Anti–Prostate Stem Cell Antigen Monoclonal Antibody 1G8 Induces Cell Death *In vitro* and Inhibits Tumor Growth *In vivo* via a Fc-Independent Mechanism

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Abstract

Prostate stem cell antigen (PSCA), a 123–amino acid cell surface glycoprotein, is highly expressed in both local and metastatic prostate cancers as well as in a large proportion of bladder and pancreatic cancers. PSCA overexpression correlates with a high risk of recurrence after primary therapy for prostate cancer. We have reported previously that anti-PSCA monoclonal antibody (mAb) 1G8 inhibits tumor growth, prevents metastasis, and prolongs the survival of mice inoculated with human prostate cancer cell lines and xenografts. The current study was undertaken to elucidate the mechanism of action of anti-PSCA antibody therapy. In particular, we asked whether antitumor activity resulted from recruitment of an immune response or a direct effect on the tumor cell itself. *In vitro* assays show that both intact 1G8 and $F(ab')_2$ fragments of 1G8 induce prostate cancer cell death. The anti-PSCA antibody-induced cell death is caspase independent and requires antigen cross-linking. These results were confirmed in *in vivo* models in which both 1G8 and $F(ab')_2$ fragments were able to inhibit prostate tumor formation and growth equally. These results suggest that the anti-PSCA mAb 1G8 acts by a direct, Fc-independent mechanism to inhibit prostate tumor growth both *in vitro* and *in vivo*. (Cancer Res 2005; 65(20): 9495-500)

Introduction

Prostate cancer is the most common malignancy and the second leading cause of cancer-related mortality among men, with ~200,000 new cases and 30,000 deaths yearly in the United States. The advent of prostate-specific antigen (PSA) screening in the 1980s has led to considerable stage migration with increasing numbers of cancers being diagnosed at a potentially curable juncture. However, a substantial number of patients that have undergone definitive treatment with the intent to cure (i.e., radical prostatectomy or radiation therapy) suffer biochemical recurrence and eventually progress to metastatic disease. Unfortunately, the only therapeutic options presently available for advanced prostate cancer are temporizing or extend life only modestly. Thus, novel treatment strategies are urgently needed.

Prostate stem cell antigen (PSCA) is a member of the Thy-1/Ly-6 family of glycosylphosphatidylinositol (GPI)–anchored cell surface antigens (1). PSCA is expressed in most prostate cancer specimens, including high-grade prostatic intraepithelial neoplasia, primary androgen-dependent tumors, and hormone-refractory metastases. PSCA levels are positively correlated with Gleason grade, tumor stage, and biochemical recurrence. They are also particularly elevated in bone metastasis (2–5). Finally, PSCA is strongly expressed by nonprostatic malignancies, including most bladder and pancreatic cancers (6, 7). Each of these findings makes PSCA a compelling therapeutic target.

We have reported previously that 1G8, a monoclonal antibody (mAb) we developed against PSCA, inhibits tumor growth, prevents metastasis, and prolongs the survival of mice inoculated with human prostate cancer cell lines and xenografts (8). However, the mechanism of action has yet to be elucidated. In particular, it is not known whether anti-PSCA antibodies work by recruitment of the immune system or by direct modification of antigen-regulated signaling at the cell surface. Elucidation of the molecular pathway(s) involved in PSCA-targeted antibody therapy may be critical for translation of this treatment into the clinic. In particular, knowledge of the mechanism of action may affect antibody selection (e.g., epitope selection), timing of therapy (e.g., early or late), therapeutic setting (e.g., androgen dependent versus androgen independent), or rational selection of agents for combination therapy (e.g., chemotherapy or hormonal ablation). In the current study, we provide evidence that 1G8 acts by a Fc-independent mechanism to inhibit tumor growth both *in vitro* and *in vivo*.

Materials and Methods

Cell Lines

The LAPC-9 xenograft, which expresses PSA and PSCA, was passaged in 6- to 8-week-old male severe combined immunodeficient (SCID) mice (Taconic Farms, Germantown, NY) by s.c. implantation (9). Single-cell suspensions of LAPC-9 were prepared as described (10). The LNCaP cell line was obtained from American Type Culture Collection (Manassas, VA). LNCaP-PSCA was produced by transfecting LNCaP cells with a pcDNA3 expression vector (Invitrogen, Carlsbad, CA) containing the PSCA coding sequence (2). Transfectants with stable PSCA expression were selected by G418 (400 μg/mL, Invitrogen) for 2 weeks.

