Aspirin-triggered proresolving mediators stimulate resolution in cancer

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Edited by Solomon H. Snyder, Johns Hopkins University School of Medicine, Baltimore, MD, and approved February 13, 2019 (received for review March 6, 2018)

Inflammation in the tumor microenvironment is a strong promoter of tumor growth. Substantial epidemiologic evidence suggests that aspirin, which suppresses inflammation, reduces the risk of cancer. The mechanism by which aspirin inhibits cancer has remained unclear, and toxicity has limited its clinical use. Aspirin not only blocks the biosynthesis of prostaglandins, but also stimulates the endogenous production of anti-inflammatory and proresolving mediators termed aspirin-triggered specialized pro-resolving mediators (AT-SPMs), such as aspirin-triggered resolvins (AT-RvDs) and lipoxins (AT-LXs). Using genetic and pharmacologic manipulation of a proresolving receptor, we demonstrate that AT-RvDs mediate the antitumor activity of aspirin. Moreover, treatment of mice with AT-RvDs (e.g., AT-RvD1 and AT-RvD3) or AT-LXA4 inhibited primary tumor growth by enhancing macrophage phagocytosis of tumor cell debris and counter-regulating macrophage-secreted proinflammatory cytokines, including migration inhibitory factor, plasminogen activator inhibitor-1, and C-C motif chemokine ligand 2/monocyte chemotactic protein 1. Thus, the pro-resolution activity of AT-resolvins and AT-lipoxins may explain some of aspirin’s broad anticaner activity. These AT-SPMs are active at considerably lower concentrations than aspirin, and thus may provide a nontoxic approach to harnessing aspirin’s anticaner activity.

Significance

Strong epidemiologic evidence indicates that aspirin is a powerful antitumorigenic agent. We now demonstrate that aspirin-triggered resolvins achieve the antitumor and chemopreventive activity of aspirin without toxicity, identifying a mechanism for aspirin’s anticaner activity. Our results suggest that differentiating between suppression and resolution of inflammation is highly relevant in cancer biology, revealing a class of endogenous antitumor mechanisms. These results have pivotal implications for cancer therapy and chemoprevention; unlike anti-inflammatory drugs, aspirin-triggered resolvins are active at nanogram doses and are not immunosuppressive. The antitumorigenic activity of aspirin-triggered resolvins may be harnessed to “mimic” aspirin without incurring aspirin-induced toxicity, such as bleeding, to contain minimal residual disease.

Author contributions: M.M.G., A.G., V.P.S., S.H., M.W.K., C.N.S., and D.P. designed research; M.M.G., A.G., M.L.S., P.C.N., D.R.B., and D.P. performed research; M.M.G., A.G., P.C.N., V.P.S., S.H., M.W.K., C.N.S., and D.P. analyzed data; P.C.N. performed LC-MS-MS profiling and prepared the MS-MS figures and tables; and M.M.G., A.G., S.H., M.W.K., C.N.S., and D.P. wrote the paper.

Conflict of interest statement: M.W.K. is now an employee of Bristol-Myers Squibb. His position at Bristol-Myers Squibb is not related to this work. This article is a PNAS Direct Submission. Published under the PNAS license.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1804000116/-/DCSupplemental.

Published online March 12, 2019.
the resolution of inflammation by stimulating phagocytosis of cellular debris and counter-regulating proinflammatory cytokines without being immunosuppressive (16).

Aspirin-acetylated COX facilitates the biosynthesis of aspirin-triggered specialized proresolving mediators (AT-SPMs) from omega-3 polyunsaturated fatty acid substrates, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (17). Human plasma resolvins levels are increased and detectable at 0.1–0.4 ng/mL in healthy individuals after dietary intake of EPA and aspirin (18). While AT-resolvins exhibit potent anti-inflammatory actions characteristic of native resolvins, the aspirin-triggered forms (R epimers) resist rapid inactivation by oxidoreductases and have longer half-lives (16). AT-RvD1 exhibits an approximate one log order increased potency in reducing total leukocyte infiltration in murine peritonitis compared with RvD1 (19). Intriguingly, AT-SPMs are increased in humans who respond to the anti-inflammatory activity of aspirin compared with those that do not respond to aspirin (20). Aspirin uses endogenous biosynthetic mechanisms to trigger transcellular biosynthesis of lipid mediators such as AT-lipoxins by human endothelial cell-leukocyte interactions or aspirin-stimulated neutrophils cocultured with human lung adenocarcinoma tumor cells (13, 14). AT-lipoxins also modulate tumor-associated macrophages and reduce bone cancer pain (21, 22). We recently demonstrated that SPMs, such as resolvins, enhance cytotoxic cancer therapy by promoting the clearance of therapy-generated tumor cell debris by macrophages (23). The antitumor mechanisms of the aspirin-triggered form of SPMs, such as AT-resolvins, remain to be addressed.

