Biochemical and Biophysical Research Communications 428 (2012) 292–297

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Keng-Hsueh Lan [a], Yi-Sheng Shih [b], Cheng Allen Chang [c,d], Sang-Hue Yen [b,c], Keng-Li Lan [b,c,*]

[a] Division of Radiation Oncology, Department of Oncology, National Taiwan University Hospital, Taipei 100, Taiwan
[b] Cancer Center, Taipei Veterans General Hospital, Taipei 112, Taiwan
[c] Department of Biomedical Imaging and Radiological Sciences, National Yang-Ming University, Taipei 112, Taiwan
[d] School of Biomedical Science and Engineering, National Yang-Ming University, Taipei 112, Taiwan

ARTICLE INFO

Article history:
Received 9 October 2012
Available online 22 October 2012

Keywords:
Epidermal growth factor (EGF)
Epidermal growth factor receptor (EGFR)
Cytosine deaminase
Fusion protein
5-Fluorocytosine (5-FC)

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.

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

* Corresponding author. Address: Cancer Center, Taipei Veterans General Hospital, No. 201, Shih-Pai Road, Pei-Tou District, Taipei City 112, Taiwan. Fax: +886 2876 0606.
E-mail address: kllan@vghtpe.gov.tw (K.-L. Lan).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.10.050

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 = $20 \times (0.185 \times A255 - 0.049 \times A290)$; [5-FC] mM = $20 \times (0.119 \times A290 - 0.025 \times A255)$. 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

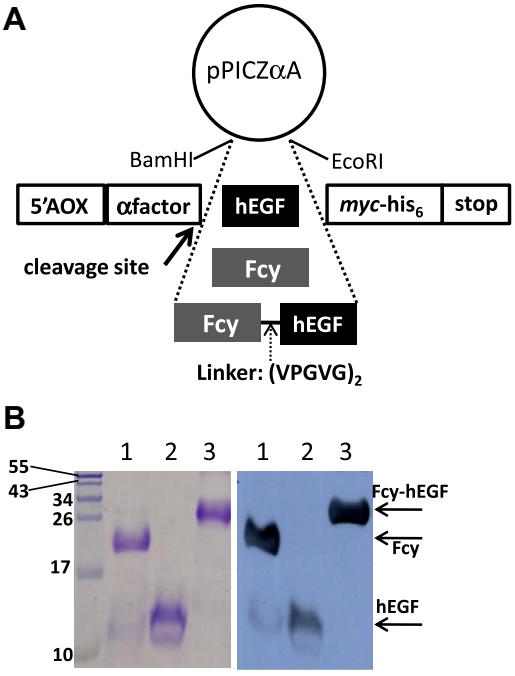

Fig. 1. Schematic diagram and purification of proteins. (A) The genes encoding Fcy–hEGF, hEGF–myc–his_6, and Fcy were cloned into the yeast vector, pPCIZ-αA, and digested with the restriction enzymes, BamHI and EcoRI. (B) Coomassie staining (left panel) and Western blot analysis (right panel) of purified Fcy (lane 1), hEGF (lane 2) and Fcy–hEGF (lane 3) proteins.

dodecyl sulfate in 50% dimethyl formamide). After a 4-h incubation at 37 °C, the optical densities were measured at 570 nm, and the extraction buffer alone served as a blank.

2.8. Data analysis

Data are given as a means (±SEM) of at least two independent experiments. The results were analyzed by ANOVA, and the significance of differences between each treatment group was determined using Newman–Keuls multiple comparison method. Data fitting and statistical analyses were computed using GraphPad Prism (GraphPad Software, San Diego, CA).

