# Ionizing Radiation Affects Epidermal Growth Factor Receptor Signalling and Metalloproteinase Secretion in Glioma Cells

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**Abstract.** *Background: The effect of different doses of X(-)rays on apoptosis, proliferation, epidermal growth factor receptor (EGFR) and matrix metalloproteinase (MMP-2) expression was investigated in a human glioblastoma cell line. Materials and Methods: The cell line LN18 was irradiated at room temperature with doses ranging from 0.5 to 15 Gy using 6 MV X(-)rays. Apoptosis was assessed using the annexin V binding assay, proliferation by the methyl tetrazolium (MTT) assay and MMP-2 secretion with zymography. The levels of phosphorylated (pEGFR) were estimated using a commercially available ELISA kit. Results: Cell proliferation decreased in a dose-dependent manner, while apoptosis was increased after radiation. Doses below 2 Gy did not affect proliferation or apoptosis. MMP-2 levels were increased 48 h after radiation in a dose-dependent manner. In contrast, EGFR signaling was significantly activated 15 min after radiation in a dose-dependent manner. Conclusion: Ionizing radiation activates EGFR signalling and enhances MMP-2 secretion, suggesting that the molecular pathways involved may contribute to the invasiveness and malignant behaviour of glioma cells and help to explain the response of gliomas to ionizing radiation.*

Malignant glioma (GBM) is the most common primary malignant tumor of the central nervous system (CNS) and is a challenging disease to treat since despite multimodal therapy protocols, the outcome of GBM remains dismal (1). The current standard treatment for managing GBM includes maximally safe surgical resection, followed by fractionated radiation therapy in combination with chemotherapy (temozolomide) (2). Since total surgical excision is almost impossible to achieve because of tumor invasiveness into the surrounding brain, radiotherapy remains an important treatment modality (3, 4).

However, one of the major problems associated with treatment failure is the radioresistance of gliomas (5). The response of tumor cells to radiation is complicated, with reports suggesting that glioblastoma cells may express sensitivity or resistance to both high and low doses (6, 7). This discrepancy may reflect differential triggering or induction of repair mechanisms (8, 9). The introduction of highly conformal techniques in radiotherapy redefines the role of low doses of radiation in the therapy of gliomas (10).

The resistance of glioma cells has been considered to reflect an intrinsic biological property of these cells regarding their ability to invade (3). Invasion of the tumor cells involves degradation of the surrounding extracellular matrix (ECM) of the brain, including activity of matrix metalloproteinases (MMPs). Levels of MMPs are elevated types in many tumor and are believed to play an important role in cellular invasion and metastasis, thus contributing to tumor aggressiveness (11, 12). In particular, the increased expression and activity of MMP-2 and MMP-9 has been proposed as being correlated with an increased grade of glioma malignancy (13-18). Furthermore, glioma invasiveness might also be related to genetic events. Mutation of tumor suppressor genes phosphatase and tensin homolog (*PTEN*) and protein 53 (*p53*) as well as the overexpression of oncogenes, such as epidermal growth factor receptor (*EGFR*) in up to 50% of gliomas cells (19, 20) seems to in turn activate invasiveness-related signal transduction pathways, such as Ras (RAT sarcoma)/mitogen activated protein kinase (MAPK), focal adhesion kinase (FAK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) (21, 22). The latter pathway is regulated by PTEN, which has been shown to inhibit cell migration, spreading, and focal adhesion (13-25).

Using an *in vivo* choriallantoic membrane model of angiogenesis and C6 rat glioma cells, our group has previously demonstrated that X-rays have an initial inhibitory

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effect on angiogenesis through ECM rearrangement. Subsequently the cells induced angiogenesis when they were inoculated into radiated tissue. This effect could be in part attributed to the increased release of MMP-2 from cells, as well as to the radiation-induced increase in the levels of integrin subunit $a_v$ (26, 27).

The aim of the current study was to investigate the effect of different doses of radiation on proliferation, apoptosis, EGFR activation and MMP-2 secretion in glioblastoma cells.