Anti-PSCA Monoclonal Antibody (1G8) Production

The anti-PSCA hybridoma 1G8 (IgG1κ) was cultured in Integra CL 1000 flasks (IBS Integra Biosciences, Ijamsville, MD) using complete serum-free medium (Mediatech Cellgro, Herndon, VA) plus penicillin/streptomycin/glutamine. 1G8 mAb was purified by protein G affinity chromatography (Amersham Pharmacia Biotech, Buckinghamshire, England). Antibody concentration was determined by UV (A_{280}) absorbance and verified by SDS-PAGE and flow cytometry.

1G8 $F(ab')_2$ and F(ab′) Fragment Purification

1G8 $F(ab')_2$ and F(ab′) fragments were prepared using the ImmunoPure $F(ab')_2$ Preparation kit (Pierce, Rockford, IL). Briefly, 1G8 was incubated on an immobilized ficin column in the presence of 1 mmol/L cysteine for $F(ab')_2$ or 10 mmol/L cysteine for F(ab′) for 4 hours at 37°C. Digested

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fragments were eluted and the fractions corresponding with peak A_{280} absorbance were collected. The collections were subsequently passed through a protein A column to remove undigested intact antibody as well as the Fc fragments. The concentration of the F(ab′)$_2$ and F(ab′) fragments was determined by UV (A_{280}) absorbance. The purity and activity of the fragments were analyzed by SDS-PAGE and flow cytometry.

Prostate Cancer Xenograft Models

Tumor take model. Male nude mice or SCID mice were inoculated in the right flank with 1×10^6 LAPC-9 or LNCaP-PSCA cells diluted in a 1:1 ratio with Matrigel (BD Biosciences, Bedford, MA), respectively. For experiments requiring antibody administration, 200 μg intact 1G8 antibody or 147 μg F(ab′)$_2$ fragments (molar equivalents) were injected i.p. on the day before tumor cell inoculation. Equivalent dosages were given i.p. thrice weekly for 4 weeks. The negative control was injected with PBS.

Established tumor model. Male nude mice or SCID mice were inoculated in the right flank with 1×10^6 LAPC-9 or LNCaP-PSCA cells diluted in a 1:1 ratio with Matrigel, respectively. Antibody administration was initiated [200 μg 1G8 or 147 μg F(ab′)$_2$ fragments] when PSA levels became detectable in the serum (1-2 ng/mL) or when the tumors became palpable, depending on the experiment. Repeat doses were given thrice weekly until the conclusion of the experiment.

Cell Killing Assays

LNCaP-PSCA cells (1.5×10^5-4×10^5) were cultured in triplicate in six-well plates in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) for 24 hours at 37°C. Fresh medium with or without 1G8 (20 μg/mL) was then applied and the cells were incubated for varying amounts of time. Viable and dead cells were stained with trypan blue and counted under conventional light microscopy. Culture supernatants were used for cell death assays using the Cellular DNA Fragmentation ELISA (Roche Applied Sciences, Indianapolis, IN).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

Cellular proliferation was measured via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using the Cell Proliferation Kit 1 (Roche Applied Sciences). Briefly, LNCaP-PSCA cells were cultured in 96-well plates for 24 hours in RPMI 1640/10% FBS. 1G8 (20 μg/mL) was added and the cells were incubated at 37°C for an additional 24 or 48 hours. After the incubation period, the MTT colorimetric reaction was carried out as described by the manufacturer and measured the absorbance at 560 nm using a plate reader.

Flow Cytometry

To assess antibody binding activity, LNCaP-PSCA cells (1×10^6) were incubated for 30 minutes at 4°C with 1 μg of either 1G8 or its corresponding F(ab′)$_2$ fragment in 100 μL PBS/2% FBS. After washing with PBS, cells were stained with a 1:500 dilution of FITC-conjugated rabbit anti-mouse IgG F(ab′)$_2$ (Jackson ImmunoResearch Laboratories, West Grove, PA). For cell death analysis, cells were first treated with the appropriate anti-PSCA antibody as described above and then stained with Annexin V and propidium iodide (PI) using the Vybrant Apoptosis Assay Kit 2 (Molecular Probes, Eugene, OR). Data were acquired on a FACScan (Becton Dickinson, San Jose, CA) and analyzed by FCS Express (De Novo Software, Ontario, Canada).

Cell Morphology Analysis

LNCaP-PSCA cells were grown on poly-L-lysine–treated coverslips for 24 hours and then incubated for an additional 24 hours with or without 1G8 antibody (20 μg/mL). Images of living cells were taken by phase-contrast light microscopy. For nuclear staining, cells were first fixed with cold methanol and then treated with RNase A (10 μg/mL) in PBS for 30 minutes at room temperature. Finally, cells were stained with PI (1 μg/mL) for 15 minutes. Images were obtained with confocal microscopy.

