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Cytotoxic drugs up-regulate epidermal growth factor receptor (EGFR) expression in colon cancer cells and enhance their susceptibility to EGFR-targeted antibody-dependent cell-mediated-cytotoxicity (ADCC)

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ABSTRACT

Cetuximab is a human–murine chimeric IgG1 monoclonal antibody to epidermal growth factor-receptor (EGFR) which exerts synergistic antitumour interactions with several cytotoxic drugs. Therefore, it is presently recommended in combination with chemotherapy in the treatment of colon, head and neck and non-small cell lung cancer. Cetuximab has been designed to inhibit EGFR signalling; however, preclinical evidence suggests that its anticancer effects *in vivo* are also related to the ability of its human IgG1 backbone to trigger immunological mechanisms. Here we have investigated whether the exposure to different cytotoxic drugs may affect the susceptibility of colon cancer cells *in vitro* to cetuximab immuno-targeting and related lymphokine-activated killer (LAK)-mediated antibody-dependent cell cytotoxicity (ADCC).

Five colon cancer cell lines expressing a different k-ras mutational status were evaluated for: (i) EGFR-expression, (ii) susceptibility to LAK cells and (iii) cetuximab-mediated ADCC, before and after exposure to 5-flurouracil (5-FU), gemcitabine (Gem), irinotecan (Iri) alone or in multiple two/three drug combinations.

These drugs were able to up-regulate EGFR expression on the surface of all the colon cancer cell lines with a maximal effect observed few hours after the exposure to GILF regimen (Gem, Iri, Levofolinic acid and 5-FU). Chemotherapy was able to greatly enhance the sensitivity to either LAK cells or cetuximab-mediated ADCC in all the colon cancer cell lines with a mechanism independent from k-ras status.

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The results of our study suggest that chemotherapy may enhance cetuximab-mediated immuno-targeting and ADCC thus providing the rationale to design novel immuno-biochemotherapy regimens.

1. Introduction

Epidermal growth factor-receptor (EGFR), also designated as HER1 or ERB-B1, is one of the four known members of the ErbB family of tyrosine kinase receptors, which is often over-expressed on the membrane of epithelial cancer cells. EGFR binding to its natural ligands (EGF, TGF-alpha, etc.) leads to the receptor dimerisation and tyrosine auto-phosphorylation, thus activating intracellular biochemical pathways that are critical in maintaining the malignant phenotype.[1,2] EGFR-activated signal cascades lead to increased proliferation, resistance to pro-apoptotic stimuli, as well as to production and release of pro-angiogenic factors.[2–5]

Cetuximab is a human/murine chimeric IgG1 monoclonal antibody (moAb) to the EGFR considered as a target-specific inhibitor since it binds ERB-B1/EGFR, competing with its ligands. Therefore, the binding leads to inhibition of the downstream effectors including k-ras and interfering with both tumour cell proliferation and drug resistance.[5–8] The results of pre-clinical studies suggest a synergistic anti-tumour interaction among cetuximab and several cytotoxic drugs including taxanes, gemcitabine, irinotecan and 5-fluorouracil.[8–10] Moreover, the results from clinical trials have demonstrated the efficacy of cetuximab in combination with chemotherapy in the treatment of patients with metastatic colon carcinoma and non-small cell lung cancer.[11–14] However, in these cases, cetuximab is reserved for patients whose tumour expresses a wild-type k-ras status. In fact, this population seems to gain the best benefit from the combined treatment in terms of both time to progression and survival.[15–18]

Although more specific and potent EGFR biochemical inhibitors have been produced in the last few years, none of them has shown a significant activity in colon cancer patients,[7,19–21] with the exception of Panitumumab[22], which is another (IgG2) moAb to EGFR, which does not induce ADCC, but which can still exert immunological activity for its high binding affinity to Fc receptors (FcγIR and FcγIIRa) expressed on granulocytes, dendritic cells and activated monocytes.[23–27]

On these bases, further mechanisms have been proposed for cetuximab-mediated anti-tumour activity. Cetuximab as an IgG1 moAb can ignite different immunological mechanisms with potential anti-tumour activity *in vivo*. Preclinical results have already shown cetuximab ability of inducing either complement mediated tumour cell killing (CDC) or antibody-dependent cell-mediated cytotoxicity (ADCC) by interacting with natural killers (NKs), monocytes and granulocytes by means of their specific Fc receptors.[23–27]

This immunological potential of cetuximab, which is obviously not shared by the small molecules with EGFR pathway inhibitory properties, might have been largely underestimated and further investigation is needed on therapeutic implications as well as cytotoxic drug interaction and immuno-targeting properties.

We have investigated whether cetuximab immuno-targeting and consequent ADCC in colon cancer cells *in vitro* might be enhanced by tumour cell exposure to cytotoxic drugs with different mechanisms of action. We have then investigated whether drug-related changes in cetuximab-mediated antitumour activity and ADCC *in vitro* might be related to changes in the EGFR expression and/or to the presence of a wild-type k-ras status in the target cells.

