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c-Myc is required for transformation of FDC-P1 cells by EGFRvIII

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Abstract In contrast to wtEGFR, its truncated version EGFRvIII transformed non-tumorigenic FDC-P1 cells only when c-Myc was coexpressed. In nude mice, EGFRvIII/c-Myc coexpressing cells induced tumors, whereas wtEGFR-expressing EGF-dependent FDC-P1 cells did not. EGFRvIII function was required for both the induction and maintenance of tumor growth. Cellular proliferation was inhibited by a selective EGFR tyrosine kinase inhibitor indicating intrinsic tyrosine kinase activities for both receptors. Unlike wtEGFR, constitutive signaling by EGFRvIII was refractory to stimulation by the EGFR ligands EGF and TGF-α. Summarized, EGFRvIII is a constitutively active receptor tyrosine kinase whose transforming capacity is lower than that of EGF-stimulated wtEGFR.

Keywords: EGFRvIII; c-Myc; Transformation; Tyrosine kinase inhibitor

1. Introduction

EGFR is a receptor tyrosine kinase involved in the proliferation, differentiation and survival of epithelial cells, astrocytes, and neurons [1]. The signal transduction cascade emanating from the EGFR is induced by the binding of activating ligands such as EGF and TGF-α, resulting in EGFR homo-/hetero dimerization, autophosphorylation and subsequent activation of downstream signal transduction molecules [2]. Using various cellular model systems (both fibroblastic and hemopoietic), it has been shown that EGFR signaling can contribute to cell transformation. In this process the EGFR is permanently stimulated, for example through the autocrine production of stimulating ligands [3,4]. Excess EGFR activity resulting from such an autocrine loop or from a high EGFR overexpression is implicated in a significant proportion of human carcinoma types such as non-small cell lung cancer [5], breast cancer [6], and prostate cancer [7]. EGFR is also involved in the development of glioblastoma multiforme [8,9]. There, the predominant mechanism is the amplification of the EGFR gene leading to EGFR overexpression, which is found in about 40% of patients. Interestingly, only in about half of these cases the amplified EGFR gene encodes the wild-type growth factor receptor (wtEGFR). In the other half, mutant EGFR forms are coexpressed with the wtEGFR. Thus, the EGF-receptor is involved in two distinct progression steps in glioblastoma where amplification of the wtEGFR is followed by acquisition of activating alterations in the EGFR gene structure [10]. The most common mutation of EGFR found in glioblastoma is the N-terminal truncation known as variant III (EGFRvIII). Epidermal growth factor receptor variant III (EGFRvIII) lacks the amino acids 6–273 encoded by exons 2–7 [11]. Over the last 10 years the transforming potential as well as the mechanisms underlying transformation by EGFRvIII and wtEGFR have been investigated [12]. One striking difference between the two EGFR forms is that EGFRvIII displays signaling activity in the absence of stimulating ligands. In accordance with this observation, EGFRvIII can transform cells of various types without the involvement of EGFR-stimulating ligands [13–17]. However, very little is known about binding of EGF or TGF-α to EGFRvIII [16,18]. Only one study reported a weak binding of TGF-α to EGFRvIII that resulted in cellular stimulation [13].

The objective of this study was to compare the transforming potential of EGFRvIII with that of wtEGFR. For this purpose, a doxycycline-regulated expression system was established for both receptors in IL-3-dependent, non-tumorigenic FDC-P1 cells. The resulting clones were characterized with respect to their sensitivity to a specific EGFR inhibitor, response to exogenous growth factor, and tumorigenic potential.

2. Materials and methods

2.1. Transfection and cloning of FDC-P1 cell derivatives expressing EGFRvIII and wtEGFR

Modified FDC-P1 cells derived from mouse myelomonocytic progenitors [19] were used as recipient cells; two different cell lines (FtL and FtLm) were used as parentals (for generated and used cell lines see Table 1). Additionally to their normal growth medium [19], untransfected cells received 1% (v/v) IL-3 supernatant of X63Ag8-653-IL3 cells [20], wtEGFR-transfected cells 20 ng/ml EGF (Promega).

Plasmids were introduced into cells via electroporation and antibiotic selection with 400 μg/ml hygromycin B (Invitrogen) in presence of 1% (v/v) IL-3 was performed for 7–10 days. After antibiotic

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Abbreviations: wtEGFR, wild-type growth factor receptor; EGFRvIII, epidermal growth factor receptor variant III; tTA, tetracycline-regulated transactivator protein; siRNA, small interfering RNA; VEGF2R, vascular endothelial growth factor receptor

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Table 1
Description of cell lines generated

FDC-P1 derivative	Introduced genes	Growth factor dependence	Tumorigenicity	Drug sensitivity towards BIBX1382BS (in absence of IL-3)[a]
FDC-P1	–	IL-3	–	n.a.
FtL	tTA	IL-3	–	n.a.
FtLm	tTA, *c-MYC*	IL-3	–	n.a.
F/Lauto	wtEGFR, TGF-α	–	+	+
FtL-VEGF2R	VEGF2R, tTA	–	+	–
FtL-wtEGFR	wtEGFR, tTA	EGF	–	+
FtL-EGFRvIII	EGFRvIII, tTA	IL-3	–	n.a.
FtLm-wtEGFR	wtEGFR, tTA, *c-MYC*	EGF	–	+
FtLm-EGFRvIII	EGFRvIII, tTA, *c-MYC*	–	+	+

tTA tetracycline-regulated transactivator protein; n.a., not applicable.
[a]Drug sensitivity determined using both Cytosensor™ and MTS-assays.

selection, IL-3 was gradually removed. The obtained bulk cultures were cloned in 1% (w/v) Methocel (Invitrogen). Finally, clonal identity was verified by Southern blotting.

