

Radiation-Enhanced Vascular Targeting of Human Lung Cancers in Mice with a Monoclonal Antibody That Binds Anionic Phospholipids

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Abstract Purpose: New treatment strategies aimed at damaging tumor vasculature could potentially improve tumor response to radiation therapy. We recently showed that anionic phospholipids, principally phosphatidylserine, are specifically exposed on the luminal surface of tumor blood vessels. Here we tested the hypothesis that radiation therapy can increase phosphatidylserine exposure on lung tumor vasculature, thereby enhancing the antitumor properties of the anti-phosphatidylserine antibody 2aG4.

Experimental Design: The therapeutic efficacy of radiation therapy plus 2aG4 was tested in nude mice bearing radiation-resistant A549 human lung tumors. Radiation-induced phosphatidylserine exposure on endothelial cells and A549 tumor cells was analyzed by immunofluorescence staining. The mechanism of the enhanced antitumor effect was examined by histology and antibody-dependent cell-mediated cytotoxicity experiments.

Results: Focal irradiation of A549 human lung cancer xenografts increased the percentage of tumor vessels with exposed phosphatidylserine from 4% to 26%. Treatment of mice bearing A549 tumors with 2aG4 plus focal radiation therapy inhibited tumor growth by 80% and was superior to radiation therapy or 2aG4 alone ($P < 0.01$). Combination therapy reduced blood vessel density and enhanced monocyte infiltration into the tumor mass beyond that observed with individual treatments. *In vitro*, 2aG4 enhanced the ability of macrophages to kill endothelial cells with exposed phosphatidylserine in an Fc'-dependent manner.

Conclusion: These results suggest that 2aG4 enhances the antitumor effects of radiation therapy by increasing antibody-dependent cell-mediated cytotoxicity toward tumor vessels with externalized phosphatidylserine. Baviximab, a chimeric version of 2aG4 in clinical trials, has the potential to enhance the therapeutic efficacy of radiation therapy in lung cancer patients.

Lung cancer is a worldwide health care problem with an estimated 1.35 million new cases diagnosed in 2002 (1). It is also the leading cause of cancer death in the United States, more than breast, colon, and prostate cancer combined. Even with all available therapeutic modalities, the overall 5-year survival rate is only ~ 15% (2). New systemic therapies directed against rational targets have entered clinical trials for the treatment of lung cancers. Among these targeted agents, drugs targeting tumor blood vessels such as combretastatin A4 phosphate (3) and 5,6-dimethylxanthenone-4-acetic acid (4) have shown considerable promise (5–7).

We recently showed that anionic phospholipids, principally phosphatidylserine, are specifically externalized on tumor endothelial cells (8), likely in response to oxidative stress conditions present in the tumor microenvironment (9). To target tumor endothelial cells with exposed phosphatidylserine, we developed a novel mouse immunoglobulin G3 (IgG3) antibody, 3G4 (10), which binds to anionic phospholipids complexed with serum protein β 2-glycoprotein 1 (11). This antibody inhibits tumor growth as a single agent and enhances the antitumor effects of clinical chemotherapeutic drugs. Specifically, 3G4 enhances the antitumor effect of docetaxel in a human breast cancer model (12). It also significantly reduces tumor burden and metastatic lesions when coadministered with gemcitabine in an orthotopic model of pancreatic cancer (13). The enhanced therapeutic effects seem to be due to the ability of these chemotherapeutic drugs to increase exposure of phosphatidylserine on tumor blood vessels, amplifying the target for 3G4 (14).

Radiation therapy is a primary treatment for patients with lung cancer. Unfortunately, many lung cancers become radiation resistant and refractory to radiation therapy. However, studies in mice have shown that combining radiation therapy with tumor vascular targeting agents can enhance the therapeutic effect (15). Furthermore, recent studies indicate that damage to tumor endothelial cells may determine the tumor response to radiation therapy (16), suggesting that enhanced

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targeting of the tumor endothelial cells may lead to enhanced antitumor effects. Thus, we tested the hypothesis that radiation therapy can enhance the antitumor effects of phosphatidylserine-targeting antibodies in a mouse model of radiation-resistant human lung cancer. We show that radiation therapy enhances localization of our anti-phosphatidylserine antibodies to lung tumor vasculature. We show that the antitumor effect of 2aG4, a mouse IgG2a version of 3G4, is enhanced by radiation therapy, likely by stimulating antibody-dependent cell-mediated cytotoxicity (ADCC) against tumor endothelial cells with exposed phosphatidylserine.

Materials and Methods

Cells. The human lung cancer cell lines A549 (CCL-185) and H460 (HTB-177), the hybridoma cell line C44 (CRL-1943), and the mouse macrophage cell line Raw264.7 (TIB-71) were obtained from the American Type Culture Collection. Human umbilical vascular endothelial cells (HUVEC) were obtained from Clonetics.

Antibodies. A mouse IgG2a version of 3G4 (10), named 2aG4, was constructed using recombinant DNA techniques. The resulting plasmid was transfected into NS0 myeloma cells, which were cultured in DMEM (Mediatech), supplemented with 10% fetal bovine serum, 10 mmol/L sodium pyruvate, and nonessential amino acids (Mediatech). Stable clones were screened for high-level antibody production. 2aG4 and C44, an isotype-matched control antibody for 2aG4, were purified from cell culture supernatant as previously described (17). Rat anti-mouse CD31, rat anti-mouse CD11b (M1/70, Mac-1, integrin M-chain, complement receptor CR3), and rat anti-mouse FcγRIII (CD16) monoclonal antibodies were purchased from BD Pharmingen. Hamster anti-mouse CD31 was obtained from Pierce Biotechnology. A rabbit polyclonal antibody specific for cleaved caspase-3 was obtained from Cell Signaling Technologies. Secondary antibodies were purchased from Jackson ImmunoResearch.

