CSP

Prevention of local tumor growth with paclitaxel-loaded microspheres

Solomon M. Azouz, MS,[a] Joseph Walpole, BS,[a] Sepideh Amirifeli, MD,[a] Kendra N. Taylor, PhD,[a] Mark W. Grinstaff, PhD,[b] and Yolonda L. Colson, MD, PhD[a]

Objectives: Lung cancer is associated with a significant rate of locoregional recurrence after surgical resection, particularly when nonanatomic wedge resections are performed. The primary aim of this study was to assess the feasibility of a microsphere drug delivery system to locally deliver chemotherapy and prevent the establishment and growth of lung cancer cells and establish proof of concept for a potential future approach to target occult microscopic disease remaining at the surgical resection margin.

Methods: Poly-(D,L-lactic-co-glycolic acid) (PLGA) microspheres loaded with the antineoplastic agent paclitaxel were prepared and tested for antitumor efficacy in an in vitro cell proliferation assay for tumor inhibition and induction of apoptosis. The in vivo prevention of Lewis lung carcinoma cell establishment and growth in subcutaneous tissues of mice was also assessed by comparing 4 treatment groups: Lewis lung carcinoma cells alone, Lewis lung carcinoma cells combined with 100×10^6 unloaded (carrier alone) PLGA microspheres, and Lewis lung carcinoma cells combined with 50×10^6 or 100×10^6 paclitaxel-loaded PLGA microspheres. After the coinjection of Lewis lung carcinoma cells with or without microspheres, in vivo tumor growth was monitored, and tumor weight was recorded on death.

Results: Paclitaxel-loaded PLGA microspheres were found to effectively prevent growth of tumor cells in culture through the induction of apoptosis. Similarly, paclitaxel-loaded PLGA microspheres significantly inhibited tumor growth in vivo at both the 50×10^6 and 100×10^6 microsphere dose (0.497 ± 0.183 and 0.187 ± 0.083 g total tumor weight, respectively) compared with 2.91 ± 0.411 g for Lewis lung carcinoma cells with unloaded microspheres and 3.37 ± 0.433 g for untreated tumor ($P < .001$). Toxicity was not clinically apparent in any animal treated with paclitaxel-loaded PLGA microspheres.

Conclusions: Paclitaxel-loaded PLGA microspheres induce tumor apoptosis and inhibit the establishment and growth of lung cancer cells both in vitro and in vivo without obvious systemic toxicity. By using models consistent with localized microscopic tumor burdens, these results suggest that local delivery of paclitaxel through a microsphere system might lead to an effective future method of decreasing local tumor recurrence in non–small cell lung cancer when applied to the surgical margins at risk for microscopic tumor foci. Such an approach might be particularly efficacious after wedge resection in the setting of poor pulmonary reserve or significant comorbidity, where local recurrence rates are increased and acceptable alternative treatment options are limited.

From the Division of Thoracic Surgery, Brigham and Women's Hospital,[a] Boston, Mass; and the Departments of Biomedical Engineering and Chemistry, Boston University,[b] Boston, Mass.

Received for publication Oct 24, 2007; revisions received Dec 18, 2007; accepted for publication Dec 22, 2007.

Address for reprints: Yolonda L. Colson, MD, PhD, Brigham and Women's Hospital, 75 Francis St, Boston, MA 02115 (E-mail: ycolson@partners.org).

J Thorac Cardiovasc Surg 2008;135:1014-21

0022-5223/$34.00

doi:10.1016/j.jtcvs.2007.12.011

The Lung Cancer Study Group demonstrated that the locoregional recurrence rate in patients with stage I lung cancer (T1 N0 M0) is increased 3-fold after limited resections compared with that after lobectomy.[1] Landreneau and colleagues[2] demonstrated similar results in which local recurrence rates increased from 9% after lobectomy to 24% when more limited wedge resections were performed because of poor pulmonary reserve. Previous approaches aimed at decreasing local

Abbreviations and Acronyms

BSA = bovine serum albumin
DMEM = Dulbecco's modified Eagle's medium
DMSO = dimethyl sulfoxide
ELISA = enzyme-linked immunosorbent assay
FBS = fetal bovine serum
IC_{50} = amount of paclitaxel necessary to inhibit the growth of lung cancer tumor cells by 50%
LLC = Lewis lung carcinoma cell
MTT = 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide
Pax-PLGA = paclitaxel-loaded poly-(D,L-lactic-co-glycolic acid)
PI = propidium iodide
SEM = scanning electron microscopy

recurrence rates after lung resection have used external radiation treatment or placement of radioactive seeds along the stapled surgical margin but have met with limited success because of concerns about radiation risks to both the patient and operating personnel, impaired postoperative healing, and increased radiation fibrosis to the bystander lung tissue.[3,4] Similarly, the toxicity of systemic chemotherapy has outweighed the potential benefit of improved local control in this select population. Therefore the current therapy of choice for local tumor recurrence is repeat surgical resection when possible. However, because of tumor location and patient morbidity, only one third of patients are candidates for resection, and if distant recurrence develops, less than 10% of patients will have their disease controlled. More than half of all deaths after resection are attributed to recurrent disease.[5,6]

Therefore the prevention of tumor recurrence at the suture line, especially when patients cannot tolerate a lobectomy or further lung resection, has the potential to improve surgical management of lung cancer in the future. The overall goal of this study was to evaluate the feasibility of a delayed drug delivery system to prevent the local growth and establishment of lung cancer cells in vivo, so as to assess the potential for future intraoperative application to achieve distribution of antineoplastic agents to sites at increased risk for occult microscopic malignant disease. In this article we have established proof of concept for this approach by successfully demonstrating that bioabsorbable 1- to 5-μm-diameter paclitaxel-loaded poly-(D,L-lactic-co-glycolic acid) (Pax-PLGA) microspheres exhibit delayed drug release as quantified by using a paclitaxel enzyme-linked immunosorbent assay (ELISA), induce apoptosis in lung cancer tumor cells in vitro, and prevent the local growth and establishment of lung cancer tumor cells in vivo.

