RESEARCH

Nimotuzumab-mediated antibody-dependent cellular cytotoxicity activity on target tumor cell lines depends on the expression level of the epidermal growth factor receptor

Lisset Chao, Dayana Pérez, ✍Belinda Sánchez

Tumor Immunology Direction, Center of Molecular Immunology, CIM
POBox 16040, Havana 11600, Cuba
✍belinda@cim.sld.cu

ABSTRACT

The epidermal growth factor receptor (EGFR) is an attractive target for cancer treatment due to its major role in tumor development. For this reason, the nimotuzumab monoclonal antibody (mAb) was developed. This is a humanized monoclonal antibody specific for the extracellular domain of EGFR that inhibits its signaling cascades and arrest the cell cycle in epithelial tumor cell lines. At the same time, it is known that the antibody dependent cellular cytotoxicity (ADCC) response is one of the critical mechanisms underlying the clinical efficacy of some therapeutic anticancer mAbs. However, there were no reports on the capacity of nimotuzumab to mediate this effect. Therefore, in this work, the ability of nimotuzumab to induce ADCC was investigated, as well as the influence of EGFR expression levels on this mechanism of action. It was demonstrated that this mAb is able to mediate ADCC against cultured cell lines, which increases with an increase in mAb's concentration and the effect directly depending on the level of EGFR expression. These results suggest ADCC activity as one of the potential therapeutic mechanisms mediating the action of nimotuzumab in patients with high EGFR expression tumors.

Keywords: Nimotuzumab, antibody-dependent cellular citotoxicity, human epidermal growth factor receptor, tumor cell culture, cancer therapeutics

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RESUMEN

Citotoxicidad celular dependiente de anticuerpos inducida por el nimotuzumab en líneas tumorales depende de los niveles de expresión del receptor del factor de crecimiento epidérmico humano. El receptor del factor de crecimiento epidérmico humano (EGFR) se ha convertido en uno de los blancos más atractivos para el tratamiento del cáncer, debido a su papel trascendental en el desarrollo tumorigénico. Nimotuzumab es un anticuerpo monoclonal (mAb) específico contra el dominio extracelular del EGFR, del cual inhibe su cascada de señalización y arresta el ciclo celular en células tumorales de origen epitelial. Es conocido que la citoxicidad celular dependiente de anticuerpo (ADCC) es un mecanismo que contribuye a la efectividad clínica de algunas drogas terapéuticas antitumorales. Sin embargo, no existen reportes sobre la posible ocurrencia de respuesta ADCC mediada por el nimotuzumab y su relevancia para el efecto antitumoral. Por tales razones, en este estudio se analizó la capacidad del nimotuzumab de inducir respuesta ADCC y la influencia de los niveles de expresión del EGFR en dicho mecanismo de acción. Se demostró que este mAb induce ADCC en células tumorales en cultivo, la cual se incrementa con el aumento en la concentración del anticuerpo y es dependiente de los niveles de EGFR. Estos resultados sugieren que la ADCC puede ser uno de los mecanismos potenciales a través de los cuales el nimotuzumab ejerce su efecto terapéutico en pacientes con tumores de alta expresión del EGFR.

Palabras clave: Nimotuzumab, citotoxicidad celular dependiente de anticuerpos, receptor del factor de crecimiento epidérmico humano, cultivo de células tumorales, terapia del cáncer

*I*ntroduction

The epidermal growth factor receptor (EGFR) has become one of the most promising therapeutic targets in the treatment of various solid tumors. This tyrosine kinase receptor is involved in a complex signaling cascade that modulates metabolism, growth, differentiation, adhesion, migration, survival and escape in tumor cells [1]. In addition, recent studies have demonstrated that EGFR signaling is involved not only in the malignant behavior of tumor cells, but also in the modification of the tumor microenvironment to favor cancer progression [2].