Detection of phosphatidylserine on irradiated cells in vitro. To induce exposure of phosphatidylserine, HUVECs and A549 cells were grown to ~70% confluence and irradiated with 2 Gy using a Mark I Cesium-137 irradiator (J.L. Shepherd) at a dose rate of 1.74 Gy/min. Cells were irradiated in the presence of 2aG4 or control antibody C44 (10 μg/mL). Exposure of phosphatidylserine was determined by immunofluorescence staining or flow cytometry at various time points after irradiation. Cells grown on eight-well chamber slides were washed gently five times with PBS and fixed with 4% paraformaldehyde at room temperature for 15 min. Cells were then incubated with a Cy2-labeled goat anti-mouse antibody for 1 h at room temperature. Next, cells were washed, permeabilized with 0.1% Triton-X100 for 5 min, and counterstained with Texas red-labeled phalloidin (Invitrogen). To examine DNA fragmentation, nuclei were stained with 4',6-diamidino-2-phenylindole. Images were captured using a Coolsnap digital camera mounted on a Nikon microscope and analyzed with MetaVue software (Universal Imaging Corporation). For flow cytometry, cells were harvested and washed gently thrice with ice-cold 1% bovine serum albumin/PBS. Cells were incubated with a Cy2-labeled goat anti-mouse antibody for 30 min and 7-amino-actinomycin D for 15 min at 4°C. Next, cells were washed thrice with ice-cold 1% bovine serum albumin and analyzed using a BD FACSCalibur flow cytometer. Data were acquired and analyzed by CellQuest software (BD Biosciences). 7-Amino-actinomycin D-positive cells were excluded from analysis.

Subcutaneous A549 and H460 human lung cancer models. Subconfluent cultures of A549 or H460 tumor cells were harvested by brief exposure to 0.25% (w/v) trypsin and 0.02% EDTA. Cells were then washed in serum-free medium and resuspended in PBS. Cell viability was >95% as determined by trypan blue dye exclusion. Tumor cells (1×10^7) in 0.2 mL PBS were injected s.c. into the flank of 8-week-old male nude mice or severe combined immunodeficient (SCID) mice

(National Cancer Institute). Tumors were allowed to grow until they reached a diameter of 5 to 7 mm (~7 days after tumor cells injection). All procedures were conducted in accordance with institutional guidelines.

Detection and quantification of anionic phospholipid exposure in vivo. SCID mice bearing 5- to 7-mm A549 tumors were irradiated with daily fractions of 2 Gy given for 5 consecutive days. Radiation therapy was delivered using an XRAD320 X-ray generator (Precision X-ray) operating at 250 kV, 15 mA, with a 3.1-mm copper filter (source-to-skin distance, 35 cm), at a dose rate of 106.5 cGy/min. Nonanesthetized mice were briefly restrained in a custom jig constructed to position and expose the tumor for radiation therapy. Nontumor areas were shielded using a sheet of lead 3 mm thick. Twelve hours after the final radiation therapy fraction, 100 μg of 2aG4 or control antibody C44 were injected i.v. and allowed to circulate for 1 h. Mice were then anesthetized, exsanguinated, and perfused with heparinized saline. Tumors were removed and snap-frozen for preparation of cryosections. Sections were fixed with 4% paraformaldehyde and blocked with PBS containing 10% goat serum. Vascular endothelium was stained using a rat anti-mouse CD31 antibody followed by Cy3-labeled goat anti-rat IgG. 2aG4 or C44 was detected with biotinylated goat anti-mouse IgG followed by Cy2-labeled streptavidin. Apoptotic cells were detected using a rabbit polyclonal antibody specific for cleaved caspase-3 followed by Cy3-labeled donkey anti-rabbit IgG. Images were captured and analyzed as described above. Doubly labeled endothelial cells (i.e., CD31⁺/2aG4⁺ or CD31⁺/caspase-3⁺) were identified by yellow fluorescence on merged images. The percentage of doubly positive vessels was calculated as follows: (mean number of yellow vessels per field / mean number of total vessels) × 100. Ten random 0.079-mm² fields were evaluated for each section.

Treatment of mice bearing A549 or H460 human lung tumor xenografts. Nude mice bearing 5- to 7-mm-diameter A549 tumors were randomly divided into four groups ($n = 15$). Mice were given the following treatments: group 1, 2aG4; group 2, untreated; group 3, radiation therapy + C44; group 4, radiation therapy + 2aG4. Radiotherapy was administered in a fractionated manner as described above. Antibodies were given i.p. in doses of 100 μg thrice a week for 3 weeks. Tumor sizes and the general physical status of the animals were assessed twice a week. Tumor volume was calculated according to the formula $\pi/6 \times D \times d^2$, where D is the larger tumor diameter and d is the smaller tumor diameter measured in two perpendicular directions. On day 49, all mice were anesthetized, exsanguinated, and perfused with heparinized saline. Tumors were harvested and snap-frozen for sectioning and following analysis.