3. Results

3.1. Cloning, expression and purification of Fcy–hEGF

To localize the enzymatic activity of Fcy to EGFR-expressing cancer cells, we cloned the coding sequence of a fusion protein, Fcy–hEGF, into a yeast expression vector, pPICZ-αA. The constructs were designed in such a way that an alpha-secreting signaling peptide would be present to assist in the secretion of the desired proteins from the yeast strain, *P. pastoris*, after cleavage of this signal peptide. At the C-terminus, c-myc and hexa-histidine (myc-his_6) tags were added for convenient protein detection and purification. Schematic representations of Fcy–hEGF, hEGF, and Fcy in the pPICZ-αA vector are shown in Fig. 1. Fcy and hEGF are connected with the $(VPGVG)_2$ linker. The host yeast strain, wild-type X-33 *P. pastoris*, was transformed with the plasmids, and high-expressing colonies were selected with Zeocin. Proteins secreted into the medium were then loaded onto a nickel-resin affinity column and eluted with a high concentration of imidazole. The typical yield of Fcy–hEGF was approximately 2–5 mg/L from yeast culture medium, whereas the yields of both hEGF and Fcy were approximately 10–20 mg/L. The purified proteins were detected in Coomassie stained gels (left panel) and Western blots using an antibody specific for c-myc (Fig. 1B).

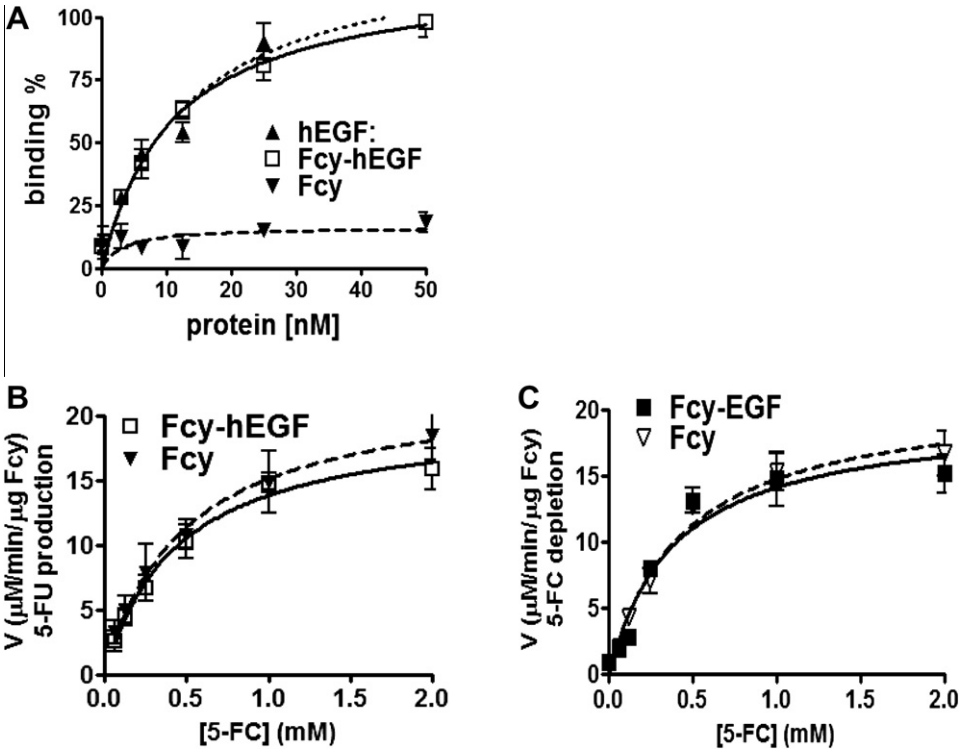

Fig. 2. Bioactivities of Fcy–hEGF. (A) *In vitro* binding of Fcy–hEGF, hEGF and Fcy to purified EGFR immobilized on an ELISA plate was examined using an HRP-tagged anti–his_6 antibody. The binding affinities of Fcy–hEGF and hEGF for EGFR are 11 ± 2 and 13 ± 3 nM, respectively, whereas no discernible coupling between Fcy and EGFR was noted. (B) The rates of 5-FU production by 10 pmol of Fcy–hEGF (0.28 μg) and Fcy (0.20 μg) were determined in the presence of increasing concentrations of 5-FC (0–3 mM). The 5-FU production rates were plotted against the concentrations of 5-FC. Each experiment was repeated in duplicate, in at least three experiments.

