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

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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 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 EGFR 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 GBM (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 extracelluar 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...
irradiation. Clonogenic survival assays were performed as described previ-
ously (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 PI3K inhibitor, LY294002 (41), and the MAPK kinase 1 inhibitor, 
PDK90809 (42), were purchased from Cell Signaling Technology. Doses of 50 
μM of PDK90809 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.

The GBME3 cell line was verified by both immuno-

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 immu-

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/ml 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 
hour 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 RAS GDP 
(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 with 1 μg/ml total 
cell protein with magnesium-containing lysis buffer. Five to 10 μg of Raf-1 RBD, 
agarose was added to 500 μg–1 mg of cell lysate/assay. After incubation for 30 
min, the agarose beads were collected, washed, and resuspended in sample 
buffer. Immuno blotting was then performed with a pan-RAS antibody (Santa 
Cruz Biotechnology), as described above.

Annexin V Apoptosis Assay. Apoptosis was assayed by detection of 
membrane externalization of phosphatidylserine with Annexin V-FITC con-
jugate (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 cytom-
etry 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 
5 cell lines were then pretreated with chemotherapy 
with doses of 10–50 μM, followed by irradiation in doses ranging from 2 
into enhanced clonogenic survival in the BCNU pretreat-
ment arms with BCNU alone, BCNU preceded by radiation, and 
Administration of AG1478 significantly enhanced both 
sequences and antagonistic effect between radiation and BCNU when administered sequen-
the cells pretreated by chemotherapy compared with those treated by radiation 
alone (Figs. 1, 2, 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. Fur-
thermore, 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 admin-
istered 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 pretreat-
ment 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 radia-

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Fig. 3 also demonstrates that this effect translates into significantly reduced clonogenic survivals when AG1478 is coadministered with combined radiation and BCNU compared to when combined with either radiation or BCNU on an individual basis. Indeed, it appears that inhibition of EGFR signaling not only abrogated the antagonism between radiation and BCNU, but it also appeared to lead to at least an additive effect between the treatment modalities in the GBME3–5 cell lines.

To further investigate the possible role of EGFR in mediating cross-resistance between radiation and chemotherapy using a different approach, the EGFR-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.

Fig. 1. Antagonism between sequential administration of radiation and BCNU chemotherapy in the GBME3–5 primary human glioblastoma (GBM) cell lines. A–C show peak apoptosis values of GBME3–5 cells, respectively, when pretreated by radiation at different levels (e.g., 2 Gy versus 10 Gy), followed by BCNU chemotherapy at doses ranging from 0 to 50 μM in the presence of exogenous EGF (50 μM). It was found that in the radiation pretreatment arms, there was a significant reduction in peak apoptosis values compared with the BCNU alone arm without any pretreatment (gray-shaded cross; P < 0.0001). Upon treatment with the highly potent and selective EGFR inhibitor AG1478, there was a significant increase in peak apoptosis values in the RT (2 Gy) + AG1478 → BCNU arm compared with the RT (2 Gy) → BCNU arm, and in fact was also significantly higher than the BCNU and BCNU + AG1478 arms (P < 0.0001). This suggests that: EGFR has a role in mediating antagonism between sequential administration of radiation and chemotherapy in the GBME3 cell line; and enhancement of apoptosis upon EGFR inhibition is greater in the setting of combined modality therapy than when combined with radiation alone in the GBME3 cell line. Bars, 95% confidence interval.

Fig. 2. Antagonism between sequential administrations of BCNU and radiation in the GBME3–5 primary human glioblastoma (GBM) cell lines. A–C show peak apoptosis values of GBME3–5 cells when pretreated by BCNU at different dose levels (0–50 μM), followed by RT at doses ranging from 0 to 10 Gy in the presence of exogenous EGF (50 μM). Pretreatment with BCNU followed by RT results in significantly lower values than with no BCNU pretreatment (RT-alone arm). Administration of AG1478 appeared to not only abrogate this cross-resistance but in fact led to significantly higher apoptosis levels in the BCNU (25 μM) + AG1478 → RT arm than in the RT + AG1478 arm (P < 0.0001). Again suggesting that anti-EGFR strategies may have greater effects in the setting of combined modality therapy than single-agent therapy. 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/H11001 AG1478, RT/H11001 FTI, RT/H11001 LY294002+FTI, and RT/H11001 AG1478, RT/H11001 FTI, RT/H11001 LY294002+FTI,

![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.](image)

![Fig. 4. EGFR activation patterns in the GBME3–5 cell lines and increased EGF stimulation reduces apoptosis values in sequentially administered RT+BCNU in the GBME3 cell line. A demonstrates on a Western blot comparable degrees of EGFR signaling, as measured by phospho-EGFR levels, in GBME3–5 cell lines compared with A431 controls in the presence of EGF stimulation. Note that the GBME4 and E5 cell lines demonstrate signaling through EGFR, even when grown in serum-free medium without exogenous EGF, whereas the GBME3 cell line demonstrated strong signaling through EGFR only in the presence of exogenous EGF when grown in serum-free medium. B demonstrates that signaling through EGFR, as measured by phospho-EGFR levels, is increased upon administration of either BCNU or RT. When AG1478 is administered with either BCNU or RT, this appears to abrogate signaling through EGFR. C, peak apoptosis values of GBME3 cells grown in serum-free medium with increasing levels of exogenous stimulation, either pretreated by BCNU followed by RT or pretreated by RT followed by BCNU. In both arms, peak apoptosis values decreased significantly upon exogenous EGF (P < 0.0001). Bars, SD.](image)
and RT+LY294002+PD98059 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 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 did not only fail 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.
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 radiation, 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, EGF stimulation 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 to the extent seen with its ability to suppress RT-induced apoptosis. Furthermore, pretreatment with RT+PD98059 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...
BCNU through activation of both MAPK (p44/p42) and PI3K/AKT signaling. These findings also support the previous observation that RAS activation, via EGFR, appears to be an important mechanism by which radiation activates both MAPK (p44/p42) and PI3K/AKT pathways.

**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 in these primary, 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, PI3K/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 PI3K/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., PI3K/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 PI3K/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 IκB 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 PI3K/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 treat-
ment 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 sequen-
tially 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, clin-
ical trials will be needed to determine the feasibility of this approach.

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