2. Material and methods

2.1. Tumour cell cultures

HT29, WiDr, COLO-205, LoVo and SW620 human colon carcinoma cell lines were purchased from American Type Culture Collection. The EL-4/HHD cell lines (mouse β2 microglobulin-deficient thymoma cells transfected with the HHD HLA-A2.1 monochain construct) were provided by Antonio Scardino (INSERM, Goustav Roussy Institute, Villejuif, France). All the tumour cells were maintained in complete RPMI-1640 (Bio Whittaker, Lonza, Belgium) medium with the addition of 10% heat-inactivated foetal calf serum, 2 mM L-glutamine and 100 U/mL penicillin/streptomycin and cultured at 37 °C in 5% CO_2 humidified atmosphere. Adherent cells were removed using trypsin-EDTA solution (0.05% trypsin and 0.02% EDTA in phosphate-buffered saline without calcium and magnesium).

2.2. Human mononuclear cell cultures

Peripheral blood mononuclear cells (PBMCs) for the *in vitro* generation of the lymphokine-activated killer (LAK) cells were obtained by Ficoll-Hypaque (Celbio S.P.A., Italy) gradient centrifugation of heparinised blood collected from healthy donors. Lymphokine-activated killer (LAK) cells were generated by culturing PBMCs in the presence of IL-2 (1000 UI/ml), for 24 h.

2.3. Cytotoxic assays

Cytotoxicity of LAK cells was evaluated with 24 h-LDH release experiments (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega, Madison, USA), according to the procedure furnished by producers.

Target tumour cells (HT29, WiDr, COLO-205, LoVo and SW620) were seeded at a concentration of 1×10^5 cells/ml in 25-cm flasks. After 48 h, cells were exposed to different chemotherapy drugs used alone or in combination: control (no treatment), GEM (gemcitabine), 5-FU (levofolinic acid + 5-fluoruracil), IRI (irinotecan), GF (gemcitabine + levofolinic acid + 5-fluoruracil), IFL (irinotecan + 5-fluoruracil + levofolinic acid) and GILF (gemcitabine + irinotecan + levofolinic acid + 5-fluoruracil). The medium was replaced after 24 h of

incubation at 37 °C and 5% CO_2. Subsequently, gemcitabine at final concentration of 50 μg/ml was added to the tumour cells in the experimental groups GEM, GF and GILF. After 30 min of incubation, levofolinic acid was added at a concentration of 2 μg/ml (10^{-4} M), to the experimental groups 5-FU, GF, IFL and GILF; after 30 min, the medium was replaced in all groups. 5-FU was added at a concentration of 500 μg/ml (10^{-3} M) in experimental groups 5-FU, GF, IFL and GILF and incubated for 5.5 h. Then, irinotecan at a concentration of 100 μg/ml was added to cells in experimental groups IRI, IFL and GILF. After 3.5 h, the medium was replaced. Finally 5-FU was added again at the same concentration to experimental groups 5-FU, GF, IFL and GILF. After 48 h from the beginning of cells treatments, target tumour cells were detached, washed and seeded at a concentration of 5×10^3 cells/well in 96-well plates and cetuximab was added at various concentrations (no Cetuximab, 1 μg/ml, 10 μg/ml and 100 μg/ml). After 1 h effector cells (LAK from normal donor) were added to wells at each effector/target cells concentration (E/T ratios 50:1, 25:1, 12.5:1 and 1:1).

Cytotoxicity was evaluated with the following formula: % cytotoxicity = (Experimental – Effector Spontaneous – Target Spontaneous)/(Target Maximum – Target Spontaneous) × 100.

2.4. Inhibition of cell recovery: MTT experiment

The effects of cetuximab on colon cancer cells were evaluated by means of a colorimetric assay (MTT assay).

Tumour cells (human colon carcinoma cell lines WiDr, COLO-205, LoVo, SW620, HT29 and mouse-lymphoma cell line EL-4/HHD) were seeded at a concentration of 1×10^5 cells/ml in 25-cm flasks. After 48 h, cells were exposed to different drugs as previously described. Two days after drug exposure, 1×10^3 cells per well were seeded in flat-bottomed 96-well plates and then Cetuximab was added at various concentrations (no Cetuximab, 1 μg/ml, 10 μg/ml and 100 μg/ml). After 96 h of incubation, 20 μl of MTT solution (5 mg/ml in PBS) was added to each well, then all media were replaced with 200 μl/well of dimethyl sulfoxide (DMSO) after 3.5 h. Absorbance data were recorded at 570 and 650 nm wave-length using a 96-well plate reader.

