

Combining Cetuximab with Killer Lymphocytes Synergistically Inhibits Human Cholangiocarcinoma Cells *In Vitro*

TAKASHI MORISAKI¹, MASAYO UMEBAYASHI¹, AKIFUMI KIYOTA¹, NORIHIRO KOYA¹, HIROTO TANAKA¹, HIDEYA ONISHI² and MITSUO KATANO²

¹Fukuoka General Cancer Clinic, Fukuoka, Japan;

²Departments of Cancer Therapy and Research, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

Abstract. *Aim: We explored the possibility of combining adoptive immunotherapy with cytokine-activated killer (CAK) cells and the epidermal growth factor receptor monoclonal antibody, cetuximab, as a treatment for cholangiocarcinoma. Materials and Methods: CAK cells were cultured with a high-dose of interleukin-2 and anti-CD3 monoclonal antibodies. This cell population contained both activated CD16⁺/CD56⁺ (NK) cells and CD3⁺/NKG2D^{high+} T-cells. The effect of CAK cells and cetuximab, alone and in combination, on the viability of human cholangiocarcinoma cells was evaluated. Results: Culture of CAK cells alone, but not cetuximab alone, exhibited modest cytotoxicity toward cholangiocarcinoma cells. However, combining CAK cells with cetuximab significantly enhanced cytotoxicity. This enhancement was inhibited by the addition of excess human immunoglobulins, suggesting that antibody-dependent cytotoxicity, mediated by activated NK cells in the CAK cell culture was involved in this mechanism. Conclusion: Cetuximab may be used to enhance CAK cell therapeutic activity in patients with cholangiocarcinoma, by potentiating antibody-dependent cellular cytotoxicity.*

Cholangiocarcinoma (CC) originates from the neoplastic transformation of the epithelial cells that line the intra- and extrahepatic bile ducts (1, 2). As CC is difficult to diagnose at an early stage and no effective therapy other than complete resection has been established, its prognosis is very poor (3). The incidence and mortality rates of CC, especially those of intrahepatic CC, are increasing globally (1, 3). Clearly, there is an urgent need for new therapies for this aggressive disease.

Correspondence to: Dr. Takashi Morisaki, Fukuoka General Cancer Clinic, 3-1-1 Sumiyoshi, Hakata-ku, Fukuoka 812-0018, Japan. Tel: +81 922827696, Fax: +81 924056376, e-mail: tmorisaki@cancer-clinic.jp

Key Words: Cetuximab, ADCC, killer lymphocytes, cholangiocarcinoma.

There is no standard chemotherapy option for patients with unresectable or metastatic CC. Recently, gemcitabine was proven to have good efficacy as a single agent for CC with a response rate of around 30% (4, 5). Response rates between 9 and 50% and median survival of 6-16 months have been reported for several gemcitabine combinations, including cisplatin, capecitabine, and oxaliplatin (5-7). However, this rare malignancy is still associated with a poor prognosis.

The epidermal growth factor (EGFR), which is overexpressed in many types of human cancer, including CC, has been associated with poor prognosis (8-10). Accumulating evidence suggests that EGFR-targeting therapy is able to inhibit CC (10, 11). Blocking the interaction between EGFR and its endogenous ligand, and thus inhibiting the downstream signal transduction cascade, can achieve antitumour effects, cell-cycle arrest and inhibition of metastasis. Cetuximab, a monoclonal antibody (mAb), binds to the extracellular domain of EGFR and demonstrates clinical efficacy in treating CC (12). However, *K-Ras* mutations, which are frequently associated with CC cells, may cause cetuximab-resistance (13).

Cytokine-activated killer (CAK) cells include activated natural killer (NK) cells and activated T-cells, both of which have shown clinical benefits when used for adoptive immunotherapy (14-18). CAK cells can be expanded *ex vivo* in the presence of high doses of recombinant human interleukin-2 (IL-2) and anti-CD3 agonistic antibodies (OKT3). The cytotoxicity of both NK cell and T-cell populations is at least partly dependent on the natural killer group 2, member D (NKG2D) and DNAX accessory molecule-1 (DNAM-1) systems (15). Importantly, NKG2D can help NK cells to overcome inhibitory signals (15). Moreover, NK cells exert antibody-dependent cellular cytotoxicity (ADCC) against immunoglobulin-coated cancer cells (16, 17). NK cells have not shown any clinical efficacy on their own (18), but when combined with a mAb, they can provide clinical benefits through antibody-dependent cytotoxicity (ADCC) (19, 20). Activated expanded CD8⁺ T-

cells can also lyse tumour cells through NKG2D systems independently of the TcR-class I system (15).

