The Epidermal Growth Factor Receptor Pathway Mediates Resistance to Sequential Administration of Radiation and Chemotherapy in Primary Human Glioblastoma Cells in a RAS-dependent Manner

Arnab Chakravarti, Abhijit Chakladar, Meaghan A. Delaney, Douglas E. Latham, and Jay S. Loeffler

ABSTRACT
Resistance to conventional adjuvant therapies (i.e., chemotherapy and radiation) has been well documented in malignant gliomas. Unlike many other tumor types, combined modality therapy involving radiation and chemotherapy has failed to appreciably enhance outcome for glioblastoma patients compared with radiation alone. In vitro, we have observed an actual antagonistic effect between sequential administration of radiation and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) chemotherapy in three primary human glioblastoma cell lines (referred to as the GBME3-5 cell lines), which also happen to demonstrate strong expression of the epidermal growth factor receptor (EGFR). Upon inhibition of EGFR with the EGFR tyrosine kinase inhibitor, AG1478, it was found that this cross-resistance between sequential administration of radiation and BCNU was abrogated. To dissect which of these pathways may be responsible for the observed antagonism, known EGFR-regulated downstream signaling pathways including RAS, phosphatidylinositol 3-kinase (PI3-K), mitogen-activated protein kinase (p44/p42), and protein kinase C were inactivated with both pharmacological inhibitors and transient transfection experiments with dominant-negative and constitutively active constructs in the presence of exogenous EGF stimulation. It was found that BCNU inhibited radiation-induced apoptosis through EGFR-mediated activation of PI3-K/AKT via RAS. On the other hand, radiation was found to inhibit BCNU-induced apoptosis through EGFR-mediated activation of both PI3-K and mitogen-activated protein kinase (p44/p42) pathways, also via RAS. Inhibition of either EGFR or RAS activity appears to not only abrogate the observed antagonism between sequentially administered radiation and chemotherapy but actually results in a greater enhancement of apoptosis in the setting of combined modality therapy than when administered with either radiation or chemotherapy as single agents. Therefore, these findings suggest that strategies to inactivate EGFR or RAS signaling may be critical to improving not only the efficacy of single-agent therapy but also of combined modality therapy in gliomas.

INTRODUCTION
Resistance to conventional adjuvant therapies, e.g., chemotherapy and radiation, has posed major challenges in the treatment of patients with malignant gliomas. Median survivals, despite aggressive surgery and radiation ± chemotherapy, remain in the 9–10-month range for patients with GBM1 (1, 2). Therapeutic strategies combining chemotherapy and radiation have failed to produce the additive or synergistic effects observed frequently in other tumor types. Indeed, clinical trials combining the two modalities in GBM patients have produced disappointing results thus far (1–5). The molecular basis of the observed lack of efficacy of combined modality therapy in GBMs has not been well understood. In vitro, we have observed an actual antagonistic effect between radiation and chemotherapy in three primary human glioblastoma cell lines (referred to here as the GBME3–5 cell lines), which happen to express the EGFR.

The EGFR pathway has been demonstrated to contribute to resistance to radiation and chemotherapy in many tumor types, including gliomas (6–20). The known downstream effectors of EGFR include: PI3-K, RAS-RAF-MAPK p44/p42, and protein kinase C signaling pathways, among others (21–36). It has been demonstrated that DNA damage (i.e., ionizing radiation) can activate these critical pathways through EGFR (37). Because activation of these pathways has been found to enhance cell survival, we investigated whether EGFR-mediated activation of these pathways in response to DNA damage may be responsible for the antagonistic effect observed upon combined administration of radiation and chemotherapy in a sequential manner in these cell lines.

Our findings suggest that both radiation and chemotherapy can activate EGFR signaling, leading to increased cross-resistance between sequential administration of the two treatment modalities. It appears that RAS-mediated activation of PI3-K/AKT and MAPK (p44/p42), via EGFR, may play a critical role in mediating this effect. Activation of PI3-K/AKT was found to be especially critical in mediating resistance to radiation-induced apoptosis with BCNU pretreatment, whereas activation of both PI3-K/AKT and MAPK (p44/p42) pathways appeared to contribute to resistance to BCNU-mediated apoptosis, via EGFR, in a RAS-dependent manner.