In a separate study, nude mice bearing 5- to 7-mm-diameter H460 tumors were randomly divided into four groups ($n = 15$). Mice were given the following treatments: group 1, 3G4; group 2, control antibody; group 3, radiation therapy; group 4, radiation therapy + 3G4. Radiotherapy and antibody were administered as described above for 2 weeks. Tumor sizes and the general physical status of the animals were assessed twice a week.

Quantification of tumor vessels after combination therapy (Chalkley method). To quantify the number of tumor vessels, frozen tumor sections were stained with an anti-CD31 antibody as described above. Images from 10 random 0.079-mm² fields at ×200 magnification for each tumor section were captured and analyzed to determine the total area of CD31-positive microvessel-like structures as previously described (8). Five tumors from each group were analyzed. Tumor vascularity was expressed as the area of vessels per square millimeter of section (18).

Quantification of monocyte infiltration after combination therapy. To determine a time course for macrophage infiltration after combination treatment, a separate experiment was done. Twelve A549-bearing nude mice were focally irradiated with 2 Gy for 5 consecutive days. Mice were given 100 μg 2aG4 on days 2 and 5. At various time points after the last treatment, A549 tumors were harvested and frozen tumor sections prepared. Frozen tumor sections from the

time course experiment and the previous tumor treatment experiment were stained with antibodies specific for the mouse monocyte/macrophage markers M1/70 (CD11b, Mac-1, and M-chain) and FcγRIII (CD16), followed by Cy2-labeled goat anti-rat IgG. Blood vessels were detected with hamster anti-mouse CD31 followed by Texas red-labeled goat anti-hamster IgG. Ten fields for each treatment group were analyzed. The number of macrophages in tumor sections from each treatment group was quantified using Metaview software.

Antibody-dependent cell-mediated cytotoxicity assay. Antibody-dependent cell-mediated cytotoxicity was measured with a ^{51}Cr release assay. Briefly, HUVEC and A549 cells were irradiated with a dose of 2 Gy using a Mark I Cesium-137 irradiator as described above. Twenty-four hours later, irradiated cells were incubated with 100 μL of ^{51}Cr for 3 h (1 mCi/mL; GE Healthcare Bio-Sciences). Labeled cells (1×10^4) were mixed with 10 $\mu\text{g}/\text{mL}$ 2aG4, 2aG4 F(ab')₂, or C44 and seeded into 96-well microplates. Raw264.7 mouse macrophages were added to each well such that effector-to-target ratios ranged from 40:1 to 1:1. After overnight incubation, supernatants were collected and release of ^{51}Cr was measured with a gamma counter (Packard Cobra II). Spontaneous release (SR) is defined as ^{51}Cr released from labeled target cells in the absence of Raw264.7 effector cells. Maximum release (MR) was determined by measuring ^{51}Cr released from labeled target cells treated with 1% Triton X-100. Percent lysis was calculated as (effector-mediated release - target SR) / (target MR - target SR) \times 100.

Statistical analyses. Tumor volume, tumor vascularity, and ADCC data were compared by Student's *t* test. $P < 0.05$ was considered significant.

Results

Radiation-induced exposure of phosphatidylserine on endothelial cells. Recent studies indicate that endothelial cells may be more sensitive to radiation therapy than tumor cells, and that endothelial cells may expose phosphatidylserine after irradiation (16). Therefore, we irradiated HUVECs and A549 human lung cancer cells and monitored phosphatidylserine exposure at several time points as determined by 2aG4 binding. As expected, 2aG4 did not bind to nonirradiated cells (data not shown). Irradiated A549 lung tumor cells were not stained by C44 or 2aG4 (Fig. 1A and B). Irradiation of HUVECs caused the appearance of small membrane blebs that were not stained by the isotype-matched control antibody C44 (Fig. 1C) but stained intensely by 2aG4 (Fig. 1D). Similar radiation-induced phosphatidylserine exposure was also observed on a mouse endothelial cell line, bEnd3.¹ 2aG4 binding to HUVECs first became detectable 2 h after a 2-Gy dose of irradiation. Forty-eight hours after irradiation, ~50% of HUVECs were stained by 2aG4 as quantified by flow cytometry (Fig. 1E). Irradiation with 10 Gy did not increase the percentage of HUVECs, which were stained by 2aG4 (data not shown). Fewer than 10% of A549 cells were stained by 2aG4 48 h after irradiation (Fig. 1E).

Enhanced exposure of phosphatidylserine on tumor blood vessels after irradiation. The exposure of phosphatidylserine on tumor vessels *in vivo* was determined by i.v. injection of 100 μg 2aG4 into SCID mice bearing s.c. A549 human lung tumors. Tumors were irradiated with 2 Gy for 5 consecutive days to mimic a clinical radiation therapy schedule. Antibody was given 12 h after the last dose of radiation therapy and allowed to circulate for 1 h before sacrifice. Localization of 2aG4 to tumor