2.5. Western blot analysis

HT29, WiDr and SW620 sub-confluent cells were trypsinised, counted and seeded in appropriate culture disks. Cells were incubated for 24 h at 37 °C and 5% CO_2. Subsequently, fresh medium was added to control tumour cells, whereas fresh medium containing drugs was added as described. Thereafter, the cells were incubated for 24 h before being detached. For cell extract preparation, the cells were washed twice with ice-cold PBS/BSA, scraped and centrifuged for 30 min at 4 °C in 1 ml of lysis buffer (1% Triton, 0.5% sodium deoxycholate, 0.1 NaCl, 1 mM EDTA, pH 7.5, 10 mM Na_2HPO_4, pH 7.4, 10 mM PMSF, 25 mM benzamidin, 1 mM leupeptin, 0.025 U/ml aprotinin). Equal amounts of cell proteins were separated by SDS–PAGE. The proteins on the gels were electro-transferred to nitrocellulose and reacted with MAbs anti-EGFR. Expression of EGF-R on cancer cells was measured at baseline (0 h), and after 3, 6, 24 and 48 h after treatments.

2.6. Cytofluorimetric assay

HT29, WiDr and SW620 cells were treated as described. The cells were then washed three times with cold Ca^{2+} and Mg^{2+} free Dulbecco's phosphate buffer saline (DPBS) and stained for 1 h with fluorescent-labelled monoclonal antibodies against EGFR. They were then washed three additional times with cold DPBS, resuspended in DPBS and analysed with flow cytometry (FACScan, Becton Dickinson). For each sample, 2×10^4 events were acquired. Analysis was carried out by triplicate determination on at least three separate experiments.

2.7. Statistical considerations

The between-mean differences were statistically analysed using Stat View statistical software (Abacus Concepts, Berkeley, CA). The results were expressed as the mean SD of four determinations made in three different experiments, and significance was determined using the 2-tail Student's *t*-test for paired samples. A *p*-value of $\leqslant 0.05$ was considered statistically significant.

3. Results

3.1. Chemotherapy modulates EGFR expression on cell membrane of colon cancer cells in vitro

Anti-cancer drugs with different mechanisms of action were assessed for their ability of modulating EGFR expression in colon cancer cells.

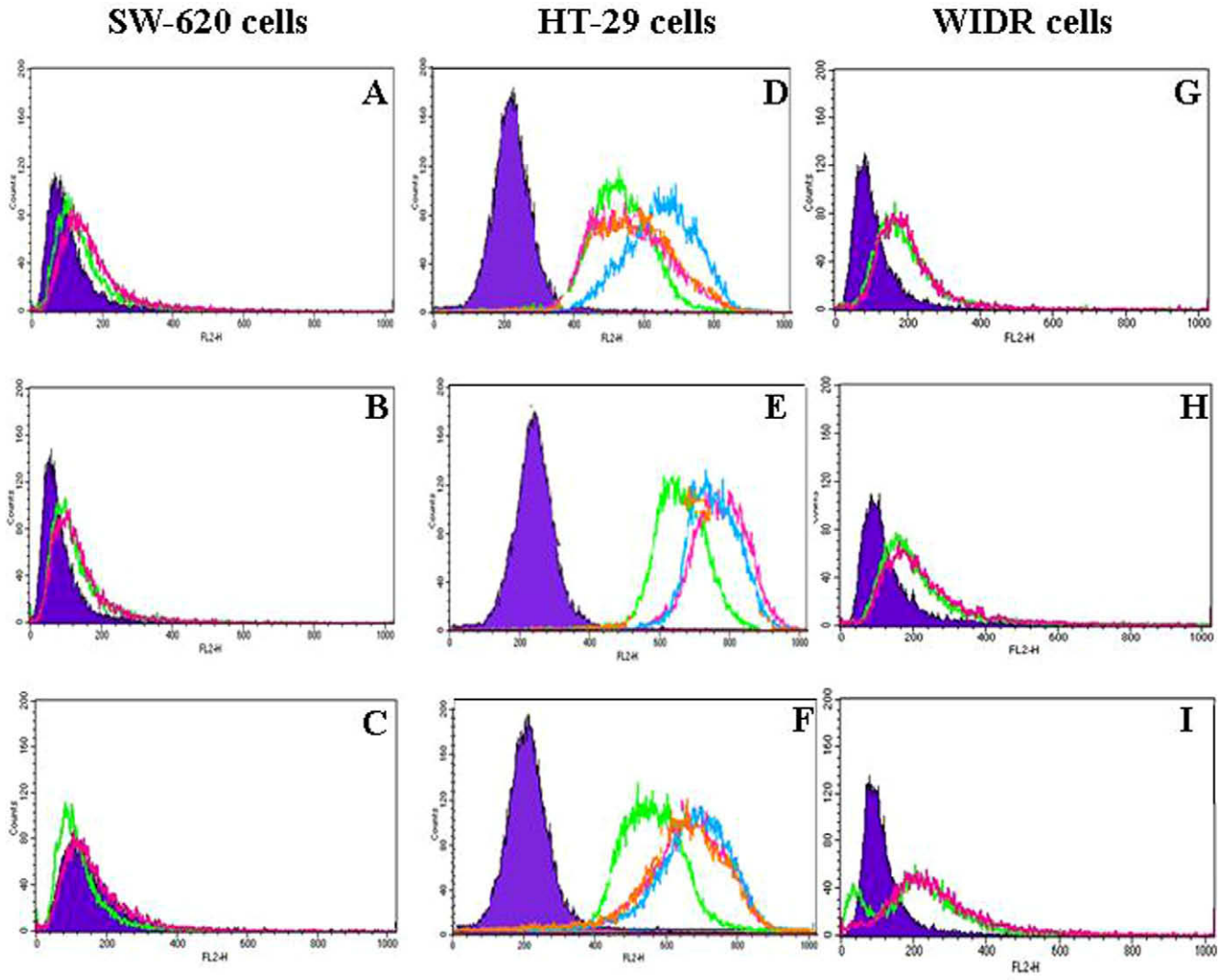
We have investigated if the treatment with GEM, 5-FU and/or IRI used alone or in two/three multi-drug combinations plays a role in the modulation of EGFR. Three colon carcinoma