MATERIALS AND METHODS
Cell Culture/Administration of Pharmacological Agents. The GBME3–5 cell lines were established in culture from glioblastoma tumor specimens using techniques described in detail elsewhere (38). Briefly, the GBM tissues were obtained during open resection and mechanically dissociated, and the dispersed cells and fragments were cultured and passaged as described (38). Molecular profiling revealed that all cell lines had absence of p16 expression. Sequencing revealed the presence of full-length EGFR for the E3 cell line and mutant VIII variant in the E4 and E5 cell lines. All cell lines demonstrated activation of EGFR signaling as measured by phospho-EGFR levels (see Fig. 4A). Because the VIII mutants lack extracellular binding domains and therefore demonstrate constitutive activation of EGFR signaling that was not modulated by exogenous EGFR ligands (e.g., EGF and transforming growth factor-α), the GBME3 cell line was used for experiments where exogenous ligands (e.g., EGF) was used to incrementally stimulate signaling through EGFR to examine downstream pathways contributing to cross-resistance between radiation and chemotherapy. Radiation treatments were done using a 250 kVp photon beams and were delivered using a dose rate of 2 Gy/min. BCNU was purchased from Sigma. In experiments in which radiation preceded BCNU, cells were irradiated within 5 min of BCNU administration, and apoptosis was measured at various time points ranging from 15 min to 24 h after the time of initial BCNU administration to assess peak apoptotic indices. In experiments where BCNU preceded radiation, cells were incubated with BCNU for 1 h before radiation and irradiated, and the medium was promptly changed to one that was BCNU free. Peak apoptosis levels were likewise assessed at 15 min to 24 h after

Received 1/23/02; accepted 6/3/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by NIH/National Cancer Institute Grant K08CA82163 and the Massachusetts General Hospital Brian Silber Fund (both to A.C.)

2 To whom requests for reprints should be addressed, at Massachusetts General Hospital, Laboratory of Molecular Oncology, 133rd Street, Building 149, Room 7330, Charlestown, MA 02129. Phone: 617-726-2241, i.d. 30601; Fax: 617-726-5637; E-mail: achakravarti@partners.org

3 The abbreviations used are: EGF, epidermal growth factor receptor; PI3-K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; FTI, farnesyltransferase inhibitor; HA, hemagglutinin; RBD, Ras binding domain; RT, radiotherapy; RTK, receptor tyrosine kinase.
irradiation. Clonogenic survival assays were performed as described previously (39). Experiments described in Figs. 1, 2, 3, 4A, 5, 6A, and 6B, were performed in the presence of exogenous EGF (50 μM). Experiments shown in Figs. 4C, 6C, and 6D were performed using incremental concentrations of exogenous EGF (purchased from Sigma).

AG1478, a highly potent and selective EGFR tyrosine kinase inhibitor, is reported to have an IC50 > 100 μM versus Her-2 and platelet-derived growth factor receptor kinase (purchased from Calbiochem; Ref. 40). AG1478 doses of 20 μM were found to diminish EGFR tyrosine activity by >95% in the GBME3 cell line using phospho-EGFR levels as “read outs” and were, therefore, used. AG1478 was administered concomitantly with whichever therapy was sequentially first to order to suppress activation of the EGFR signaling. The medium was then changed to one that was free of AG1478 before administration of the subsequent therapy.

The PI3-K inhibitor, LY294002 (41), and the MAPK kinase 1 inhibitor, PD98059 (42), were purchased from Cell Signaling Technology. Doses of 50 μM of PD98059 and LY294002 were found to be the lowest doses that resulted in >95% reduction of phospho-MAPK and phospho-Akt levels under normal culture conditions for GBME3 cells and therefore were used to inactivate these pathways. Higher doses did not appear to have additional effect in this regard. Doses of 60 μM of protein kinase C inhibitor (Calbiochem; Ref. 43) were used with the same rationale. The FTI used in this study was FTI-276 (44), which is a highly potent and selective CAAX peptidomimetic of the COOH terminal of Ras proteins (Calbiochem). It was found that 10 μM inhibited Ras-GTP levels by >95%; hence, this concentration was used. Administration of LY294002, PD98059, protein kinase C inhibitor, and FTI-276 was identical to that of AG1478 described above.

Transfections. Transient transfections of AKT constructs were carried out using 4 μg of DNA and Lipofectamine (Life Technologies, Inc.) according to the protocol suggested by the manufacturer. The AKT constructs were kind gifts of A. Bellacosa (Fox Chase Cancer Center, Philadelphia, PA). These included DNA of the following cytomegalovirus promoter-based constructs: HA-tagged myristylated AKT (constitutively active) and HA-tagged c-AKT K179M (kinase-deficient or dominant-negative form) as described previously. The transfection efficiency of the GBME3 cells was verified by both immunofluorescence detection of HA expression (HA probe purchased from Santa Cruz Biotechnology; SC-805) and by an AKT Kinase Assay kit (Cell Signaling Technology).

