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β_1 Integrin Inhibitory Antibody Induces Apoptosis of Breast Cancer Cells, Inhibits Growth, and Distinguishes Malignant from Normal Phenotype in Three Dimensional Cultures and *In vivo*

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Abstract

Current therapeutic approaches to cancer are designed to target molecules that contribute to malignant behavior but leave normal tissues intact. β_1 integrin is a candidate target well known for mediating cell-extracellular matrix (ECM) interactions that influence diverse cellular functions; its aberrant expression has been implicated in breast cancer progression and resistance to cytotoxic therapy. The addition of β_1 integrin inhibitory agents to breast cancer cells at a single-cell stage in a laminin-rich ECM (three-dimensional IrECM) culture was shown to down-modulate β_1 integrin signaling, resulting in malignant reversion. To investigate β_1 integrin as a therapeutic target, we modified the three-dimensional IrECM protocol to approximate the clinical situation: before treatment, we allowed nonmalignant cells to form organized acinar structures and malignant cells to form tumor-like colonies. We then tested the ability of β_1 integrin inhibitory antibody, AIIB2, to inhibit tumor cell growth in several breast cancer cell lines (T4-2, MDA-MB-231, BT474, SKBR3, and MCF-7) and one nonmalignant cell line (S-1). We show that β_1 integrin inhibition resulted in a significant loss of cancer cells, associated with a decrease in proliferation and increase in apoptosis, and a global change in the composition of residual colonies. In contrast, nonmalignant cells that formed tissue-like structures remained resistant. Moreover, these cancer cell-specific antiproliferative and proapoptotic effects were confirmed *in vivo* with no discernible toxicity to animals. Our findings indicate that β_1 integrin is a promising therapeutic target, and that the three-dimensional IrECM culture assay can be used to effectively distinguish malignant and normal tissue response to therapy

Introduction

Development of monoclonal antibody therapies designed to target aberrant cell surface signaling receptors, such as HER-2 and epidermal growth factor receptor (EGFR), have shown great promise in cancer therapy (1,2). One other class of cell surface receptors that is critical

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in mediating cell-extracellular matrix (ECM) interactions is β_1 integrin, a major contributor for growth factor receptor signaling. β_1 integrins belong to a family of heterodimeric transmembrane receptors that transmit biomechanical cues that critically mediate cell-ECM interactions (reviewed in ref. 3). β_1 integrin is aberrantly expressed in human breast carcinomas and has been shown to play a central role in growth, apoptosis, invasion, and metastasis (4–8). In addition to its role in cancer progression, an emerging body of evidence indicates that β_1 integrin signaling plays a significant role in mediating resistance to cytotoxic chemotherapies by enhancing cell survival in hematologic malignancies, lung, and breast cancers (9–12). Inhibition of β_1 integrin has also been shown to abrogate the formation of metastasis in gastric and breast cancer models (13–15). Thus, several aspects of β_1 integrin signaling point to it as a multifaceted target for breast cancer therapy.

Using a three-dimensional IrECM cell culture model, which emulates a more physiologically relevant microenvironment (16), we showed previously that down-modulation of β_1 integrin and growth factor signaling pathways resulted in reversion of the malignant phenotype (17), leading to growth arrest and reformation of tissue polarity (18). In addition, β_1 integrin and growth factor signaling were found to be integrated in the context of the three-dimensional IrECM but not on tissue culture plastic (18,19).

We reasoned that a modified version of this culture model could provide an accurate surrogate for testing therapies for human breast cancer cells and tumors. We developed the modified three-dimensional IrECM assay and show that inhibition of β_1 integrin results not only in antiproliferative and proapoptotic effects in malignant cell lines in three-dimensional cultures, but that these results were recapitulated also *in vivo*. β_1 integrin inhibition preferentially affected malignant cells both in culture and *in vivo*; the nonmalignant acini and normal tissues were not affected, and remarkably, there was little or no toxicity to the animals.

Materials and Methods

Cell culture

HMT-3522-S1 (S-1) mammary epithelial cells were originally derived from a woman with nonmalignant fibrocystic breast disease (20) and cultured in H14 medium as previously described (17). S-1 cells were propagated on plastic in medium containing 10 ng/mL EGF, and T4-2 cells were grown on collagen type I-coated flasks in the absence of EGF (17). Human breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from the American Type Culture Collection (Manassas, VA), and SKBR-3 and BT474 were a gift from Dr. Joe Gray (University of California in San Francisco, UCSF). Three-dimensional cultures were plated with cells trypsinized from monolayer cultures and plated on top of commercially available matrix produced from Englebreth-Holm-Swarm tumors (Matrigel, Collaborative Research, Waltham, MA). Cell lines were maintained in media described above, conditioned with 5% Matrigel. This assay is distinct from previously published reversion assays that were done with cells completely embedded within Matrigel (17). Cells were plated on day 0. For S-1 cultures, AIIB2 was added on day 6 of culture, after acinar formation had occurred. For malignant cell lines, AIIB2 was added on day 4 of culture, after cells had undergone several population doublings. All cultures were analyzed after 3 days of AIIB2 treatment.

β_1 integrin and HER-2 inhibitory antibodies

AIIB2, a β_1 integrin function-blocking antibody (originally a gift from C. Damsky, UCSF) was isolated and prepared from a hybridoma cell line (Sierra Biosources, Milipitas, CA). AIIB2 is a rat monoclonal IgG1 that was originally isolated from a human choriocarcinoma hybridoma that specifically binds β_1 integrin extracellular domain (21–23). Experiments using F(ab')₂ fragments of enzyme-digested AIIB2 indicated that the epitope-binding portion of the antibody

was active and resulted in down-modulation of β_1 integrin-mediated signaling (17,19). AIIB2 was added to culture medium on alternate days. Herceptin is a humanized monoclonal antibody against the erbB2 or HER-2 receptor (24) that was used (20 $\mu\text{g}/\text{mL}$) to treat SKBR3 cells on day 6. Control cultures for all experiments were treated with the same concentration of nonspecific IgG.

Immunofluorescence

Cells from three-dimensional cultures were fixed onto a glass slide using 4% paraformaldehyde or methanol/acetone. Nonspecific sites were blocked with 0.5% casein/PBS solution for 1 hour at room temperature. Primary β_1 integrin monoclonal rat anti-mouse antibody (PharMingen, San Diego, CA; 1:100) was diluted in blocking buffer and was applied for 1 hour at room temperature in a humidified chamber. Slides were washed in PBS containing 0.1% bovine serum albumin, before incubating in secondary antibody conjugated to FITC (Molecular Probes, Eugene, OR) for 1 hour in a dark humidified chamber at room temperature. The slides were then washed and counterstained with 4',6-diamidino-2-phenylindole before mounting with Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

Confocal microscopy

Confocal images were acquired by using a Zeiss LSM 410 inverted laser scanning confocal microscope equipped with an external argon/krypton laser. Using a Zeiss Fluor $\times 40$ (1.3 numerical aperture) objective, images were captured at the colony midsection. Relative immunofluorescence intensity of images was standardized by comparing only cultures that were processed identically and stained in the same experiment.

