Cetuximab induce antibody-dependent cellular cytotoxicity against
EGFR-expressing esophageal squamous cell carcinoma

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To evaluate the possibility of treatment with antiepidermal growth factor receptor (EGFR) mAb, Cetuximab against esophageal squamous cell carcinoma (SCC), we performed detail analysis of the antibody-dependent cellular cytotoxicity (ADCC) mediated by Cetuximab against esophageal SCC. Esophageal SCC cell lines with various levels of EGFR ($n = 8$) were evaluated for their Cetuximab-mediated ADCC by $^{51}$Cr-release assay. As a result, Cetuximab was able to induce ADCC against EGFR-expressing esophageal SCC and the activities reflected the degree of EGFR expression on the esophageal SCC. The activities of Cetuximab-mediated ADCC by patients’ PBMC were impaired in comparison with those by healthy donors’ PBMC. Moreover, the inhibition of transforming growth factor (TGF)-$\beta$ could enhance Cetuximab-mediated ADCC against TGF-$\beta$-producing SCC. In conclusion, Cetuximab was able to induce ADCC against EGFR-expressing esophageal SCC. Some modalities aiming at enhancing the Cetuximab-mediated ADCC may be necessary for successful Cetuximab treatment of patients with esophageal SCC.

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Key words: cetuximab; EGFR; esophageal cancer; ADCC; TGF-$\beta$

Esophageal cancer is the sixth most frequent cause of cancer death worldwide. Most patients with esophageal cancer in Japan have squamous cell carcinoma (SCC), while most of those in Western countries have adenocarcinoma. Despite improvements in surgical techniques and perioperative management, and surgery combined with chemotherapy and radiotherapy, the prognosis remains poor. Therefore, for esophageal SCC patients, novel therapies such as molecular targeted therapy including small molecules, and is able to induce the internalization and downregulation of EGFR. Many mechanisms are thought to contribute to the antitumor activity of Cetuximab, including the direct inhibition of EGFR tyrosine kinase activity, the inhibition of cell cycle progression, increased levels and activities of proapoptotic molecules, and enhanced cytotoxicity of chemotherapy and radiotherapy. In addition, it has been shown to inhibit angiogenesis, invasion and metastasis. However, there is no previous report describing the application of Cetuximab for esophageal SCC with a detailed analysis of antibody-dependent cellular cytotoxicity (ADCC) mediated by Cetuximab.

In the present study, we performed detail analysis of Cetuximab-mediated ADCC activity against esophageal SCC cell lines with various levels of EGFR, and furthermore, the synergic effect of Cetuximab and anti-TGF-$\beta$$_2$ (TGF, transforming growth factor) blocking mAb in a SCC cell line producing TGF-$\beta$$_2$.

Material and methods

Cell lines

Esophageal SCC cell lines TE1, TE2, TE3, TE4 and TE5 were a kind gift from Dr. Nishihara (Institute of Development, Aging and Cancer, University of Tohoku, Sendai, Japan). Esophageal SCC cell lines KYSE30, KYSE50 and KYSE110 were purchased from the Health Science Research Resources Bank (Osaka, Japan). Epidermoid carcinoma A431 was obtained from the American Type Culture Collection (Rockville, MD). All cells were cultured in RPMI 1640 medium with 5% fetal bovine serum, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin and 2 mmol/l-glutamine.

Chemicals and antibodies

Humanized mouse anti-human EGFR antibody, Cetuximab (Erbitux) was purchased from Merck (Dietikon, Switzerland), and anti-CD20 mAb Rituxan, which is an isotype-matched control mAb for Cetuximab, was purchased from Roche (Basel, Switzerland). Anti-TGF-$\beta$$_2$ neutralizing antibody was purchased from R&D Systems (Minneapolis, MN). EGFR tyrosine kinase inhibitor, Gefitinib (Iressa) was purchased from AstraZeneca (Osaka, Japan).

Flow cytometry

For the cytometry of EGFR expression by flow cytometry, mouse anti-human EGFR mAb (DakoCytomation) as the primary mAb and a FITC-conjugated polyclonal rabbit anti-mouse mAb as the secondary mAb (DakoCytomation) were used. As a negative control for the primary mAb, mouse immunoglobulin G1 mAb (Beckman-Coulter, Miami, FL) was used. Each step of the incubation with mAb was performed at 4°C for 30 min. After cells were washed twice in PBS, the stained cells were analyzed on a flow cytometer.