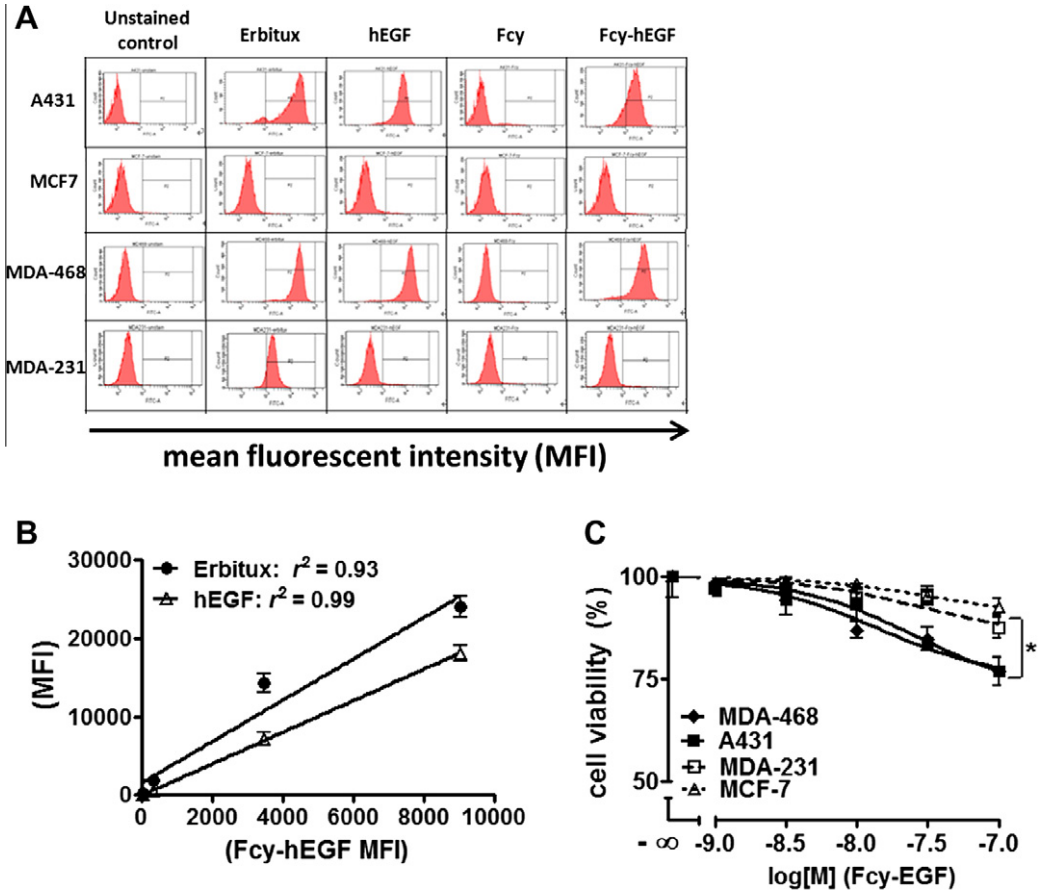

Fig. 3. Fcy–hEGF, hEGF, and Fcy bind to cells with various EGFR expression levels. (A) The expression level of EGFR was analyzed in A431, MDA-MB-468, MDA-MB-231 and MCF-7 cells with FACS using an anti-EGFR antibody, erbitux. The binding of Fcy–hEGF, hEGF, and Fcy to cell surface EGFR was determined using an anti-his_6-tag antibody labeled with FITC. (B) The mean fluorescent intensity (MFI) obtained from the binding of erbitux and hEGF was plotted against that of Fcy–hEGF binding to individual cell lines. (C) Fcy–hEGF alone exhibits suppressive effects on EGFR-overexpressing MDA-468 and A431 cells compared with MDA-231 and MCF-7 cells (*$p < 0.05$). Representative data from three independent experiments are shown.

