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BIOLOGY CONTRIBUTION

AUGMENTATION OF RADIATION RESPONSE BY PANITUMUMAB IN MODELS OF UPPER AERODIGESTIVE TRACT CANCER

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Purpose: To examine the interaction between panitumumab, a fully human anti-epidermal growth factor receptor monoclonal antibody, and radiation in head-and-neck squamous cell carcinoma and non–small-cell lung cancer cell lines and xenografts.

Methods and Materials: The head-and-neck squamous cell carcinoma lines UM-SCC1 and SCC-1483, as well as the non–small-cell lung cancer line H226, were studied. Tumor xenografts in athymic nude mice were used to assess the *in vivo* activity of panitumumab alone and combined with radiation. *In vitro* assays were performed to assess the effect of panitumumab on radiation-induced cell signaling, apoptosis, and DNA damage.

Results: Panitumumab increased the radiosensitivity as measured by the clonogenic survival assay. Radiation-induced epidermal growth factor receptor phosphorylation and downstream signaling through mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription 3 (STAT3) was inhibited by panitumumab. Panitumumab augmented radiation-induced DNA damage by 1.2–1.6-fold in each of the cell lines studied as assessed by residual γ-H_2AX foci after radiation. Radiation-induced apoptosis was increased 1.4–1.9-fold by panitumumab, as evidenced by Annexin V-fluorescein isothiocyanate staining and flow cytometry. *In vivo,* the combination therapy of panitumumab and radiation was superior to panitumumab or radiation alone in the H226 xenografts ($p = 0.01$) and showed a similar trend in the SCC-1483 xenografts ($p = 0.08$). *In vivo,* immunohistochemistry demonstrated the ability of panitumumab to augment the antiproliferative and antiangiogenic effects of radiation.

Conclusion: These studies have identified a favorable interaction in the combination of radiation and panitumumab in upper aerodigestive tract tumor models, both *in vitro* and *in vivo*. These data suggest that clinical investigations examining the combination of radiation and panitumumab in the treatment of epithelial tumors warrant additional pursuit.

Panitumumab, Radiation, Epidermal growth factor receptor, EGFR.

INTRODUCTION

The epidermal growth factor receptor (EGFR) is overexpressed or mutated in most epithelial malignancies (1). The widespread overexpression of this receptor in epithelial cancers has led to the development of a series of EGFR inhibitors, including the anti-EGFR monoclonal antibodies (mAbs) cetuximab and panitumumab. EGFR-targeted therapies have recently gained Food and Drug Administration approval in the treatment of patients with head-and-neck squamous cell carcinoma (HNSCC), non–small-cell lung cancer (NSCLC), and pancreatic and colorectal cancer.

The interaction between the EGFR and ionizing radiation was initially explored in the early 1990s. Investigators noted an increase in EGFR expression in various cell lines after radiation exposure (2), as well as an inverse relationship between EGFR expression and radiosensitivity (3). A series of studies on the radiosensitizing effects of the human-mouse chimeric mAb cetuximab (C225) established the capacity of EGFR inhibition to enhance the effects of radiation, both *in vitro* and *in vivo,* in various tumor model systems (4, 5).

In 2006, an international Phase III trial that combined the EGFR inhibitor mAb cetuximab with radiation in HNSCC

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Conflict of interest: R. Radinsky and D. J. Freeman are employees of Amgen, Incorporated. P. M. Harari holds a laboratory research agreement with Amgen, Incorporated.

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patients demonstrated a 10% improvement in overall survival for patients receiving the EGFR inhibitor (6). This trial provided primary support for Food and Drug Administration registration of cetuximab for use in conjunction with radiation in patients with locoregionally advanced HNSCC.

Panitumumab is a fully human monoclonal IgG2 antibody that has been demonstrated to bind EGFR, block binding of EGF to the receptor, and inhibit receptor autophosphorylation (7). A Phase III trial using panitumumab has demonstrated a significant improvement in progression-free survival in patients with chemorefractory metastatic colorectal cancer (8), leading to Food and Drug Administration approval in this setting.

In HNSCC, panitumumab is being investigated in combination with chemoradiotherapy. In a recently completed Phase I trial studying the addition of panitumumab to carboplatin, paclitaxel, and intensity-modulated radiotherapy for locally advanced HNSCC, panitumumab did not appear to increase the rate of acute and late toxicities compared with that expected from chemoradiotherapy alone (9). To date, the interaction between panitumumab and radiation has not been thoroughly investigated in preclinical models. The objective of the present study was to examine the interaction between panitumumab and radiation in xenograft models of HNSCC and NSCLC. In addition, we sought to explore the mechanisms by which panitumumab modulates the antitumor efficacy of radiation.

METHODS AND MATERIALS

Cell lines, compounds, and antibodies

The human NSCLC line NCI-H226 was obtained from the American Type Culture Collection (Rockville, MD). The human HNSCC line UM-SCC1 (floor of the mouth) was provided by Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI), and the SCC-1483 cells were provided by Dr. Jennifer Grandis (University of Pittsburgh, Pittsburgh, PA).

Panitumumab (ABX-EGF, Vectibix) was generously provided by Amgen (Thousand Oaks, CA). All other antibodies were purchased from commercial sources as follows: EGFR, pEGFR(Tyr1173), and horseradish peroxidase-conjugated goat-anti-rabbit IgG, goat-anti-mouse IgG, and donkey-anti-goat IgG antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Poly (ADP-ribose) polymerase (PARP), histone H3, phosphorylated EGFR(Tyr845), phosphorylated signal transducer and activator of transcription 3 (STAT3)(Tyr705), and phosphorylated mitogen-activated protein kinase(Thr202/Tyr204) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Tubulin antibody was from Calbiochem (San Diego, CA).

Subcellular fractionation and immunoblotting

Whole cell lysates were obtained using Tween-20 lysis buffer. For subcellular fractionation, cytoplasmic and nuclear extracts were prepared according to the instructions of the NE-PER nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL). Proteins were fractionated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were detected by incubation with the primary antibodies, horseradish peroxidase-conjugated secondary antibodies, and the enhanced chemiluminescence (ECL+) detection system (Amersham Biosciences, Piscataway, NJ). Where necessary, the protein bands were quantitated using ImageJ software (National Institutes of Health, Bethesda, MD).