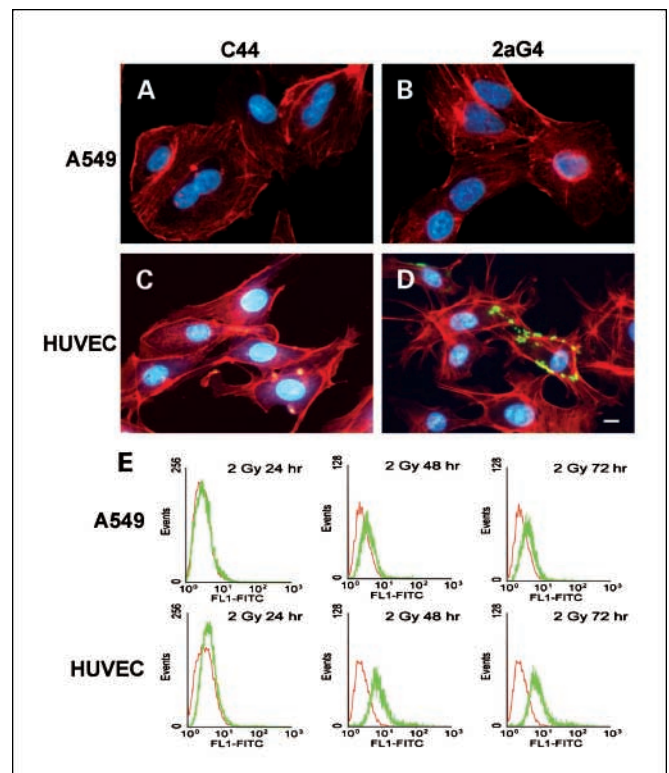


Fig. 1. Irradiation induces phosphatidylserine exposure on HUVECs but not A549 tumor cells *in vitro*. A549 cells (A and B) or HUVECs (C and D) were grown on chamber slides and irradiated with 2 Gy in the presence of 2aG4 (B and D) or control antibody C44 (A and C). Two hours later, cells were fixed and stained for antibody binding. 2aG4 binding to externalized phosphatidylserine appears green. The cytoskeleton appears red, and nuclei appear blue. Bar, 10 μm . E, flow cytometric analysis of intact cells (7-amino-actinomycin D negative) stained with 2aG4 (green) or C44 (red) 24, 48, or 72 h after irradiation.

endothelial cells was judged by coincident staining of 2aG4 (green) and CD31 (red), which appeared yellow (Fig. 2A). No coincident staining was observed in normal organs or in tumors from mice injected with control antibody C44.

The average percentage of 2aG4-positive tumor vessels in irradiated tumors was 26%, as compared with 4% in nonirradiated tumors (Fig. 2B; $P < 0.01$). To determine if 2aG4-positive endothelial cells are apoptotic, tumor sections were also stained with an anti-cleaved caspase-3 antibody. Only 1% of endothelial cells were stained by the anti-cleaved caspase-3 antibody in either irradiated or nonirradiated tumors. Thus, the fractionated radiation therapy schedule induces phosphatidylserine exposure on tumor endothelial cells, but not apoptosis. This is consistent with reports showing that a radiation therapy dose >10 Gy is required to induce apoptosis of tumor endothelial cells (19). Importantly, the 2aG4-positive/caspase-3-negative endothelial cells appeared morphologically intact and functional.

Combination therapy inhibits the growth of human lung tumor xenografts in mice. A549 cells are relatively radiation resistant (20) and a good model of radiation-resistant human lung cancer. As expected, growth of A549 tumors was unaffected by fractionated radiation therapy of 2 Gy for 5 consecutive days (Fig. 3A). However, when radiation therapy was combined with 2aG4 treatment, marked tumor growth inhibition was observed. Tumors from mice given radiation therapy + 2aG4

¹ Unpublished data.

grew by only 1.8-fold over the course of the experiment. In contrast, tumors from mice given radiation therapy + C44 grew by 5-fold, the same as in untreated mice. The radiation therapy + 2aG4 treatment thus reduced tumor growth by ~80% relative to the growth in the radiation therapy + C44 group or untreated controls. Treatment with radiation therapy + 2aG4 was significantly superior to treatment with 2aG4 alone ($P < 0.01$). The combination therapy was well tolerated, and no gross side effects such as skin desquamation or body weight loss were observed. These results show that 2aG4 significantly enhances the therapeutic efficacy of radiation therapy for radiation-resistant human lung tumors in mice.

We also examined the H460 model of human lung cancer, which has intermediate radiosensitivity (Fig. 3B). Again, treatment with radiation therapy + 3G4 was significantly superior to treatment with antibody or irradiation alone ($P < 0.05$).

Decreased vascular density in tumors after combination therapy. Frozen A549 tumor sections were prepared from tumor tissue collected at the end of the previous experiment and stained for CD31 to analyze vascular density. Irradiation alone had no effect on vascular area, whereas 2aG4 alone reduced vascular area by only 20% relative to that in untreated tumors (Fig. 4). In contrast, radiation therapy + 2aG4 reduced

tumor vascularity by 91% relative to control tumors ($P < 0.01$). These findings indicate that the combination of 2aG4 + radiation therapy has a strong antivascular effect.

Increased monocyte/macrophage infiltration into tumors after combination therapy. While conducting the vascular density analysis, we observed an increase in immune cell infiltration in the combination group. The infiltrating cells were positive for both monocyte/macrophage markers (CD11b and CD16) and negative for the neutrophil marker Ly-6G or natural killer cell marker Ly-49 (results not shown). To gain a better understanding of the infiltration process, we examined the infiltration of immune cells at various time points. A549 tumors were treated with 2aG4 + radiation therapy as described above. Mice were sacrificed 24, 48, 72, and 96 h after the last dose of radiation therapy and analyzed for immune cell infiltration. CD11b- and CD16-positive cells (monocytes/macrophages) started attaching to endothelial cells 24 to 48 h after the last treatment (Fig. 5A and B). At 96 h, these cells were clearly moving out of the vessels into the tumor interstitium (Fig. 5C). By the end of the original experiment (21 days after the last treatment), strikingly greater numbers of monocytes/macrophages were present in the tumor interstitium of mice treated with 2aG4 + radiation therapy (Fig. 5D) than in mice treated with 2aG4 or radiation therapy alone (results not shown). A time course showed that the number of infiltrating CD11b- and CD16-positive cells started to peak 72 h after the first treatment ($P < 0.01$; Fig. 5E). This time frame is similar to that of peak phosphatidylserine exposure on cultured HUVECs after irradiation (see Fig. 1E). This prolonged monocyte/macrophage infiltration may provide a proinflammatory microenvironment which could be partly responsible for the inhibitory effect of the combination therapy (Fig. 5F).