Materials and Methods

The Harvard School of Public Health Standing Committee on Animals approved this animal study. All animals received humane care in compliance with the "Guide for the care and use of laboratory animals" published by the National Institutes of Health.

Cell Culture

The murine Lewis lung carcinoma cell (LLC) line was a gift from J. Folkman (Children's Hospital, Boston, Mass). LLCs were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (all reagents from Invitrogen, Carlsbad, Calif) at 37°C in a 5% CO_2 humidified atmosphere, and greater than 95% viability was confirmed before use.

Preparation of PLGA microspheres

The fabrication techniques used to create the PLGA microspheres were modified from Edlund and Albertsson[7] and Wang and coworkers.[8] Briefly, 0.5 g of 75:25 PLGA (Absorbable Polymer International, Birmingham, Ala) was dissolved in 4 mL of dichloromethane (Sigma–Aldrich, St Louis, Mo), and 42.5 mg of paclitaxel (MP Biomedical, Irvine, Calif) solubilized in 85 μL of dimethyl sulfoxide (DMSO; Sigma–Aldrich) was added and vortexed. Only DMSO was added for unloaded control PLGA microspheres. The polymer solution was placed in 10 mL of 5% polyvinyl alcohol (Spectrum, Gardena, Calif), vortexed, and stirred overnight. The microspheres were collected and washed 3 times in 50 mL of deionized water before being lyophilized and stored at −35°C.

Scanning Electron Microscopy

Several million spheres were diluted in diH_2O, placed on a silicon slide, and allowed to dry overnight before coating with gold. Scanning electron microscopy (SEM) was performed at 40 kV by using a JSM 6400 Scanning Electron Microscope at ×75 and ×750 objective (JEOL, Tokyo, Japan).

Paclitaxel ELISA

The concentration of paclitaxel released from microspheres into the surrounding media was assessed by using a solid-phase competitive inhibition ELISA kit (Hawaii Biotech, Aiea, Hawaii). One million microspheres suspended in 50 μL of minimal essential medium (Cellgro, Herndon, Va) were placed on a 0.2-μm Anopore membrane tissue culture insert (Nalge Nunc, Rochester, NY) in 150 μL of media. Media were removed for analysis after 1, 3, 5, 7, 10, 15, and 20 days, with replacement of media in the bottom well to ensure a constant concentration gradient. Anti-paclitaxel antibody with bound free paclitaxel was washed from the plates, and remaining anti-paclitaxel antibody was bound to alkaline phosphatase–labeled anti-mouse immunoglobulin conjugate. The resultant color change was measured by using a VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, Calif) at an absorbance of 405 nm, with greater intensity correlating to less competitive inhibition and thus lower paclitaxel concentrations.

In Vitro Tumor Cell Proliferation Assays

Inhibition of LLC cell growth was determined by using an MTT (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide) cell proliferation assay. Briefly, 3×10^3 per well LLCs were seeded in 96-well plates in DMEM without FBS. After being serum starved overnight, the cells were treated with paclitaxel alone, control PLGA microspheres, or Pax-PLGA microspheres in fresh media containing

10% FBS. Positive and negative controls, with or without 10% FBS, were present in each experiment. At 3, 5, and 7 days, individual wells were treated for 2 hours at 37°C with 50 μL of thiazolyl blue tetrazolium bromide (MTT, Sigma–Aldrich) dissolved in PBS (Sigma–Aldrich). Absorbance values measured at 570 nm were normalized to a standard curve to determine the number of viable cells in situ.

Apoptosis Assay

Induction of apoptosis was assessed by means of propidium iodide (PI) staining, followed by flow cytometric analysis, as described previously.[9,10] One million LLC cells alone, LLCs with 2×10^6/mL Pax-PLGA microspheres or control PLGA microspheres, or LLCs in media containing 100 ng/mL paclitaxel were incubated for 4 days in DMEM containing 10% FBS before harvesting. After washing in PBS–0.05% bovine serum albumin (BSA; Fisher Scientific, Houston, Tex), cells were fixed in 3 mL of 70% ethanol on ice for 2 hours, centrifuged at 400*g*, washed with cold PBS–BSA, and resuspended in 300 μL of PI staining solution containing 1 μg/mL RNAase A (ABgene, Rochester, NY) and 1.6 μg/mL PI (Sigma–Aldrich) dissolved in PBS–BSA. Cells were incubated for 30 minutes at 37°C before flow cytometric analysis was performed, whereby cells containing fragmented DNA were detected and quantified as apoptotic.