Results

Anti-PSCA antibody induces cell death *in vitro*. We have reported previously that the anti-PSCA mAb 1G8 impedes tumor growth and prevents progression to metastatic disease of human prostate cancer xenografts (8). To gain insight into the mechanism of action of 1G8, we first asked if there was evidence of antibody activity *in vitro*. The prostate cancer cell line LNCaP was transfected with a PSCA expression vector, resulting in a cell line (LNCaP-PSCA) with stable expression of PSCA. These cells were incubated in the presence or absence of 1G8. Treatment with 1G8 led to growth inhibition as established by two separate techniques—viable cell counting and MTT assay (Fig. 1*A* and *B*, respectively). This growth inhibition seemed to result from induction of cell death rather than from an effect on proliferation. There was no decrease in uptake of tritiated thymidine after antibody exposure, whereas there was a 5- to 6-fold increase in the percentage of nonviable (i.e., dead) cells (Fig. 2*A*). In addition, Annexin V/PI staining (Fig. 2*B* and *C*) and an ELISA test measuring cellular DNA fragmentation both confirmed 1G8's induction of cell death (Fig. 2*D*). Loss of plasma membrane integrity was seen as early as 6 hours after treatment with 1G8 (Fig. 2*C*). These results suggest that PSCA antibody 1G8 may work through a direct effect on PSCA-expressing prostate cancer cells *in vitro*.

To rule out the possibility of cytotoxicity secondary to nonspecific antibody-antigen interactions, we tested the effects of 1G8 on LNCaP cells transfected only with vector. No increase in cell death was observed in the absence of PSCA, showing that 1G8-mediated cytotoxicity is PSCA specific (data not shown). To rule out the possibility of complement-mediated cytotoxicity as the cause of cell death in tissue culture, we compared the effects of 1G8 in doubly heat-inactivated and non-heat-inactivated serum. No differences were seen in either condition, suggesting that the cell death seen in tissue culture is not mediated by complement. This was further substantiated by substituting the native 1G8 antibody with bivalent F(ab′)$_2$, thereby removing the CH2

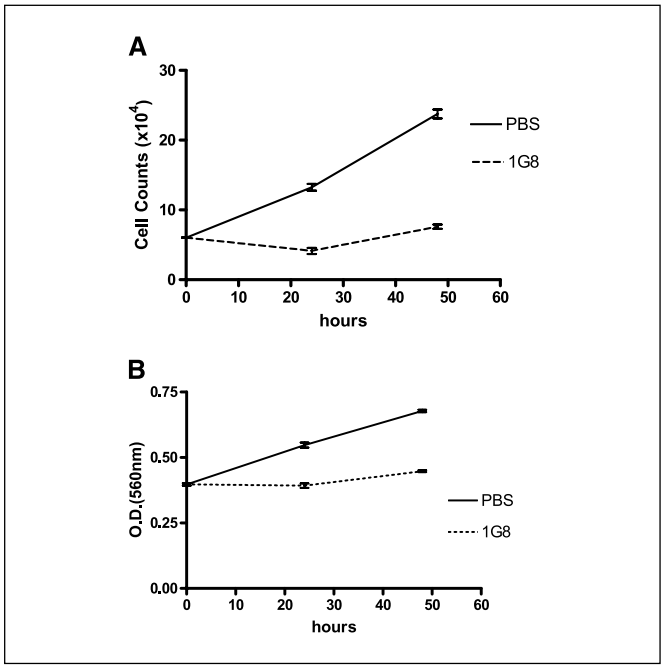

Figure 1. Treatment with 1G8 leads to cell growth inhibition. *A*, LNCaP-PSCA cells were incubated with PBS or 1G8 (20 μg/mL) for the indicated time. Viable cells were counted after trypan blue staining. *B*, LNCaP-PSCA cells were treated with or without 1G8 (20 μg/mL) for the indicated time. Cell proliferation was measured via MTT assay.