3.2. Fcy–hEGF retains the characteristics of both Fcy and hEGF

To confirm that Fcy–hEGF still preserves the functional properties of both Fcy and hEGF, we examined the EGFR binding and enzymatic activity of this fusion protein. The binding affinities of Fcy–hEGF and hEGF, for purified EGFR immobilized on an ELISA plate, were similar, whereas no discernible binding of Fcy to EGFR was noted (Fig. 2A). The enzymatic activity of the Fcy moiety of Fcy–hEGF was evaluated by measuring the change in fluorescent intensities contributed by 5-FU. The rates of 5-FU production by both Fcy–hEGF and Fcy were accelerated in the presence of increasing concentrations of 5-FC (Fig. 2B). The deduced V_{max} values of 5-FU production by Fcy–hEGF and Fcy were 20 ± 2 and 22 ± 3 μM/min/μg, respectively, whereas the k_m values were 0.45 ± 0.10 mM and 0.49 ± 0.15 mM, respectively. Likewise, the calculated rates of 5-FC depletion, expressed by the changes in absorbance at 255 and 290 nm, were in agreement with 5-FU production. The V_{max} of 5-FC depletion for Fcy–hEGF and Fcy were 20 ± 2 and 21 ± 2 μM/min/μg, respectively, whereas the k_m values were 0.40 ± 0.10 mM and 0.44 ± 0.09 mM, respectively (Fig. 2C). There was no significant difference in EGFR binding between Fcy–hEGF and hEGF or in enzymatic activity between Fcy–hEGF and Fcy.

3.3. Fcy–hEGF binds to EGFR-expressing cancer cells in the absence of mitogens

We used FACS to examine the binding of Fcy–hEGF to cell surface EGFR. EGFR expression levels on A431, MDA-MB-468, MDA-MB-231 and MCF-7 cells were analyzed using the anti-EGFR antibody, erbitux, which was subsequently bound by a FITC-tagged goat anti-human IgG antibody. The binding of Fcy–hEGF, hEGF, and Fcy to cell surface EGFR was determined using an anti-his_6-tag antibody labeled with FITC (Fig. 3A). The mean fluorescent intensity (MFI) obtained from binding of erbitux and hEGF was plotted against that of Fcy–hEGF binding to individual cell lines. The linear regression exhibited good correlation between the MFI of Fcy–hEGF binding and EGFR detection with the EGFR-specific antibody, erbitux, or the native ligand, EGF (Fig. 3B). MTT assays were performed to evaluate the potential mitogenic effect of Fcy–hEGF on cells with various EGFR-expression levels. Although the EGF moiety contributes to the binding of the Fcy–hEGF fusion protein to EGFR, it does not enhance the proliferation of EGFR-expressing MDA-468 and A431 cells. On the contrary, Fcy–hEGF demonstrates an inhibitory effect on cell proliferation, in a dose-dependent manner, especially in MDA-468 and A431 cells (Fig. 3C).

3.4. The combination of 5-FC and Fcy–hEGF preferentially suppresses the viability of EGFR-expressing cells

The dose-dependent effect of Fcy–hEGF and Fcy, combined with 1 mg/mL of 5-FC, on A431, MDA-MB-468, MDA-MB-231 and MCF-7 cells was evaluated using an MTT assay. We observed a dose-dependent suppressive effect of Fcy–hEGF on both MDA-468 and A431, whereas the inhibitory effect on MCF-7 and MDA-231 reached a plateau at 58% and 40% cell viability, respectively (Fig. 4A). In contrast, the suppressive effects of Fcy did not demonstrate specificity be-