In Vivo Model of Subcutaneous Tumor Growth

C57BL/6J (B6) female mice (Jackson Laboratory, Bar Harbor, Me) of 18 to 22 g were anesthetized by means of isoflurane inhalation in all experiments. LLCs (7.5×10^5) were coinjected into the subcutaneous tissues of the upper back of B6 mice with 100×10^6 unloaded PLGA microspheres, Pax-PLGA microspheres (50×10^6 and 100×10^6 doses), or 100 μL of PBS alone. Paclitaxel-loaded microspheres were kept separate from the LLC tumor cells until immediately before loading of each individual syringe. This method ensured that tumor cells and microspheres were colocalized at the injection site similar to the approximation of microspheres and occult microscopic disease at the resection margin. To control for the effects of paclitaxel exposure during injections, control samples of LLCs were also coinjected with 2.5 μg of paclitaxel (greater than the amount of paclitaxel necessary to inhibit the growth of lung cancer tumor cells by 50% [IC_{50}]) suspended in 100 μL of Cremophor EL [BASF Corporation, Florham Park, NJ]/ethanol (50/50 by volume, as is done clinically). Animals were monitored for associated chemotherapy toxicity and weight loss. After 3 weeks, mice were killed, and a blinded observer assessed tumor weight and animal toxicity.

Statistics

Data are expressed as means ± standard error unless otherwise indicated. Differences between treatment groups were evaluated by using the Student *t* test.

Results

Pax-PLGA Microspheres

Pax-PLGA microspheres were prepared as detailed above and analyzed with SEM for size, porosity, and integrity. SEM revealed that microspheres were intact spheres of uniform shape ranging in size from 1 to 5 μm in diameter (Figure 1). There was little evidence of porosity, thus suggesting the capacity for prolonged drug release over an extended period of time. A greater than 90% encapsulation efficiency of paclitaxel within Pax-PLGA microspheres was confirmed through high-performance liquid chromatography analysis.

Pax-PLGA Microspheres Exhibit High-dose, Long-term Release of Paclitaxel

Timely and sufficient drug release is critical for effective antitumor activity. In vitro MTT cell proliferation assays with tumor LLCs were performed with growth in 10% and 0% serum, serving as positive and negative controls, respectively, to determine the IC_{50}. The IC_{50} for growth of tumor LLCs incubated with paclitaxel alone was approximately 10 ng/mL (Figure 2). This was observed for the 3-, 5-, and 7-day assays and is indicative of the estimated paclitaxel dose that must be released from Pax-PLGA microspheres to inhibit LLC tumor growth. Complete inhibition of tumor growth required higher doses of paclitaxel, as evidenced by minimal to no growth at paclitaxel concentrations of greater than 100 ng/mL.

The kinetics of paclitaxel release over 20 days was measured by using a quantitative paclitaxel ELISA assay to determine whether sufficient levels of paclitaxel were released from Pax-PLGA microspheres. Figure 3 shows that Pax-PLGA

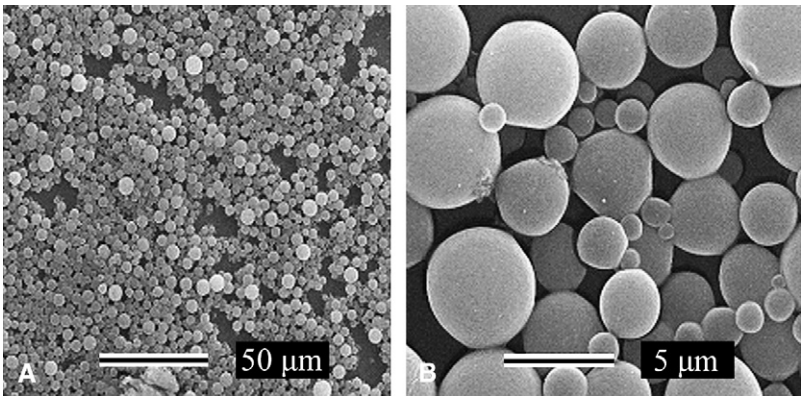

Figure 1. Poly-(D,L-lactic-co-glycolic acid) microsphere characteristics imaged by means of SEM. A, Microspheres are of uniform shape (×75 magnification). B, SEM imaging reveals microspheres to be 1 to 5 μm in diameter (×750 magnification).

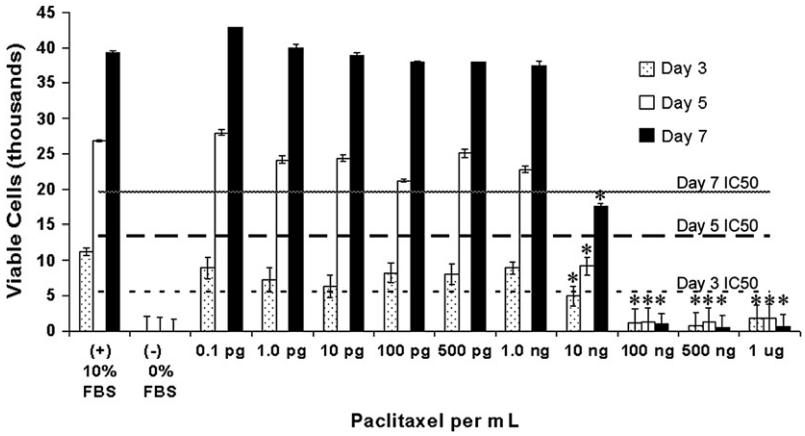

Figure 2. Dose–response curve for in vitro inhibition of tumor cell growth by paclitaxel. Three-, 5-, and 7-day proliferation assays demonstrating effective growth inhibition of LLCs when paclitaxel is added to the media are shown. Viable cell numbers are the average of 8 wells in this representative experiment (n = 3). The plotted IC_{50} values are the concentration at which cell growth is inhibited by 50% compared with the positive control. *Error bars* reflect the standard error of the mean. *Statistical significance versus positive control: $P < .01$ by Student *t* test. *FBS*, Fetal bovine serum.