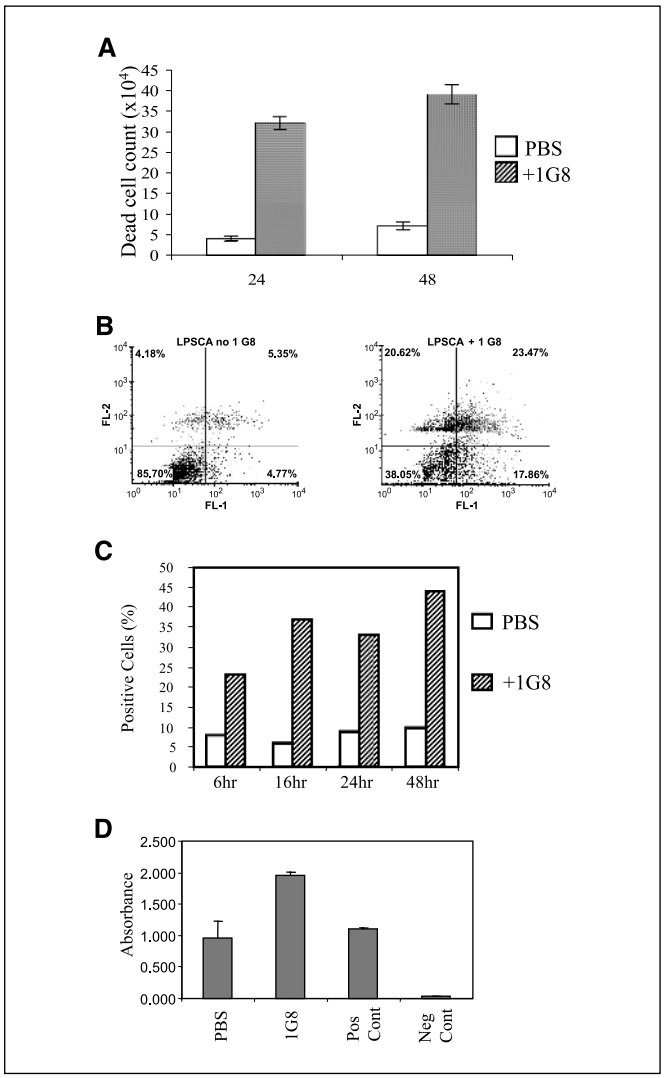

Figure 2. 1G8 induces cell death. *A,* LNCaP-PSCA cells were treated with PBS or 1G8 (20 μg/mL) for 24 and 48 hours; dead cells were counted after trypan blue staining. *B,* after treatment with PBS or 1G8 (20 μg/mL) for 24 hours, cells were stained with Annexin V and PI and analyzed by FACS, FL-1 for Annexin V and FL-2 for PI. *C,* LNCaP-PSCA cells were treated with 1G8 at various times, stained with PI, and analyzed by FACS. *D,* after LNCaP-PSCA cells were treated with antibody for 24 hours, culture supernatants were measured for DNA fragmentation by ELISA. Positive (*Pos*) and negative (*Neg*) controls were provided by manufacturer.

domain-binding site of complement. Again, no difference in cell killing was seen using either $F(ab')_2$ or whole antibody, suggesting that 1G8 can mediate cell death by a direct mechanism of action.

1G8-induced cell death in tissue culture is caspase independent. 1G8-treated LNCaP-PSCA cells manifested dramatic morphologic changes, including a flattened "stressed" appearance as well as pronounced intercellular adherence (Fig. 3*A*). In addition, after 1G8 treatment, most of the cells exhibited irregular, "broken" nuclei and abnormal chromatin structure (Fig. 3*B*). ELISA cell death analysis showed DNA leakage from the nuclei to the cytoplasm (Fig. 2*D*). Although the precise significance of these structural changes is unknown, it is notable that we did not observe the trademark chromatin condensation and degradation of apoptotic cells.

Aspartate-specific cysteinyl proteases, or caspases, have been shown to play an integral role in apoptosis. zVAD-fmk (caspase inhibitor VI, Calbiochem, La Jolla, CA) is a cell-permeable, broad-spectrum caspase inhibitor, which has been shown to block various forms of apoptosis by irreversibly binding to the catalytic site of several caspases. Incubation of LNCaP-PSCA cells with zVAD-fmk did not interfere with 1G8-induced cell death (Fig. 3*C*). These results suggest that cell death triggered by 1G8 in tissue culture may be caspase independent.