We previously reported the effects of gemcitabine on the expression of MICA/B RNA and protein in the HepG2 tumour-cell line, and the susceptibility of these cells to cytotoxicity, exerted by activated NK cells and activated T-cells (21). We hypothesized that a combinational approach might be more effective than the use of either CAK cells or mAb alone. In the present study, we tested whether combining cetuximab with CAK cells delivered synergistic cytotoxicity toward CC cells and aimed at determining the underlying mechanism.

Materials and Methods

Reagents. Humanized mouse anti-human EGFR antibody cetuximab (Erbixax™) was purchased from ImClone Systems, Inc. (NY, USA). For cell imaging, Calcein-AM was purchased from Dojindo (Osaka, Japan).

Culture of tumour cells and viability assay. Human cholangiocarcinoma cell lines, Hucct1 cells and OZ cells were obtained from the RIKEN Cell Bank (Osaka, Japan) and were cultured in RPMI medium supplemented with 5% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, USA) and 1% penicillin/ streptomycin (Meiji Seika, Japan). Viable cells were counted using Cell Counting Kit-8 (Dojindo, Osaka, Japan). Cells were treated with different amounts of cetuximab, or with DMSO vehicle control. DMSO concentrations were less than 0.1%. For WST-8 proliferation assays, cells were cultured in 96-well dishes (NUNC) in a volume of 100 µl, at a density of 5×10^4 cells/ml. Assays were performed over 48 h, using a minimum of five replicates. For co-incubation with drugs, cells were treated with different concentrations of cetuximab (100 pg/ml-100 µg/ml). Proliferation assays were performed using the WST-8 cell proliferation kit (Dojindo, Osaka Japan), according to the manufacturer's instructions.

Generation of ex vivo expanded CAK cells. CAK cells were induced from peripheral blood mononuclear cells (PBMCs) obtained from two patients with CC, having acquired written informed consent. Briefly, PBMCs were incubated with a high concentration of recombinant IL-2 (rIL-2, 2000 U/ml; Chiron, USA) and 5 µg/ml OKT3 (Orthoclone® OKT3, Janssen Pharmaceutical, Tokyo, Japan) and expanded for 10-14 days to obtain sufficient numbers of CAK cells.

mAb reagents and flow cytometry. Surface markers on CAK cells were labelled by direct or indirect immunofluorescence using monoclonal antibodies [CD3-FITC, CD16-FITC, CD56-PE; Immunotech, Beckman Coulter, France. NKG2D-PE; R&D Systems, USA]. Fluorescence was detected using an FC500 flow cytometer (Beckman Coulter, France) and expressed as relative mean fluorescence intensity or the percentage above baseline.

Cytotoxicity assay. We modified an adherent target detachment (ATD) assay described previously (21-23), in order to measure the cytotoxicity of CAK cells. Target cells (5,000 per well) were seeded in a 96-well flat bottom plate and incubated for 24 h to allow adherence. CAK cells and cetuximab, alone or in combination, were then added to the wells, and the cells were incubated for a further 24 h prior to the addition of effector cells at an effector:target cell (E:T) ratio of 10:1 or

20:1. Cells were then incubated for 4 h, after which dead target cells were detached from the culture surface by washing and recovered together with the added effector cells. To quantify viable adherent cells, WST-8 reagent solution (from Cell Counting Kit-8) was added to the washed wells and incubated for 1 h at 37°C. The absorbance at 450 nm was then measured using a microplate reader (ImmunoMini NJ-2300; Nalge Nunc International). Detached cells were stained with 7-amino-actinomycin D (7-ADD; Beckman Coulter) to confirm that detached tumour cells were indeed non-viable.

Calcein-release cytotoxicity assay and cell imaging. ADCC assays were also performed using calcein-AM release. Briefly, target cells (OZ and Hucct1) were resuspended at 1×10^5 cells/ml in complete medium and left to adhere to a plastic culture surface. After overnight culture, cells were incubated at 37°C for 1 h in the presence of calcein-AM, before CAK cells and/or cetuximab were added to the culture. After 4 h of incubation at 37°C, the plate was washed and adherent cells were subjected to analysis under a fluorescence microscope driven by the Lumina Vision software (version 2.4.2; Mitani Corp, Fukui, Japan). Images were captured using a conventional fluorescence microscope (IX81; Olympus, Tokyo, Japan), equipped with a colour CCD camera (DPI72; Olympus) and an objective lens (LUC plan FLN; Olympus). All procedures were performed at 20-25°C. Images were analysed using the Lumina Vision software. During observation, cells were warmed to 37°C on a thermoplate (MATS-U55R30; Tokai Hit, Shizuoka, Japan).