2aG4 mediates enhanced ADCC of irradiated endothelial cells. To determine whether infiltrating monocytes/macrophages may be able to kill 2aG4 opsonized endothelial cells, a ^{51}Cr release assay was done. HUVECs were irradiated to induce phosphatidylserine exposure, labeled with ^{51}Cr , then incubated with C44, 2aG4, or 2aG4 F(ab')₂ in the presence of the murine macrophage line Raw264.7. The mean percentage lysis mediated by the isotype-matched control antibody C44 was arbitrarily set to one. 2aG4 increased the relative amount of HUVEC lysis by 13.8-fold ($P < 0.01$; Fig. 6). Lysis was dependent on the Fc part of the antibody because 2aG4 F(ab')₂ was unable to mediate a significant increase in HUVEC lysis. Specific lysis of irradiated A549 tumor cells was not observed (Fig. 6), likely due to a lack of phosphatidylserine exposure of A549 cells after 2-Gy irradiation (see Fig. 1). These findings suggest that 2aG4 may target endothelial cells with exposed phosphatidylserine for ADCC.

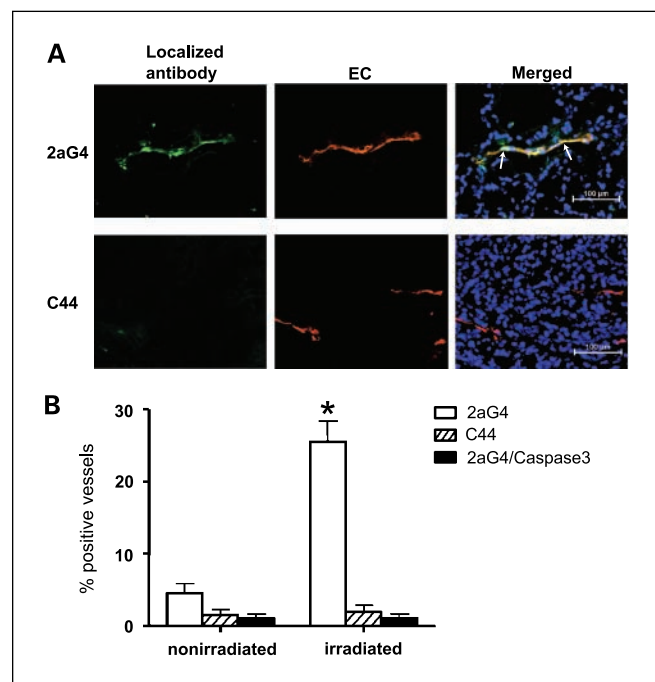


Fig. 2. Radiation therapy enhances exposure of phosphatidylserine on blood vessels in A549 tumors. SCID mice bearing A549 tumors were irradiated using a fractionated dosing schedule of 2 Gy for 5 consecutive days. Exposure of phosphatidylserine on tumor vascular endothelium was determined by i.v. injection of 2aG4 or control antibody C44 followed by perfusion of mice with saline 1 h later. Frozen tumor sections were analyzed by immunofluorescence. **A**, tumor sections from mice injected with 2aG4 (upper) or C44 (lower). Injected antibody appears green. Endothelial cells (EC) stained with anti-CD31 appear red. Localization of 2aG4 to tumor endothelial cells in merged images appears yellow (arrows). **B**, quantification of the number of 2aG4-positive or C44-positive vessels relative to the number of CD31-positive vessels for irradiated and nonirradiated tumors. Also shown is the number of 2aG4-positive/cleaved caspase-3-positive tumor endothelial cells relative to the number of CD31-positive vessels to show that radiation therapy induces phosphatidylserine exposure but not endothelial cell apoptosis. Columns, average percentages ($n = 5$); bars, SE.

Discussion

Approximately half of all patients with lung cancer receive radiation therapy (21). High-dose stereotactic radiotherapy is being used to treat early-stage lung tumors and is achieving satisfactory local tumor control with tolerable toxicities (22). However, even with all available therapeutic modalities, the 5-year survival rate for lung cancer patients remains at only 15% (23), indicating that new treatment strategies are