Western Blot Analysis. Lysates were generated by placing these cells in RIPA lysis buffer. Bradford assays were performed to determine total protein concentrations, which were normalized to 1 μg/μl for all samples. Samples were then prepared in sample buffer and heated to 95°C for 5 min. Samples were run on 10–12% polyacrylamide gels. Fifteen μl of protein lysates in sample buffer from each tissue were loaded within each well. Positive controls were loaded on each gel and represented lysates from U87 cells for phospho- and total MAPK and AKT. Gels were run at constant current (30 mA) for 2 h for maximum separation. Semidry transfer was then performed. The membrane was then blocked for 1 h in 5% milk in 0.2% TBST (Tris buffer solution with 0.2% Triton X). The membranes were then washed in 0.2% TBST three times for 15 min each. The membranes were then incubated overnight with primary antibody directed against Total MAPK (Cell Signaling Technology), Total Akt (Cell Signaling Technology), Phospho-MAPK (Cell Signaling Technology), Phospho-Akt (Cell Signaling Technology), Phospho-EGFR (Cell Signaling Technology), Total Protein Kinase C (Santa Cruz), and Phospho-Protein Kinase C (Cell Signaling Technology). Subsequently, the membranes were washed in 0.2% TBST three times for 15 min each. Membranes were then incubated with secondary antibody for 45 min and subsequently washed. Chemiluminescent (Bio-Rad) detection was then used to detect expression of these proteins and phospho-proteins, the levels of which were quantitated using densitometry.

RAS-GTP Assay. RAS-GTP levels were assessed by affinity precipitation and immunoblotting using a GST fusion protein corresponding to total RBD (residues 1–149) of Raf-1, which were bound to glutathione agarose (Raf-1 RBD agarose; Upstate Biotechnology). The cells were lysed with magnesium-containing lysis buffer. The lysate was diluted to 1 μg/μl total cell protein with magnesium-containing lysis buffer. Five to 10 μg of Raf-1 RBD agarose was added to 500 μg-1 μg of cell lysate/assay. After incubation for 30 min, the agarose beads were collected, washed, and resuspended in sample buffer. Immunoblotting was then performed with a pan-RAS antibody (Santa Cruz Biotechnology), as described above.

Annexin V Apoptosis Assay. Annexin V was assayed by detection of membrane externalization of phosphatidylserine with Annexin V-FITC conjugate (Caltag). To assess peak apoptotic index, cells were harvested at various intervals after treatment and resuspended in PBS solution. Both adherent and floating cells were harvested for the apoptosis assay. The cells were then suspended in 1–2 ml of FITC-Annexin V solution. Propidium iodide was added to a final concentration of 1 μg/ml. This was analyzed by flow cytometry using blue light excitation, and green fluorescence of FITC was measured at 530 ± 20 and red fluorescence of was measured at >600.

RESULTS

Antagonism between Radiation and Chemotherapy in the GBME3 Cell Line. The GBME3–5 cell lines were irradiated from 2 to 10 Gy followed by BCNU chemotherapy in doses ranging from 10 to 50 μM in the presence of exogenous EGF (50 μM). Peak apoptosis was then assessed at various time points, ranging from 15 min to 24 h after initial chemotherapy administration to assess peak apoptotic index. Compared with cells treated by BCNU alone at virtually each BCNU dose level, apoptosis was significantly lower in the cells pretreated by radiation compared with BCNU alone (Figures 1A–C).

The GBME3–5 cell lines were then pretreated with chemotherapeutic agents in doses of 10–50 μM, followed by irradiation in doses ranging from 2 to 10 Gy in the presence of exogenous EGF (50 μM). Peak apoptosis was likewise assessed at time points from 15 min to 24 h after irradiation. At each radiation dose level >2 Gy, there also appeared to be a significant reduction of peak apoptosis values in the cells pretreated by chemotherapy compared with those treated by radiation alone (Figures 2). This observed reduction of peak apoptosis levels translated into enhanced clonogenic survival in the BCNU pretreatment group compared with the radiation-alone group (Figures 3). Therefore, it appears in these cells that there is an antagonistic effect between radiation and BCNU when administered sequentially in either order.

Role of EGFR in the Cross Resistance between Radiation and Chemotherapy. It was found that the GBME3–5 cell lines had evidence of signaling through EGFR as measured by phospho-EGFR levels (Fig. 4A). It was also found that administration of either BCNU or radiation led to up-regulation of signaling through EGFR, as measured by phospho-EGFR levels (Fig. 4B) for GBME3 cells. Furthermore, it was found that this up-regulation of signaling through EGFR could be diminished by the administration of the selective and potent EGFR tyrosine kinase inhibitor, AG1478 (Fig. 4B).

To investigate the possible role of EGFR in mediating this antagonistic effect between chemotherapy and radiation, AG1478 was used in combination with radiation (RT) alone and BCNU alone. Coadministration of AG1478 significantly enhanced both radiation and BCNU-mediated apoptosis, respectively, in these cell lines (Figs. 1 and 2, respectively) when these agents were administered individually.

The cells were then pretreated with AG1478 + RT, followed by BCNU to investigate the possible role of EGFR in mediating the observed antagonism between sequential administration of these agents. Peak apoptosis values were compared with those of pretreatment arms with BCNU alone, BCNU preceded by radiation, and combined BCNU and AG1478. Pretreatment with AG1478 appeared to abrogate the antagonistic effect observed when radiation preceded BCNU; and peak apoptosis values were significantly increased when compared with the treatment arms mentioned above (Fig. 1 A and B).

