Innate Immunity

http://ini.sagepub.com

Cetuximab-mediated cellular cytotoxicity is inhibited by HLA-E membrane expression in colon cancer cells

Estrella Mariel Levy, Gabriela Sycz, Juan Martin Arriaga, María Marcela Barrio, Erika María von Euw, Sergio Bayo Morales, Mariana González, José Mordoh and Michele Bianchini

Innate Immunity 2009; 15; 91
DOI: 10.1177/1753425908101404

The online version of this article can be found at:
http://ini.sagepub.com/cgi/content/abstract/15/2/91

Published by:
SAGE
http://www.sagepublications.com

On behalf of:
International Endotoxin & Innate Immunity Society

Research article

Innate Immunity

15(2) (2009) 91–100

ISSN 1753-4259 (print)
10.1177/1753425908101404

Cetuximab-mediated cellular cytotoxicity is inhibited by HLA-E membrane expression in colon cancer cells

Estrella Mariel Levy[1], Gabriela Sycz[1], Juan Martin Arriaga[1], María Marcela Barrio[1], Erika María von Euw[1], Sergio Bayo Morales[3], Mariana González[4], José Mordoh[1,2], Michele Bianchini[1]

[1]*Centro de Investigaciones Oncológicas (CIO–FUCA), Buenos Aires, Argentina*
[2]*Fundación Instituto Leloir, IIBBA – CONICET, Buenos Aires, Argentina*
[3]*Hospital Municipal Dr Bernardo Houssay, Buenos Aires, Argentina*
[4]*Academia Nacional de Medicina, IIHEMA, Buenos Aires, Argentina*

Cetuximab, an anti-epidermal growth factor receptor monoclonal antibody, has been shown to increase the median survival of colorectal cancer patients. We previously reported that the expression of HLA-E is significantly increased in primary human colorectal cancer, perhaps contributing to tumour escape from immune surveillance. To establish if HLA-E could be a factor that renders colorectal cancer cells less susceptible to antibody-dependent cellular cytotoxicity (ADCC), in the present study we analysed Cetuximab-mediated cytotoxicity against several colorectal cancer cell lines expressing, or not, HLA-E at the cell surface. We first observed that colorectal cancer cells treated with Cetuximab were killed more efficiently by ADCC. Interestingly, treatment of target cells with recombinant human-β_2-microglobulin inhibits Cetuximab-mediated ADCC through HLA-E membrane stabilization. The specific immunosuppressive role of HLA-E was confirmed using an anti-NKG2A monoclonal antibody, that restored the ability of immune cells to kill their target. This result demonstrates that HLA-E at the cell surface can reliably suppress the ADCC effect. On the other hand, Cetuximab induced a direct growth inhibition but only at high concentrations; furthermore, the CDC effect was quite moderate, and we failed to observe a pro-apoptotic effect. Taking into account that our findings suggest that ADCC activity is the main anti-tumour effect observed at clinically achievable concentrations of Cetuximab at the tumour site, we suggest that determination of HLA-E in colorectal cancer could be relevant to predict success of Cetuximab treatment.

Keywords: Cetuximab, colorectal cancer, antibody-dependent cellular cytotoxicity, HLA-E

Introduction

Colorectal cancer is one of the most common malignancies in the Western world, and remains a major cause of cancer-related death in both men and women.[1] After surgery for locally advanced primary tumour (Dukes' B and C), 5-fluorouracil-based adjuvant chemotherapy and radiation therapy significantly improve the overall survival rate; however, at 5 years, 40–50% of patients will still die of the disease.[2,3] Virtually all patients with metastatic stage IV colorectal cancer will die of their cancer.

Toxicity and lack of tumour specificity are the most important limits of conventional approaches, such as chemotherapy and radiation therapy.[4] Novel therapeutic options such as immunotherapeutic approaches, which exploit the naturally occurring defence system, and are capable of evoking tumour-specific immune responses,

Received 20 October 2008; Revised 27 November 2008; Accepted 30 November 2008

Correspondence to: Michele Bianchini PhD, Centro de Investigaciones Oncológicas (CIO–FUCA), Zabala 2836, 1426 Buenos Aires, Argentina. Tel: +54 11 63232900 ext. 1114; Fax: +54 11 6323299; E-mail: biomike72@hotmail.com

are being investigated.[5] The availability of new biological agents such as monoclonal antibodies for the treatment of patients with metastatic disease has led to improved survival.[6] Since epidermal growth factor receptor (EGFR) is overexpressed in a variety of solid tumours and it is correlated with the progression of the disease, targeting EGFR is a promising approach for cancer treatment.[7] Cetuximab (Erbitux®, Merck), a human–mouse chimeric IgG_1 antibody with a high affinity for EGFR,[8] has now been approved for use in patients with colorectal cancer.[9] Many mechanisms are thought to contribute to the antitumour activity of Cetuximab, including the direct inhibition of EGFR tyrosine kinase activity,[10] the inhibition of cell cycle progression,[11,12] increased levels and activities of pro-apoptotic molecules[13] and enhanced cytotoxicity of chemotherapy and radiotherapy.[14] In addition, it has been shown to inhibit angiogenesis, invasion and metastasis[15,16] and mediate antibody-dependent cellular cytotoxicity (ADCC).[17]

However, EGFR expression alone is not the only determinant of Cetuximab response; recent findings report that all patients with K-RAS point mutations are resistant to the treatment.[18] On the other hand, only 40% of patients with wild-type K-RAS respond to Cetuximab therapy; this observation implies that, for the remaining 60% of patients, other unknown mechanisms involved in resistance to Cetuximab therapy exist and require investigation.

In this regard, we previously demonstrated that HLA-E expression at the cell membrane of colon cancer cells prevents NK lytic activity. In addition, we also demonstrated that patients whose tumours had a high percentage of HLA-E positive cells were associated with shorter disease-free survival than those whose tumours had low HLA-E.[19] HLA-E is a non-polymorphic MHC class I (class Ib) molecule that is specifically recognized by CD94/NKG2A heterodimer; this receptor is a type II transmembrane protein of the C-type lectin-like family, mainly expressed on NK and NKT cells.[20,21] Its interaction with HLA-E transmits an inhibitory signal that reduces NK cytotoxicity.[22] Our hypothesis is that patients with low expression of HLA-E may be candidates for antibody therapies such as Cetuximab, since their tumour cells should be more susceptible to NK lysis. In contrast, patients with high levels of HLA-E may not be good responders to ADCC-mediated killing. To establish if HLA-E expression could be a factor that renders colorectal cancer cells less susceptible to Cetuximab-mediated ADCC, in the present study we analysed the ADCC activity against several EGFR-positive colorectal cancer cell lines, expressing different amount of HLA-E at the cell surface.

