PTEN reconstitution alters glioma responses to c-Met pathway inhibition

C. Rory Goodwin[a,c], Bachchu Lal[a,b], Sandra Ho[a], Crystal L. Woodard[e], Xin Zhou[a], Alexandra Taeger[a], Shuli Xia[a,b] and John Laterra[a,b,c,d]

Mutations/deletions of the tumor-suppressor phosphatase and tensin homolog PTEN result in PI3K/Akt pathway hyperactivation and potentially alter oncogenic responses to targeted receptor tyrosine kinase inhibitors. We previously showed that hepatocyte growth factor (HGF): c-Met pathway inhibition decreases tumor growth and oncogenic signaling responses in PTEN-null/Met+ gliomas. Here, we use two tet-on PTENwt-inducible glioma cell lines and xenograft models to examine the influence of PTEN on oncogenic signaling responses to HGF:c-Met pathway inhibitors. Reconstitution of PTEN inhibited Akt by more than 80% and inhibited cell growth by approximately 70–75% in both cell lines *in vitro*. C-Met inhibition alone inhibited in-vitro cell growth by approximately 80–85% and the magnitude of growth inhibition was not altered by combining PTEN reconstitution with c-Met inhibition. Combining PTEN reconstitution with Met inhibition arrested a higher percentage of cells in G_1/G_0 phase of the cell cycle when compared with either PTEN reconstitution or c-Met inhibition alone. Both PTEN reconstitution alone and inhibiting autocrine HGF:c-Met signaling alone, using anti-HGF mAb, robustly inhibited the growth of subcutaneous and intracranial glioma xenografts. Combining anti-HGF therapy with PTEN reconstitution did not significantly alter the magnitude of xenograft growth inhibition. Semiquantitative immunohistopathological analyses revealed that the inhibition of glioma xenograft angiogenesis and cell proliferation by anti-HGF mAb was greatest in conjunction with PTEN reconstitution. In contrast, xenograft cell apoptosis was greatest in response to anti-HGF therapy alone and PTEN reconstitution abrogated the apoptotic response to anti-HGF therapy. These results provide new insights into how PTEN modulates glioma responses to the inhibition of HGF:c-Met signaling and possibly other receptor tyrosine kinase pathways. *Anti-Cancer Drugs* 22:905–912 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2011, **22**:905–912

Keywords: Akt, angiogenesis, apoptosis, hepatocyte growth factor, xenograft

[a]The Hugo Moser Research Institute at Kennedy Krieger, Departments of [b]Neurology, [c]Neuroscience, [d]Oncology and [e]Pharmacology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Correspondence to Dr C. Rory Goodwin, MD, PhD, The Kennedy Krieger Research Institute, 707 N. Broadway, Baltimore, MD 21205, USA
Tel: +1 443 923 2683; fax: +1 443 923 2695; e-mail: rory@jhmi.edu

Received 11 December 2010 **Revised form accepted** 28 April 2011

Introduction

Mutations/deletions of tumor suppressors, such as the phosphatase and tensin homolog deleted on chromosome 10*q* (PTEN), lead to Akt hyperactivation and are commonly found in malignant neoplasms [1]. Receptor tyrosine kinase (RTK) systems such as those involving c-Met, epidermal growth factor receptor (EGFR), and platelet-derived growth factor receptor commonly influence PTEN-associated oncogenic signaling [2–5]. Amplifications and/or activating mutations in RTKs lead to hyperactivation of downstream signaling pathways, such as the PI3K/Akt pathway, which mediates oncogenic phenotypic responses. Dysfunction of these oncogenic molecular pathways contributes to uncontrolled tumor cell proliferation, survival, invasiveness, tumor angiogenesis, and poor patient prognoses in a variety of solid malignancies [1].

PTEN is a polypeptide with dual lipid and protein phosphatase activities. PTEN dephosphorylates the lipid second messenger phosphatidylinositol 3,4,5 triphosphate (PIP3) to create phosphatidylinositol 3,4 biphosphate. Conversion of PIP3 to phosphatidylinositol 3,4 biphosphate diminishes the downstream effects of PI3K including the extent of Akt activation [6]. Somatic deletions/mutations of PTEN are commonly found in a wide variety of solid tumors and are detected in 40–50% of glioblastomas [7,8]. PTEN expression in PTEN-null glioma cells suppresses glioma growth *in vitro* and *in vivo* inhibiting cell migration and invasion while also inducing apoptosis [9–12]. PTEN is shown to coregulate RTK-mediated gene expression in glioma models and influences the response to targeted kinase inhibitors [13,14].

C-Met and its cognate ligand hepatocyte growth factor (HGF) have been implicated in the formation and progression of a variety of solid tumor types [15]. C-Met is a potent activator of the Ras/Map kinase and PI3K/Akt pathways and targeting this RTK/ligand system has antioncogenic effects in several preclinical model systems [16–18]. Preclinical evidence supporting the role of HGF/c-Met in tumorigenesis has led to the development of a plethora of c-Met pathway inhibitors, now entering phase

0959-4973 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e3283484750

I/II clinical trials [19]. Previous studies have suggested that the therapeutic efficacy of RTK inhibitors is predicted by PTEN activity, with PTEN loss rendering tumors unresponsive [14]. Other evidence suggests that tumor responses to RTK inhibitors are not predicted by the activity of any other single pathway component including PTEN, but rather are determined by the integrated activity of multiple pathway components that together determine total oncogenic 'flux' [20]. With the emergence of novel therapeutic agents aimed at the HGF/c-Met pathway, the influence of PTEN on the tumor response to HGF:c-Met pathway inhibition requires further attention.

