies seemed to be inconsequential. Taken together, our results and observations indicate fibrinogen-coated oil droplets markedly improve the therapeutic efficacy of docetaxel for the treatment of a mammary tumor grown in ascites form, a consequence of thrombin-mediated retention of the drug-loaded droplets within the tumor microenvironment.

INTRODUCTION

Fibrinogen, of all of the plasma proteins, binds avidly to hydrophobic surfaces (including those of hydrophobic liquids) in contact with blood (1, 2). The bound protein can remain functional in the classic sense of fibrin gelation. Earlier, we exploited the affinity of fibrinogen for hydrophobic liquids to develop fibrinogen-coated olive oil droplets as vehicles with which to deliver lipophilic drugs to sites of fibrin(ogen) deposition *in vivo* (3). Subsequently, we showed that fibrinogen-coated droplets of olive oil administered intravenously accumulate at an imposed fibrin(ogen)-rich inflammatory site. As a consequence of that demonstration, we proposed such droplets might be used advantageously to deliver oil-soluble drugs to any site of fibrin(ogen) deposition, including malignant tumors (4, 5). In support of our proposal, we showed that fibrinogen-coated droplets of olive oil loaded with docetaxel, a lipophilic taxane, significantly prolong the survival of mice bearing a solid tumor, the B16F10 melanoma (6).

The effectiveness of fibrinogen-coated, docetaxel-loaded oil droplets against a solid tumor like the melanoma prompted us to consider whether the droplets might also be effective against less consolidated tumors (*e.g.*, ascites tumors) that are especially prone to fibrin(ogen) deposition (7). An example is the TA3/St ascites tumor, which derived from a spontaneous mammary carcinoma in a mouse of the A/HeHa strain (8, 9). Because its microenvironment is so fibrin(ogen)-rich, we felt the TA3/St tumor might be ideally suited to treatment with fibrinogen-coated olive oil droplets loaded with docetaxel, an agent particularly effective against mammary carcinomas (10, 11).

In this report, we describe studies in which the TA3/St mammary tumor grown in ascites form was used to compare the anticancer activity of docetaxel delivered in fibrinogen-coated olive oil droplets to that of docetaxel administered as its standard formulation, Taxotere. Because the therapeutic benefit conferred by the fibrinogen-coated oil droplets was substantial, additional studies were done to elucidate the role of thrombin activity/fibrin formation in the operation of the droplets, and to determine whether the droplets elicit antifibrinogen antibodies that are of obvious clinical consequence.

MATERIALS AND METHODS

Reagents and Chemicals. Docetaxel and Taxotere were purchased from Aventis Pharmaceuticals (Bridgewater, NJ). Murine fibrinogen was obtained from Sigma Chemical Co. (St. Louis, MO). Unless specified otherwise, it was used as received. Human fibrinogen was obtained from Enzyme Research Laboratories (Indianapolis, IN). The buffer composition of the human fibrinogen was changed by gel permeation chromatography using Sephadex G-25 as matrix material and 0.02 mol/L Tris-

Received 1/20/04; revised 6/24/04; accepted 7/7/04.

Grant support: This work was supported by grants to G. Retzinger from the Susan G. Komen Foundation for Breast Cancer Research; the United States Department of Defense (BC023913); Emerging Concepts, Inc.; and the Johnson & Johnson Focused Giving Program. It was also supported by grants to P. Desai and G. Retzinger from Aventis and the Ruth Lyons Cancer Fund.

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DOI: 10.1158/1078-0432.CCR-04-0118

HCl (pH 7.40) as eluent. The human fibrinogen was then aliquoted and stored frozen at −20°C until use. Fibrinogen concentration was determined with 5.12×10^5 $m^{-1} \cdot cm^{-1}$ as the molar absorptivity of the protein at 280 nm. Docetaxel was tritium custom-labeled by Amersham Biosciences (Piscataway, NJ). Labeling of the drug was limited to its aryl rings, yielding a final product of specific activity 259 GBq · $mmol^{-1}$. Fatty acid-free BSA was purchased from ICN (Irvine, CA). Pluronic F68 (F68) was obtained from BASF Corporation (Mt. Olive, NJ). Olive oil, casein, murine thrombin (>1,000 NIH units · mg^{-1}), and hirudin (3,000 units · mg^{-1}) were obtained from Sigma. Refludan was purchased from Aventis Pharma (Frankfurt, Germany). Rabbit antihuman fibrinogen IgG was purchased from American Diagnostica (Greenwich, CT). Rabbit antihuman thrombin IgG that cross-reacts with murine thrombin was obtained from American Diagnostica. Horse radish peroxidase-conjugated goat antimouse IgG, and alkaline phosphatase (AP)-conjugated goat antirabbit IgG were purchased from Promega (Madison, WI). Polystyrene-divinylbenzene beads of diameter 6.4 ± 1.9 μm, and polystyrene-divinylbenzene beads of diameter 0.945 ± 0.0064 μm were obtained from Seradyn (Indianapolis, IN). Before use, beads were washed and lyophilized as described elsewhere (12). Freund's incomplete adjuvant was obtained from Difco Laboratories (Detroit, MI). All other reagents and chemicals were of the highest quality available commercially.

Mice. Eight- to 10-week-old female A/Jax mice weighing between 17 and 20 g were from The Jackson Laboratory (Bar Harbor, ME). Before use, mice were acclimated to the local environment for a period of 1 week. The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

Tumor Cells. TA3/St ascites tumor cells were from Drs. Janice Nagy and Harold Dvorak (Boston, MA). Tumor cells were propagated as stock cultures within the abdominal cavities of A/Jax mice. Tumor cells intended for administration to test animals were collected from the abdominal cavities of three mice that had carried tumor cells for 5 days. Tumor cells from the three animals were pooled, and then washed once using 20 mL of normal saline (NS) and centrifugation at 1,500 × *g* for 3 minutes. Before inoculating them into naive mice, tumor cells were dispersed in NS to a final concentration of 1×10^6 cells · mL^{-1}. The volume of a tumor cell inoculum was always 1 mL.

Preparation of Emulsions. When included in an olive oil preparation, docetaxel was first dissolved in the oil phase to a final concentration of 1 mg · mL^{-1} with an ultrasonic water bath and intermittent sonication over the course of 1 hour. As determined with high performance liquid chromatography, such sonication does not result in any discernible deterioration of docetaxel (6).