microspheres demonstrate an initial burst effect that results in 6.8 μg/mL of paclitaxel delivered within the first 5 days of incubation. However, after 10 days, release becomes more steady, with an increase in concentration of approximately 1.4 μg/mL per 5-day incubation period. At 20 days, the cumulative release of paclitaxel from 1×10^6 Pax-PLGA microspheres was 11.9 μg/mL. These results demonstrate that Pax-PLGA microspheres exceed the dose of paclitaxel release required for LLC cytotoxicity by nearly 1000-fold.

Dose-dependent Inhibition of LLC Proliferation In Vitro Through Pax-PLGA Microspheres

LLC cells (3×10^3) per well were grown in 10% serum and incubated with increasing doses of Pax-PLGA microspheres to test the hypothesis that Pax-PLGA microspheres can effectively prevent growth of lung cancer tumor cells because of paclitaxel release,. Tumor viability was determined by using the standard MTT assay after coincubation for 3, 5, and 7 days. As evident in Figure 4, *A*, cultures treated with Pax-PLGA microspheres demonstrated a marked dose-dependent inhibition of tumor growth. Although cell numbers are small in the day 3 positive control cultures (10% serum), the number of viable tumor cells that remain after coculture with all doses of Pax-PLGA microspheres is less than the day 3 IC_{50} because of effective early growth inhibition. This early effect on tumor cell growth is likely the result of the initial burst effect, at which even a concentration of 0.3 Pax-PLGA microspheres per tumor cell releases sufficient paclitaxel (10.4 ng/mL) to exceed the 10 ng/mL paclitaxel concentration required for tumor inhibition at day 3 (Figure 4, *B*). However, similar to the results obtained with paclitaxel alone, more definitive prevention of tumor cell growth in day 5 and day 7 cultures required an initial local paclitaxel concentration of greater than 100 ng/mL. Given the delayed release of paclitaxel from Pax-PLGA microspheres, Figure 4, *B*, demonstrates that early paclitaxel concentrations do not exceed 100 ng/mL until concentrations of 4 to 8 microspheres per tumor cell ($12–24 \times 10^3$ per well) are used, and this coincides with the complete prevention of tumor growth noted in vitro at concentrations of 8 or more Pax-PLGA microspheres per tumor cell ($P < .001$; Figure 4, *A*).

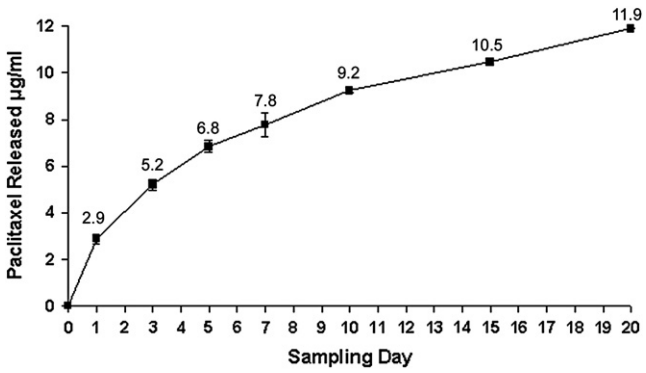

Figure 3. Cumulative paclitaxel release from poly-(D,L-lactic-co-glycolic acid) (Pax-PLGA) microspheres quantitatively determined by means of paclitaxel enzyme-linked immunosorbent assay. After Pax-PLGA microspheres release an initial burst of drug, consistent release is maintained for at least 20 days. *Error bars* represent the standard deviation of the mean. These data are the result of 3 separate experiments.

Unloaded PLGA Microspheres Alone Are Not Cytotoxic

Unloaded PLGA microspheres containing only the DMSO carrier were coincubated with tumor LLCs in a standard MTT proliferation assay for 3, 5, and 7 days to establish that tumor growth was not inhibited due to the PLGA microspheres alone. As shown in Figure 5, inhibition of tumor growth did not occur with unloaded PLGA microspheres alone, demonstrating that tumor cytotoxicity was not due to the polymer itself. In addition, control PLGA microspheres treated for 5 days with MTT did not bind MTT nonspecifically, and thus the polymer did not interfere with calculations of tumor cell viability (data not shown).

