Effect of Yeast-derived β-glucan in Conjunction With Bevacizumab for the Treatment of Human Lung Adenocarcinoma in Subcutaneous and Orthotopic Xenograft Models

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Summary: Human lung cancer is the leading cause of cancer death worldwide. Bevacizumab, a monoclonal antibody against vascular endothelial growth factor (VEGF), in combination with chemotherapy showed significant therapeutic efficacy in human lung cancer patients. However, increased adverse effects limit its clinical utilization. Previous studies demonstrated that polysaccharide β-glucan significantly augments antitumor monoclonal antibody-mediated efficacy via stimulation of the innate effector neutrophil complement receptor 3. Here, we explored combined β-glucan with bevacizumab therapy for human lung cancer using murine xenograft models. To that end, human lung adenocarcinomas were screened for membrane-bound VEGF expression. Both subcutaneous and orthotopic lung cancer xenograft models were used to evaluate the combination therapy. We found that PC14PE6 adenocarcinoma cells express membrane-bound VEGF both in vitro and in vivo. Bevacizumab bound to surface VEGF on PC14PE6 cells and activated complement. In the subcutaneous PC14PE6 tumor model, β-glucan plus bevacizumab showed augmented efficacy in terms of tumor progression and long-term survival compared with bevacizumab-treated alone. These effects were accompanied with massive complement deposition and neutrophil infiltration within tumors. However, this effect was not observed in surface-bound VEGF-negative human lung tumors. Therapeutic efficacy of β-glucan with bevacizumab was further demonstrated in an orthotopic lung cancer model. Thus, our data suggest that β-glucan enhances bevacizumab-mediated efficacy and may provide therapeutic benefits for lung cancers with membrane-bound VEGF expression.

Key Words: angiogenesis, antitumor monoclonal antibody, β-glucan

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Lung cancer is the leading cause of cancer-related death both in the United States and worldwide.[1,2] In 2008, the American Cancer Society estimated 208,600 Americans would be newly diagnosed with lung cancer and that nearly 161,000 Americans would die because of lung cancer.[3] Among lung cancers, 80% to 85% are nonsmall cell lung cancers (NSCLCs) including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma; small cell lung cancer accounts for 15% to 20%.[4] Despite medical and scientific progress against cancer over the past decades and great improvements in survival, the overall 5-year survival rate for lung cancer only improved marginally from 13% in 1975 to 16% in 2003.[3,5] This is primarily attributed to the majority of lung cancers being diagnosed with locally advanced stage or metastatic disease and NSCLCs not being highly responsive to chemotherapy.[6] Thus, additional treatment options for lung cancer are drastically needed.

Angiogenesis is a hallmark of solid tumor development,[7] as this growth requires a rich and vast supply of nutrition. Previous studies demonstrate that tumors secrete angiogenic factors including vascular endothelial growth factor (VEGF) to stimulate angiogenesis and increase vascular permeability.[8] VEGF is also an antiapoptotic factor and may promote tumor invasion and metastasis.[9] VEGF is expressed in the majority of patients with NSCLC,[10] making tumor VEGF an attractive target for lung cancer therapy. Anti-VEGF monoclonal antibody [monoclonal antibody (mAb), bevacizumab, Avastin] is a mouse-derived humanized monoclonal IgG1 antibody (Ab) against human VEGF. It binds to the secreted VEGF isoforms and prevents VEGF binding to its receptors.[11,12] It also normalizes the blood flow and changes the osmotic pressure within the tumor to improve the delivery of chemotherapy medications.[13,14] Although bevacizumab uses the human IgG1 framework which itself is capable of potently activating complement, it has not been shown that immunologic effects such as antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity are involved in the bevacizumab-mediated antitumor effect. A landmark phase III clinical trial conducted by the Eastern Cooperative Oncology Group 4599 established the role of biologic- targeted therapy using bevacizumab together with systemic chemotherapy (paclitaxol plus carboplatin) for locally advanced or metastatic nonsquamous NSCLC. An improvement of median overall survival climbed from 10.3 to 12.3 months.[15] The efficacy of bevacizumab was confirmed by a recent report of the international randomized phase III trial Avastin in Lung cancer which combined systemic chemotherapy gemcitabine plus cisplatin with or

without bevacizumab in locally advanced and metastatic nonsquamous NSCLC.[16,17] Despite the positive results in these studies, clinical improvement of long-term survival for advanced lung cancer patients is still a daunting task.

Oral and intravenous (IV) fungal β-glucans have been used as an adjuvant tumor therapy for decades with varying and unpredictable success owing to a lack of understanding of their action mechanism.[18] Our previous studies have demonstrated the mechanism of action of β-glucan by which soluble, low-molecular weight yeast-derived β-glucan binds to leukocyte complement receptor 3 (CR3; CD11b/CD18, Mac-1, αMβ2-integrin), priming this iC3b receptor of neutrophils, macrophages, and natural killer cells to mediate the cytotoxicity of iC3b-opsonized tumor cells.[19,20] In vitro studies demonstrate that dual ligation of CR3 by I-domain ligand iC3b, which is mediated by complement-activating antitumor Abs, and lectin-like domain ligand β-glucan leads to degranulation and cytotoxic responses.[21,22] The therapeutic efficacy using combined yeast-derived or barley β-glucan with complement-activating antitumor mAbs has been demonstrated in a variety of murine syngeneic tumor models[22–25] and xenograft human tumor models.[26–29] In these, successful therapy required tumor-reactive antibodies that activated complement and deposited iC3b on tumor cells. The requirement for iC3b on tumors and CR3 on leukocytes was highlighted by therapy failures in complement 3 (C3)- and CR3-knockout mice.[23,24]

Our recent study demonstrated that yeast-derived β-glucan could augment bevacizumab-mediated therapeutic efficacy for human ovarian cancer with membrane-bound VEGF expression.[30] In this study, we explored the possible synergistic effect of β-glucan and bevacizumab in the treatment of human lung carcinomas. Here, we show that in xenograft mouse models of human lung adenocarcinoma expressing tumor surface VEGF, IV administration of yeast-derived β-glucan significantly enhanced the effect of bevacizumab in inhibiting tumor growth and improving overall survival. This effect was achieved in both the subcutaneous (SC) and the orthotopic human lung adenocarcinoma xenograft murine models.