Here we provide evidence that AT-SPMs, including AT-resolvins and AT-lipoxins, are critical for the anticancer activity of low-dose aspirin by stimulating the resolution of tumor-promoting inflammation in mice. Both low-dose aspirin and AT-SPMs inhibit experimental primary tumor growth and metastasis by stimulating the clearance of therapy-generated tumor cell debris. Given that traditional cancer therapies (e.g., chemotherapy and radiation) induce inflammation and generate tumor cell debris (23–25), aspirin-triggered lipid autacoids that stimulate endogenous inflammation-clearing (resolution) mechanisms may offer a novel therapeutic approach to harness aspirin’s anti-cancer activity while avoiding the toxicity of aspirin.

Results

Antitumor Activity of Aspirin Is Resolvin-Receptor Dependent. To evaluate the potential anticancer activity of AT-RvDs, we used AT-SPMs in an aggressive murine Lewis lung carcinoma (LLC) tumor model (26). Systemic treatment with AT-RvD1, AT-RvD3, or AT-LXA4 (0.6 μg/kg/d) inhibited LLC tumor growth (generated with an inoculum of 10⁶ cells/mouse) for up to 12 treatment days compared with vehicle-treated mice (Fig. L4). To confirm that the inhibition of primary tumor growth by AT-SPMs was not strain-, cell line-, or sex-specific, we next examined the tumor cell lines MC38 colon adenocarcinoma in male C57BL/6 mice and 4T1 mammary carcinoma in female BALB/c mice. AT-RvD1, AT-RvD3, or AT-LXA4 also inhibited MC38 and 4T1 tumor growth (10⁶ MC38 or 4T1 cells/mouse) compared with control mice for up to 19 and 25 treatment days, respectively (SI Appendix, Fig. S1 A–C).

We next evaluated whether aspirin has antitumor activity in these models and whether AT-SPMs can be detected in mice treated with aspirin. To do so, we treated orthotopic and spontaneous tumor models with low-dose aspirin (30 mg/kg/d) (27, 28). Aspirin suppressed lung (LLC) tumor growth, with a threefold reduction in tumor burden (lung weight) and a fivefold reduction in metastases, as well as orthotopic mammary carcinoma (4T1) growth (Fig. 1B and SI Appendix, Fig. S2 A and B). Moreover, aspirin inhibited spontaneous tumor growth in a genetically engineered mouse model [mouse mammary tumor virus (MMTV)-PyMT] (SI Appendix, Fig. S2 C and D). To determine whether aspirin induces AT-SPM production in our tumor models, we quantified AT-SPMs via LC-MS/MS profiling of tumor lysates and plasma isolated from LLC tumor-bearing mice and 4T1 mammary carcinoma in female BALB/c mice. AT-RvD1, AT-RvD3, or AT-LXA4 also inhibited MC38 and 4T1 tumor cell lines MC38 colon adenocarcinoma in male C57BL/6 mice and 4T1 mammary carcinoma in female BALB/c mice. AT-RvD1, AT-RvD3, or AT-LXA4 also inhibited MC38 and 4T1 tumor growth (10⁶ MC38 or 4T1 cells/mouse) compared with control mice for up to 19 and 25 treatment days, respectively (SI Appendix, Fig. S1 A–C).