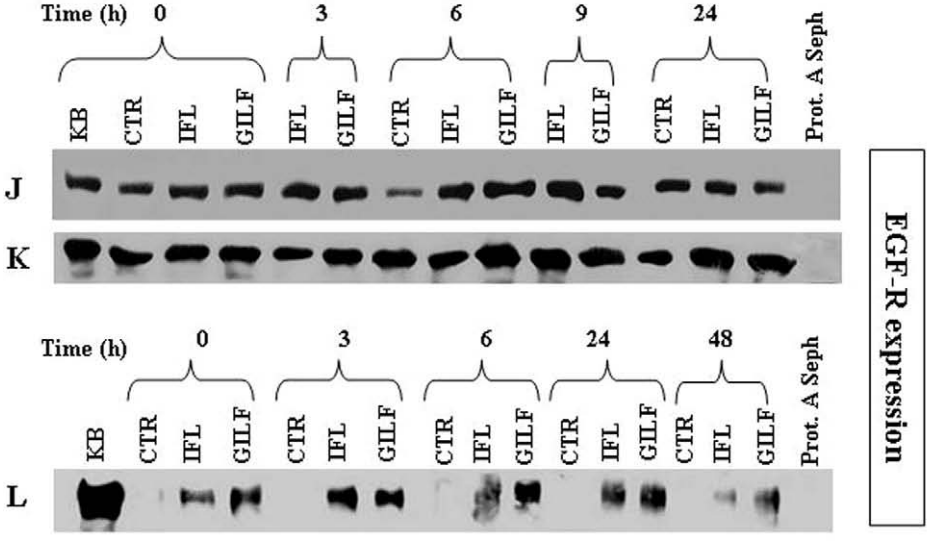
Table 1 – K-ras status and EGFR expression of tumour cell lines.

Cell line	K-ras status	EGFR gene c.EX12, 13, 14, 15 del	EGFR expression Baseline[a]	EGFR expression Post IFL[a]	EGFR expression Post GIFL[a]
COLO-205	Wild-type	13	80	170	220
HT29	Wild-type	13, 14, 15	250	500	750
LOVO	13/(Gly > Asp)	15	150	250	300
SW620	12/(Gly > Asp)	–	90	150	180
WiDr	Wild-type	–	100	200	250

[a] Results are expressed as main fluorescence intensity per cells.

cell lines that differ from k-ras mutational status and EGFR expression were used as experimental models: HT29 and WiDr cell lines show a wild-type k-ras while SW620 shows a mutated k-ras. Moreover, SW620 and WiDr cell lines show a very low or undetectable EGFR expression while HT29 cell line constitutively over-expresses EGFR (Table 1).





We have evaluated the EGFR expression by cytofluorimetric and Western blot analysis at the end and at 3, 6, 9, 24 and 48 h from the beginning of treatment.

Interestingly, up-regulation of EGFR was observed on the membrane of SW620 (Fig. 1A–C) and WiDr cell lines which in the absence of drug exposure show a very low or undetectable EGFR expression (Fig. 1G–I).

In SW620 cell line (Fig. 1A–C) which does not show baseline EGFR expression, IFL and GILF regimens were able to up-regulate EGFR expression early after the end of chemotherapy (25% and 46%, respectively) (Fig. 1A) and such effect still lasted after 3 h (48% and 50%, respectively) (Fig. 1B) until return to basal levels (lack of expression) (Fig. 1C). We found a statistically significant difference ($P < 0.05$) when EGFR expression was compared between chemotherapy and control (cells grown in fresh medium) groups; on the other hand, there was no difference when the experimental groups of cells exposed to IFL and GILF regimens were compared.