Statistical analysis. All data are expressed as the mean±standard error of the mean. Differences between groups were assessed for statistical significance using the Mann-Whitney test or the paired Student's *t*-test, depending on the distribution of the data. $p < 0.05$ was taken to indicate statistical significance.

Results

Effects of cetuximab on CC cell growth. We first examined the effect of cetuximab on the viability of CC cells using the WST-8 assay. Hucct1 and OZ cells were cultured with cetuximab (0.0001, 0.001, 0.01, 1, 10, 100 µg/ml) for 48 h. Treatment with cetuximab had no effect on the viability of either cell line, as shown in Figure 1.

Killing of CC cells by CAK cells alone or in combination with cetuximab. We next investigated whether cultured CAK cells possessed cytotoxicity toward CC cell lines either alone or in combination with cetuximab. CAK cells were obtained by culturing PBMCs in the presence of high-dose IL-2 and OKT-3 for 10-14 days. The cell surface expression levels of FcR IIIa (CD16) and NKG2D were examined from two different donors, as shown in Figure 2. CAK cells from the two donors consisted of two effector populations containing CD16⁺/CD56⁺ NK cells and CD3⁺/NKG2D^{high} TNK cells. We examined the cytotoxic activity of CAK cells against CC using an ATD assay. Although the cytotoxicity of CAK cells alone was modest and varied by donor, cetuximab (at doses of 1 µg/ml and 10 µg/ml) significantly augmented the cytotoxicity of CAK cells in both cases (Figure 3). When

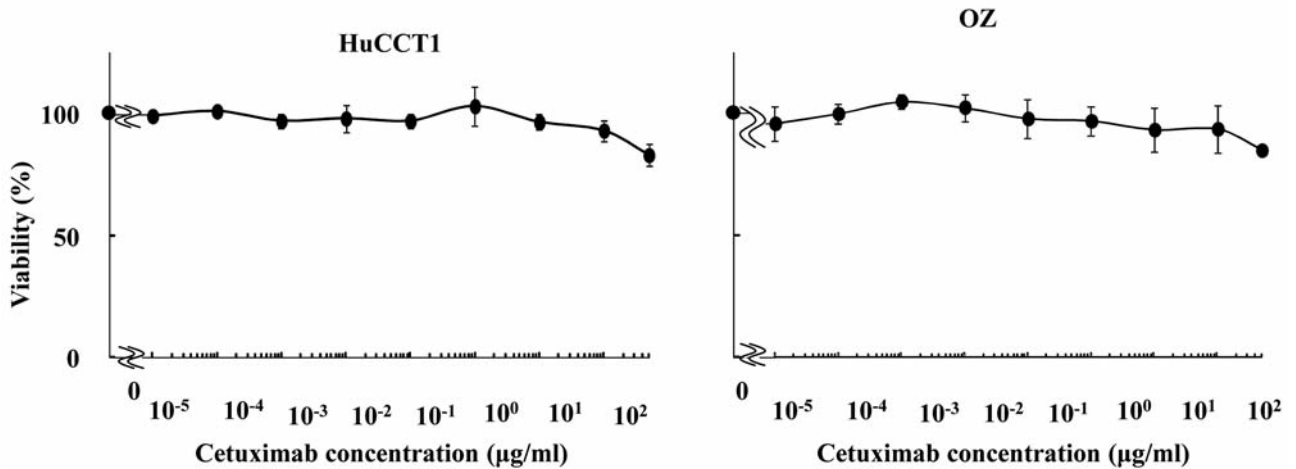


Figure 1. Cetuximab alone does not inhibit the growth of cholangiocarcinoma cells. Cells were treated with gemcitabine at 0.0001-100 µg/ml for 48 h, and growth inhibition was quantified by the WST-8 assay. Left panel: Representative data from Hucct1 cells. Right panel: Representative data from OZ cells.