## Materials and Methods

*Cell culture and radiation*. The LN18 glioblastoma cell line was purchased from the American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 2 mM L-glutamine and supplemented with 5% fetal bovine serum (FBS), 2.5 μg/ml amphoterecin B, 100 U/ml penicillin-streptomycin and 50 μg/ml gentamycin (all Biochrom, Berlin, Germany) at 37˚C, 5% $CO_2$ and 100% humidity. The cells were exposed to different doses of radiation ranging from 0.5 to 15 Gy comitted through a 6 MV linear accelerator source (SL75 Philips, 6 MV; Medical Systems, Crawley, West Sussex, UK).

*Cell proliferation assay*. To determine whether radiation affects the proliferation of LN18 cells, the 3-[4,5-dimethylthiazol-2-yl]-2,5-dimethyltetrazolium bromide (MTT; Applichem, Darmstadt, Germany) a may was used, as previously described (28). The proliferation assay was performed in medium with 5% FBS, 48 h after radiation.

*Apoptosis assay*. Apoptosis was detected using the annexin V/propidium iodide detection assay as previously described (28). The cells were exposed to different doses of radiation and 24 h later cells were analyzed by flow cytometry (EPICS-XL; Coulter, Miami, FL, USA) using XL-2 software, according to the manufacturer's instructions (rh Annexin V/FITC kit; Bender MedSystems GmbH, Vienna, Austria). The experiments were performed in medium with 5% FBS.

*EGFR phosphorylation*. The levels of phosphorylated EGFR (pEGFR) were determined using an ELISA kit system (Bender MedSystems GmbH), according to the manufacturer's instructions. Briefly the cells were seeded in 100 mm petri-dishes at a density of $10^6$ cells per dish in medium with 5% FBS. The medium was replaced by serum-free medium after cell attachment. Twenty-four hours later the cells were exposed to radiation as described above. The supernatant was removed 15 min later and the cells were collected with a scraper. Lysis of the cells was followed by adding receptor binding buffer.

The samples were transferred onto a 96-microwell plate coated with a monoclonal antibody to human active EGFR and incubated for 1 h at 37˚C. The microwells were emptied and washed three times with washing buffer. An anti-phosphotyrosine monoclonal antibody (horseradish peroxidase conjugated) was added and the samples were incubated for 1 h at 37˚C. The microwells were washed four times and a suitable substrate solution was added to each sample for 15 min. The reaction was stopped by adding a stop solution and the samples were immediately measured on a microplate reader (Magellan 2; Sunrise, Tecan, Switzerland) at a wavelength of 450 nm. The results were normalized by measuring the quantity of total proteins using a Bradford assay (Sigma, Steinheim, Germany).

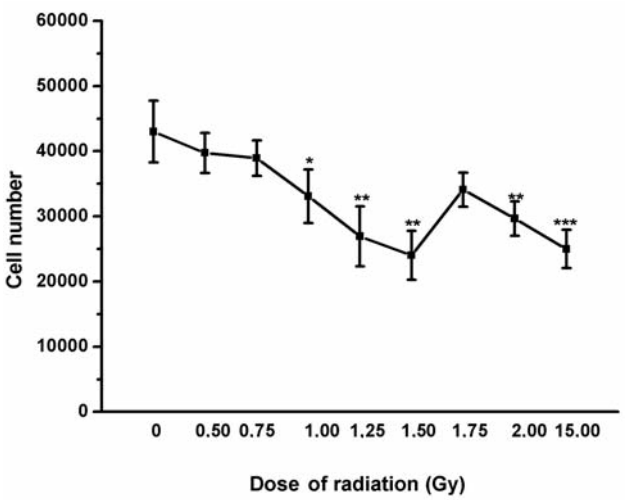


Figure 1. *LN18 cell proliferation 48 h after radiation. Mean±SEM of the number of cells, for three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 (unpaired t-test) compared to untreated cells.*