Both drug-free and drug-loaded olive oil were emulsified with the method of high pressure extrusion (3, 6). To a clean, 12 × 75-mm glass tube was added 2 mL of drug-free or drug-loaded olive oil and 3 mL of NS containing F68 at a concentration of 1 mg · mL^{-1}. The oil/water mixture was gently agitated and was then extruded 5 times under high pressure, 103.5 MPa, through the aperture of a homogenizer (EmulsiFlex-20,000-B3; Avestin, Ottawa, Canada). To the resulting oil-in-water emulsion was immediately added either 2 mL of NS or 2 mL of NS containing murine fibrinogen, human fibrinogen or BSA. Because murine fibrinogen from commercial source is exceedingly expensive, we reduced the concentration of all proteins used to coat droplets from 4 mg · mL^{-1} (6) to 2 mg · mL^{-1}. After sonicating the emulsion for 10 to 15 seconds with an ultrasonic water bath, centrifugation at 1,500 × *g* for 15 minutes was used to float the oil droplets. The resulting infranatant was removed, and an ultrasonic water bath was used to disperse the residual cream layer in 3 mL of NS. The volume of emulsion used for an injection was 1 mL, of which 40% was olive oil. Oil droplets prepared in this fashion are polydisperse in size, ranging in diameter from ~1 to ~100 μm, with a mean diameter of ~12 μm (6).

Unless specified otherwise, NS as diluent was used to prepare Taxotere for injection to a final concentration of 0.40 mg · mL^{-1}.

Tumor Model and Treatments, and Toxicity Assessment. To begin an experiment, mice received an injection intraperitoneally with tumor cells. Three days later, a single inoculum of test or control material was then administered intraperitoneally to the animals. Whether in oil droplets or as Taxotere, the amount of docetaxel administered per test inoculum was always 0.40 mg (*i.e.*, ~20 mg/kg animal body mass). Regardless of material/vehicle, the total volume per injection was always 1 mL.

For some experiments, Refludan was administered to tumor-bearing mice. The first dose was administered intraperitoneally ~2 hours before treating animals with test or control materials. Thereafter, tumor-bearing mice received a similar bolus at 24 hour intervals on all subsequent days. Before injection, the Refludan was diluted with NS. The amount of Refludan administered each time was 40 μg in 0.2 mL.

To assess toxicity, a separate experiment was done in which healthy, tumor-free mice of known weights were treated in groups of five, each with a single intraperitoneal injection of one of several preparations. Using a top-loading balance, we measured the weights of the animals every day for 15 days. Statistical significances of differences between mean weights of mice from the various treatment groups were determined with Student's *t* test. We took as significant $P \leq 0.05$.

Analysis of Antitumor Efficacy Data. The number of mice surviving in an experimental group was monitored as a function of time after tumor cell inoculation. Results of survival studies were plotted as the percentage of animals remaining alive in a group *versus* day after tumor cell inoculation. For purposes of inter-group comparisons, the percentage of increase in life span (%ILS) because of a treatment was defined as [(median days of survival of treated group − median days of survival of NS-treated group)/median days of survival of NS-treated group] × 100%. Statistical significances of differences between mean survival times of various treatment groups were determined with Student's *t* test. Again, we took as significant $P \leq 0.05$.

Abdominal Cavity Lavage and Recovery and Evaluation of Ascites/Lavage Fluid. Mice that had been treated variously were euthanized and, immediately thereafter, each was given an intraperitoneal inoculum of 2 mL NS. Three-milliliter plastic syringes equipped with 18 gauge needles were then used to collect as much of the lavage fluids and their

accompanying materials as possible from the abdominal cavities of the animals. Lavage fluid recovered from an animal was used in various ways. Sometimes it was immediately spread onto glass microscope slides and either covered with a glass coverslip for viewing using phase-contrast microscopy or stained with oil red O (13) and covered with a glass coverslip for viewing. For one set of experiments, centrifugation at 1,500 × *g* for 10 minutes was used to clear lavage fluids of cells, and a turbidimetric method (14) was used to assess the thrombin activities in 20–50 μL aliquots of the resulting cell-free fluids. In brief, the turbidimetric method involves adding a test sample to a stirred dispersion of fibrinogen-coated, microscopic latex beads. Such beads agglutinate in the presence of thrombin, a consequence of interbead fibrin formation. A platelet aggregometer or other photometric device can be used to monitor agglutination. The rate of change of light-scattering of the stirred bead dispersion is proportional to the thrombin activity in the sample. Thrombin-inducible agglutination of fibrinogen-coated beads is inhibited in stoichiometric fashion by hirudin, a thrombin specific inhibitor (14).

Monitoring the Association of Docetaxel with Tumor Cells. Both oil droplets and Taxotere were added to [^{3}H]docetaxel. When included in oil droplets, [^{3}H]docetaxel was added along with unlabeled docetaxel to the oil just before emulsification of that phase. When included in Taxotere, [^{3}H]docetaxel was added to the ethanolic polysorbate 80 of the commercial preparation just before dilution of that material with NS.

Three days after administering tumor cells to mice, tumor-bearing animals were each given an intraperitoneal injection of the radioactively spiked version of either Taxotere or docetaxel-loaded oil droplets. Some oil droplet-treated mice were also treated with Refludan as described above. Twenty-four or 48 hours later, all of the animals of a group were sacrificed, their abdominal cavities were lavaged, and the lavage fluids and accompanying materials were collected as described above. After determining the number of cells in a lavage sample, centrifugation at 1,500 × *g* for 2 minutes was used to sediment the cells in the sample. The radioactivity associated with the cells was then measured after diluting them with liquid scintillation medium. Results were recorded as nanograms of docetaxel per 1,000 recovered tumor cells.

Immunodetection of Proteins. Immunoblotting was used to detect thrombin in fluids recovered from the abdominal cavities of tumor-bearing mice. Mice that had received injection with tumor cells were euthanized after various lengths of time. Immediately after euthanizing an animal, its abdominal cavity was inflated with 2 mL of NS. The lavage and accompanying ascites fluid were then collected with a 3-mL plastic syringe equipped with an 18 gauge needle. Centrifugation at 1,500 × *g* for 15 minutes was used to sediment tumor cells, and the cell-free fluid was aspirated and saved. Ten microliters of the aspirate were blotted alongside control materials onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA). After drying a membrane at room temperature for ~1 hour, it was immersed for 30 minutes in 60 mL of buffer containing 0.02 mol/L Tris-HCl (pH 7.5), 0.5 mol/L NaCl, 0.05% (v/v) Tween 20, and 40 mg · mL^{-1} of BSA. Subsequently, membranes were rinsed with water and treated, first with rabbit antithrombin IgG as primary (1°) antibody and then with AP-conjugated, goat antirabbit IgG as secondary (2°) antibody. Substrates for AP activity were from Promega.