Two major approaches that inhibit EGFR functions have being successfully used in cancer treatment: tyrosine kinase inhibitors (TKI) and mAbs [3]. Several monoclonal antibodies (mAbs) have been evaluated in the past years for tumors of different origins. Among them, cetuximab (a chimeric IgG1) and panitumumab (a fully human IgG2) have been approved by the Food and Drug Administration for colorectal carcinoma and head and neck cancer [4, 5]. In Cuba, a humanized therapeutic mAb named nimotuzumab was developed against EGFR at the Center of Molecular Immunology [6]. This mAb has been approved for head and neck, nasopharyngeal tumors, adult high-grade glioma, pediatric glioma, and advanced esophageal cancer, in combination with radiotherapy and radio-chemotherapy, or as monotherapy [7-12]. Clinical trials are ongoing globally to evaluate nimotuzumab in other indications.

Clinical benefit is provided by therapeutic EGFR directed mAbs, but many patients fail to respond to these therapies, what emphasizes the relevance of completely understanding their mechanisms of action. Multiple investigations have been focused on the capacity of anti-EGFR mAbs, as nimotuzumab,

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to inhibit the receptor signaling cascade [13]. However, other studies with cetuximab described the role of the Fc region in the mechanism of action through the induction of antibody-dependent cell cytotoxicity (ADCC) [14]. Studies in patients with colorectal cancer demonstrated that mAb-binding Fcγ receptor (FcγR) polymorphisms correlate with the clinical outcome of cetuximab [15, 16].

Different effects of nimotuzumab over tumor cells have been already described, as cell cycle arrest, inhibition of Akt activation and reduced vascular endothelial growth factor (VEGF) production. All of them are directly related with its property of inhibiting EGFR activation. However, the role of the Fc region of this mAb has not been elucidated [13].

In the present study we demonstrate that nimotuzumab not only induces ADCC on tumor cell lines but also this cytotoxicity positively associates with EGFR expression levels.

Materials and methods

Cell lines

Human epidermoid carcinoma A431 (ATCC CRL-1555), breast carcinoma MDA-MB468 (ATCC HTB-132), human lung adenocarcinoma H125 (CRL-5801, ATCC) and prostate carcinoma PC3 (ATCC CRL-1435) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The human small cell lung cancer U1906 [17] was gently donated by the Molecular Biology Department of MPI (Germany). All these cell lines were grown in Dulbecco's minimal essential medium (DMEM; Gibco, United Kingdom) supplemented with 10 % fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin.

Western blot analysis

Cells were lysed in RIPA buffer (1× PBS, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS) with 50 mM NaF, 1 mM Na3VO4, 5 mM EDTA and 1 mM henylmethylsulphonylfluoride. Protein concentrations were determined according to bicinchoninic acid protein assay (BCA) kit (Santa Cruz Biotechnology, USA). Thirty micrograms of protein extracts were applied to 7.5 % SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes during 1 h (Millipore, USA). The membranes were blocked in TBS-T buffer (10 mM Tris, 15 mM NaCl, 0.01 % Tween 20) with 5 % skim milk powder and incubated with EGFR-specific primary antibodies (Cell Signaling, USA), washed, and incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling). Proteins were visualized by ECL (Santa Cruz Biotechnology). To corroborate similar transfer and equal loading, membranes were incubated with a β-actin specific antibody (Cell Signaling). The ratio represented the relation between the densitometry units of EGFR and β-actin bands (ImageJ program).

Flow cytometry analysis for EGFR recognition

EGFR surface expression on human tumor cell lines was analyzed by flow cytometry using nimotuzumab at 10 µg/mL, followed by FITC-conjugated anti-human IgG (Fab specific, 1:200 dilution) (Sigma-Aldrich, USA). Data were obtained with a Gallios Flow cytometer (Beckman Coulter, USA) by collecting a minimum of 10 000 events and analyzed using the Kaluza software v2.1 (Beckman Coulter). The data were expressed as fluorescence mean intensity (FMI).