Likewise, pretreatment with AG1478 + BCNU followed by radiation led to significantly enhanced levels of apoptosis compared with RT alone, to RT preceded by BCNU, and to combined AG1478 + RT.
To further investigate the possible role of EGFR in mediating cross-resistance between radiation and chemotherapy using a different approach, the EGF-responsive GBME3 cells were cultured in serum-free medium with increasing amounts of exogenous EGF added to determine whether EGF stimulation could enhance the antagonistic effect between radiation and chemotherapy. These results confirm a significant reduction of peak apoptosis values with increasing levels of exogenous EGF, regardless of sequence of radiation or BCNU administration (Fig. 2C). The above experiments, using EGFR inhibition and exogenous EGF stimulation, suggest strongly that the EGFR pathway may play an important role in antagonism between sequential administration of radiation and chemotherapy in EGFR-expressing glioma cells.
Downstream Pathways Regulated by EGFR in the GBME3 Cell Line. The mechanism by which EGFR mediates resistance between radiation and chemotherapy in this cell line was further investigated. We proceeded to first investigate which downstream signaling pathways are regulated most strongly by EGFR and which are most strongly up-regulated after radiation and chemotherapy in an EGF-dependent manner. The EGF-responsive GBME3 cell line (which contains wild-type full-length EGFR) was used for these experiments. The GBME4–5 cell lines had the vIII EGFR mutant, which lacks the extracellular ligand binding domain. Hence, these cell lines had constitutive EGFR activation without EGF stimulation and were not used for the experiments using EGF stimulation. It becomes evident that RAS activity, as measured by RAS-GTP levels, and activities of MAPK (p44/p42), PI3-K, and protein kinase C, as measured by phospho-specific antibodies, are all strongly regulated by EGF in the GBME3 cell line (Fig. 5A).

It was then investigated whether radiation and chemotherapy could up-regulate signaling through these pathways in an EGF-dependent manner. GBME3 cells were cultured in serum-free medium with or without exogenous EGF added and followed by either chemotherapy or radiation. The activation states of these pathways were then determined using the methods described above. It appears that RAS, MAPK (p44/p42), and PI3-K/AKT activities become up-regulated after either radiation or chemotherapy in an EGF-dependent manner (Fig. 5, A and B). PKC activity appeared to be more modestly regulated by radiation and chemotherapy. Fig. 5, C–F, also suggest that RAS may play a critical role in up-regulation of both MAPK (p44/p42) and PI3-K pathways upon EGF stimulation in this cell line in the presence of both RT and BCNU.

Downstream EGFR Targets Involved in Cross-Resistance between Radiation and Chemotherapy in the GBME3 Cell Line. To determine which of these EGF-regulated pathways is involved in antagonizing the effects of sequential administration of radiation and chemotherapy, these pathways were inhibited using selective pharmacological inhibitors. BCNU-mediated apoptosis was most significantly enhanced in the RT+AG1478, RT+FTI, RT+LY294002+FTI, RADIATION AND BCNU ANTAGONISM VIA EGFR

Fig. 3. In GBME3–5 cell lines, decreased levels of apoptosis is associated with increased clonogenic survival in the BCNU-pretreated cells after irradiation. A–C demonstrate that reduced levels of apoptosis seen in Fig. 2 in the BCNU pretreatment groups correspond to increased levels of clonogenic survival. For each cell line, pretreatment with BCNU chemotherapy is associated with enhanced clonogenic survival compared with radiation alone (P < 0.0001). Antagonism of EGFR signaling not only appears to be more effective in the setting of combined chemotherapy and radiation compared with radiation alone (P < 0.0001). Bars, SD.

**Downstream Pathways Regulated by EGFR in the GBME3 Cell Line.** The mechanism by which EGFR mediates resistance between radiation and chemotherapy in this cell line was further investigated. We proceeded to first investigate which downstream signaling pathways are regulated most strongly by EGFR and which are most strongly up-regulated after radiation and chemotherapy in an EGF-dependent manner. The EGF-responsive GBME3 cell line (which contains wild-type full-length EGFR) was used for these experiments. The GBME4–5 cell lines had the vIII EGFR mutant, which lacks the extracellular ligand binding domain. Hence, these cell lines had constitutive EGFR activation without EGF stimulation and were not used for the experiments using EGF stimulation. It becomes evident that RAS activity, as measured by RAS-GTP levels, and activities of MAPK (p44/p42), PI3-K, and protein kinase C, as measured by phospho-specific antibodies, are all strongly regulated by EGF in the GBME3 cell line (Fig. 5A).