Immunoblotting was also used to detect fibrinogen both on washed tumor cells and on washed oil droplets. Mice that had received injection intraperitoneally 3 days earlier with tumor cells were treated, each with 1 mL of a test emulsion. One hour later, the mice were euthanized, after which each was given an intraperitoneal injection of 2 mL of NS. Three-milliliter plastic syringes equipped with 18 gauge needles were used to collect lavage fluids from the abdominal cavities of the mice. Centrifugation at 1,500 × *g* for 15 minutes was used to sediment and float tumor cells and oil droplets in fluids, respectively. Tumor cell and oil droplet fractions were isolated, and a stirred flow cell (10 mL, Amicon, Danvers, MA) equipped with a polycarbonate filter (Sigma) with pores of diameter 1.2 μm was used to wash the cells or oil droplets 8 times. The washing medium was NS containing 10 mg · mL^{-1} of BSA. On milliliter of NS was used to rinse washed oil droplets from a filter. Ten microliters of the resulting dispersion of oil droplets were blotted alongside positive and negative control materials onto a polyvinylidene difluoride membrane. NS was used to rinse washed tumor cells from a filter, and then cells were resuspended in that medium to a final concentration of 1 × 10^6 cells · mL^{-1}. Ten microliters of tumor cell suspension was blotted alongside control materials onto a polyvinylidene difluoride membrane. After drying a membrane at room temperature for ~1 hours, it was immersed for 30 minutes in 60 mL of buffer containing 0.02 mol/L Tris-HCl (pH 7.5), 0.5 mol/L NaCl, 0.05% (v/v) Tween 20, and 40 mg · mL^{-1} of BSA. Subsequently, membranes were rinsed with water and treated first with rabbit antifibrinogen IgG as 1° antibody and then with AP-conjugated, goat antirabbit IgG as 2° antibody. Substrates for AP activity were from Promega.

Elicitation and Assessment of Antifibrinogen Antibodies. Mice in groups of 3 to 6 received injection intraperitoneally at time 0 and after 7, 14, and 21 days with one of several preparations. The preparations included (*a*) NS, 1 mL; (*b*) emulsion containing docetaxel-free, fibrinogen-free oil droplets, 1 mL; (*c*) emulsion containing docetaxel-free oil droplets coated with murine fibrinogen, 1 mL; (*d*) emulsion containing docetaxel-free oil droplets coated with human fibrinogen,1 mL; (*e*) Freund's incomplete adjuvant, 0.4 mL, containing 1 mg of murine fibrinogen in NS; and (*f*) Freund's incomplete adjuvant, 0.4 mL, containing 1 mg of human fibrinogen in NS. After 38 days, heparinized Natelson capillary tubes were used to collect blood samples from the retro-orbital plexuses of treated mice. Blood was also collected in similar fashion from age-matched untreated mice. After centrifugation of the blood samples at 1,500 × *g* for 12 minutes, ELISA was used to isolate and assess the plasma from each for antifibrinogen antibodies.

For the ELISA, 96-well flat-bottomed microtiter plates (PRO-BIND, Becton Dickinson, Franklin Lakes, NJ) were prepared and used as follows. Into each well of a plate was added 150 μL of 0.02 mol/L Tris-HCl (pH 7.40), containing 100 μg · mL^{-1} of fibrinogen. Depending on the experiment, either human or murine fibrinogen was used. Before using the murine fibrinogen, we removed the IgG that contaminated the protein by using a 1 mL Protein G Sepharose column (HiTrap Protein G HP, Amersham Biosciences) according to the column's manufacturer. The solution containing the appropriate species-

specific fibrinogen was incubated in the wells of a plate for 2 hours at room temperature. After that time, the fibrinogen-containing solution was forcibly ejected from the wells. "Blocking" medium, 250 μL, containing 0.02 mol/L Tris-HCl (pH 7.40), 0.1% (w/v) Tween 20, 0.5 mg · mL^{-1} of casein and 1 mg · mL^{-1} of BSA was next added to each well of the plate, which was then left undisturbed for 60 minutes. After forcibly ejecting the blocking medium and rinsing the wells thrice with water, the plate was ready for the addition of plasma samples. Plasma samples to be assessed for antifibrinogen antibody content were prediluted 1:5, v/v, with NS. A 250 μL aliquot of such a dilution was added to the first well of a row of wells on a fibrinogen-coated plate. Two-fold serial dilution of the content of the first well was then done, out to a maximum of 24 wells. The dilutions of plasma were incubated in the wells for 1 hours at room temperature, after which the dilutions were forcibly ejected, and the wells were rinsed 4 times with buffer containing 0.1% (w/v) Tween 20. One hundred and fifty micorliters of a 1:2,500 dilution of horse radish peroxidase-conjugated goat antimouse IgG was next added to each well and incubated there at room temperature for 1 hour. After that time, the IgG-containing solution was ejected from the wells, and the plates were rinsed 4 times with buffer containing 0.1% (w/v) Tween 20 before adding *o*-phenylenediamine (Sigma) as colorimetric substrate for horse radish peroxidase. The end point titer of the antifibrinogen antibodies was determined from visual inspection of the intensity of color in the wells after 20 minutes.

Assessment of the Coagulability of Citrated Plasma Samples. Blood samples from the retro-orbital plexuses of mice were collected with plain Caraway blood collecting tubes that had each been preloaded with ~5 μL of 0.105 mol/L (*i.e.*, 3.2%, w/v) buffered citrate solution. The volume of blood collected within a tube was ~270 μL. Within 3 seconds of having collected a blood sample, it was discharged into a 0.5 mL conical polypropylene tube containing 30 μL of 0.105 mol/L buffered citrate. Centrifugation at 15,000 × *g* for 5 minutes was used to separate citrated plasma from blood cells. The activated partial thromboplastin times (aPTTs) of the citrated plasma samples were determined by technologists of the Investigative Hemostasis Laboratory of the University of Cincinnati Medical Center who used for those determinations actin FSL (Dade Behring, Deerfield, IL) and an automated coagulation analyzer (BCS, Dade Behring).

RESULTS

Antitumor Efficacy of Docetaxel Formulations and Preliminary Assessment of Toxicity. Earlier, we showed that docetaxel delivered within fibrinogen-coated droplets of olive oil significantly prolongs the survival of mice bearing a solid, intra-abdominal form of the B16F10 melanoma (6). With respect to docetaxel delivered as Taxotere, docetaxel delivered within oil droplets coated with human fibrinogen increased the median survival time of melanoma-bearing mice by 330%. We set out to determine whether docetaxel delivered within fibrinogen-coated oil droplets would, with respect to Taxotere, provide a survival benefit to mice bearing a fibrin(ogen)-rich ascites tumor. In the past, we used only human fibrinogen to coat oil droplets because the human protein is much less expensive than murine fibrinogen. Now, however, and despite its much greater cost, we wanted to use murine fibrinogen for at least some of our experiments to show the operational equivalence of the murine protein. Additionally, we anticipated murine fibrinogen would *a priori* be less likely than human fibrinogen to elicit an immune response in oil droplet-treated mice (see below).