MATERIALS AND METHODS

Cell Culture

The human lung adenocarcinoma cell line PC14PE6 was kindly provided by Dr Isaiah Fidler (MD Anderson Cancer Center, Houston, TX). Other human NSCLC cell lines A549, H1299, and NCI-H23 were obtained from American Type Culture Collection (Manassas, VA). The PC14PE6 cells were cultured in high glucose Dulbecco modified essential medium (Caisson Labs, North Logan, UT), plus 10% fetal bovine serum (Equitech Biotechnology, Kerrville, TX), 1 × pyruvate, 100 UI/mL penicillin (Mediatech, Manassas, VA), 100 μg/mL streptomycin (Mediatech), 2 mmol/L L-glutamine (Mediatech), 1 × MEM Vitamins and 1 × nonessential amino acids (Mediatech). Cells were maintained in a 5% CO_2 incubator at 37°C. The NCI-H23, A549, and H1299 cells were cultured in McCoys 5a (Caisson Laboratories) with 10% fetal bovine serum.

Antibodies and Other Reagents

The mAb against VEGF bevacizumab was from Genentech (San Francisco, CA) and the chimeric mAb against epidermal growth factor receptor (EGFR) cetuximab was from ImClone Systems (New York, NY). Anti-mouse C3-fluorescein isothiocyanate (FITC) and anti-human C3-FITC were purchased from Cappel (ICN, Costa Mesa, CA). Anti-mouse Gr-1-phycoerythrin (PE), streptavidin-PE, streptavidin-FITC, and relevant isotype mAb controls were purchased from eBioscience (San Diego, CA). Therapeutic soluble poly-(1,6)-β-D-glucopyranosyl-(1,3)-β-D-glucopyranose (PGG) β-glucan, a pharmaceutical-grade β-glucan with an average molecular mass of 150 kDa, was obtained from Biothera (Imprime PGG, Eagan, MN). Bovine serum albumin powder was purchased from Equitech Bio (Kerrville, TX). Biotin and oregon green labeling kits were obtained from Molecular Probes (Invitrogen, Carlsbad, CA).

Flow Cytometry Analysis and Immunofluorescence Staining

To detect the surface expression of VEGF and EGFR, lung cancer cells (5×10^5) were stained with biotin-labeled bevacizumab, or biotin-labeled Cetuximab or isotype controls on ice for 30 minutes. Cells were washed with phosphate buffered saline (PBS) and then stained with streptavidin-PE or streptavidin-FITC. Cells were washed with PBS and analyzed by flow cytometry. For tissue immunofluorescence staining to detect VEGF expression, neutrophil infiltration and complement activation in tumors, solid tumors were excised and snap frozen in tissue freezing medium (OCT, Sakura Finetechnical Co Ltd, Tokyo, Japan) and kept in −80°C freezer until used. Tissue blocks were cut at 8 μm thickness and fixed in cold acetone for 30 minutes. Tissue slides were first blocked with 3% bovine serum albumin/1 × PBS (blocking medium) and were then stained with biotin-labeled bevacizumab or PE-labeled anti-mouse Gr-1 with FITC-labeled anti-mouse C3 in the blocking medium at room temperature for 1 hour. Slides were washed in blocking medium 3 times for 5 minutes each time. The biotin-bevacizumab stained slides were stained again with strepavidine-PE for 1 hour and washed accordingly. All slides were air dried and mounted with antifading medium (Biomeda, Inc, Foster City, CA). Images were acquired by fluorescence microscopy (Nikon Eclipse TE300 confocal cell images, Nikon Corporation, Tokyo, Japan). The exposure was 1000 ms unless otherwise specified.

Detection of Complement Activation

In vitro activation of complement and deposition of C3b on the cell surface were carried out. Human serum was freshly collected and kept on ice. Tumor cells (1×10^6) were incubated with 100 μL of either isotype control Ab or 10 μg/mL working dilution of bevacizumab alone, diluted human serum (1:10) alone, or diluted human serum (1:10) containing 10 μg/mL of bevacizumab at 37°C for 30 minutes. Cells were washed and resuspended in 100 μL of solution containing detecting Ab goat anti-human C3-FITC. Cells were incubated on ice for 30 minutes and washed twice as above. Propidium iodide was used to exclude the dead cells. Cells were then analyzed by flow cytometry.

Mice and Tumor Models

The murine tumor therapy protocols were conducted in compliance with all guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Louisville. Fox Chase Institute for Cancer Research severe combined immunodeficient (SCID) mice were purchased from Taconic (Hudson, NY). For the SC xenograft model, 6 to 8 week-old SCID mice were injected SC in the lower side of abdomen with 10×10^6

PC14PE6 cells or NCI-H23 cells mixed with Matrigel (BD Biosciences, San Jose, CA). Treatment was started when tumor diameter reached ~7 mm for up to 4 weeks, during which time tumor diameters were recorded twice weekly. Tumor-bearing mice were randomized and divided into 4 groups and received PBS, PGG β-glucan alone (1.2 mg IV twice weekly), or bevacizumab (0.2 mg IV twice weekly) with or without PGG β-glucan (1.2 mg IV twice weekly). Tumor diameter was measured by caliper. Mice were killed when tumors reached 15 mm in diameter as recommended by Institutional Animal Care and Use Committee guidelines. In the treatment groups, survival was monitored up to 110 days beyond tumor implantation. In an orthotoptic animal model, 1×10^6 PC14PE6 cells mixed with Matrigel and were injected into the lung as described previously.[31] Treatment was started on day 7 after tumor implantation and lasted for a total of 8 weeks. Animal survival was monitored daily over 110 days.

