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The epidermal growth factor receptor modulates DNA double-strand break repair by regulating non-homologous end-joining

Malte Kriegs, Ulla Kasten-Pisula, Thorsten Rieckmann, Katharina Holst, Jarob Saker, Jochen Dahm-Daphi, Ekkehard Dikomey*

Laboratory of Radiobiology & Experimental Radiooncology, Hubertus Wald Tumorzentrum – University Cancer Center Hamburg, University Medical Center Hamburg – Eppendorf, Martinistr. 52, D-20246 Hamburg, Germany

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ABSTRACT

In mammalian cells repair of radiation-induced DNA damage appears to be also controlled by the epidermal growth factor receptor (EGFR) with a special impact on DNA double-strand break (DSB) repair. Aim of this study was to demonstrate this interaction between EGFR signalling and DNA DSB repair and to identify the underlying downstream pathways. We especially wanted to know in how far non-homologous end-joining (NHEJ) as the most important DSB repair pathway is involved in this interaction. Overall DSB repair was determined by counting γH2AX foci remaining 24 after irradiation, while NHEJ activity was monitored by using a specially designed repair construct stably integrated into the genome. The overall DSB repair capacity was clearly enhanced when EGFR was activated by its natural ligand EGF and, vice versa, was reduced when EGFR was blocked either by the specific antibody Cetuximab or the tyrosine kinase inhibitor erlotinib, whereby reduction was clearly stronger for erlotinib. There was also a difference in the pathways affected. While erlotinib lead to a block of both, MAPK as well as AKT signalling, Cetuximab only affected MAPK. As demonstrated by specific inhibitors (PD98059, AKTIII) EGFR interacts with DSB repair mostly via MAPK pathway. Also for NHEJ activity, there was a substantial increase, when EGFR was activated by EGF as determined for two different reporter cell lines (A549.EJ and H1299.EJ) and, vice versa, a reduction was seen when EGFR signalling was blocked by Cetuximab or erlotinib. There was, however, no difference for the two inhibitors used. This regulation of NHEJ by EGFR was only blocked when ERK was affected by siRNA but not when AKT was knocked down. These data indicate that EGFR modulates DSB repair by regulating NHEJ via MAPK signalling.

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1. Introduction

In mammalian cells repair of DNA damage induced by ionizing radiation appears to be controlled also by the epidermal growth factor receptor (EGFR). This is considered to be of especial importance for tumour cells, since several tumour entities are characterised by a substantial over-expression of EGFR [1,2]. This trans-membrane tyrosine kinase receptor, which belongs to the ErbB-family, is primarily located in cell membrane and is activated by ligands such as epidermal growth factor (EGF), amphiregulin, TGFα but also by irradiation [3,4]. Ligand binding leads to dimerization, which induces several down-stream signal cascades. The most prominent EGFR dependent signal cascades are the Ras/Raf/MEK/ERK dependent MAPK cascade, the PI3K dependent AKT kinase cascade, the JAK/STAT and PKC dependent signalling (reviewed in Ref. [3]). Using these pathways, EGFR is considered to modulate cell proliferation, differentiation as well as apoptosis but also DNA repair [5].

The modulation of DNA repair is suggested especially to occur for radiation-induced DNA double-strand breaks (DSB). There was an increase in the number of residual DSBs as detected by the number of γH2AX foci measured 24 h after irradiation, when EGFR signalling was blocked either by tyrosine kinase inhibitor BIBX or the monoclonal antibody (mAB) Cetuximab [6,7].

The effects seen after EGFR blockage indicate that the overall DSB repair depends on EGFR signalling, but does not allow an identification of the specific pathway involved. In mammalian cells there are several different pathways, by which radiation-induced DSB are repaired. Non-homologous end-joining (NHEJ) is generally the dominant pathway. This pathway, which is active in all phases of the cell cycle, is characterized by the binding of Ku70/80 heterodimer to the DSB forming a complex with the catalytic subunit DNA-PKcs, which will then recruit several other proteins such as Artemis, polymerase μ and λ as well as the LigIV/XRCC4/XLF complex (reviewed by Shrivastav et al. [8]). DSB can also be repaired by homology-directed repair (HDR), which is active only in S and

* Corresponding author. Tel.: +49 40 42803 3593; fax: +49 40 42803 5139.
E-mail address: dikomey@uke.uni-hamburg.de (E. Dikomey).

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G2 [8]. HDR starts with strand resection by the MRN complex creating single-strands, which than interacts with RPA protein and Rad51 as the central protein of HDR. This protein facilitates the invasion of the single-strand into the homologous counterpart followed by repair synthesis and complex reorganisation of the DNA [9]. Beside HDR and NHEJ, mammalian cells are also endowed with the two back-up pathways "single-strand annealing" (SSA) [10,11] and PARP dependent NHEJ [12], which come into play, when the other pathways are either defective or blocked [11].

The relevance of one specific DSB repair pathway can be studied by using specifically designed repair substrates stably integrated into the genome. Generally, DSBs are induced in these substrates by using rare cutting restriction enzymes such as I-SceI. Repair of these enzymatically induced DSBs will alter the substrate in such a way, that a previously inactive reporter gene encoding the green fluorescence protein (GFP) becomes active, so that cells which have successfully completed this repair can be identified by their GFP fluorescence intensity. By using such specific constructs, it was possible to demonstrate that a lack of XRCC4 resulted in a substantial reduction of the NHEJ activity [13].