*Zymography assay*. The release of MMP-2 into the culture medium of the LN18 cells was measured by zymography as previously described (24). In brief, the LN18 cells were plated in 24-well plates at a density of $3\times10^4$ cells per well in medium with 5% FBS. The cells were treated as described above for the proliferation studies. After 48 h, 50 μl of the medium from each group were diluted with 2× Laemmli sample buffer without β-mercaptoethanol. The samples were analysed in 10% SDS-PAGE gels containing 0.1% gelatin. Following electrophoresis, the gels were washed four times in 2.5% Triton-X 100 for 15 min at room temperature and incubated for 24 h at 37˚C in developing buffer (50 mM Tris-HCl pH 7.6, 200 mM NaCl and 10 mM $CaCl_2$). The gels were stained with Coomassie Brilliant Blue R (Applichem) for 4 h at room temperature, de-stained in methanol acetic acid-water (4.5:1:4.5 v/v) for 2 h and photographed using a digital camera.

*Statistical analysis*. Descriptive statistics were computed for all the variables. Differences between groups and controls were tested by unpaired *t*-test. Each experiment included at least triplicate measurements for each condition tested unless otherwise indicated. All the results are expressed as mean±the standard error of the mean (SEM) from at least three independent experiments.

## Results

*Cell proliferation*. Following cell exposure to radiation doses ranging from 0.5 to 15 Gy, a statistically significant and dose-dependent reduction in the cell number was observed. More specifically, the *p*-values were 0.049, 0.006, 0.002, 0.006 and <0.0001 for the radiation doses of 1, 1.25, 1.5, 2 and 15 Gy

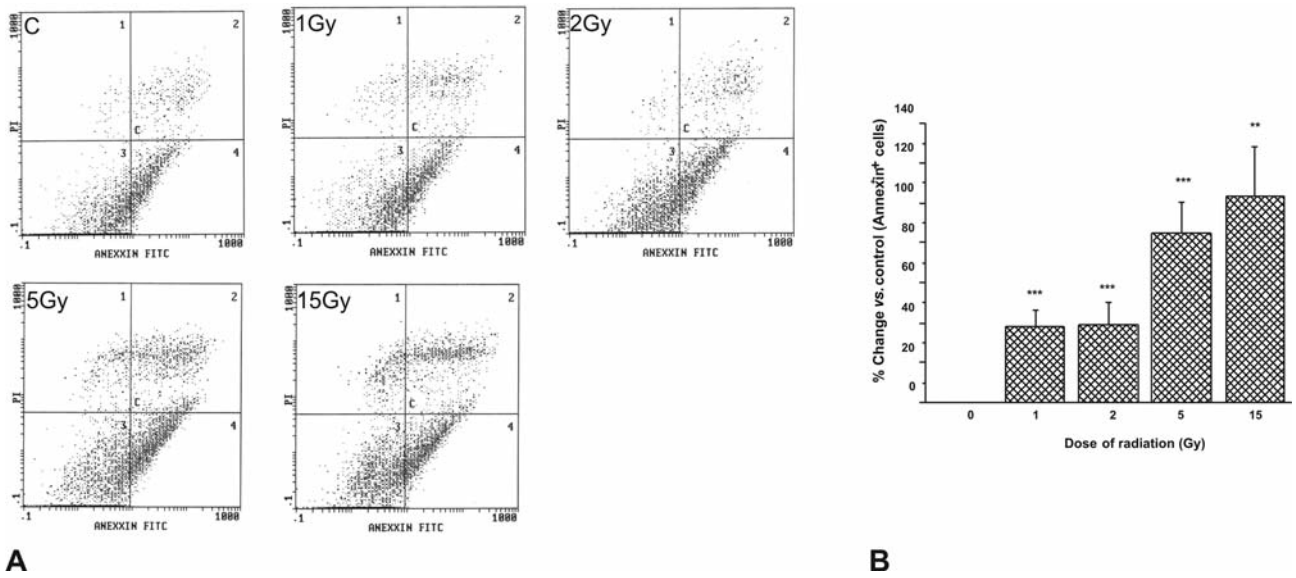


Figure 2. *Effect of radiation on LN18 cell apoptosis. A: Cells were irradiated and 24 h later stained with annexin/propidium iodide and analyzed by flow cytometry. C, Untreated LN18 cells; 1, 2, 5 and 15 Gy: LN18 cells radiated with 1, 2, 5 and 15 Gy. B: Percentage change compared to the control of annexin+ cells ±SEM, for three independent experiments. **p<0.01 and ***p<0.001 (unpaired t-test) compared to untreated cells.*

respectively, compared to the unirradiated cells. A reduction in cell number was also observed even at the radiation dose of 0.5 Gy, although it was not statistically significant (Figure 1).