Immunohistochemical Analysis

In an orthotopic mouse model, the lungs were harvested from mice treated with different regimens as described above. Tissues were processed at the pathology laboratory of the University of Louisville. Paraffin-embedded tissues were sliced at 8 μm thickness. Samples were stained with hematoxlyin-eosin or stained with anti-Ki67 Ab (Lab Vision, Fremont, CA). Briefly, slides were hydrated and placed in citrate (Dako Glostrup, Denmark) overnight at 72°C, rinsed in distilled water, placed in Tris Buffer (DakoCytomation, Carpinteria, CA) for 5 minutes and transferred to an Autostainer (DakoCytomation). The following incubation times with washes in Tris buffer following each step were used: 5 minutes in 3% hydrogen peroxide, 60 minutes in primary Ab (Ki67 at 1:50, Lab Vision, clone SP6), 30 minutes in a polymer (Dako Envision Plus Rabbit), and 10 minutes in Liquid DAB (DakoCytomation). Nuclei were counter-stained with Aqueous Hematoxylin M10 (Biomeda, Foster City, CA) for 1 minute and neutralized in automation buffer for 1 minute, rinsed in distilled water, dehydrated in 95% and 100% ethanol, cleared in xylene and mounted with Permount.

Statistical Analysis

Data from mouse therapy protocols were entered into Prism 4.0 (GraphPad Software, La Jolla, CA) to generate graphs of tumor regression or survival and to determine the survival significance of differences between data sets. One-way analysis of variance with Fisher least significant difference was used to compare tumor sizes at the end of treatment. The log-rank test was used to determine the significance of differences between 2 survival curves.

RESULTS

Expression of Surface-bound VEGF and Complement Activation on Human NSCLC Cell Lines

To detect VEGF expression on human NSCLC cell surfaces, the 4 human lung adenocarcinoma cell lines A549, NCI-H23, H1299, and PC14PE16 were stained with anti-VEGF mAb. As shown in Figure 1 left panel, PC14PE6 cells, but not other NSCLC cell lines, express membrane-bound VEGF. This is consistent with previous findings.[32,33] In contrast, all the 4 NSCLC cell lines express EGFR (erbB-1, Her-1) on their surfaces at various levels (Fig. 1, right panel). To confirm that PC14PE6 tumor expresses surface-bound VEGF in vivo, PC14PE6 or NCI-H23 cells were inoculated in SCID mice to form SC tumors. The tumor specimens were cryosectioned and stained with anti-VEGF mAb (red). Anti-EGFR mAb (green) was also used for staining to reveal tumor cells. Indeed, surface-bound VEGF was expressed on PC14PE6 tumors and colocalized with EGFR expression (Fig. 2, left panel). In contrast, NCI-H23 tumors expressed abundant EGFR. However, VEGF was not expressed on these tumors (Fig. 2, right panel).

Next, we examined whether this binding of bevacizumab to cell surface VEGF could activate the complement system and cause deposition of iC3b on the tumor cell

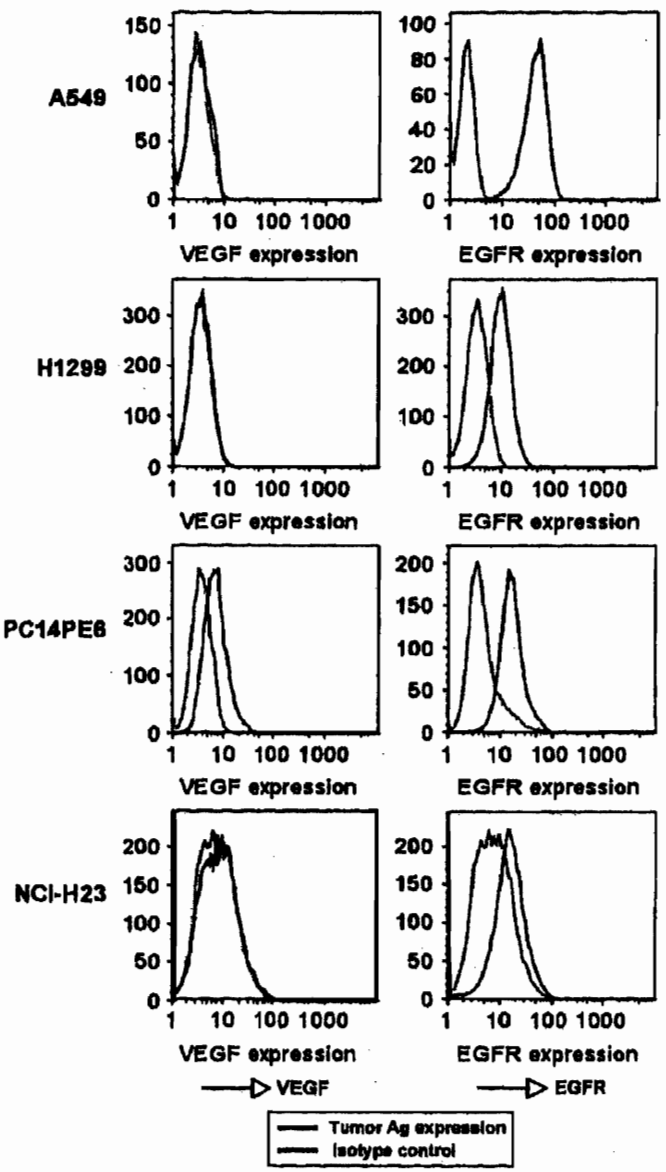

FIGURE 1. Expression of surface VEGF and EGFR on human lung adenocarcinomas. Human lung adenocarcinoma cell lines A549, NCI-H23, H1299, and PC14PE16 were examined for cell surface VEGF and EGFR expression by flow cytometry. Cells were stained with bevacizumab-PE (left panel), cetuximab-PE (right panel) or PE-labeled isotype control antibody (grey line). EGFR indicates epidermal growth factor receptor; PE, phycoerythrin; VEGF, vascular endothelial growth factor.

