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Modulation of signal transduction via EGFR and its effect on the radiosensitivity of glioblastoma cells in vitro

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Glioblastoma multiforme (GBM) frequently involves amplification and alteration of the EGFR gene, resulting in overexpression or autocrine stimulation. Signalling via EGFR can promote cell survival or radioresistance through the activation of downstream signaling including the RAF/ERK and PI3K/AKT pathways. EGFR inhibitors are commonly considered as candidates for modifying the response of tumour cells to anticancer agents such as radiotherapy

Based on previous in vitro studies into modulation of radiosensitivity in glioblastoma cell lines U343 and U251 by the EGFR inhibitor, erlotinib, the aim of the present study was to investigate the molecular mechanisms of modulation by erlotinib. In particular, the effect of erlotinib on colony formation, proliferation rate, EGFR receptor activation, and downstream activation of the RAF/ERK MAP kinase pathway, were studied. Furthermore, the effect of PI3K inhibitors and AKT1 knock-down on PI3K/AKT signaling and radiosensitivity was investigated. The following major results were obtained:

1. Erlotinib significantly reduced the rate at which colonies grew in size in the colony formation assay (U251). Similarly, erlotinib reduced the proliferation rate in a mass culture growth assay (U343, U251). However, erlotinib did not downregulate constitutively activated AKT which is normally considered to mediate protection against ionizing radiation. Furthermore, erlotinib or the EGFR inhibitor, AG1478 did not downregulate activation of downstream MAP kinase ERK1/2 but produced a biphasic response with increasing activation at higher concentrations of the inhibitor depending on cell type and culture conditions. These results strongly suggest that erlotinib did not enhance radiosensitivity by inhibiting the PI3K/AKT survival pathway in the glioblastoma cell lines tested but rather by an unspecific inhibitory effect on the proliferation rate of the cells.

2. Activation of EGFR was low despite the constitutively high expression of EGFR protein in U251 glioblastoma cells kept in full medium. EGFR inhibitor erlotinib efficiently inhibited EGF-induced phosphorylation of EGFR and ERK1/2 in starving U251 cells, whereas it did not inhibit ERK1/2 phosphorylation induced by serum in starving cells or in standard culture conditions. Analysis of receptor tyrosine kinase (RTK) activation using a phospho-RTK array showed that serum stimulation significantly upregulated phosphorylation of IGF-1R but not EGFR. Furthermore, the IGF-1R inhibitor, AG1024, inhibited serum-induced ERK1/2, implying that IGF-1R rather than EGFR plays the main role in ERK1/2 phosphorylation under standard culture conditions *in vitro*. Constitutive phosphorylation of AKT was demonstrated to be independent of EGFR, IGF-1R or PDGFR regulation in glioblastoma cells. These results have important implications for drug testing in vitro because they show that RTK signaling via the EGFR, thought to be important in vivo, may not be activated in vitro despite high levels of EGFR expression.
3. While erlotinib did not downregulate activation of PI3K/AKT signaling, the role of this pathway in mediating radioprotection was studied further using PI3K inhibitors and knock-down of AKT1. The relative resistance of U343 glioblastoma cells to Wortmannin compared with U87 and U251 was found to be associated with a 2.6 and 3.1-fold increase, respectively, in the Rictor/Raptor ratio of U343 cells. Inhibition of AKT phosphorylation by specific concentrations (50 nM) of PI3K inhibitors or AKT1 knock-down showed no radiosensitizing effect on glioblastoma cells. Thus the data of the present studies did not support the putative protective role of PI3K/AKT signaling in modulating radiosensitivity. By contrast, radiosensitivity of glioblastoma cells was enhanced by high concentrations (5-20 μM) of PI3K inhibitor Wortmannin which inhibited DNA-PK phosphorylation and γ-H2AX foci formation after irradiation. Thus the radiosensitizing effect of micromolar concentrations of Wortmannin could be explained by the effect on DNA double-strand break repair by nonhomologous end joining.