Isolation of peripheral blood mononuclear cells

Fifty milliliters of blood samples were obtained from healthy donors and immediately heparinized. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Paque PLUS centrifugation (Amersham Biosciences, United Kingdom) [18] and cultured overnight in RPMI medium (Gibco) supplemented with 10 % of FBS. Written informed consents were received from all the donors. The study protocol was conducted in accordance to the principles of the Declaration of Helsinki and Good Clinical Practices guidelines [19, 20].

Cytotoxicity assays

To perform ADCC assays, tumor cell lines A431, MDA-MB-468, H125, PC3 and U1906 were plated on 96-well plates at a concentration of 5×10^3, 5×10^3, 1×10^4, 2.5×10^3 and 5×10^3, respectively. Nimotuzumab was added at 1, 10, 50 and 100 µg/mL and peripheral blood mononuclear cells (PBMC) were added at E:T ratio 100:1. Cetuximab mAb was used as positive control of ADCC at 10 µg/mL. Target cells without treatment were includes as control for spontaneous lysis. Cells treated with 1 % Triton X were used as positive control of maximal lysis. The mAb C5hT was used at 10 µg/mL as isotype control [21]. Lysis percentages induced by nimotuzumab and cetuximab were normalized by subtraction of the lysis values induced of mAb C5hT control and plotted on a graph.

Each reaction was done in triplicate. The plates were incubated for 4 h at 37 °C, and the absorbance of the supernatants at 490 nm was recorded to determine the release of lactate dehydrogenase with the lactate dehydrogenase-based cytotoxicity detection kit (Roche Diagnostics, Germany). The percentage of cytotoxicity was calculated using the following formula: Lysis (%) = (experimental release – target cell spontaneous release – effector cell spontaneous release)/(maximal release – target cell spontaneous release) × 100.

Results

EGFR binding of nimotuzumab in different human tumor cell lines

To assess the capacity of the antibody nimotuzumab to bind to the EGFR of human tumor cell lines of varied origin, we first analyzed the protein expression of this receptor in A431, MDA-MB-468, H125 and PC-3 cell. As shown in Figure 1A, A431 expressed higher levels of EGFR and progressively decreased from MDA-MB-468 to H125. Conversely, PC-3 cells expressed the lowest levels of the receptor.

In order to evaluate the EGFR binding of nimotuzumab in the cell surface of human tumor cell lines, we used flow cytometry analysis with nimotuzumab at 10 µg/mL. The data were expressed as mean fluo-