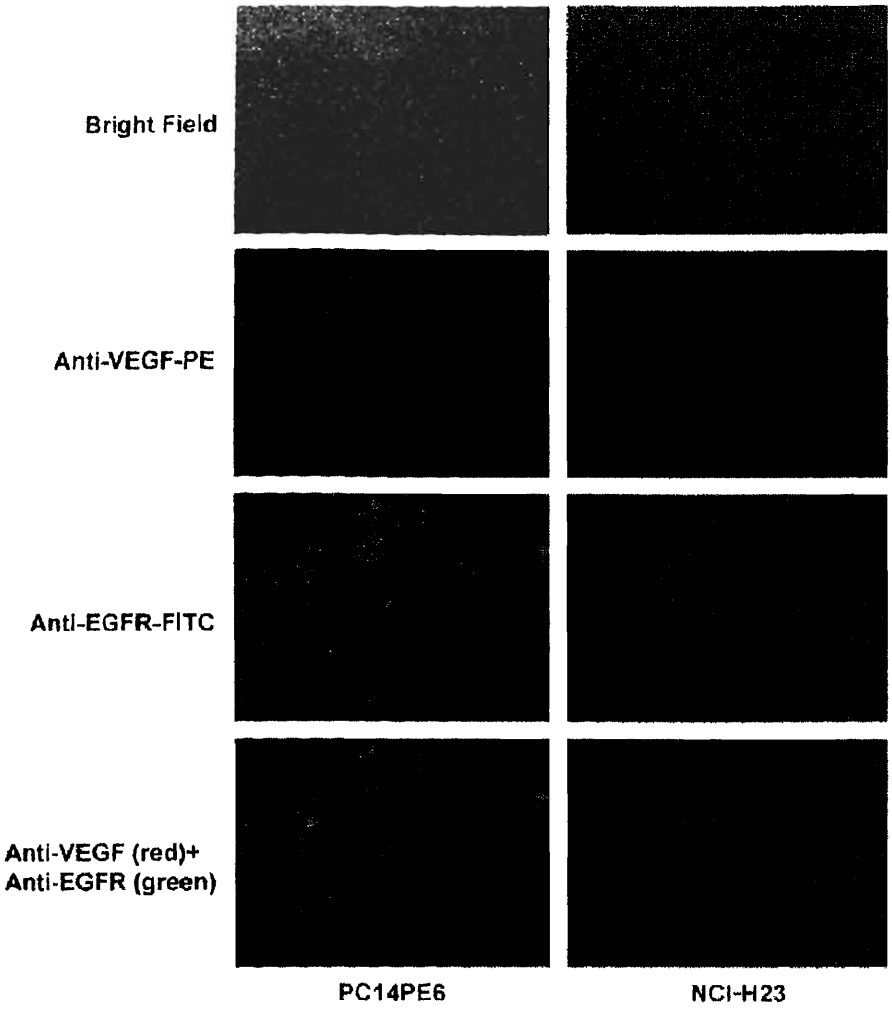

FIGURE 2. In vivo expression of vascular endothelial growth factor and epidermal growth factor receptor by xenograft tumors. Severe combined immunodeficient mice were implanted with PC14PE6 cells or NCI-H23 cells. After tumor sizes reached 5 to 7 mm in diameter, mice were killed and tumors were removed and snap frozen. Frozen section slides from PC14PE6 tumors (left panel) or NCI-H23 tumors (right panel) were stained with phycoerythrin-bevacizumab (red) and fluorescein isothiocyanate-cetuximab (green). Images were acquired by fluorescence microscopy. Original magnification = × 400.

surface. To this end, all the 4 NSCLC cell lines were incubated with bevacizumab plus fresh human serum as the source of complement and then stained with anti-C3b-FITC mAb. As shown in Figure 3, anti-VEGF mAb could bind membrane-bound VEGF on PC14PE6 cells and efficiently activate complement as assessed by flow cytometry. All other tumor cell lines that were negative for membrane-bound VEGF expression were also negative for complement activation.

PGG β-glucan in Combination With Anti-VEGF mAb Therapy in the SC Xenograft Lung Cancer Models

To determine whether the combined PGG β-glucan with bevacizumab therapy has an augmented antitumor effect compared with the bevacizumab alone therapy in vivo, human NSCLC xenografts on SCID mice were carried out. Because in vitro study showed that PC14PE6 cells express membrane-bound VEGF, PC14PE6 xenografts on SCID mice were used to assess therapeutic efficacy with different therapeutic regimens. In addition, human NSCLC NCI-H23 cells, which do not express membrane-bound VEGF, were used as a control. To this end, SCID mice were injected SC with 10×10^6 cultured PC14PE6 cells or NCI-H23 cells mixed with Matrigel. When the tumor diameter reached ~7 mm, mice were randomly assigned to 4 groups and IV-treated twice per week with either PBS, bevacizumab alone, PGG β-glucan alone, or bevacizumab plus PGG β-glucan for a total of 4 weeks. The growth of tumors was closely monitored. In the PC14PE6 tumor model, tumor-bearing mice treated with PBS were all killed early owing to the large sizes of tumors (Fig. 4A), whereas the tumor-bearing mice treated with IV bevacizumab alone had slower growth of tumors as compared with PBS-treated control mice ($P < 0.05$) and lived up to 48 days (Figs. 4A, B). PGG β-glucan treatment only did not significantly impact tumor growth or long-term tumor-free survival compared with saline-treated mice (data not shown). Strikingly, tumor-bearing mice treated with PGG β-glucan in combination with bevacizumab showed a much slower growth of tumors during the treatment period compared with bevacizumab treatment only ($P < 0.05$, Fig. 4A). More importantly, approximately 10% of these mice achieved long-term tumor-free survival whereas mice treated with bevacizumab, although exhibiting delayed tumor growth, did not survive beyond 50 days

