The Radiation Induced Migration of Human Malignant Glioma Cells can be Blocked by Inhibition of the EGFR Downstream Pathways

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Introduction

Astrocytoma is the most frequent tumor in the central nervous system. Malignant glioblastomas are the end point of astrocytoma tumor progression. This tumor entity has a high morbidity and lethality with an overall survival of less than one year after diagnosis [1]. Key biological features of glioblastoma are excessive proliferation, disseminated tumor growth, resistance towards apoptotic stimuli, neovascularization, and suppression of antitumor immune surveillance. After surgical therapy, involved-field radiotherapy is the most important therapeutic option. This therapy prolongs the average survival for 6-8 month [2]. Recently, concomitant and adjuvant chemoradiotherapy with temozolomide has become the standard treatment for newly diagnosed glioblastoma [3]. Targeted therapy itself is confounded by the heterogeneous expression of drug targets, however several active agents have been identified, bevacizumab, cilengitide and cediranib. All of these agents have undergone multiple clinical trials and have demonstrated benefits for patients [4]. Radiosensitization is a new therapeutic strategy in the treatment of malignant gliomas. Still, it has been shown that sublethal irradiation of glioma cells promotes migration and invasiveness [5].

The aim of the current study was to investigate the signal pathways of irradiation induced migration of glioma cells. Therefore, we investigated the role of the EGFR and the downstream PI3K and MAPK signaling pathways and their impact on migration and proliferation.

Material and Methods

Cell culture and irradiation

The cell lines LN18, LN229 and LNZ308, described previously were used [11]. Cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen, Karlsruhe, Germany) with 10% fetal calf serum, 2 mM glutamine, and 100 µg/ml penicillin/streptomycin and maintained at 37°C in an atmosphere of 5% CO₂ to 70-90% confluence and irradiated in a linear particle accelerator (MX2, Siemens; Erlangen, Germany) at 2Gy, 5Gy and 8Gy. Therefore, the cell culture plates were placed between two perspex-glasses. After irradiation cells were cultured for another 24 hours.

Abstract

Background: It is well documented that low dose ionizing radiation induces migration of glioma cells, but the mechanisms are still poorly understood. The aim of the current study was to elucidate the intracellular signal transduction pathways of radiation induced migration in human glioma cells.

Methods: Migration was assessed via a wound healing assay. In addition, tumor cell proliferation was evaluated with a MTT colorimetric assay using 3 glioma cell lines (LN18, LN229, LNZ308). The cells were treated with increasing doses of irradiation (2Gy, 5Gy, 8Gy) in the presence or absence of EGF or inhibitors of the EGFR or downstream pathways (AG1478, LY294002, PD98059). Biochemical activation of EGFR, Akt/PKB and MAPK/ERK was examined by Western blot analysis.

Results: Irradiation induced a dose dependant intense increase of migrating cells and a decrease of proliferation. The inhibition of PI3K by LY294002 (50 µmol/L) reduced the radiation-induced migration (LN18: p<0.001, LN229: p=0.16, LNZ308: p=0.13), the blockade of MEK1 by PD98059 (50 µmol/L) was also effective (LN18: p=0.036, LN229: p=0.021, LNZ308: p=0.021). After irradiation, no effect on EGFR or the downstream pathways was observed in Western blot analysis.

Conclusion: Our results demonstrate that the downstream pathways of EGFR are involved in radiation induced migration of glioma cells.

Keywords: EGFR; Radiation; Migration; Glioma

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Wound healing assay

Investigation of cell migration capability after irradiation treatment was done by a modified wound healing assay: Briefly, treated and untreated cells were grown to confluent monolayers. Immediately before irradiation the inhibitors LY294002 (50 μM) (Calbiochem, Darmstadt, Germany), PD98059 (50 μM) (Biomol, Hamburg, Germany), tyrphostin AG1478 (10 μM) (Merck, Darmstadt, Germany) and the stimulator epidermal growth factor (EGF) (10 ng/ml) (Upstate, Billerica, USA) were added to the medium. Next, the monolayers were wounded by scratching the surface as uniformly as possible with a pipette tip and irradiated. This initial wounding (0 hr) and the movement of the cells in the scratched area were photographically monitored. 12 hrs after irradiation all cells and migrating cells were counted. The following formula shows the calculation of the migration index: MI = (M - (Mx((T1-T2)/T1)x100 (MI: migration index, T1: cell number at 0 hr, T2: cell number at 12 hr, M: migrating cells). The migration index had to be corrected by considering the proliferation rate of cells, because proliferating cells push into the scratched area mimicking an artificial migration. Therefore we calculated the proliferation index and substracted this index from the migration index.

