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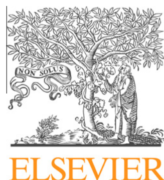

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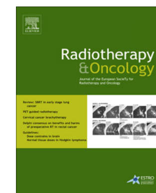

Original article

Radiosensitization of NSCLC cells by EGFR inhibition is the result of an enhanced p53-dependent G1 arrest

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ABSTRACT

Purpose: How EGF receptor (EGFR) inhibition induces cellular radiosensitization and with that increase in tumor control is still a matter of discussion. Since EGFR predominantly regulates cell cycle and proliferation, we studied whether a G1-arrest caused by EGFR inhibition may contribute to these effects.

Materials and methods: We analyzed human non-small cell lung cancer (NSCLC) cell lines either wild type (wt) or mutated in p53 (A549, H460, vs. H1299, H3122) and HCT116 cells (p21 wt and negative). EGFR was inhibited by BIBX1382BS, erlotinib or cetuximab; p21 was knocked down by siRNA. Functional endpoints analyzed were cell signaling, proliferation, G1-arrest, cell survival as well as tumor control using an A549 tumor model.

Results: When combined with IR, EGFR inhibition enhances the radiation-induced permanent G1 arrest, though solely in cells with intact p53/p21 signaling. This increase in G1-arrest was always associated with enhanced cellular radiosensitivity. Strikingly, this effect was abrogated when cells were re-stimulated, suggesting the initiation of dormancy. In line with this, only a small non-significant increase in tumor control was observed for A549 tumors treated with fractionated RT and EGFR inhibition.

Conclusion: For NSCLC cells increase in radiosensitivity by EGFR inhibition results from enhanced G1-arrest. However, this effect does not lead to improved tumor control because cells can be released from this arrest by re-stimulation.

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The receptor of the epidermal growth factor (EGFR) regulates cell proliferation, differentiation and survival [1]. Many tumor entities such as non-small cell lung cancer (NSCLC) and head and neck squamous cell carcinomas (HNSCC) are characterized by EGFR mutations or increased EGFR expression [2]. This EGFR-deregulation is associated with increased resistance towards X-irradiation (IR), poor prognosis and a high number of recurrences [2–5]. As a consequence, targeting of the EGFR by either specific antibodies (AB) or tyrosine kinase inhibitors (TKI) was considered to be a promising strategy to improve local control as well as overall survival (OS) when combined with radio-(RT) or radiochemotherapy (RCT).

In fact, Bonner et al. [6] reported an increased OS by about 8% compared to RT alone when advanced HNSCC patients were treated with the anti-EGFR AB cetuximab additionally. However, in a recent DAHANCA study no benefit was seen for HNSCC patients when RT was combined with the anti-EGFR AB zalutumumab [7].

For NSCLC patients it is also still unclear, whether EGFR inhibition may lead to tumor radiosensitization and therefore to an increase in tumor control. Whereas EGFR inhibition is well established for NSCLC as monotherapy, it is not yet approved in combination with RT [8]. However, several phase I and II studies indicate that gefitinib and erlotinib combined with RT or RCT are well tolerated and might yield good responses [9–13]. In contrast to the monotherapy situation the response was found to depend neither on EGFR nor on K-Ras mutations [14].

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So far preclinical data investigating radiosensitization of NSCLC cells by EGFR inhibition are inconclusive [15–22]. In case sensitization was observed it was associated with increased induction of apoptosis [19,20] or impaired repair of DNA double strand breaks (DSB) [15,16,21]. Additionally, many studies reported an inhibition of cell growth and an accumulation of cells in G1 phase of the cell cycle when the EGFR was inhibited [18–20,23]. The accumulation in G1 was shown to be associated with a decrease in the expression of cyclins and an increase in p27 expression [23]. However, so far it is not known, whether such a G1 arrest is only transient or permanent. Only a permanent cell cycle arrest will actually lead to cell inactivation and will therefore affect cellular or tumor radiosensitivity. In case of human fibroblasts a permanent G1 arrest induced by radiation is suggested to account for almost half of the inactivation seen after IR [26] and clearly depends on intact p53/p21 signaling [24,25].

It is now shown here for the first time for NSCLC cells that inhibition of EGFR causes an increase of the IR-induced permanent G1 arrest but only in cells with intact p53/p21 signaling. This effect was always associated with an enhanced cellular radiosensitivity. Strikingly, the G1 arrest and with that the radiosensitization were reversed by re-stimulation. In line with this only a minor improvement in tumor control was measured for a p53 wt A549 tumor model treated by fractionated RT combined with EGFR inhibition.