*Apoptosis assay*. Apoptosis was induced at doses ranging from 1 to 15 Gy in a manner proportional to the arrest of proliferation (Figure 2). The *p*-values were <0.001 for the doses of 1, 2, 5 and 0.002 for 15 Gy, respectively.

*EGFR phosphorylation*. In contrast to the results obtained for cell proliferation and apoptosis, the EGFR proliferation pathway was activated at 2 and 15 Gy (*p*-values of 0.007 and 0.04, respectively) (Figure 3).

*Zymography assay*. A dose-dependent increase of MMP-2 secretion in the supernatant medium was detected 48 h after cell irradiation (Figure 4). The *p*-values were compared to unirradiated cells <0.01 at the 1 Gy dose, 0.01 at 2 Gy and <0.001 at 5 Gy and 15 Gy. This increase was in line with the activation of the EGFR pathway.

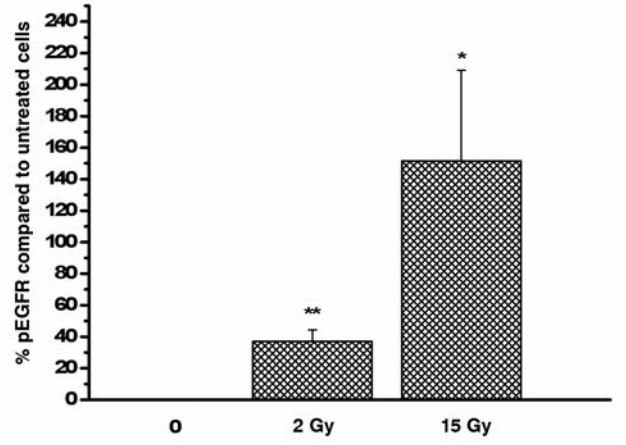


Figure 3. *Effect of radiation on pEGFR levels. Cells were collected 15 min after irradiation with 2 or 15 Gy. Percentage change compared to control pEGFR/mg of total protein±SEM for three independent experiments, *p<0.05 and ** p<0.01.*

## Discussion

Radiation-induced cell and tissue effects following relatively high dose exposure have been previously demonstrated in several experimental models. However, over the last two decades, research has been focused on the investigation of the effect of low doses irradiation, mainly because of the development and application of refined and sensitive analytical procedures (30). According to the definition by Beauchesne *et al*. and Short *et al*., doses below 1 Gy are regarded as 'low', whereas doses over 5 Gy are 'high' (8, 31). The study of low-dose irradiation for the treatment of cancer seems very attractive, since the biological and health effects of low doses are thus far conflictingly addressed. Some data support the hypothesis that low doses activate defense mechanisms,