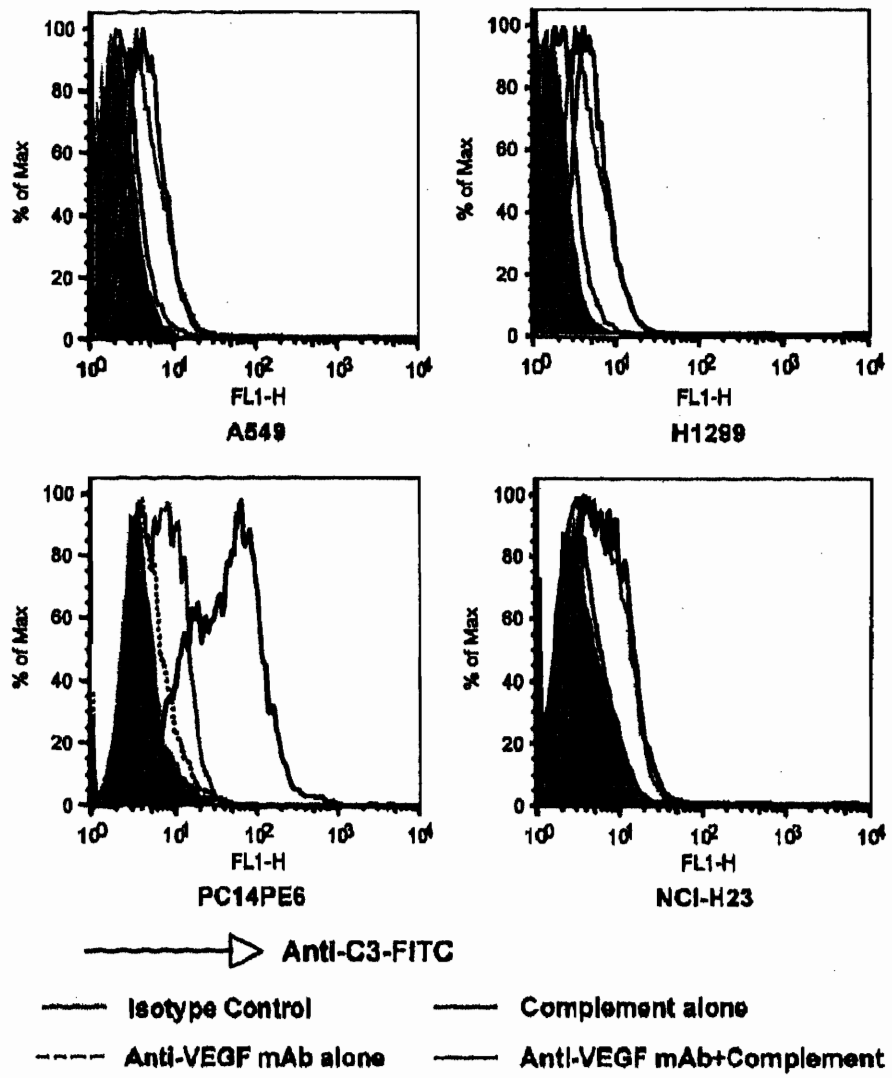

FIGURE 3. Detection of complement deposition on human nonsmall cell lung cancer cells. Human lung adenocarcinoma cells, H1299, PC14PE6, A549, and NCI-H23 were incubated with control isotype Ab (grey filled line), bevacizumab alone (black dotted line), complement alone (grey dotted line) or a mixture of bevacizumab and complement (black filled line) and then stained with fluorescein isothiocyanate-labeled goat anti-human C3b Ab. Cells were analyzed by flow cytometry. Data suggest that bevacizumab could bind to surface vascular endothelial growth factor on PC14PE6 cells and activate complement. Ab indicates antibody.

posttumor inoculation. In the NCI-H23 xenograft model, tumor-bearing mice treated with anti-VEGF mAb showed significantly reduced tumor burden compared with PBS-treated control mice or PGG β-glucan-treated mice. However, no significant difference was observed in tumor-bearing mice treated with anti-VEGF mAb alone or anti-VEGF mAb plus PGG β-glucan (data not shown). These data suggest that the addition of PGG β-glucan to bevacizumab therapy significantly enhances the regression of the membrane-bound VEGF-positive PC14PE6 tumors and achieves long-term survival.

Deposition of Complement Component and Infiltration of Neutrophils in the SC Xenograft Tumor Tissues

Our previous studies have demonstrated that complement activation and neutrophil priming and infiltration within tumors are key events for successful therapy using β-glucan in conjunction with antitumor Ab therapy.[22,25] Thus, the frozen sections of SC tumor tissues were stained with anti-mouse-Gr-1 mAb to reveal neutrophil infiltration together with anti-mouse-C3b Ab for complement activation. As shown in Figure 5, PBS saline-treated tumors displayed minimal complement deposition and very little neutrophil infiltration. Similar observations occurred in PGG β-glucan-treated only tumor sections (data not shown). In contrast, tumors from anti-VEGF mAb-treated or anti-VEGF mAb plus PGG β-glucan-treated mice exhibited massive complement deposition and neutrophil infiltration. These studies suggest that bevacizumab indeed binds to tumor VEGF and subsequently activates complement. The complement activation may release potent chemoattractants such as C5a, which recruit β-glucan-primed neutrophils into tumors.[25]

Enhanced Therapeutic Efficacy of PGG β-glucan Plus Bevacizumab in the PC14PE6 Lung Adenocarcinoma Orthotopic Xenograft Model

Although the SC xenograft mouse model provides a convenient way to monitor tumor progression, it is less clinically relevant for lung tumor development. To overcome potential shortcomings of the SC tumor model, an