The chemotherapy-induced up-regulation was equally detected in HT29 cells expressing wild-type k-ras status (Fig. 1D–F). IFL and GILF regimens were able to induce a significant up-regulation (about 70% and 80%, respectively) which could be detected early after the chemotherapy reaching a maximal effect at 24 h from the beginning of treatment (Fig. 1F). We found a significant statistical difference ($P < 0.05$) when EGFR expression was compared between chemotherapy and control (HT29 cells grown in fresh medium) groups. There was a significant difference between HT29 cells exposed to GILF and IFL regimens only when the assay was performed early after the end of the treatment (time 0, G). Analogous results were obtained in WiDr cells (Fig. 1G–I).

These results were confirmed by Western blotting with a specific Mab in SW620, WiDr and HT29 cell lines (Fig. 1J–L). Therefore, the increased expression of EGFR on cell membrane was paralleled by enhanced whole EGFR protein expression in tumour cells treated with anti-cancer drugs.

3.2. *Cetuximab-mediated ADCC and immune-sensitisation*

The colon carcinoma cell lines were assessed in cytotoxic assays for their sensitivity to cetuximab-mediated ADCC. In this study we used interleukin-2 (IL-2) activated PBMCs (LAK) cells as immuno-effectors by taking into consideration the results of previous studies showing the ability of these cytokine activated-lymphocytes to kill target cells in ADCC experiments with cetuximab *in vitro*.[26]

Our experiments were performed by exposing colon cancer target cells to allogeneic LAK cells at different effector (E)/target (T) ratios (50/1, 25/1, 12.5/1 and 1/1) in a fresh medium, in the presence of escalating doses of cetuximab (1, 10 and 100 μg/ml), or in the presence of escalating doses of the anti-CD20 rituximab moAb (1, 10 and 100 μg/ml) which was used as a negative control antibody. Before being used as targets in the cytotoxic assays, tumour cells had received no treatment or had been previously exposed to sub-lethal doses of the above described cytotoxic drugs.

WiDr and HT29 target cells expressing a wild-type k-ras profile showed a basal resistance to the immune-effector cells (<20%). However, the cytolytic effects of these lymphocytes became evident at higher E/T ratios (50/1 and 25/1) when cetuximab was added in the assays (ADCC). When these target cells had been previously exposed to the chemotherapy agents their sensitivity to either lymphocytes alone or cetuximab-mediated ADCC was significantly enhanced ($P < 0.04$). In our experiments, the IFL and GILF regimens showed the most efficient immune-sensitising effect (Fig. 2).

Untreated LoVo and SW620 target cells, which conversely express a mutated k-ras profile, were very sensitive to the LAK cells at the baseline. The killing of these target cells was again significantly increased by the addition of cetuximab in a dose-dependent manner. In the latter case, the exposure to chemotherapy agents increased only their susceptibility to

Fig. 1 – (Panels A–I). Cytofluorimetric analysis of EGFR expression on the cell surface of SW620 (A–C), HT29 (D–F) and WiDr (G–I) colon carcinoma cell lines. Figures show the ability of IFL and GILF poly-chemotherapy regimens to up-regulate EGFR expression early after the end of chemotherapy (time 0, A and G) and still lasting after 3 (B and H) and 24 h (Panels C and I) in SW620 and WiDr cells, respectively. We found a significant statistical difference ($P < 0.05$) when EGFR expression was compared between chemotherapy and control (cells grown in fresh medium) groups; conversely, there was no difference when the groups of cells exposed to IFL and GILF regimens were compared. HT29 cell line constitutively over-expresses EGFR on the membrane, however, IFL and GILF regimens were able to induce a significant up-regulation which starts early after the chemotherapy (E) and is maintained for 48 h (F) after the end of chemotherapy. We found a significant statistical difference ($P < 0.05$) when EGFR expression was compared between drug-exposed and control HT29 cells grown in fresh medium experimental groups. There was a significant difference between HT29 cells exposed to GILF and IFL regimens only when the test was performed early after the end of the treatment (time 0 and D). SW620 and WiDr cells: mean fluorescence intensity in cells at the end of chemotherapy (CTR, violet curve), or exposed to IFL (green line) and GILF (red line) regimens. HT29 cells: mean fluorescence intensity with an irrelevant IgG1 labelling used as a negative control (violet curve); HT29 cells at the end of chemotherapy (green line), or exposed to IFL (red line) and GILF (blue line) regimens. Each histogram corresponds to the analysis of at least 20,000 events. The experiments were repeated at least three times and gave always similar results. (Panels J–L) Analysis of EGFR expression by Western blot analysis in SW620 (J), WiDr (H) and HT29 (L) colon carcinoma cell lines. HT29 cell line constitutively over-expresses EGFR on the membrane. IFL and GILF regimens induce a significant up-regulation of EGFR which starts early after drug exposure and are maintained for 48 h after the end of chemotherapy (L). The last lane is the whole cell lysate incubated with protein A sepharose without the specific anti-EGFR antibody. The experiments were repeated three times with similar results.

