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Expression of EGFR variant vIII promotes both radiation resistance and hypoxia tolerance

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Abstract

***Background and purpose*: EGFRvIII has been described to function as an oncoprotein with constitutive activation promoting neoplastic transformation and tumorigenicity. The present study was undertaken to test whether EGFRvIII also contributes to hypoxia tolerance.**

***Material and methods*: The human glioma cell line U373 was genetically modified to stably express EGFRvIII. Western blotting and immunohistochemistry verified the expression of EGFRvIII. Tumour xenografts were produced by injecting U373 control and EGFRvIII positive cells subcutaneously into the lateral flank of recipient mice. Colony formation assays were performed after ionizing radiation at 4 Gy and after exposure to anoxia for 1–4 days.**

***Results*: EGFRvIII accelerated tumour growth leading to a 3.5-fold increase in tumour size compared to control tumours at 40 days after cell injection. EGFRvIII promoted clonogenic survival by almost 2-fold and 4-fold after 4 Gy and 4 days of anoxia, respectively. EGFRvIII was also associated with a substantially bigger colony size after anoxic treatment.**

***Conclusions*: EGFRvIII expression stimulates the growth of tumour xenografts and strongly promotes survival after irradiation and under hypoxic stress.**

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Keywords: EGFRvIII; Radiation resistance; Radiotherapy; Hypoxia tolerance; Targeted therapy

The epidermal growth factor receptor (EGFR) is a well-characterized proto-oncogene that is expressed in multiple cancers where it has been shown to promote tumour progression and therapy resistance [1,3,12,19]. EGFR targeted strategies are actively under investigation and EGFR-specific tyrosine kinase inhibitors (TKI) and monoclonal antibodies (mAb) have shown great promise [4,6–8,17]. This is illustrated by the high number of currently running clinical trials investigating anti-EGFR strategies in cancer treatment. However, some of the first clinical reports failed to corroborate the promising anti-tumour effects seen in preclinical studies, implicating persistent growth pathways despite blockade of wild-type EGFR [5,21]. The presence of naturally occurring mutations of EGFR may account for the limited clinical response to EGFR-targeted therapies [10,13,18]. A commonly described variant harbours an in-frame deletion of exons 2–7 resulting in a truncated version of the receptor which lacks a portion of the extracellular ligand binding domain. This variant, called EGFRvIII, has not been detected in normal tissue, but is found in many malignancies, such as glioblastoma, non-small lung cell carcinoma, breast cancer, prostate cancer and just recently also in head and neck cancer [15,16,20]. Ligand-independent activation of EGFRvIII may explain the relative inability of blocking mAbs to downregulate the receptor [2,26]. The present study was undertaken to test the hypothesis that EGFRvIII expressing tumour cells not only contribute to therapy resistance but also to hypoxia tolerance. This would have important implications for our understanding of the tumour microenvironment and for the optimization of EGFR-targeted strategies in cancer therapy.

Materials and methods

Generation of a stable EGFRvIII expressing cell line

U373 human glioma cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in MEMα medium (Invitrogen, Breda, NL) supplemented with 10% fetal calf serum (FCS). The phβAc.EGFRvIII

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plasmid, a generous gift from D.Bigner (Duke University, NC, USA), was transfected into U373 cells using PolyFect (Qiagen, Venlo, NL) according to the manufacturer's directions. Forty-eight hours after transfection, cells were trypsinized and seeded at low density for selection in 300 μg/ml Geneticin (Invitrogen, Breda, NL). The plates were incubated for two weeks to allow formation of resistant colonies. Several colonies were chosen for expansion and labeled U373-vIII clones A–G.

Immunoblotting

The cell pellet obtained from a 6 cm plate was lysed in 50 μl RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 7.5, 1× protease inhibitor cocktail) and incubated on ice for 30 min. Cell debris was removed by centrifugation at 10,000*g* for 15 min. The protein concentration in the supernatant was determined using the *DC* Assay (BioRad, Veenendaal, NL). Forty micrograms of each sample was resolved on an 8% SDS–PAGE gel and blotted onto a Hybond ECL nitrocellulose membrane (GE Healthcare, Deigem, BE) by electrotransfer. The membrane was blocked with 5% milk-TBST (20 mM Tris–HCl, pH 7.6, 140 mM NaCl, 0.1% Tween 20) and incubated overnight in a 1:1000 dilution of EGFR (15F8) rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA). The membrane was washed with TBST and incubated for 1 h with a goat anti-rabbit secondary antibody (Cell Signaling Technology) at 1:3000 dilution. Immobilized proteins were detected using SuperSignal West Pico chemiluminescent substrate (Perbio, Etten-Leur, NL) and by exposing the blot to X-ray film.