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Antibody-dependent cell-mediated cytotoxicity assay

Peripheral blood mononuclear cells (PBMC) were separated from peripheral blood obtained from healthy donors and esophageal SCC patients prior to treatment by centrifugation with Ficoll-Paque (Pharmacia, Uppsala, Sweden). After the target cells were labeled with 50 μCi of 51Cr for 60 min, target cells (5 × 10^5/well) and PBMC from healthy donors or esophageal cancer patients as effector cells were coincubated at various effector/target ratios in 200 μl of X-VIVO medium in a 96-well U-bottomed plate in triplicate with indicated doses of Cetuximab or a control antibody, Rituxan. After 6 hr of incubation, the radioactivity of the supernatant (100 μl) was measured with a γ-counter. The percentage of specific lysis = 100 × (experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm).

Apoptosis

Each cell line (2 × 10^5 cells) was incubated in 2 ml of X-VIVO with Cetuximab (0.5 μg/ml), Gefitinib (0.1 μM), control mAb or Cetuximab (0.5 μg/ml) plus Gefitinib (0.1 μM) at 37°C in a 6-well plate. After incubation for 24 hr, apoptosis in each well was measured by staining with FITC-conjugated Annexin-V and Propidium Iodide (PI) using a MEBCYTO Apoptosis kit (MBL, Nagoya, Japan) following the manufacturer’s recommendations. The populations of early apoptotic cells (annexin-V positive/PI negative) and late apoptotic cells (PI positive) as a percent of total cells were evaluated.

MTT cell proliferation assay

Each cell line (2,500 cells) was incubated in 2 ml of X-VIVO with Cetuximab (0.5 μg/ml), Gefitinib (0.1 μM), control mAb or Cetuximab (0.5 μg/ml) plus Gefitinib (0.1 μM) in a 96-well flat-bottomed plate in triplicate. After incubation for 24 hr, the culture supernatants in each well were collected and tested for their contents using a Quantikine ELISA kit for Human EGF, TGF-α, TGF-β1 or TGF-β2 (R&D Systems) following the recommendations of the manufacturer.

Treatment with anti-TGF-β2 neutralizing mAb of Cetuximab-mediated ADCC

After the target cells were labeled with 50 μCi of 51Cr for 60 min, target cells (5 × 10^5/well) and PBMC were coincubated at various ratios in 200 μl of X-VIVO medium in a 96-well U-bottomed plate in triplicate with or without anti-TGF-β2 mAb (5 ng/ml, R&D Systems) in the presence of Cetuximab (0.5 μg/ml). After incubation for 6 hr at 37°C, the radioactivity of the supernatant (100 μl) was measured with a γ-counter.

IL-2 treatment of PBMC from the patients

PBMC (1 × 10^6 cell/ml) from esophageal cancer patients were incubated with X-VIVO medium in 48-well culture plates in the presence or absence of IL-2 (500 IU/ml, Shionogi, Japan) for 5 days.

Statistics

To evaluate statistical differences between groups, Student’s t test was performed. Significant differences were considered at p < 0.05.

Results

EGFR expression and production of EGF and TGF-α on esophageal SCC

We examined EGFR expression on esophageal SCC cell lines (n = 8) and epidermoid carcinoma A431, which is a well known cell line with EGFR-overexpression by flow cytometry. There were variable levels of EGFR expression on the esophageal SCC, and the levels of KYSE30 and TE3 were comparable to the EGFR-overexpressing A431 cell line (Table I).

The production of EGF and TGF-α, which are ligands for EGFR, were marginal in all tested cell lines (Table I).
The antiproliferative activity and apoptosis-inducing activity of Cetuximab against EGFR-expressing esophageal SCC

To examine the antiproliferative activity and apoptosis-inducing activity of Cetuximab, an MTT assay and Annexin-PI assay were performed for high-EGFR expressing cell lines (KYSE30, TE3 and TE5) and low EGFR expressing cell lines (KYSE110, TE1 and TE4).

Significant inhibition of tumor cell growth induced by Cetuximab was found in cells with high-EGFR expression (TE3 and TE5) at comparable levels observed in A431, while inhibition in cells with low-EGFR expression was marginal (Fig. 1).