Clonogenic radiation survival

The cells were exposed to either panitumumab or human IgG for 72 h and subsequently irradiated at 0–8 Gy with a Shepherd & Associates Model 109 irradiator (San Fernando, CA) and a ^{137}Cs hotbox source. The plates were rinsed with phosphate-buffered saline (PBS)/0.02% ethylenediaminetetraacetic acid (EDTA), and the cells were detached using 0.05% Trypsin/EDTA, counted, and seeded for colony formation in six-well plates at 100–8,000 cells/well. The cells were incubated for 14–21 days with medium changes every 48–72 h. At the end of the experiment, the colonies were stained with crystal violet in methanol and counted manually. Colonies consisting of $\geq$50 cells were scored, and six replicate wells containing 10–150 colonies/well were counted for each condition.

Flow cytometry assessment of apoptosis and surface EGFR expression

Apoptosis was detected using the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit I (BD Biosciences, San Jose, CA). Specifically, the cells were treated with either panitumumab or control for 24 h and then exposed to radiation, 0–9 Gy. At 48 h after irradiation, the culture medium was collected (to retain detached cells), the plates were rinsed with PBS/0.02% EDTA, and the cells were detached using 0.05% Trypsin/EDTA and combined with their medium and floating cells. The cells were washed twice in cold PBS and resuspended in the provided binding buffer at 10^6 cells/mL. Each sample was incubated with 5 μL each of the provided Annexin V-FITC and propidium iodide (PI) solutions for 30 min in the dark; the volumes were increased to 500 μL, and the samples were run using a BD FACScan flow cytometer using CellQuest software (Becton Dickinson, Franklin Lakes, NJ) for acquisition and analysis.

Surface EGFR expression on live cells was semiquantitatively assessed by flow cytometry. In brief, the cells were detached with PBS/0.2% EDTA (no trypsin) and then resuspended in PBS/0.2% bovine serum albumin/0.1% sodium azide. Subsequently, 1×10^6 cells were incubated for 30 min with 1 μg of either FITC-conjugated anti-EGFR or FITC-conjugated isotype-matched IgG (sc-101 and sc-2857, Santa Cruz Biotechnology). PI was then added, and gated analysis was performed on live cells by excluding PI-positive cell populations.

Quantification of γ-H_2AX Foci

The cells were plated on chamber slides and exposed to 25 nM panitumumab or human IgG for 1 h before irradiation. Cells were irradiated, fixed 24 h after irradiation in 2% paraformaledhyde/3% sucrose solution for 10 min, and then permeabilized for 5 min in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.4), 50 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose, and 0.5% Triton X-100. The cells were incubated with anti-phospho-γ-H_2AX antibody (Upstate, Billerica, MA) dissolved in 2% bovine serum albumin (BSA) Fraction V 1:800 for 20 min. The cells were then incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG dissolved in 2% BSA Fraction V 1:100 for 20 min, and the cells were then mounted using ProLong Gold antifade reagent with 46′-diamidino-2-phenylindole-2 HCl (Invitrogen, Carlsbad, CA). Fluorescence images were captured using an Olympus BX51 Epifluorescent microscope fitted with a SPOT RT color camera and analyzed with the SPOT Advanced software (Diagnostic