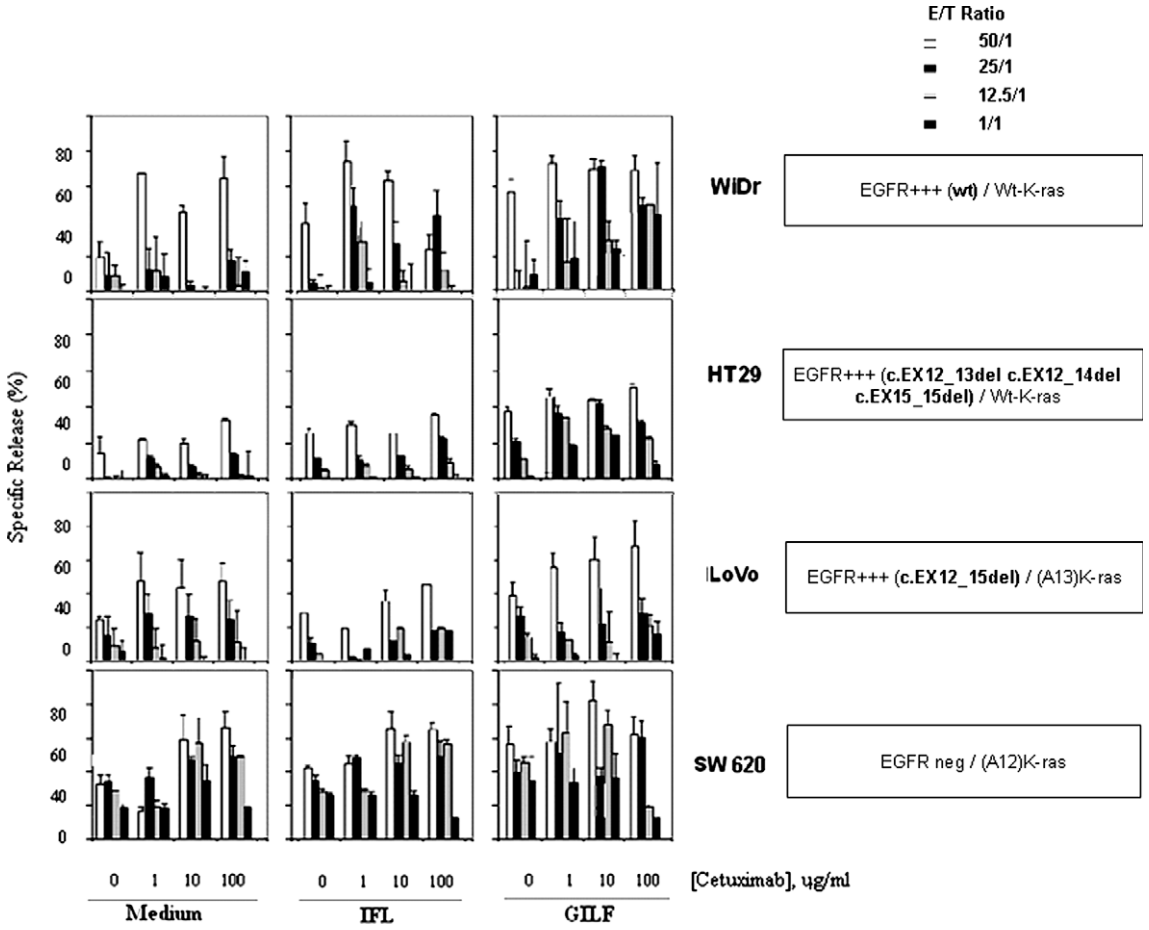


Fig. 2 – The figure shows the results of cytotoxic assays designed to test the *in vitro* anti-tumour activity of IL-2-activated human PBMCs against different colon carcinoma target cell lines expressing a different k-ras mutational status. The cytotoxic tests were performed at different effector/target (E/T) ratios in the presence of no antibody or escalating cetuximab concentrations. The target cells used in these experiments had been previously exposed to no treatment (first picture column) or ILF (second column) or GILF drug combinations (third column), respectively. When rituximab was used as a negative control antibody in the place of cetuximab the results were comparable with those obtained with the activated lymphocytes alone. Tumour cells exposed to IFL and GILF regimens showed the greatest sensitivity to the immune-effectors and to cetuximab-driven ADCC ($P < 0.05$). Tumour cells with a mutated k-ras showed the greatest sensitivity to the immune-effectors ($P < 0.05$). Bars, SEs. The experiments were repeated five times with similar results.

cetuximab-mediated ADCC ($P < 0.05$), but not to LAK cells alone (Fig. 2).

HT29 target cells that over-express a non-functional EGFR presenting multiple mutations (Table 1), resulted constitutively very sensitive to the LAK cells and cetuximab-mediated ADCC. In these cells, exposure to chemotherapy induced a further up-regulation of EGFR and enhanced their sensitivity to both LAK cells and cetuximab-mediated ADCC ($P < 0.05$). The maximal effect was achieved with IFL and GILF poly-chemotherapy regimens (Fig. 3). This effect started early after the end of the chemotherapy and was maintained for 48 h (data not shown).