After first demonstrating that the survival benefit conferred to TA3/St tumor-bearing mice by docetaxel-loaded oil droplets coated with either murine or human fibrinogen was the same ($P = 0.40$, data not shown), we then compared that survival benefit to those conferred by other preparations, including NS, docetaxel-free oil droplets coated with either BSA or murine fibrinogen, Taxotere, docetaxel-loaded oil droplets coated with BSA, or docetaxel-loaded oil droplets that had not been coated with any protein. As shown in Fig. 1, the survival plots related to NS and docetaxel-free droplets of olive oil were virtually superimposable, the differences in mean survival times not being statistically significant ($P \geq 0.80$). For mice in those groups, the median time to death (MTTD) was 9.5 days. Taxotere-treated mice lived significantly longer than vehicle-treated mice ($P = 0.0002$), their MTTD being 14.5 days (%ILS = 53). Mice treated with BSA-coated droplets of docetaxel-loaded olive oil lived significantly longer ($P = 0.035$, MTTD = 20 days, %ILS = 111) than Taxotere-treated mice, but not as long as mice treated with either uncoated droplets of docetaxel-loaded olive oil ($P = 0.007$, MTTD = 25 days, %ILS = 163) or docetaxel-loaded oil droplets coated with murine fibrinogen ($P = 0.0001$, MTTD = 29.5 days, %ILS = 211). Although the MTTD's of the last 2 groups were different and their survival profiles overlapped only slightly, the *mean* times to death of animals in the 2 groups (*i.e.*, 24.8 ± 2.8 days for mice treated with uncoated droplets and 28.4 ± 4.7 days for mice treated with fibrinogen-coated droplets) was not significantly different, $P = 0.082$. This last finding was not unexpected (6), and is addressed below. We conclude that droplets of olive oil, particularly ones precoated with fibrinogen, are better vehicles for the intraperitoneal delivery of docetaxel to the ascites form of the

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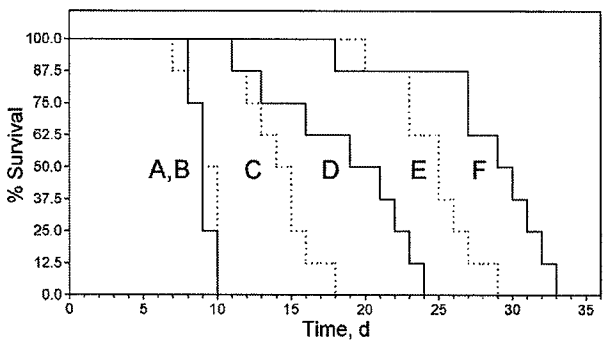

Fig. 1 Survival profiles of TA3/St ascites tumor-bearing A/Jax mice treated with NS (*A*, dotted line); docetaxel-free oil droplets coated with murine fibrinogen (*B*); Taxotere (*C*); docetaxel-loaded oil droplets coated with BSA (*D*); docetaxel-loaded oil droplets not coated with any protein (*E*); and docetaxel-loaded oil droplets coated with murine fibrinogen (*F*). Not shown is the profile related to treatment with docetaxel-free oil droplets coated with BSA. It is indistinguishable from *A* and *B*. There were eight animals in each treatment group. See the Results section for details.

TA3/St mammary tumor than ethanolic polysorbate 80, the medium within which docetaxel is formulated commercially.

Unless indicated otherwise, the fibrinogen we used for all subsequent experiments was of human origin.

As a preliminary assessment of toxicity, we monitored for 15 days the weights of healthy, tumor-free mice treated with either NS, Taxotere, or fibrinogen-coated droplets of docetaxel-loaded olive oil. At each daily weighing, there were no statistically significant differences between the average weights of animals of any of the treatment groups. With respect solely to recipient weight, we conclude that a single intraperitoneal injection of fibrinogen-coated droplets of docetaxel-loaded olive oil is no more harmful to mice in the short term than is a single intraperitoneal injection of NS.

Thrombin Activity in Ascites Fluid from Tumor-Bearing Mice. Fibrinogen-coated droplets of olive oil were developed by us as vehicles with which to deliver oil-soluble drugs to sites of fibrin formation. We expected thrombin activity existing at such sites would facilitate incorporation there of the droplets into nascent fibrin clots. Because fibrin accumulates within the abdominal cavities of A/Jax mice bearing the ascites form of the TA3/St mammary tumor (7), we anticipated thrombin would be present in ascites fluids of tumor-bearing animals. Indeed, thrombin was present both antigenically (data not shown) and
F2 functionally, Fig. 2, in cell-free ascites/lavage fluids recovered from tumor-bearing mice. On average ($n = 3$), the thrombin activity recovered from a tumor-bearing mouse after 3 days was 0.019 ± 0.009 units/mL of recovered fluid. After 5 days, the thrombin activity had increased significantly ($P \leq 0.05$) to 0.053 ± 0.015 units/mL of recovered fluid. No thrombin activity was detected in lavage fluids recovered from healthy, tumor-free mice ($n = 3$).

Measures that Reduce the Therapeutic Efficacy of Droplets of Docetaxel-Loaded Olive Oil. If thrombin-catalyzed fibrin formation of droplet-bound fibrinogen contributes to therapeutic efficacy, then measures that prevent/limit that formation should prevent/limit therapeutic efficacy. Two such measures include inhibiting thrombin activity by using, for example, hirudin; precoating droplets with a protein other than fibrinogen (*e.g.*, BSA). In fact, both measures prevented/limited the therapeutic efficacy of docetaxel-loaded oil droplets, Figs. 1
F3 and 3. Because the half-life of hirudin *in vivo* is short, $t_{1/2} = 1.3$ hours (15) and because we found the thrombin activity in the abdominal cavities of tumor bearing-mice to be high, we administered a rather large dose of hirudin (Refludan), 40 μg, each day to each test animal. On the basis of drug mass per unit recipient mass, that amount of hirudin corresponds to approximately 4 times the usual loading dose of the drug when it is used in humans (15). As shown in the figure, whereas the daily administration of hirudin by itself had no statistically significant effect ($P = 0.25$) on the survival of tumor-bearing mice, it did reduce the survival benefit of mice treated with *any* of the docetaxel-loaded oil droplets to that of mice treated with Taxotere ($P \geq 0.80$). Taken together, these results suggest fibrin formation at the surface of the oil droplets contributes to the survival benefit conferred by docetaxel-loaded droplets of olive oil, whether the droplets are precoated with fibrinogen or not.