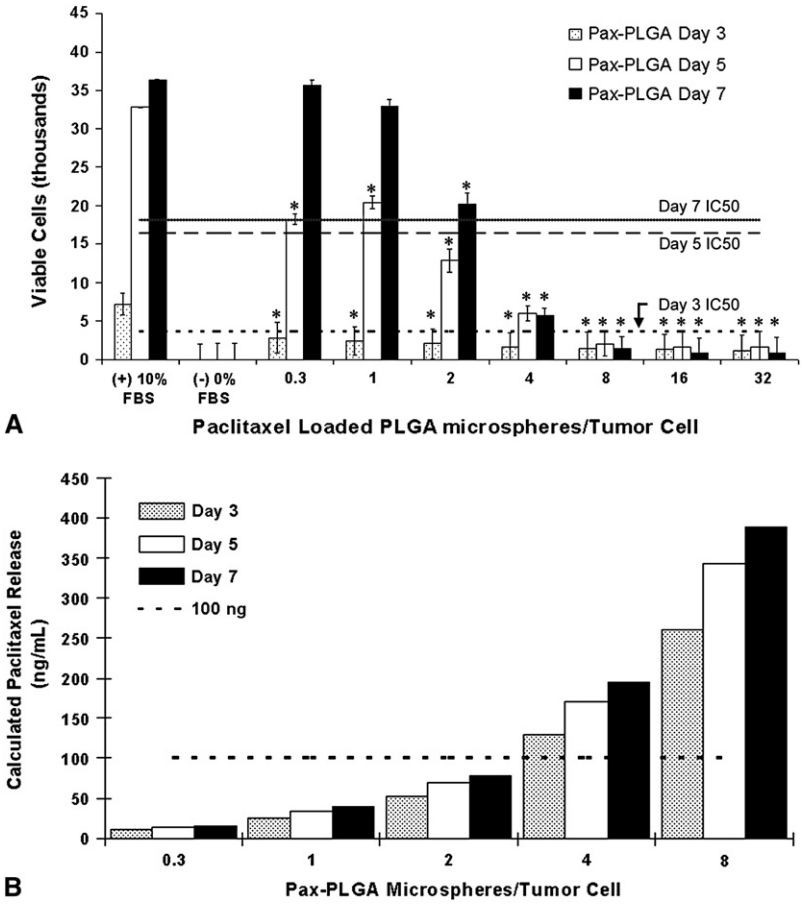

Figure 4. Dose–response curve for paclitaxel-loaded poly-(D,L-lactic-co-glycolic acid) *(Pax-PLGA)* microsphere inhibition of lung cancer growth in vitro. A, Proliferation assay demonstrating that effective growth inhibition of LLCs by paclitaxel microspheres occurs at concentrations of greater than 8 microspheres per tumor cell. The plotted IC_{50} values represent 50% of the proliferation of the positive control proliferation from the specified day. *Error bars* for viable cell numbers represent the standard error of the mean for 8 wells. These data are representative of 3 separate experiments. *$P < .001$ compared with the positive control. B, Estimated paclitaxel released from Pax-PLGA microspheres based on ELISA drug release curve at days 3, 5, and 7. *FBS*, Fetal bovine serum.

Pax-PLGA Microspheres Induce Apoptosis of Tumor Cells In Vitro

LLC morphology was examined after incubation with one million Pax-PLGA microspheres versus unloaded PLGA microspheres per milliliter of media to determine whether tumor apoptosis was induced by coculture with Pax-PLGA microspheres. As shown in Figure 6, *A*, phase contrast microscopy of tumor LLCs after a 4-day coculture with Pax-PLGA microspheres was consistent with significant LLC apoptosis, as evident by membrane blebbing, chromatin condensation, and haloing. These changes were not evident in LLCs cultured with control unloaded PLGA microspheres (Figure 6, *B*). Similarly, cell-cycle analysis of LLCs by using PI flow cytometric staining demonstrated a significant increase in the percentage of apoptotic LLCs after a 4-day coculture with Pax-PLGA microspheres or paclitaxel alone compared with LLCs cultured with control PLGA microspheres or media alone ($P < .05$; n = 3; Figure 6, *C*).

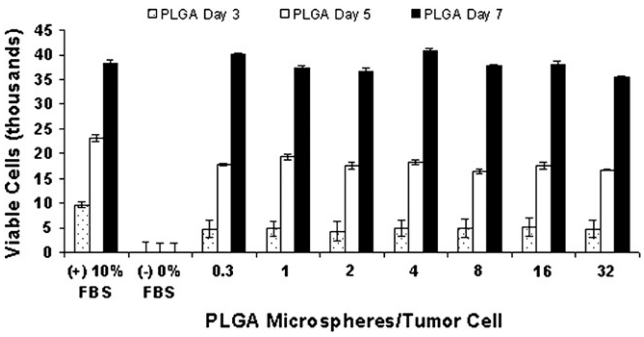

Figure 5. Poly-(D,L-lactic-co-glycolic acid) *(PLGA)* microspheres alone are not cytotoxic. Proliferation assay over 3, 5, and 7 days demonstrating no effective growth inhibition by control PLGA microspheres. *Error bars* represent the standard error of the mean for viable cell numbers in 8 wells. These data are representative of 3 separate experiments. *FBS*, Fetal bovine serum.