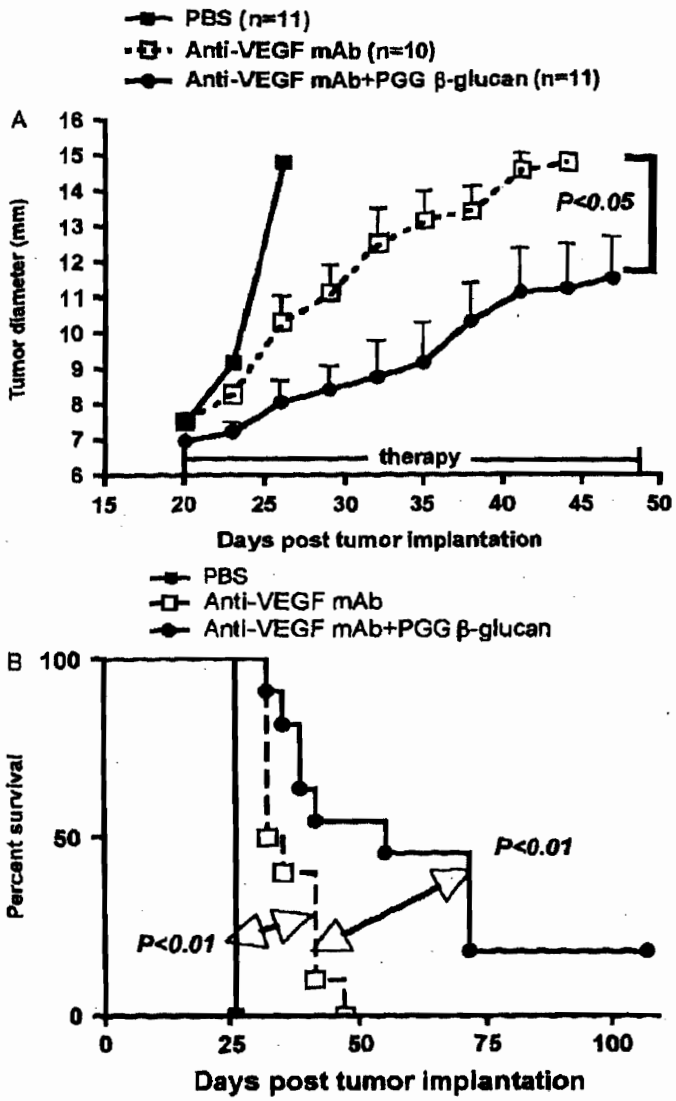

FIGURE 4. Combined PGG β-glucan with bevacizumab therapy in SC xenograft mouse model. A and B, Institute for Cancer Research severe combined immunodeficient mice were injected SC with PC14PE6 cells mixed with Matrigel. Tumors were allowed to form ~7 mm in diameter before therapy. Mice were then treated intravenously twice a week with phosphate buffered saline (n=11), bevacizumab alone (0.2 mg, n=10) with or without β-glucan (1.2 mg, n=11), or PGG β-glucan alone for a total of 4 weeks. Both tumor growth (A) and survival (B) were monitored. Tumor measurements were made at the indicated time. Points, mean; bar, SE. PGG indicates poly-(1,6)-β-D-glucopyranosyl-(1,3)-β-D-glucopyranose; SC, subcutaneous.

orthotopic lung cancer model was further used to evaluate the therapeutic efficacy of PGG β-glucan in combination with bevacizumab. To this end, 1×10^6 PC14PE6 cells were mixed with Matrigel and directly injected into each mouse's left lung. Mice were divided into 4 groups and treated with different regimens 7 days after tumor cell inoculation. As shown in Figure 6A, mice treated with PBS or PGG β-glucan alone died at approximately 25 to 30 days after tumor cell inoculation. Bevacizumab treatment significantly prolonged the survival compared with PBS or PGG β-glucan-treated alone mice ($P < 0.01$). However, none of these mice survived beyond day 100. Strikingly, approximately 85% of mice treated with PGG β-glucan in combination with bevacizumab survived at the cessation of treatment. About 35% of these mice were eventually tumor-free and survived long-term. As compared with bevacizumab treatment alone, mice treated with PGG β-glucan in addition to bevacizumab survived significantly longer (Fig. 6A, $P < 0.001$). To further examine lung tumor development, mouse lungs were isolated from different regimen-treated animals at day 25 after tumor cell inoculation. As shown in Figure 6B, mice treated with PBS or PGG β-glucan had huge tumor burdens and multiple lung tumor nodules. Bevacizumab-treated alone mice showed much smaller tumor nodules at the site of tumor cell inoculation. No apparent tumor nodules were observed in mice treated with PGG β-glucan in combination with bevacizumab.

Next, to characterize the proliferative status of these tumor cells in lungs, we stained tissues with Ki67 expression, a marker of cell proliferation. In PBS or PGG β-glucan-treated mouse lung tissues, Ki67-positive tumor cells were detected in all fields (Fig. 6C). In contrast, much less Ki-67 nuclear staining was detected in the lung tissues from anti-VEGF mAb-treated or anti-VEGF plus PGG β-glucan-treated mice. Hematoxlyin-eosin staining revealed that the lungs from mice treated with PBS or PGG β-glucan alone were full of tumor cells and that lung architecture was extensively destroyed (Fig. 6D). Lung tissues from anti-VEGF mAb-treated alone mice displayed limited levels of tumor necrosis and few inflammatory cell infiltrations. Strikingly, lung tissues from anti-VEGF mAb in combination with PGG β-glucan-treated mice exhibited extensive tumor necrosis and moderate inflammatory cell infiltration with limited normal lung architecture remaining.

DISCUSSION

In this study, we demonstrated that yeast-derived PGG β-glucan augmented bevacizumab-mediated therapeutic efficacy for human nonsquamous NSCLC tumors with membrane-bound VEGF expression. Although the proposed antitumor mechanism of bevacizumab occurs via blockade of circulating VEGF thereby preventing its binding to receptors on the vascular endothelium, we showed that bevacizumab could bind to tumor surface VEGF and then activate complement leading to massive intratumor complement deposition and neutrophil infiltration. The deposition of complement along with soluble β-glucan primes CR3-positive neutrophils to elicit CR3-DCC. Thus, this approach may provide a novel therapeutic avenue for lung cancer patients with membrane-bound VEGF expression.

Bevacizumab in combination with chemotherapy has been approved for advanced and metastatic nonsquamous NSCLC on the basis of Eastern Cooperative Oncology Group 4599 trial.[15] Although the combination therapy has proven to be more efficacious than chemotherapy alone,[34] the response to this combination is not uniform and efforts to improve therapy are still drastically needed.[35] The most predominant and physiologically relevant VEGF isoform (VEGF165) secreted by both cancerous and noncancerous cells has been shown to remain membrane-bound.[36] Human VEGF exerts its functions through binding to 2 related receptors, VEGF receptor 1 (Flt-1) and VEGF receptor 2 (Flk-1 or KDR), which are expressed predominately on endothelial cells.[37,38] We showed that the human lung adenocarcinoma cell line PC14PE6 expresses VEGF