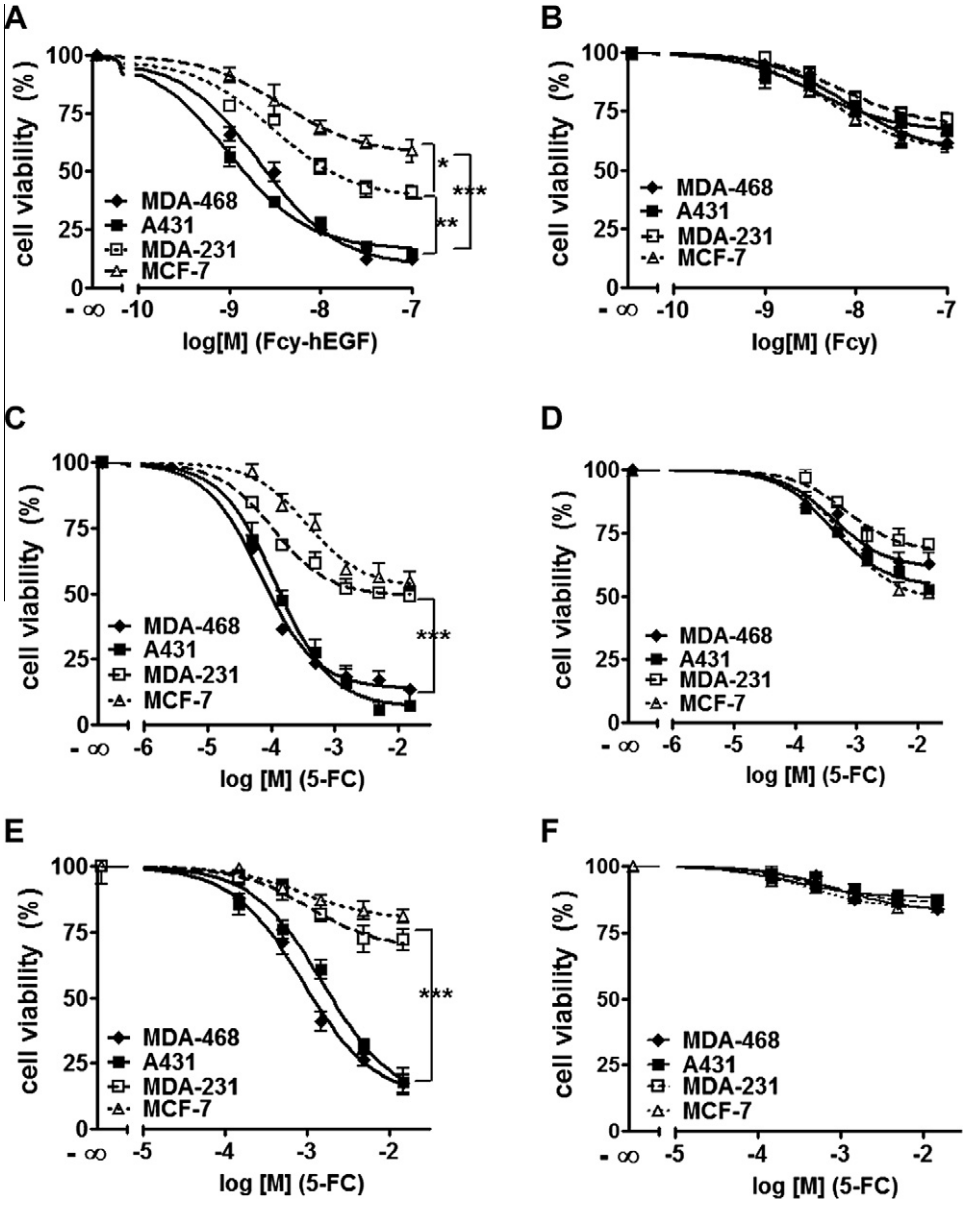

Fig. 4. Fcy–hEGF combined with 5-FC displays a significant inhibitory effect on EGF-overexpressing MDA-468 and A431 cells. (A) In the presence of 5-FC (1 mg/ml), we observed marked dose-dependent inhibition by Fcy–hEGF in MDA-468 and A431 cells, with IC_{50} values of 2.1 and 1.0 nM, respectively. In contrast, the inhibitory effect of Fcy–hEGF on MDA-231 and MCF-7 plateaued at 40% and 58% cell viability (p value of MDA-468/A431 vs. MDA-231 and MCF-7: <0.05 and <0.001, respectively). (B) No discernible difference was detected with increasing concentrations of Fcy on the cell lines tested. (C and D) Dose–response curves of 5-FC were obtained by continuously treating cells with either Fcy–hEGF (C) or Fcy (D). As opposed to the Fcy-treated group, significant differences were observed between cell lines that expressed high (MDA-468 & A431) and low (MDA-231 & MCF-7) levels of EGFR (***$p < 0.005$ in Fcy–hEGF treated group). (E and F) Similar experiments were conducted as in Fig. 4(C) and (D), except the cells were incubated with either Fcy–hEGF (E) or Fcy (F) for only 1 h before washing with PBS and the addition of 5-FC. In the Fcy–hEGF treated group, 5-FC treatment resulted in a more significant suppressive effect on both MDA-468 and A431 cells compared with MDA-231 and MCF-7 cells (***$p < 0.005$). These experiments were conducted at least three times, with similar results.