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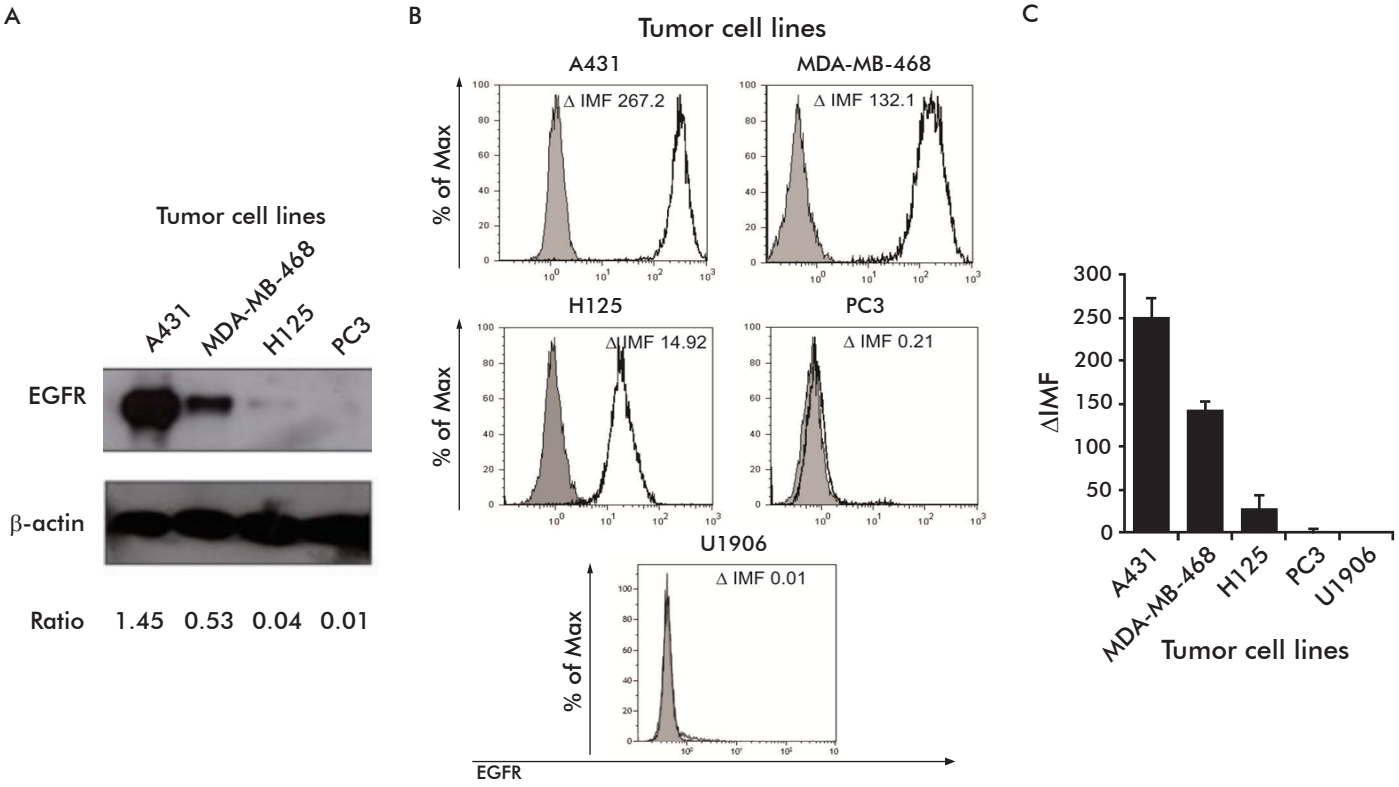

Figure 1. Binding of nimotuzumab to human tumor cell lines with different EGFR expression. A) EGFR protein levels determined by a Western Blot assay. The ratio represents the relation between the densitometry units of EGFR and β-actin bands. B) Representative flow cytometry experiment of 3 experiments, evaluating EGFR binding of 10 μg/mL nimotuzumab in the cell surface of tumor cells lines, expressed as ΔMFI values. Isotype controls staining are shown as filled histograms. C) Summary of the ΔMFI values obtained for each tumor cell line from 3 replicates experiments (mean ± SD are represented), calculated as MFI [nimotuzumab] - MFI [isotype control Ab].

rescence intensity (MFI). As shown in Figure 1B and C, EGFR binding of nimotuzumab was higher in A431 cells and continuously decrease in MDA-MB-468, H125 and PC3, with no binding detected in the PC3 cell line, similar to U1906 cells, used as a negative control of EGFR expression [17].

ADCC activity of nimotuzumab

The capacity of nimotuzumab to induce ADCC in A431 tumor cells was evaluated by treating it with the mAb and further comparison with cetuximab, used as a positive control. U1906 tumor cell lines were used as negative control of ADCC due to its EGFR negative expression. To evaluate the impact of the EGFR expression levels in this effect, A431, MDA-MB-468, H125 and PC-3 cells were compared for concentration-dependent ADCC mediated by nimotuzumab. Lysis percentages of nimotuzumab were normalized against the lysis values of de IgG control and plotted on a graph.

Nimotuzumab was capable to induce ADCC at 10 μg/mL in A431, an EGFR+ tumor cell line. In U1906 tumor cells, a negative cell control of EGFR expression, no cytotoxic activity was observed with this mAb. The positive control cetuximab, mediated ADCC up to 60 % of lysis in the A431 tumor cell line (Figure 2A).