Materials and Methods

Cell lines

Five colorectal cancer cell lines were obtained from the American Tissue Culture Collection (ATCC): HCT-116, Caco-2, HT-29, DLD-1 and T84 (derived from lung metastasis). All cell lines were grown at 37°C in a humid atmosphere containing 5% CO_2 with Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies), supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 3.5 mg/ml sodium carbonate and 4.5 mg/ml glucose (Colon Medium).

Immunofluorescence analysis by FACS

Indirect immunofluorescence was performed on colorectal cancer cell lines using a specific mouse anti-human monoclonal antibody for HLA-E (clone MEM-E/08; BioVendor, Czech Republic) or HLA-Ia (clone W6/32; Dako Cytomation) and a human–mouse chimerized IgG_1 antibody for EGFR (Cetuximab; Merck); as negative controls, we used matched isotype antibodies (mouse IgG_1-k [BD Biosciences, Pharmingen] or human IgG [Dako Cytomation]). Primary antibodies were incubated for 45 min at 4°C. After washing, cells were incubated for 30 min with a secondary anti-mouse FITC-labelled for HLA-E or anti-human FITC-labelled for EGFR (Dako Cytomation) and analysed on a FACSCalibur flow cytometer using the CellQuest software (BD Biosciences) for data analysis. Intensity was calculated with the formula: mean intensity level (MIL) = % positive cells × mean fluorescence intensity.

MTT Cell viability assay

Each cell line (HCT-116 and DLD-1, 7500 cells; T84, 10,000 cells; HT-29, 6000 cells; and CACO-2 7000 cells) was incubated in 200 µl of Colon Medium with Cetuximab (concentrations ranging from 1×10^{-3} to 1×10^{2} µg/ml) or anti-CD20 mAb (Rituximab, MabThera/Rituxan, Roche), in a 96-well flat-bottomed plate in triplicate. After incubation for 96 h at 37°C, 100 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 2 mg/ml; Sigma, St Louis, MO, USA) diluted in colon medium were added to each well and incubation was carried out for 90 min. Then, the supernatant was discarded and the crystal products were eluted with isopropyl alcohol (200 µl/well; Sigma, St Louis, MO, USA). Colorimetric evaluation was performed with a spectrophotometer at 570 nm. The inhibition of proliferation was shown as percentage of cell growth induced by Cetuximab in comparison with that induced by control mAb.

Apoptosis

To evaluate concentration-dependent apoptosis in the presence of Cetuximab (1, 10 and 100 μg/ml), each cell line (1.5×10^5 cells) was incubated in 2.5 ml of Colon Medium; Rituximab (10 μg/ml) was used as irrelevant antibody. After incubation for 24 h at 37°C, apoptosis was measured by staining with FITC-conjugated Annexin-V and propidium iodide using the Annexin V–FITC Apoptosis Detection Kit I (BD Biosciences), following the manufacturer's recommendations. The populations of early apoptotic cells (annexin-V positive/propidium iodide negative) and late apoptotic cells (annexin-V positive/propidium iodide positive) as a percentage of total cells were evaluated. To evaluate time-dependent apoptosis, each cell line (1.5×10^5 cells) was incubated in 2.5 ml of Colon Medium in the presence of Cetuximab (10 μg/ml) or Rituximab (10 μg/ml), as irrelevant antibody. After incubation at 37°C for 24 h, 48 h or 72 h, apoptosis in each cell line was measured as before.

Quantitative real-time PCR (qPCR) measurement of BAX and BCL-xL

Total RNA was recovered from both human neutrophils (from 10 healthy donors) and colorectal cancer cells using Trizol reagent (Invitrogen Technologies) following the manufacturer's instructions. The cDNA was synthesized from 2.5 μg of total RNA using 200 U of M-MLV reverse transcriptase (Promega, Madison, WI, USA) and 2 μg of random primers (Biodynamics) at 37°C for 1 h. For the detection of BAX and BCL-xL, LC-FastStart DNA Master SYBR Green I Kit (Roche Diagnostic) was used in a Roche Light Cycler 2.0 instrument. Duplicate experiments were conducted using the following primers: BAX forward, 5′-GGGACGAACTGGADAGTAACA-3′; BAX-reverse, 5′-CCGCCACAAAGATGGTCAC-3′; BCL-XL forward, 5′-ACTGTGCGTGGAAAGCGTAG-3′; BCL-xL reverse, 5′-GGTTCTCCTGGTGGCAATG-3′. We calculate the relative expression of transcripts as the ratio between the level of BAX and the level of BCL-xL to determine the sensitivity to apoptosis of tumour cells.

Complement dependent cytolysis assay

Target cells grown under regular culture conditions were harvested and resuspended in Colon Medium without FCS at a concentration of 1×10^6 cells/ml of medium. Calcein-acetyoxymethyl (Calcein-AM) purchased from Molecular Probes (Invitrogen Life Technology, USA) as a 1 mg/ml solution in dry dimethyl sulphoxide was added to a final concentration of 10 μM and the cells were incubated for 30 min at 37°C. Cells were then washed twice in 1× PBS and resuspended in prewarmed colon medium (37°C) to a final concentration of 62,500 cells/ml. To each 160 μl of cell suspension, 20 μl of cold human serum (obtained from healthy donors) and 20 μl of 1 : 2 serial dilutions of Cetuximab were added. In all experiments, 4 concentrations of Cetuximab ranging from 10^{-1} to 10^2 μg/ml, plus a blank control, were used. After incubation of the cell cultures for 45 min at 25°C, cells were sedimented and 130 μl of the supernatant analysed by measuring the fluorescence intensity to estimate cell death (through calcein release) at 485/535nm using a DTX880 fluorometer (Beckman Coulter). Total lysis of the cells was achieved by solubilising a non-Cetuximab-treated control sample with 1% Triton X-100. The percentage of specific lysis was calculated as: (experimental fluorescence – spontaneous fluorescence)/(maximum fluorescence – spontaneous fluorescence) × 100. Data analyses were carried out using Excel (Microsoft). All experiments were performed in triplicate.