We previously reported that anti-HGF therapy inhibits glioma growth *in vitro* and *in vivo* in an HGF^+/$c\text{-}Met^+$/PTEN-null glioma model. Here, we investigated whether PTEN reconstitution alters tumor sensitivity to anti-HGF therapeutics using previously characterized glioma cell lines that express wild-type PTEN under the control of a tet-responsive promoter [21]. Specifically, we investigated whether PTEN function alters glioma xenograft growth, cell proliferation, angiogenesis, and apoptosis responses to c-Met inhibition.

Methods

Reagents

SU11274 and PHA665752 are c-Met inhibitors shown to block the catalytic domain of the c-Met receptor. L2G7 is an anti-HGF monoclonal antibody shown to inhibit the direct interaction of HGF and c-Met, and 5G8 is the isotype-matched control monoclonal antibody.

Tet-on inducible cell lines

The tet-on PTENwt inducible cell lines U87-PTENwt and U251-PTENwt were a kind gift from Dr Maria Georgescou. Radu *et al.* [21] developed a tetracycline-inducible method for expressing PTENwt using a retroviral vector in PTEN-deficient U87 and U251 glioma cell lines. In their system, two retroviral constructs pCX_n/tetracycline repressor (TR2) and pCX_bR(TO)-PTEN were introduced simultaneously into U87 or U251 glioma cells by infection. The pCX_n/TR2 construct encodes TR of the tetracycline operon that is found in the second construct. The second construct contains the inducible promoter that controls transcription of the desired gene, in this case PTEN. When tetracycline is absent, the repressor prevents PTEN expression from the second retroviral construct. When tetracycline, or its derivative doxycycline, is present, the repressor is inhibited and catalytically active PTEN is expressed.

Immunoblot analysis

Total protein was extracted from glioma xenografts and cells using radioimmunoprecipitation assay buffer (1% igepal, 0.5% sodium deoxycholate, and 0.1% SDS in phosphate buffer solution) containing fresh 1X protease and 1X phosphatase inhibitors (Calbiochem, Darmstadt, Germany) at 4°C. Tissue extracts were sonicated on ice and centrifuged at 5000 rpm at 4°C for 5 min. Supernatants were assayed for protein concentrations by the Coomassie protein assay (Pierce, Rockford, Illinois, USA) according to the manufacturer's recommendations. Aliquots of 40 or 60 μg of total protein were combined with Laemmli loading buffer containing β-mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis according to the method of Towbin *et al.* [22] with some modifications [23]. For immunoblot analyses, proteins were electrophoretically transferred to nitrocellulose with a semidry transfer apparatus (GE Healthcare, Piscataway, New Jersey, USA) at 50 mA for 60 min. Membranes were incubated for 1 h in Odyssey Licor blocking buffer (Lincoln, Nebraska, USA) at room temperature and then overnight with primary antibodies at 4°C in 5% bovine serum albumin in tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS/T). Membranes were then washed 3X with TBS/T, incubated with secondary antibody at 1 : 10 000 for 1 h in TBS/T, again washed 3X with TBS/T, followed by washing 2X with TBS. Proteins were detected and quantified using the Odyssey Infrared Imager (LI-COR Biosciences).

Cell-viability assay

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Cells were plated at 50 000/well in 24-well tissue culture plates and cultured for 24 h before treatment with specified reagents. Twenty-four to 72 h after treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide was added to each well at a final concentration of 150 μg/ml, and the cells were incubated for 2 h at 37°C. The medium was then removed and the cell layer was dissolved with dimethylsulfoxide. The formazen reaction product was quantified spectrophotometrically at 570 nm using a Spectra MAX 340pc plate reader (Molecular Devices, Sunnyvale, California, USA). The results are expressed as the percentage of absorbance measured in control cultures after subtracting the background absorbance from all values.

Flow cytometry analysis

Cell cycle analysis was quantified using propidium iodide (BD Biosciences, San Diego, California, USA) according to the manufacturer's instructions. In brief, cells were trypsinized, pelleted by centrifugation, and resuspended in annexin V-binding buffer [NaCl (150 mmol/l), $CaCl_2$ (18 mmol/l), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (10 mmol/l), KCl (5 mmol/l), and $MgCl_2$ (1 mmol/l)]. Fluorescein isothiocyanate-conjugated annexin V (1 μg/ml) and propidium iodide (50 μg/ml) were added to cells and incubated for 30 min at room temperature in the dark. Nonimmune mouse IgG was used as the negative control. Analyses were performed on a FACscan (Becton-Dickinson, Mountain View, California, USA). Data were analyzed with CellQuest software (Becton-Dickinson).