Materials and methods

Cell lines

Cells were grown in medium containing 10% FCS (PAN Biotech) and 2 mM glutamine (Invitrogen) at 37 °C and 100% humidification; NSCLC cell lines A549 (squamous cell carcinoma) and H1299 (large-cell carcinoma) and the colorectal carcinoma cell line HCT116 were grown in D-MEM (Invitrogen) under 10% CO_2, the NSCLC cell lines H460 (large-cell carcinoma) and H3122 (adenocarcinoma) were grown in RPMI (Invitrogen) under 5% CO_2. All cells were identified by a short tandem repeat multiplex assay (Applied Biosystems). A549, H460 and H1299 cells express wt EGFR [27] (H3122 not characterized). The p53 status of A549 and H460 is wt, H1299 cells harbor a homozygote p53 deletion mutation (p53 negative). The sequencing of exons 5–8 of p53 revealed a homozygote 854A > T mutation in exon 8 (E285V) in H3122 cells.

Substances

Erlotinib (Tarceva®; Roche) & BIBX1382BS (BIBX; Boehringer), cetuximab (Erbitux®; Merck), spindle fiber toxin colcemid (Merck), DMSO (vehicle; Roche), propidium iodide (Merck), RNase A (Serva).

Irradiation (IR)

Cells were irradiated at room temperature with 200 kV X-rays (Gulmay RS225, Gulmay Medical Ltd.; 15 mA, 0.8 mm Be+ 0.5 mm Cu filtering; dose rate of 1.2 Gy/min).

Western blotting

Proteins from whole cell extracts were detected by Western blot according to standard protocols using SuperSignal West Dura Reagents (Thermo Scientific), the Night OWL system and Win Light 32 software (Berthold) for signal detection and quantification. Antibodies: anti-EGFR, anti-pEGFR, anti-ERK1/2, anti-pERK, anti-Akt and anti-pAkt (Cell Signaling Technology), anti-p53 (Novocastra), anti-p21 (Pharmingen), anti-actin (Sigma-Aldrich), anti-GAPDH, anti-rabbit and anti-mouse HRP-conjugated secondary antibody (Amersham).

G1 kinetics

Twenty-four hours after seeding, cells were irradiated using X-ray with or without 10 h pre-incubation with EGFR inhibitors. Thirty minutes before irradiation, 0.2 μg/ml colcemid was added to block cells in metaphase and therefore to hinder the cell to re-enter the G1 phase. At different time intervals thereafter cells were harvested and fixed with ethanol, washed with PBS (0.1% Tween) and stained with propidium iodide solution (10 μg/ml, RNase A 0.1 μg/ml) for 30 min at room temperature. DNA histograms as obtained by flow cytometry (FACS Scan with CellQuestTM Pro software, Becton Dickinson) were used to determine the fraction of G1 cells using ModFit LTTM software (Verity Software House, Inc.). For re-stimulation, cells were re-seeded 24 h after irradiation and colcemid was added 3 h later. An experimental scheme is given in Fig.S1A.

Cell proliferation and survival

To measure proliferation, cells were seeded, treated with EGFR-inhibitors 24 h later and cell numbers were determined at the indicated time points. Cell survival was measured by colony formation. Therefore cells were either seeded 24 h before (pre-plating) or 24 h after (re-stimulation) irradiation. Cells were allowed to grow for 10–14 days and were fixed when the colonies reached equal size. Colonies of more than 50 cells were scored as 'survivors' and the surviving fraction was normalized to the plating efficiency of the non-irradiated controls.

siRNA

A549 cells were transfected with 10 nM siRNA using HiPerFect (Quiagen) according to the manufacturer's instructions (siRNA p21, Cell Signaling Technology; GAPDH control siRNA, Thermo Scientific). Media were changed 14 h after transfection and inhibitors were added immediately. Ten hours later, colcemid was added prior to irradiation and the resulting G1-kinetics was subsequently analyzed as described above.

Xenograft experiments

For tumor control experiments A549 xenografts were grown on nude mice according to conditions published by the Experimental Centre of the Medical Faculty Carl Gustav Carus; Technische Universität Dresden. Experiments and animal facilities were approved according to German animal welfare regulations and institutional guidelines [28]. For detailed experimental settings see Supplementary Experimental Procedures.

Data evaluation

If not indicated cell culture experiments were repeated up to three times. All data are represented as mean values (±SEM). Prism software (GraphPad Prism) was used for analyzing and graphing the data, while Student's t-test was instrumental in the statistical analysis. *P*-values were calculated using one-sided tests (statistically significant p values <0.05).

For xenografts, the medians of tumor growth time were compared by means of the Mann-Whitney *U*-test using GraphPad Prism (GraphPad Prism). For detailed experimental settings see Supplementary Experimental Procedures.

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