Effects of a human compact anti-ErbB2 antibody on prostate cancer

ANGELA ELIANA MALARA[1], CARMINE FEDELE[1], LUIGI ALOJ[2], CLAUDIO ARRA[3], PAOLO LACCETTI[1], GIUSEPPE D'ALESSIO[1] and CLAUDIA DE LORENZO[1]

[1]Department of Structural and Functional Biology, University of Naples Federico II, I-80126 Naples; [2]Department of Nuclear Medicine, [3]SSD Animal Facility, IRCCS National Cancer Institute, G. Pascale Foundation, I-80131 Naples, Italy

Abstract. Prostate cancer is the most commonly diagnosed malignancy in men in developed countries. ErbB2, a tyrosine kinase receptor overexpressed in many human cancer types, contributes to prostate cancer progression by activating the androgen receptor in a steroid poor environment, thus promoting androgen-independent cell growth. The consequent development of hormone refractory tumors is a major obstacle in prostate cancer therapy. The inhibition of ErbB2 signal transduction pathways by the use of human antibodies could be a valuable alternative strategy for cancer therapy. We performed a comparative analysis *in vitro* and *in vivo* of the antitumor effects of three different antibodies targeting different epitopes of ErbB2: Herceptin (trastuzumab), 2C4 (pertuzumab) and Erb-hcAb (human anti-ErbB2-compact antibody), a novel fully human compact antibody produced in our laboratory. Herein, we demonstrate that the growth of both androgen-dependent and independent prostate cancer cells was efficiently inhibited by Erb-hcAb. The antitumor effects induced by Erb-hcAb on some cell lines were more potent than those observed for either Herceptin or 2C4. Thus, Erb-hcAb could be a promising candidate in the immunotherapy of prostate cancer for which no obvious treatment has been reported so far.

Introduction

Prostate cancer is the second leading cause of cancer-related death among men in the USA. In 2005 it was the most frequently diagnosed cancer, accounting for approximately 33% of incident cases in men and 10% of deaths (1). Most of the patients with an advanced form of the disease at diagnosis are treated by systemic androgen ablation therapy. Initially, the cancer is responsive to this type of treatment because it exhibits an androgen-sensitive phenotype (2). Unfortunately, at a later stage the cancer typically progresses in a form that no longer responds to androgen deprivation, thus becoming androgen-independent. Androgen-independent prostate cancer is resistant to therapeutic interventions and ultimately leads to the demise of the patient.

The mechanisms through which prostate cancer converts to androgen independence are still unclear. It has been reported that the onset of ErbB2 protein overexpression may coincide with the emergence of androgen independence as the androgen receptor can be activated by ErbB2 through the Akt pathway, thus promoting androgen-independent prostate cancer growth and survival (3).

ErbB2 (also known as HER2) is a transmembrane tyrosine kinase member of the epidermal growth factor receptor (EGFR) family, overexpressed in many human cancer types. In particular, ErbB2 is implicated in malignant transformation and tumorigenesis (4,5), and is overexpressed in breast, ovary, prostate, non-small cell lung cancer and in several other carcinomas (6-8), whereas on normal tissues it is not detectable or it is expressed at very low levels (9). Owing to their role in cancer pathogenesis, ErbB2 and EGFR have become important targets of anticancer therapy: many drugs have been developed against one or both of these receptors.

Herceptin (trastuzumab) is the only humanized monoclonal antibody available for the treatment of ErbB2-positive breast cancer since 1998 when it was approved by FDA, and is now used for the treatment of both metastatic and early-stage breast cancer (10,11). Although Herceptin significantly improves the outcome for patients with ErbB2-positive breast cancer, not all the patients benefit from the treatment. A significant fraction of the patients do not respond at all, and most of the patients who initially respond to Herceptin generally tend to develop resistance during treatment (12).

Furthermore, carcinomas with a high expression of ErbB2, such as non-small cell lung carcinoma, gastric and prostate tumors, have been found to be resistant or much less sensitive to Herceptin treatment (13-15). Finally, large-scale clinical studies with Herceptin have shown that it engenders cardiotoxicity (16,17), which occurs more frequently in patients treated

Correspondence to: Dr Claudia De Lorenzo, Department of Structural and Functional Biology, University of Naples Federico II, via Cinthia, I-80126 Naples, Italy
E-mail: cladelor@unina.it

previously or concurrently with anthracyclines (18). Thus, there is an urgent need to identify new antibodies capable of disrupting the ErbB2 signaling pathway in cancer patients.