Tumor xenografts

Glioma xenografts were generated as previously described [24]. Female mice (6–8 week old; National Cancer Institute, Frederick, Maryland, USA) were anesthetized by intraperitoneal (i.p.) injection of ketamine (100 mg/kg) and xylazine (5 mg/kg). For subcutaneous xenografts, Nu/nu mice received 4×10^6 cells in 0.05 ml of plain media subcutaneously in the dorsal flank. When tumors reached approximately 200 mm^3, the mice were randomly divided into groups ($n = 5$ per group) and received the indicated doses of L2G7, an anti-HGF-neutralizing antibody or an isotype-matched control mAb (5G8) in 0.1 ml of phosphate buffer solution i.p. as previously described [17]. Tumor volumes were estimated by measuring two dimensions [length (a) and width (b)] and by calculating the volume as $V = ab^2/2$ [17,25]. At the end of each experiment, tumors were excised, frozen in liquid nitrogen, and protein was extracted for immunoblot analysis.

For intracranial xenografts, Scid/beige mice received 1×10^5 cells per 2 µl by stereotaxic injection into the right caudate/putamen [24]. L2G7 or 5G8 mAb was administered as above. Groups of mice ($n = 5$) were killed by perfusion fixation at 24 h after the last injection and the brains were removed for histological studies. Tumor volumes were quantified by measuring tumor cross-sectional areas on hematoxylin and eosin-stained cryostat sections using computer-assisted image analysis as previously described [24]. Tumor volumes were estimated based on the formula: volume = (square root of maximum cross-sectional area)^3 [26].

The Johns Hopkins University Institutional Animal Care and Use Committee approved all animal protocols used in this study.

Immunohistochemistry

Cryostat sections were stained with anti-cleaved caspase-3, anti-MIB-1, or anti-laminin antibodies as previously described [26]. Biotinylated-conjugated secondary antibodies followed by incubation with 3,3′-diaminobenzidine peroxidase substrate was used to detect primary antibodies. Anti-MIB-1-stained sections were counterstained with Gill's hematoxylin solution. Anticleaved caspase 3 and antilaminin stained sections were counterstained with methyl green. Proliferation, apoptotic, and microvessel density indices were determined by computer-assisted quantification using Image J Software (*rsb.info.nih.gov/ij/*) essentially as previously reported [17].

Statistical methods

Statistical analysis consisted of one-way analysis of variance followed by the Tukey or Dunnet's multiple-comparison test using Prism (GraphPad software Inc., San Diego, California, USA). A P value of less than 0.05 was considered significant. All experiments reported here represent at least three independent replications. For the in-vivo studies reported in Figs 3 and 4, a representative experiment was chosen to summarize at least three independent replications with a total of $n = 15$ animals per group). Data are represented as mean values ± standard deviation.

Results

PTEN reconstitution and c-Met inhibition decrease Akt activation and glioma cell growth

We found that PTEN reconstitution, c-Met pathway inhibition, or their combination significantly decreased Akt activation and glioma growth as assessed by immunoblot analysis and cell viability assays, respectively. C-Met inhibition with SU11274 (10 µmol/l) decreased Akt activation by approximately 30% in both U87-PTENwt and U251-PTENwt glioma cell lines ($P < 0.05$), whereas PTEN reconstitution alone and in combination with SU11274 (10 µmol/l) decreased Akt activation by 90% in both cell lines ($P < 0.05$; Fig. 1a and c). Reconstituting PTEN in U87-PTENwt and U251-PTENwt glioma cells inhibited cell growth by approximately 70 and 75%, respectively when measured 72 h after initiating the treatment. C-Met inhibition alone or combined with PTEN reconstitution both inhibited cell growth by approximately 80% in U87-PTENwt cells and by approximately 85% in U251-PTENwt cells (Fig. 1b and d). Thus, c-Met inhibition alone had a potent inhibitory effect on cell growth that was not altered by PTEN reconstitution.

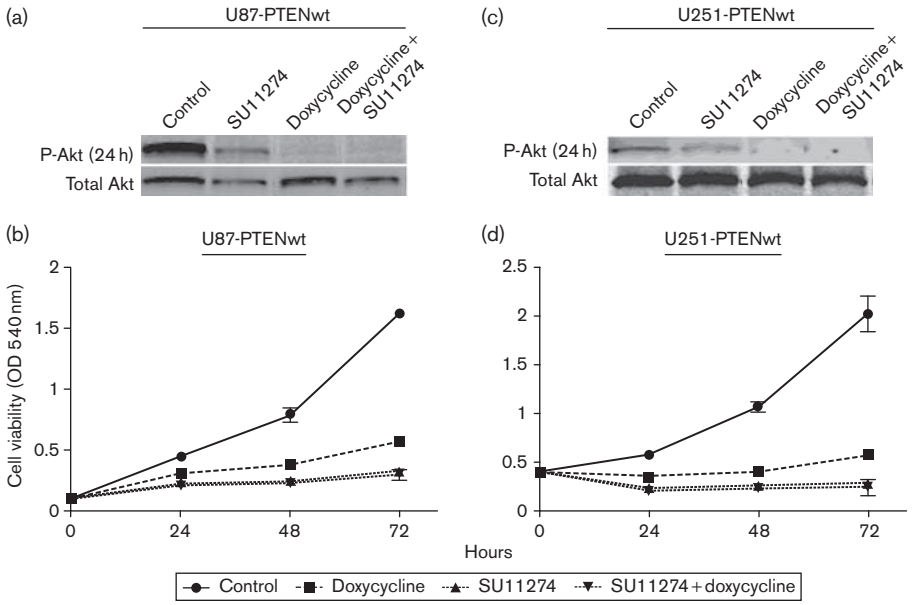
PTEN reconstitution and c-Met inhibition alter glioma cell cycle progression