Antibody-dependent cell-mediated cytotoxicity assay

To enhance HLA-E surface expression, 8 μg/ml recombinant human-β_2-microglobulin (BD Biosciences) were added to 1×10^5 colon cancer cells and kept for 16 h in 800 μl of colon medium at 37°C. Each cell line pretreated or not with recombinant human-β_2-microglobulin was used as target (T) and labelled with Calcein-AM. Effector PBMCs (E) were obtained from peripheral blood of healthy human donors by density gradient centrifugation. Target cells were resuspended in complete Colon Medium at a final concentration of 1×10^6 cells/ml and incubated with 10 μM calcein-AM for 30 min at 37°C with occasional shaking. After two washes in AIM-V® medium, the cytotoxicity assay was performed using different E : T ratios ranging from 100 : 1 to 25 : 1, in triplicate, in a total volume of 150 μl, with indicated doses of Cetuximab or control antibody (Rituximab). Three replicate wells for spontaneous (only target cells in AIM-V® medium) and maximum release (only target cells in medium plus 1% Triton X-100) were also measured. Monoclonal antibody anti-NKG2A (clone 131411, R&D systems) or isotype control IgG2a (Sigma, St Louis, MO, USA) were added (1 μg/ml) to PBMCs immediately before the cytotoxicity assay, with the aim of blocking the HLA-E specific receptor. After incubation at 37°C in 5% CO_2 for 4 h, 130 μl aliquots of the supernatant were analysed by fluorometry to measure cell death (calcein release) at 485/535nm using a DTX880 spectrophotometer (Beckman Coulter). The percentage of specific lysis was calculated as before.

Statistical analysis

To evaluate significant differences between groups, Student's *t*-test was performed. Significance was considered at $P < 0.01$.

RESULTS

Epidermal growth factor receptor status of tumour cell lines

We measured EGFR protein expression on each cell line by flow cytometric analysis. As shown in Figure 1, all colorectal cancer cell lines were positive for EGFR expression even if at variable levels. Although HCT-116, HT-29 and DLD-1 showed a comparable percentage of EGFR-positive cells (>95%), the mean intensity level (MIL, see Materials and Methods) was significantly higher for DLD-1 cells.

Direct growth inhibitory effect of Cetuximab on tumour cell lines

To examine the antiproliferative activity of Cetuximab, the MTT assay was performed. Each cell line was incubated in 200 µl of Colon Medium with six different concentrations of Cetuximab (ranging from 1×10^{-3} to 1×10^{2} µg/ml), and control mAb (Rituximab). The inhibition of proliferation induced by Cetuximab in comparison with that induced by control mAb is shown as a percentage of cell growth. Of note, in all cell lines the antiproliferative effect of Cetuximab was observed only between 1×10^{1} and 1×10^{2} µg/ml (Fig. 2); at 1×10^{2} µg/ml, Cetuximab inhibition of proliferation was 93.71% (average of the five colorectal cancer cell lines).

Apoptosis

To examine the apoptosis-inducing activity of Cetuximab, an Annexin–propidium iodide assay was performed on the five colorectal cancer cell lines using three different concentrations of Cetuximab (1, 10 and 100 µg/ml) or control mAb (Rituximab). No significant levels of apoptosis were induced by any Cetuximab concentration after 24 h of treatment on tested cell lines (data not shown). Since the pro-apoptotic effect of Cetuximab could increase with time, we repeated the Annexin–propidium iodide assay using 10 µg/ml during 24, 48 or 72 h. We observed that even after 72 h of treatment, no significant or weak levels of apoptosis were induced by Cetuximab in all analyzed cell lines (Table 1). The BCL-2 family genes are the major regulators of mitochondrial apoptotic homeostasis.[23] Several members of this family (including BCL-xL) promote survival while other members such as BAX promote cell death. The relative ratios of these pro- and anti-apoptotic members, rather than the expression level of any single BCL-2 family gene, have been shown to determine the ultimate apoptotic sensitivity. In all cases, BAX/BCL-xL ratios (Fig. 3) were significant lower than in normal human neutrophils used as reference samples (reference samples [mean ± SD], 4.54 ± 1.24; DLD-1, 0.03 ± 0.01; T-84, 0.10 ± 0.02; HT-29, 0.11 ± 0.01; HCT-116, 0.02 ± 0.001; and CACO-2, 0.11 ± 0.07).

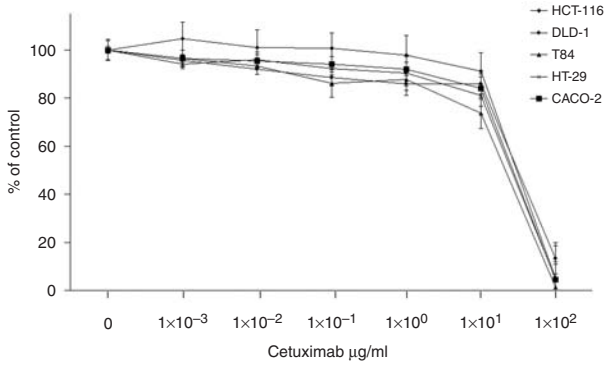

Fig. 2. Growth inhibitory effect of Cetuximab on colorectal cancer cell lines. The figure shows the dose-dependent growth inhibitory effect of Cetuximab (0–1×10^{2} µg/ml). Cell growth was inhibited only at the highest concentration (1×10^{2} µg/ml). Results are expressed as percentage of the Rituximab treated (control) values. The data shown are the mean ± SD values from triplicate experiments.

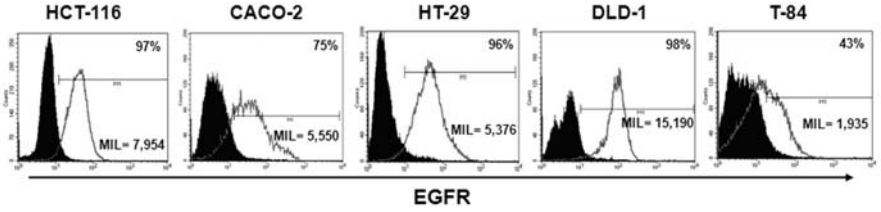

Fig. 1. Expression of EGFR in the five colorectal cancer cell lines by flow cytometric analysis. The histograms show EGFR membrane expression in each colorectal cancer cell line. Isotype control is indicated in the black fill histogram. EGFR positive expression is indicated in the grey empty histogram. EGFR expression is expressed both in percentage of positive cells and as mean intensity level (MIL = % positive × mean fluorescence intensity) for each cell line.

Complement dependent cytolysis by Cetuximab on five colon cancer cell lines

We next assessed whether Cetuximab could induce colon cancer cell lysis via complement dependent cytolysis (CDC). The only cell line susceptible to Cetuximab-mediated CDC was DLD-1 (Fig. 4). No CDC was observed when DLD-1 cells were incubated with Rituximab (data not shown). None of the other cell lines tested (HT-29, HCT-116, CACO-2 and T84) showed any CDC by Cetuximab, suggesting that neither CDC nor direct receptor-mediated mechanisms could substantially contribute to cell lysis under our experimental conditions. The response of DLD-1 cells to Cetuximab-induced CDC appeared to correlate positively with the expression of EGFR (Fig. 1).