tween cell lines and was less significant than the effect of Fcy–hEGF (Fig. 4B). MTT assays were also conducted to determine the dose-dependent effect of increasing concentrations of 5-FC in the presence of Fcy–hEGF and Fcy (100 nM). Similar to our previous results, the viability of EGFR-overexpressing MDA-468 and A431 cells displayed the most significant reduction in response to the mixture of 5-FC and Fcy–hEGF. This prodrug system suppressed the viability of MDA-231 and MCF-7 cells to a lesser extent (Fig. 4C). Importantly, this specificity toward EGFR-expressing cells was not observed with Fcy (Fig. 4D). To better demonstrate the inhibitory effect of Fcy–hEGF in conjunction with 5-FC, the medium containing either Fcy–hEGF (Fig. 4E) or Fcy (Fig. 4F) was removed after 1 h of incubation, and the cells were washed with PBS three times before the addition of increasing concentrations of 5-FC. Cell viability was suppressed to a similar extent as was observed in the experiments conducted under the continuous presence of the indicated proteins, as shown in Fig. 4C and D, albeit with a rightward shift of IC_{50} values.

4. Discussion

Given that metastatic epithelial cancers account for a significant portion of all cancer deaths, it is imperative to develop therapeutics that specifically treats this type of cancer. One of the signatures of these epithelial cancers is the overexpression of EGFR, which contributes to many hallmark features of cancer, including resistance to apoptosis, unlimited growth, angiogenesis, unresponsiveness to contact inhibition, etc. [7].

In the present study, we investigated the potential of an Fcy–hEGF fusion protein combined with 5-FC as a therapeutic agent for EGFR-expressing cancer cells. Fcy is the yeast cytosine deaminase (CD), which is capable of converting a less toxic antifungal medicine, 5-FC, into a widely administered chemotherapeutic, 5-FU [8]. Several research groups have obtained promising results while investigating recombinant cytosine deaminase proteins fused with targeting molecules for the tumor or its microenvironment [9–12]. The anticancer effects of the combination of CD/Fcy and 5-FC has also been explored as a prodrug system using gene therapy approaches with different vehicles, ranging from liposomes, anaerobic bacteria, and viruses to human mesenchymal stem cells [13]. However, due to the complex nature of gene therapy, the safety and effectiveness of a variety of carriers and vectors, gene transduction and expression efficiency, and tumor specificity may present challenges for the success of this combination [14].

We cloned the genes encoding Fcy–hEGF or its control proteins, Fcy and EGF, into a *P. pastoris* yeast expression vector. These hexahistidine-tagged proteins were secreted into the culture medium and purified using a nickel-resin column. Fcy–hEGF retained both its ability to bind EGFR and to convert 5-FC to 5-FU, with affinity and enzymatic activity similar to that of EGF and Fcy, respectively. The deduced k_m and V_{max} values of 5-FU production were 0.45 ± 0.10 mM and 20 ± 2 μM/min/μg, respectively, which were similar to those of 5-FC depletion (Fig. 2B and C). The k_m values of Fcy-EGF and Fcy for 5-FC were comparable, while they were smaller than the k_m of 0.8 mM derived from a glutathione-S-transferase (GST)-fused Fcy expressed by *Escherichia coli* [8]. Additionally, a difference in the V_{max} values of Fcy-EGF and Fcy-GST was also noted (20 ± 2 vs. 68 ± 12 μM/min/μg, respectively). This discrepancy might be partly because different hosts were used to express the Fcy–hEGF and Fcy–GST proteins.