As representative data of Annexin-PI assay was shown in Figure 2, significant levels of apoptosis induced by Cetuximab were found in high-EGFR expressing cells (KYSE30, TE3 and TE5) as
well as A431, while the remaining cells did not show apoptosis induced by Cetuximab (Table I). Taken together, the antiproliferative activity and apoptosis-inducing activity of Cetuximab was found in high-EGFR expressing SCC.

Since it has been shown that the combination of Cetuximab with Gefitinib could induce a complementary impact on apoptosis and proliferation, the combination therapy of Cetuximab with Gefitinib for esophageal SCC was performed in MTT and apoptosis assay. As a result, some degrees of additional effect were seen in antiproliferative activity on only TE3 (Fig. 1). However, there was no complementary effect in apoptosis-inducing activity on tested cell lines (Table I).

**Cetuximab-mediated ADCC for esophageal SCC in healthy donors**

To investigate whether Cetuximab induces ADCC against esophageal SCC, Cetuximab-mediated ADCC for esophageal SCC with different levels of EGFR was determined by PBMC isolated from healthy donors (n = 7). Representative data of ADCC indicated that Cetuximab induced much higher cytotoxicity against high-EGFR expressing KYSE30 in comparison to that against low-EGFR expressing TE4 (Fig. 3). Summarized data from healthy donors (n = 7) showed that Cetuximab-induced ADCC reflected the degree of EGFR expression by flow cytometric analysis on the esophageal SCC cell lines (Fig. 4). There was no direct cytotoxicity for the targets using Cetuximab (Fig. 4). Furthermore, there was a dose-dependency of Cetuximab in Cetuximab-mediated ADCC against high-EGFR expressing TE5 (Fig. 5).

**Cetuximab-mediated ADCC for esophageal SCC in the patients**

Next, we examined Cetuximab-mediated ADCC by PBMC isolated from esophageal SCC patients (n = 7) who belong to stage III (n = 5) and IV (n = 2) (UICC-TNM classification). None of the patients received radiotherapy, chemotherapy or other medical interventions during our study.
As a result, Cetuximab also induced ADCC activity by the patients’ PBMC against esophageal SCC and the activities of ADCC reflected the degree of EGFR expression on esophageal SCC (Fig. 6). However, the levels of ADCC by the patients’ PBMC were weaker than those by healthy donors’ PBMC (Figs. 4 and 6). For example, the Cetuximab-mediated ADCC for TE3 in the patients (48.3 ± 13.1 at 40:1 E/T ratio, 29.1 ± 8.0 at 20:1 ratio and 14.9 ± 5.2 at 10:1 ratio) were significantly weaker than those in healthy donors (79.5 ± 13.2 at 40:1 E/T ratio (p < 0.01), 54.2 ± 10.1 at 20:1 ratio (p < 0.01) and 27.5 ± 6.6 at 10:1 ratio (p < 0.02)). Also, the Cetuximab-mediated ADCC for TE1 in the patients (37.6 ± 15.3 at 40:1 E/T ratio, 22.4 ± 11.7 at 20:1 ratio and 13.6 ± 9.6 at 10:1 ratio) were significantly impaired in comparison to those in healthy donors (59.1 ± 5.9 at 40:1 E/T ratio (p < 0.02), 42.3 ± 8.7 at 20:1 ratio (p < 0.02) and 24.1 ± 6.5 at 10:1 ratio (p < 0.03)). These results indicated that the levels of ADCC by the patients’ PBMC were weaker than those by healthy donors’ PBMC.

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Since we previously reported that NK cell dysfunction contributed to impaired Trastuzumab-mediated ADCC in gastric cancer patients,29 we next investigated if the impairment of Cetuximab-mediated ADCC in the patients can be improved by the addition of exogenous IL-2 to activate the NK cells. Representative data showed that the amount of Cetuximab-mediated ADCC by IL-2-treated PBMC was elevated in comparison to that by nontreated PBMC (Fig. 7). These results were confirmed in PBMC derived from 5 different patients with esophageal cancer.

**Treatment with anti-TGF-β2 neutralizing mAb of Cetuximab-mediated ADCC**

Since we reported previously that the inhibition of TGF-β2 enhanced an ADCC activity for cancer cells producing TGF-β2,30 we next investigated if treatment with anti-TGF-β2 neutralizing mAb enhanced Cetuximab-mediated ADCC. The amount of production of TGF-β2 from TE1 (440.9 ± 25.5 pg/ml) was much higher than TE5 (30.2 ± 29.5 pg/ml) and KYSE30 (90.2 ± 8.8 pg/ml). We found that the activities of Cetuximab-mediated ADCC for high TGF-β2 producing TE1 was enhanced by the addition of anti-TGF-β2 neutralizing mAb, while no significant effect for TE5 or KYSE30 was seen (Fig. 8). These observations were confirmed in the different E/T ratio settings (Fig. 8).