Western immunoblot

Cells propagated in three-dimensional IrECM were first treated with ice-cold PBS/EDTA [0.01 mol/L sodium phosphate (pH 7.2) containing 138 mmol/L sodium chloride and 5 mmol/L EDTA] to isolate the cells and then lysed in radioimmunoprecipitation assay buffer as previously described (17). Equal amounts of protein were loaded onto reducing SDS gels. After transfer onto nitrocellulose membrane (Invitrogen, Carlsbad, CA), blots were blocked with 5% nonfat milk and probed. Primary antibodies used include β_1 integrin, clone 18 (1:1,000), phospho-FAK, clone 14 (1:1,000; BD Transduction Laboratories, Lexington, KY); phospho- β_1 integrin (1:1,000; Biosource, Camarillo, CA); β -actin, clone AC-15 (1:5,000; Sigma, St. Louis, MO). Blots were washed, incubated with secondary antibody, and exposed to X-ray film.

Fluorescence-activated cell sorting analysis

Cells were propagated on tissue culture plastic and harvested using 0.25% trypsin. After resuspending in 1 mL DMEM/F-12 media with trypsin inhibitor, cells were spun down and washed in $1 \times$ PBS, 5% fetal bovine serum, and 0.1% sodium azide on ice. Cells were incubated with primary antibody (AIIB2, 1:10) at 4°C for 30 minutes to 1 hour, washed, and incubated with a fluorescein-conjugated IgG secondary antibody (1:100) for 30 minutes. After washing, 1 mL of 1% paraformaldehyde solution was added to the pellet and suspended immediately. Cells were analyzed using a Beckman-Coulter EPICS XL-MCL Analyzer. System II Data Acquisition and Display software, version 2.0 was used for data analysis.

Apoptosis and proliferation assays

Apoptosis was assayed in cell culture using a commercially available kit (In Situ Cell Death Detection kit, fluorescein; Roche, Nutley, NJ) designed to detect terminal deoxynucleotidyl transferase (TdT)-mediated nick end labeling (TUNEL). Cells were fixed in 4% paraformaldehyde and permeabilized in cold 0.1% Triton X-100 in 0.1% sodium citrate. After

washing in PBS, cells were incubated in TUNEL reaction mixture at 37°C for 60 minutes, washed, and mounted. Proliferation was detected by indirect immunofluorescence of Ki-67 nuclear antigen. Cells were fixed in methanol/acetone and blocked using 10% goat serum, then incubated in primary rabbit antibody against Ki-67, clone MIB-1 (1:200; Novocastra Laboratories, Norwell, MA) for 1 hour and washed before FITC-conjugated anti-rabbit secondary antibody (The Jackson Laboratory, Bar Harbor, ME) was applied. Nuclei were counterstained with DAPI.

For assay of apoptosis in paraffin-embedded tissues, Apoptag In Situ Apoptosis Detection kit (Intergen, Burlington, MA) was used to detect TUNEL reaction. Paraffin-embedded xenograft tumors were sectioned at 5- to 10- μ m-thick sections. Sections were deparaffinized and rehydrated using xylene and ethanol washes. Tissues were then treated with proteinase K at room temperature, washed, and quenched using 3% hydrogen peroxide. Buffer solution was applied, and sections were incubated in TdT enzyme at 37°C for 1 hour. Stop/wash buffer was used before anti-digoxigenin peroxidase conjugate was applied. Proliferation was assayed in paraffin-embedded tissues using indirect immunohistochemistry. Sections were deparaffinized as above and blocked using 10% normal horse serum, then incubated with mouse monoclonal antibody against Ki-67 (Oncogene, San Diego, CA) overnight at 4°C, and washed in PBS. They were then serially incubated with biotinylated anti-mouse antibody, and streptavidin-horseradish peroxidase and 3,3'-diaminobenzidine (DAPI) medium. After counterstaining with hematoxylin, sections were dehydrated in serial concentrated ethanol and xylene and mounted. Cells were scored by counting the total number of nuclei in five high-power microscopic fields ($\times 40$) using a $\times 10$ objective, or a minimum of 200 nuclei per tumor section.

Tumor growth and toxicity assessment *in vivo*

Female $nu^{-/-}$ mice were obtained from Charles River (Wilmington, MA) or Taconic (Germantown, NY) and housed five per cage with chow and water *ad libitum* in a controlled animal barrier. Animals were injected s.c. with 5 to 10×10^6 T4-2 cells or 10^7 MCF-7 cells into the upper back posterior to the right front limb. Estradiol pellets were inserted s.c. above the tail for animals bearing MCF-7 xenografts. AIIB2 antibody or nonspecific rat IgG was injected into the i.p. cavity biweekly beginning on day 4 or day 28 after cell implantation. Tumor dimensions (width, height, and depth) were measured biweekly. At the time of sacrifice, animals were euthanized, and tumors were harvested and either immediately frozen in ornithine carbamyl transferase or fixed in formalin. Serum was collected using cardiac puncture techniques.

Animals were monitored for evidence of toxicity by measuring weight, assessing overall activity, and necropsy. Additional toxicity studies were done using β_1 integrin inhibitory antibody, clone Ha 2/5 (PharMingen), which specifically recognizes murine β_1 integrin. Antibody was administered at doses of 1 to 20 mg/kg biweekly over 4 weeks. All experimental procedures were followed according to the UCSF, and LBNL Animal Welfare Committees approved policies and guidelines.

Statistical analysis

For each dose of AIIB2 or control IgG in culture, pairwise differences in Ki-67 or TUNEL were tested among the six cell lines using Student's *t* test (25). Multivariate ANOVA was used for analysis of tumor volume at each time point. For each dose of AIIB2 or control IgG *in vivo*, pairwise Student's *t* test or χ^2 comparison was used to analyze differences between TUNEL and Ki-67 expression. MINITAB (Minitab, Inc., State College, PA) statistical software was used for all calculations.

Results

β_1 integrin inhibition results in cytotaxis and apoptosis in breast cancer cell colonies treated in three-dimensional cultures

We showed previously that down-modulation of β_1 integrin downstream signaling pathways in *single* cancer cells embedded within three-dimensional lrECM was associated with phenotypic reversion, exemplified by growth arrest and acinar differentiation (17,26), whereas *single* nonmalignant mammary epithelial cells underwent apoptosis (27). We sought to explore whether there is a role for β_1 integrin as a molecular target in breast cancer, which relies on the differential response between normal and malignant tissues. In patients, tumors are commonly discovered *after* a multicellular three-dimensional tumor has already been formed, and normal cells are found in an organized three-dimensional context. We reasoned that this scenario could be emulated also in the three-dimensional lrECM assay. In addition, we wanted to know whether we could then distinguish between the response of normal and malignant structures. Accordingly, we modified the three-dimensional lrECM assay to test these concerns.