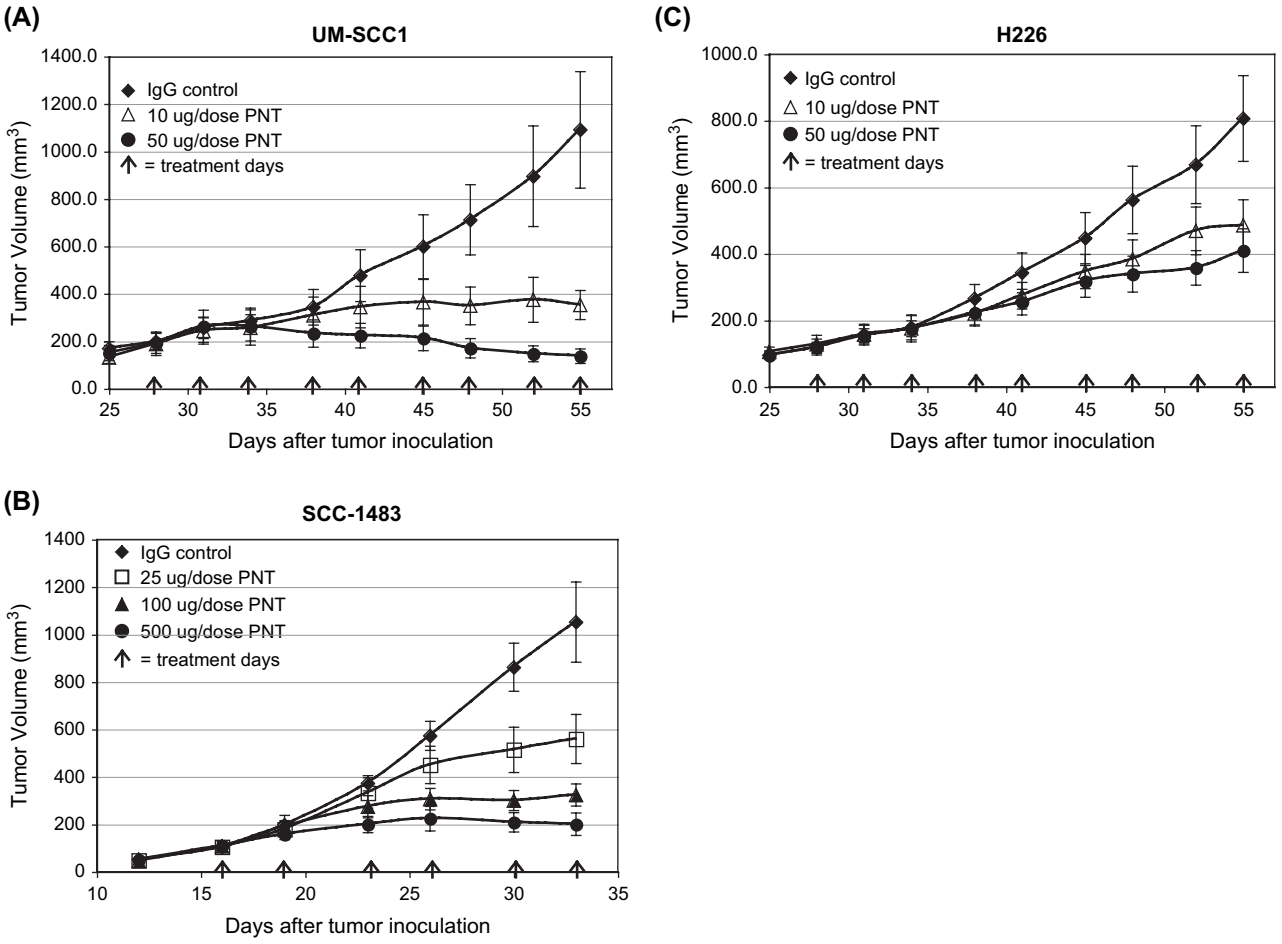

Fig. 1. In vivo efficacy of panitumumab (PNT) in (A,B) head-and-neck squamous cell carcinoma and (C) non–small-cell lung cancer xenograft models. Mice were treated with indicated doses of PNT twice weekly by intraperitoneal injection (vertical arrows). Points indicate mean tumor size (n = 8/group in H226 and UMSCC-1, n = 10/group in SCC-1483); bars indicate standard error of mean.

Instruments, Sterling Heights, MI). Visual scoring of ≥100 random cells for each treatment condition was performed manually.

PARP cleavage analysis

Plates containing exponentially growing cells were exposed to either panitumumab (25 nM) or human IgG for 6 h and subsequently irradiated with 0–9 Gy. At 48 h after irradiation, the media were collected to retain the detached cells, centrifuged at 4°C, and the cell pellets were resuspended in Tween-20 lysis buffer, as described in the section ''Subcellular fractionation and immunoblotting.'' The suspension was then added back to the appropriate PBS-rinsed plate to lyse the remaining adherent cells. Immunoblotting was performed to detect endogenous levels of full-length PARP1 (116 kDa), as well as the large fragment (89 kDa) of PARP1 resulting from caspase cleavage.

Tumor growth in athymic nude mice

Athymic nude mice (3–4-week-old males) were obtained from Harlan Bioproducts for Science (Indianapolis, IN). The care and treatment of the experimental animals was in accordance with institutional guidelines. Cells ($\sim 2 \times 10^6$) from the respective human cancer lines were injected subcutaneously into the flank area on Day 0. The tumor volume was determined by direct measurement with calipers and calculated using the formula $(\pi)/6 \times$ (large diameter) $\times$ (small diameter)^2. Panitumumab and control IgG were administered by intraperitoneal injection at the specified doses twice weekly. Radiation was delivered using a Philips RT-250 orthovoltage unit (Philips Medical Systems, Bothell, WA) and custom-designed mouse jigs, which specifically exposed the dorsal flank (harboring tumor xenografts) for irradiation.

Immunohistochemistry

The expression of proliferating cell nuclear antigen (PCNA) and von Willebrand factor (VWF) was detected in histologic sections of tumor xenografts. In brief, the excised tumor specimens were fixed in 10% neutral buffered formalin. After embedding in paraffin, 5-μm sections were cut, and the tissue sections were mounted. The sections were dried, deparaffinized, and rehydrated. PCNA antigen unmasking was performed by heat-induced epitope retrieval with 10 mM sodium citrate buffer (pH 6.0) in a steamer for 20 min. VWF antigen was unmasked with Proteinase K at 37°C for 20 min. After quenching endogenous peroxidase activity and blocking nonspecific binding sites, the slides were incubated at 4°C overnight with the primary antibody (PCNA, 1/4,000, Cell Signaling Technologies, Danvers, MA; VWF, 1/150, Dako, Glostrup, Denmark) followed by a 30-min incubation of secondary antibody. The slides then were incubated with streptavidin peroxidase, visualized using the