1G8-induced cell death in tissue culture requires antigen cross-linking. Many antibodies kill cells by cross-linking antigen on the cell surface (11). To determine whether 1G8-mediated cell death in tissue culture is caused by antigen cross-linking, we compared the ability of whole antibody, bivalent $F(ab')_2$, and monovalent $F(ab')$ to inhibit LNCaP-PSCA cell growth *in vitro*. $F(ab')_2$ and $F(ab')$ fragments were prepared as described in Materials and Methods, confirmed to retain binding to PSCA by fluorescence-activated cell sorting (FACS; Fig. 4*A*), and assayed for

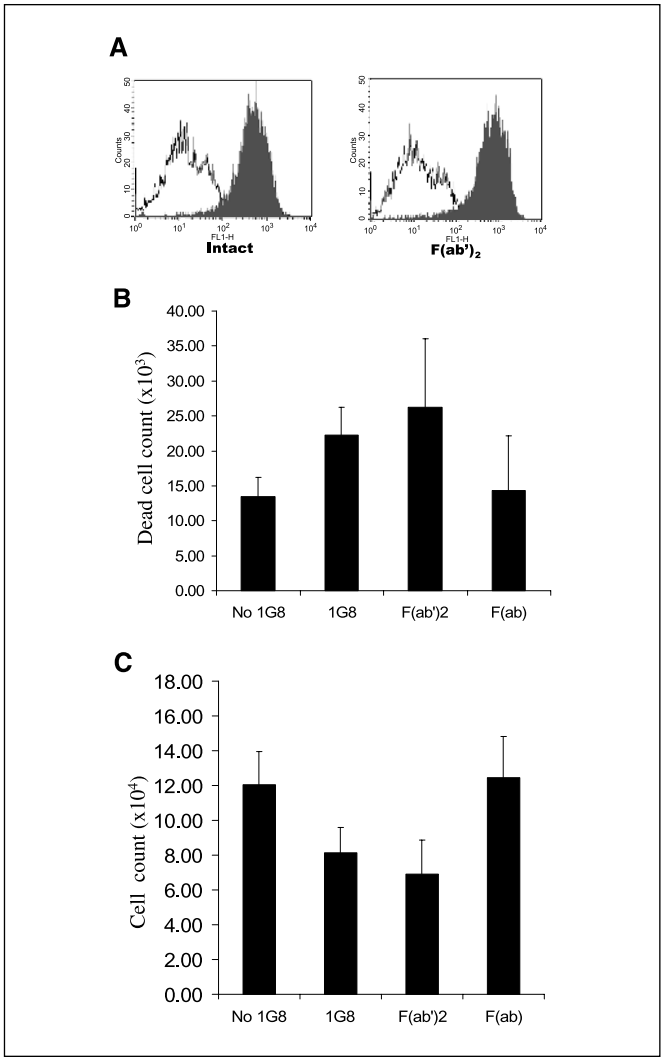

Figure 3. 1G8-mediated cell death requires antigen cross-linking. *A,* FACS analysis of 1G8 and $F(ab')_2$ binding activity. Open peak is negative control (secondary antibody alone). *B,* LNCaP-PSCA cells were treated with PBS, 1G8 (20 μg/mL), $F(ab')_2$ (14.7 μg/mL), or $F(ab')$ (14.7 μg/mL) for 24 hours. Dead cells were counted. *C,* viable cells were counted.