When cultured on top of three-dimensional lrECM gels with 5% Matrigel conditioned media, nonmalignant breast cells undergo morphogenesis and, after 6 days, form acini with polarized cells oriented around a central lumen with a well organized basement membrane, recapitulating normal acinar structures found *in vivo* (Fig. 1A; for review, see ref. 28). In contrast, all malignant breast cell lines tested (T4-2, MDA-MB-231, SKBR3, BT474, and MCF-7) continued to proliferate and formed disorganized tumor colonies (Fig. 1A). Our previous studies have shown that β_1 integrin inhibitory monoclonal antibody, AIIB2, or its F(ab')₂ fragments applied to single cells were capable of down-modulating β_1 integrin signaling pathways (17,19). In the present studies, breast cancer cell lines were propagated in three-dimensional lrECM until colonies were formed (4 days) and were then treated with AIIB2 at doses ranging from 0.08 to 0.24 mg/mL, or with isotype-matched nonspecific rat IgG1 as control (Fig. 1B). Using confocal microscopy, we show that β_1 integrin was appropriately localized to the basolateral surfaces of the S-1 cells within the acini, as is the case *in vivo*. In contrast, it was diffusely distributed around the surfaces of each cell within T4-2 and MDA-MB-231 colonies in three-dimensional lrECM, and little expression was seen on three of the other cell lines (Fig. 1B).

In assays starting from single cells, AIIB2 concentrations of 0.10 to 0.16 mg/mL were sufficient to induce reversion (17,19). In the current procedure, colonies were analyzed for percentage of proliferating cells using Ki-67 nuclear antigen and for apoptosis by TUNEL assay. After 3 days of treatment, all but one of the malignant cell lines showed a significant proportional decrease in the percentage of proliferating cells (46–54% of Ki-67 expressing cells at 0.08 mg/mL AIIB2 and 0.24 mg/mL AIIB2, respectively; $P < 0.02$, Student's *t* test for any AIIB2 dose compared with controls for T4-2, MDA-MB-231, BT-474, and MCF-7; Fig. 1C). The only exception among the five malignant cell lines was SKBR3, which did not show a significant decrease in the percentage of Ki-67-positive cells with AIIB2 treatment. Apoptosis was assayed simultaneously; there was a dramatic increase in TUNEL-positive nuclei for all malignant cell lines (85–88% at 0.08 mg/mL AIIB2 and 0.24 mg/mL AIIB2, respectively; Fig. 1D; $n = 3$). For the MDA-MB-231 cell line, the higher dose of AIIB2 was associated with a statistically significant increase in TUNEL-positive nuclei, whereas the *P* approached significance for the lower dose (hence the absence of the *).

In contrast, the nonmalignant cell line S-1 formed acinar structures when cultured on top of three-dimensional lrECM for 6 days and, unlike colonies made of malignant cells, did not undergo increased apoptosis or cytotaxis upon addition of AIIB2 regardless of the dose used (Fig. 1C and D). Similar results were obtained for S-1 cells treated at day 4 (data not shown).

In addition, there was no significant change in the distribution of the size or number of total colonies (data not shown). Previous studies have shown that AIIB2 applied to single S-1 cells induce apoptosis (27); however, in the present study, we show that when S-1 cells are in the context of organized structures, they are resistant to apoptosis. This indicated that the signaling context of β_1 integrin is critical to response to AIIB2 treatment: nonmalignant mammary epithelial cells with intact cell-cell and cell-ECM interactions were resistant to the inhibitor. These results confirm and extend studies of conventional apoptotic and chemotherapeutic agents tested previously in the single-cell assay in three-dimensional IrECM (29).

Coexpression of total β_1 integrin, phosphorylated β_1 integrin, and phosphorylated ^{397}FAK among breast cell lines cultured in three-dimensional IrECM

β_1 Integrin expression detected by immunofluorescence was characterized by basolateral localization in nonmalignant S-1 acinar structures and disorganized and aberrant expression in the malignant cell lines. To further characterize β_1 integrin expression, we analyzed cell lysates for total β_1 integrin levels using Western immunoblotting. Total β_1 integrin expression corresponded to that detected using immunofluorescence; three cell lines (S-1, T4-2, and MDA-MB-231) showed relatively higher levels of β_1 integrin compared with SKBR3, BT474, and MCF-7 (Fig. 2A). In addition, fluorescence-activated cell sorting (FACS) analysis confirmed the surface expression of β_1 integrin reflected that detected using immunofluorescence and Western blot (Fig. 2B). We concluded that β_1 integrin expression was variable, and response to β_1 integrin inhibitory antibody did not seem to correlate with total levels of β_1 integrin expression in individual cell lines.

We reasoned that signaling proteins that are critical in β_1 integrin signaling, such as phosphorylation of β_1 integrin cytoplasmic tail (30), or focal adhesion kinase (FAK; refs. 31, 32), may correlate with response to AIIB2 treatment. To test this, protein lysates from the six cell lines propagated in three-dimensional IrECM were used to detect relative coexpression of β_1 integrin, phosphorylated β_1 integrin (p- β_1 integrin), and phosphorylated ^{397}FAK (p- ^{397}FAK). We found that p- β_1 integrin levels were relatively lower in S-1, T4-2, and MDA-MB-231 cells compared with SKBR3, BT474, and MCF-7 cell lines, which inversely correlated with total β_1 integrin levels (Fig. 2A). p- ^{397}FAK levels did not seem to correlate with total β_1 integrin or p- β_1 integrin levels. Interestingly, p- ^{397}FAK levels were lowest in SKBR3 cells, which were refractory to AIIB2-induced cytostasis.

SKBR3 colonies respond to a combination of AIIB2 and Herceptin

The SKBR3 cell line overexpresses erbB2 (HER-2), a member of the EGF family of growth factor receptors. β_1 integrin has been shown to cooperate with other members of the EGF family, such as erbB1 (19); however, the relationship between HER-2 and β_1 integrin signaling is not well understood. We reasoned that HER-2 signaling was one factor that could contribute to the decreased cytostatic response of SKBR3 cells treated with AIIB2. Herceptin is a monoclonal antibody directed against HER-2 and has a significant role in treatment of patients with HER-2 overexpressing breast cancer (33). Therefore, we tested the effect of Herceptin and AIIB2 in combination in SKBR3 cells. Compared with colonies treated with nonspecific control IgG, SKBR3 colonies treated with AIIB2 or Herceptin alone showed a proportional decrease in Ki-67–positive cells (44.8% for AIIB2 and 39.1% for Herceptin). However, colonies that were treated with both AIIB2 and Herceptin had an augmented proportional decrease in Ki-67–positive cells (68.8%; $P < 0.05$, χ^2 ; Supplementary Fig. S1).