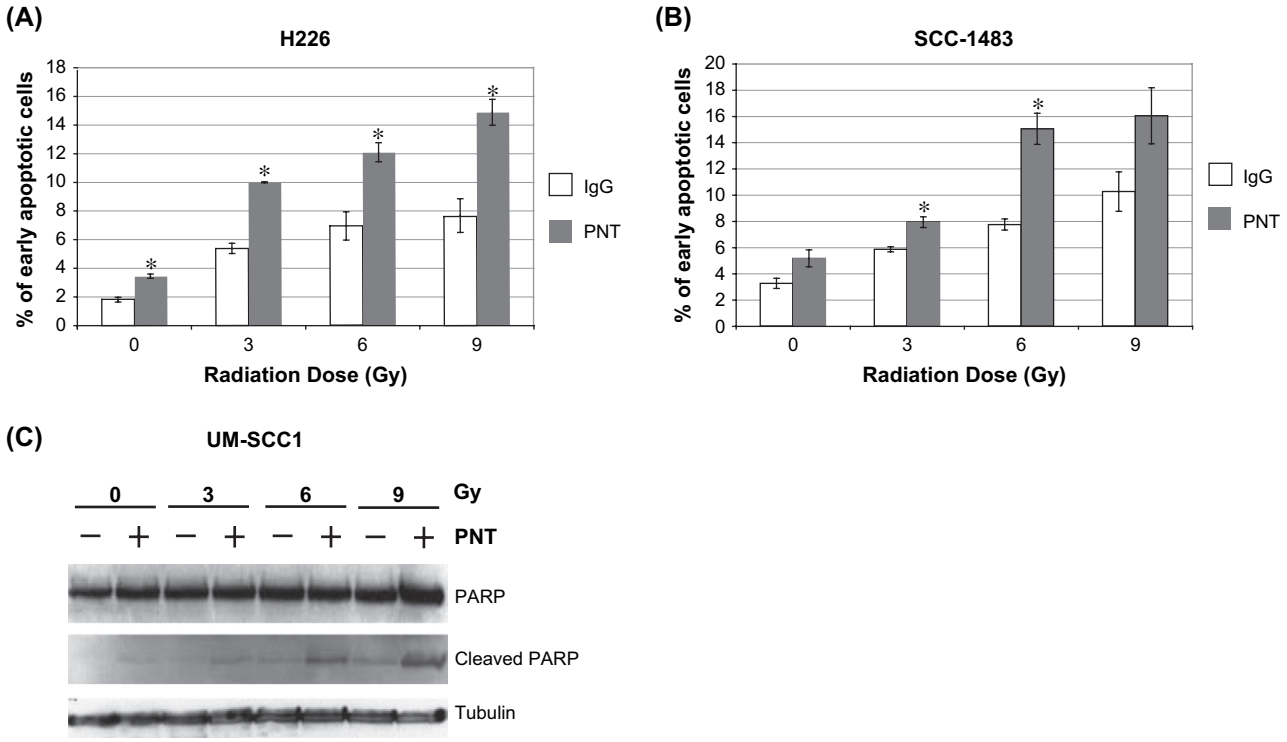

Fig. 2. Panitumumab (PNT) augmented radiation-induced apoptosis. (A) H226 and (B) SCC-1483 cells treated with either 25 nM PNT or IgG control 24 h before radiation. At 48 h after radiation, cells were stained with Annexin V-fluorescein isothiocyanate. Plotted values are mean percentage of cells undergoing early apoptosis (Annexin V positive, propidium iodide [PI] negative) in three replicates for each condition. Bars indicate standard error of mean. Asterisks indicate sets in which PNT augmented response ($p < 0.05$, Student's t test). (C) UM-SCC1 cells exposed to either 25 nM PNT or human IgG for 6 h before radiation (0–9 Gy). At 48 h after radiation, cells were harvested for cleaved poly (ADP-ribose) polymerase (PARP) analysis as described in the ''Methods and Materials'' section.

3,3′-diaminobenzidine chromogen (Lab Vision, Fremont, CA), counterstained with hematoxylin, dehydrated, and mounted.

RESULTS

Panitumumab-induced tumor growth inhibition

Antitumor activity of panitumumab has been demonstrated in pancreatic, renal, breast, prostate, ovarian, and colon tumor xenografts expressing varying levels of EGFR (7). To assess the antitumor efficacy in xenograft models of HNSCC and NSCLC, mice bearing established xenografts were treated with increasing doses of panitumumab (Fig. 1). After 4 weeks of treatment, 10-μg doses of panitumumab induced a 1.6-fold reduction in volume in the H226 tumors ($p = 0.05$) and a 3.1-fold reduction in the UM-SCC1 tumor volumes ($p = 0.01$). In the SCC-1483 tumors, 3 weeks of 25-μg doses of panitumumab induced a 1.9-fold reduction in the tumor volumes ($p = 0.02$). These respective drug doses in the less-sensitive cell lines (SCC-1483 and H226) were chosen for the subsequent *in vivo* studies with radiation.

Panitumumab augments radiation-induced apoptosis

Flow cytometric analysis was performed on SCC-1483 and H226 cells using Annexin V-FITC staining after a dose-dependent induction of apoptosis was demonstrated with radiation (Fig. 2). Panitumumab alone caused a mild increase in the percentage of cells undergoing early apoptosis (high Annexin V-FITC, low PI staining) in SCC-1483 and H226 cells. Panitumumab pretreatment also augmented the effect of radiation on the induction of apoptosis. At doses of 3–9 Gy, panitumumab increased the percentage of cells undergoing early apoptosis by 1.7–1.9-fold in H226 cells ($p < 0.02$). In SCC-1483 cells, at doses of 3 and 6 Gy, panitumumab resulted in a 1.4–1.9-fold increase in cells undergoing apoptosis ($p < 0.02$). Furthermore, in UMSCC-1 cells, we analyzed cleavage of the caspase substrate PARP, which is indicative of cellular commitment to a pathway of programmed cell death. Radiation doses of 3, 6, and 9 Gy caused modest PARP cleavage (Fig. 2C), and pretreatment with panitumumab increased the level of cleaved PARP at each radiation dose.

Panitumumab augments radiation-induced DNA damage

Immunostaining for γ-H_2AX foci to determine the effect of panitumumab on radiation-induced DNA double-strand breaks was performed (Fig. 3). Histone H_2AX is phosphorylated in response to DNA double-strand breaks (10), and pretreatment with panitumumab significantly increased the number of residual foci in SCC-1483 cells by a factor of 1.3–1.5 after 2, 4, and 6 Gy radiation ($p < 0.03$). A similar effect of panitumumab combined with radiation on residual