ADCC by Cetuximab against three colorectal cancer cell lines

The effector cell-mediated cytotoxic efficacy of Cetuximab was analysed using the cell lines HT-29, HCT-116 and CACO-2. To identify the optimal Cetuximab concentration for ADCC activity, we performed a 4-h calcein-AM release assay with Cetuximab concentrations ranging from 1×10^{-2} to 1×10^{1} μg/ml using an E : T ratio of 10 : 1. As shown in Figure 5A, Cetuximab-mediated ADCC activity against the three colorectal cancer cell lines was already detectable at a concentration of 1×10^{-2} μg/ml, expressed as a percentage of control mAb. Interestingly, we found that when Cetuximab doses higher than 1 μg/ml were used, a significant reversal of lysis was consistently observed (Fig. 5A, top-right panel). As shown in Figure 5B, in the case of HT-29 (used as an illustrative case), the activity of effector cells was proportionally reduced at 10, 50 and 100 μg/ml of Cetuximab. This effect could be attributable to direct interaction of the Fc region of IgG with FcR on NK cells due to the excess of antibody in the medium that induces a down-regulation of NK activity.[24] Therefore, we used 1 μg/ml for subsequent assays.

Using this optimal antibody concentration, lytic activity against three colorectal cancer cell lines (HT-29, HCT-116 and CACO-2) was evaluated at various E : T ratios, by a 4-h Calcein-AM release assay using healthy human PBMCs ($n = 3$). As shown in Figure 6, at all the E : T ratios used, Cetuximab exhibited an enhanced cell lysis about 3-fold above the PBMCs activity with control antibody ($P = 0.005$). These data suggest that Cetuximab

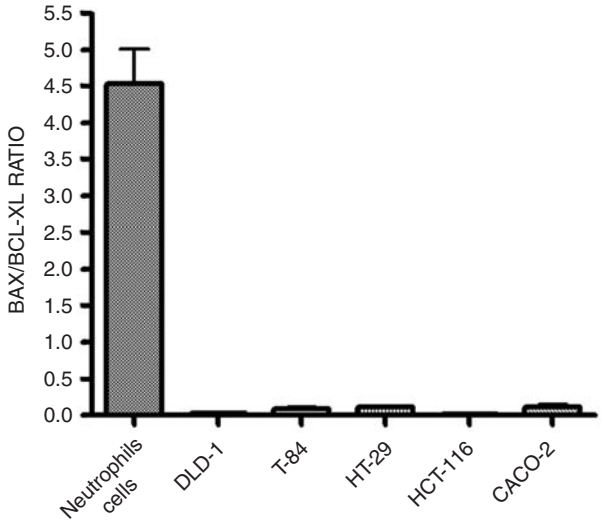

Fig. 3. Quantitative PCR analysis. Bar graph shows BAX/BCL-xL ratios determined by qRT-PCR analysis in 10 neutrophils samples from healthy donors and the five colorectal cancer cell lines. The data shown are the mean ± SD values from duplicate experiments.

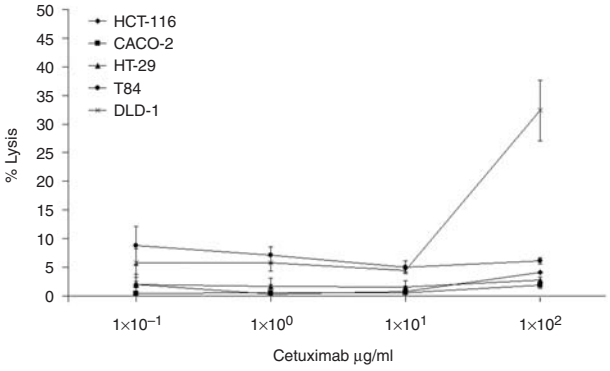

Fig. 4. Complement dependent cytolysis activity in colorectal cancer cell lines mediated by Cetuximab. Antibody concentrations up to 1×10^{2} μg/ml were tested. A specific cell lysis was observed only for DLD-1 cells at the highest antibody concentration. Results are expressed as percentage of the untreated control values. The data shown are the mean ± SD values from triplicate experiments.

Table 1. Apoptotic effect against colorectal cancer cell lines

Apoptosis	HCT-116	HT-29	CACO-2	T84	DLD-1
Rituximab (10 μg/ml) 72 h	4.66%	4.88%	4.84%	29.36%	7.76%
Cetuximab (10 μg/ml) 24 h	3.70%	9.57%	5.16%	17.50%	8.76%
Cetuximab (10 μg/ml) 48 h	4.11%	8.63%	6.71%	16.91%	9.22%
Cetuximab (10 μg/ml) 72 h	3.71%	8.92%	5.27%	22.44%	8.16%