Immunohistochemistry

Cells grown in chamber slides *in vitro* were fixed with 10% formalin and stained for EGFR using a 1:200 dilution of EGFR (sc-03) rabbit polyclonal antibody (Santa Cruz Biotechnology, Heidelberg, DE). The primary antibody was detected by an EnVision peroxidase-linked secondary antibody (DAKO, Heverlee, BE) used in combination with DAB+ chromogen and substrate (DAKO, Heverlee, BE). Slides were counterstained with hematoxylin and mounted in DPX medium (Brunschwig chemie, Amsterdam, NL).

Seven micrometer thick tumour sections were stained using the same primary antibody as above at 1:100 dilution. A biotinylated goat anti-rabbit secondary (DAKO) was used at a 1:200 dilution followed by Vectastain ABC solution (Brunschwig chemie) and detection with DAB.

Radiation of cells

Cells were seeded in 6 cm dishes in order to reach ~80% confluence at the time of irradiation. Growth medium containing 0.5% FCS was added to cells 16 h before irradiation. During irradiation, dishes were placed in a Plexiglas jig filled with water at 37 °C. Cells were irradiated using an MCN 225 industrial X-ray tube (Philips, Eindhoven, NL) operated at 225 kV and 10 mA to deliver a dose of 4 Gy at a rate of 0.85 Gy/min. Immediately following irradiation, cells were returned to the incubator for 24 h at which time they were plated in MEMα + 10% FCS for the clonogenic survival assay.

Clonogenic survival assay

Cells were counted using a Coulter Z Series particle counter (Beckman Coulter, Mijdrecht, NL) and seeded in triplicate 6 cm dishes. For hypoxia experiments, cells were seeded for clonogenic survival prior to hypoxic exposure at 0% oxygen for 1–4 days. After the hypoxic treatment, the plates were removed from the hypoxic chamber (a MACS VA500 microaerophilic workstation supplied by Don Whitley Scientific, Shipley, UK) and incubated under standard culture conditions until colonies formed (~14 days in total). Colonies were fixed and stained with 2% bromophenol blue in 70% ethanol. Plating efficiency was determined by counting colonies consisting of $\geqslant$50 cells and correcting for the number of cells seeded.

Tumour xenograft growth

Animal experiments were performed using adult NMRI (nu/nu) female mice (28–32 g) from the animal facility of the Catholic University of Leuven in Belgium. The animal facilities and experiments were in accordance with local institutional guidelines for animal welfare and were approved by the Animal Ethics Committee of the university. Three million U373 and U373-vIII(+) cells were resuspended in 100 μl growth medium and injected subcutaneously into the lateral flank of recipient mice. Tumours were measured with callipers in three orthogonal diameters and used to calculate tumour volume based on the formula $A \times B \times C \times \pi/6$. Animals were followed until the ethically allowed tumour burden was reached, at which time tumours were excised, fixed in 1–4% formaldehyde, and embedded in paraffin. Mean tumour volumes were calculated for each group.

In vitro growth under aerobic and hypoxic conditions

For growth under aerobic conditions, 10^5 cells were seeded in triplicate on 6 cm dishes. The plates were incubated under normal culture conditions in a 5% CO_2 incubator for 1–6 days. To monitor growth under hypoxia, 5×10^5 cells were seeded in 10 cm dishes. The following day (day 0), dishes were placed in the hypoxic chamber for 1–4 days. Plates were harvested by washing two times with phosphate-buffered saline followed by trypsinization. Total cell numbers were enumerated using a Coulter Z Series particle counter (Beckman Coulter, Mijdrecht, NL). Cell numbers were normalized to the amount of cells present on day 0. Doubling times were calculated from the slope of the best-fit line during the exponential phase of growth.

Statistics

Statistical analysis was carried out using the program SPSS 12.0.1 for Windows (SPSS Inc., 2003, Chicago, IL, USA). A non-parametric Mann–Whitney *U* test was used to assess differences in xenograft tumour growth. A Student's *t*-test was used to assess differences in radiation survival and a one-way ANOVA was used to determine differences in growth under hypoxia. *P* values <0.05 were considered to be significant.

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