The results of the cytotoxic assays carried out with a control moAb (rituximab) at the place of cetuximab were comparable to those obtained with the use of LAK cells alone (data not shown). The use of cetuximab alone without immune-effectors, or the addition of complement to cetuximab did not exert any cytolytic effect in our assay (data not shown).

4. Discussion

The results of this preclinical study show for the first time the ability of several cytotoxic drug combinations to up-regulate EGFR expression on the surface of colon carcinoma cells, enhancing their sensitivity to cetuximab-mediated ADCC by LAK cells. We also show that this mechanism is independent by either pre-treatment EGFR expression or k-ras mutational status in the tumour cells. Finally, we observed that the expression of an activating k-ras mutation in the tumour cells is correlated with a higher susceptibility to either LAK-mediated cytotoxicity or cetuximab-mediated ADCC.

Experimental evidence has already been provided that IgG1 antibodies mainly kill cancer cells by activating CDC

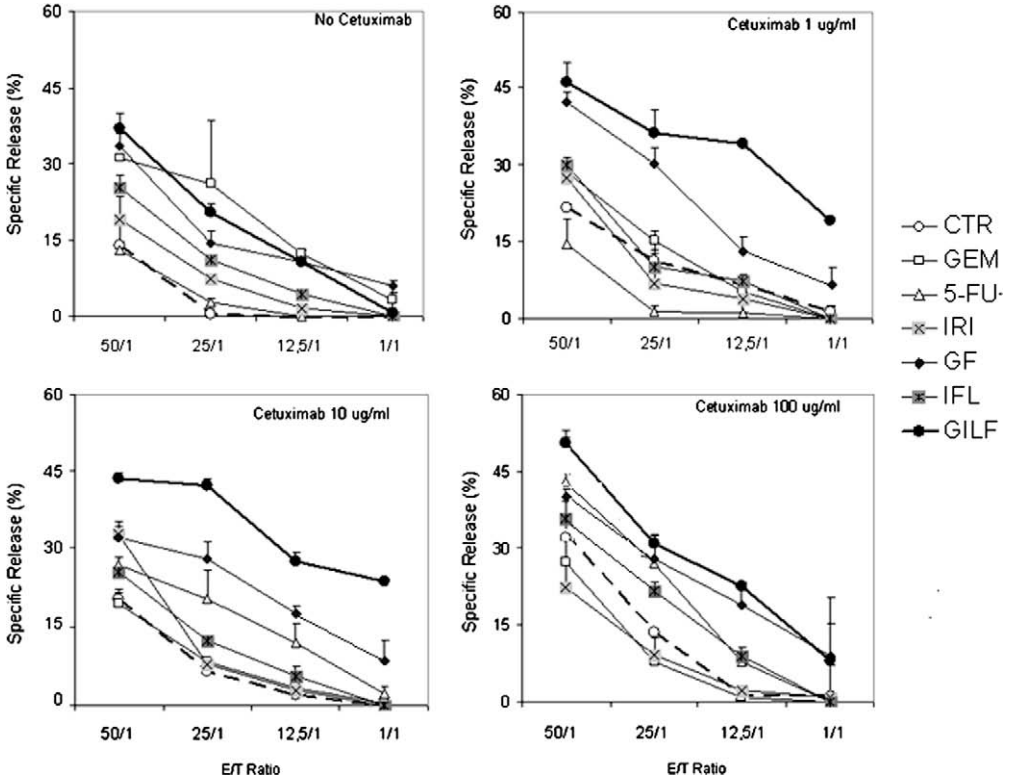


Fig. 3 – The figure shows the results of cytotoxic assays designed to assess the *in vitro* anti-tumour activity of IL-2-activated human PBMCs against the HT29 colon carcinoma target cells which over-express the EGFR. The cytotoxic tests were performed at different effector/target (E/T) ratios in the presence of no antibody or escalating cetuximab concentrations. The target cells used in these experiments had been previously exposed to no treatment, gemcitabine (G), 5-fluorouracil (F), irinotecan (I), GF, IFL and GILF. When rituximab was used as a negative control antibody in the place of cetuximab, the results were comparable with those obtained with the activated lymphocytes alone. Tumour cells exposed to GF and GILF regimens showed the greatest sensitivity to the immune-effectors and to the cetuximab driven ADCC ($P < 0.05$). These experiments were repeated five times with similar results.