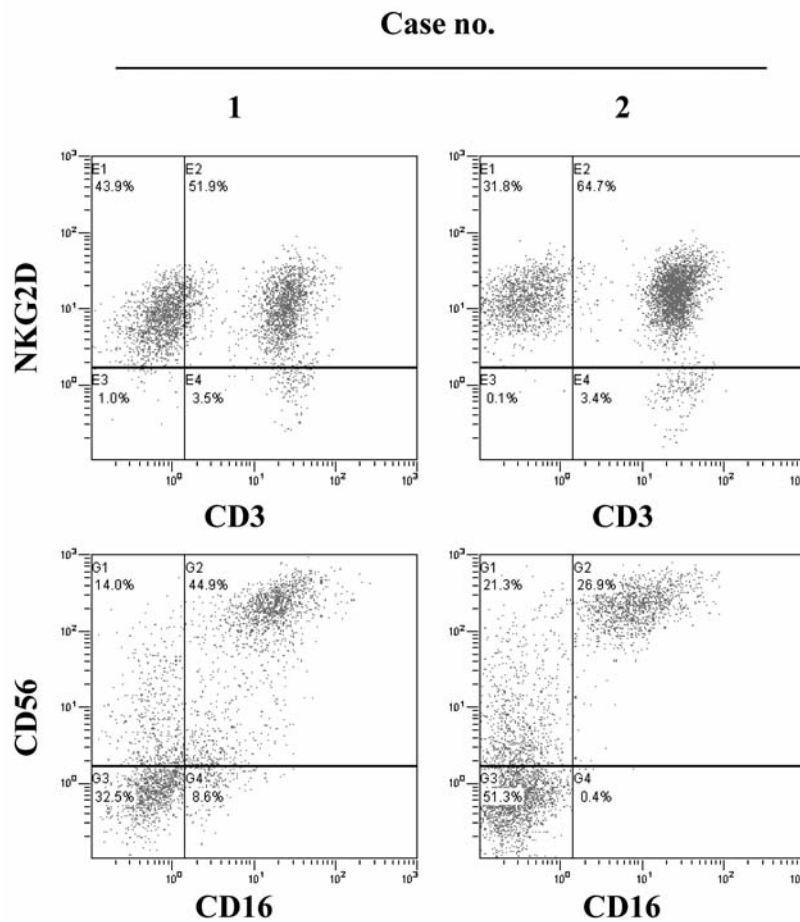


Figure 2. Cell surface antigen expression in cytokine activated killer cells. Human peripheral blood mononuclear cells (PBMC) were treated with high dose IL-2 (2,000 U/ml), and coated with OKT-3 (5 µg/ml). On Day 14 of cell culture, CAK cells were washed and subjected to FACS analysis. Lymphocytes were gated depending on their expression of CD3⁺/NKG2D⁺ and CD16⁺/CD56⁺. The percentages of each of these cell populations in each sample are shown.