Cell cycle responses were examined to determine whether PTEN alters cell proliferation responses to c-Met inhibition. U87 and U251 glioma cells treated with either PHA665752 (100 nmol/l) or doxycycline (100 nmol/l) to induce PTEN expression, or their combination, were subjected to cell cycle analysis by flow cytometry. PTEN reconstitution alone, Met inhibition alone, or the combination each arrested cell cycle progression in the G_1/G_0 phase and decreased the percentage of cells in the G_2/M phase in comparison with controls ($P < 0.05$; Fig. 2a, c, d and f). However, combining c-Met inhibition and PTEN restoration in both cell lines led to a modest statistically significant increase in the G_1/G_0 cell cycle arrest compared with either c-Met inhibition or PTEN reconstitution alone ($P < 0.01$; Fig. 2a and d).

PTEN reconstitution and anti-hepatocyte growth factor therapy alter tumor growth responses in subcutaneous glioma xenografts

We examined the effects of PTEN reconstitution, c-Met pathway inhibition, and their combination on the growth characteristics of glioma xenografts. U87-PTENwt cells were implanted subcutaneously in Nu/Nu mice and tumors were allowed to reach a size of approximately 200 mm^3. Animals were then treated ± doxycycline (2 mg/ml in drinking water) with either control mAb

Fig. 1



PTEN reconstitution and c-Met inhibition decrease Akt activation and glioma cell growth responses. U87-PTENwt and U251-PTENwt glioma cells were acclimated overnight in low serum (0.1% fetal bovine serum), and then treated with the c-Met inhibitor SU11274 (10 μmol/l) ± doxycycline (2 μg/ml). (a and c) Immunoblot analysis of phospho-Akt^{Ser473} and total Akt were performed 24 h after treatment. Cells were assayed 24, 48, or 72 h after treatment for cell viability by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (b and d).

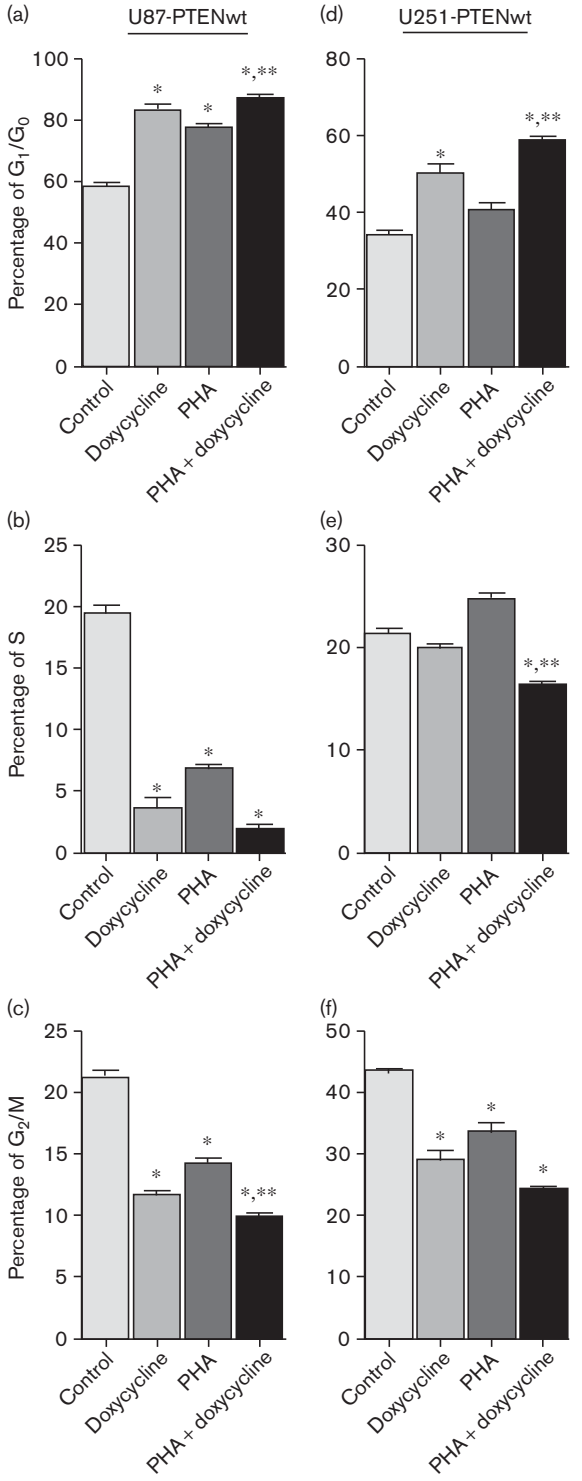
(5G8) or neutralizing anti-HGF mAb (L2G7; 1.25 mg/kg i.p.) every alternate day for 10 days. We have previously shown that L2G7 therapy effectively inhibits c-Met receptor activation (phosphorylation) and downstream signaling in wild-type U87 glioma xenografts [17]. L2G7 alone, PTEN reconstitution alone, and combination of L2G7 and PTEN reconstitution markedly inhibited tumor growth (Fig. 3a). At day 10, corresponding with the last day of treatment, L2G7 alone or PTEN reconstitution alone inhibited U87 tumor xenograft growth by approximately 75%. Semiquantitative immunoblot analysis of tumor extracts revealed a substantial reduction in Akt activation (serine 473 phosphorylation) in response to PTEN reconstitution (Fig. 3b). Combining L2G7 and PTEN reconstitution also markedly inhibited tumor growth by approximately 85% ($P < 0.05$). There was a trend, which did not reach statistical significance, toward greater tumor growth inhibition in response to PTEN reconstitution and L2G7 when compared with either treatment alone.