β_1 integrin inhibition preferentially affects larger tumor masses with a global redistribution in colony size and morphology

To determine the effect of treatment on the colony population as a whole, we counted the total number of cells and then scored for individual colonies by size. Using T4-2 cells as a prototype,

we found that β_1 integrin inhibition resulted in a significant decrease in total cell number (Fig. 3A, mean \pm SE; $P < 0.05$, χ^2). To further examine how the treatment influenced the global composition of colonies in the population, we counted the number of cells within each colony after 3 days of treatment. The mean colony size decreased from 12 to 6 cells with treatment reflected by the distribution of the size of colonies (Fig. 3B, mean \pm SE; $P < 0.05$, t test). Similar results were seen for all other cancer cell lines (data not shown). To investigate the time course and mechanism of these changes, we counted the average number of Ki-67- and TUNEL-positive nuclei in the T4-2 cultures as a function of time after addition of AIIB2 (Fig. 3C, mean \pm SE; $P < 0.05$, t test). The number of proliferating cells decreased dramatically even within 1 hour after addition of AIIB2, indicating an immediate growth arrest. The percentage of TUNEL-positive nuclei increased from 24 to 72 hours.

Treatment with AIIB2 results in decreased tumor formation, increased apoptosis, and cytostasis *in vivo*

We have shown previously that breast cancer cells that have been pretreated with β_1 integrin inhibitors before injection into nude mice have decreased ability to form tumors *in vivo* (17, 26). To determine the efficacy and optimal dose of AIIB2 that effectively inhibits tumor formation *in vivo*, we tested the ability of AIIB2 to inhibit untreated T4-2 cells to form tumors in adult female $nu^{-/-}$ mice. Animals were implanted with 5 to 10×10^6 T4-2 cells or 10^7 MCF-7 cells either s.c. or into the mammary fat pad on day 0. Three groups of mice ($n = 9$) received biweekly i.p. injections of (a) isotype-matched nonspecific rat IgG1, (b) 1 mg/kg AIIB2, or (c) 5 mg/kg AIIB2 in a blinded fashion beginning on day 4. Tumors were measured biweekly, and volume was estimated by multiplying width \times length \times depth. Compared with tumors propagated in animals that received control IgG, there was a significant dose-dependent decrease in the volume of treated tumors (Fig. 4A) and in the number of animals harboring tumors (Fig. 4A'; $P < 0.03$, χ^2). After 4 weeks, animals were sacrificed, serum was analyzed for AIIB2 levels, and tumors were analyzed for histology. Compared with controls, treated animals had a dose-dependent level of AIIB2 detectable in serum samples (Fig. 4A'', mean \pm SE; $P < 0.05$, χ^2). Representative micrographs of the same coregistered region of a tumor stained with H&E, Ki-67, and TUNEL are shown (Fig. 4B)

Sections from each tumor were evaluated for apoptosis by TUNEL assay and proliferation by Ki-67 (Fig. 4B'). Compared with controls, treated tumors had significantly decreased percentage of Ki-67-positive cells and a significantly increased level of TUNEL-positive nuclei (mean \pm SE; $P < 0.05$, χ^2). In addition, tumors treated with 5 mg/kg AIIB2 had significantly higher percentage of caspase-3-positive cells ($11 \pm 2\%$) compared with controls ($3.13 \pm 0.7\%$; $P < 0.05$, χ^2). Similar results were obtained for MCF-7 xenografts treated with AIIB2 *in vivo* (data not shown).

AIIB2 is effective against established tumors *in vivo*

To further evaluate the efficacy of AIIB2 *in vivo*, we allowed MCF-7 cells to continue to grow for ~4 weeks and then randomized animals to receive nonspecific rat IgG1, 1 mg/kg AIIB2, or 5 mg/kg AIIB2 for four additional weeks. Compared with controls, treated animals had significantly less tumor growth (Fig. 5A). In addition, histologic analysis showed that treated tumors had significantly fewer Ki-67-positive cells compared with controls (Fig. 5B, mean \pm SE; $P < 0.001$, χ^2) and significantly decreased TUNEL-positive nuclei (Fig. 5B, mean \pm SE; $P < 0.01$, χ^2). Similar results were found for T4-2 xenografts treated *in vivo* (data not shown).

There is no discernible toxicity with β_1 integrin inhibition *in vivo*

Animals were monitored for any signs of toxicity by measuring weekly weight and assessing activity and general appearance. There was no difference in animal weight between the treated

or control groups (Fig. 5C), and no discernible toxicity among any groups, up to AIIB2 doses of 20 mg/kg administered biweekly over 4 weeks (data not shown).

Although AIIB2 seems to cross react with murine β_1 integrin,⁶ we sought to further evaluate the potential toxicity of broad β_1 integrin inhibition *in vivo*. Therefore, we used clone Ha2/5, a β_1 integrin function-blocking antibody that recognizes murine β_1 integrin. Adult female $\nu^{-/-}$ mice were treated with serially increasing doses of antibody from 1 to 20 mg/kg over 4 weeks via biweekly i.p. injection. There were no differences in body weight, activity, overall appearance, or examination at necropsy among animals receiving antibody compared with controls, and no evidence of toxicity among any groups (data not shown).

Discussion

Recent advances in cancer therapy have taken advantage of the aberrant receptors in tumor cells to inhibit growth and enhance the efficacy of conventional cytotoxic treatments (2). β_1 Integrin belongs to a class of cell surface receptors that not only facilitates growth factor receptor signaling but also plays diverse roles in mediating multiple aspects of malignant cell behavior. Indeed, expression of β_1 integrin was shown recently to be necessary for formation of mammary tumors in engineered murine models (4). In addition, β_1 integrin has been shown to enhance survival by mediating resistance to cytotoxic treatment in several cancers (9,34). The success of any therapy depends on its ability to distinguish between malignant and normal tissues or the therapeutic index. Taking advantage of the modified three-dimensional IrECM culture assay, we show that β_1 integrin inhibitory monoclonal antibody effectively distinguishes between normal and malignant tissue structures. Treatment of malignant colonies with AIIB2 resulted in a dramatic loss in total cell number with a concomitant decrease in proliferation and increase in apoptosis. In addition, there was a global redistribution in the malignant colony size and morphology, reflected by a decrease in mean colony size. In contrast, nonmalignant epithelial cells that were capable of forming organized and polar structures with appropriate cell-ECM interactions remained intact and were resistant to β_1 integrin inhibition. *In vivo*, AIIB2 treatment inhibited tumor growth with an associated decrease in proliferation and increase in cell death in early treated tumors and a decrease in proliferation in treatment of established tumors, with no measurable toxicity to the host. Overall, these results indicate that β_1 integrin inhibition is a potentially viable therapeutic approach in the treatment of breast cancer.