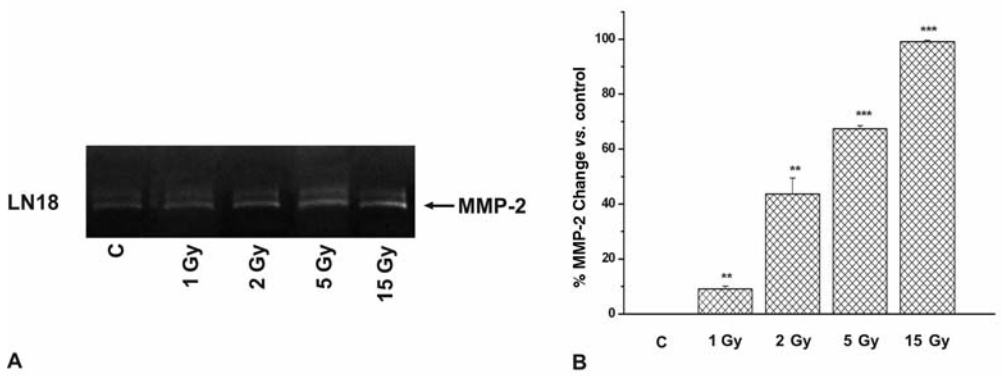


Figure 4. *Effect of radiation on MMP-2 secretion from LN18 cells. A: Zymography assay 48 h after irradiation. Representative gel of three independent experiments. B: Densitometric quantification of the corresponding MMP-2 bands. Mean±SEM of the % change compared to the control. Error bars: SEM for three independent experiments, **p<0.01 and ***p<0.001.*

including DNA repair mechanisms and elimination of potentially mutant cells by apoptosis, senescence, or lack of repair (32), through intracellular and intercellular signaling and trigger adaptive responses. Short *et al*. have shown that glioblastoma cells lines commonly show an increase in radiosensitivity to low acute doses, suggesting that in a clinical setting very low doses per fraction could produce increased tumor cell kill (31).

To assess this controversial and debatable effect of low doses of radiation and to possibly provide new information on the topic, we analyzed the impact of different doses of X rays on apoptosis, proliferation and EGFR expression in a single glioblastoma cell line. The use of a single cell line represents a notable methodological limitation of the current study. However, the LN18 cell line has been extensively used in previous radiation research regarding activation of EGFR and MMP expression (19, 33).

The main finding was that low doses of ionizing radiation activated EGFR signalling and MMP secretion in the glioma cells. Recent studies regarding the role of MMP in tumor progression revealed a more complicated and important role of MMP besides ECM degradation. MMP proteolysis regulates cell signaling that controls homeostasis of the extracellular microenvironment (34). While research on the therapeutic manipulations of MMP-2 and MMP-9 appears to have a solid rationale (15, 17, 18), the use of MMP inhibitors in clinical trials has failed so far (35). Many factors may have contributed to this failure. These inhibitors were tested in patients with advanced disease despite the fact that the experiments in animals showed benefits at the early stage of the disease (36). The tested MMP inhibitors were broad-spectrum drugs without being specific for a single MMP. Furthermore, some patients developed severe side-effects that forced dose reduction below the minimal inhibitory concentration (37-39). Although the clinical trials failed, third-generation MMP inhibitors are being developed (36). In addition to tumor radioresistance, the efficacy of radiation in treating gliomas has lately been questioned even more due to ionizing radiation enhancement of glioma cell invasiveness through the induction of MMP-2 expression in *PTEN* mutated glioma cell lines (19). In line with this view, the current results demonstrated potential enhancement of LN18 glioma cell invasiveness through increased MMP-2 levels, with doses of radiation as low as 1 Gy.

EGFR has been found in various studies to be activated by the irradiation of various cancer cells, including gliomas (40, 41), thus contributing to radioresistance, while in a recent study, high EGFR expression predicted benefit from radiotherapy (42). In agreement with the previous studies, the present data clearly demonstrated the enhancement of the aggressive behavior of LN18 by ionizing radiation even at extremely low doses of radiation.

Since both MMP-2 and EGFR expression in glioma cells were enhanced after radiation, an increase in cell proliferation would also be expected. In contrast, in the current study, a quite significant arrest of proliferation was observed even when small doses of radiation were applied. Furthermore, a significant induction of apoptosis was observed throughout the whole range of radiation doses used and in a manner proportional to the arrest of proliferation. These data were in line with previous results from our group in the *in vivo* choriallantoic membrane model of angiogenesis, where radiation induced tumor angiogenesis although it had also caused extensive damage to vessels (26). It seems that a cell population in the glioma culture is resistant to radiation, or alternatively, this population might be able to repair radiation-induced damage and become more aggressive.

In summary, doses of ionizing radiation ranging from 0.5 to 15 Gy can reduce cell proliferation in the short term, even for highly radioresistant tumors, such as gliomas. These

findings may have important implications in experimental and clinical situations and warrant further evaluation firstly in animal models and subsequently in the clinical setting in patients suffering from these highly malignant tumors.

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