Pax-PLGA Microspheres Inhibit Tumor Growth In Vivo

In vivo efficacy of Pax-PLGA microspheres was assessed in a murine subcutaneous tumor model, whereby injection of 750,000 tumor LLCs results in the development of palpable subcutaneous tumor nodules within 1 week, with rapid tumor growth requiring death within 2 to 3 weeks.[11] In this model

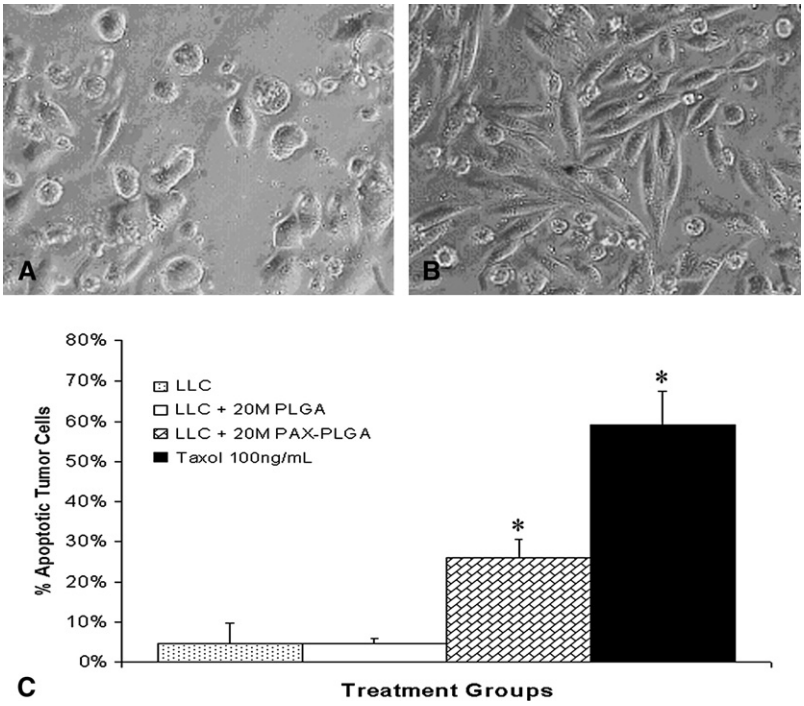

Figure 6. Propidium iodide *(PI)* staining apoptosis analysis. Phase contrast microscopy of day 4 LLC cultures in the presence of paclitaxel-loaded poly-(D,L-lactic-co-glycolic acid) *(PAX-PLGA)* microspheres (A) or control poly-(D,L-lactic-co-glycolic acid) *(PLGA)* microspheres (B) before PI staining is shown (×10 magnification). C, Percentage of tumor LLCs with apoptotic genomic DNA, as determined by means of flow cytometric propidium iodide staining and cell-cycle analysis, after 4-day coculture with Pax-PLGA or control PLGA microspheres.

the subcutaneous injection of tumor LLCs with 50×10^6 or 100×10^6 Pax-PLGA microspheres significantly inhibited tumor growth (0.497 ± 0.183 and 0.187 ± 0.083 g) compared with 2.91 ± 0.411 g for LLCs with control unloaded PLGA microspheres and 3.37 ± 0.433 g for LLCs alone ($P < .001$, Figure 7). In fact, more than 70% of the animals treated with Pax-PLGA microspheres had minimal to no evidence of tumor implantation or growth after coinjection of 100×10^6 Pax-PLGA microspheres. No mice coinjected with LLCs and paclitaxel in Cremophor EL/ethanol exhibited significant inhibition of tumor growth compared with that seen in untreated control animals ($P = .175$, n = 6). These findings indicate that the brief exposure of LLCs to paclitaxel within the syringe during injection was not responsible for preventing primary tumor establishment in animals receiving Pax-PLGA. Furthermore, toxicity was not clinically apparent in any animals treated with Pax-PLGA microspheres, as witnessed by changes in hair loss, weight, appetite, or activity level. Thus locally delivered chemotherapy through Pax-PLGA microspheres can significantly reduce the growth and establishment of lung carcinoma in vivo in this subcutaneous model of microscopic malignant disease, without clinical evidence of systemic toxicity.

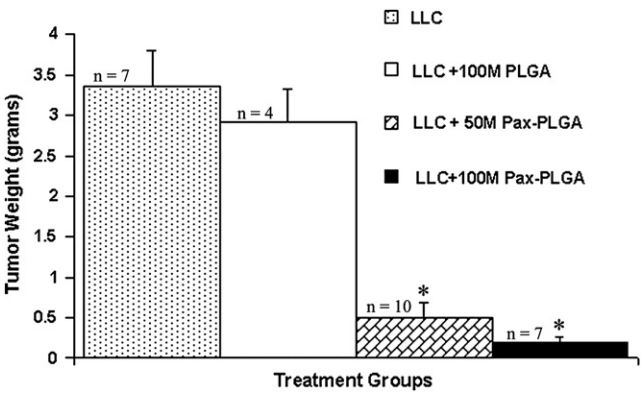

Figure 7. In vivo inhibition of tumor growth in the presence of paclitaxel-loaded poly-(D,L-lactic-co-glycolic acid) *(Pax-PLGA)* microspheres. Tumor weight at 3 weeks after subcutaneous injection of 750,000 tumor LLCs alone, tumor with unloaded poly-(D, L-lactic-co-glycolic acid) *(PLGA)* microspheres, and tumor with low or high doses of Pax-PLGA microspheres in C57BL/6 mice are shown. *Error bars* represent the standard error of the mean. *$P < .001$ compared with LLCs alone by means of the Student *t* test.