Comparison among analyzed tumor cell lines revealed that the highest cytotoxic activity was obtained against A431 cells, which express the highest EGFR levels, followed by MDA-MB-468 and H125 cells. In contrast, no toxicity was observed against PC3 cells which expressed the lowest EGFR levels (Figure 2B). Hence, increased EGFR was accompanied by enhanced ADCC response triggered by nimotuzumab.

Additionally, irrespective of EGFR expression levels, the nimotuzumab-induced ADCC response increase in a mAb concentration-dependent manner (Figure 2B). In A431, 30 % of lysis was observed at 1 μg/mL of nimotuzumab, reaching up to 60 % with 100 μg/mL of Ab. Likewise, increased lysis was detected in MDA-MB-468 cells with 100 μg/mL as compared to 1 μg/mL nimotuzumab. In H125 cells, no effect was detected at 1 μg/mL nimotuzumab, requiring a concentration higher than 10 μg/mL to achieve the cytotoxic effect.

Discussion

In this study, the capacity of nimotuzumab to induce ADCC and the impact of the EGFR expression on this effector mechanism were determined. First, there were evaluated the protein levels of EGFR in the different tumor cell lines used in the study. It was corroborated by Western blot that A431 and MDA-MB-468 cells express high levels of EGFR, which were moderate in the case of H125 cells and low for PC-3 cells. Previous reports demonstrated that A431 cells and MDA-MB-468 tumor cells express 2,6 × 10^6 and 1.3 × 10^6 EGFR molecules per cell,

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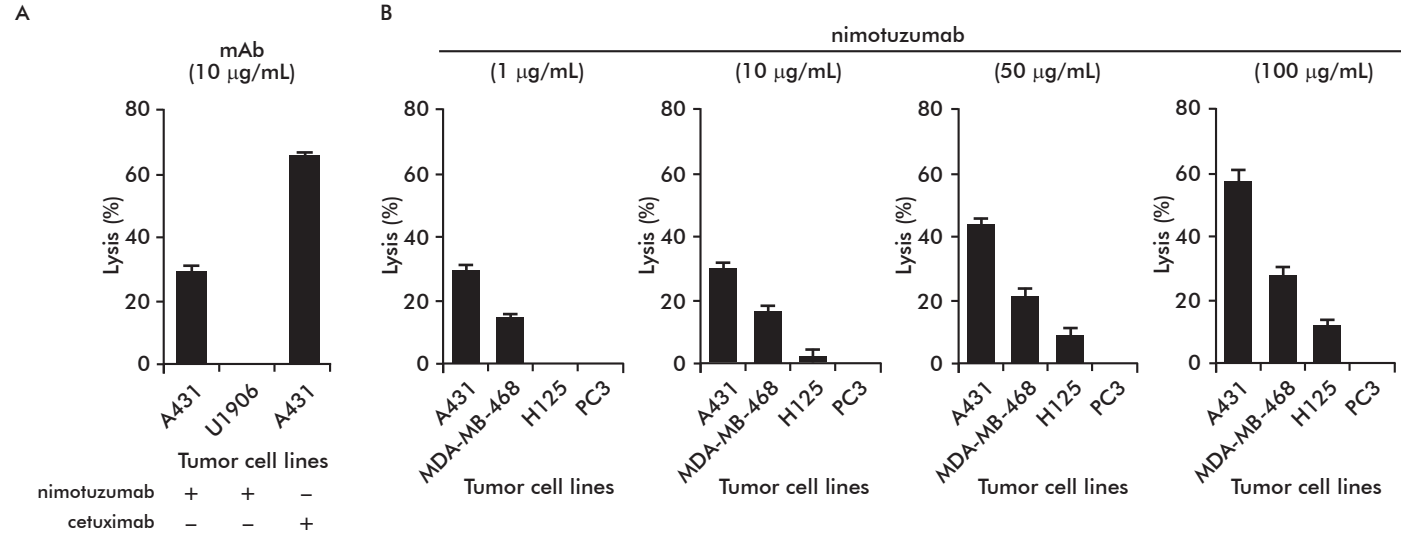