Fig. 1. AT-SPMs or aspirin inhibits primary tumor growth. (A) AT-RvD1, AT-RvD3, or AT-LXA4 (0.6 μg/kg/d) with primary LLC tumor growth. Treatment was initiated on the day of tumor cell injection throughout. Values are represented as mean ± SEM; n = 5–10 mice/group. The two-tailed Student’s t test was used for final tumor measurements. *P < 0.05 vs. control. (B) Aspirin (30 mg/kg/d used throughout) with orthotopic LLC tumor growth. n = 4–5 mice/group. *P < 0.05 vs. control. Lungs were resected and weighed, and visible metastatic nodules were counted at 19 d after LLC injection. *P < 0.05 vs. control. (C) LC-MS/MS fragmentation spectra of AT-RvD1, AT-RvD3, and AT-LXA4 in LLC tumor tissue from mice systemically treated with aspirin (30 mg/kg/d) for 9 d.
9 d compared with control mice (Fig. 1C and SI Appendix, Tables S1 and S2). Specifically, AT-RvD1 in tumor tissue isolated from tumor-bearing mice increased from 4.4 pg/100 mg of tissue to 65.9 pg/100 mg of tissue following aspirin treatment compared with controls (SI Appendix, Table S1). Importantly, aspirin induced tumor cell apoptosis when administered systemically to mice injected with GFP-labeled LLC tumors (10^6 cells/mouse) (SI Appendix, Fig. S3A). In contrast, aspirin did not exhibit direct tumor cell cytotoxicity in cell cultures, suggesting a stroma-dependent cell-killing mechanism (SI Appendix, Fig. S3B).

To further assess whether aspirin’s antitumor activity is mediated by AT-SPMs, we used WRW4, a pharmacologic antagonist of the AT-RvD1, RvD1, and the AT-LXA4 receptor ALX/FPR2 (29). The antitumor activity of aspirin in tumor-bearing mice (10^6 LLC cells/mouse) was neutralized by coadministration with the ALX/FPR2 antagonist (WRW4) (Fig. 2C). WRW4 further neutralized aspirin’s antitumor activity in orthotopic (4T1) and spontaneous (MMTV-PyMT) tumor models (Fig. 2B and SI Appendix, Fig. S4A and B). To confirm that these results are not specific to WRW4, we used an additional blocking peptide to neutralize ALX/FPR2 function. Consistent with WRW4, the antitumor activity of aspirin was abrogated by coadministration with an anti-ALX/FPR2 blocking peptide (SI Appendix, Fig. S4C). We further characterized the role of ALX/FPR2 in aspirin’s antitumor activity by systemically treating established tumors (10^6 LLC cells/mouse) in genetically engineered ALX/FPR2 knockout (KO) or wild-type (WT) mice with low-dose aspirin or vehicle. Consistent with the antitumor activity of resolvins (23, 30–34), LLC tumor growth was accelerated in the ALX/FPR2 KO mice compared with WT mice (Fig. 2C). While systemic treatment with low-dose aspirin markedly inhibited LLC tumor growth in WT mice, it produced drastically reduced antitumor activity in ALX/FPR2 KO mice (Fig. 2C). The minimal residual antitumor activity of aspirin in ALX/FPR2 KO mice may be due to endogenous production of aspirin-triggered SPMs that act via receptors other than ALX/FPR2.

We next evaluated whether aspirin’s antitumor activity was limited to primary tumor sites by studying spontaneous lung metastasis following primary tumor resection. Using a well-established model of minimal residual disease in which resection of a primary tumor reproducibly stimulates the development of distant lung metastasis at 14–17 d after resection (26), we investigated whether aspirin exhibits antitumor activity mediated by resolvins after LLC resection. Aspirin alone inhibited spontaneous LLC metastatic growth triggered by primary tumor resection (Fig. 2D). In contrast, autopsies of moribund mice at 14 d after primary LLC tumor resection revealed a dramatic increase in lung weight and number of surface lung metastases in mice systemically administered the combination of low-dose aspirin and the pharmacologic resolin receptor (ALX/FPR2) antagonist WRW4 compared with low-dose aspirin alone (Fig. 2D).

AT-SPMs Stimulate Macrophage Phagocytosis of Therapy-Generated Tumor Cell Debris. A critical function of resolvins and lipoxins is stimulation of nonphlogistic macrophage phagocytosis of debris (16). Therefore, we examined whether aspirin or AT-SPMs can stimulate the endogenous clearance of therapy-killed tumor cells, which, as we recently showed, promote tumor growth and recurrence (23). Using annexin V and propidium iodide staining, we confirmed the generation of apoptotic/necrotic tumor cells, hereinafter referred to as “tumor cell debris,” in cell cultures treated with chemotherapy (etoposide) or targeted therapy (erlotinib) (SI Appendix, Fig. S5 A–C). AT-RvD3 (100 pM–100 nM) stimulated human monocyte-derived macrophage phagocytosis of etoposide-generated human lung carcinoma (H460) debris or erlotinib-generated human lung carcinoma (HCC827) debris (Fig. 3A). Similarly, AT-RvD3 stimulated RAW264.7 murine macrophage phagocytosis of erlotinib-generated murine LLC tumor cell debris (Fig. 3A). Both AT-RvD1 and AT-LXA4 (100 pM–100 nM) also enhanced RAW264.7 murine macrophage or human monocyte-derived macrophage phagocytosis of therapy-generated LLC or H460 tumor cell debris, respectively, by 30–40% above vehicle (Fig. 3 B and C). Thus, aspirin-triggered lipoxins and resolvins stimulate phagocytosis of tumor cell debris in a dose-dependent manner, with activity diminishing at doses above 1 nM, a behavior characteristic of ligands that signal via G protein-coupled receptors (35).