The use of three-dimensional cultures provides a physiologically relevant context in which to emulate cells *in vivo* (35,36) and has been used previously to investigate novel mechanisms of drug resistance in cancer cells that are demonstrable specifically only in a three-dimensional setting when the appropriate basement membrane molecules are present (37,38). To model the differences between normal and malignant tissues, we took advantage of the ability of a nonmalignant cell line, HMT-3522-S-1, to undergo normal morphogenesis in three-dimensional IrECM, in contrast to malignant cells that continue to form disorganized invasive colonies. This allowed us to examine the effects of β_1 integrin inhibition on the morphology of cancer cell colonies as a population and to distinguish the potential effects on nonmalignant acini. We had shown previously that nonmalignant cells that were treated with β_1 integrin inhibition as single cells were susceptible to apoptosis (27,39). However, the response of cells within acinar-like tissue structures where β_1 integrin function is relatively intact has not been investigated. We found that in response to 3 days of AIIB2 treatment, all but one malignant cell line in three-dimensional IrECM showed a dramatic loss in total number of cells, coupled with a significant increase in the percentage of apoptotic cells and a significant decrease in the percentage of proliferating cells. In contrast, S-1 cells that formed polar acinar-like structures

⁶Unpublished data.

were entirely resistant to AIIB2. These results indicate that most malignant cells that form colonies in three-dimensional IrECM rely on β_1 integrin signaling for proliferation and survival, whereas in the context of an organized structure, cells were either no longer dependent on β_1 integrin signaling for survival, or that β_1 integrin was not accessible to the antibody.

Further analysis of cell cultures during and after AIIB2 treatment revealed that the largest cancer cell colonies were being affected, resulting in a global change in the morphology and distribution of proliferating cells, reflected in a decrease in mean colony size. This pattern of multiple residual “tumor foci” was seen also *in vivo* (data not shown). The morphologic characteristics of these clones were distinctly different from the untreated tumors, as were features of cell-cell and cell-ECM interactions. These results have implications for clinical treatment. β_1 Integrin has been implicated in mediating resistance to cytotoxic chemotherapies (9,10), and inhibition of different tumor types may enhance response by abrogating resistance. In addition, ionizing radiation was shown to up-regulate β_1 integrin in cancer cells (40,41), and our preliminary studies of β_1 integrin inhibition combined with ionizing radiation are promising and may lead to novel strategies for combinatorial therapies to eradicate or further reduce tumor viability *in vivo*.

Several promising biological therapies aimed at signaling pathways have entered clinical trials; however, despite evidence of response to treatment, useful biomarkers have frequently been difficult to validate (1,42,43). For example, the current treatment of cancers with EGFR inhibition illustrates the complexity of some molecular targets and the lack of robust predictive markers that would aid in the selection of individuals for treatment (42). The mechanisms that are involved in cytoskeleton and apoptosis associated with β_1 integrin inhibition in malignant cells are likely to involve interactions between multiple signaling pathways. For example, our previous studies have shown that β_1 integrin signaling pathway integrates and cooperates with the EGFR signaling pathway via mitogen-activated protein kinase and phosphatidylinositol 3-kinase (18,19). In the present study, we found that β_1 integrin expression on the six breast cell lines used was variable. We probed the cell lines for p- β_1 integrin and p-³⁹⁷FAK to investigate potential markers for β_1 integrin signaling activity. Interestingly, p- β_1 integrin expression was inversely correlated with total β_1 integrin, suggesting that either species is required for β_1 integrin signaling to occur. Although p-³⁹⁷FAK is a requisite protein for focal adhesion formation, its role in β_1 integrin signaling in the context of the cell lines we investigated remains unclear. We recognize that β_1 integrin signaling involves several steps, including activation, heterodimerization, ligand binding, and clustering (44,45); these functional aspects of β_1 integrin signaling activity may not be reflected by the level of receptor expression and/or status of any single signaling protein alone. The major factor that distinguished the nonmalignant S-1 cells and the malignant cell lines is the organization and polarity of β_1 integrin localization, indicating that the context of signaling may be the most important feature that enhances the therapeutic window. Studies are ongoing to investigate which pathways may be the most robust predictors of response in to β_1 integrin inhibition in the clinical setting.

SKBR3 cells were less responsive to β_1 integrin inhibition compared with other cancer cell lines. This cell line is characteristically devoid of E-cadherin and overexpresses growth factor receptor HER-2 features that could contribute to uncoupling of β_1 integrin signaling and survival (46,47). Interestingly, BT474 cells, which overexpress HER-2 and estrogen receptor (ER), remain sensitive to AIIB2. In contrast, SKBR3 cells overexpress HER-2 but are ER negative, a phenotype that has implicated growth factor signaling pathways with resistance to tamoxifen (48). Herceptin, a monoclonal antibody against HER-2, has been shown to down-modulate the HER-2 receptor, resulting in cytoskeleton (24). We found that the addition of Herceptin to AIIB2 in SKBR3 cells in three-dimensional IrECM resulted in a significantly decreased percentage of Ki-67-positive cells compared with cultures treated with AIIB2 alone. These data indicate that an additive cytostatic effect is achieved by using the combination of

inhibitory antibodies. Further investigations of the features of SKBR3 that may confer resistance to AIIB2 are warranted and may help identify subsets of tumors that may respond to a combination of β_1 integrin inhibition and Herceptin or hormonal therapy.

We found that β_1 integrin inhibition was effective in both T4-2 and MCF-7 xenografts in nude mice *in vivo*, confirming our results in three-dimensional IrECM. Similar to the response observed in culture, tumor xenografts treated *in vivo* showed decreased proliferation and increased apoptosis compared with controls in the animals that received treatment beginning 4 days after tumor implantation. In animals where the tumors were treated after 4 weeks of implantation, there was a significant decrease in tumor size and proliferation and a decrease in apoptosis in treated animals compared with controls. The decrease in observed TUNEL-positive cells in the larger tumors could be due to the increased amounts of necrosis, an alternate mechanism of cell death, seen in larger tumors (data not shown).

Toxicity studies using AIIB2 and clone Ha2/5 revealed no discernible toxicity in animals, even with 20 mg/kg doses. These results indicate that β_1 integrin signaling confers growth and survival advantages in cancer cells *in vivo* that can be discriminated from normal β_1 integrin signaling by AIIB2. Other mechanisms also should be considered. For example, immune-mediated secondary effects of the antibody have been shown to play a significant role in antibody-mediated therapies (47). We have previously shown that the F(ab')₂ fragments of AIIB2 are active in three-dimensional IrECM assays (17), and others have shown that AIIB2 binds to a region of β_1 integrin extracellular domain between two putative ligand binding sites that are thought to induce a conformational change (48), resulting in down-modulation of signaling. The activity or presence of Fc-directed immune response *in vivo* has not been isolated from the activity of the F(ab')₂ region per se. These studies, in addition to humanization of the AIIB2 clone, are necessary next steps towards clinical drug development.

In summary, β_1 integrin inhibition using monoclonal antibody AIIB2 results in cytostasis and apoptosis in malignant breast cancer colonies but not normal tissue structures propagated on top of three-dimensional IrECM gels. The three-dimensional IrECM assay appropriately distinguishes the difference in response between normal structures and malignant colonies and reveals global changes in morphology associated with treatment. In addition, AIIB2 inhibits breast cancer growth *in vivo* by eliciting increased apoptosis and decreased proliferation, with no discernible toxicity to animals. We conclude that β_1 integrin inhibition using monoclonal antibodies is a promising approach to breast cancer treatment, and that the modified three-dimensional IrECM assay and protocol is an appropriate assay for testing differences in malignant and normal cell response to targeted therapeutic agents.

