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5-Fluorocytosine combined with Fcy–hEGF fusion protein targets EGFR-expressing cancer cells

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ABSTRACT

Human epithelial cancers account for approximately 50% of all cancer deaths. This type of cancer is characterized by excessive activation and expression of the epidermal growth factor receptor (EGFR). The EGFR pathway is critical for cancer cell proliferation, survival, metastasis and angiogenesis. The EGF–EGFR signaling pathway has been validated as an important anticancer drug target. Increasing numbers of targeted therapies against this pathway have been either approved or are currently under development. Here, we adopted a prodrug system that uses 5-fluorocytosine (5-FC) and human EGF (hEGF) fused with yeast cytosine deaminase (Fcy) to target EGFR-overexpressing cancer cells and to convert 5-FC to a significantly more toxic chemotherapeutic, 5-fluorouracil (5-FU). We cloned and purified the Fcy–hEGF fusion protein from *Pichia pastoris* yeast. This fusion protein specifically binds to EGFR with a similar affinity as hEGF, approximately 10 nM. Fcy–hEGF binds tightly to A431 and MDA-MB-468 cells, which overexpress EGFR, but it binds with a lower affinity to MDA-MB-231 and MCF-7, which express lower levels of EGFR. Similarly, the viability of EGFR-expressing cells was suppressed by Fcy–hEGF in the presence of increasing concentrations of 5-FC, and the IC_{50} values for A431 and MDA-MB-468 were approximately 10-fold lower than those of MDA-MB-231 and MCF-7. This novel prodrug system, Fcy–hEGF/5-FC, might represent a promising addition to the available class of inhibitors that specifically target EGFR-expressing cancers.

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1. Introduction

The majority of human epithelial cancers are marked by functional activation of growth factors and receptors of the epidermal growth factor receptor (EGFR) family. Signaling pathways governed by the EGF–EGFR axis play central roles in cancer cell proliferation, survival, metastasis and angiogenesis [1]. Several EGFR antagonists are currently available for the treatment of four metastatic epithelial cancers: non-small-cell lung cancer, squamous-cell carcinoma of the head and neck, colorectal cancer, and pancreatic cancer [2]. These types of cancer account for more than 50% of all cancer deaths.

The overexpression of EGFR on these cancer cells is a well-characterized drug target. Two classes of EGF–EGFR inhibitors, monoclonal antibodies and small-molecule tyrosine kinase inhibitors, have been successfully tested and are now in clinical use. Anti-EGFR monoclonal antibodies, such as cetuximab (erbitux), bind to the extracellular domain of EGFR and compete for receptor binding by occluding the ligand-binding region, blocking ligand-induced EGFR tyrosine kinase activation. Small-molecule EGFR tyrosine kinase inhibitors, such as erlotinib and gefitinib, compete with ATP for the intracellular catalytic domain of the EGFR tyrosine kinase, inhibiting EGFR autophosphorylation and downstream signaling [1]. Although these inhibitors of the EGF–EGFR signaling pathway have achieved significant success in treating variable epithelial cancers, resistance occurs in a significant proportion of patients through various mechanisms, including mutation of the tyrosine kinase domain, compensation by other oncogenic pathways, etc. [3,4].

To design a novel therapeutic targeting the EGFR signal pathway, we constructed a fusion gene encoding human EGF linked to a yeast cytosine deaminase (CD), Fcy. This approach takes advantage of the enzymatic ability of cytosine deaminase to convert a relatively safe molecule, 5-fluorocytosine (5-FC), into a very commonly administered chemotherapeutic, 5-fluorouracil (5-FU), which is 1000-times more toxic than 5-FC. 5-FC has been administered at a dose of 150–200 mg/kg for the treatment of

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fungal infections with a favorable safety profile [5]. Given that many EGFR-overexpressing cancers, such as head and neck, pancreatic, colon-rectal cancers, etc., were often treated by 5-FU, this EGFR targeting prodrug system could circumvent the high systemic cytotoxicity of 5-FU by concentrating the production of 5-FU at the EGFR-expressing tumor sites. 5-FU is a small molecule capable of diffusing in and out of cells, leading to a significant bystander effect without the requirement of direct cell-to-cell contact.

In this study, we have cloned and purified a fusion protein, Fcy–hEGF–myc–his_6 (Fcy–hEGF), expressed by a yeast host, *Pichia pastoris*. This fusion protein retains both EGFR binding activity and the capability to convert 5-FC to 5-FU, similar to the effects of EGF and Fcy, respectively. More importantly, Fcy–hEGF binds more readily to and exhibits a more significant cytotoxic effect on EGFR-expressing cancer cells compared to cells with less EGFR expression.

2. Materials and methods

2.1. Cell lines and reagents

MCF-7, MDA-MB-468, and MDA-MB-231 human breast cancer cells and a human epidermoid carcinoma cell line, A431, were purchased from American Type Culture Collection. 5-Flourocytosine was purchased from Sigma Aldrich (St. Louis, MO).