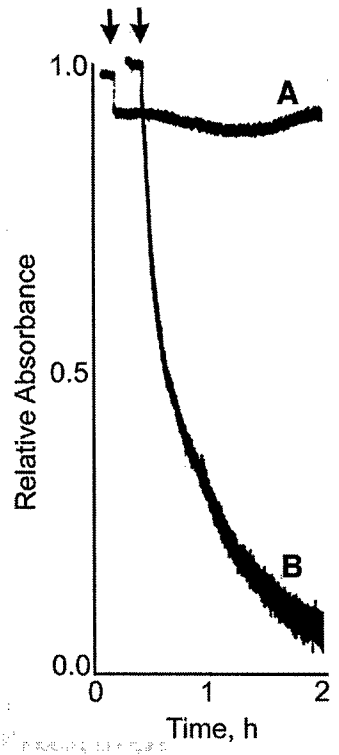

Fig. 2 Five units of hirudin (*A*) inhibits the decrease in apparent absorbance that otherwise (*B*) accompanies the addition of ascites fluid to a stirred dispersion of fibrinogen-coated microscopic beads. The decreasing apparent absorbance is attributable to bead aggregation, a thrombin-dependent phenomenon. *Arrows* indicate the addition of cell-free ascites fluid to reaction cuvettes containing beads in aqueous dispersion. See text and reference 14 for details.

Association of Docetaxel with Tumor Cells *in Vivo*. Previously, we presented preliminary evidence that the association of docetaxel with intra-abdominal solid tumor tissue is significantly greater when the drug is delivered intraperitoneally in fibrinogen-coated oil droplets than when the drug is delivered intraperitoneally as Taxotere (6). The availability of [^{3}H]docetaxel as well as the ease with which ascites tumor cells can be collected from the abdominal cavities of mice prompted us to determine more directly the relationship between delivery vehicle and the association of docetaxel with tumor cells. Mice inoculated 3 days earlier with TA3/St tumor cells were given [^{3}H]docetaxel either as Taxotere or in fibrinogen-coated oil droplets. Some droplet-treated mice were also given hirudin as described above. Twenty-four or 48 hours after treating them with [^{3}H]docetaxel, animals of a group were sacrificed, and their tumor cells were harvested and washed to remove any unbound materials. Subsequently, radioactivities associated with the cells were measured. We then determined for each group the mean ($\pm$ SD) mass, in nanograms, of docetaxel per 1,000 tumor cells per animal. After 24 hours, the value was 0.062 ± 0.043 for mice ($n = 5$) treated with Taxotere and 0.446 ± 0.116 for mice ($n = 5$) treated in the absence of hirudin with docetaxel-loaded oil droplets coated with fibrinogen. After 48 hours, the value was 0.010 ± 0.001 for mice ($n = 5$) treated

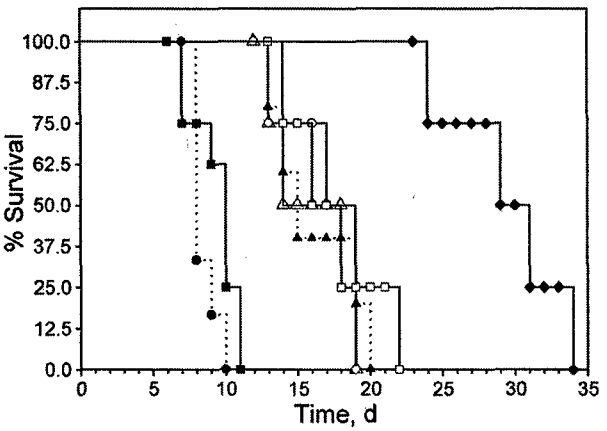

Fig. 3 Hirudin reduces the therapeutic efficacy of docetaxel-loaded oil droplets to that of an equivalent dose of docetaxel administered as Taxotere. For this experiment, the following treatments were administered to tumor-bearing mice: (■), NS alone (n = 8); (◆), NS and hirudin (n = 6); (▲), Taxotere alone (n = 5); (△), BSA-coated droplets of docetaxel-loaded olive oil plus hirudin (n = 4); (○) human fibrinogen-coated droplets of docetaxel-loaded olive oil plus hirudin (n = 4); (□), uncoated droplets of docetaxel-loaded oil droplets plus hirudin (n = 4); and (◆), human fibrinogen-coated droplets of docetaxel-loaded olive oil minus hirudin (n = 4). Whereas the three distinct groups of profiles are all significantly different from each other ($P \leq 0.03$), none of the individual profiles of a group is significantly different from the other profiles of that group ($P \geq 0.80$). See text for details.

with Taxotere, 0.149 ± 0.046 for mice (n = 5) treated in the absence of hirudin with docetaxel-loaded oil droplets coated with fibrinogen, and 0.047 ± 0.050 for mice (n = 4) treated with both hirudin and docetaxel-loaded oil droplets coated with fibrinogen. We conclude that, at least during the first 2 days after treatment and in the absence of hirudin, about an order of magnitude more docetaxel is associated with tumor cells if the drug is administered in fibrinogen-coated oil droplets than if it is administered as Taxotere ($P \leq 0.001$). Furthermore, and consistent with the results of the survival studies, if hirudin is coadministered with fibrinogen-coated droplets of docetaxel-loaded olive oil, the association after 2 days of the drug with tumor cells is reduced significantly ($P \leq 0.02$) from that observed in the absence of the thrombin inhibitor.

Association of Oil Droplets with Tumor Cells *in Vivo*. If fibrin does mediate the binding of drug-loaded oil droplets to tumor cells, then one might expect to see oil droplets adhered to tumor cells recovered from oil droplet-treated mice. Furthermore, hirudin should prevent such adherence. As shown in Fig. 4, rosettes consisting of oil droplets and tumor cells were abundant in ascites removed from tumor-bearing mice shortly after having administered to the animals either fibrinogen-coated droplets or uncoated droplets. Coadministration of hirudin with either of those droplets prevented rosette formation. Although the association of BSA-coated droplets with tumor cells was much less obvious than that seen when using either fibrinogen-coated droplets or uncoated droplets, such association was still present and sensitive to hirudin. We conclude that fibrin does indeed mediate the binding of oil droplets to tumor cells.