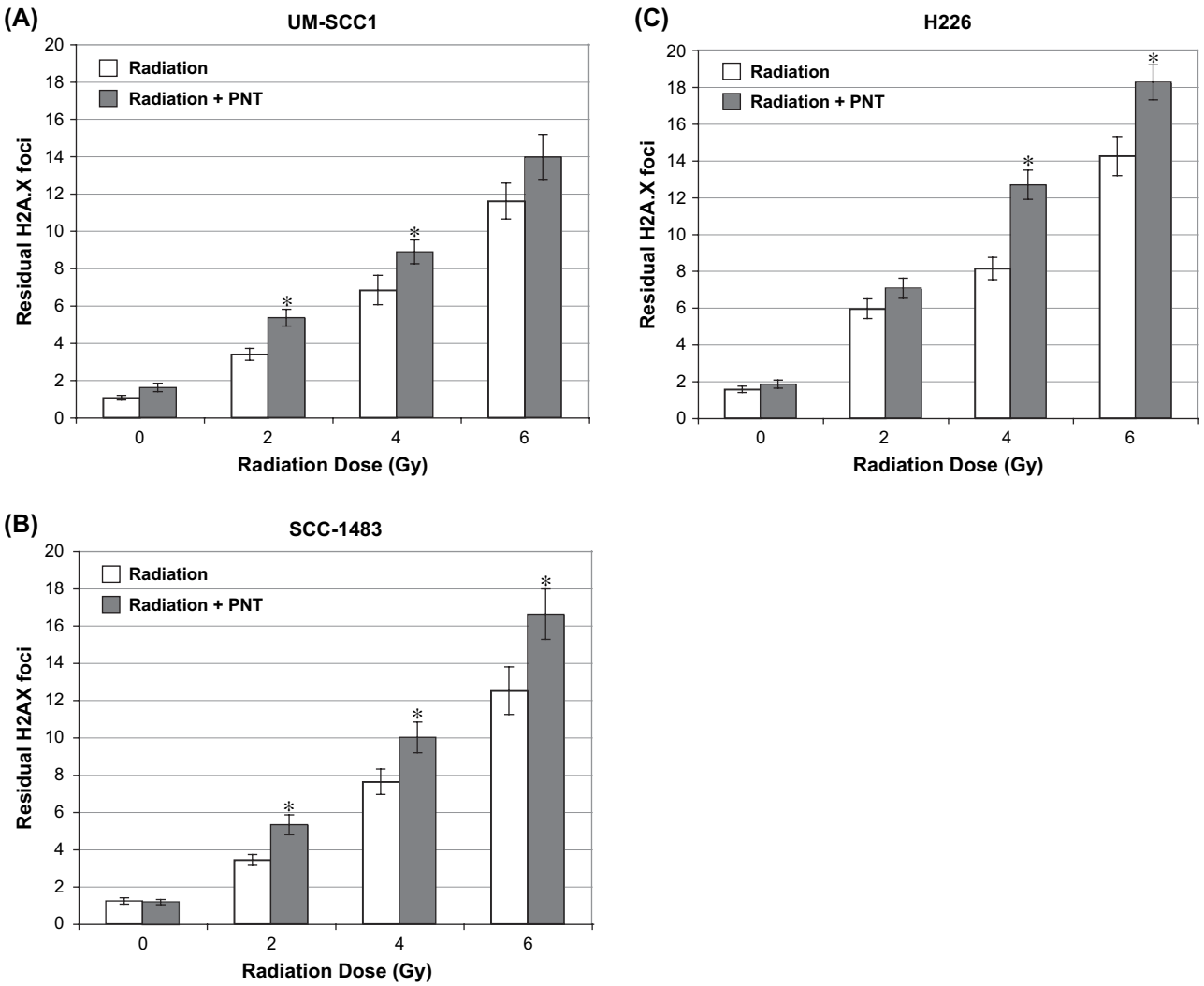

Fig. 3. Panitumumab (PNT) augmented radiation-induced DNA damage. Cells from head-and-neck squamous cell carcinoma lines (A) UM-SCC1 and (B) SCC-1483 and from (C) NSCLC line H226 exposed to either human IgG or PNT (25 nM) for 1 h before radiation. Cells were then incubated for 24 h and analyzed for residual H_2AX foci. Foci were counted in >100 cells for each condition. Bars indicate standard error of mean. Asterisks indicate sets in which PNT resulted in significantly more residual foci ($p < 0.05$, Student's t test).

γ-H_2AX foci (1.2–1.6-fold increases) were seen in UM-SCC1 cells at 2 and 4 Gy ($p < 0.05$) and in H226 cells at 4 and 6 Gy ($p < 0.01$).

Panitumumab modulates in vitro radiation response

We conducted experiments assessing the effect of panitumumab on clonogenic survival after radiation. As shown in Fig. 4, panitumumab pretreatment induced modest, but consistent, radiosensitization in UM-SCC1 at 2, 4, 6, and 8 Gy ($p < 0.01$) and in H226 at 4, 6, and 8 Gy ($p < 0.02$). No significant radiosensitization was observed in the SCC-1483 cells (data not shown). After irradiation, EGFR is known to move to the nucleus and subsequently enhance the kinase activity of nuclear DNA-dependent kinase, a key enzyme responsible for repair of DNA double-strand breaks (11). To examine the ability of panitumumab to affect this nuclear translocation of EGFR after radiation, we separated the nuclear and cytosolic fractions of cell lysate after irradiation of UMSCC-1 cells. Figure 4c demonstrates that the fraction of cellular EGFR in the nucleus was increased 20 min after irradiation and that pretreatment with panitumumab limited this radiation-induced nuclear translocation.

Panitumumab inhibits radiation-induced EGFR activation

We examined whole cell lysates from irradiated UM-SCC1 cells that were pretreated with panitumumab. Irradiated cells without panitumumab pretreatment demonstrated a dose-responsive augmentation of EGFR phosphorylation at tyrosine sites 845 and 1173 (Fig. 4D). Furthermore, it appeared that the increased activation of EGFR after irradiation can increase downstream signaling, as demonstrated by an enhancement of the phosphorylated version of STAT3, and that preadministration of panitumumab is able to inhibit radiation-induced EGFR signaling.