Recently, we produced a novel fully human anti-ErbB2 immunoagent (19), engineered by fusing Erbicin, a human anti-ErbB2 single-chain antibody fragment (scFv) (20), with the Fc region of human IgG1.

The human anti-ErbB2 antibody construct has been called Erb-hcAb for its 'compact' size (100 kDa), compared with the full size (155 kDa) of a natural IgG. It has been reported that Erb-hcAb is capable of selectively binding to malignant cells that express ErbB2, and of inhibiting their growth *in vitro* and *in vivo*, with no effects on ErbB2-negative cells. Moreover, Erb-hcAb is endowed with both antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) effects (19,21).

More recently, it has been shown that Erb-hcAb does not display the cardiotoxic effect of Herceptin *in vitro* on rat cardiomyocytes and *in vivo* on a mouse model, whereas Herceptin was strongly toxic. This difference was found to be due to their different mechanism of action, which can explain their different effects: Herceptin, in contrast to Erb-hcAb, induces apoptosis in cardiac cells (22). Finally, Erb-hcAb is active *in vitro* and *in vivo* against some Herceptin-resistant, ErbB2-positive breast cancer cell lines (23).

In the present study, we investigated for the first time the antitumor effects of Erb-hcAb towards androgen-dependent and androgen-independent ErbB2-positive prostate cancer cells. The analyses were carried out in comparison with Herceptin and 2C4, the mouse antibody version of pertuzumab, a new humanized anti-ErbB2 monoclonal antibody recently tested in phase II clinical trials for breast (24), ovarian, prostate and non small-cell lung cancer (25,26).

Materials and methods

Cell cultures and antibodies. The hybridoma cells producing 2C4 antibody (LGC Standards, Sesto San Giovanni, Italy) were grown in DMEM-RPMI-1640 medium in a 1:1 ratio (Gibco-BRL, Life Technologies, Paisley, UK).

The PER.C6® cell line (Crucell N.V., Leiden, The Netherlands), transfected with the recombinant plasmid pIgPlus (19), was cultured according to the manufacturer's recommendations.

The LNCaP, PC-3 (LGC Standards), SKBR3 and A431 cell lines (from ATCC, Rockville, MD) were cultured in RPMI-1640 medium.

DU145 cells (LGC Standards), were cultured in EMEM (Eagle's minimal essential medium) containing sodium pyruvate and non-essential amino acids at a concentration of 1 mM. The 22Rv1 cells (LGC Standards) were cultured in DMEM containing HEPES at a concentration of 15 mM. All the cell lines were supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin (all from Gibco), and were cultured at 37°C in 5% CO_2 atmosphere.

The antibodies used were: Herceptin (Genentech, South San Francisco, CA, USA); horseradish peroxidase-conjugated goat anti-human affinity isolated IgG1 (Fc-specific, Sigma, St. Louis, MO, USA), horseradish peroxidase-conjugated

monoclonal anti-human IgG (Fc-specific, Sigma), horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (Pierce, Rockford, IL, USA), horseradish peroxidase-conjugated anti-β actin antibody (Pierce).

Erb-hcAb was produced by transfected PER.C6® cells and purified as previously described (19).

2C4 was produced and purified as follows. 2C4 hybridoma cells were expanded to near confluence in complete medium, and then grown for 3-4 days in serum-free medium. The secreted antibody was purified from culture medium by affinity chromatography on a protein G Sepharose loaded with 300-500 ml of conditioned medium. Wash and elution steps were carried out as described for Erb-hcAb (19).