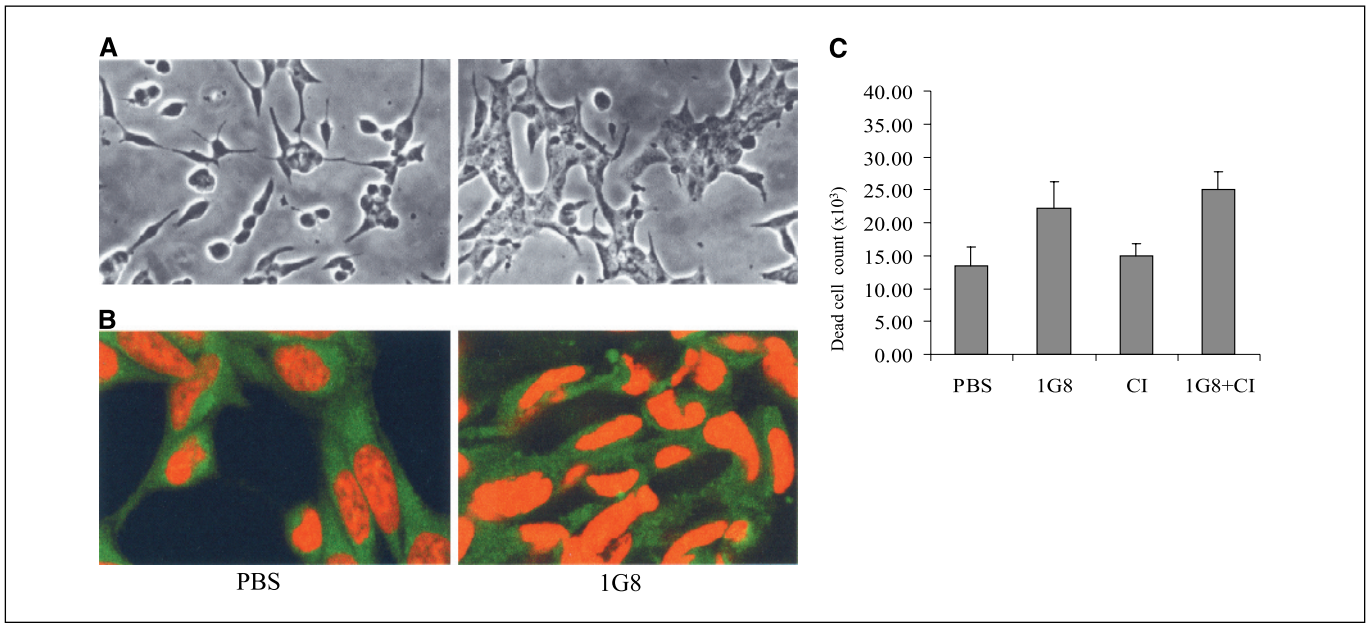

Figure 4. 1G8-induced cell death in tissue culture is not a typical caspase-dependent event. *A,* phase-contrast microscopic picture of LNCaP-PSCA cells treated with PBS or 1G8. *B,* confocal microscopic picture of LNCaP-PSCA cells treated with 1G8 and stained with PI. *C,* dead cell count after LNCaP-PSCA cells were treated with PBS, 1G8 (20 μg/mL), zVAD-fmk [caspase inhibitor (*CI*); 50 μmol/L], or 1G8 + CI.

purity by SDS-PAGE. As shown in Fig. 4*B* and *C*, both whole antibody and $F(ab')_2$ were able to induce cell death, whereas F(ab′) had no effect whatsoever. These results suggest that 1G8-mediated cell death requires antigen cross-linking.

1G8 inhibits *in vivo* prostate cancer tumor growth by a direct, Fc-independent mechanism. Our tissue culture results suggest that 1G8 may act by direct cross-linking of PSCA on the cell surface. Demonstration of a particular mechanism *in vitro*, however, does not guarantee that the same pathway mediates *in vivo* responses. Thus, we next asked whether PSCA antibody 1G8 activity *in vivo* was dependent or independent of the antibody's Fc domain. To answer this question, we compared the ability of intact 1G8 versus purified $F(ab')_2$ fragments to (*a*) prevent new tumor formation ("tumor take" model) and (*b*) inhibit growth of preexisting LAPC-9 prostate cancer xenografts ("established tumor" model). Previous studies from our group and others have shown that anti-PSCA antibodies can both block new tumor formation and slow growth of established tumors. Molar equivalents of 1G8 versus $F(ab')_2$ fragments were given i.p. into nude mice beginning 1 day before tumor cell inoculation or when serum PSA and/or palpable tumor were detected (usually around day 15 following tumor inoculation). As seen in Fig. 5*A* and *B*, both 1G8 and its $F(ab')_2$ counterpart inhibited tumor take and slowed the growth of established LAPC-9 tumors, consistent with a Fc-independent mode of action.

To determine if this "direct" activity was exclusive to LAPC-9 or was generalizable to other PSCA-expressing prostate tumors, we repeated both experiments using the LNCaP-PSCA cell line. As shown in Fig. 5*C*, both 1G8 and $F(ab')_2$ inhibited LNCaP-PSCA tumor take in SCID mice. Similar results were also obtained in an established tumor model of LNCaP-PSCA (data not shown). Thus, the Fc moiety does not seem to be necessary for 1G8's antitumor effects in tissue culture nor in multiple preclinical models of PSCA-positive prostate cancer.

Discussion

Treatment of prostate cancer cells with anti-PSCA antibody 1G8 results in cell death and *in vivo* growth suppression by a Fc-independent mechanism of action. These results are similar to those reported for the anti–epidermal growth factor receptor (EGFR) antibody 225, the murine parental antibody of the chimeric clinical antibody Erbitux (12). Fan et al. showed that both native 225 and its $F(ab')_2$ fragment could inhibit *in vivo* growth of EGFR-positive tumors, although the activity was less sustained and less complete (~50%) than native antibody. The partial loss of 225's activity was attributed to the more rapid pharmacodynamic turnover of $F(ab')_2$. In contrast, 1G8's $F(ab')_2$ was equal to or even superior to native 1G8 both *in vitro* and *in vivo*. The reasons for this phenomenon are not known, as we too predicted that $F(ab')_2$ should be less active because of its higher rate of turnover. One possible explanation that we have noted is that $F(ab')_2$ of 1G8 is internalized more rapidly into the cell than native 1G8.[1] Because receptor internalization can play a role in the biological activity of some antibodies, this may be one possible explanation for the robust activity of the $F(ab')_2$ fragment that needs to be explored further.