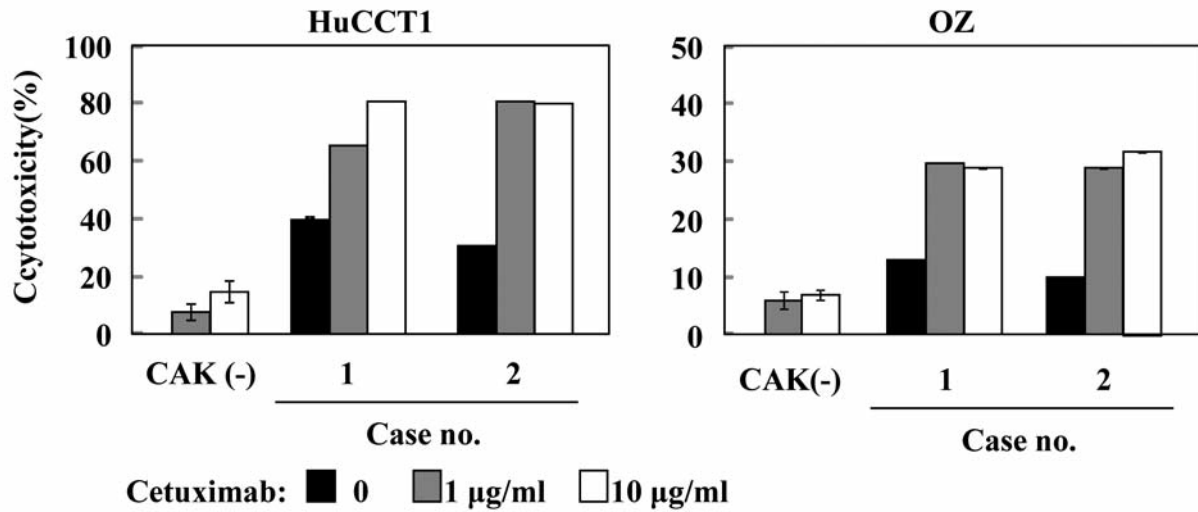


Figure 3. Cytotoxic activity of cytokine activated killer cells against cholangiocarcinoma (CC) cells with and without cetuximab. Adherent target detachment (ATD) assay was used (as described in the Materials and Methods) to detect cytotoxicity of CAK cells against CC cells following treatment of CC cells without and with cetuximab (0, 1, 10µg/ml). The ratio of effector (CAK):target (Hucct1 and OZ) cells was 20:1. All data are expressed as the mean percentage of specific cytotoxicity±SEM. Data are from triplicate wells and are representative of three independent experiments.

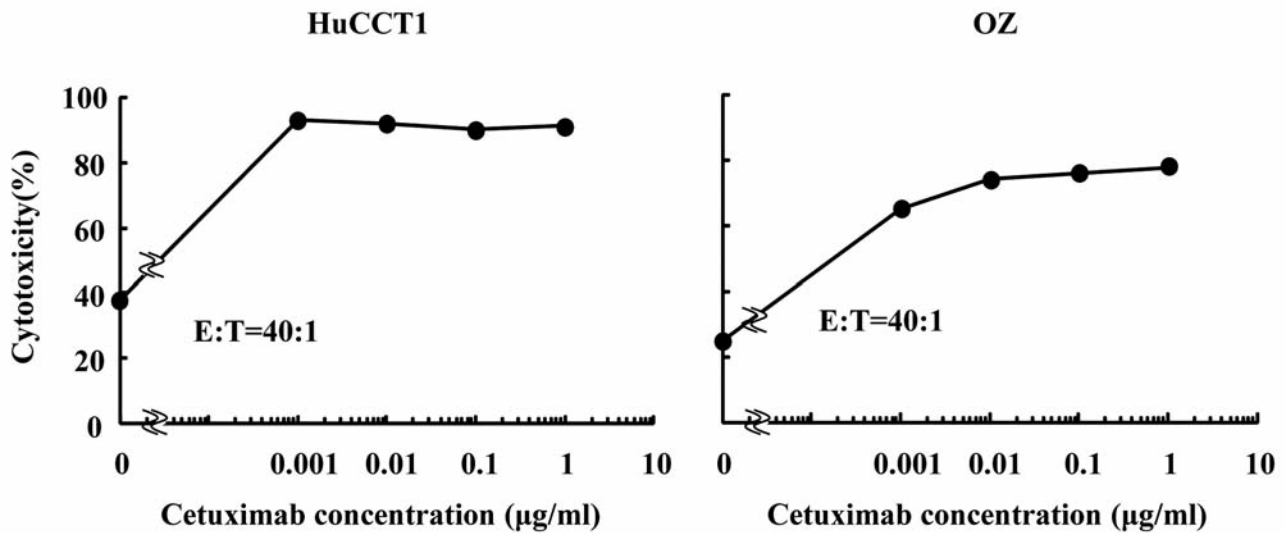


Figure 4. Concentration-dependence of cetuximab-mediated antibody mediated cellular cytotoxicity activity against Hucct1 cell. Hucct1 cells were incubated with CAK cells at an effector (CAK):target (Hucct1) ratio of 20:1, together with various concentrations of cetuximab (0.001-1 µg/ml). Data are representative of three independent experiments, and show mean values from triplicate wells within a single experiment. Error bars denote the standard deviation.

subjected to ADCC testing at increasing doses of cetuximab, we found that doses of 0.001-10 µg/ml significantly increased the susceptibility of both CC cell lines to cytotoxicity by CAK cells, reaching a plateau at 0.01 µg/ml (Figure 4). These data indicate that cetuximab at concentrations in excess of 0.01 µg/ml is sufficient for maximum ADCC activity.

Cell imaging of ADCC of CAK cells in combination with cetuximab toward CC cells. We aimed to confirm the cytotoxic activity of CAK cells using a calcein release and imaging assay. As shown in Figure 5, the augmentation of CAK cell-mediated cytotoxicity by cetuximab was confirmed in both cell lines. The synergistic effect of the cetuximab plus CAK cell therapy was evident in imaging analysis using Lumina Vision.