Endogenous Fibrin(ogen) Both on Oil Droplets and on Tumor Cells *in Vivo*. One of us showed earlier that BSA molecules coated onto a hydrophobic particle that is subsequently implanted into a mouse are digested and replaced with endogenous protein, most notably fibrinogen (16). Thus, whereas BSA molecules preadsorbed to oil droplets can, in the short term, block/limit the binding of endogenous fibrinogen, the latter protein ultimately coats the droplets *in vivo* yielding, in effect, fibrinogen-coated particles. It came as no surprise to us, then, that not only uncoated oil droplets but also BSA-coated droplets of docetaxel-loaded olive oil elicited a significant improvement over Taxotere (see Fig. 1). Using an immunoblotting technique, we found, as expected, that both uncoated droplets and BSA-coated droplets acquire *in vivo* a layer of endogenous

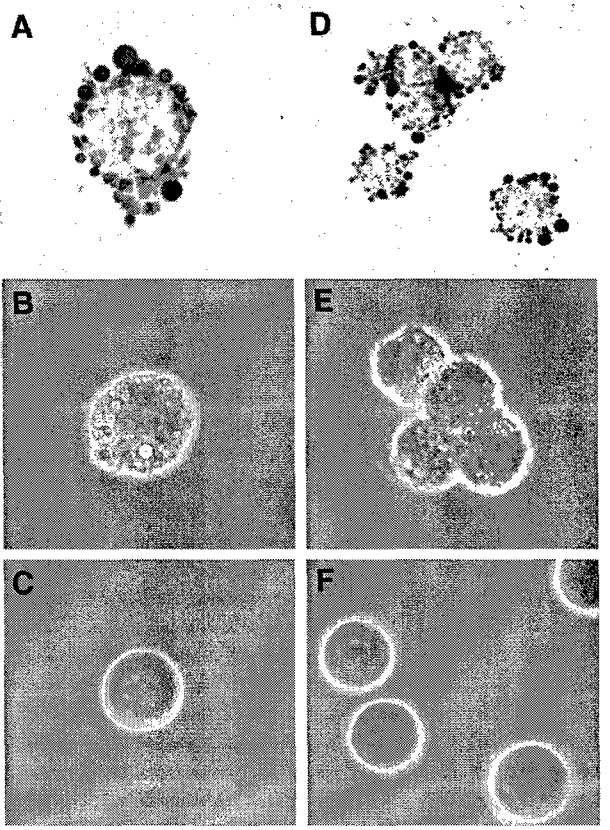

Fig. 4 Fibrinogen-coated droplets of docetaxel-loaded olive oil and/or TA3/St tumor cells recovered from oil droplet-treated, tumor-bearing mice. Tumor cells were washed extensively before plating them onto a slide for photomicroscopy. In *A* and *D*, oil droplets are stained with oil red O. *B*, *C*, *E*, and *F* are photos taken with phase-contrast microscopy of unstained oil droplets and/or cells. *A*, *B*, *D*, and *E* are photos taken of material recovered from tumor-bearing mice treated with only fibrinogen-coated, docetaxel-loaded oil droplets. As shown, tumor cells and fibrinogen-coated droplets from such animals adhere to each other, forming rosettes. Similar results are obtained even if docetaxel-loaded droplets are not precoated with fibrinogen before their administration. *C* and *F* are photos of material recovered from tumor-bearing mice treated with both hirudin and fibrinogen-coated, docetaxel-loaded oil droplets. The last dose of hirudin was given ~2 hours before the collection of ascites/lavage fluid. As shown, hirudin prevents the association of fibrinogen-coated oil droplets with the cells. See text for details.

F5 fibrin(ogen), Fig. 5*A*. Importantly, not only the droplets, but also the ascites tumor cells are coated with fibrin(ogen), Fig. 5*B*. Because fibrin molecules on juxtaposed microparticles can interact to cause the mutual adhesion of the particles (2, 14), our results suggest oil droplets are tethered to tumor cells by fibrin polymers that are themselves anchored to the two surfaces.

Production of Antifibrinogen Antibodies in Mice Treated with Olive Oil Droplets. Many proteins become immunogenic when they are bound to oil droplets or other hydrophobic particles (16, 17). For this reason, we looked for antifibrinogen antibodies in plasmas from healthy, tumor-free mice that had been treated with 4 weekly doses of olive oil droplets. One group of mice was treated with droplets that had been coated with human fibrinogen, another was treated with droplets that had been coated with murine fibrinogen, and another was treated with uncoated oil droplets. Untreated mice served as negative controls, and mice treated with Freund's incomplete adjuvant containing either human fibrinogen or murine fibrinogen served as positive controls. Whereas none of the mice in the untreated control group had demonstrable antibodies directed against fibrinogen of either murine or human origin, all of the mice in the positive control groups and all of the mice in the groups treated with fibrinogen-coated oil droplets had substantial amounts of antifibrinogen antibodies, Table 1. Although T1 significantly higher titers of antifibrinogen antibodies were AQ:A achieved when human fibrinogen was used as coating than when murine fibrinogen was used as coating ($P \leq 0.001$), the levels of antifibrinogen antibodies in both cases were appreciable. Perhaps not surprisingly, two of five mice treated with uncoated oil droplets had demonstrable, albeit low, levels of antibodies directed against murine fibrinogen. These findings prompted investigation of the biological consequences of antifibrinogen antibodies in mice treated with fibrinogen-coated oil droplets.

Effect of Droplet-Induced Antifibrinogen Antibodies on Survival of Tumor-Bearing Mice Treated with Taxotere. Because docetaxel administered in oil droplets conferred a significant benefit to survival in comparison to docetaxel delivered as Taxotere, we considered the possibility that antifibrinogen antibodies might somehow contribute to the therapeutic efficacy of docetaxel when the drug is administered in oil droplets. If so, then Taxotere administered to tumor-bearing mice with high titers of droplet-induced antifibrinogen antibodies might be expected to survive longer than tumor-bearing mice treated with Taxotere alone. To test this, Taxotere was administered both to tumor-bearing mice that had no demonstrable antifibrinogen antibodies and to age-matched, tumor-bearing mice in which high titers of antihuman fibrinogen antibodies had been elicited by the time of tumor cell inoculation. As shown in Fig. 6, the F6 presence of even very high levels of droplet-induced antifibrinogen antibodies did not prolong the survival of tumor-bearing animals beyond that yielded by Taxotere alone ($P = 0.26$). Such a result suggests antifibrinogen antibodies play no role in the therapeutic efficacy of docetaxel when the drug is delivered within fibrinogen-coated droplets of olive oil.