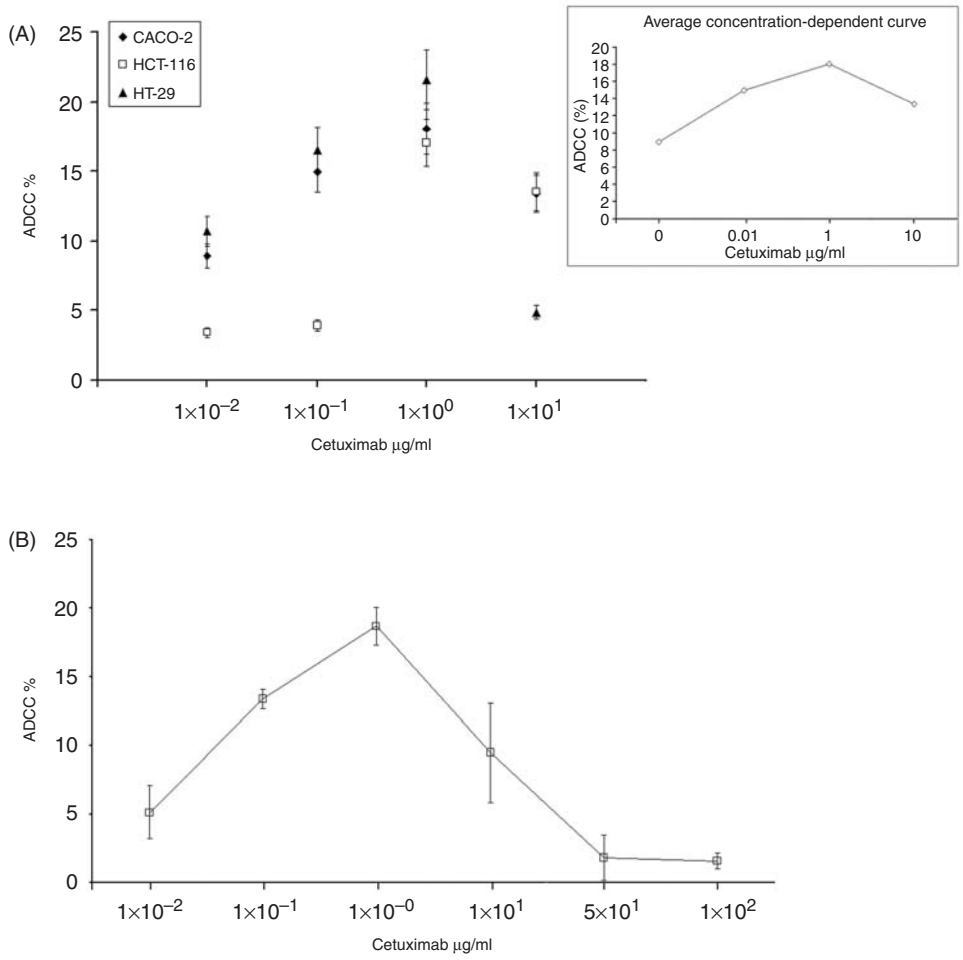

Fig. 5. Concentration-dependent curve of Cetuximab-mediated ADCC activity with PBMCs from healthy donors. (A) ADCC activity against HCT-116, HT-29, CACO-2 cell lines incubated with PBMCs at an E : T ratio of 10 : 1 along with various concentrations of Cetuximab (1×10^{-2} to 1×10^{1} μg/ml). Figures represent three independent experiments. Each point denotes the mean value of a triplicate experiment (bars, SD). The percentage of ADCC/PBMCs was calculated for each condition as the percentage of lysis in the presence of Cetuximab minus the percentage of lysis in the presence of control antibody (Rituximab). (B) Dose-dependent inhibition of ADCC/PBMCs activity of high doses of Cetuximab. In this illustrative example, HT-29 target cells were incubated along with PBMCs (E : T ratio of 10 : 1) and various concentrations of Cetuximab (1×10^{-2} to 1×10^{2} μg/ml).

was capable of activating ADCC activity efficiently even against colon cancer cells which weakly express EGFR, as in the case of CACO-2 cells (Fig. 6C).

Surface expression of HLA-E is responsible for inhibition of Cetuximab-mediated ADCC by NK cells

Since we reported previously that a stable level of membrane HLA-E suppresses NK cell-mediated target lysis for DLD-1 cells,[19] we next investigated if HLA-E surface stabilization by recombinant human-β_2-microglobulin would inhibit Cetuximab-mediated ADCC. Upon incubation of the four colorectal cancer cell lines for 16 h in the presence of exogenous recombinant human-β_2-microglobulin, only HLA-E and not HLA-Ia cell surface expression was reconstituted (Fig. 7). We also verified that recombinant human-β_2-microglobulin addition did not modify EGFR expression (data not shown). Three colorectal cancer cell lines pre-treated or not with recombinant human-β_2-microglobulin were co-incubated with PBMCs cells from healthy donors for 4 h. As a result, we observed that Cetuximab-mediated ADCC was significantly inhibited by the membrane expression of HLA-E (Fig. 8A–C). For example, the Cetuximab-mediated ADCC (at the E : T ratio of 50 : 1) for HCT-116 (Fig. 8A) in the presence of membrane HLA-E ($5.9 \pm 0.9\%$) was significantly weaker than those in the absence of surface HLA-E ($26.1 \pm 2.9\%$). These observations were confirmed at different E : T ratio settings using PBMCs derived from three different healthy donors. To confirm that the reduction of Cetuximab-induced lysis is specifically due to HLA-E membrane stabilization on target cells, we incubated effector cells with a blocking antibody specific for NKG2A, the inhibitory receptor for HLA-E (Fig. 8D).

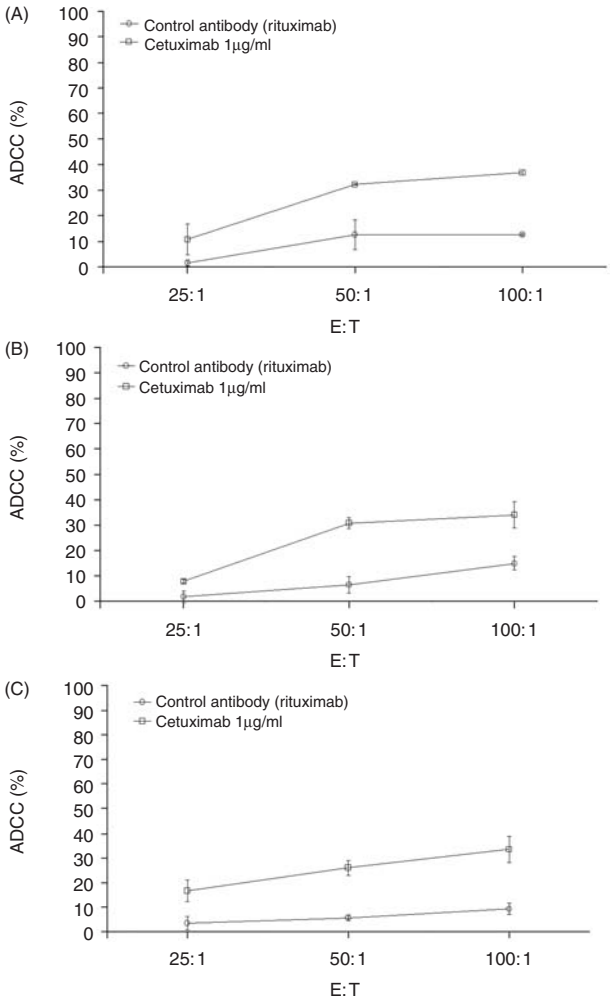

Fig. 6. Cetuximab-mediated ADCC/PBMCs activity against HCT-116 (A), HT-29 (B), CACO-2 (C) cell lines at different E : T ratios. Colorectal cancer cell lines were analyzed for ADCC using PBMCs from healthy donors in the presence of Cetuximab or control mAb (Rituximab). Each graph depicts results from one representative donor. A minimum of three normal donors was tested per tumour cell line. Each point denotes the mean value of a triplicate experiment (bars, SD).

When HCT-116 cells were co-incubated with PBMCs in the presence of anti-NKG2A antibody, the percentage of lysis ($14.7 \pm 1.9\%$) was restored to the same levels observed in the absence of HLA-E ($17.2 \pm 2.5\%$), while the addition of an isotypic control antibody was not able to produce the same effect ($2.3 \pm 0.4\%$). This result confirms that the presence of HLA-E at the cell surface is the mechanism by which the addition of recombinant human-β_2-microglobulin inhibits Cetuximab-dependent ADCC.

Moreover, to confirm that NK cells are responsible for Cetuximab-mediated ADCC activity, NK cells ($CD3^-$ $CD56^+$) were separated from PBMCs by the MACS system (Fig. 8E) to obtain pure NK cells (>95%). As shown in Figure 8F, when purified NK cells were co-incubated with DLD-1 cells in the presence of Cetuximab (1 μg/ml), we observed a higher percentage of lysis (>60%) when compared to the cytotoxic assay performed using total PBMCs. As expected, NK cytotoxicity was also reduced by the addition of recombinant human-β_2-microglobulin to DLD-1 cells (Fig. 8F). These results show that colon cancer cells expressing stable levels of membrane HLA-E are less susceptible to NK cell attack even in the presence of Cetuximab.