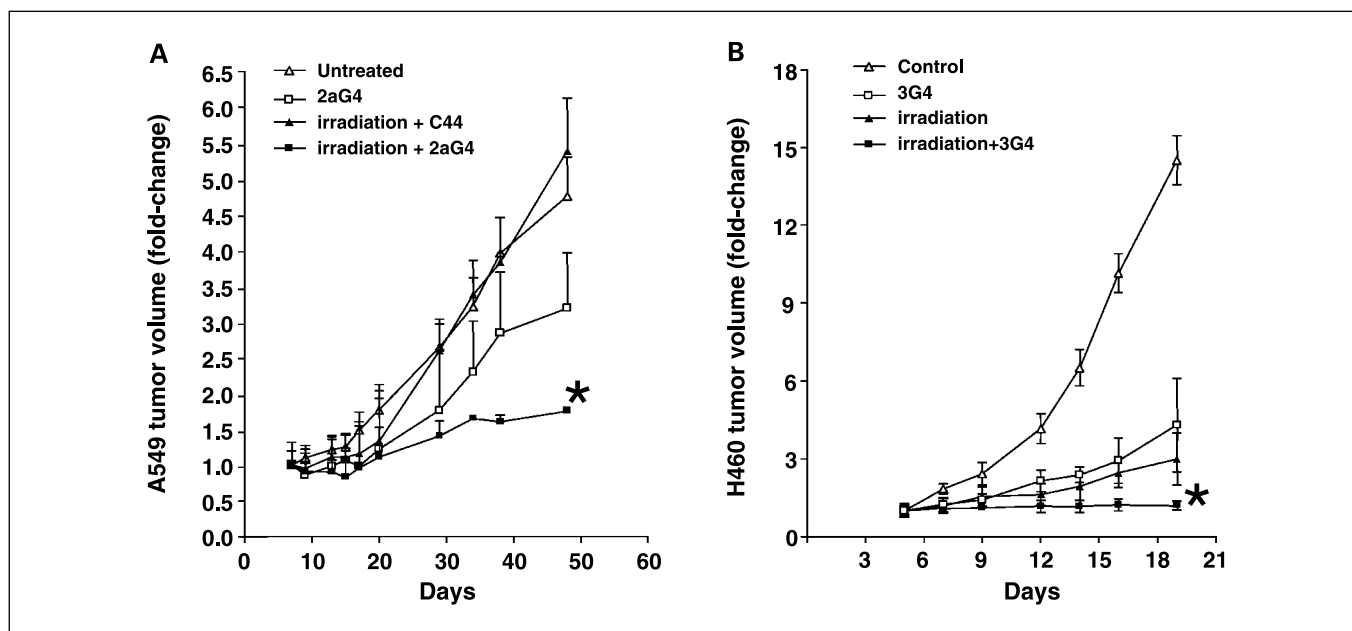


Fig. 3. Radiation therapy enhances the antitumor effect of antibody on human lung tumor xenografts in mice. *A*, nude mice bearing s.c. A549 human lung tumors (5–7 mm in diameter) were treated with 2aG4 alone, radiation therapy + C44, or radiation therapy + 2aG4. Each group contained 15 mice. Radiation therapy was given in 2-Gy fractions for 5 consecutive days. Antibody (100 μ g) was given thrice a week for 3 wks. Points, mean tumor volumes; bars, SE. By day 49, tumor volumes in the radiation therapy + 2aG4 group were significantly smaller than all other groups (*, $P < 0.01$). *B*, nude mice bearing H460 human lung tumors growing s.c. (5–7 mm in diameter) were treated with antibody alone, radiation therapy alone, or radiation therapy + 3G4. Each group contained 10 mice. Points, mean tumor volumes; bars, SE. By day 19, tumor volumes in the radiation therapy + 3G4 group were significantly smaller than in all other groups (*, $P < 0.05$).

needed. A promising recent development is the demonstration that the therapeutic efficacy of radiation therapy can be significantly enhanced when combined with inhibitors of angiogenesis (24) or tumor vascular targeting agents (25). Specifically, combretastatin A4 phosphate combined with radiotherapy effectively shuts down tumor vasculature in human non-small cell lung cancer (3). 5,6-Dimethylxanthone-4-acetic acid combined with carboplatin and paclitaxel also increases median survival in patients with lung cancer (4).

The objective of this study was to determine whether the tumor vascular targeting antibody 2aG4 could enhance the antitumor effects of radiation therapy. 2aG4 targets anionic phospholipids, primarily phosphatidylserine, exposed on the outer surface of cellular membranes. Phosphatidylserine is normally sequestered to the inner surface of the membrane but becomes specifically externalized on tumor vascular endothelial cells in response to oxidative stresses present in the tumor microenvironment (8). Importantly, irradiation is a well-known generator of reactive oxygen species (26, 27). Oxidation of membrane lipids by reactive oxygen species leads to elevated intracellular calcium levels, which inhibit the major importer of phosphatidylserine, aminophospholipid translocase, and simultaneously activate phosphatidylserine exporters (28–31). Thus, we hypothesized that radiation therapy would increase the amount of phosphatidylserine present on tumor endothelial cells, enhancing the antitumor effects of 2aG4.

To determine whether radiation therapy induces phosphatidylserine exposure on endothelial cells *in vitro*, we irradiated HUVECs and monitored phosphatidylserine externalization with 2aG4. Phosphatidylserine exposure occurred as early as 2 h after 2-Gy irradiation, without evidence of other apoptotic

characteristics such as cell shrinkage and nuclear fragmentation. Similar radiation-induced phosphatidylserine exposure was observed in a mouse endothelial cell line, bEnd3.¹ Forty-eight hours after irradiation, more than half of the HUVECs were