2.2. MTS-assay

Cells were seeded in triplicates in 96-well assay plates at 20.000 cells per well in 100 μl standard medium. Clones overexpressing wtEGFR were supplemented with 8 ng/ml EGF, whereas clones overexpressing EGFRvIII did not receive any additional growth factors as pilot experiments have shown that addition of EGF to EGFRvIII-expressing cells resulted in no change of proliferation rate. Determination of IC_{50} values was done using seven dilution steps. Doxycycline (Sigma) concentrations ranged from 10^{-10} to 10^{-3} g/l in steps of one magnitude. BIBX1382BS is a proprietary EGFR tyrosine kinase inhibitor from Boehringer-Ingelheim [21]. After 48 h, viable cells were determined using a MTS-mix (Promega).

2.3. FACS-analysis

FACS-analysis was performed as previously described [17]. Primary antibodies were Ab-1 – detects both wtEGFR and EGFRvIII and Ab-5 – recognizes exclusively wtEGFR (both from CALBIOCHEM), the secondary antibody was F 261 (DAKO). Evaluation was done using the software "Cell Quest" (Becton Dickinson).

2.4. Cytosensor™ experiments

A suspension of 4×10^6 cells per 300 μl was prepared and mixed gently with 100 μl freshly molten agarose cell entrapment medium at 37 °C as described in the manufacturer's protocol. 10 μl of this mix was transferred into the center of each cell capsule. After solidification of the cell/agarose mixture, the capsules were placed into the Cytosensor™ Microphysiometer (Molecular Devices). Prior to measurement, cells were equilibrated with medium for 1–2 h until a steady baseline was observed. Afterwards inhibitor/growth factors were added to the media and the pH-change was monitored.

2.5. RT-PCR

Total RNA was prepared with TRIzol (Invitrogen) according to the manufacturer's protocol. Using the Superscript Kit (Invitrogen) cDNA was prepared. Approximately 1 μg cDNA was used for PCR, in which a fragment of 145 bp length specific for EGFRvIII (exon 2–7 overlap) is amplified.

Primers: 5′-GGAGGAAAAGAAAGGTAATTATGTGGT-3′ and 5′-TATTCCGTTACACACTTTGCGG-3′.

2.6. RNA interference

Cells that were used as IL-3 controls were grown for 48 h in presence of IL-3. wtEGFR-expressing cells also received EGF. 5000 cells per 96-well were transfected with small interfering RNA (siRNA) using OligofectAMINE according to the manufacturer's instruction. Forty-eight hours later the number of proliferating cells was determined using the MTS-assay as described above.

siRNA oligonucleotides (Dharmacon) sequences were anti-human myc: 5′-AACUUCUACCAGCAGCAGCAG-3′ and a scrambled (negative) control: 5′-AAUACCCUUCAGCAGCAGCAG-3′.

To exclude a general toxicity of the applied c-MYC siRNA, cells were also treated in the presence of IL-3. The level of c-MYC down-regulation was determined using quantitative PCR. Western blotting could not be performed as there is no antibody available that does not cross-react with mouse c-MYC.

2.7. Tumorigenicity in athymic mice

wtEGFR, EGFRvIII and FtLm cells were tested for tumorigenicity in 5–10 week old female Hsd:NMRI-nu/nu mice (Harlan, The Netherlands). For this, 100 μl of a cell suspension (at 10^7 cells per ml in PBS + 5% FCS) of each cell line was injected s.c. into the right hind flank of three mice. Tumors were measured three times a week with a calliper. To monitor side effects of treatment, mice were inspected daily for abnormalities and weighed three times a week. The experiment ran for 3 months. HP-β-CD (hydroxypropyl-beta-cyclodextrin) was used as vehicle.

2.8. Therapeutic experiment

When tumors were established and had reached diameters of approximately 5–10 mm, administration of compounds was started. Doxycycline was administered via drinking water whereas BIBX1382BS was given once per day by gavage needle. Animals were killed or taken out of study when tumors had grown to large volumes (between 1200 and 2000 mm^3) or when the animals' general condition had deteriorated (e.g., progressive weight loss), possibly as a consequence of compound administration.

Absolute tumor volumes were expressed as relative tumor volumes (V_{rel}), i.e., normalized with respect to the tumor volume at the start of the treatment ($V_{rel} = T/T_{start}$) and as specific relative tumor volumes (referred to as T/C [%]), i.e., as the mean relative volume of a group of compound-treated tumors in % of the mean relative volume of vehicle control tumors (T/C [%] = 100 · V_{rel} substance-treated/V_{rel} vehicle-treated). Evaluation of responses was based on V_{rel}- and T/C [%]-values determined on the day following the final treatment. Tumor-bearing animals that died during an experiment or were removed from the experiment prematurely for other reasons were not considered for the calculation of mean tumor volumes. A final T/C [%]-value below 50% was regarded as tumor response.

3. Results

3.1. EGFRvIII expression is insufficient to render FDC-P1 cells IL-3-independent

The transient and massive cell death that occurred in all transfected FDC-P1 cultures in response to IL-3-withdrawal indicates that an efficient selection process preceded the emergence of IL-3-independent cells (for generated and used cell lines see Table 1). To distinguish between a general heterogeneity of the transfected FDC-P1 cells *per se* – which might be responsible for this efficient selection process – or secondary mutational events that occur stochastically in some cells,