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Gefitinib enhances radiotherapeutic effects of ^{131}I–hEGF targeted to EGFR by increasing tumor uptake of hEGF in tumor xenografts

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ABSTRACT

Gefitinib is an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor which has been proven effective for cancer treatment. In this study, we sought to determine whether gefitinib could increase the in vivo tumor uptake of human ^{131}I–EGF (^{131}I–hEGF), thereby enhancing the potential of hEGF as a vehicle for EGFR-targeted radionuclide therapy. Western blot analysis was conducted to detect the effects of gefitinib on EGFR expression in human head and neck squamous carcinoma cell line UM-SCC-22B. Nude mice bearing UM-SCC-22B tumor xenografts were pretreated via i.p. injection of gefitinib or DMSO (vehicle control), followed by i.v. injection of ^{125}I–hEGF; the animals were then subjected to ex vivo biodistribution or injection of ^{131}I–hEGF for planar γ-imaging using SPECT, respectively. Targeted radionuclide therapy using ^{131}I–hEGF combined with gefitinib as a vehicle targeting EGFR was also performed in UM-SCC-22B tumor xenografts. The EGFR level was unchangeable in cells pretreated with gefitinib, but after gefitinib pretreatment, the uptake of ^{125}I–hEGF in 22B tumor xenografts increased substantially while the uptake of ^{125}I–hEGF in normal organs was effectively unchanged. ^{131}I–hEGF as a vehicle for EGFR-targeting therapy combined with gefitinib therefore showed strong therapeutic effects against 22B tumor xenografts tolerant to gefitinib. The uptake of hEGF to EGFR-positive tumors was enhanced significantly after gefitinib pretreatment, suggesting that ^{131}I–hEGF is a potential vehicle for EGFR-targeting radionuclide therapy when combined with gefitinib.

1. Introduction

The epidermal growth factor receptor (EGFR) family is critical in the etiology and progression of solid tumors. The epidermal growth factor (EGF) and its receptor are involved in the regulation of cellular proliferation, apoptosis, cell motility, and many other cellular processes [1,2]. EGFR is overexpressed in many tumors, including head and neck, renal, lung, glioma, breast, colorectal, prostate, ovarian, bladder, and pancreas, and prompts highly aggressive malignant phenotypes and poor prognoses [3]. Human EGF (hEGF) is a peptide with a molecular weight of about 6 kDa which is a natural ligand to the EGFR. For this reason, EGFR is an attractive therapeutic target – in fact, several hEGF radiopharmaceuticals have been developed to target EGFR-overexpressing cancer cells for tumor therapies.

Therapeutic strategies against EGFR mainly include two types of EGFR inhibitors: The small molecular tyrosine kinase inhibitor (TKI) and the monoclonal antibody (mAb) for the treatment of cancers such as non-small cell lung cancer and colorectal cancer. Gefitinib (ZD1839, Iressa™) is an anilino-quinazoline derivative and a notable EGFR tyrosine kinase inhibitor that undermines EGFR kinase activity by binding the adenosine triphosphate (ATP) pocket of the catalytic domain [4]. Clinical trials of EGFR inhibitors have failed, however, due to low response rates in treating tumors such as those found in breast cancer patients [5]. It is be necessary to study effective strategies to enhance the efficacy of EGFR inhibitors to develop an effective combination therapy to this patient population.

Recent research has shown that gefitinib can increase the amount of EGFR dimers 3.0–3.8 fold in the absence of hEGF in A431 cells [2]. Receptor dimers have higher affinity for the ligand, so in U343 cells, the uptake of ^{211}At-hEGF is markedly increased (up to 3.5 fold) in cells pretreated with gefitinib (1 μM) [6]. In a previous study, we found that gefitinib mediation significantly increases the tumor uptake of hEGF likely due to the increased binding numbers of hEGF to EGFR dimers induced by gefitinib [7]. Gefitinib treatment also increases EGFR Bmax, possibly by membrane stabilization of the inactive receptor dimers in the absence of hEGF [8].

Radionuclide targeted therapy is a promising form of molecular radiotherapy that has recently made the transition from the laboratory bench to the clinic [9]. In this study, we investigated whether ^{131}I–hEGF radiotherapy combined with gefitinib is an effective radiotreatment for EGFR-positive tumors with special focus on gefitinib-nonresponsive tumors.

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2. Materials and methods

2.1. Cell culture and animal models

One cell line was used for the purposes of this study: Human head and neck squamous carcinoma cell line UM-SCC-22B. Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C under a humidified atmosphere containing 5% CO_2. For UM-SCC-22B tumor-bearing animal models, cells (2×10^6) were inoculated s.c. into the right upper flanks at a volume of 100 μL in five-week-old female BALB/c nude mice. All animals were purchased from the Department of Experimental Animals, Peking University Health Science Center (Beijing, China) and were performed in accordance with the Guidelines of Peking University Health Science Center Animal Care and Use Committee. The tumor-bearing animals were utilized once the tumor size reached ~50 mm^3 for radiotherapy studies and ~200 mm^3 for biodistribution and imaging studies.

2.2. Western blotting

Cells were grown in 6-well dishes until subconfluence and then incubated in serum-free medium for 24 h. The monolayer was pretreated with gefitinib at varying doses (0.05 μM, 0.1 μM, 0.5 μM, 1 μM) for 2 h and then treated with hEGF (100 ng/ml) for 15 min. The cells were then collected and lysed in cell lysis buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF) containing protease inhibitors: 1 mM PMSF (Sigma), 1 mg/mL Leupeptin (Sigma) and 1 mg/ml Pepstatin A (Sigma). After the insoluble portion of the lysates was cleared by centrifugation, protein concentration was determined with a BCA Protein Assay Kit (Pierce). Forty micrograms of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose (NC) membrane. The primary antibodies used for western blot analysis were against EGFR (SC-05, Santa Cruz). GAPDH was used as the loading control and the bands were detected using an enhanced chemiluminescent western blotting detection system (GE Healthcare). The films were scanned in grayscale mode and the resulting images analyzed in ImageJ (http://rsb.info. nih.gov/ij/).