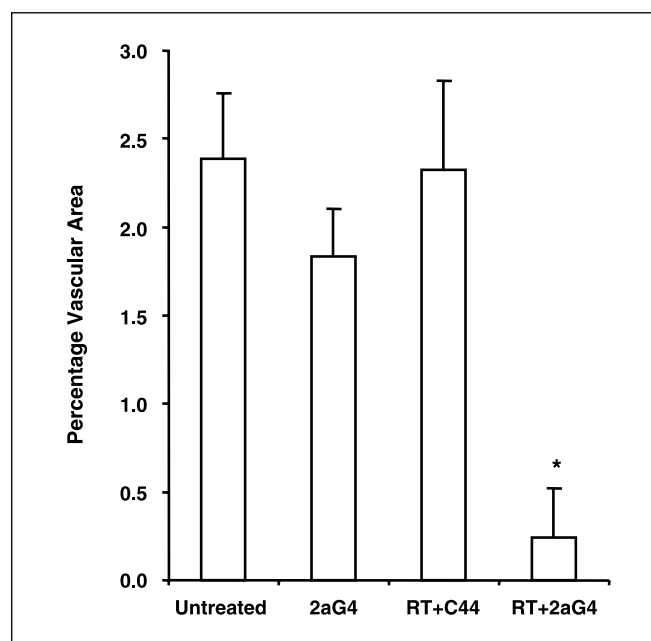


Fig. 4. Decreased vascular density in A549 tumors treated with radiation therapy (RT) plus 2aG4. At the conclusion of the experiment shown in Fig. 3, frozen tumor sections were prepared and stained for CD31 to determine the vascular density. Five tumors from each treatment group were analyzed by capturing 10 random fields ($\times 200$) of each section. Columns, mean percentage of CD31-positive vessel area per square millimeter of section; bars, SE. *, $P < 0.01$, combination treatment reduced vascularity significantly more than either agent alone.

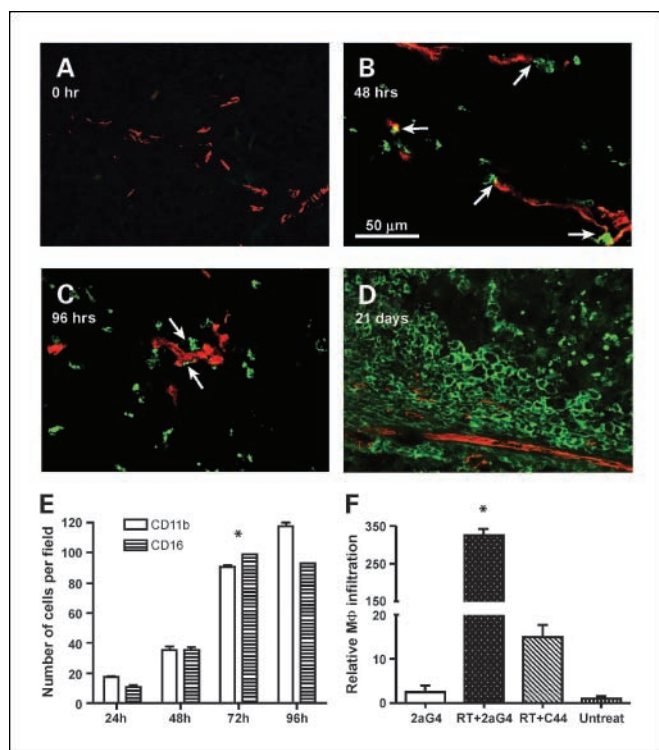


Fig. 5. Increased monocyte/macrophage infiltration into A549 tumors treated with radiation therapy plus 2aG4. Nude mice bearing A549 tumors were treated with radiation therapy + 2aG4 as described in Fig. 3. Tumors were harvested 0, 24, 48, 72, 96, or 21 d after the last treatment. *A* to *D*, mouse monocytes/macrophages expressing CD11b (Mac-1) were detected in tumor sections by immunohistochemistry and appear green. These cells also showed coincident expression of CD16 (FcγRIII; data not shown). Endothelial cells were stained for CD31 expression and appear red. Very few CD11b-positive cells are seen in tumors before treatment (*A*). CD11b-positive cells (*arrows*) are seen in the tumor vasculature 48 h after combination treatment (*B*). CD11b-positive cells are seen migrating out the blood vessels 96 h after combination treatment (*C*). By the end of experiment shown in Fig. 3 (21 d after treatment), monocytes/macrophages filled the tumor interstitium (*D*). *E*, quantification of monocyte/macrophage infiltration over time. Sections from tumors harvested at the indicated times after combination treatment were stained for the presence of infiltrating CD11b/CD16-positive cells. The number of infiltrating cells at 72 h was significantly higher than at 24 h (*, $P < 0.01$). *F*, quantified results of macrophage infiltration in different treatment groups. *, $P < 0.01$.

stained by 2aG4. These 2aG4 positively stained HUVECs were intact as judged by forward/side scatter characteristics and were by negative 7-amino-actinomycin D staining. No phosphatidylserine exposure was seen on nonirradiated cells. In contrast, phosphatidylserine exposure was not detected on cultured A549 lung cancer cells under the same experimental conditions. These results are consistent with those of Kolesnick et al. who showed that endothelial cells are more sensitive to irradiation than are tumor cells (16, 32). The sensitivity of endothelial cells is possibly explained by their abundant expression of acid sphingomyelinase (33), which is activated by irradiation. Activation of acid sphingomyelinase generates ceramide, which causes lamellar-to-nonlamellar phase transitions and loss of lipid asymmetry in the membrane bilayers (34). The mechanisms that many cancer cells have developed to evade apoptosis may also explain the lack of phosphatidylserine exposure on irradiated A549 cells.