Although our results support Fc-independent activity as the major mode of action of anti-PSCA antibody 1G8, this has not been the case for all antibodies. Clynes et al., for example, showed that Rituxan and Herceptin, two Food and Drug Administration–approved antibodies targeting CD20 and HER-2/*neu*, respectively, require the presence of functional FcγRIII receptor on macrophages and natural killer cells for their preclinical activity (13). Herceptin and Rituxan lost most of their activity in FcγRIII-deficient nude mice, suggesting that the *in vivo* activity of these

[1] Z. Gu, unpublished data.

antibodies is mediated by antibody-dependent cellular toxicity (ADCC). These investigators also speculated that the partial loss of activity of anti-EGFR mAb 225's F(ab′)$_2$ fragments *in vivo* could be attributed to the contribution of the immune system to 225's overall activity. In the case of anti-PSCA mAb 1G8, there was no loss of activity when using F(ab′)$_2$, suggesting that ADCC contributes at most a minor amount to its activity. Interestingly, however, we have noted that a chimeric 1G8 (in which the mouse IgG1 Fc domain is replaced with the human IgG1 domain) and a humanized 1G8 (in which the complementarity determining regions of 1G8 were grafted onto a human 1gG1 framework) have greater *in vivo* activity than murine 1G8[2] despite markedly lower affinities. One possible explanation for this superior activity is that chimeric and humanized 1G8 are human isotype IgG1, which can interact more effectively with mouse FcγRIII to induce ADCC than mouse 1gG1. Chimeric, humanized, and murine 1G8 need to be compared in FcγRIII-deficient mice to confirm this hypothesis. Nevertheless, these results suggest that one might be able to augment 1G8's activity by engineering its Fc domain to induce ADCC.

Although the current results support a direct, cross-linking–dependent mechanism of activity, the molecular signals elicited by anti-PSCA antibody 1G8 are not known. PSCA's function in normal and malignant cells is not known and PSCA knockout mice have no known phenotype.[3] One hint to PSCA's function may come from its structure as a GPI-anchored protein. GPI-anchored proteins characteristically localize to lipid rafts, detergent-resistant membrane microdomains believed to play a role in integrating signal transduction pathways (14–16). Several antibodies targeting GPI-anchored and raft-associated proteins have been reported to have biological activity, including antibodies against CD24 (GPI-anchored), CD20 (raft-associated), and CD48 (GPI-anchored). Anti-CD24 antibodies induce apoptosis and are dependent on cross-linking, similar to PSCA-directed antibody 1G8. CD24 antibodies increase Lyn kinase phosphorylation and may also alter membrane composition, although the precise cause of cell death has not been ascertained (17). CD20-induced cell death has been reported to be caspase independent and may be related to changes in calcium flux (18, 19). These observations raise two important considerations for future investigation. First, the role of rafts and the GPI anchor in PSCA-mediated cellular toxicity needs to be determined. Second, it needs to be determined if 1G8-mediated cell death is unique to PSCA or might be generalizable to other prostate cancer–associated, GPI-anchored membrane proteins. The latter issue, in particular, has important ramifications in terms of new target development for antibody therapy of prostate cancer.

One notable observation in our study is that PSCA mAb 1G8-induced cell death *in vitro* is not dependent on caspases and is not characterized by the classic features of apoptosis. Recently, Nagy et al. reported on the cytotoxic effects of HLA-DR-directed antibodies on malignant lymphoid cells (20). These antibodies killed tumor cells with similar kinetics to the PSCA antibody 1G8, reducing the percentage of viable cells as early as 4 hours after treatment. The cytotoxicity of these antibodies was also not dependent on caspase activity and did not produce the chromatin condensation and nuclear morphologic changes typical of classic