It was then investigated whether radiation and chemotherapy could up-regulate signaling through these pathways in an EGF-dependent manner. GBME3 cells were cultured in serum-free medium with or without exogenous EGF added and followed by either chemotherapy or radiation. The activation states of these pathways were then determined using the methods described above. It appears that RAS, MAPK (p44/p42), and PI3-K/AKT activities become up-regulated after either radiation or chemotherapy in an EGF-dependent manner (Fig. 5, A and B). PKC activity appeared to be more modestly regulated by radiation and chemotherapy. Fig. 5, C–F, also suggest that RAS may play a critical role in up-regulation of both MAPK (p44/p42) and PI3-K pathways upon EGF stimulation in this cell line in the presence of both RT and BCNU.

**Downstream EGFR Targets Involved in Cross-Resistance between Radiation and Chemotherapy in the GBME3 Cell Line.** To determine which of these EGF-regulated pathways is involved in antagonizing the effects of sequential administration of radiation and chemotherapy, these pathways were inhibited using selective pharmacological inhibitors. BCNU-mediated apoptosis was most significantly enhanced in the RT+AG1478, RT+FTI, RT+LY294002+FTI, RADIATION AND BCNU ANTAGONISM VIA EGFR

Fig. 3. In GBME3–5 cell lines, decreased levels of apoptosis is associated with increased clonogenic survival in the BCNU-pretreated cells after irradiation. A–C demonstrate that reduced levels of apoptosis seen in Fig. 2 in the BCNU pretreatment groups correspond to increased levels of clonogenic survival. For each cell line, pretreatment with BCNU chemotherapy is associated with enhanced clonogenic survival compared with radiation alone (P < 0.0001). Antagonism of EGFR signaling not only appears to be more effective in the setting of combined chemotherapy and radiation compared with radiation alone (P < 0.0001). Bars, SD.

**Downstream Pathways Regulated by EGFR in the GBME3 Cell Line.** The mechanism by which EGFR mediates resistance between radiation and chemotherapy in this cell line was further investigated. We proceeded to first investigate which downstream signaling pathways are regulated most strongly by EGFR and which are most strongly up-regulated after radiation and chemotherapy in an EGF-dependent manner. The EGF-responsive GBME3 cell line (which contains wild-type full-length EGFR) was used for these experiments. The GBME4–5 cell lines had the vIII EGFR mutant, which lacks the extracellular ligand binding domain. Hence, these cell lines had constitutive EGFR activation without EGF stimulation and were not used for the experiments using EGF stimulation. It becomes evident that RAS activity, as measured by RAS-GTP levels, and activities of MAPK (p44/p42), PI3-K, and protein kinase C, as measured by phospho-specific antibodies, are all strongly regulated by EGF in the GBME3 cell line (Fig. 5A).

It was then investigated whether radiation and chemotherapy could up-regulate signaling through these pathways in an EGF-dependent manner. GBME3 cells were cultured in serum-free medium with or without exogenous EGF added and followed by either chemotherapy or radiation. The activation states of these pathways were then determined using the methods described above. It appears that RAS, MAPK (p44/p42), and PI3-K/AKT activities become up-regulated after either radiation or chemotherapy in an EGF-dependent manner (Fig. 5, A and B). PKC activity appeared to be more modestly regulated by radiation and chemotherapy. Fig. 5, C–F, also suggest that RAS may play a critical role in up-regulation of both MAPK (p44/p42) and PI3-K pathways upon EGF stimulation in this cell line in the presence of both RT and BCNU.

**Downstream EGFR Targets Involved in Cross-Resistance between Radiation and Chemotherapy in the GBME3 Cell Line.** To determine which of these EGF-regulated pathways is involved in antagonizing the effects of sequential administration of radiation and chemotherapy, these pathways were inhibited using selective pharmacological inhibitors. BCNU-mediated apoptosis was most significantly enhanced in the RT+AG1478, RT+FTI, RT+LY294002+FTI, RADIATION AND BCNU ANTAGONISM VIA EGFR

Fig. 3. In GBME3–5 cell lines, decreased levels of apoptosis is associated with increased clonogenic survival in the BCNU-pretreated cells after irradiation. A–C demonstrate that reduced levels of apoptosis seen in Fig. 2 in the BCNU pretreatment groups correspond to increased levels of clonogenic survival. For each cell line, pretreatment with BCNU chemotherapy is associated with enhanced clonogenic survival compared with radiation alone (P < 0.0001). Antagonism of EGFR signaling not only appears to be more effective in the setting of combined chemotherapy and radiation compared with radiation alone (P < 0.0001). Bars, SD.