Coagulability of Plasma from Mice Treated with Fibrinogen-Coated Droplets of Olive Oil. Antibodies directed against fibrinogen, a protein critical to coagulation, might be expected to interfere with blood clotting *in vivo* and/or *in vitro*. In cancer patients, such interference would be undesirable. Although tumor-bearing mice treated with fibrinogen-coated oil droplets did not seem to be any more predisposed to hemorrhage than tumor-bearing mice treated otherwise, we could not, by direct observation alone, exclude the possibility of a more subtle coagulation defect in droplet-treated mice. In a preliminary attempt to determine what effect, if any, antifibrinogen antibodies elicited by fibrinogen-coated oil droplets have on coagulation, the aPTT's of healthy tumor-free droplet-treated mice ($n = 7$) were compared with those of healthy tumor-free untreated mice ($n = 6$). To ensure the presence of very high titers of antifibrinogen antibodies, the oil droplets used for the experiment were coated with human fibrinogen. Citrated plasmas were used to determine both antifibrinogen antibody titers and aPTT's. Whereas none of the plasmas from untreated mice had demonstrable antihuman fibrinogen antibodies, all of the plasmas from droplet-treated mice were positive for such antibodies, out to a dilution of one part in 8×10^7. Consistent with published data (18), the mean $\pm$ SD of the aPTT of untreated mice was 24.3 $\pm$ 3.9 seconds. The mean $\pm$ SD of the aPTT of droplet-treated mice was not significantly different, 21.2 $\pm$ 2 seconds ($P = 0.09$). Taken together, these results and our gross

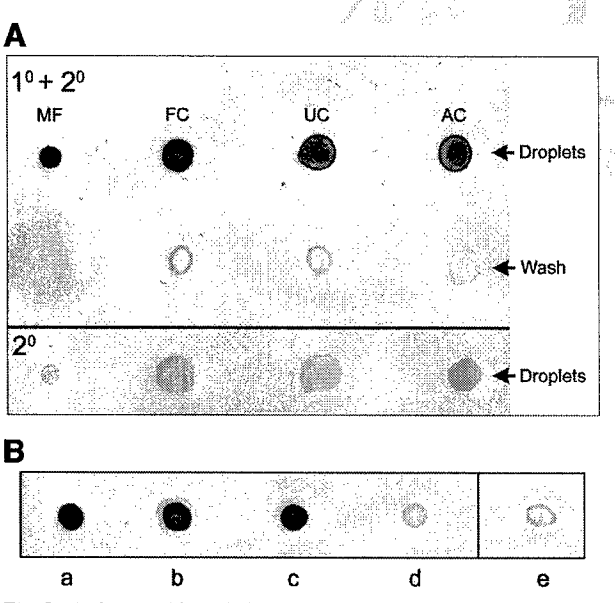

Fig. 5 *A*, immunoblot of fibrinogen bound to docetaxel-loaded oil droplets. Tumor-bearing mice were treated intraperitoneally with either murine fibrinogen-coated (FC), uncoated (UC), or BSA-coated (AC) droplets of docetaxel-loaded olive oil. One hour later, the droplets were recovered, washed extensively, and then immunostained for fibrinogen. Materials in the *top two rows* were exposed to both the 1° antibody and the AP-conjugated 2° antibody. Those materials included authentic murine fibrinogen (MF), FC oil droplets, UC oil droplets, AC oil droplets (*first row*); and the last washes of the oil droplets (*second row*). The materials in the *third row* (*i.e.*, MF, FC oil droplets, UC oil droplets, and AC oil droplets) were exposed to only the 2° antibody (as negative controls). See text for details. *B*, immunoblot of fibrinogen bound to TA3/St ascites tumor cells. Both 1° and AP-conjugated 2° antibodies were used to visualize murine fibrinogen in samples of *a*, authentic murine fibrinogen; *b*; intact ascites fluid; *c*, washed ascites tumor cells; and *d*, the last wash of the ascites tumor cells. In *e*, washed ascites tumor cells were exposed to only the AP-conjugated 2° antibody (as a negative control). See text for details.

Table 1 Antifibrinogen antibodies elicited by various oil preparations

	Last positive well	
	Anti-MF	Anti-HF
FIA/MF	3,3,3,3	NM
Oil droplets/MF	2,5,6,6,7,11	NM
FIA/HF	NM	21,21,22
Oil droplets/HF	NM	≥24,≥24,≥24,≥24
Oil droplets/uncoated	<1,<1,<1,<1,2,2	NM

NOTE. Mice in groups of three to six were treated intraperitoneally with one of the oil preparations listed in the left column. Depending upon the species from which the fibrinogen used in a formulation derived, the plasmas were assessed for antibodies directed against either MF or HF. Mice treated with uncoated (*i.e.*, fibrinogen-free) oil droplets were assessed for antibodies directed against MF. See text for details. For a given plasma, 2-fold serial dilutions were made, with the first well of a series being a 1:5, v/v, dilution. The numbers in the table refer to the last obviously positive wells of the plasmas of the mice in a group.

Abbreviations: FIA, Freund's incomplete adjuvant; NM, not measured; MF, murine fibrinogen; HF, human fibrinogen.

observations suggest antihuman fibrinogen antibodies elicited in mice by oil droplets coated with that protein do not interfere overtly with coagulation *in vitro* or *in vivo*.

DISCUSSION

In a previous paper, we described the preparation, characterization and anticancer activity of fibrinogen-coated droplets of docetaxel-loaded olive oil (6). We proposed there that the fibrinogen coating facilitated the retention of the particles within the fibrin(ogen)-rich tumor microenvironment, thereby increasing the therapeutic efficacy of the docetaxel with respect to that of Taxotere, the existing commercial formulation of the drug. In the present report, we used a fibrin(ogen)-rich, ascites tumor model to (*a*) extend our earlier studies on the therapeutic efficacy of the droplets, (*b*) show, by various means, the role of fibrin formation in that efficacy, and (*c*) document and explore immunologic consequences of the new therapy. We address these three items next.

If left untreated, mice bearing the ascites form of the TA3/St mammary tumor all die by 10 days. Whereas Taxotere prolongs the median survival of tumor-bearing mice by 53%, docetaxel delivered in microscopic droplets of olive oil prolong median survival by as much as 211% (*i.e.*, a 4-fold improvement over Taxotere). Several experiments indicate droplet-bound fibrinogen and its conversion to fibrin are responsible for the efficacy of docetaxel-loaded oil droplets. Briefly, maximal survival benefit is achieved when droplets are precoated with fibrinogen, and a measure that inhibits thrombin activity (*i.e.*, a measure that inhibits fibrinogen-to-fibrin conversion) reduces the survival benefit of docetaxel-loaded oil droplets to that of an equivalent dose of docetaxel administered as Taxotere. It is not surprising that even uncoated or BSA-coated droplets confer a hirudin-sensitive benefit to survival that is greater than that conferred by Taxotere, because endogenous fibrinogen coats those droplets, rendering them equivalent operationally to droplets precoated with fibrinogen. The survival benefits conferred by uncoated droplets and BSA-coated droplets are somewhat less than that conferred by droplets precoated with fibrinogen because in the former cases extraneous proteins limit the packing density of endogenous fibrinogen. We conclude that microscopic droplets of olive oil, particularly ones precoated with fibrinogen, are a more effective means by which to deliver docetaxel to the ascites form of the TA3/St mammary tumor than is Taxotere. It seems the conversion of droplet-bound fibrinogen to fibrin by thrombin acting locally facilitates the specific adherence of drug-loaded droplets to tumor cells and, perhaps, the general retention of droplets within the tumor microenvironment. We speculate that measurement of thrombin activity existing within malignant tissue may prove a useful means by which to identify tumors that might be most responsive to the new therapy.