Low-Dose Aspirin Triggers Macrophage Clearance of Therapy-Generated Tumor Cell Debris in a Receptor-Dependent Manner. Macrophages express COX and LOX enzymes required for SPM biosynthesis, including resolvins (36, 37), and aspirin stimulates macrophage in vitro production of SPMs (16). Given that AT-SPMs stimulate the clearance of tumor cell debris, we also examined whether aspirin can stimulate macrophage phagocytosis of tumor cell debris. Consistent with AT-SPMs, aspirin significantly stimulated human monocyte-derived macrophage phagocytosis of therapy-generated LLC or H460 tumor cell debris up to 116% above vehicle (Fig. 4A and B). Similarly, aspirin stimulated RAW264.7 murine macrophage phagocytosis of erlotinib-generated LLC debris up to 163% above vehicle (Fig. 4C). Again, there was a biphasic response in which aspirin at high doses (2 or 5 μM) did not stimulate macrophage phagocytosis of tumor cell debris (Fig. 4C).

To determine whether the stimulation of macrophage phagocytosis by aspirin was mediated by AT-SPMs, including AT-LXA4 and AT-RvD1, macrophages were treated with the ALX/FPR2 antagonist WRW4 before treatment with aspirin and coincubation with tumor cell debris. While aspirin (1 nM) significantly stimulated human monocyte-derived macrophage phagocytosis, treatment with WRW4 neutralized aspirin-stimulated macrophage phagocytosis of debris (Fig. 4D). Macrophages treated with WRW4...
significant, reduction in MIF secretion by human monocyte-derived macrophages in the presence of etoposide-generated H460 tumor cell debris (Fig. 5B, black bars). Treatment of macrophages with the combination of aspirin and the ALX/FPR2 antagonist WRW4 did not suppress MIF secretion by debris-stimulated macrophages (Fig. 5B, black bars). AT-RvD3 treatment also inhibited plasminogen activator inhibitor-1 (PAI-1) secretion by human monocyte-derived macrophages in the presence of erlotinib-generated HCCS27 debris (Fig. 5C, black bars). While aspirin also inhibited secretion of PAI-1 by macrophages in the absence or presence of erlotinib-generated HCCS27 tumor cell debris, WRW4 again neutralized this aspirin-mediated inhibition (Fig. 5D). Both AT-LXA4 and AT-RvD3 also inhibited macrophage secretion of C-C motif chemokine ligand 2 (CCL2)/monocyte chemoattractant protein 1 (MCP-1) in the presence or absence of tumor cell debris (Fig. 5E). Consistent with MIF and PAI-1, aspirin inhibited macrophage secretion in the absence of tumor cell debris, whereas treatment of macrophages with both aspirin and WRW4 did not inhibit cytokine production (Fig. 5F). Thus, AT-SPMs and low-dose aspirin stimulate the resolution of inflammation by increasing

AT-SPMs and Low-Dose Aspirin Suppress Macrophage Secretion of Proinflammatory Cytokines. In addition to stimulating phagocytosis of cellular debris, SPMs and AT-SPMs (e.g., AT-LXA4, AT-RvD1, AT-RvD3) actively promote the resolution of inflammation by counter-regulating proinflammatory cytokines/chemokines (16). On screening for a broad panel of 36 proinflammatory cytokines, we identified several macrophage-produced cytokines whose secretion was suppressed by aspirin or AT-SPMs in the presence or absence of tumor cell debris. Both AT-LXA4 and AT-RvD3 (1 nM) inhibited baseline human monocyte-derived macrophage secretion of the proinflammatory cytokine macrophage migration inhibitory factor (MIF) (Fig. 5A, gray bars). Aspirin (1 nM) also significantly inhibited baseline macrophage secretion of MIF (Fig. 5B, gray bars). Coincubation of macrophages with etoposide-generated human H460 tumor cell debris drastically increased macrophage secretion of MIF by 4.5-fold, which was dampened by 40–50% by both AT-LXA4 and AT-RvD3 (Fig. 5A, black bars). Aspirin (1 nM) also exhibited a minimal, not statistically
macrophage phagocytosis of tumor cell debris and counter-regulating protumorigenic cytokines.