PTEN reconstitution alters glioma responses to anti-hepatocyte growth factor therapeutics in orthotopic glioma xenografts

Glioma responses to PTEN reconstitution ± HGF:c-Met pathway inhibition were examined in more detail using an orthotopic intracranial model. U87-PTENwt cells were implanted into the caudate/putamen of Scid/beige mice. Beginning on postimplantation day 15, animals were treated ± doxycycline (2 mg/ml in drinking water) with either 5G8 or L2G7 (1.25 mg/kg i.p.) administered every alternate day for 6 days. Animals were killed and tumor sizes were quantified by morphometric histological analysis. PTEN reconstitution alone and L2G7 alone both reduced tumor xenograft size by approximately 90 and 80%, respectively (Fig. 4a). PTEN reconstitution and L2G7 inhibited tumor growth by approximately 95% (Fig. 4a). The effects of PTEN reconstitution alone, HGF:c-Met pathway inhibition alone, or their combination on tumor growth were similar in orthotopic and subcutaneous U87PTENwt xenografts.

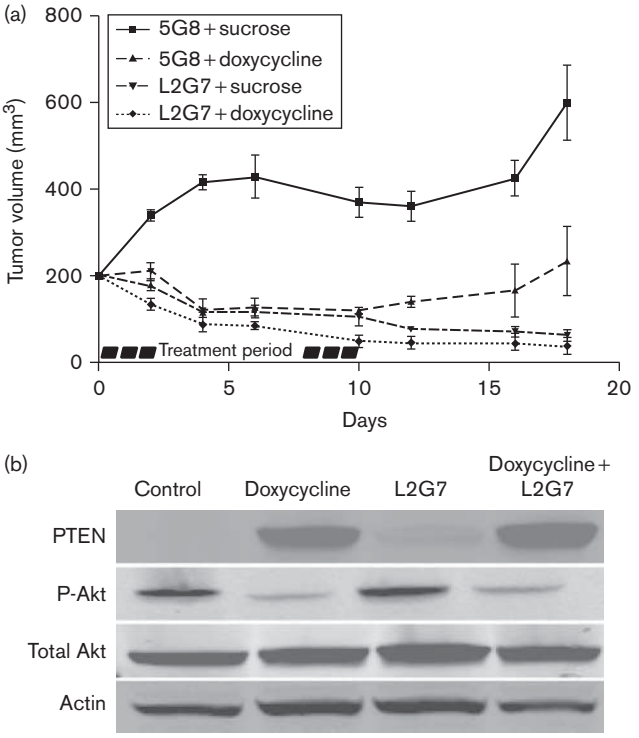
We analyzed orthotopic xenograft histological sections for the effects of PTEN reconstitution, HGF:c-Met pathway inhibition, or both on cell proliferation using anti-Ki67 staining (Fig. 4b), angiogenesis using antilaminin staining (Fig. 4c), and apoptosis using anticleaved caspase 3 staining (Fig. 4d). Neither PTEN reconstitution nor L2G7 monotherapy affected tumor Ki-67 labeling or tumor vascular density when compared with controls. However, combining PTEN reconstitution and L2G7 inhibited cell proliferation and angiogenesis by approximately 60 and 65%, respectively, consistent with cooperativity ($P < 0.05$). There was a trend of two-fold increase in apoptosis in response to PTEN reconstitution alone that was not statistically significant. L2G7 therapy alone statistically significantly increased tumor cell apoptosis four-fold ($P < 0.05$). Glioma cell apoptosis in response to combining PTEN reconstitution and L2G7 was significantly less than that seen in response to L2G7

Fig. 2



PTEN reconstitution and Met inhibition alter glioma cell cycle progression. U87-PTENwt or U251-PTENwt cells were acclimated overnight in low serum (0.1% fetal bovine serum) and treated with the c-Met inhibitor PHA665752 (100 nmol/l) ± doxycycline (2 μg/ml) for 24 h before cells were fixed and labeled with propidium iodide. Cell cycle analysis was performed by flow cytometry and the percentage of cells in the G_1/G_0 phase (a and d), S phase (b and e), and G_2/M phase were determined. *$P<0.05$ compared with controls, **$P<0.01$ compared with PHA alone or doxycycline alone.