Whether droplets are precoated with fibrinogen of human origin or of murine origin, they elicit in recipient mice a significant antibody response against the protein. Indeed, two of five mice treated with uncoated oil droplets developed measurable titers of antifibrinogen antibodies. Our observations and preliminary studies suggest such antibodies are of no obvious short term consequence in A/Jax mice bearing the ascites form of the TA3/St tumor; neither do they contribute to, nor do they detract from, the therapeutic benefit that derives from a single injection of docetaxel. But because even droplet-treated tumor-bearing A/Jax mice all succumb to tumor by 35 days, we are unable to make any claim regarding the long-term effects of the antibodies in those animals. It is conceivable such antibodies might limit the effectiveness of subsequent oil droplet treatments. Whereas that consideration was not apropos to the single treatment model used here by us (*i.e.*, antibodies or not, droplets of docetaxel-loaded olive oil provided a benefit to survival significantly greater that that provided by Taxotere), it would be relevant to treatment regimens in which multiple doses of drug-loaded oil droplets were administered over time. We note anecdotally that the antifibrinogen antibody response of droplet-treated C57BL/6

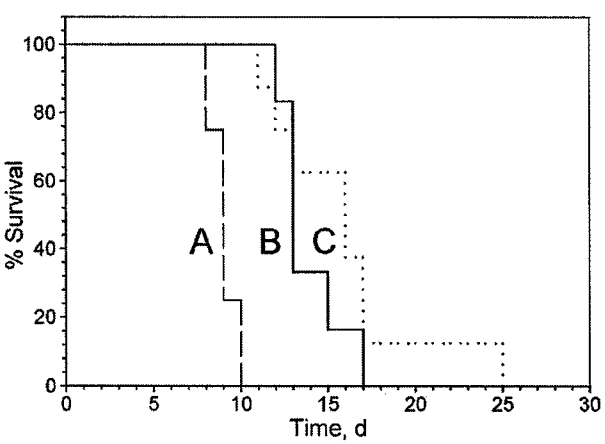

Fig. 6 Survival profiles of TA3/St tumor-bearing A/Jax mice treated with NS (*A*) or with Taxotere (*B* and *C*). The mice in groups *A* ($n = 4$) and *C* ($n = 4$) had no antifibrinogen antibodies preexisting in their plasma before treatment. Each mouse in group *B* ($n = 6$), however, had an exceedingly high titer (*i.e.*, positive past a plasma dilution of $1:8 \times 10^7$, v/v) of antihuman fibrinogen antibodies at the time of treatment. *B* and *C* are statistically different from *A* ($P \leq 0.001$) but not from each other ($P = 0.26$). See text for details.

mice is essentially the same as that of droplet-treated A/Jax mice.[8] Importantly, two of nine C57BL/6 "cured" of an intraperitoneal form of B16F10 melanoma by treatment with fibrinogen-coated droplets of docetaxel-loaded olive oil (6) had no demonstrable antifibrinogen antibodies 1 year after treatment.[8]

To be sure, much still needs to be done to delineate both the benefits and limitations of the new docetaxel formulation, in particular, and of the delivery vehicle, in general. To date, we have used fibrinogen-coated droplets of docetaxel-loaded olive oil to treat only tumors that are, by intention, confined to the abdominal cavity. Whereas we suspect tumors confined to other closed spaces (*e.g.*, the pleural cavity, pericardial sac, or brain case, will benefit similarly from treatment with the droplets), we have not yet tested that suspicion. Neither have we tested the efficacy of docetaxel-loaded droplets against tumors that either are located within a specific organ or are widely metastatic. We do know that, after their intravenous administration, fibrinogen-coated oil droplets are retained in organs of the reticuloendothelial system (3). Thus, intravenous administration might be especially good for the delivery of droplets to tumors in organs that typically harbor metastatic disease. Regardless, we are now developing smaller versions of fibrinogen-coated oil droplets that might better circulate and percolate through tissues. We are also testing the suitability of fibrinogen-coated oil droplets for the delivery of a number of other lipophilic anticancer agents, both individually and in combination. Eventually, we hope to develop a single formulation that delivers several mechanistically dissimilar agents.

For many reasons, cancer patients are predisposed to thrombosis (19), and thus they occasionally need to be treated with an anticoagulant or even a fibrinolytic agent. Our results suggest concurrent administration of such agents with fibrinogen-coated droplets of docetaxel-loaded olive oil might reduce the therapeutic efficacy of the droplets. On the other hand, if anticoagulants and fibrinolytics reduce the therapeutic efficacy of the droplets, then perhaps pro-coagulants and antifibrinolytics might improve efficacy. Clearly, before the full potential of fibrinogen-coated oil droplets as delivery vehicles for lipophilic anticancer agents can be realized, much more needs to be done to delineate the influence of coagulation-related medicinals on the new modality.

The use of docetaxel in its current clinical formulation is attended by several adverse side effects attributable to either the drug itself (*e.g.*, fluid retention, neurotoxicity, musculoskeletal toxicity and neutropenia; ref. 20), or to the solvent system used in the drug's commercial preparation (*e.g.*, hypersensitivity; ref. 21). One would hope any new formulation of docetaxel would not only better target the drug to malignant tissue but would also be attended by fewer or less severe side effects. Although we addressed some toxicity issues in the studies presented here, we focused more on the anticancer efficacy of the new formulation and on understanding the basis of that efficacy than on adverse side effects. We hope in the near future to compare specific indices of toxicity (*e.g.*, neutrophil count) after treatment of mice with docetaxel-loaded oil droplets to those same indices after treatment with Taxotere.

Aside from their ability to target tumors as sites of fibrin formation, fibrinogen-coated oil droplets (or other fibrinogen-coated microparticulates) might be ideally suited to delivering anticancer agents to malignancies for yet another reason. Work in one of our laboratories supports the hypothesis that macrophages transport particulate materials and even tumor cells in fibrinogen-dependent extracellular fashion (22),[9] and one of us has proposed that metastasis is, at least in part, a manifestation of extracellular trafficking of tumor cells by macrophages (22, 23). If the macrophage-directed "traffic patterns" of particulate materials and metastatic tumor cells are the same, then it is not unreasonable to suppose anticancer agents delivered as/within fibrinogen-coated particulate materials might be exploited to target metastatic disease.

ACKNOWLEDGMENTS

G. Retzinger thanks Ruth Mary Retzinger for inspiration.

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