Proliferation assessment

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to assess cell proliferation (Roche Diagnostics GmbH, Penzberg) as previously described [12]. Therefore the cells were plated into 96-well plates at a concentration of 1000 cells/well. The above mentioned inhibitors were added 12 hours prior to irradiation. Cells were incubated 12, 24 and 72 hrs after radiation. Then 10 μl of the MTT solution was added to each well, and the cells were incubated at 37°C for about 4 hrs. Subsequently, 100 μl of dimethyl sulfoxide was added to each well, yielding purple solution. The optical density was measured at 590 nm using an ELISA reader (ASYS Hitech GmbH, Eugendorf).

Immunoblot analysis

24 hrs after irradiation cells were harvested in lysis buffer (Cell Lysis Buffer, New England Biolabs, Ipswich, USA) at 4°C. Lysate was centrifuged (10000 rpm) for 15 min at 4°C to remove insoluble components. Protein concentration was quantified by the Bio-Rad DC protein assay. Equal amounts of protein were separated on SDS-PAGE 10% gels. Proteins were transferred to Immobilon-P PVDF membrane (Millipore, Billerica, USA). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST). The membrane was then incubated with primary antibody in 5% nonfat dry milk in TBST, followed by secondary antibody linked to rabbitradish peroxidase diluted in 5% nonfat dry milk in TBST. ECL Detection System for Western blot Analysis (Amersham, Freiburg, Germany) was followed according to the manufacturer’s instructions for antibody detection. The Imager SRX-101° (Konica Minolta, Langenhagen, Germany) was used to detect bands of appropriate sizes.

The following antibodies were used: phospho-EGFR, EGFR, phospho-PKB/Akt (Ser473), PKB/Akt (Ser473), phospho-p44/42 MAPK (Thr202/Tyr204), and p44/42 MAPK (Thr202/Tyr204) from Cell Signaling Technology, Boston, USA at a dilution of 1:1000.

Statistical analysis

The effect of the radiation dose on migration/proliferation in control cells was assessed in a linear regression model with the migration/proliferation as dependent variable and the radiation dose as a metric predictor. The potential inhibition or enhancement of radiation-based migration/proliferation was investigated based on a generalized least squares model fitted with the R function ‘glm’ with the migration/proliferation as dependent variable. The predictors were: the radiation dose (metric predictor, coefficient β_m), the stimulus (categorical predictor with coefficients β_Sph, β_LY, etc. and controls as reference category), and their interactions (coefficients β_Sph×LY, β_LY×LY, etc.). A compound symmetry correlation structure was assumed within each experiment. The coefficients were tested based on the Wald test. Separate analyses were conducted for the three cell lines LN18, LN229 and LNZ308. All statistical analyses were performed using the R statistical software (www.r-project.org), version 2.8.1.

Results

Radiation induces migration and decreases proliferation

First we tested the migration and proliferation behavior with and without radiation in 3 cell lines (LN18, LN229, LNZ308) by the acute wound healing assay. The cells were irradiated with increasing doses of 2Gy, 5Gy and 8Gy and monitored for 72 hrs. We found a dose-dependent increase of migrating cells in all 3 cell lines after irradiation (Figure 1) (tested hypothesis β_rad=0; LN18: p=0.001, LN229: p=0.028, LNZ308: p=0.083). In contrast, proliferation was dose-dependently decreased by radiation, using the proliferation index by the wound healing assay (tested hypothesis β_rad=0; LN18: p=0.008, LN229: p=0.42, LNZ308: p=0.59).

EGF receptor is not involved in radiation induced migration

Stimulation of the EGF receptor by EGF showed no apparent increase of migrating cells after EGF application. Accordingly, inhibition of the EGF receptor by adding the EGF-inhibitor AG1478 showed no modification in cell migration.