**Downstream Pathways Regulated by EGFR in the GBME3 Cell Line.** The mechanism by which EGFR mediates resistance between radiation and chemotherapy in this cell line was further investigated. We proceeded to first investigate which downstream signaling pathways are regulated most strongly by EGFR and which are most strongly up-regulated after radiation and chemotherapy in an EGF-dependent manner. The EGF-responsive GBME3 cell line (which contains wild-type full-length EGFR) was used for these experiments. The GBME4–5 cell lines had the vIII EGFR mutant, which lacks the extracellular ligand binding domain. Hence, these cell lines had constitutive EGFR activation without EGF stimulation and were not used for the experiments using EGF stimulation. It becomes evident that RAS activity, as measured by RAS-GTP levels, and activities of MAPK (p44/p42), PI3-K, and protein kinase C, as measured by phospho-specific antibodies, are all strongly regulated by EGF in the GBME3 cell line (Fig. 5A).

It was then investigated whether radiation and chemotherapy could up-regulate signaling through these pathways in an EGF-dependent manner. GBME3 cells were cultured in serum-free medium with or without exogenous EGF added and followed by either chemotherapy or radiation. The activation states of these pathways were then determined using the methods described above. It appears that RAS, MAPK (p44/p42), and PI3-K/AKT activities become up-regulated after either radiation or chemotherapy in an EGF-dependent manner (Fig. 5, A and B). PKC activity appeared to be more modestly regulated by radiation and chemotherapy. Fig. 5, C–F, also suggest that RAS may play a critical role in up-regulation of both MAPK (p44/p42) and PI3-K pathways upon EGF stimulation in this cell line in the presence of both RT and BCNU.

**Downstream EGFR Targets Involved in Cross-Resistance between Radiation and Chemotherapy in the GBME3 Cell Line.** To determine which of these EGF-regulated pathways is involved in antagonizing the effects of sequential administration of radiation and chemotherapy, these pathways were inhibited using selective pharmacological inhibitors. BCNU-mediated apoptosis was most significantly enhanced in the RT+AG1478, RT+FTI, RT+LY294002+FTI,
and RT+LY294002 pretreatment arms (Fig. 6A). The RT+PD98059 and the RT+LY294002 pretreatment arms were also found to enhance BCNU-mediated apoptosis to a similar degree as each other, albeit to a lesser degree than the other pretreatment arms mentioned above. These results suggest that resistance to BCNU-mediated apoptosis, resulting from RT pretreatment in the presence of EGF stimulation, may be mediated equivalently through PI3-K and MAPK signaling pathways. Furthermore, because EGFR was found to regulate both of these pathways in a RAS-dependent manner in this cell line (Fig. 5, C–F), it is not surprising that pretreatment of RT+FTI abrogated the negative sequential interaction of RT with BCNU.

When the treatment sequence was reversed and BCNU preceded radiation, it was apparent that radiation-induced apoptosis was most significantly increased in the AG1478+BCNU, FTI+BCNU, and LY294002+FTI+BCNU pretreatment arms in almost an equivalent manner (Fig. 6B). PD98059+BCNU did not appear to abrogate the antagonistic effect between radiation and chemotherapy, nor did the addition of PD98059 further enhance the proapoptotic effect of the LY294002+BCNU arm. These observations suggest that BCNU-mediated stimulation of PI3-K/AKT, via EGFR, appears to most significantly increase resistance to radiation-induced apoptosis. Inhibition of RAS appeared to have a similar effect. Interestingly, inhibition of MAPK (p44/p42) in the presence of EGF stimulation/BCNU not only failed to produce an additive effect between BCNU and radiation, it failed to abrogate the antagonistic effect observed when BCNU preceded radiation. Therefore, although in this cell line both PI3K/AKT and MAPK (p44/p42) activities were increased after BCNU administration in an EGF-dependent manner (Fig. 5B), the PI3K/AKT pathway appears to be the dominant EGFR-regulated pathway that mediates resistance to radiation-induced apoptosis.

Fig. 5. Radiation can activate PI3-K/AKT and MAPK (p44/p42) pathways via EGFR in a RAS-dependent manner in GBME3 cells. A demonstrates that under the same culture conditions, radiation enhances activation of RAS, PI3-K/AKT, MAPK (p44/p42), and EGFR signaling in an EGF (50 μM)-dependent manner. Likewise, B demonstrates that BCNU activates these same pathways via EGFR stimulation. C. GBME2 cells grown in serum-free medium and irradiated in the presence or absence of exogenous EGF. Administration of the MAPK kinase 1 inhibitor, PD98059, or FTI-276 was able to abrogate radiation-induced activation of phospho-MAPK in the presence of EGF. This suggests that radiation may lead to increased signaling through MAPK (p44/p42) via increased signaling through EGFR in a RAS-dependent manner. Likewise, D suggests that radiation may lead to increased signaling through PI3-K/AKT via increased signaling through EGFR in a RAS-dependent manner. E and F demonstrate likewise for BCNU administration.
Sequential Effects of Radiation and Chemotherapy in AKT-Myr and AKT-KD-transfected GBME3 Cells in the Presence of Pharmacological Inhibitors of RAS and MAPK (p44/p42). The GBME3 cells were transfected with the constitutively active (AKT-Myr) and kinase-deficient (AKT-KD) forms of AKT. The objectives of these experiments were to confirm the findings above using an alternative approach and also to confirm the relative importance of PI3-K/AKT as a RAS effector in mediating radiation resistance.