So far, little is known regarding the effect of EGFR signalling on repair substrates specific for NHEJ or HDR. For HDR, it was shown by Li et al. [14] that blockage of EGFR by erlotinib for 16 h resulted in a substantial reduction, which was attributed to the translocation of BRCA1 into the cytoplasm so that less of this protein is present in the nucleus to perform HDR. A regulation of HDR by EGFR was also demonstrated by Golding et al. [15] showing that HDR was stimulated by EGF or when EGFR variation III (EGFRvIII) was present in the reporter cell line. In line with this, inhibition of MAPK (ERK1/2), which is one of the main downstream pathways of EGFR, was reported to reduce HDR [16]. Also the increase of Rad51 seen in A549 cells after treatment with MMC and Cisplatin, which is taken as a surrogate of active HDR, was found to be completely abolished, when EGFR is blocked by the tyrosin kinase inhibitor gefitinib [17]. However, when interpreting these results it should be noted that some of the EGFR inhibitors used are found not only to affect EGFR but also other receptors of the ErbB-family [18] and, in addition, that generally down regulation of HDR has only a minor effect on overall DSB repair [19,20].

A regulation of NHEJ by EGFR was first demonstrated by Golding et al. [15] using a specific repair construct. It was found that NHEJ was stimulated by EGF as well as by the presence of EGFRvIII and reduced when EGFR was blocked by specific inhibitors. These observations are in line with data indicating such specific interaction. For instance, it was shown by Dittmann et al. that EGFR regulates the activity of the DNA-PK complex [21]. In line with this, block of EGFR by the mAB Cetuximab resulted in a reduced DNA-PK activity and sequestration of DNA-PK into the cytosol [6,21,22]. Similar data was found when EGFR tyrosine kinase function was depressed by the small molecule inhibitor BIBX1382BS [7].

There are also data suggesting that EGFR might interact with NHEJ via XRCC1. This protein plays a central role in base excision as well as single-strand break repair [23–25], but might also interfere with NHEJ, because of its interaction with DNA-PKcs [12]. It was reported that an activation of EGFR by X-irradiation resulted in a rapid up-regulation of XRCC1 expression, which was mediated via MAPK signalling [26].

The hypothesis that EGFR signalling modulates DSB repair by specifically affecting NHEJ is now tested in this report. Overall DSB repair was measured by scoring the number of γH2AX foci 24 h after X-irradiation, while NHEJ activity was determined by using an especially designed repair substrate stably integrated into the genome of two different cell lines (A549 and H1299). It is shown here that activation or blockage of EGFR signalling affects overall DSB repair by regulating NHEJ. This regulation was found to depend on MAPK signalling.

2. Materials and methods

2.1. Substances

EGF, Sigma; erlotinib (tyrosine kinase inhibitor, Tarceva®), Roche; Cetuximab (monoclonal anti-EGFR antibody, Erbitux®), Merck; PD98059 (MEK inhibitor), Calbiochem; AKT inhibitor VIII, Calbiochem; DMSO for cell culture (vehicle), Sigma; NU7026 (DNA-PK inhibitor), Sigma.

2.2. Cell line

Bronchial carcinoma cells A549 and H1299 were grown in D-MEM (Gibco) containing 10% FBS (Biochrom AG) and 4 mM glutamine (Gibco) at 37 °C, 10% CO_2 and 100% humidification.

2.3. X-irradiation

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).

2.4. Western blot

Proteins of whole cell extracts were detected by Western blot according to standard protocols. Primary antibodies from Cell Signaling Technology: anti-Akt, anti-p42 MAPK, anti-EGFR, anti-phospho-AKT (Ser473), anti-phospho-p44/42 MAPK (Thr202/204); from Upstate: anti-phospho-EGFR (Tyr1173); from Sigma–Aldrich: anti-actin. Secondary antibody from Amersham: anti-rabbit and anti-mouse HRP conjugated. Signals were determined via chemiluminescence, using the ECL™ Western Blotting Detection Reagents (Amersham).

2.5. γH2AX foci

Experiments were performed with 2.4×10^5 cells seeded on culture slides (BD-Falcon). Twenty-four hours after irradiation or sham-treatment cells were fixed in neutral buffered 4% formalin for 15 min. After permeabilization of cell membranes with Triton X-100 (0.2% in PBS, v/v 10 min RT), bovine serum albumin (3%, w/v in PBS, RT, 30 min) was used to block unspecific reactions before primary antibody was added for 1 h (anti-phospho Histone H2A.x (Ser139), Millipore; 1:100 in 1% BSA/PBS; RT). Samples were washed three times (PBS/1% BSA/0.5% Tween20) and were incubated with secondary antibody ALEXA fluor® 594 (Invitrogen; 1:600; RT, 60 min) subsequently. Slides were washed again three times and nuclei were labelled using DAPI/Antifade (QBiogene). The γH2AX foci were counted visually using a Zeiss fluorescence microscope (Zeiss Axioplan 2) with 630-fold magnification. The cells were selected according to morphological criteria and only intact nuclei were analysed. For evaluation, 100 nuclei of each dose group were randomly chosen and the number of foci per nucleus was recorded.

2.6. Generation of NHEJ reporter cells lines

Generation of A549 and H1299 cell lines with stably transfected NHEJ reporter constructs was performed according to the protocol described elsewhere [27]. In brief: 1×10^6 A549 cells were electroporated in the presence of XmnI linearized 0.2 μg NHEJ reporter plasmid (pEJ) [11]. Cells were cultivated in the presence of penicillin, streptomycin and puromycin (A549) or G418 (H1299) and monoclonal colonies were isolated approximately two weeks later. Cells were tested for positive NHEJ and clones were chosen for further experiments. These are two

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