Figure 1: The numbers of migrating cells after irradiation with 0, 2, 5 and 8Gy were determined by the wound healing assay for the glioma cell lines LN18, LN229 and LNZ308. Cells were grown to confluent monolayers, wounded by scratching the surface as uniformly as possible with a pipette tip and afterwards irradiated. The number of migrated tumor cells in the wound was determined. The means of 4 tests per time point and dose were calculated after 12 hrs.
EGFR downstream pathways mediate radiation induced migration

The inhibition of PI3-kinase with LY294002 reduced the radiation-induced migration (tested hypothesis $\beta_{\text{rad}}=0$; LN18: $p=0.001$, LN229: $p=0.16$, LN2308: $p=0.13$). Furthermore we found a significant decrease of radiation-induced migration with isochronic inhibition of MEK1 by PD98059 (tested hypothesis $\beta_{\text{radPD}}=0$; LN18: $p=0.036$, LN229: $p=0.021$, LN2308: $p=0.021$) (Figure 2).

**Inhibition and simultaneously radiation reduce proliferation marginally**

By a MTT test we found a diminution of proliferation by inhibition of the pathways. In this context LY294002 offered main consequence at the proliferation. PD98059 was less effective. After EGF stimulation and simultaneously radiation we could show an increase of proliferating cells (Figure 3). These results were confirmed with the proliferation issues of the wound healing assay.

**EGF receptor is not phosphorylated by radiation**

Summarizing the results, it was shown that radiation did not activate the EGF receptor. No difference between irradiated and untreated cells was seen on protein level by western blot analysis for the markers phospho-PKB/Akt and phospho-MAP-kinase. In detail, after inhibition with LY294002 a decrease of phospho-PKB/Akt was observed. The inhibition of MEK1 with PD98059 caused a decrease of phospho-MAP-kinase in all three cell lines.

**Discussion**

The EGF receptor, that is overexpressed in many tumor entities, is involved in bad prognosis and shortened overall survival [13,14]. Some studies also showed that EGFR overexpression is correlated with a worse response to radiation and chemotherapy [15,16]. As it was also shown that the EGF receptor was activated through autophosphorylation after radiation [17]. We focused on this receptor and investigated its possible contribution to radiation induced migration.

We could not find significant inhibition of migration cells after treatment with EGFR inhibitor AG1478. Also we saw no activation of the EGFR in western blot assays after stimulation with EGF or radiation. Therefore, we assume that the EGF receptor seems to be irrelevant for the radiation induced migration.

It is known that the autophosphorylation of the EGFR receptor produces an activation of the downstream signal pathways [16-19] and it was reported that the PI3K pathway seems to be responsible for migration [20,21]. So we focused on the downstream pathways of EGFR, the PI3K and the MAPK pathway.

Analyses of the pathways downstream EGFR gave the following results: inhibition of the PI3K by LY294002 showed a decrease of migrating cells. The inhibition of MEK1 by PD98059 showed manifestly a decrease of migration. When blocking the downstream pathways of EGFR the biggest impact on inhibition of proliferation was found by using the PI3K inhibitor LY294002. However, there was no significance. The MEK1 inhibitor PD98059 was less effective.

Further studies identified PI3K as regulator of the cellular answer to ionizing rays. Thereby, the inhibitor of the PI3K, LY294002 boost the antineoplastic effect of the simultaneous radiation [22-24], which was also found in our study in proliferation assays. In addition, we observed the inhibitory effect of PD98059 at migration under radiation doses. Li et al. found that the activation of MAPK by radiation was not only caused by phosphorylation of the EGFR [25] but also through a Src-dependent pathway. This might be the reason why migration is
primarily decreased by inhibition with PD98059. Furthermore, Hwang et al. [26] could show that PD98059 has no impact on migration without radiation.

As glioma cells typically invade the surrounding area, the micro-surgical extirpation in combination with adjuvant irradiation therapy is the gold standard [27] and radiation induced migration becomes clinically relevant. In the last few years, many attempts were made for optimizing radiotherapy, such as increasing the dose (over 60 Gy yield), whole brain irradiation, and the adoption of a stereotactic therapy [28, 29]. Unfortunately, all these therapy concepts led to an increase of recrudescences in the surrounding area and a combination with chemotherapy must be discussed.

Conclusion

Glioblastoma cannot be removed completely because of the infiltrative growth in the surrounding area. The edge and infiltrative zone are the main problems because most recurrences extinguish there. Therefore, radiation therapy is applied but the effect is mostly temporary. Also, recent studies show an increase of migrating cells there. Therefore we postulate that the downstream pathways and not the EGFR inhibition of the EGFR was not effective.

Therefore we postulate that the downstream pathways and not the EGFR itself might be responsible for the radiation induced migration in glioma.

References