Furthermore, the inhibition of TGF-β2 in Cetuximab-mediated ADCC was also confirmed by the exogenous addition of TGF-β2 in the ADCC against non-TGF-β2 producing TE5 cell, in which the Cetuximab-mediated ADCC activities for TE5 in the presence of TGF-β2 (100 pg/ml) were significantly decreased than those in the absence of TGF-β2 (60.3% ± 5.7% vs. 67.7% ± 7.9%, p < 0.05). Collectively, these results indicated that the inhibition of...
TGF-β2 enhanced the Cetuximab-mediated ADCC activity for esophageal SCC producing TGF-β2.

Discussion

The present study contains novel and important findings relevant to the Cetuximab-mediated ADCC against EGFR-expressing esophageal SCC. First, Cetuximab was able to induce ADCC against EGFR-expressing esophageal SCC and the activities reflected the degree of EGFR expression on the esophageal SCC. Second, the activities of Cetuximab-mediated ADCC by patients’ PBMC were impaired in comparison with those by healthy donors’ PBMC. Third, the inhibition of TGF-β could enhance Cetuximab-mediated ADCC against TGF-β-producing SCC. Since we have recently shown that EGFR-overexpression was observed in 50% of esophageal SCCs and was correlated with disease progression, a therapeutic approach with Cetuximab is promising for the treatment of esophageal SCC.

Since EGFR is known to be overexpressed in variety of solid tumors with relatively high incidence and is correlated with the progression of disease,15–18 anti-EGFR targeting therapy is a promising approach for cancer treatment. Recently, 2 anti-EGFR therapies have been in clinical development: small-molecule EGFR tyrosine kinase inhibitors such as Gefitinib and humanized antibodies against EGFR represented by Cetuximab.24,25 Cetuximab (Erbitux), a human-mouse chimerized IgG1 antibody with a high affinity to EGFR, is now approved by the FDA for use in patients with colorectal cancers.35,36 This antibody blocks the binding of ligands, and is able to induce internalization of EGFR, and downregulate EGFR expression,19,20 leading to the direct inhibition of EGFR tyrosine kinase activity,21 the inhibition of cell cycle progression,22,23 increased levels and activities of proapoptotic molecules22,24 and enhanced cytotoxicity of chemotheraphy and radiotherapy.25 As a new finding in the present study, we clearly showed that EGFR-expressing esophageal SCC could be killed by Cetuximab-mediated ADCC. Moreover, the activities of Cetuximab-mediated ADCC reflected the degree of EGFR expression on esophageal SCC. However, the levels of EGFR expression on esophageal SCC lines was not only factor predicting the sensitivity to Cetuximab-induced ADCC, since SCC cells with almost same amount of Cetuximab-induced ADCC had a different level of EGFR expression. It has been shown that Cetuximab-induced ADCC activity did not correlate directly with the levels of EGFR expression in Human tumor xenograft models. These observations suggest that molecular events other than EGFR levels, EGFR such as the combination of Cetuximab with Gefitinib could induce a complementary impact on EGFR-downstream signaling, apoptosis and proliferation. However, the present study failed to show the additional effect induced by the combination of Cetuximab with Gefitinib in antiproliferative activity and apoptosis-inducing activity against esophageal SCC. Alternatively, other studies indicated that combinations of Cetuximab with radiation and chemotherapeutic agents produced synergistic growth inhibition in EGFR-expressing tumors.26 Since esophageal SCC is well known to be sensitive to radiation and chemotherapy, anti-EGFR targeting with Cetuximab combined with chemoradiation may be a promising approach for esophageal SCC patients.

In conclusion, we reported that Cetuximab was able to induce ADCC against EGFR-expressing esophageal SCC and the activities correlated with EGFR expression. These results, some modalities aiming at reversing NK dysfunction might encourage the application of Cetuximab in the treatment of esophageal SCC. Some modalities aiming at enhancing the Cetuximab-mediated antitumor effect may be necessary for the successful Cetuximab treatment of patients with esophageal SCC.

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