The Fcy–hEGF protein binds to EGFR expressed on the cell surface to a similar extent as the EGFR-specific antibody, erbitux, and native ligand, EGF. Several tumor-targeting fusion genes/proteins have been generated by linking cytosine deaminase to molecules with antiangiogenic properties, such as endostatin [15], or those specific for markers of the tumor [11,12,16] and its microenvironment [10]. To our knowledge, the Fcy–hEGF protein produced here is the first attempt to use the native ligand of a receptor abundantly expressed by cancer cells for targeting cytosine deaminase to cancer cells. This approach may offer some advantages compared to previous examples of CD-conjugated molecules. Overexpression of EGFR on cancer cells, such as MDA-468 and A431 [17], could reach well over one million proteins per cell, thereby providing an enormous amount of binding sites for Fcy–hEGF and the potential for generating high local concentrations of 5-FU. Additionally, EGF coupled with EGFR could be internalized into the nucleus [18], where 5-FU exerts its functions. However, the mitogenic effect of Fcy–hEGF might be incurred by the EGF moiety. We examine the potential proliferative effect of Fcy–hEGF on cancer cells with various EGFR expression levels using the MTT assay. The viability of EGFR-overexpressing MDA-468 and A431 cells was unexpectedly suppressed by increasing concentrations of Fcy–hEGF alone, to a level reaching statistical significance compared with those of MDA-231 and MCF-7 cells (Fig. 3C). This distinct effect was not observed while testing the response of cells to Fcy (data not shown).

Although Fcy–hEGF and Fcy exhibited comparable enzymatic activity in cell-free assays, our cell viability assay displayed significantly divergent results when comparing cells that express high and low levels of EGFR, following treatment with 5-FC combined with either Fcy–hEGF or Fcy and 5-FC (Fig. 4A and C vs. B and D). Fcy–hEGF in combination with 5-FC demonstrates a preferential suppressive effect on MDA-468 and A431 cells compared to MDA-231 and MCF-7 cells, even though Fcy, similar to Fcy–hEGF, was continuously present in the culture medium. This result is due to the differential interaction between Fcy–hEGF and the cells expressing high vs. low levels of EGFR rather than a difference in vulnerability to 5-FU, given that no significant difference in the IC_{50} values of 5-FU was observed among these cell lines (data not shown). Furthermore, a significant dose-dependent response to 5-FC was only observed in MDA-468 and A431 cells after a 1-h incubation and removal of Fcy–hEGF, suggesting that Fcy–hEGF/5-FC specifically targets cells that expression high levels of EGFR.

Our results demonstrate the availability of a purified, unique Fcy–hEGF fusion protein that is capable of binding with high affinity to its receptor, EGFR, and converting 5-FC to 5-FU to preferentially inhibit EGFR-overexpressing cancer cells. Thus, this therapeutic agent merits further development and might be a valuable addition to the arsenal of therapeutics targeting EGFR-expressing cancers.

Acknowledgments

This work was supported by Grants from the National Science Council of Taiwan (NSC99-2627-M-010-004 and NSC99-2314-B-075-001-MY3) and the Taipei Veterans General Hospital (V101C-075).