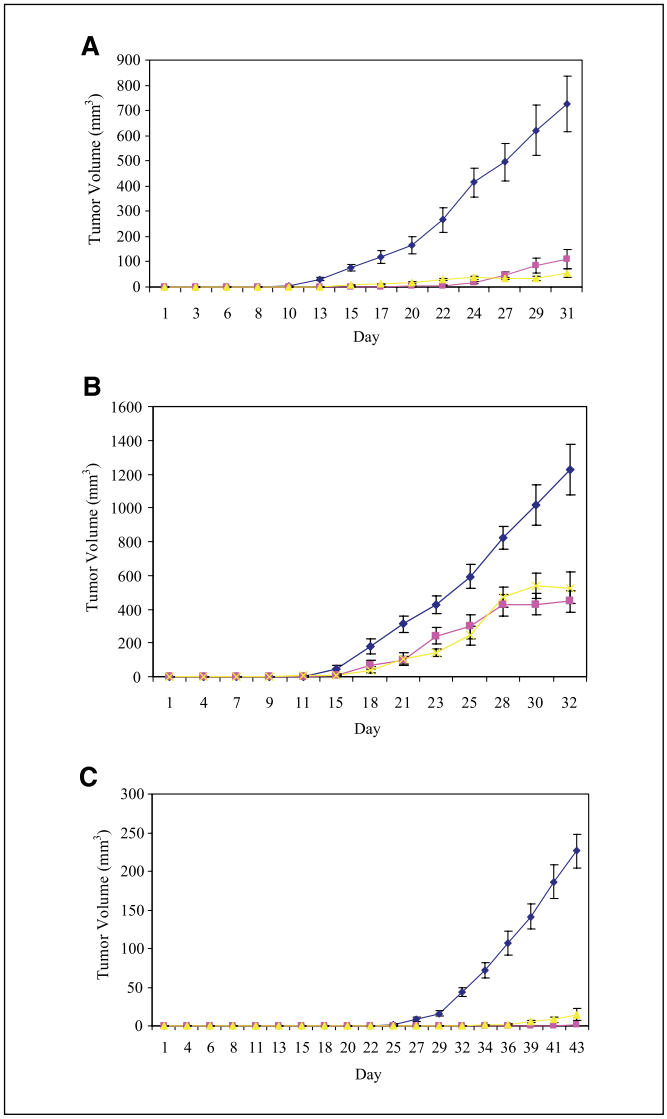

Figure 5. Both 1G8 and its F(ab′)$_2$ fragments inhibit tumor growth *in vivo*. *A,* LAPC-9 tumor take model in nude mice xenografts. *B,* LAPC-9 established tumor model in nude mice xenografts. *C,* LNCaP-PSCA tumor take model in SCID mice. *Blue,* PBS; *yellow,* 1G8; *red,* F(ab′)$_2$.

apoptosis. The mechanism underlying this nonclassic apoptosis is not known, but the similarities between the two responses suggest that HLA-DR-directed and PSCA-directed antibodies may signal through similar pathways.

It is not known whether the cytotoxic effects of PSCA antibody 1G8 are epitope specific. To date, a total of three anti-PSCA antibodies have been shown to have antitumor activity, among them the 1G8 and 3C5 antibodies developed by our laboratory. 1G8 and 3C5 recognize distinct epitopes and both inhibit *in vivo* growth of prostate cancer (8, 21). 3C5's activity, however, was notably lower than 1G8, with less growth reduction of established tumors and less effect on overall survival. 3C5 has lower affinity than 1G8 for PSCA and is an IgG2a antibody rather than IgG1. Because we now know that the Fc domain does not play a major role in 1G8's activity, it remains to be determined whether 1G8's superior activity is attributed to its higher affinity or the unique epitope it recognizes. Likewise, it remains to be determined if

[2] Z. Gu and T. Olafsen, unpublished work.
[3] R. Reiter and O. Witte, unpublished data.

3C5's activity is Fc dependent or Fc independent, because murine isotype γ2a can induce profound ADCC in mouse models. The answer to these questions is particularly important as PSCA-directed antibody therapy nears the clinic, because antibody selection might have profound effects on clinical efficacy.

PSCA-directed antibody therapy is currently under development for translation into the clinic. Our group has developed chimeric and humanized versions of 1G8, which are currently being evaluated preclinically. Our results show that 1G8 is active against both LAPC-9 and LNCaP-PSCA tumors, which express comparable levels of PSCA. We have reported previously that at least 50% of metastatic prostate cancers express levels of PSCA equal to or higher than LAPC-9, supporting the clinical relevance of our current findings. We have also seen *in vivo* activity using cell lines that express one-tenth of LAPC-9's level of expression,[4] suggesting that anti-PSCA therapy may be broadly applicable to most prostate cancers. The current results are also likely relevant to therapy in other cancers, as we have also recently shown activity of PSCA antibody 1G8 in pancreatic cancer (22). These results suggest that further elucidation of PSCA function and the mechanism of action of PSCA antibodies at the molecular level may have a major effect on the clinical management of prostate and other cancers.

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[4] J. Yamashiro, unpublished data.

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