Figure 2. ADCC of the monoclonal antibody nimotuzumab on human tumor cell lines. A) Immune effector cells (PBMCs) were incubated in the absence or presence of nimotuzumab at 10 μg/mL, with A431 or U1906 human tumor cells line at an effector to target ratio of 100:1 for 4 h. Cetuximab was used as positive control of ADCC in A431 tumor cells, at 10 μg/mL. Supernatants were analyzed for lactate dehydrogenase as a measure of cytotoxicity. Means ± SD from at least three independent experiments with different blood donors are presented. B) Immune effector cells (PBMC) were incubated in the absence or presence of nimotuzumab, at increasing concentrations, with human tumor cells line at an effector to target ratio of 100:1 for 4 h. Supernatants were analyzed for lactate dehydrogenase as a measure of cytotoxicity. Data were analyzed with regard to EGFR binding level, as well as nimotuzumab concentrations used in the experiments. Means ± SD from at least three independent experiments with different blood donors are presented.

respectively [22, 23], while H125 cells express 2.1 × 10^5 receptors [24] and PC-3 cells only ~3 × 10^4 [25].

We also show that nimotuzumab has de capacity to recognize the EGFR in three out of the five tumor cell lines tested, but neither in PC3 cells which display low levels of the receptor, nor in U1906, this last cell line reported with negative expression of the receptor. Garrido et al. described this effect of nimotuzumab by the "affinity window" hypothesis [26]. Based in a mathematic model previously constructed they predicted an "affinity window" (K_D between 10^{-7} and 10^{-9} M) for optimal therapeutic index of the anti-EGFR antibodies [27]. Nimotuzumab K_D for the Fab fragments is 2.1 × 10^{-8} [28] and would fall within the predicted optimal affinity window. That study includes an avidity component and suggests that nimotuzumab binds with both arms when EGFR expression is moderate to high, but a monovalent interaction is transient at low EGFR density. This model would explain why nimotuzumab could preferentially bind tumor tissues overexpressing the receptor, relying on bivalent binding for stable attachment to the cellular surface [26].

To first evaluate the nimotuzumab capacity to mediate ADCC on tumor cells, we selected A431 cell line and U1906 as a negative control. A strong lysis capacity of nimotuzumab was obtained on A431 cells, even when it was lower than that of cetuximab. Such a response could be explained by affinity differences between both antibodies. It was also demonstrated that nimotuzumab-induced ADCC was dependent on EGFR expression levels. High EGFR expression on tumor cells was linked with a higher Fc-mediated killing, further suggesting that differential EGFR binding by nimotuzumab determines the levels of ADCC. Pre-clinical data support the idea of the dependence of EGFR levels for antitumoral effect of this mAb. In this line, Akashi et al. found that the inhibitory effect of nimotuzumab on EGFR signaling depends on the expression level of EGFR on the cell surface [29]. Additionally, Garrido *et al*. demonstrated that EGFR expression is crucial for Fab-mediated antitumor mechanism [26].

Similar results have been found in the clinic. Coincidently, EGFR expression showed a significant association with survival in patients receiving nimotuzumab in combination with chemoradiation and radiotherapy, as compared to radiotherapy alone, in a multicenter, open label Phase IIb, randomized clinical trial in patients with squamous cell carcinomas of the head and neck [30].

In summary, here we provide evidence of a new mechanism of action of nimotuzumab, ADCC, which can contribute to better understand its effect in the clinical setting and emphasize the role of EGFR expression on this mAb antitumor effect. Moreover, these results also bring new elements to consider the evaluation on whether the EGFR expression level is a predictive marker of nimotuzumab's clinical efficacy, providing a subset of patients that might be benefited with this mAb. In future experiments, it should be demonstrated whether NK cells could be involved in nimotuzumab-induced ADCC, as has been demonstrated for other IgG1 human antibodies [31]. Potentially, NK cells activation due to ADCC could be translated into the activation of the adaptive immune system [31, 32].

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