2.3. Radiolabeling

Human epidermal growth factor (hEGF, Chemicon International, USA) was labeled with ^{125}I or ^{131}I (Beijing Atom High Tech, Beijing, China) using the Chloramine-T protocol for all experiments. The methods were performed as previously described [10–12].

2.4. Cell activity assay

UM-SCC-22B tumor cells were plated in 24-well plates. The cells were treated with gefitinib alone (10 μM), radiation alone (10 μCi ^{131}I–hEGF) and the combination of gefitinib (10 μM) and radianuclide labeled agents (10 μCi ^{131}I–hEGF) for 24 h at 37 °C. Subsequently, cells were washed with ice 1× cold PBS and their cell activity detected by MTT.

2.5. Planar γ-imaging

Nude mice bearing UM-SCC-22B tumor xenografts were pretreated via i.p. injection of gefitinib (50 mg/kg/day) or DMSO (vehicle control) for 4 days, followed by i.v. injection of ^{131}I–hEGF. The animals were then individually subjected to planar gamma imaging studies. Each animal was injected i.v. with 300 μCi of ^{131}I–hEGF via tail vein after anesthetization, then placed prone on a two-head γ-camera (Siemens, E. Cam) equipped with a parallel-hole, high-energy, and high-resolution collimator. Imaging was conducted at 4 and 24 h post-injection. The mice were euthanized ethically once the imaging process was complete.

2.6. Biodistribution

Nude mice bearing UM-SCC-22B tumor xenografts were pretreated via i.p. injection of gefitinib (50 mg/kg/day) or DMSO (vehicle control) for 4 days, followed by i.v. injection of ^{125}I–hEGF. The animals were then subjected to ex vivo biodistribution assay. UM-SCC-22B tumor-bearing mice (20–25 g) were randomly divided into groups (n = 4/group). Each animal was injected i.v. with 100 μL of ^{125}I–hEGF (5 μCi). At selected time points (2 h, 4 h, 24 h), animals were anesthetized and organs of interest were harvested, weighed, and measured for radioactivity in a gamma counter. Each organ uptake was calculated as the percentage of injected dose per gram of tissue (% ID/g). The results are expressed as mean ± SD (n = 4 per group). To determine the specific accumulation of ^{125}I–hEGF in UM-SCC-22B tumors, four nude mice bearing UM-SCC-22B tumor xenografts were pretreated via i.p. injection of anti-EGFR mAb Vectibix at a volume of 100 μL (500 μg) for 24 h, following i.v. injection of ^{125}I–hEGF (5 μCi), and the biodistribution data (4 h post-injection) was calculated as described above. At the 4 h point, we also performed ex vivo biodistribution of SCC1 tumor xenografts using the same method.

2.7. Targeted radiotherapy

Therapy studies were investigated in UM-SCC-22B tumor-bearing mice with a uniform tumor size of ~50 mm^3 (9 days after inoculation). For the ^{131}I–hEGF-based TR study, the mice were randomly divided into four groups of seven mice each. The mice in the control group were administered DMSO, the mice in the gefitinib-alone treatment group were given i.p. injections of gefitinib (50 mg/kg/day), the mice in the ^{131}I–hEGF-alone treatment group were given a single i.v. dose (400 μCi) of ^{131}I–hEGF via the tail vein, and the mice in the gefitinib and ^{131}I–hEGF combination treatment group were pretreated via i.p. injection of gefitinib (50 mg/kg/day) for 4 days followed by a single dose (400 μCi) of ^{131}I–hEGF by i.v. injection. The tumor size and body weight of the animals were measured daily throughout the experiment. The tumor volume was calculated, assuming the tumors were ellipsoid, as volume = $4/3\pi$ (1/2 length × 1/2 width × 1/2 height). Mice were culled and euthanatized if the tumor size reached >1500 mm^3 or the body weight was reduced >20%. Tumors, livers, and kidneys were isolated after the therapeutic studies and subjected to histopathological analyses. Survival curve plots and Kaplan–Meier analysis were performed in Prism 4.0 software.

2.8. Histologic analysis

The UM-SCC-22B tumors, livers and kidneys of each group mice were obtained after targeted radiotherapy studies to assess their therapeutic efficacy. The tumors were immediately resected and then frozen within OCT medium (Sakura Finetek USA, Inc.) and cut into 5-μm-thick slices for immunofluorescence staining. CD31, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), and Ki67 staining were performed to detect tumor vasculature, tumor apoptosis status, and tumor cell proliferation, respectively. Briefly, frozen tumor sections after TR studies were fixed in cold acetone for 15 min, blocked in 10% donkey serum for 1 h at 37 °C, and incubated in rat anti-mouse CD31 antibody (1:100; BD Biosciences) and rabbit anti-human Ki67 (1:100, NeoMarkers) overnight at 4 °C. The tumor sections were incubated after washing them with PBS, FITC-conjugated goat anti-rat, or Cy3-conjugated donkey anti-rabbit secondary antibodies (1:200; Jackson ImmunoResearch Laboratories). Finally, the slides were mounted and imaged on a Leica TCS-NT confocal microscope. To assess the toxicity of TR, livers and kidneys were fixed with 10% formalin/PBS, embedded in paraffin, and sections 5- to 8-μm in thickness were stained with hematoxylin and eosin (H&E) for routine histopathologic examination.