When BCNU preceded radiotherapy, transfection with AKT-KD was able to abrogate the antiapoptotic effect of exogenous EGF and resulted in an additive effect between BCNU and radiation (Fig. 6C). Because there was no detectable enhancement in apoptosis observed in the AKT-KD+FTI or AKT-KD+PD98059 arms compared with the AKT-KD arm, this seems to confirm the above findings that in the GBME3 cell line, EGFR signaling appears to mediate resistance to radiation, primarily through the PI3-K/AKT pathway. Cells transfected with AKT-Myr demonstrated significantly reduced levels of radiation-induced apoptosis compared with nontransfected cells. Furthermore, pretreatment with FTI+BCNU and PD98059+BCNU failed to significantly increase apoptosis compared with BCNU alone in AKT-Myr-transfected cells. This suggests that EGF-mediated stimulation of MAPK (p44/p42) appears to be a less potent inhibitor of radiation-induced apoptosis in this cell line.

When radiation preceded BCNU, transfection of AKT-KD abrogated the antiapoptotic effect of exogenous EGF to a lesser degree than described above for the reverse treatment sequence, where BCNU preceded RT. Pretreatment of AKT-KD cells with either RT+PD98059 or RT+FTI significantly enhanced BCNU-mediated apoptosis compared with pretreatment with RT alone (Fig. 6D). This confirms the findings above that indicate that radiation can enhance resistance to BCNU-mediated apoptosis through activation of both PI3-K/AKT and MAPK (p44/p42) signaling. As further evidence of this, transfection with AKT-Myr was not able to suppress BCNU-mediated apoptosis to the extent seen with its ability to suppress RT-induced apoptosis. Furthermore, pretreatment with RT+P98059 and RT+FTI enhanced BCNU-mediated to a greater degree than seen with pretreatment with RT alone for AKT-Myr-transfected cells. This again suggests that radiation pretreatment of GBME3 cells mediates resistance to
DISCUSSION

This study demonstrates several important findings: (a) EGFR signaling in primary GBM cells can result in an antagonistic effect between combined administration of radiation and chemotherapy, when these agents are applied sequentially; (b) inhibition of EGFR signaling not only abrogates the sequential antagonism between radiation and BCNU but results in an additive effect that exceeds that observed with EGFR antagonism combined with either radiation or chemotherapy alone. These findings therefore suggest that therapeutic strategies involving EGFR antagonism in gliomas may have optimal effects when used in the setting of combined modality, rather than in concert with single agent therapy alone; (c) in a more general light, these findings shed further insight into the molecular mechanisms of resistance to conventional treatments in gliomas. It was demonstrated here that either radiation or chemotherapy can enhance signaling through EGFR, leading to activation of downstream pathways involving RAS, PI3K/AKT, and MAPK (p44/p42), which, once activated, can mediate resistance to the other therapeutic modality. Fig. 7 illustrates some of the pathways up-regulated by radiation and chemotherapy administration via EGFR. BCNU-mediated activation of PI3K/AKT, most likely through EGFR-mediated up-regulation of RAS, appears to greatly enhance resistance to radiation-induced apoptosis. Radiation-mediated activation of both PI3K/AKT and MAPK (p44/p42) pathways, also likely through RAS, appears to significantly enhance resistance to chemotherapy-induced apoptosis.

There is growing preclinical and even clinical evidence of the potential efficacy of combining anti-EGFR therapies with single-agent radiation and/or chemotherapy (7–13, 15–17, 19, 20, 45). Indeed, the data suggest that response to conventional therapy may be significantly enhanced by this approach. This study demonstrates that at least in certain situations, anti-EGFR therapy may produce optimal effects when combined with both radiation and chemotherapy. Whether this is the case only for tumors such as glioblastomas, for which the lack of efficacy of combined radiation and chemotherapy has been well-documented, or whether this holds true for other tumors, where there is documented benefit from combined modality therapy, remains unclear.

There have been previous studies demonstrating the antagonistic effect of radiation and chemotherapy in established GBM cell lines (46). We found this to be the case for the majority of the tumor-derived GBM cell lines as well, which happen to have strong expression of EGFR. One of the significant findings of this study is that EGFR signaling may, indeed, have a role in mediating the observed antagonism between radiation and chemotherapy in at least a certain subset of glioblastomas. However, previous studies have shown that only ~40% of glioblastomas have overexpression or amplification of EGFR (47). Given that the vast majority of glioblastoma patients, many of whom have non-EGFR-expressing tumors, fail to respond to combined administration of radiation and chemotherapy, suggests that EGFR signaling may be just one of several molecular mechanisms leading to the observed lack of synergism, and even to antagonism, between radiation and chemotherapy.