Fig. 3



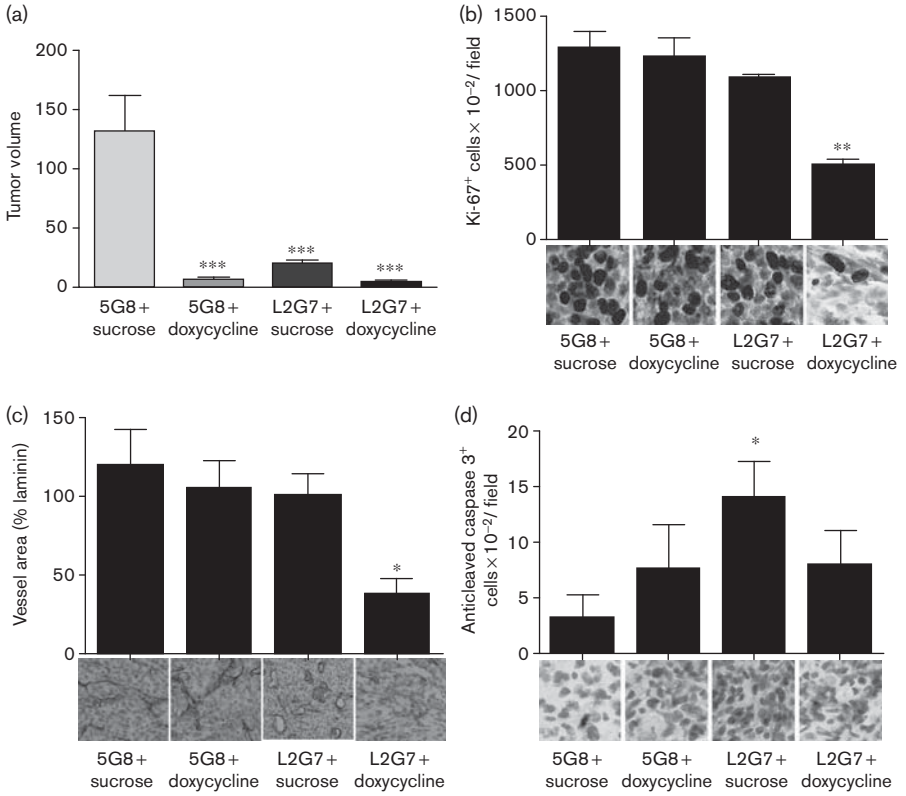
PTEN reconstitution and anti-hepatocyte growth factor (HGF) therapy alter tumor growth responses in subcutaneous glioma xenografts. Pre-established U87-PTENwt subcutaneous xenografts (200 mm^3) were treated with ± doxycyline (2 mg/ml in drinking water) with either anti-HGF L2G7 or control mAb 5G8 (1.25 mg/kg, intraperitoneally) on days 0, 2, 4, 6, and 8. Doxycycline was withdrawn on day 10. Xenografts were measured every alternate day and tumor volumes were calculated as described methods. (b) Immunoblot analysis of PTEN and phospho-Akt^{Ser473} in subcutaneous xenografts sampled on day 6.

alone. Thus, although PTEN reconstitution and anti-HGF therapy co-operated to inhibit tumor cell proliferation and angiogenesis, PTEN reconstitution rendered tumor cells less sensitive to developing apoptosis in response to anti-HGF therapy.

Discussion

Hyperactivation of the oncogenic serine/threonine kinase Akt occurs in up to 65% of glioblastoma and is very common in other brain and systemic malignancies [1,27]. Akt hyperactivity most commonly results from amplifications, mutations, deletions, or hyperactivation of upstream signaling molecules such as the RTKs EGFR, platelet-derived growth factor receptor, and c-Met, the intracellular second messengers PI3K, and through the loss of the tumor-suppressor PTEN [1,6,7,28]. Activating Akt mutations occur less commonly and independent of RTK gains and PTEN loss [1,28]. Considerable effort toward developing targeted therapeutics has focused on inhibiting RTK-Akt signaling due to its importance in regulating tumor cell growth, invasion, survival, and

Fig. 4



PTEN reconstitution alters cell responses to anti-hepatocyte growth factor (HGF) therapeutics in orthotopic glioma xenografts. Animals bearing U87-PTENwt intracranial xenografts were treated with ± doxycycline (2 mg/ml in drinking water) for 6 days and with either anti-HGF or control mAb 5G8 (1.25 mg/kg, intraperitoneally) for 6 days. Intracranial tumor xenograft sections were analyzed for tumor volume (a), cell proliferation (b), angiogenesis (c), and apoptosis (d) as described in Materials and methods. ***$P<0.001$ compared with controls, *$P<0.05$ compared with controls, L2G7 alone, or doxycycline alone, and **$P<0.01$ compared with control, L2G7 alone, or doxycycline alone.

sensitivity to DNA-damaging agents [1,29]. The influence of PTEN on tumor responses to RTK inhibition remains incompletely characterized. We previously reported that inhibiting HGF/c-Met signaling in an HGF$^+$/Met$^+$/PTEN-null glioma model robustly inhibits tumor-initiating capacity, tumor xenograft growth, and resistance to cytotoxic therapeutics [2,17,24]. Here, we show that reconstituting PTEN alters the response of a glioblastoma xenograft model to HGF/c-Met pathway inhibition, namely by enhancing the inhibition of glioma cell proliferation and tumor angiogenesis, but by diminishing tumor cell apoptosis.

One explanation for the lack of an additive in-vivo antitumor effect from combining PTEN restoration and c-Met pathway inhibition could be attributed to the high dependency of these cell lines on c-Met and PTEN loss. In our experiments, we aimed to isolate the effects of c-Met and PTEN on the tumor growth response, whereas clinical specimens demonstrate heterogeneity in their drivers of tumor progression. Under conditions in which PTEN and c-Met/HGF are predominant drivers of tumor malignancy, additive antitumor effects might have been detected.