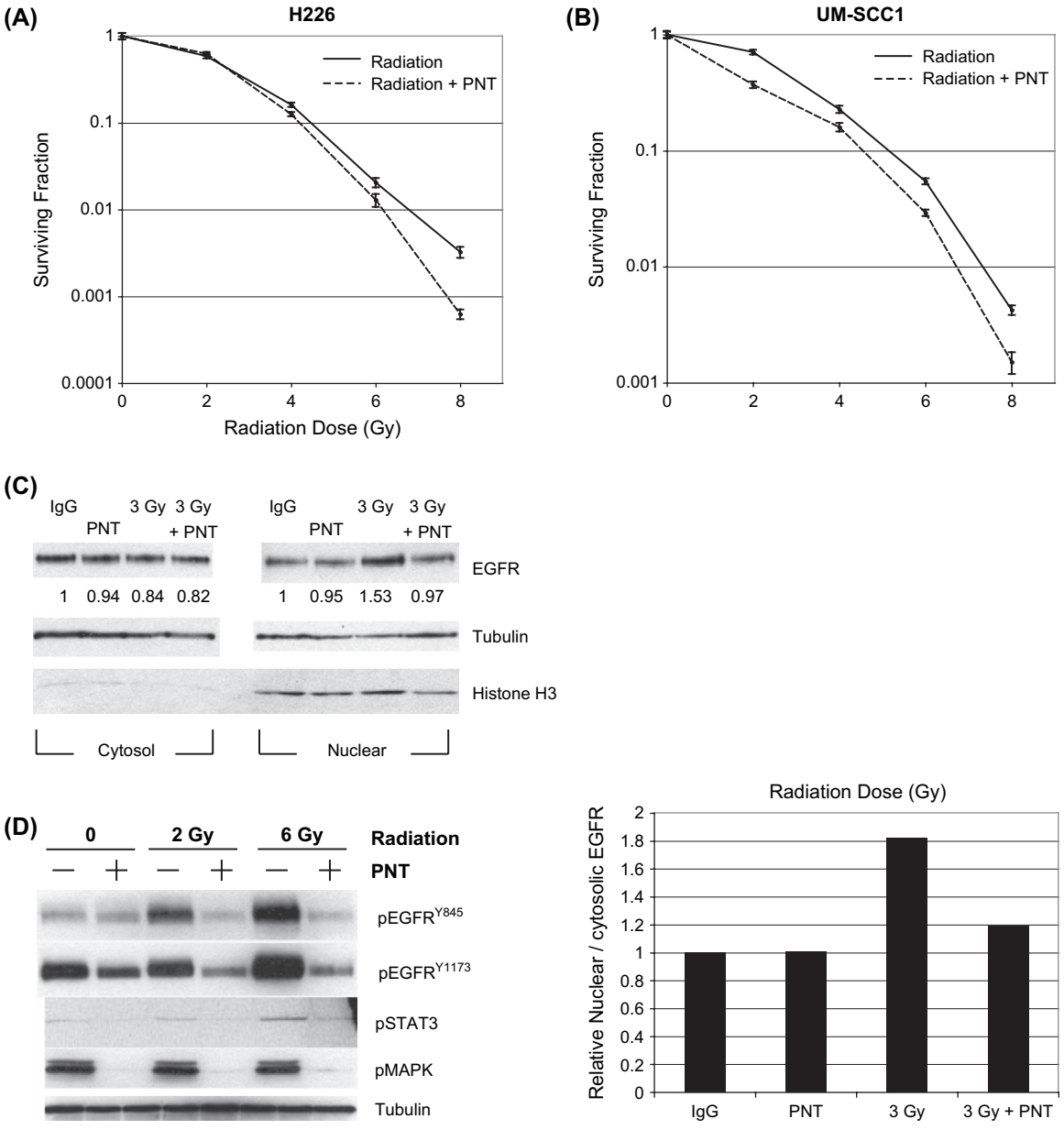

Fig. 4. Effect of panitumumab (PNT) on radiation response. Clonogenic survival was assessed in (A) H226 and (B) UM-SCC1. Cells were exposed to either PNT (25 nM) or nonspecific IgG for 72 h before irradiation. Points indicate mean values from six seeded wells; bars indicate standard error of mean. (C) PNT blocked radiation-induced epidermal growth factor receptor (EGFR) nuclear translocation in UM-SCC1 cells. PNT (25 nM) or nonspecific IgG was administered 1 h before radiation. Cells were lysed 20 min after radiation and cytosolic and nuclear fractions analyzed by Western blot. EGFR bands were quantitated and relative amounts to IgG control bands calculated. Histone H3 blotting demonstrated adequate separation of nuclear and cytosolic fractions. (D) PNT blocked radiation-induced EGFR phosphorylation and downstream signaling. UM-SCC1 cells were exposed to either PNT (25 nM) or human IgG for 6 h before radiation and harvested 24 h after radiation. STAT3 = signal transducer and activator of transcription 3; MAPK = mitogen-activated protein kinase.

Panitumumab exhibited differential effects on EGFR phosphorylation in SCC-1483

We examined the status of EGFR in each of the studied cell lines. SCC-1483 demonstrated robust expression of EGFR both in whole cell lysates and on the surface of live cells (Fig. 5). Furthermore, panitumumab demonstrated the ability to bind SCC-1483 EGFR and block EGF stimulation. However, in this *in vitro* setting, the administration of panitumumab resulted in a paradoxic phosphorylation of tyrosine 1173 in SCC-1483 cells; in contrast, panitumumab downregulated EGFR phosphorylation in H226 and UM-SCC1 cells (Fig. 5C).

Panitumumab augments in vivo *radiation response*

To examine the effects of combining panitumumab and radiation *in vivo,* mice bearing established xenografts were treated with control IgG, radiation alone, panitumumab