Radiation therapy (2 Gy \times 5) of A549 tumors boosted phosphatidylserine exposure on tumor vessels from 4% to 26% as detected by 2aG4 staining. However, very few 2aG4-positive

tumor endothelial cells under this radiation treatment were apoptotic as judged by staining with an anti-cleaved caspase-3 antibody. These observations are also consistent with published data from Garcia-Barros et al. (16) who showed that the apoptosis of tumor endothelial cells was not observed after a single irradiation dose of <11 Gy. The phosphatidylserine-positive/caspase-3-negative tumor endothelial cells appeared morphologically intact and functional, unlike caspase-3-positive endothelial cells. These phosphatidylserine-positive endothelial cells might maintain their lining function for a few days, as shown in other studies (35, 36). The vasculature of normal skin in the path of the irradiation beam showed negligible phosphatidylserine exposure. Endothelial cells in normal organs are generally quiescent, whereas tumor endothelial cells are angiogenic and may contain cytogenetic abnormalities (37). Dividing cells are generally more sensitive to radiation therapy than resting cells, which may explain the lack of phosphatidylserine exposure on normal endothelial cells.

The combination of radiation therapy plus 2aG4 inhibited the growth of radiation-resistant A549 human lung tumor xenografts by 80%. Tumor growth inhibition was significantly better with the combination than with the individual treatments. The combination of radiation therapy with control antibody had no effect on A549 tumor growth, showing the antigen specificity of the radiation therapy/2aG4 combination. Because the A549 tumor cells did not expose a significant amount of phosphatidylserine in response to irradiation *in vitro* or *in vivo*, this result cannot be explained by a direct effect on tumor cells. These data suggest that tumor stromal cells, probably the endothelial cells, are the major target for the

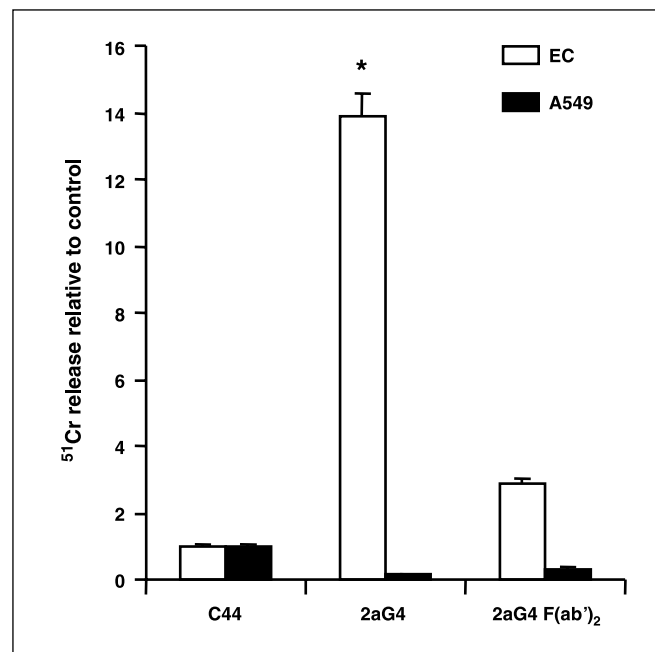


Fig. 6. 2aG4 mediates ADCC of irradiated HUVECs *in vitro*. HUVECs and A549 were irradiated with 2 Gy and labeled with ⁵¹Cr 24 h later. Cells were then incubated with RAW264.7 effector cells in the presence of C44, 2aG4, or 2aG4 F(ab')₂ for 24 h. Supernatants were collected and assayed for ⁵¹Cr release using a gamma counter. Cell lysis in the presence of the control antibody C44 was arbitrarily set as one. Optimal specific lysis of phosphatidylserine-positive endothelial cells in the presence of 2aG4 was observed at an effector-to-target ratio of 5, which is the ratio shown here. Lysis in the presence of 2aG4 was significantly greater than in the presence of 2aG4 F(ab')₂. *, $P < 0.01$.

combination therapy. In this regard, a 91% reduction in tumor vascularity was observed when 2aG4 was combined with radiation therapy, whereas antibody or radiation therapy alone had relatively little effect. The observed decrease in vascular density was accompanied by infiltration of host immune cells. The majority of these cells expressed M1/70 (CD11b, Mac-1) and Fc γ RIII (CD16), identifying them as monocytes/macrophages. Although tumor-infiltrating macrophages have been shown to promote tumor angiogenesis, progression, and metastasis (38–42), macrophage infiltration correlated with tumor growth inhibition in the present study. A high level of Fc γ RIII (CD16) expression is a characteristic of macrophages with antitumor activity, known as M1 macrophages (38). In addition, macrophages exposed to phosphatidylserine-expressing cells produce TGF- β , which, in turn, suppresses TNF- α secretion and proinflammatory responses (43). 2aG4 could block phosphatidylserine and promote the tumoricidal actions of macrophages. Thus, it is possible that

the combination of radiation therapy plus 2aG4 results in the recruitment of M1 type differentiated macrophages, which damage phosphatidylserine-positive tumor endothelial cells. In support of this, 2aG4 enhanced macrophage-mediated lysis of phosphatidylserine-positive endothelial cells in ADCC assays in an Fc-dependent manner. This result is consistent with numerous reports showing that tumor-targeting antibodies can activate monocytes/macrophages with the ability to inhibit tumor growth (44–49).

The results presented here show that targeting phosphatidylserine on tumor vasculature can improve the efficacy of radiation therapy without additional toxicity. A chimeric version of 2aG4, known as bavituximab, has entered clinical trials in patients with various solid tumors. The present study suggests that clinical evaluation of bavituximab in combination with radiation therapy should be considered for lung cancer patients and patients with other cancers commonly treated with radiation therapy.

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