HGF/c-Met pathway signaling is recognized to support the malignant behavior of subsets of multiple solid malignancies [15]. Understanding how distinct molecular backgrounds influence tumor responses to HGF/c-Met pathway inhibitors is of considerable importance. On the basis of an elegant phase 2 trial in patients with glioblastoma, Mellinghoff *et al.* [30] concluded that PTEN loss renders tumors unresponsive to the inhibition of EGFR and potentially other RTKs. Our findings in this study using a well-characterized isogenic glioblastoma model are consistent with previous findings showing that malignancies can robustly respond to RTK inhibition, even in the absence of PTEN [17,20,31]. Differences in the RTK pathways examined, c-Met versus EGFR, differences in tumor genetic heterogeneity, or differences in the magnitudes of RTK inhibition might account for the differences in these clinical and preclinical results. The sensitivity of PTEN-null U87 xenografts to RTK inhibition suggests that downregulating PI3K activity can

compensate for the absence of the lipid phosphatase PTEN in reducing tumor levels of PIP3 and thereby reduce the overall flux to Akt activation. In previous studies, we showed that expressing the constitutively active RTK EGFRvIII renders Met-dependent tumors insensitive to Met pathway inhibitors [31]. This effect is likely to be mediated at least in part by the fact that EGFRvIII signaling is parallel and redundant with c-Met signaling and maintains the flux through oncogenic PI3K/Akt and Ras/MAPK relatively independent of c-Met.

Our in-vitro findings confirm the results of Li *et al.* [32] showing that HGF:c-Met pathway inhibition and adenoviral-based PTEN restoration additively inhibit proliferation and cell cycle progression in PTEN-null glioblastoma cells. Our results in this study expand on these findings by examining specific tumor cell responses within the more complex orthotopic in-vivo context. We were surprised that PTEN reconstitution and anti-HGF therapy in combination co-operatively inhibited tumor cell proliferation and angiogenesis in the absence of a comparable effect on tumor growth. This is likely explained by the apoptotic response that was greatest in response to anti-HGF therapy alone, approximately two-fold greater than the response to the combination. The greater apoptotic response to anti-HGF alone suggests that in the absence of PTEN, c-Met inhibition preferentially inhibits tumor growth by a proapoptotic mechanism. This finding is consistent with our previous studies in U87 glioma xenografts and HGF^+/Met^+ medulloblastoma models [31,33].

Conclusion

Taken together, our findings in this study reveal potentially important differences in the antitumor response to c-Met inhibition depending on PTEN status. Although PTEN status may not necessarily alter the magnitude of tumor growth inhibition to anti-HGF therapy, PTEN status does influence the magnitude of proliferation inhibition relative to apoptosis promotion. These results provide new insights into the molecular basis for different tumor responses to HGF:c-Met pathway inhibition and suggest that the relative benefits of combining HGF/c-Met pathway inhibitors with other anti-proliferative agents or proapoptotic agents will be influenced by tumor PTEN status.

Acknowledgements

Funding Sources: this study was supported by National Institutes of Health grants NS43987 and CA129192 (JL) the United Negro College Fund/Merck Science Initiative (C.R.G.).

Conflicts of interest

There are no conflicts of interest.