Cell lysis and immunoblot analyses. The cells, washed with PBS, were collected by centrifugation at 1200 rpm for 7 min. The pellet was resuspended in lysis buffer (10 mM Tris-HCl, pH 7.4, 0.5% Nonidet P-40, 150 mM NaCl), containing Complete™ protease inhibitor (Boehringer Mannheim, Germany). After 20 min at 0°C, the extracts were clarified by centrifugation at 12,000 rpm at 4°C for 15 min. Aliquots of 20 μg were run on 7.5% SDS-PAGE, followed by electroblotting onto PVDF membranes (Millipore, Bedford, MA, USA). The ErbB2 protein was detected using Neu anti-ErbB2 mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by rabbit anti-mouse horseradish peroxidase conjugated antibody. The signal intensity of the reactive bands was quantitatively measured by a phosphorimager (45-710, Bio-Rad Laboratories, Hercules, CA, USA).

ELISA assays. ErbB2-positive prostate tumor cells, harvested in non-enzymatic dissociation solution (Sigma), were washed and transferred to U-bottom microtiter plates ($1x10^5$ cells/well). After blocking with PBS containing 6% bovine serum albumin (BSA), cells were treated with the antibodies as previously described (18). For detection of human or mouse antibodies, peroxidase-conjugated anti-human IgG (Fc-specific, Sigma) or anti-mouse IgG antibody (Pierce) were used, respectively. Binding values were determined from the absorbance at 450 nm, and reported as the mean of at least three determinations (standard deviation ≤5%).

Cell growth inhibition assays. Tumor cells were seeded in 96-well, flat-bottom plates at a density of $7.5x10^3$ cells/well. After addition of the protein under examination, viable cells were tested by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (MTT) (Sigma) or counted by the trypan blue-exclusion assay at suitable time intervals. Cell survival was expressed as percent of viable cells in the presence of the protein under test with respect to control cultures grown in the absence of the protein. Typically, cell survival values were obtained from at least three independent experiments in which triplicate counts were determined; standard deviations were <5%.

In vivo antitumor activity. In vivo experiments were performed with 6-week-old male Balb/c nude mice (Charles River Laboratories, Calco, Italy). LNCaP or PC-3 cells ($2x10^6$) were suspended in 0.2 ml sterile PBS and injected subcutaneously (Day 0) in the right paw. On Day 20, when tumors were clearly

detectable, Erb-hcAb, Herceptin or 2C4 dissolved in PBS were administered intraperitoneally to three different groups of 5 mice at doses of 5 mg/kg of body weight for seven times at 72-h intervals. Another group of control mice was treated with identical volumes of sterile PBS. During the period of treatment, tumor volumes (V) were measured with a calliper and calculated by the formula of rotational ellipsoid $V = A \times B^2/2$ (A is the axial diameter and B the rotational diameter). SD values were <10%. All mice were maintained at the animal facility of the Department of Cellular and Molecular Biology and Pathology, University of Naples Federico II. The animal experimentations described herein were conducted in accordance with the Italian regulation for experimentation on animals. All *in vivo* experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Pharmacokinetics and biodistribution of Erb-hcAb. For assessing the pharmacokinetics and biodistribution properties of Erb-hcAb, *in vivo* experiments were performed in a mouse model. To this aim, CD1 nude mice were subcutaneously injected with LNCaP cells (1x10^6 cells) in the flank to generate xenografts. After 3 weeks tumor weights were in the range of 0.2-0.5 g. Animals were divided into 3 groups of 5 mice each and ^{125}I labeled Erb-hcAb was injected intraperitoneally (1-2x10^6 cpm/animal). At preset times ranging from 5 min to 96 h retro-orbital sinus sampling was performed by alternating group of animals for each time point in order to evaluate blood clearance of the radiolabeled compact antibody. At 96 h animals were sacrificed and residual radioactivity in organs was determined by gamma counting and weighing. Radioactivity levels are expressed as percentage of injected dose per gram (% ID/g) normalized for a 20-g mouse.

The blood clearance parameters of the labeled protein were fitted using Prism 4.0 software (Macintosh version, GraphPad Software, San Diego, CA, USA, www.graphpad.com) according to the equation: $X(t) = A \exp(-(\alpha t)) + B \exp(-(\beta t))$, where X(t) is the %ID/g of radiolabeled antibody at time t. This biexponential blood clearance profile was calculated from the time of maximal concentration of blood radioactivity.