DISCUSSION

Antibody therapies are a major approach in the treatment of various cancer types.[25] Direct growth inhibition, apoptosis induction, CDC and ADCC mediated by antibodies are modes of action for this therapy.[26] It has been shown that Cetuximab-induced antitumour activity did not correlate directly with the level of EGFR expression in human cancer cells;[27,28] these observations suggest that molecular events other than EGFR levels are true determinants of responsiveness and that further investigation is necessary to determine the factors that affect the antitumour activity of Cetuximab. In this regard, new studies are focused on searching for molecular factors associated with resistance to Cetuximab. Among these, Bcl-x(L) and, to a lesser extent, Mcl-1, are important anti-apoptotic factors that confer resistance to Cetuximab-mediated apoptosis in colorectal cancer.[29] Moreover, it has been shown that the simultaneous activation of the RAS and PIK3CA pathways confers maximal resistance to this agent.[30] Recently, several clinical studies simultaneously demonstrated that KRAS mutations provide an independent predictive factor of the response to Cetuximab.[18,31] Taking into account that monoclonal antibody therapies may also act through immunological effectors as in ADCC, we explored the possibility that other immune-related molecules could also mediate Cetuximab resistance. Considering the immunomodulatory function assigned to HLA-E molecule,[32] its expression in malignant cells may represent one of various mechanisms used by tumour cells to escape immune surveillance[33] and a possible determinant of ADCC inhibition. Accordingly, we recently reported[19] that primary human colorectal cancer spontaneously expresses significant levels of HLA-E molecules, probably contributing to the escape of these tumours from immune surveillance by inhibiting NK cell target lysis. These results suggested that it is necessary to establish if the membrane expression of HLA-E on colon cancer cells could have an inhibitory effect of Cetuximab-mediated ADCC. Consequently, we designed a model based on HLA-E structural stabilization on the cell surface by

Cell line	mHLA-E expression spont.	mHLA-E expression + r*hu*-β_2m	HLA-Ia expression spont.	HLA-Ia expression + r*hu*-β_2m
DLD-1	25	4,389	212	140
HCT-116	42	1,822	3,020	1,299
HT-29	110	1,456	5,205	1,845
CACO-2	98	816	7,059	3,393

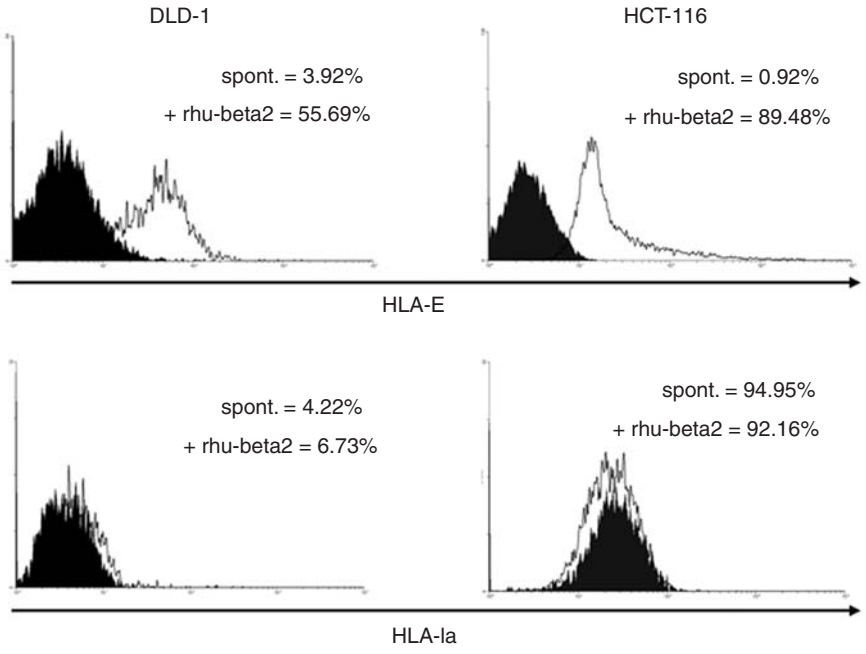

Fig. 7. Flow cytometric analysis of HLA-E cell membrane stablization. In the table are reported HLA-E and HLA-Ia expression values (MIL) from four colorectal cancer cell lines (HCT-116, DLD-1, CACO-2 and HT-29) upon addition or not of recombinant human-β_2-microglobulin during 16 h. The graphs show two representative examples (DLD-1 left panels and HCT-116 right panels) of HLA-E membrane stablization (upper) and HLA-Ia expression (lower panels). by recombinant human-β_2-microglobulin (8 μg/ml) in two colorectal cancer cell lines. Spontaneous expressions of HLA-E or HLA-Ia are represented by black filled histograms while induced expressions, after treatment with recombinant human-β_2-microglobulin, are represented by empty histograms.

recombinant human-β_2-microglobulin addition, since most colorectal cancer cell lines produce significant amounts of HLA-E molecules that are retained inside the cells.[19]

As a new finding, in the present study we clearly showed that EGFR-expressing colorectal cancer cells could be protected from Cetuximab-mediated ADCC through HLA-E membrane expression induced *in vitro* by exogenous recombinant human-β_2-microglobulin. The activities of Cetuximab-mediated ADCC did not reflect the degree of EGFR expression on colorectal cancer cells; in fact, low EGFR expression levels are sufficient for maximum Cetuximab-mediated ADCC activity. However, when HLA-E was stabilized on the cell surface, all cell lines showed a significant reduction in the Cetuximab-mediated ADCC (about 50%). The critical role of HLA-E was further confirmed by the addition of a blocking antibody (anti-NKG2A) that determines the recovery of Cetuximab-mediated ADCC levels, comparable to those obtained in non HLA-E expressing cells.