**Discussion**

Chronic inflammation has emerged as a critical factor in tumorigenesis and cancer progression (2). We recently demonstrated that tumor cell debris generated by cancer therapy stimulates tumor growth and metastasis, a process mediated by proinflammatory cytokines (23). Along with blocking the bio-synthesis of prostaglandins from omega-6 fatty acid substrates, aspirin covalently interacts with COX-2 and alters COX-2 activity to trigger the production of anti-inflammatory and pro-resolving AT-SPMs (13, 19) from omega-3 fatty acid precursors. Here we demonstrate that both low-dose aspirin and AT-SPMs, including resolvins and lipoxins (AT-RvD1, AT-RvD3, and AT-LXA4), inhibit primary tumor growth and metastasis by enhancing endogenous macrophage clearance of tumor cell debris and quelling tumor-associated inflammation by counter-regulating protumorigenic cytokines. Importantly, we demonstrate via genetic and pharmacologic ablation that the antitumor activity of aspirin is resolvin receptor-dependent, thereby identifying a previously unknown mechanism for the unique chemopreventive activity of aspirin.

Genetic or pharmacologic ablation of ALX/FPR2 did not completely abrogate the antitumor activity of aspirin. This could potentially result from the diverse actions of SPMs. Aspirin-triggered SPMs encompass not only AT-RvD1, AT-RvD3, and AT-LXA4, each of which activates the ALX/FPR2 and GPR3 receptors (15, 38), but also AT-protectin D1 (AT-PD1), whose receptor remains to be identified, and the AT-resolvins E series, which act via the ChemR23 and BLT1 receptors (18, 39). Thus, aspirin-triggered PD1 and the resolvins E series may account for the residual antitumor activity of aspirin in the ALX/FPR2 KO mouse or in the presence of the ALX/FPR2 antagonist WRW4. However, AT-SPMs (AT-RvD1, AT-RvD3, and AT-LXA4) that act via the receptor ALX/FPR2 collectively may be more potent than AT-PD1 or E series AT-resolvins, as pharmacologic and genetic ALX/FPR2 ablation almost completely neutralized both aspirin inhibition of tumor growth and aspirin stimulation of phagocytosis.

Aspirin reduces cancer risk and mortality by up to 50%; however, its use in chemoprevention of cancer is not recommended due to the increased risk of hemorrhagic events, such as gastrointestinal bleeding and stroke (3). Intriguingly, humans who respond to aspirin treatment of inflammatory lesions show elevated SPMs compared with those who do not respond to aspirin, suggesting that SPMs may play a critical role in the anti-inflammatory activity of aspirin (20). Dietary intake of EPA and DHA together with aspirin increases circulating resolvin levels in humans (17, 19). Moreover, low-dose aspirin administered to healthy volunteers for cardioprevention is also capable of producing bioactive levels of aspirin-triggered lipoxins (40). Our study demonstrates that aspirin-triggered SPMs are increased in tumor tissues and plasma following low-dose aspirin treatment, suggesting that SPMs may mediate aspirin’s broad anti-inflammatory and anti-cancer activities.

Our results demonstrate that systemic administration of aspirin or AT-SPMs inhibit the growth of primary tumors and metastasis in multiple murine tumor models, including orthotopic and genetically engineered models. The dose of aspirin used in this study (30 mg/kg/d) may be slightly higher than the equivalent dose of standard low-dose aspirin used in cardioprevention and may need to be adjusted to body size (41, 42). Importantly, the aspirin dose used in our in vivo tumor studies induced tumor cell apoptosis in mice compared with controls, in accordance with previous reports (43). However, aspirin treatment of cell cultures did not induce tumor cell apoptosis across a broad range of doses (10^0–10^4 μM). Thus, aspirin did not exhibit direct tumor cell cytotoxicity; rather, aspirin induction of tumor cell death was determined to be tumor stroma-dependent.