This directed us to examine the downstream pathways regulated by EGFR to more mechanistically understand how EGFR may lead to this observed antagonism. Because many different pathways are known to converge onto these more central signaling pathways (i.e., RAS, PI3-K/ AKT, MAPK (p44/p42)), identifying which of these downstream EGFR-regulated pathways underlie its ability to mediate cross-resistance between radiation and chemotherapy may serve as a pivot point to help us understand this phenomenon on a more universal level (i.e., for both EGFR and non-EGFR-expressing cells).

Although several critical signaling pathways are known to be EGFR regulated, it appears that RAS-mediated activation of PI3K/ AKT and MAPK (p44/p42) may be particularly important in mediating resistance to radiation and chemotherapy, whether individually or sequentially administered. Previous studies have demonstrated that although oncogenic mutation of RAS is in gliomas, levels of activated RAS are frequently elevated (48–52). Furthermore, in established glioma cell lines, it was demonstrated that pharmacological antagonism of RAS could lead to enhanced chemotherapy effect (49, 50). Certainly, it could be extrapolated that any agent further up-regulating RAS activity (i.e., ionizing radiation) may exaggerate this anti-apoptotic effect, as was found in this study. Although the RAS/RAF/ MAPK cascade has been studied extensively, data are emerging that the ability of RAS to activate PI3-K/AKT signaling may, indeed, be a more potent suppressor of radiation-induced apoptosis in certain cases (53, 54). Indeed, the data presented here suggest that antagonism of RAS (i.e., with FTIs) may be another approach to abrogate antagonism between radiation and chemotherapy in glioblastomas and, similar to anti-EGFR therapies, may also be best administered in the setting of combined modality, rather than single-agent, therapy. This approach is also presently being investigated for non-EGFR-expressing glioblastoma cell lines.

Strategies to antagonize other more central EGFR-regulated signaling pathways [i.e., PI3-K/AKT and MAPK (p44/p42)] appear to be another attractive strategy to enhance therapeutic effect in gliomas. However, caution must be exercised when considering such strategies because these pathways, especially PI3-K/AKT, have been demonstrated to be essential survival pathways for normal astrocytes and neurons as well as for glioblastomas (53–57). In fact, when treating cultures of primary human astrocytes with LY294002, we have found enhanced levels of radiation and chemotherapy-induced apoptosis in these cells as well. Therefore, preferable strategies would be to target tumor-specific upstream activators or downstream mediators of these critical signaling pathways, which have the greatest potential to effect therapeutic gain.

Identifying and targeting tumor-specific downstream mediators of these central signaling pathways may, however, prove to be more difficult. For example, regulation of the apoptotic machinery by AKT has been found to be quite complex and involves regulation of both mitochondria-dependent mechanisms (through regulation of caspase 9, Bad, and IкB kinase) and mitochondria-independent mechanisms (through phosphorylation of forkhead receptor; Refs. 58–65). Targeting such common mediators of apoptosis is likely to affect normal as well as tumor cells. Targeting the upstream activators of PI3-K/AKT and MAPK (p44/p42) such as RTKs and RAS may prove to be more optimal in terms of inactivating these pathways in a tumor-specific manner. Because malignant gliomas are known to express a number of...
different RTKs in addition to EGFR such as platelet-derived growth factor, insulin-like growth factor I, Trk-A, among others, this treatment strategy would require a careful survey of RTK expression on a case-by-case basis (47).

In summary, there is growing clinical and preclinical data that EGFR signaling enhances resistance to conventional therapies in many tumor types. Because EGFR has been found to not only increase resistance to apoptosis but also increase angiogenic potential, DNA repair capacity, cellular invasion, and motility, its ability to mediate cross-resistance between radiation and chemotherapy, when sequentially administered, likely represents only one of several mechanisms by which EGFR enhances the malignant potential of gliomas. It is encouraging, however, that in this preclinical model that this cross-resistance can be abrogated using EGFR inhibition. Ultimately, clinical trials will be needed to determine the feasibility of this approach.

ACKNOWLEDGMENTS

We acknowledge Peter M. Black, Department of Neurosurgery, Brigham and Women’s Hospital, for kindly providing the primary glioblastoma cell line and Ms. Lisa E. Kenney for typing assistance. We also acknowledge A. Bellacosa for kindly providing the AKT-KD and AKT-Myr constructs used in this study.

REFERENCES


The Epidermal Growth Factor Receptor Pathway Mediates Resistance to Sequential Administration of Radiation and Chemotherapy in Primary Human Glioblastoma Cells in a RAS-dependent Manner

Arnab Chakravarti, Abhijit Chakladar, Meaghan A. Delaney, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/15/4307

Cited articles
This article cites 61 articles, 32 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/15/4307.full#ref-list-1

Citing articles
This article has been cited by 29 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/62/15/4307.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.