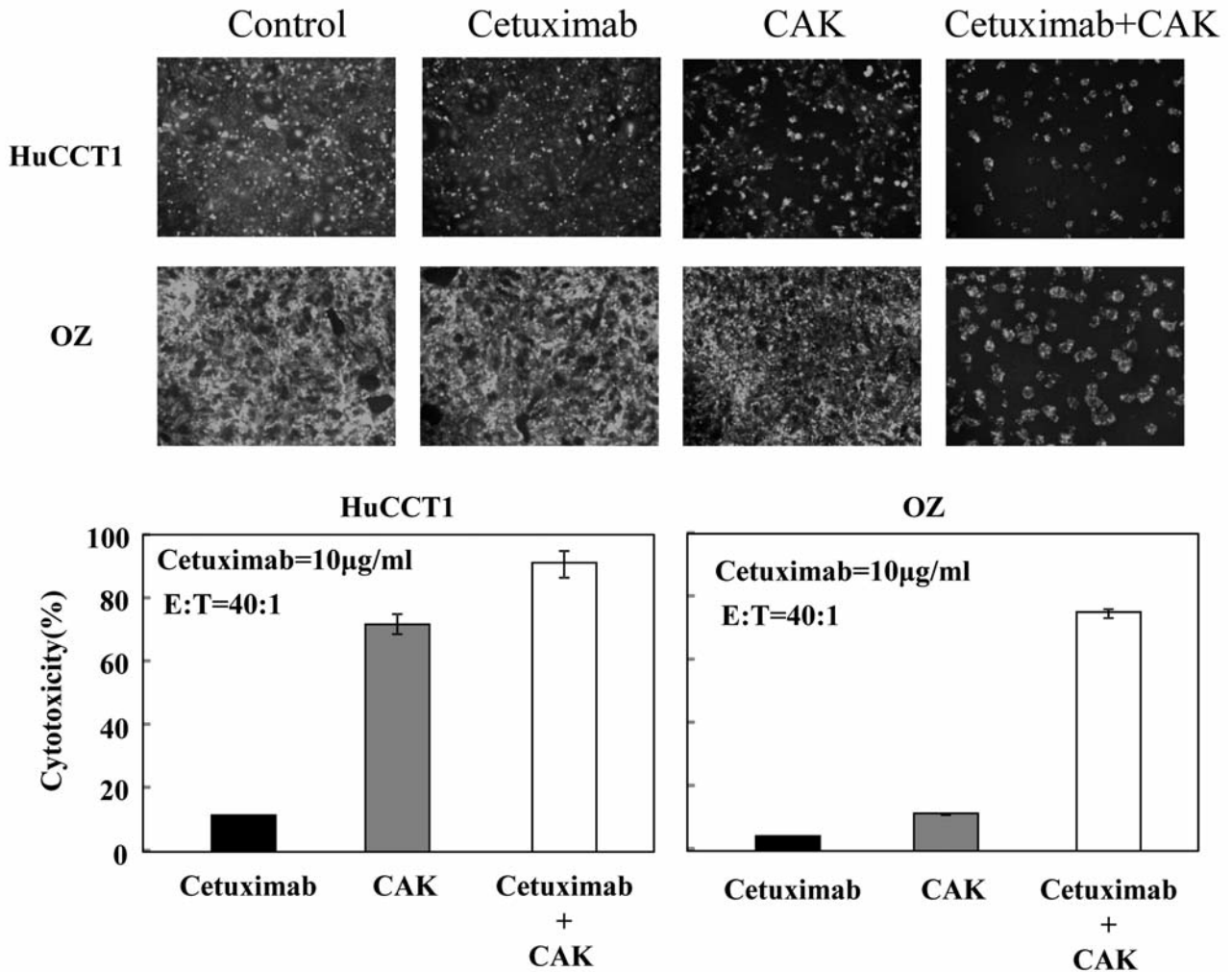


Figure 5. Imaging of cell cytotoxicity toward cholangiocarcinoma cells by cytokine activated killer cells and cetuximab. *Hucct1* or *OZ* cells were stained with calcein-AM, combined with CAK cells and/or cetuximab (10 μ g/ml) and were cultured for 4 h. Cells were then observed by fluorescence microscopy, with images analysed with Lumina Vision, as described in the Materials and Methods.