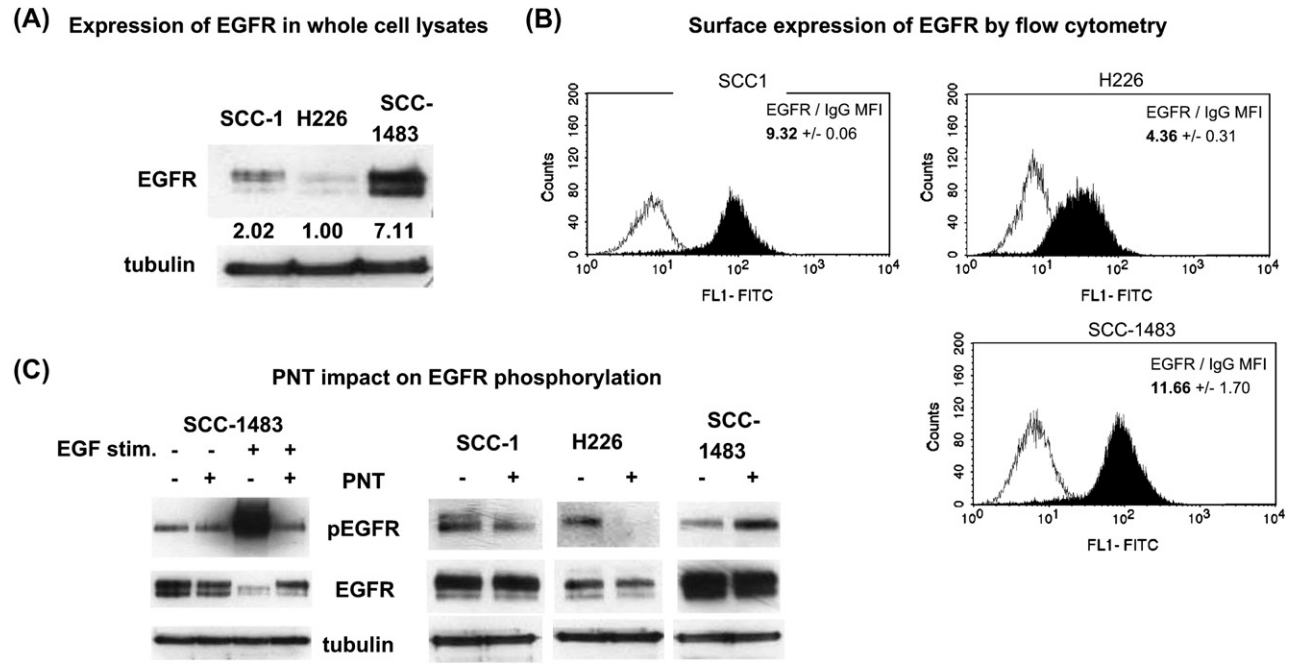

Fig. 5. Epidermal growth factor receptor (EGFR) expression and panitumumab (PNT) activity. Relative expression of EGFR (A) in whole cell lysates and (B) on cell surface determined by Western blot and flow cytometry. Surface EGFR expressed as ratio of EGFR to IgG control mean fluorescence intensity (MFI). (C) Western blot analysis revealed that PNT pretreatment can block EGF stimulation (10 ng/mL × 20 min) in SCC-1483 cells but also resulted in paradoxic phosphorylation of EGFR at tyrosine 1173. stim. = stimulation.

alone, or panitumumab and radiation combined. After 3 weeks of treatment, the difference between the radiation-alone and combined radiation and panitumumab arms was significant in H226 tumors ($p = 0.01$; Fig. 6). At 3 weeks after treatment initiation, SCC-1483 xenografts treated with combination therapy also showed significant growth inhibition compared with panitumumab alone ($p = 0.02$) and a trend compared with radiation alone ($p = 0.08$).

To further study the variance between the *in vitro* clonogenic results and the *in vivo* radiosensitization by panitumumab in SCC-1483 tumors, we examined these tumors histologically. Examination of PCNA by immunohistochemistry revealed a large percentages of cells with dark, positive nuclear staining for PCNA in control tumors in SCC-1483 (Fig. 6). The combination of panitumumab and radiation markedly reduced the fraction of cells with detectable PCNA and the intensity of PCNA staining. A similar pattern was noted in H226 tumors (data not shown). We also examined a surrogate marker of tumor vasculature by staining for VWF. The centers of the control tumors demonstrated large areas of proliferating tumor cells with pronounced vasculature coursing throughout the tumor core. Radiation alone disrupted this architecture to some degree, and tumors receiving combination therapy manifested scant islands of tumor cells, with complete disruption of large vessels traversing the interior of the tumor.

DISCUSSION

This study presents evidence that the fully human EGFR mAb panitumumab augments radiation efficacy in models of upper aerodigestive tract cancer. We identified consistent *in vivo* activity of panitumumab in xenograft models of HNSCC and NSCLC and demonstrated the ability of panitumumab to enhance the growth-inhibitory effects of radiation. To examine the underlying mechanisms behind these observations, we have demonstrated that panitumumab reduces clonogenic survival and blocks radiation-induced EGFR signaling. We have also confirmed that panitumumab augments radiation-induced apoptosis and DNA damage and have presented evidence that inhibition of EGFR nuclear translocation after radiation could underlie these findings. Additionally, we have presented data demonstrating *in vivo* activity of panitumumab with radiation in a cell line that does not show radiosensitization *in vitro* with panitumumab.

Strong evidence has indicated that accelerated tumor cell proliferation contributes to treatment failure in HNSCC and NSCLC (12). Radiation-induced activation of EGFR has been proposed as a mechanism contributing to accelerated cellular repopulation after radiotherapy (13). We have shown that panitumumab can prevent radiation-induced phosphorylation of EGFR and downstream signaling pathways (Fig. 4D) and that PCNA expression is reduced in xenografts treated with the combination of panitumumab and radiation compared with either modality alone (Fig. 6). The ability of panitumumab to inhibit accelerated EGFR-dependent proliferative signaling after radiation might contribute to the observed augmentation of the antitumor efficacy of radiation in these models.

Epidermal growth factor receptor signaling is known to induce antiapoptotic effects, and EGFR blockade has been