2.2. Cloning of DNA in the yeast expression vector

The DNA sequence encoding Fcy was PCR amplified using a cDNA library that was obtained from yeast as template, whereas human EGF was PCR amplified using a cDNA library derived from a human cancer cell line, SKOV3-ip1. The resulting PCR products were cut with BamHI and EcoRI, which were introduced in PCR primers, and ligated into the protein expression vector, pPICZ-αA, which was cut with the same enzymes. Fcy and hEGF were individually cloned into this vector after the α-secreting signal peptide at the N-terminus, and the C-terminus of the pPICZ-αA vector has a c-myc and hexa-histidine (myc-his_6) tag for convenient protein recognition and purification. Another Fcy PCR product, containing a BamHI cloning site engineered at both the 5′ and 3′ ends, was digested with BamHI and cloned into the pPICZ–αA-hEGF–myc–his_6 vector that was previously cut with BamHI and treated with calf intestinal alkaline phosphatase (CIAP) to prevent self-ligation of the vector.

2.3. Expression and protein purification

Fcy–hEGF–myc-his_6, hEGF–myc-his_6, or Fcy–myc–his_6 were transformed into wild-type X-33 *P. pastoris*, plated on Zeocin-containing (200 μg/ml) agar plates and incubated for 3–4 days until the appearance of colonies. Individual colonies were screened for high expression of the individual proteins, as detected using an antibody against c-myc. For large-scale expression, 0.5 L of BMD medium was inoculated with the selected colony and grown in shaker flasks to an OD_{600} of 8–10. Protein expression was induced with the daily addition of upto 1% methanol. Three days after induction, the protein-containing culture medium was collected and subjected to filtering before being loaded onto a nickel-resin column (Qiagen, Valencia, CA). The column was washed with 10 column volumes of PBS buffer containing 5 mM imidazole, and the bound proteins were eluted with increasing concentrations of imidazole, using an ÄKTAprime plus purification system (GE Healthcare, Piscataway, NJ). The proteins were characterized on SDS–PAGE gels by staining with Coomassie-blue and Western blot analysis using an antibody specific for c-myc.

2.4. In vitro binding of Fcy–hEGF, hEGF, and Fcy to purified EGFR

Purified EGFR (R&D Systems, Minneapolis, MN) was diluted in coating buffer (0.2 M sodium carbonate/bicarbonate pH 9.4, 0.5 μg/mL) and immobilized on an ELISA plate by incubation at 4 °C overnight. Various concentrations of Fcy–hEGF, hEGF, and Fcy (0–50 nM) were incubated with immobilized EGFR at room temperature for 1 h, followed by washing the ELISA plate three times with PBS buffer. The *in vitro* binding of each his_6-tagged proteins with EGFR was detected using an HRP-tagged, anti-his_6 antibody and developed by the addition of the HRP substrate (100 μL/well), 3,3′,5,5′-tetramethylbenzidine (TMB). The peroxidase reaction was stopped 30 min after the addition of 0.5 M H_2SO_4 (50 μL/well), and the absorbance was measured at 450 nm with a multichannel microtiter plate reader.

2.5. In vitro enzymatic activity of Fcy–hEGF and Fcy

The enzymatic activities of Fcy–hEGF and Fcy were determined by measuring the production of 5-FU in the presence of 5-FC. Ten picomoles of either Fcy–hEGF (0.28 μg) or Fcy (0.20 μg) was mixed with increasing concentrations of 5-FC (0, 0.03, 0.1, 0.3, 1, and 3 mM) in 1 ml PBS buffer to initiate the conversion of 5-FC to 5-FU at room temperature. Two microliters of the reaction was collected every three minutes and the fluorescent intensities of 5-FC and 5-FU were measured using a *NanoDrop* 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). The absorbance at 255 and 290 nm was used to calculate the concentrations of 5-FU and 5-FC, using a formula deduced by Senter et al. [6]: [5-FU] mM = 20 × (0.185 × A255 − 0.049 × A290); [5-FC] mM = 20 × (0.119 × A290 − 0.025 × A255). The rates of 5-FU production and 5-FC depletion under various conditions of either Fcy–hEGF or Fcy admixed with increasing concentrations of 5-FC were used to calculate the V_{max} and k_m.

2.6. Fcy–hEGF, hEGF, and Fcy binding to EGFR-expressing cells

The expression level of EGFR in A431, MDA-MB-468, MDA-MB-231, and MCF-7 cells was analyzed with Fluorescence Activated Cell Sorting (FACS). Cell surface EGFR was detected with an anti-EGFR antibody, cetuximab (erbitux), which was subsequently bound by a FITC-tagged goat anti-human IgG antibody. To demonstrate the binding ability of purified Fcy–hEGF, hEGF, and Fcy to cell surface EGFR, A431, MCF-7, MDA-MB-468, and MDA-MB-231 cells were incubated with the indicated his_6-tagged protein for 1 h. A FITC-labeled antibody specific for his_6-tag was later incubated with the cells, which were subjected to FACS analysis. The fluorescent intensities represent the amount of EGFR detected by erbitux or his_6-tagged proteins on each cancer cell line.

2.7. MTT assays for the measurement of cell viability

A431, MDA-MB-468, MDA-MB-231 and MCF-7 (5000 cells/well) cells were incubated with combinations containing different concentrations of 5-FC and Fcy–hEGF, Fcy, or hEGF. Two groups were tested at different incubation times with the proteins. One group was treated continuously with both 5-FC and the indicated proteins, whereas the proteins were removed from the other group after 1 h of incubation by washing with PBS three times before the addition of 5-FC. Cells were plated in a 96-well plate and subjected to the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay 3 days after the addition of the indicated proteins and 5-FC. Twenty-five microliters of MTT solution (5 mg/mL in PBS) was added to the cells. After a 2-h incubation, MTT was removed and the cells were washed with PBS, followed by addition of 0.1 mL of the extraction buffer (20% sodium

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