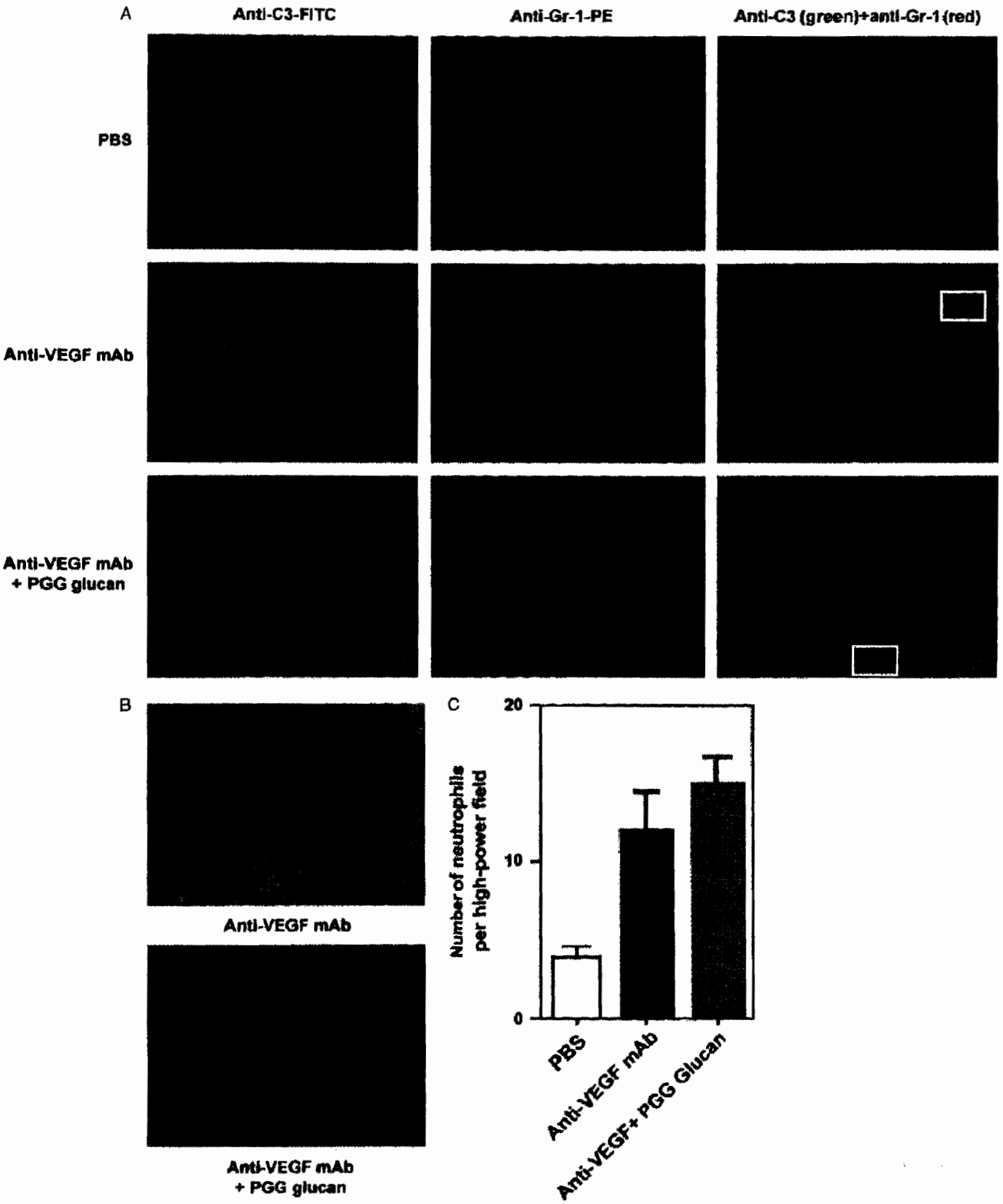

FIGURE 5. Complement deposition and neutrophil infiltration within PC14PE6 tumors. A and B, Tumors from mice treated with different regimens were isolated and tumor sections were stained with anti-mouse C3b-fluorescein isothiocyanate (green) and anti-Gr-1-phycoerythrin (red). Data suggest that tumors from bevacizumab with or without β-glucan-treated mice exhibit massive complement activation and neutrophil infiltration. Original magnifications = × 100 (A) and × 400 (B). C, Quantitative summary of the neutrophil infiltration measured as the mean number of Gr-1+ cells in 5 representative high-power fields (× 400). Mean ± standard error is shown.