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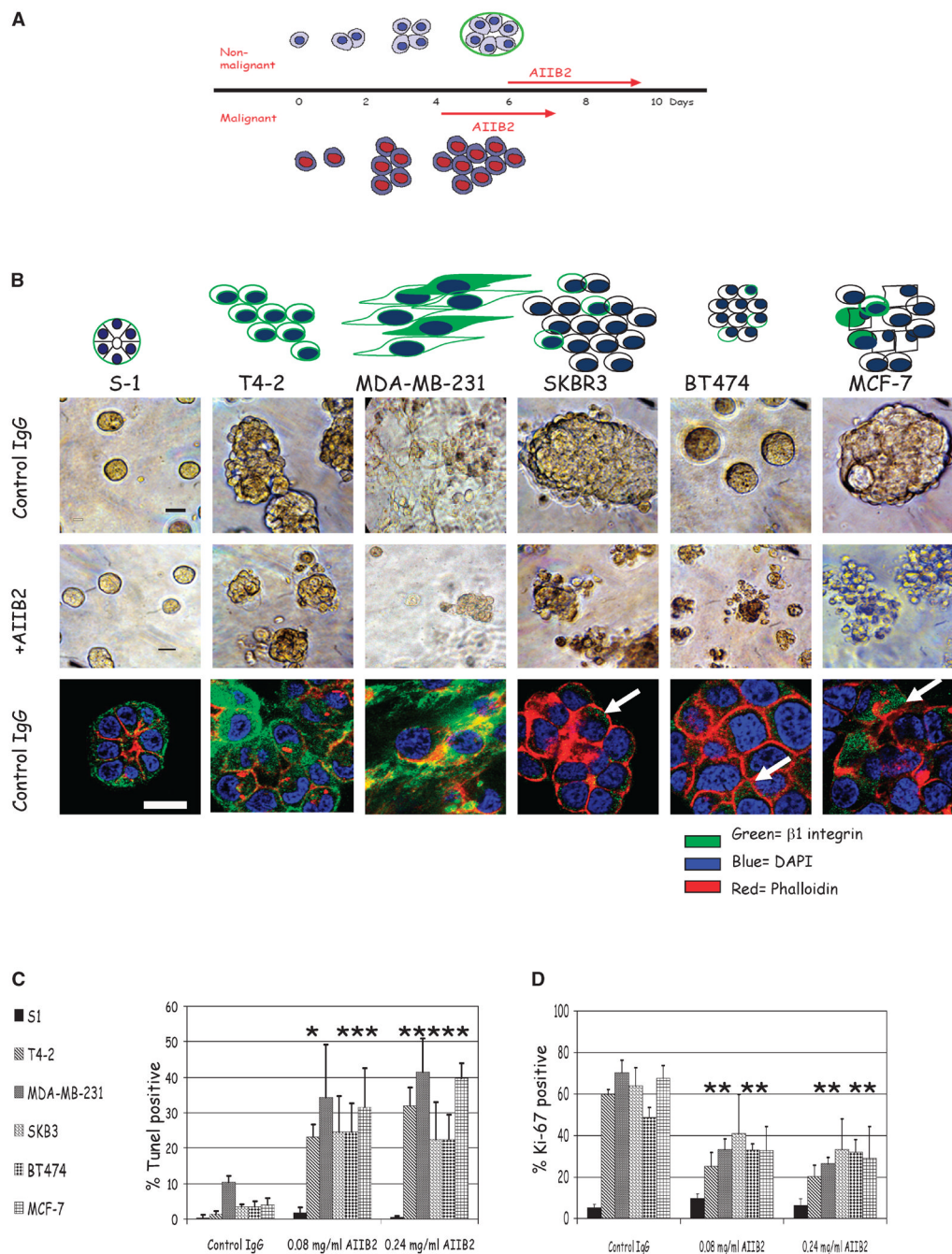


Figure 1. Morphology and response of breast cell lines to β_1 integrin inhibition in three-dimensional lrECM. Five malignant breast cell lines (T4-2, MDA-MB-231, SKBR3, BT474, and MCF-7) and one nonmalignant breast cell line (S-1) were propagated in three-dimensional lrECM and treated with β_1 integrin inhibitory antibody, AIIIB2, after colonies were formed. *A*, schema of the three-dimensional lrECM assay used in this study. *B*, *top* and *middle*, phase-contrast micrographs of cultured colonies before and after antibody treatment, respectively. Bar, 20 μ m. *Bottom*, confocal midsections of β_1 integrin immunofluorescence (—) of colonies cultured in three-dimensional lrECM. Phalloidin was used to stain actin filaments (—), and DAPI was used to stain individual nuclei (—). Bar, 13 μ m for all cell lines shown. *C*, whereas there were

no significant changes in Ki-67 or TUNEL expressing cells among nonmalignant S-1 cells after β_1 integrin inhibition, malignant cell lines, except SKBR3, had a significant decrease in Ki-67 expressing cells and a significant increase in TUNEL-positive cells after antibody treatment. *Columns*, mean ($n = 3$); *bars*, SE. $P < 0.05$, t test.