Effects of soluble polyclonal immunoglobulins on the combined cytotoxicity of CAK cells and cetuximab. Soluble immunoglobulins have been shown to inhibit FcR-mediated ADCC antitumour cell activity (24). Moreover, there is considerable evidence that saturating amounts of immunoglobulins can down-regulate Fc-R expression in NK and monocytes and can suppress FcR-positive cell activation (25). To confirm the role of CAK cell-expressed FcR in cetuximab-enhanced CC cell cytotoxicity, we performed an FcR blocking assay. When cetuximab-FcR interactions were blocked with excess amounts of immunoglobulin, the killing of cetuximab-treated CC cells by CAK cells was significantly reduced to the level seen following treatment with CAK cells alone. These results suggest that ADCC has a role in cetuximab-mediated potentiation of CAK cell cytotoxicity toward CC cells (Figure 6).

Discussion

In this report, we investigated the cytotoxicity of CAK cell cultures toward CC targets in the presence or absence of the anti-EGFR antibody, cetuximab. We showed that the use of CAK cell cultures alone has moderate cytotoxic activity *in vitro* against the CC lines, *OZ* and *Hucct1*. Most of this cytotoxicity can be attributed to the CD3⁺/NKG2D⁺ TNK and CD16⁺/CD56⁺ NK cell populations, which kill the target cells in an MHC-unrestricted manner. Furthermore, we demonstrated that cetuximab significantly enhanced CAK cell-mediated cytotoxicity toward CC cells *via* ADCC. Many studies have demonstrated the ADCC mechanism through which cetuximab induces clinical effects against various types of cancers (26-28), but this is the first to show that cetuximab-induced ADCC is relevant to human CC cells.

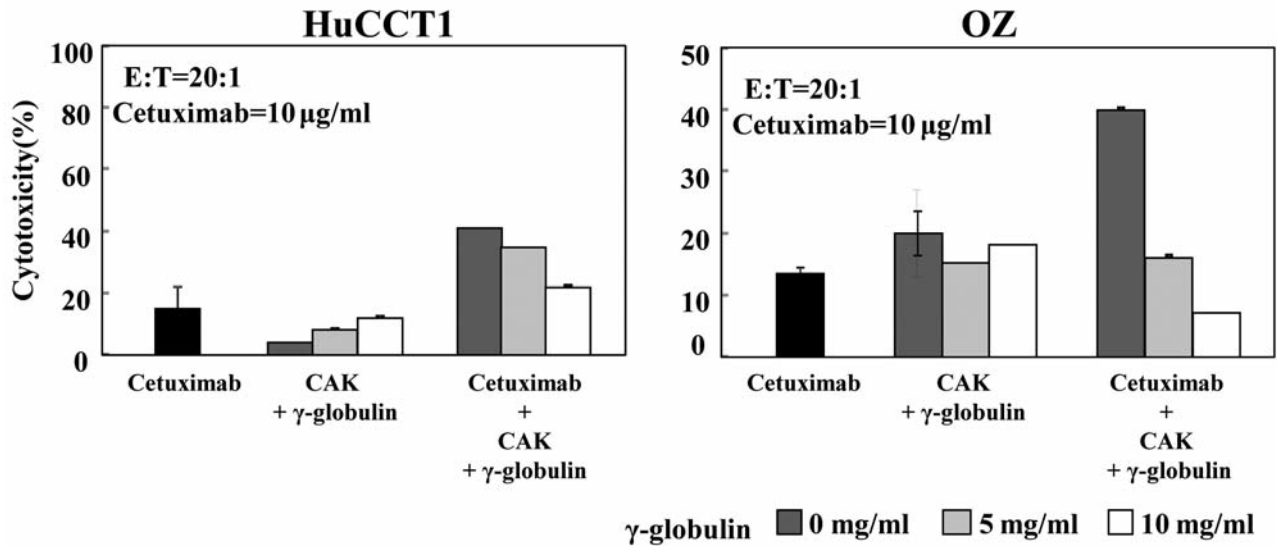


Figure 6. Effect of Fc-receptor lockade on cetuximab-mediated cytokine activated killer cell cytotoxicity. Cholangiocarcinoma cells were pre-treated with excess amounts of soluble immunoglobulins to saturate FcR expressed on those cells. CAK cells were then added to the cholangiocarcinoma cells with or without cetuximab. After 4 h culture, an adherent target detachment assay was performed, as described in the Materials and Methods. Data are expressed as the mean percentage \pm SD of three independent experiments.