Additionally, we showed in the present study that Cetuximab had no pro-apoptotic effect on the five studied colorectal cancer cell lines. Similarly, CDC activity could be induced by Cetuximab treatment only in the case of DLD-1 cells. We propose that Cetuximab-mediated CDC could be related to the amount of EGFR expressed on the membrane target cell, since DLD-1 is the more EGFR-positive cell line. When we focused on the direct growth inhibitory effect of Cetuximab against colon cancer cells, we found that this antibody could induce a potent antiproliferative effect against all

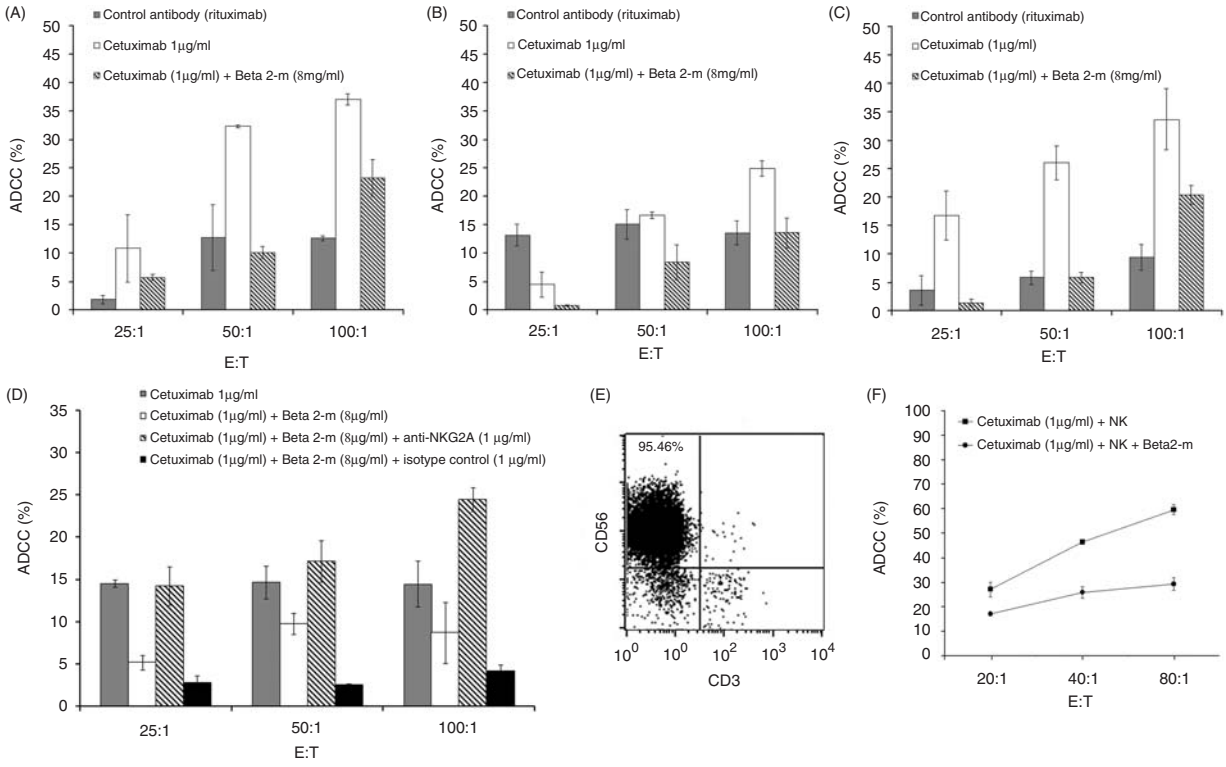

Fig. 8. (A–C) Cetuximab-mediated ADCC/PBMCs activity against colorectal cancer cell lines expressing or not HLA-E at the cell surface using different E : T ratios. ADCC assays were performed using colorectal cancer cells as target, pretreated or not with recombinant human-β_2-microglobulin (8 μg/ml) in the presence of Cetuximab (1 μg/ml). PBMCs were co-cultured for 4 h with colorectal cancer cells (A, HCT-116; B, HT-29; C, CACO-2) expressing or not HLA-E at the cell surface. Control conditions included PBMCs co-cultured with tumour cells in the presence of Rituximab. The columns represent the mean of three replicates (bars, SD). (D) Effect of NKG2A blocking monoclonal antibody or isotypic control on Cetuximab-mediated ADCC activity of PBMCs. These effector cells were tested for cytotoxicity against HCT-116 cells in a standard 4-h calcein release assay in the presence of Cetuximab (1 μg/ml). The columns represent the mean of three replicates (bars, SD). (E) Illustrative graph of NK purified from healthy donor PBMCs using a magnetic system. Upper left quadrant represents NK positive cells ($CD56^+$ $CD3^-$). (F) Effect of HLA-E stablization on Cetuximab-mediated ADCC activity of NK cells. Effector cells were tested for cytotoxicity against recombinant human-β_2-microglobulin treated or not DLD-1 cells in a standard 4-h calcein release assay, using different E:T ratios in the presence of Cetuximab (1 μg/ml). The points represent the mean of three replicates (bars, SD).

colorectal cancer cell lines, although this antitumour activity was achieved only with Cetuximab concentrations higher than 10 μg/ml. While it has been reported that antibody concentrations in the serum reach comparable levels, it is unlikely that this concentration could be reached inside the tumour mass.[34] For this reason, we suggest that Cetuximab-mediated ADCC activity could have an important antitumour effect in the tumour site since our findings demonstrate that its maximal activity is already reached at lower Cetuximab concentrations (<1 μg/ml).

Conclusions

We believe that optimal patient selection to use EGFR-targeted agents will depend on the identification of more specific biological markers of potential responsiveness to ADCC-inducing agents. Considering the immunomodulatory function assigned to HLA-E, mainly through the interaction with the inhibitory receptor NKG2A/CD94 on NK cells, its expression in malignant cells may represent one of the various mechanisms used by tumour cells to escape immune surveillance and a reliable determinant of ADCC inhibition. We believe that our data justify further studies using tumour samples from Cetuximab-treated patients to establish if high HLA-E levels could be a bad prognosis marker in primary colorectal cancer for antibody therapy.

Acknowledgements

This work was supported with funds from Fundación Sales, Fundación Mosoteguy, Fundación Cáncer (FUCA), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) and Fundación Maria Calderón de la Barca. MMB, JM and MB are members of CONICET. We are grateful to Dr Irene Larripa for

generously allowing use of the LightCycler 2.0 thermo cycler.