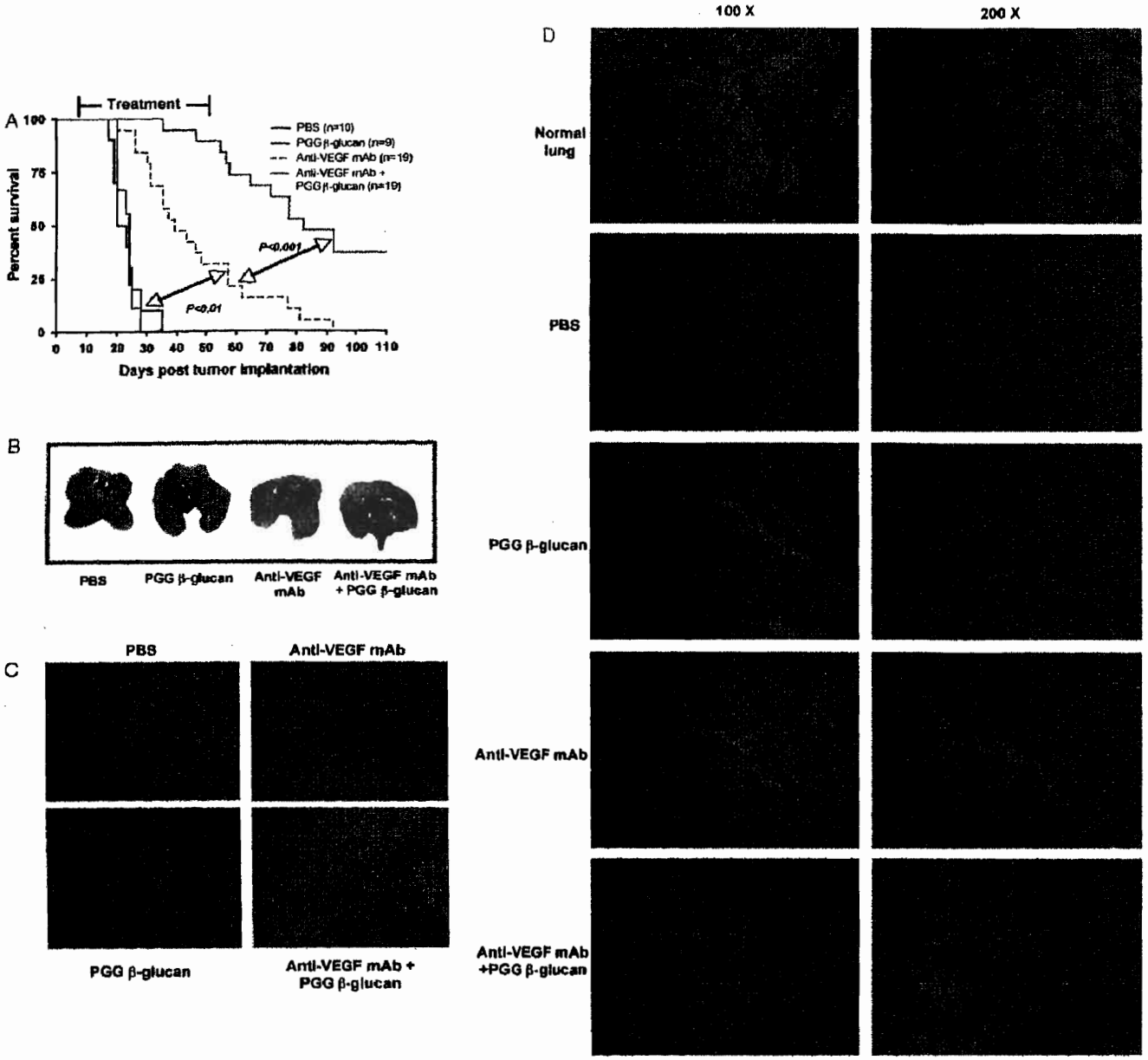

FIGURE 6. The tumoricidal activity of immunotherapy with β-glucan in combination with bevacizumab in an orthotopic lung cancer model. A, Institute for Cancer Research severe combined immunodeficient mice were inoculated with PC16PE6 cells mixed with matrigel in the left lung. Mice were treated with phosphate buffered saline (n = 10), PGG β-glucan alone (n = 9), bevacizumab alone (n = 19), or PGG β-glucan+bevacizumab (n = 19) 7 days after tumor cell injection. Mice were observed for survival over 110 days. B, Lung nodules in an orthotopic mouse model. Freshly harvested lungs from mice treated with different regimens at day 25 posttumor cell inoculation were displayed. C, Lung tissues from different groups were sectioned and stained with anti-Ki67 Ab (brown). Magnification = × 200. D, Lung tissues were sectioned for hematoxlyin-eosin staining. Magnifications = × 100 (left panel) and × 200 (right panel). Squared areas indicate tumor necrosis and inflammatory cell infiltration. PGG indicates poly-(1,6)-β-D-glucopyranosyl-(1,3)-β-D-glucopyranose.

on its surface both in vitro and in vivo (Figs. 1, 2). In addition, in vitro and in vivo studies demonstrated that anti-VEGF mAb bound to surface VEGF on PC14PE6 cells leading to complement activation (Figs. 3, 5). These results suggest that bevacizumab could elicit its immunologic effects via binding of surface VEGF. These effects may include the opsonization of target cells for efficient antibody-dependent cellular cytotoxicity and/or complement-dependent cytotoxicity. In addition, these studies further warrant the utilization of β-glucan in conjunction with bevacizumab, which elicits CR3-DCC.[18]

The therapeutic efficacy of PGG β-glucan in combination with bevacizumab was first assessed using SC SCID xenograft models. Bevacizumab itself has some protective effects. The effect of bevacizumab occurred in both membrane-bound VEGF-positive PC14PE6 tumors and VEGF-negative NCI-H23 tumors. In the clinical setting, treating lung cancer patients having metastatic disease with bevacizumab alone is not as effective as those treated in combination with chemotherapy.[39] Unfortunately, this combination therapy also results in significant bleeding such as pulmonary hemorrhage,[35] which limits general use

for most of the patients. In contrast, PGG β-glucan is a polysaccharide and has minimal toxicity.[18,40] Our study showed that the therapeutic effect of bevacizumab for membrane-bound VEGF-positive PC14PE6 tumors was greatly enhanced when PGG β-glucan was added. However, the augmented effect was not observed in VEGF-negative NCI-H23 tumors. These studies suggest that combination therapy may offer potential clinical benefits for a cohort of cancer patients with membrane-bound VEGF expression. This also raises the question of whether membrane-bound VEGF detection should be included in clinical pathologic reports. A study to detect membrane-bound VEGF on human lung cancer specimens is underway.

CR3-postitive neutrophils have been defined as effector cells for β-glucan-mediated tumor therapy.[24,25] Therefore, we examined the infiltration of neutrophils within tumor tissues. In the PBS-treated mice, minimal deposition of iC3b occurred in tumors, but there was hardly any neutrophil infiltration within tumors. Tumors treated with bevacizumab alone or in combination with PGG β-glucan had massive complement deposition and neutrophil infiltration. Thus, the enhanced tumoricidal effect mediated by combined PGG β-glucan with bevacizumab is possibly owing to CR3-DCC rendered by neutrophils, as demonstrated previously.[25]

The beneficial effect of PGG β-glucan in combination with bevacizumab was further determined using an orthotopic lung cancer model. Similarly, mice treated with PBS or PGG β-glucan alone died quickly with none of these mice surviving beyond 35 days. Bevacizumab treatment alone displayed significant protection and prolonged survival. Mice treated with PGG β-glucan plus bevacizumab exhibited significantly prolonged survival compared to mice treated with bevacizumab alone. It is worth noting that approximately 85% of mice treated with PGG β-glucan in combination with bevacizumab were still surviving at the cessation of treatment. However, some of these mice eventually died after treatment was stopped, which may be related to lung metastatic lesions that occurred in these mice. This may also suggest that PGG β-glucan in combination with bevacizumab therapy would need to be used persistently to treat lung cancer patients similar to bevacizumab treatment in the clinical setting with a maintenance dose of bevacizumab after chemotherapy.[15] In addition, most of lung tumors express surface EGFR. Therefore, future work will include evaluation of PGG β-glucan in conjunction with bevacizumab and anti-EGFR mAb (cetuximab) as cetuximab also potently activates complement. It is expected that combined PGG β-glucan with these 2 mAbs may confer additional clinical benefits for lung cancer patients.

In summary, we demonstrated that yeast-derived β-glucan augments bevacizumab-mediated therapeutic efficacy for human NSCLC tumors with membrane-bound VEGF expression. This combination therapy may provide significant clinical benefits for a cohort of human lung cancer patients. Further studies with this combination therapy need to be carried out in clinical investigation.

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