In this study, the two CC cell lines were both resistant to cetuximab. In previous studies, it has been shown that cetuximab resistance is associated with K-RAS mutation (13). Recent reports have demonstrated that these two cell lines express EGFR but not at supra-physiological levels (10, 11). Moreover, Hucct1 and OZ CC cells have been reported to possess K-RAS mutations (11). In this study, as we hypothesized, cetuximab alone had no cytotoxicity against OZ and Hucct1 cells. However, cetuximab significantly enhanced CAK cell-mediated cytotoxicity through ADCC, suggesting that even weak expression of EGFR is sufficient to mediate ADCC against CC cells. Recent clinicopathological studies have shown that approximately 20-60% of CCs are positive for EGFR expression (10, 29). Thus, clinical trials of EGFR-targeting therapies such as cetuximab show significant promise. Indeed, Gruenberger *et al.* reported that combined chemotherapy with gemcitabine, oxaliplatin, and cetuximab in a phase II trial had encouraging antitumour activity (12).

The CAK cells used in this study were a heterogeneous population including activated NK cells and activated T-cells. The cytotoxicity of both cell populations is at least partly dependent on the NKG2D-MICA/B systems. Importantly, NKG2D can help NK cells to overcome inhibitory signals, and substantially enhances the cytotoxicity of activated T-cells against tumour cells independently of any interaction with TcR-class I (30). Activated expanded CD8⁺ T-cells can also lyse tumour cells through NKG2D systems, independently of the TcR-Class I system (30, 31),

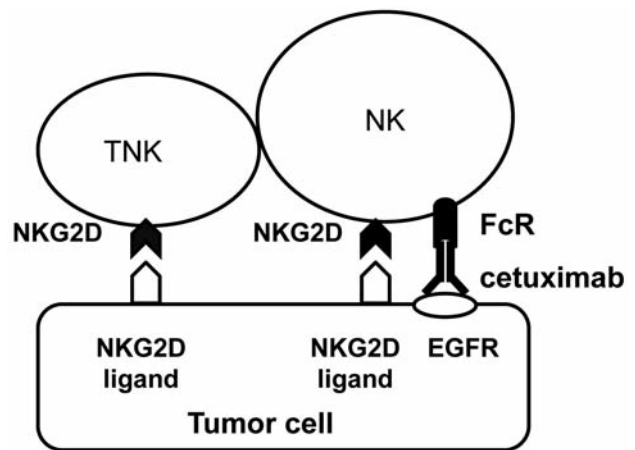


Figure 7. Multiple mechanisms of cholangiocarcinoma cell killing by cytokine activated killer cell cultures in the presence of cetuximab. CAK cells consist of CD3⁺NKG2D^{high} T-cells and NKG2D⁺CD16 (FcR11a)⁺ natural killer cells. CAK cells kill cholangiocarcinoma cells via HLA-independent mechanisms involving NKG2D systems. In the presence of cetuximab, antibody dependent cellular cytotoxicity may also occur in activated NK cells present in CAK cultures.

demonstrating that NK, $\gamma\delta$ T- and activated expanded CD8⁺ T-cells can all act *via* the NKG2D system and independently of interactions with the TcR-HLA class I system. Thus, the cytotoxic activity of CAK cell cultures alone may be associated with NKG2D expression

We have shown that cetuximab actions *via* interaction with FcR enhance the susceptibility of CC tumour cells to the cytotoxic actions of CAK cells. We conclude from this result, that the interaction between cetuximab and its receptor in CAK cell-mediated lysis of CC cells, and thus the increased susceptibility of cetuximab-treated cancer cells to CAK cell-induced cytotoxicity, may be mediated by the tight binding of cetuximab to FcR. Given the extensive distribution of FcR and NKG2D in activated immune cells (*e.g.* NK, $\gamma\delta$ T-cells) and the antitumour significance of interactions between NKG2D and its cognate ligands, it is reasonable to predict that cetuximab may augment CAK cell immunotherapies.

In conclusion, we have shown that the use of CAK cells in combination with cetuximab exerts significant cytotoxic activity towards CC cells *in vitro*. CAK cells could be combined with cetuximab to offer a novel immunotherapy paradigm for the treatment of CC.

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Received April 4, 2012

Revised May 16, 2012

Accepted May 17, 2012