References

1 Bellacosa A, Kumar CC, Di Cristofano A, Testa JR. Activation of Akt kinases in cancer: implications for therapeutic targeting. *Adv Cancer Res* 2005; **94**:29–86.
2 Bowers DC, Fan S, Walter KA, Abounader R, Williams JA, Rosen EM, *et al.* Scatter factor/hepatocyte growth factor protects against cytotoxic death in human glioblastoma via phosphatidylinositol 3-kinase- and Akt-dependent pathways. *Cancer Res* 2000; **60**:4277–4283.
3 Wang X, McCullough KD, Franke TF, Holbrook NJ. Epidermal growth factor receptor-dependent Akt activation by oxidative stress enhances cell survival. *J Biol Chem* 2000; **275**:14624–14631.
4 Franke TF, Yang SI, Chan TO, Datta K, Kazlauskas A, Morrison DK, *et al.* The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* 1995; **81**:727–736.
5 Sansal I, Sellers WR. The biology and clinical relevance of the PTEN tumor suppressor pathway. *J Clin Oncol* 2004; **22**:2954–2963.
6 Di Cristofano A, Pandolfi PP. The multiple roles of PTEN in tumor suppression. *Cell* 2000; **100**:387–390.
7 Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, *et al.* PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 1997; **275**:1943–1947.
8 Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, *et al.* Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* 1997; **15**:356–362.
9 Cheney IW, Johnson DE, Vaillancourt MT, Avanzini J, Morimoto A, Demers GW, *et al.* Suppression of tumorigenicity of glioblastoma cells by adenovirus-mediated MMAC1/PTEN gene transfer. *Cancer Res* 1998; **58**:2331–2334.
10 Furnari FB, Huang HJ, Cavenee WK. The phosphoinositol phosphatase activity of PTEN mediates a serum-sensitive G1 growth arrest in glioma cells. *Cancer Res* 1998; **58**:5002–5008.
11 Furnari FB, Lin H, Huang HS, Cavenee WK. Growth suppression of glioma cells by PTEN requires a functional phosphatase catalytic domain. *Proc Natl Acad Sci USA* 1997; **94**:12479–12484.
12 Tamura M, Gu J, Takino T, Yamada KM. Tumor suppressor PTEN inhibition of cell invasion, migration, and growth: differential involvement of focal adhesion kinase and p130Cas. *Cancer Res* 1999; **59**:442–449.
13 Abounader R, Reznik T, Colantuoni C, Martinez-Murillo F, Rosen EM, Laterra J. Regulation of c-Met-dependent gene expression by PTEN. *Oncogene* 2004; **23**:9173–9182.
14 Mellinghoff IK, Wang MY, Vivanco I, Haas-Kogan DA, Zhu S, Dia EQ, *et al.* Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. *N Engl J Med* 2005; **353**:2012–2024.
15 Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. *Nat Rev Mol Cell Biol* 2003; **4**:915–925.
16 Christensen JG, Schreck R, Burrows J, Kuruganti P, Chan E, Le P, *et al.* A selective small molecule inhibitor of c-Met kinase inhibits c-Met-dependent phenotypes *in vitro* and exhibits cytoreductive antitumor activity *in vivo*. *Cancer Res* 2003; **63**:7345–7355.
17 Kim KJ, Wang L, Su YC, Gillespie GY, Salhotra A, Lal B, *et al.* Systemic anti-hepatocyte growth factor monoclonal antibody therapy induces the regression of intracranial glioma xenografts. *Clin Cancer Res* 2006; **12**:1292–1298.
18 Martens T, Schmidt NO, Eckerich C, Fillbrandt R, Merchant M, Schwall R, *et al.* A novel one-armed anti-c-Met antibody inhibits glioblastoma growth *in vivo*. *Clin Cancer Res* 2006; **12**:6144–6152.
19 Liu X, Yao W, Newton RC, Scherle PA. Targeting the c-Met signaling pathway for cancer therapy. *Expert Opin Investig Drugs* 2008; **17**:997–1011.
20 Stommel JM, Kimmelman AC, Ying H, Nabioullin R, Ponugoti AH, Wiedemeyer R, *et al.* Coactivation of receptor tyrosine kinases affects the response of tumor cells to targeted therapies. *Science* 2007; **318**: 287–290.
21 Radu A, Neubauer V, Akagi T, Hanafusa H, Georgescu MM. PTEN induces cell cycle arrest by decreasing the level and nuclear localization of cyclin D1. *Mol Cell Biol* 2003; **23**:6139–6149.
22 Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979; **76**:4350–4354.
23 Reznik TE, Sang Y, Ma Y, Abounader R, Rosen EM, Xia S, *et al.* Transcription-dependent epidermal growth factor receptor activation by hepatocyte growth factor. *Mol Cancer Res* 2008; **6**:139–150.
24 Lal B, Xia S, Abounader R, Laterra J. Targeting the c-Met pathway potentiates glioblastoma responses to gamma-radiation. *Clin Cancer Res* 2005; **11**:4479–4486.

25 Tomayko MM, Reynolds CP. Determination of subcutaneous tumor size in athymic (nude) mice. *Cancer Chemother Pharmacol* 1989; **24**:148–154.
26 Abounader R, Lal B, Luddy C, Koe G, Davidson B, Rosen EM, *et al.* In vivo targeting of SF/HGF and c-Met expression via U1snRNA/ribozymes inhibits glioma growth and angiogenesis and promotes apoptosis. *Faseb J* 2002; **16**:108–110.
27 Chakravarti A, Zhai G, Suzuki Y, Sarkesh S, Black PM, Muzikansky A, *et al.* The prognostic significance of phosphatidylinositol 3-kinase pathway activation in human gliomas. *J Clin Oncol* 2004; **22**:1926–1933.
28 Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 2008; **455**:1061–1068.
29 Gallia GL, Tyler BM, Hann CL, Siu IM, Giranda VL, Vescovi AL, *et al.* Inhibition of Akt inhibits growth of glioblastoma and glioblastoma stem-like cells. *Mol Cancer Ther* 2009; **8**:386–393.
30 Mellinghoff IK, Cloughesy TF, Mischel PS. PTEN-mediated resistance to epidermal growth factor receptor kinase inhibitors. *Clin Cancer Res* 2007; **13**:378–381.
31 Lal B, Goodwin CR, Sang Y, Foss CA, Cornet K, Muzamil S, *et al.* EGFRvIII and c-Met pathway inhibitors synergize against PTEN-null/EGFRvIII+ glioblastoma xenografts. *Mol Cancer Ther* 2009; **8**:1751–1760.
32 Li Y, Guessous F, DiPierro C, Zhang Y, Mudrick T, Fuller L, *et al.* Interactions between PTEN and the c-Met pathway in glioblastoma and implications for therapy. *Mol Cancer Ther* 2009; **8**:376–385.
33 Binning MJ, Niazi T, Pedone CA, Lal B, Eberhart CG, Kim KJ, *et al.* Hepatocyte growth factor and sonic Hedgehog expression in cerebellar neural progenitor cells costimulate medulloblastoma initiation and growth. *Cancer Res* 2008; **68**:7838–7845.