REFERENCES

1. Smith R, Cokkinides V, Eyre HJ. American Cancer Society guidelines for the early detection of cancer. *J Clin Cancer* 2006; **56**: 11–25.
2. Goldberg RM, Gill S. Recent phase III trials of fluorouracil, irinotecan, and oxaliplatin as chemotherapy for metastatic colorectal cancer. *Cancer Chemother Pharmacol* 2004; **54**: 57–64.
3. Pasetto LM, Jirillo A, Iadicicco G, Rossi E, Paris MK, Monfardini S. FOLFOX versus FOLFIRI: a comparison of regimens in the treatment of colorectal cancer metastases. *Anticancer Res* 2005; **25**: 563–576.
4. Kelloff GJ, Lippman SM, Dannenberg AJ *et al*. The promise of molecular biomarkers for prevention of intraepithelial neoplasia and cancer – a plan to move forward. *Clin Cancer Res* 2006; **12**: 3661–3697.
5. Choudhury A, Mosolits S, Kokhaei P, Hansson L, Palma M, Mellstedt H. Clinical results of vaccine therapy for cancer: learning from history for improving the future. *Adv Cancer Res* 2006; **95**: 147–202.
6. Arsene D, Galais MP, Bouhier-Leporrier K, Reimund JM. Recent developments in colorectal cancer treatment by monoclonal antibodies. *Expert Opin Biol Ther* 2006; **6**: 1175–1192.
7. Ritter CA, Arteaga CL. The epidermal growth factor receptor-tyrosine kinase: a promising therapeutic target in solid tumors. *Semin Oncol* 2003; **30**: 3–11.
8. Kawamoto T, Sato JD, Le A, Polikoff J, Sato GH, Mendelsohn J. Growth stimulation of A431 cells by epidermal growth factor: identification of high-affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. *Proc Natl Acad Sci USA* 1983; **80**: 1337–1341.
9. Hildebrandt B, le Coutre P, Nicolaou A, Köble K, Riess H, Dörken B. Cetuximab: appraisal of a novel drug against colorectal cancer. *Recent Results Cancer Res* 2007; **176**: 135–143.
10. Sato JD, Kawamoto T, Le AD, Mendelsohn J, Polikoff J, Sato GH. Biological effects *in vitro* of monoclonal antibodies to human epidermal growth factor receptors. *Mol Biol Med* 1983; **1**: 511–529.
11. Peng D, Fan Z, Lu Y, DeBlasio T, Scher H, Mendelsohn J. Anti-epidermal growth factor receptor monoclonal antibody 225 up-regulates p27KIP1 and induces G_1 arrest in prostatic cancer cell line DU145. *Cancer Res* 1996; **56**: 3666–3669.
12. Fan Z, Lu Y, Wu X, Mendelsohn J. Antibody-induced epidermal growth factor receptor dimerization mediates inhibition of autocrine proliferation of A431 squamous carcinoma cells. *J Biol Chem* 1994; **269**: 27595–27602.
13. Liu B, Fang M, Lu Y, Mendelsohn J, Fan Z. Fibroblast growth factor and insulin-like growth factor differentially modulate the apoptosis and G_1 arrest induced by anti-epidermal growth factor receptor monoclonal antibody. *Oncogene* 2001; **20**: 1913–1922.
14. Ciardiello F, Bianco R, Damiano V *et al*. Antitumor activity of sequential treatment with topotecan and anti-epidermal growth factor receptor monoclonal antibody C225. *Clin Cancer Res* 1999; **5**: 909–916.
15. Perrotte P, Matsumoto T, Inoue K *et al*. Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. *Clin Cancer Res* 1999; **5**: 257–265.
16. Goldstein NI, Prewett M, Zuklys K, Rockwell P, Mendelsohn J. Biological efficacy of a chimeric antibody to the epidermal growth factor receptor in a human tumor xenograft model. *Clin Cancer Res* 1995; **1**: 1311–1318.
17. Kawaguchi Y, Kono K, Mimura K *et al*. Targeting EGFR and HER-2 with Cetuximab- and Trastuzumab-mediated immunotherapy in oesophageal squamous cell carcinoma. *Br J Cancer* 2007; **97**: 494–501.
18. Lièvre A, Bachet JB, Boige V. KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with Cetuximab. *J Clin Oncol* 2008; **26**: 374–379.
19. Levy EM, Bianchini M, Von Euw EM *et al*. Human leukocyte antigen-E protein is overexpressed in primary human colorectal cancer. *Int J Oncol* 2008; **32**: 633–664.
20. Brooks AG, Posch PE, Scorzelli CJ, Borrego F, Coligan JE. NKG2A complexed with CD94 defines a novel inhibitory natural killer cell receptor. *J Exp Med* 1997; **185**: 795–800.
21. Lanier LL. Follow the leader: NK cell receptors for classical and nonclassical MHC class I. *Cell* 1998; **92**: 705–707.
22. Lee N, Llano M, Carretero M *et al*. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc Natl Acad Sci USA* 1998; **95**: 5199–5204.
23. Liu FT, Goff LK, Hao JH, Newland AC, Jia L. Increase in the ratio of mitochondrial Bax/Bcl-XL induces Bax activation in human leukemic K562 cell line. *Apoptosis* 2004; **9**: 377–384.
24. Galatiuc C, Gherman M, Metes D *et al*. Natural killer (NK) activity in human responders and nonresponders to stimulation by anti-CD16 antibodies. *Cell Immunol* 1995; **163**: 167–177.
25. Heimann DM, Weiner LM. Monoclonal antibodies in therapy of solid tumors. *Surg Oncol Clin North Am* 2007; **16**: 775–792.
26. Iannello A, Ahmad A. Role of antibody-dependent cell-mediated cytotoxicity in the efficacy of therapeutic anti-cancer monoclonal antibodies. *Cancer Metastasis Rev* 2005; **24**: 487–499.
27. Saltz LB, Meropol NJ, Loehrer Sr PJ, Needle MN, Kopit J, Mayer RJ. Phase II trial of Cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. *J Clin Oncol* 2004; **22**: 1201–1208.
28. Chung KY, Shia J, Kemeny NE *et al*. Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. *J Clin Oncol* 2005; **23**: 1803–1810.
29. Schulze-Bergkamen H, Ehrenberg R, Hickmann L, Vick B. Bcl-x(L) and myeloid cell leukaemia-1 contribute to apoptosis resistance of colorectal cancer cells. *World J Gastroenterol* 2008; **14**: 3829–3840.
30. Jhawer M, Goel S, Wilson AJ *et al*. PIK3CA mutation/PTEN expression status predicts response of colon cancer cells to the epidermal growth factor receptor inhibitor Cetuximab. *Cancer Res* 2008; **68**: 1953–1961.
31. De Roock W, Piessevaux H, De Schutter J *et al*. KRAS wild-type state predicts survival and is associated to early radiological response in metastatic colorectal cancer treated with Cetuximab. *Ann Oncol* 2008; **19**: 508–515.
32. Derré L, Corvaisier M, Charreau B *et al*. Expression and release of HLA-E by melanoma cells and melanocytes: potential impact on the response of cytotoxic effector cells. *J Immunol* 2006; **177**: 3100–3107.
33. Wischhusen J, Friese MA, Mittelbronn M, Meyermann R, Weller M. HLA-E protects glioma cells from NKG2D-mediated immune responses *in vitro*: implications for immune escape *in vivo*. *J Neuropathol Exp Neurol* 2005; **64**: 523–528.
34. Luo FR, Yang Z, Dong H, Camuso A *et al*. Correlation of pharmacokinetics with the antitumor activity of Cetuximab in nude mice bearing the GEO human colon carcinoma xenograft. *Cancer Chemother Pharmacol* 2005; **56**: 455–464.