In this study, AT-SPMs administered in our murine tumor models exhibited antitumor activity at a 1,000-fold lower dose than that of aspirin. This dose of AT-SPMs is also more than log orders of magnitude lower than the doses of their omega-3 fatty acid precursors (EPA and DHA) or NSAIDs required for tumor inhibition (44, 45). SPMs, including resolvins, are currently in clinical development. Given the risks associated with chronic low-dose aspirin intake, mediators such as aspirin-triggered lipoxins and AT-SPMs may have more potent antitumor activity devoid of aspirin-related toxicity. Thus, aspirin-triggered resolvins may be optimal chemopreventive agents that represent a new treatment modality in cancer that remains to be evaluated in humans.

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**Fig. 5.** AT-SPMs and low-dose aspirin suppress macrophage secretion of proinflammatory cytokines. Shown is human monocyte-derived macrophage cytokine secretion following AT-SPM or aspirin (1 nM) treatment and/or coincubation with tumor cell debris. Values are represented as mean ± SEM throughout. The two-tailed Student t test was used throughout. *P < 0.05; **P < 0.01 vs. control (A–F) or aspirin alone (B, D, and F). n.s., not significant (C). n = 3–6/group throughout. (A and B) Baseline (gray bars) or H460 tumor cell debris-stimulated (black bars) macrophage secretion of macrophage MIF following treatment with AT-LXA4 (1 nM), AT-RvD3 (1 nM), aspirin (1 nM), or aspirin (1 nM) + WRW4 (10 μM). (C and D) Baseline (gray bars) or HCC27 tumor cell debris-stimulated (black bars) macrophage secretion of PAI-1 following treatment with AT-RvD3 (1 nM), aspirin (1 nM), or aspirin (1 nM) + WRW4 (10 μM). (E and F) Baseline (gray bars) or H460 tumor cell debris-stimulated (black bars) macrophage secretion of CCL2/MCP-1 following treatment with AT-LXA4 (1 nM), AT-RvD3 (1 nM), aspirin (1 nM), or aspirin (1 nM) + WRW4 (10 μM).
Materials and Methods

Methods used for the preparation of therapy-generated tumor cell debris (23, 46), flow cytometry (23, 46), LC-MS/MS profiling (47), isolation of human monocyte-derived macrophages and resident murine peritoneal macrophages (23, 46), and macrophage-conditioned medium for cytokine quantification (23, 46) have been described previously. ELISAs (R&D Systems) were performed according to provided recommended protocols. The protocols are described in detail in SI Appendix, Materials and Methods.

In Vivo Studies.

All animal studies were reviewed and approved by the Animal Care and Use Committee of Boston Children’s Hospital and Beth Israel Deaconess Medical Center. C57BL/6, BALB/c, and MMTV-PyMT mice were obtained from The Jackson Laboratory. ALX/FRPR2 KO mice were generously provided by Mauro Perretti, Queen Mary University of London. The mice were systemically treated with 0.6 μg/kg of AT-SPMs (AT-LX4, AT-RvD1 (Cayman Chemical), or AT-RvD3) via a mini osmotic pump (Alzet), low-dose aspirin (30 mg/kg; Sigma-Aldrich) via oral gavage, and/or WRW41 (1 mg/kg; EMD Millipore) or anti-ALX/FRPR2 blocking peptide antibody (25 μg/kg; LifeSpan Biosciences) via i.p. injection. For metastasis studies, LLC tumors were surgically resected from the mid-dorsum of 6- to 8-wk-old C57BL/6 mice at 14 d postinjection.

Statistics.

For all animal and in vitro studies, comparisons of two groups were performed using the Student two-tailed unpaired t test. P values <0.05 were considered statistically significant. Data are represented as mean ± SEM.

ACKNOWLEDGMENTS.

We thank Steve Moskovitz (Advanced Medical Graphics) for preparing the figures and photographs. We also thank Romain Colas, Jaime Chiang, Haixia Yang, Suzan Lazo, John Daley, and Lucius Xuan for their excellent technical assistance. This work was supported by the National Institute of Health (Grants R01 O1CA170549, to D.P. and C.N.S., ROCA148633, to D.P., and SP01 GM095647, to C.N.S.), the Credit Unions Kids for their excellent technical assistance. This work was supported by the National Institute of Health (Grants R01 O1CA170549, to D.P. and C.N.S., ROCA148633, to D.P., and SP01 GM095647, to C.N.S.), the Credit Unions Kids (M.W.K.), Jared Branfman Sunflowers for Life (M.W.K.), the Kamen Foundation (M.W.K.), Jared Branfman Sunflowers for Life (M.W.K.), and the Joe Andruzzi Foundation (M.W.K.).