Discussion

Surgical resection is well established as the standard of care in the treatment of early-stage non–small cell lung cancer. However, depending on the size of the tumor and the extent of surgical resection, local recurrence rates range from 9% to 24%.[1,2,5,6,12] Although sublobar resection, compared with lobectomy, appears to be associated with lower perioperative morbidity and mortality with better preservation of lung function,[13] the risk of local recurrence is directly related to the distance from the tumor to the surgical resection margin and is increased after limited wedge resection or when the surgical

margin is less than 1 cm from the tumor.[14] Unfortunately, many patients with limited pulmonary reserve cannot tolerate a lobectomy, yet wide margins are not possible given tumor size or anatomic location. In cases in which margins are less than 1 cm, El-Sherif[14] demonstrated that local recurrence was nearly twice that of patients in which the margin was greater than 1 cm, with a mean follow-up of only 20 months. Although local recurrence rates as low as 6.25% have been reported after wedge resection and external radiation treatment, patients were followed for only 7 months, the radiation field includes normal bystander lung tissue, and a comparison with equivalent surgical patients not treated with radiation therapy was not included, making it difficult to assess the benefits of intraoperative radiotherapy versus the risk of radiation exposure to both the patient and surgical staff.[3] Similarly, Lee and colleagues[4] used I^{125}-labeled brachytherapy seeds secured to the resection margin and achieved a local recurrence rate of 5.71% with a more extensive follow-up of 34 months; however, application of I^{125}-labeled seeds and the safety issues associated with the use and handling of a radioactive material were both difficult and cumbersome. Furthermore, only patients with "a minimum gross margin of grater than 1 cm" were included in the study, thereby eliminating those patients at greatest risk for local recurrence.

Given the challenge posed by recurrent disease at the surgical margin, the current article demonstrates that locally delivered chemotherapy through microspheres deterred the growth and establishment of lung carcinoma in both in vitro and in vivo tumor models used for assessing the growth potential of small tumor burdens. Furthermore, Pax-PLGA microspheres effectively release active drug and induce apoptosis in lung cancer cells at the site of delivery. As such, these results support the concept of potentially using a microsphere drug delivery system to locally treat microscopic tumor foci at the lung resection margin. Paclitaxel ELISA demonstrated a considerable burst effect previously observed in PLGA microspheres[8] that might be beneficial in early targeting of "in-transit" micrometastatic disease near the resection margin. It is clear, however, that effects on healing of the resection site in the setting of locally delivered chemotherapy will require critical assessment, particularly because breakdown of the polymer into lactic and glycolic acid monomers[15] could produce an inflammatory response and inhibit wound healing. These potential deleterious effects are minimized by the use of very small doses of polymer and drug at a given site. Subcutaneously injected mice have shown no evidence of skin irritation, hair loss, malaise, weight loss, or associated toxicity after exposure to either the unloaded PLGA or Pax-PLGA microspheres for several weeks, suggesting that the delayed nature of polymer breakdown and drug release might mitigate this risk.

Previous studies have attempted to treat large established tumors by directly injecting high doses of paclitaxel-loaded microspheres[16] or nanospheres[17,18] into the primary tumor. The limited success of this approach is likely due to the inability to reach adequate drug levels at all parts of the tumor, given the local diffusion of drug from the spheres. In contrast, this article proposes that a microsphere drug delivery system could potentially be used to treat microscopic tumor foci in a small defined area by locally delivering effective doses of paclitaxel directly to a limited number of occult tumor cells present within close proximity to the surgical margin. Continuous release of paclitaxel from the described Pax-PLGA microspheres suggests that tumor growth and establishment could be prevented when microspheres are localized and deliver paclitaxel at sufficient concentration to result in tumor cell death. The in vitro and in vivo studies presented here suggest that prevention of tumor growth is possible, with a readily achievable concentration of 10 to 100 microspheres per anticipated tumor cell. Concentrating delivery of antineoplastic drugs within 1 cm of the surgical margin can thereby ensure sufficient local drug levels within the area of the lung with the highest risk of local recurrence while requiring significantly less total drug than would be necessary after systemic administration. This is particularly true because previous murine studies have demonstrated that systemically administered paclitaxel results in limited amounts of chemotherapy being delivered to lung tissue.[19] Less than 0.4% of the systemically administered dose of paclitaxel was detectable in lung tissue 24 hours after intravenous injection, whereas the paclitaxel concentration was 3 to 13 times greater in the liver and small intestine.[19] Additionally, 40% of paclitaxel is excreted within 24 hours, further limiting the exposure of occult microscopic disease at the surgical margin in the lung to paclitaxel.[20] These findings demonstrate that minimal amounts of systemically administered paclitaxel are delivered to the site at greatest risk for lung cancer recurrence, the surgical resection margin. In contrast, Pax-PLGA microspheres release drug locally and continuously for more than 20 days. Subcutaneous injection of Pax-PLGA microspheres and lung cancer cells demonstrates that one hundred million Pax-PLGA microspheres delivers a cumulative equivalent dose of 238 μg of paclitaxel per mouse over 3 weeks at the site of microscopic disease and reliably inhibits the local establishment and growth in vivo. This total dose is comparable with the 10 mg/kg systemic dose commonly used in mouse recipients and remains well below the murine maximum tolerated dose of 1000 μg.[21] These results provide strong evidence that localization of Pax-PLGA microspheres at the surgical resection margin will reliably prevent local tumor recurrence.

The current study establishes the feasibility and proof of concept that Pax-PLGA microspheres can locally delivery chemotherapy and effectively prevent tumor cell growth and establishment in a localized area. Nevertheless, investigations must be performed to study the long-term drug release profile of the microspheres and the effective radius of tumoricidal effect caused by released drug before human application is explored. Understanding the kinetics of drug

release from polymer scaffolds and the potential migration of microspheres are both key features of ongoing study of drug delivery systems. In addition, subsequent projects are now in progress to study the effect of drug-eluting polymers on wound healing and to optimize the localization of polymers to the resection line. This proof of concept has provided a foundation for multiple research venues with the ultimate goal of decreasing local recurrence rates after the surgical resection of early stage lung cancers.