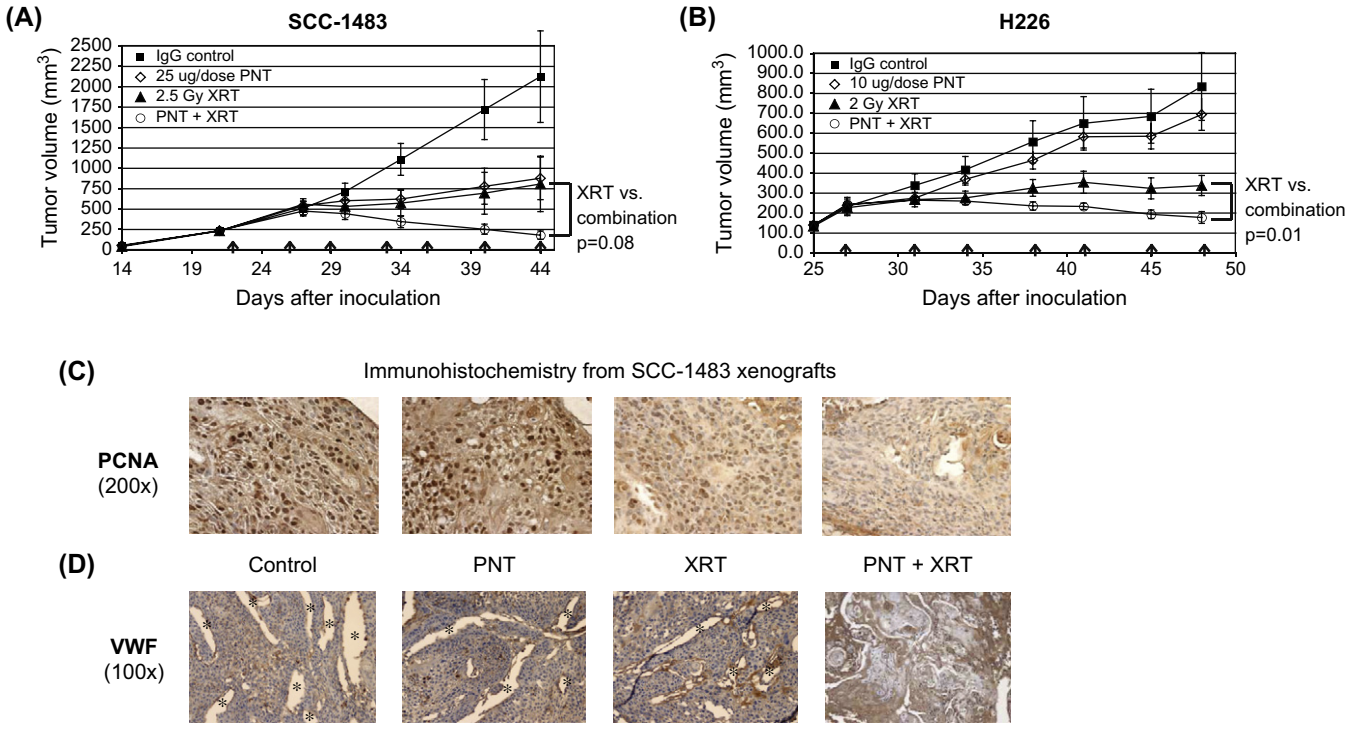

Fig. 6. *In vivo* activity of panitumumab (PNT) with or without radiation in tumor xenografts. (A,B) Mice bearing xenografts were treated with PNT, radiation (XRT), or both twice weekly (vertical arrows). Points indicate mean tumor size (n = 10/group); bars indicate standard error of mean. Effect of PNT and XRT on (C) proliferating cell nuclear antigen (PCNA) and (D) von Willebrand factor (VWF) expression in SCC-1483 xenografts. Positive staining (brown) in nucleus pronounced in control tumors; tumors treated with combination of PNT and XRT demonstrated little positive nuclear staining for PCNA. von Willebrand factor stained brown in endothelial cells, serving to demarcate tumor vasculature (asterisks).

shown to stimulate apoptosis (4). We have demonstrated the ability of panitumumab to augment radiation-induced apoptosis *in vitro*. We had previously demonstrated enhanced apoptosis with various EGFR inhibitors and radiation combinations (4, 14). Apoptosis occurs in response to DNA damage; therefore, augmentation of radiation-induced DNA damage by panitumumab (Fig. 3) could enhance the apoptotic response to irradiation.

The augmentation of radiation-induced apoptosis by panitumumab could also be mediated by blockade of radiation-induced EGFR-pSTAT3 signaling. The interaction between STAT3 and EGFR is well characterized in HNSCC (15) and NSCLC (16). Activated STAT3 plays a central role in protecting cells against apoptosis through the transcriptional modulation of survival genes, such as Bcl-xL, Bcl-2, and survivin. Our findings (Fig. 4D) are consistent with a report that STAT3 activation after either EGF stimulation or irradiation can be abolished by pretreatment with AG1478, an EGFR tyrosine kinase inhibitor (17).

Dittmann *et al.* (11) have shown that radiation induces nuclear translocation of EGFR, which results in an increase in nuclear DNA-dependent protein kinase activity. EGFR blockade with the mAb cetuximab inhibits this process and enhances radiation-induced DNA damage (11). In a similar fashion, we have demonstrated an increase in EGFR in the nucleus of UM-SCC1 cells 20 min after radiation and found that panitumumab can abrogate this shift of EGFR into the nucleus (Fig. 4C). Therefore, inhibition of nuclear translocation of EGFR by panitumumab might underlie the enhancement of DNA damage in these cells lines and contribute to enhanced apoptosis and radiosensitivity in the presence of EGFR blockade.

Although panitumumab augmented apoptosis in all three tumor cell lines tested, no clear effect on clonogenic survival was found in the SCC-1483 cells. A recent study has suggested that cell-surface EGFR expression might be an important determinant of radiosensitization by EGFR mAbs (18). However, SCC-1483 demonstrated high cell-surface EGFR expression and a lack of *in vitro* radiosensitization. We tested the activity of panitumumab in SCC-1483 cells and found that it binds the receptor but induces paradoxic phosphorylation of tyrosine 1173. This finding has been previously reported with cetuximab (5, 19) and does not result in increased mitogen-activated protein kinase phosphorylation or proliferation (data not shown). We believe this might represent altered trafficking of the receptor on EGFR mAb binding *in vitro* and likely accounts for the variance between the effect of panitumumab on SCC-1483 clonogenic survival and other endpoints studied. Subcellular location and trafficking patterns of the EGFR as predictive markers to EGFR mAb therapy is an ongoing area of research in our laboratory (20).

Nevertheless, the combination of panitumumab and radiation demonstrated enhanced antitumor efficacy in SCC-1483

xenografts, suggesting that features beyond the *in vitro* environment might contribute to the antitumor effects. For example, inhibition of tumor angiogenesis by EGFR blockade would not be apparent in culture, but it has been demonstrated to have *in vivo* importance (14, 21). We have demonstrated measurable changes in the interior of tumors receiving combination radiation and panitumumab (Fig. 6D). This finding suggests that even in a tumor cell line, such as SCC-1483, that does not demonstrate *in vitro* radiosensitization, panitumumab might still serve to augment the antitumor efficacy of radiation *in vivo*.

These preclinical results with panitumumab and radiation are promising and closely resemble the findings from preclinical studies of cetuximab and radiation. Because of its fully human sequence, a potential comparative strength of panitumumab is the lower risk of immunogenicity and allergic reactions. In contrast, cetuximab has a human IgG1 backbone and has been demonstrated to trigger antibody-dependent cellular cytotoxicity (22). Clinical evidence suggests that this could affect the efficacy of cetuximab (23); however, panitumumab has an IgG2 backbone and, thus, would not be expected to induce antibody-dependent cellular cytotoxicity. Ultimately, the therapeutic efficacies of distinct EGFR mAbs will be best evaluated in the context of controlled clinical trials.

CONCLUSION

The present studies have demonstrated that the fully human anti-EGFR mAb panitumumab can augment radiation response in HNSCC and NSCLC model systems *in vitro* and *in vivo*. These results parallel those of preclinical studies of the anti-EGFR mAb cetuximab that demonstrated favorable interaction with radiation and contributed to the Phase III trial demonstrating a survival benefit for cetuximab plus radiation in patients with advanced HNSCC. These preclinical data suggest that systematic clinical investigations to examine combinations of radiation and panitumumab in cancer therapy are warranted.

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