References

[1] F. Ciardiello, G. Tortora, EGFR antagonists in cancer treatment, N. Engl. J. Med. 358 (2008) 1160–1174.
[2] I. Vivanco, I.K. Mellinghoff, Epidermal growth factor receptor inhibitors in oncology, Curr. Opin. Oncol. 22 (2010) 573–578.
[3] C. Ma, S. Wei, Y. Song, T790M and acquired resistance of EGFR TKI: a literature review of clinical reports, J. Thorac. Dis. 3 (2011) 10–18.
[4] G. Giaccone, Y. Wang, Strategies for overcoming resistance to EGFR family tyrosine kinase inhibitors, Cancer Treat. Rev. 37 (2011) 456–464.
[5] A. Vermes, H.J. Guchelaar, J. Dankert, Flucytosine: a review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions, J. Antimicrob. Chemother. 46 (2000) 171–179.
[6] P.D. Senter, P.C. Su, T. Katsuragi, T. Sakai, W.L. Cosand, I. Hellstrom, K.E. Hellstrom, Generation of 5-fluorouracil from 5-fluorocytosine by monoclonal antibody-cytosine deaminase conjugates, Bioconjug. Chem. 2 (1991) 447–451.
[7] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell 144 (2011) 646–674.
[8] E. Kievit, E. Bershad, E. Ng, P. Sethna, I. Dev, T.S. Lawrence, A. Rehemtulla, Superiority of yeast over bacterial cytosine deaminase for enzyme/prodrug gene therapy in colon cancer xenografts, Cancer Res. 59 (1999) 1417–1421.
[9] C.T. Chen, H. Yamaguchi, H.J. Lee, Y. Du, H.H. Lee, W. Xia, W.H. Yu, J.L. Hsu, C.J. Yen, H.L. Sun, Y. Wang, E.T. Yeh, G.N. Hortobagyi, M.C. Hung, Dual targeting of tumor angiogenesis and chemotherapy by endostatin-cytosine deaminase-uracil phosphoribosyltransferase, Mol. Cancer Ther. 10 (2011) 1327–1336.
[10] B.D. Van Rite, R.G. Harrison, Annexin V-targeted enzyme prodrug therapy using cytosine deaminase in combination with 5-fluorocytosine, Cancer Lett. 307 (2011) 53–61.
[11] J.I. Park, L. Cao, V.M. Platt, Z. Huang, R.A. Stull, E.E. Dy, J.J. Sperinde, J.S. Yokoyama, F.C. Szoka, Antitumor therapy mediated by 5-fluorocytosine and a recombinant fusion protein containing TSG-6 hyaluronan binding domain and yeast cytosine deaminase, Mol. Pharm. 6 (2009) 801–812.
[12] S. Zamboni, A. Mallano, M. Flego, A. Ascione, M.L. Dupuis, M. Gellini, S. Barca, M. Cianfriglia, Genetic construction, expression, and characterization of a single chain anti-CEA antibody fused to cytosine deaminase from yeast, Int. J. Oncol. 32 (2008) 1245–1251.
[13] C. Altaner, Prodrug cancer gene therapy, Cancer Lett. 270 (2008) 191–201.
[14] C. Bonini, A. Bondanza, S.K. Perna, S. Kaneko, C. Traversari, F. Ciceri, C. Bordignon, The suicide gene therapy challenge: how to improve a successful gene therapy approach, Mol. Ther. 15 (2007) 1248–1252.
[15] F. Ou-Yang, K.L. Lan, C.T. Chen, J.C. Liu, C.L. Weng, C.K. Chou, X. Xie, J.Y. Hung, Y. Wei, G.N. Hortobagyi, M.C. Hung, Endostatin-cytosine deaminase fusion protein suppresses tumor growth by targeting neovascular endothelial cells, Cancer Res. 66 (2006) 378–384.
[16] V. Coelho, J. Dernedde, U. Petrausch, H. Panjideh, H. Fuchs, C. Menzel, S. Dubel, U. Keilholz, E. Thiel, P.M. Deckert, Design, construction, and in vitro analysis of A33scFv: CDy, a recombinant fusion protein for antibody-directed enzyme prodrug therapy in colon cancer, Int. J. Oncol. 31 (2007) 951–957.
[17] R. Taetle, J.M. Honeysett, L.L. Houston, Effects of anti-epidermal growth factor (EGF) receptor antibodies and an anti-EGF receptor recombinant-ricin a chain immunoconjugate on growth of human cells, J. Natl. Cancer Inst. 80 (1988) 1053–1059.
[18] Y.N. Wang, H. Yamaguchi, J.M. Hsu, M.C. Hung, Nuclear trafficking of the epidermal growth factor receptor family membrane proteins, Oncogene 29 (2010) 3997–4006.