Results

ErbB2 levels on prostate cancer cells. The level of ErbB2 receptor in prostate tumors was investigated by western blot analyses of cell extracts from four different prostate cancer cells lines: LNCaP, DU145, PC-3 and 22Rv1. Briefly, cells were lysed and analyzed by western blotting with a commercial anti-ErbB2 antibody (Neu, Santa Cruz Biotechnology) and an anti-actin mAb. As a positive control, we used SKBR3 human breast cancer cells, which express high levels of ErbB2, and as a negative control A431 cells from human epidermoid carcinoma expressing very low levels of ErbB2. As shown in Fig. 1, ErbB2 is expressed in all prostate cell lines tested, even though at lower levels with respect to those observed in mammary carcinoma cells.

These results were confirmed by ELISA assays, performed by incubating the prostate cancer cells with increasing concentrations of Erb-*hcAb*, Herceptin or 2C4. As shown in Fig. 2, the three antibodies were found to bind to all the prostate cells
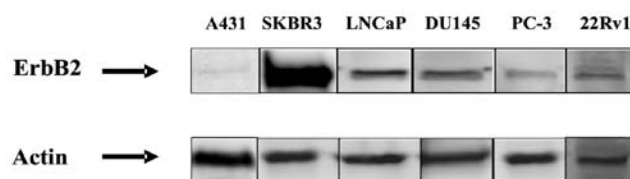

Figure 1. Analyses of ErbB2 expression on prostate cancer cell lines. Western blotting with the anti-ErbB2 (Neu) mAb of the expression levels of ErbB2 in the indicated cell lines. To normalize the band signals, an anti-actin mAb was used.
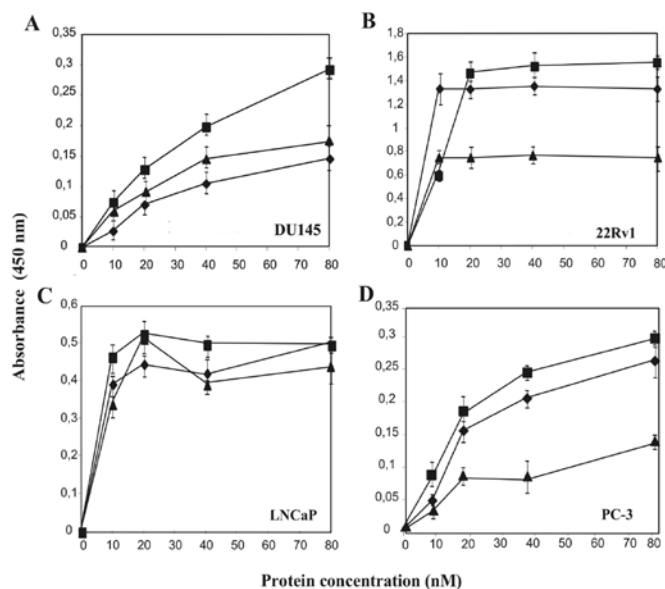

Figure 2. Binding curves of the antibodies to prostate cancer cell lines. Herceptin (triangles), Erb-hcAb (diamonds) and 2C4 (squares) were tested on (A) DU145, (B) 22Rv1, (C) LNCaP and (D) PC-3 cells.

tested with comparable affinity even though Herceptin shows a lower binding ability on 22Rv1 and PC-3 cells. As a negative control, an unrelated human anti-HIV IgG1 antibody (27) was tested in parallel ELISA assays and was found unable to bind to the prostate cells (data not shown), mentioned above.

Antitumor activity of Erb-hcAb. The antitumor effects of the antibodies were tested on ErbB2-positive prostate cancer cells by incubating LNCaP, DU145, 22Rv1 and PC-3 cells in the absence or in the presence of increasing concentrations (50-200 nM) of Erb-hcAb, Herceptin or 2C4.

After 72 h, cells were tested by MTT or counted, and their survival was expressed as percentage of viable cells in the presence of the protein under examination with respect to control cells grown in the absence of the protein. The SD was calculated on the basis of the results obtained from six different experiments.