References

1. Ginsberg RJ, Rubinstein LV. Randomized trial of lobectomy versus limited resection for T1 N0 non-small cell lung cancer. *Ann Thorac Surg*. 1995;60:615-23.
2. Landreneau RJ, Sugarbaker DJ, Mack MJ, Hazelrigg SR, Luketich JD, Fetterman L, et al. Wedge resection versus lobectomy for stage I (T1 N0 M0) non-small-cell lung cancer. *J Thorac Cardiovasc Surg*. 1997;113: 691-700.
3. Miller JI, Hatcher CR Jr. Limited resection of bronchogenic carcinoma in the patient with marked impairment of pulmonary function. *Ann Thorac Surg*. 1987;44:340-3.
4. Lee W, Daly BD, DiPetrillo TA, Morelli DM, Neuschatz AC, Morr J, et al. Limited resection for non-small cell lung cancer: observed local control with implantation of I^{125} brachytherapy seeds. *Ann Thorac Surg*. 2003;75:237-43.
5. Detterbeck FC, Rivera PM, Socinski MA, Rosenman JC. Diagnosis and treatment of lung cancer: an evidence-based guide for the practicing physician. Philadelphia: W. B. Saunders Company; 2001;177-90.
6. Martini N, Bains MS, Burt ME, Zakowski MF, McCormack P, Rusch VW, et al. Incidence of local recurrence and second primary tumors in resected stage I lung cancer. *J Thorac Cardiovasc Surg*. 1995;109:120-9.
7. Edlund U, Albertsson A. Degradable microspheres for controlled drug delivery. *Adv Polymer Sci*. 2001;157:67.
8. Wang YM, Sato H, Adachi I, Horikoshi I. Preparation and characterization of poly(lactic-co-glycolic acid) microspheres for targeted delivery of a novel anticancer agent, taxol. *Chem Pharm Bull (Tokyo)*. 1996; 44:1935-40.
9. Ormerod MG. Flow cytometry: a practical approach. 2nd ed. Oxford: IRL Press; 1994.
10. Alam M, Wang J, Coffey J, Qadri S, O'Donnell A, Aherne T, et al. Characterization of the effects of cyclooxygenase-2 inhibition in the regulation of apoptosis in human small and non–small cell lung cancer cell lines. *Ann Surg Oncol*. 2007;14:2678-84.
11. Qadri SSA, Wang J-H, Coffey JC, Alam M, O'Donnell A, Aherne T, et al. Surgically induced accelerated local and distant tumor growth is significantly attenuated by selective COX-2 inhibition. *Ann Thorac Surg*. 2005;79:990-5.
12. Thomas P, Rubinstein L. Cancer recurrence after resection: T1 N0 non-small cell lung cancer. Lung Cancer Study Group. *Ann Thorac Surg*. 1990;49:242-7.
13. Keenan RJ, Landreneau RJ, Maley RH, Singh D, Macherey R, Bartley S, et al. Segmental resection spares pulmonary function in patients with stage I lung cancer. *Ann Thorac Surg*. 2004;78:228-33.
14. El-Sherif A. Margin and local recurrence after sublobar resection of non-small cell lung cancer. *Ann Surg Oncol*. 2007;14:2400-5.
15. Wu XS, Wang N. Synthesis, characterization, biodegradation, and drug delivery application of biodegradable lactic/glycolic acid polymers. Part II: biodegradation. *J Biomater Sci*. 2001;12:21-34.
16. Xie M, Zhou L, Hu T, Yao M. Intratumoral delivery of paclitaxel-loaded poly(lactic-co-glycolic acid) microspheres for Hep-2 laryngeal squamous cell carcinoma xenografts. *Anticancer Drugs*. 2007;18: 459-66.
17. Harper E, Dang W, Lapidus RG, Garver RI Jr. Enhanced efficacy of a novel controlled release paclitaxel formulation (PACLIMER delivery system) for local-regional therapy of lung cancer tumor nodules in mice. *Clin Cancer Res*. 1999;5:4242-8.
18. Fonseca C, Simoes S, Gaspar R. Paclitaxel-loaded PLGA nanoparticles: preparation, physicochemical characterization and in vitro anti-tumoral activity. *J Control Release*. 2002;83:273-86.
19. Sparreboom A, van Tellingen O, Nooijen WJ, Beijnen JH. Tissue distribution, metabolism and excretion of paclitaxel in mice. *Anticancer Drugs*. 1996;7:78-86.
20. Monsarrat B, Alvinerie P, Wright M, Dubois J, Gueritte-Voegelein F, Guenard D, et al. Hepatic metabolism and biliary excretion of Taxol in rats and humans. *J Natl Cancer Inst Monogr*. 1993;(15):39-46.
21. Kraeber-Bodere F, Sai-Maurel C, Campion L, Faivre-Chauvet A, Mirallie E, Cherel M, et al. Enhanced antitumor activity of combined pretargeted radioimmunotherapy and paclitaxel in medullary thyroid cancer xenograft. *Mol Cancer Ther*. 2002;1:267-74.