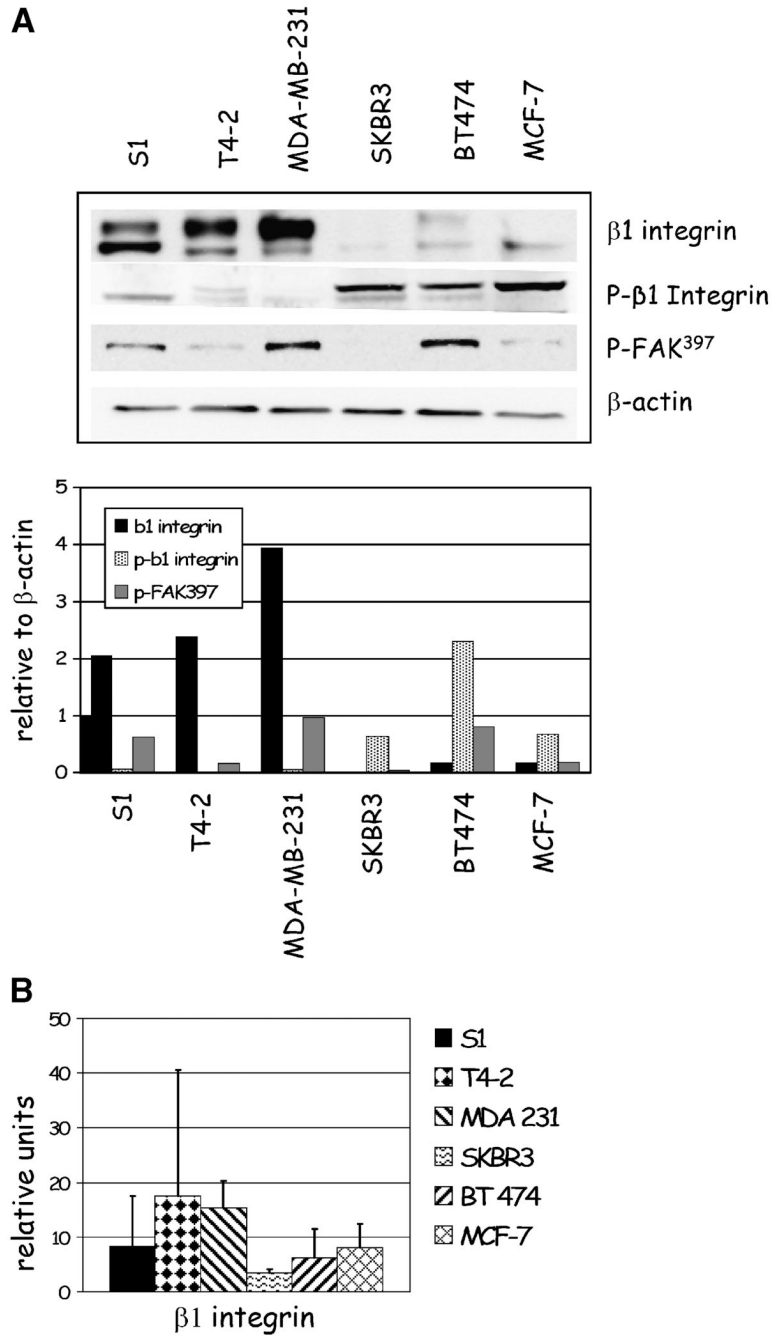


Figure 2. β_1 Integrin, p- β_1 integrin, and p-³⁹⁷FAK are expressed at different levels in breast cell lines in three dimensions. Lysates from five breast cancer cell lines (T4-2, MDA-MB-231, SKBR3, BT474, and MCF-7) and one nonmalignant cell line (S-1) in three-dimensional IrECM were probed by Western immunoblotting for total and p- β_1 integrin and p-³⁹⁷FAK and for surface β_1 integrin expression by FACS analysis. *A*, total β_1 integrin levels are relatively high in S-1, T4-2, and MDA-MB-231 cells compared with SKBR3, BT474, and MCF7 cells. Conversely, p- β_1 integrin levels are relatively low in S-1, T4-2, and MDA-MB-231 cells compared with SKBR3, BT474, and MCF7 cells. Expression of p-³⁹⁷FAK is expressed among all cell lines except SKBR3, where it is undetectable by Western blots. *B*, surface expression of β_1 integrin

by FACS analysis corresponds to that seen by immunofluorescence (Fig. 1B) and Western blots (Fig. 1A).

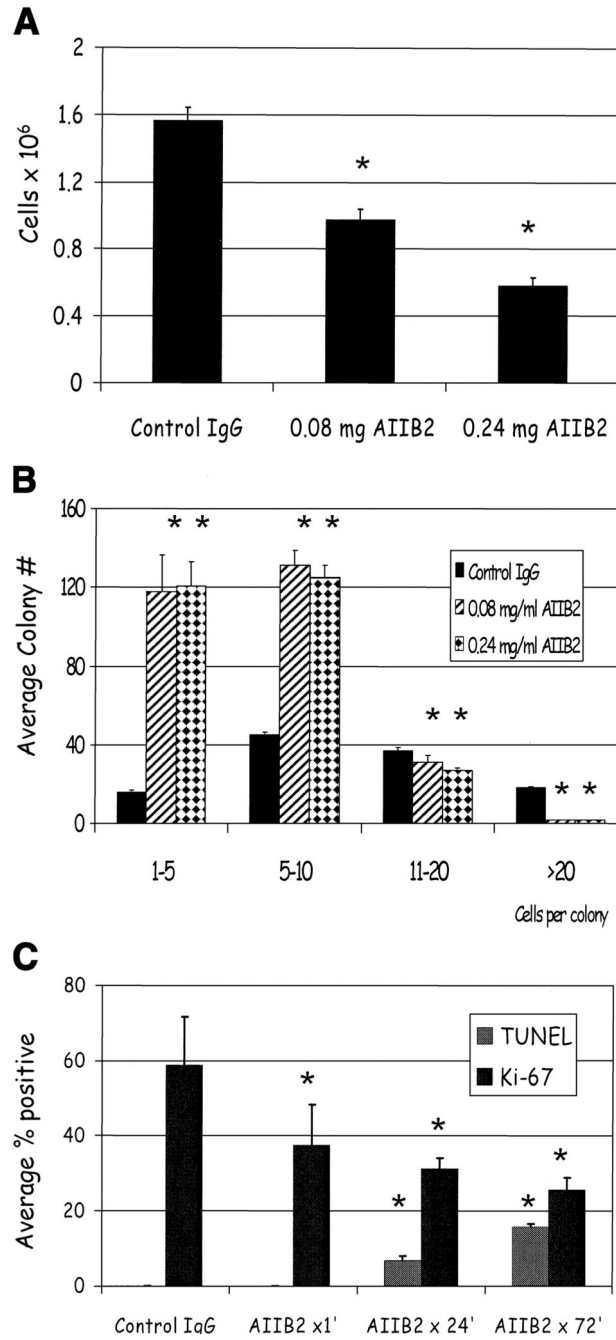


Figure 3.

AIIB2 reduces both total cell number and average colony size in malignant cell lines. *A*, average number of T4-2 cells after 3 days of treatment with AIIB2. *Columns*, mean; *bars*, SE. $P < 0.05$, χ^2 . *B*, histogram showing the average colony size decreased with AIIB2 compared with controls. *Columns*, mean; *bars*, SE. $P < 0.05$, *t* test. *C*, percentage of Ki-67 and TUNEL expressing nuclei among T4-2 cells 1, 24, and 72 hours after addition of AIIB2 to cultures. *Columns*, mean; *bars*, SE. $P < 0.05$, *t* test.