and ADCC processes; in detail, it has been demonstrated that the *in vivo* anti-tumour activity of rituximab in B cell lymphoma and trastuzumab (anti-ERB-B2) in breast and gastric cancer is largely based on ADCC processes. These IgG1 moAbs as well as cetuximab are able of igniting an ADCC process throughout the human Fc backbone which is able of binding with high affinity the Fc receptors expressed by NK (FcγIII), monocytes (FcγII) and granulocytes (FcγI).[24–30] Previous studies have also shown that the ADCC is augmented when the effector cells had been previously exposed to IL-2, a cytokine, which is able of greatly enhancing the cytolytic activity of natural killer cells and other immuno-effectors. In fact, it has been shown that the exposure of human PBMCs to high dose IL-2 (>500 mg/ml) leads to the generation of LAK cells with potent *in vitro* anti-tumour activity. These IL-2 activated immune-effectors express the FcγIII receptor and are able to ignite an efficient IgG1-mediated ADCC.[25,27] On these bases, clinical trials in cancer patients are currently investigating the use of rituximab or trastuzumab in combination with this cytokine and researchers begin to report the first intriguing results.[31,32]

The actual role of ADCC in the anti-tumour activity of cetuximab in colon and non-small cell lung cancer patients has not been fully investigated and much less information is presently available if compared with the wider knowledge concerning its ability to inhibit EGFR-mediated signalling.[8,26] However, in the latter few years a number of authors have investigated this topic and preliminary results have indeed shown the ability of cetuximab to activate an *in vitro* ADCC process. In addition, the results of recent studies have shown that a specific polymorphic variant in the Fc receptor, which leads to a higher FcγIII binding affinity for IgG1, appears to be correlated with a better outcome in colon cancer patients who had received cetuximab.[33]

In this view, we believe that EGFR significantly differs from any other target for immuno-therapy; in fact, it is a molecular structure critical for tumour cell growth and survival, thus its expression is not the same and may significantly change as a consequence of different micro-environmental conditions.

Experimental results lead us to believe that biological or environmental stress may lead to enhanced expression of EGFR that protects the tumour cells from the induction of apoptotic processes. On the other hand, the enhanced expression of EGFR makes the tumour cells also more sensitive to EGFR immune-targeting strategies. It has been previously demonstrated by our group that the exposure of cancer cells to IFN-alpha or cytotoxic anti-metabolites (i.e. cytosine arabinoside or 5-Aza 2′deoxycytidine) up-regulates EGFR expression

on epithelial tumour cells including colon cancer cells.[34–36] The increased expression of EGFR, in turn, enhanced the *in vitro* targeting of tumour cells with antibodies raised against the external domain of EGFR.[35–37] EGFR up-regulation was paralleled by an increased activation of the EGFR-dependent signal transduction pathway and was considered as a survival response of tumour cells to apoptotic and anti-proliferative stimuli induced by anti-cancer agents.[37] In detail, the EGF-dependent pathway was hyper-activated in interferon-α-treated epidermoid cancer cells and the specific disruption of this pathway potentiated the apoptosis induced by the cytokine in the cells.

In conclusion, the results of the present study demonstrate that cytotoxic drugs up-regulate EGFR expression in colon cancer cells and that this effect correlates with an enhanced sensitivity to cetuximab-mediated ADCC by LAK cells. In addition, we show that the latter effect is not related to k-ras mutational status in the target cells. The latter finding appears of specific interest if one considers that the main limitation to the use of cetuximab in the treatment of colon and non-small cell lung cancer patients is specifically represented by the presence in the tumour of activating k-ras mutations and other downstream alterations in the EGFR pathways.[15–17,38,39] The results of a number of clinical trials have shown that any possible advantage in terms of long-term survival achieved by chemotherapy + cetuximab is completely lost in patients whose tumour bears this kind of alterations. Similar results have also been reported for different anti-EGFR moAbs such as pertuzumab and panitumumab which are not able to activate ADCC, or different EGFR inhibitors such as erlotinib and gefitinib in non-small cell lung cancer.[15–17,39–41]

On the other hand, it has been recently reported the lack of response predictivity by k-ras mutation in metastatic gastric cancer treated with cetuximab in association with chemotherapy.[42] However, the absence of an association between k-ras mutations and the efficacy of treatment with cetuximab may be a result of the small sample size in such study. In addition, the low incidence of k-ras mutation in gastric cancer may also contribute to the lack of a significant association with therapeutic success.

Finally, a further consideration should be done on the finding that the exposure of target cells to the cytotoxic drugs enhances the susceptibility of target cells to the IL-2 activated immune-effectors. This phenomenon has been also reported by other authors who showed that several anticancer agents including GEM, 5-FU and IRI enhance the sensitivity of cancer cells *in vitro*, to cytotoxic mediators utilised by activated immune-effectors such as NK and antigen specific CTLs.[43–46]

On these bases, we believe that the results of the present study provide the rationale to design a new chemo-immunotherapy combination for colon cancer where cetuximab is administered just after and not concomitantly with chemotherapy, and it is followed by rIL-2/aldesleukine administration to further improve the ADCC efficiency and to overcome resistance due to k-ras mutation.

Conflict of interest statement

None declared.

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