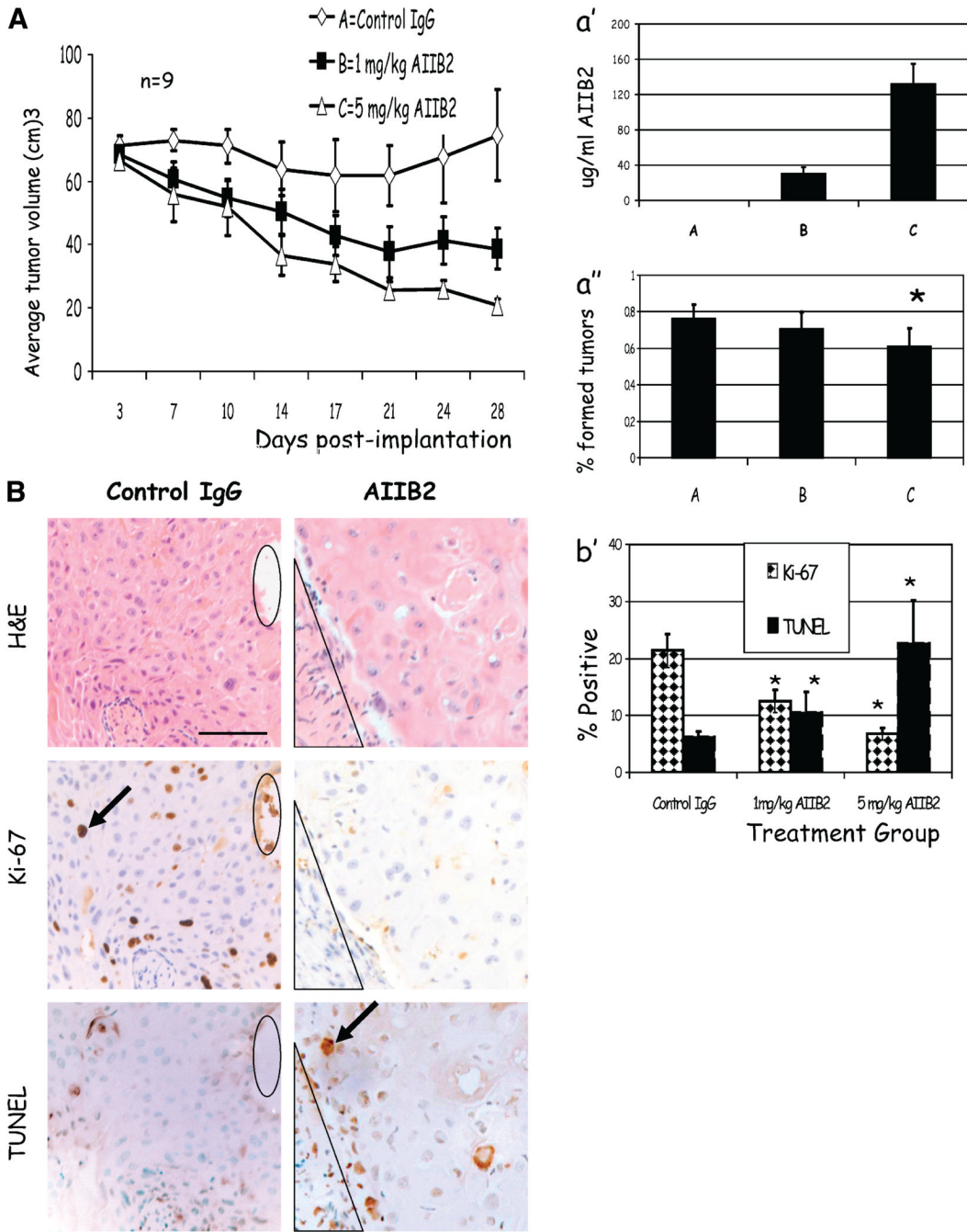


Figure 4. AIBB2 suppresses tumor growth *in vivo* by enhancing apoptosis and decreasing proliferation. A, mean tumor volumes among animals that received 1 mg/kg AIBB2 (■) or 5 mg/kg AIBB2 (△) were significantly smaller than control animals (◆). Points, mean (n = 9); bars, SE. A', average serum concentration (µg/mL) of AIBB2 at the time of sacrifice was significantly higher in animals receiving AIBB2 compared with control vehicle. Columns, mean; bars, SD. A'', average number of animals in each treatment group that had histologic evidence of tumor at the time of sacrifice was significantly lower among treated animals compared to controls. Columns, mean for three separate experiments; bars, SE. *, P < 0.03, χ^2 . B, micrographs of serial sections of tumors that were scored for apoptosis by the presence of TUNEL-positive

nuclei and for proliferation by the presence of Ki-67 nuclear antigen. *Black ovals* and *triangles*, coregistered regions in serial sections; *arrows*, examples of positive staining. *B'*, with AIB2 treatment, the average number of TUNEL nuclei increased, and the number of Ki-67–positive nuclei decreased. *Columns*, mean; *bars*, SE. *, $P < 0.05$, χ^2 . Bar, 247 μm .

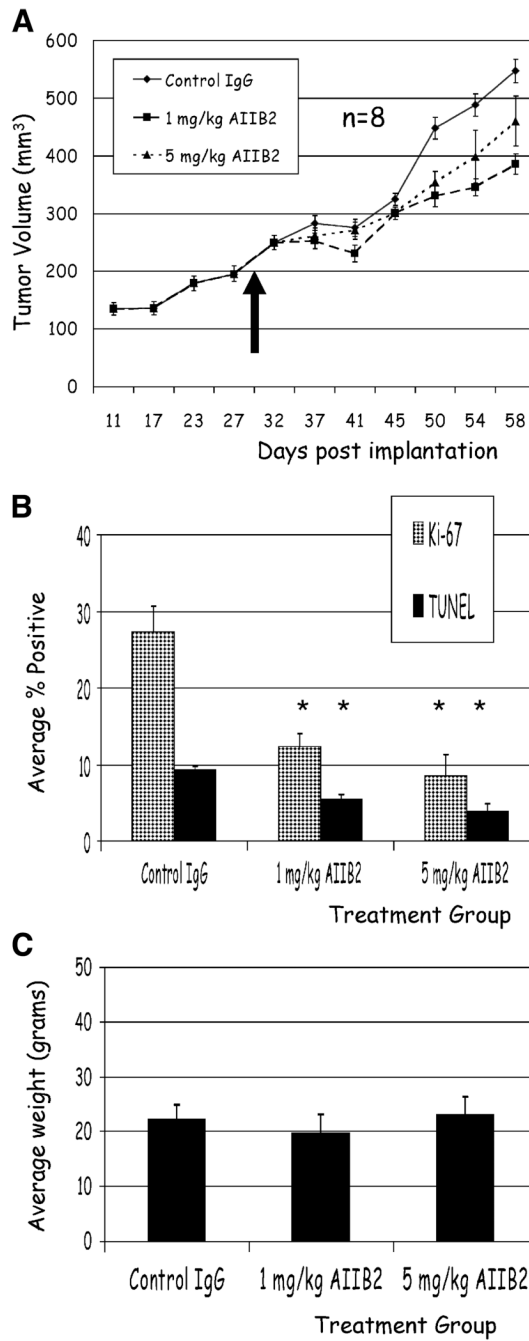


Figure 5. AIB2 effectively induces cytostasis in established MCF-7 tumors *in vivo* with no toxicity. A, animals were randomized to receive treatment or control IgG 4 weeks after MCF-7 cell implantation (arrow, time of randomization at day 30). Mean tumor volumes for animals receiving 1 mg/kg AIB2 (■) and 5 mg/kg AIB2 (△) and nonspecific rat IgG1 (◆). Points, mean ($n = 8$); bars, SE. B, compared with controls, tumors from animals that received AIB2 treatment had significantly fewer Ki-67-positive nuclei and fewer TUNEL-positive nuclei. Columns, mean; bars, SE. *, $P < 0.001$, χ^2 . C, average weight of control animals, and animals that received AIB2 treatment were not significantly different at any biweekly time point

measured throughout the experiment (data not shown) and at the end of the experiment.
Columns, mean; *bars*, SE.