As shown in Fig. 3, Erb-hcAb was found to selectively inhibit the growth of the cells tested in a dose-dependent manner, showing a stronger effect with respect to Herceptin when high protein concentrations were tested, with the only exception of DU145 cells that were only slightly affected by any antibody treatment.
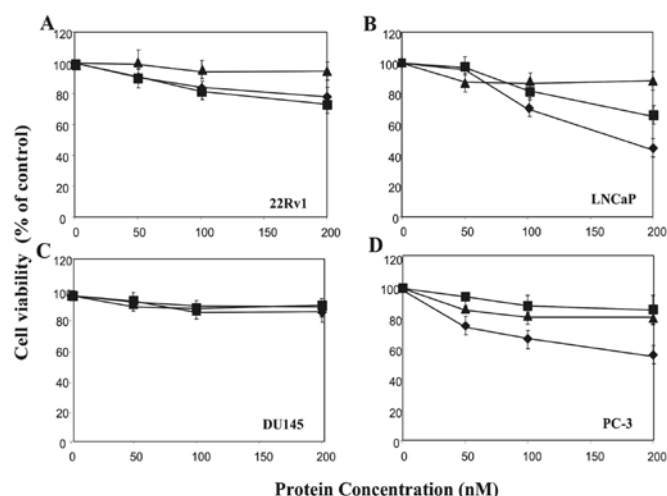
Figure 3. Effects of the antibodies on cell survival. Dose-response curves of Herceptin (triangles), Erb-hcAb (diamonds) and 2C4 (squares) on prostate cancer cells: (A) 22Rv1, (B) LNCaP, (C) DU145 and (D) PC-3.

As a positive control, the three antibodies were tested on SKBR3 human breast cancer cells, which express high levels of ErbB2, and as a negative control, they were tested on A431 cells from human epidermoid carcinoma. As expected, they were all capable of inhibiting the growth of SKBR3 cells in a similar fashion, whereas they did not show effects on ErbB2-negative A431 cells (data not shown).

For *in vivo* studies, Erb-hcAb was assessed in human prostate, LNCaP androgen-dependent and PC-3 androgen-independent cancer cells. As shown in Fig. 4, treatment of mice bearing LNCaP and PC-3 tumors with seven doses, at 72-h intervals, of 5 mg/kg body weight of Erb-hcAb induced a strong reduction in tumor volume. In parallel experiments, we tested the effects of Herceptin and 2C4, administered as indicated above for Erb-hcAb.

Surprisingly, 2C4, used at lower doses than in the previous reported experiments (28), was found to be effective for PC-3 tumors but inactive on LNCaP androgen-dependent tumors, whereas Herceptin, as expected (22), showed only slight effects on both androgen-dependent and androgen-independent

tumors. No growth inhibition was observed in mice treated with PBS. During the period of treatment, the animals did not show signs of wasting or other visible signs of toxicity.

Pharmacokinetics and biodistribution of Erb-hcAb. Erb-hcAb was labeled with Na ^{125}I and injected intraperitoneally (1-2x10^6 cpm/animal) in mice bearing LNCaP xenografts. At increasing time intervals, ranging from 5 min to 96 h retro-orbital sinus sampling was performed to evaluate blood clearance of the radiolabeled compact antibody. At 96 h animals were sacrificed and residual radioactivity in organs was determined by gamma counting and weighing.

The blood clearance profile of ^{125}I-Erb-hcAb is well described by the biexponential function with a rapid early clearance phase and a slower late phase (Fig. 5A). The values found (t$_{1/2}$ of 7.92 and 43.62 h for the α and β phase, respectively) are those expected for radiolabeled proteins of similar molecular weight (29) and indicate stability of the immuno-conjugate.

Biodistribution at the 96-h time point showed higher accumulation of ^{125}I-Erb-hcAb in the LNCaP xenografts compared to all the other organs measured (Fig. 5B). These results taken together indicate adequate biodistribution properties of the radiolabeled compound due to the extended circulating half-life which yield high concentrations in the ErbB2 expressing xenografts necessary for biological activity.

Discussion

Prostate cancer is one of the most common malignancies in men in developed countries. The developing and adult prostate is regulated by androgens acting through the androgen receptor (AR). High levels of AR expression are observed in primary prostate cancer and can be detected throughout progression in both androgen-sensitive and hormone refractory cancers (30). The development of androgen-independence creates a major challenge for treatment of prostate cancer patients because the hormone refractory tumor is minimally responsive to most of the current treatments, such as the androgen ablation therapy.

Prostate cancer progression is often associated with alteration of growth factor or growth factor receptor expression leading to androgen receptor activation. Unlike steroid
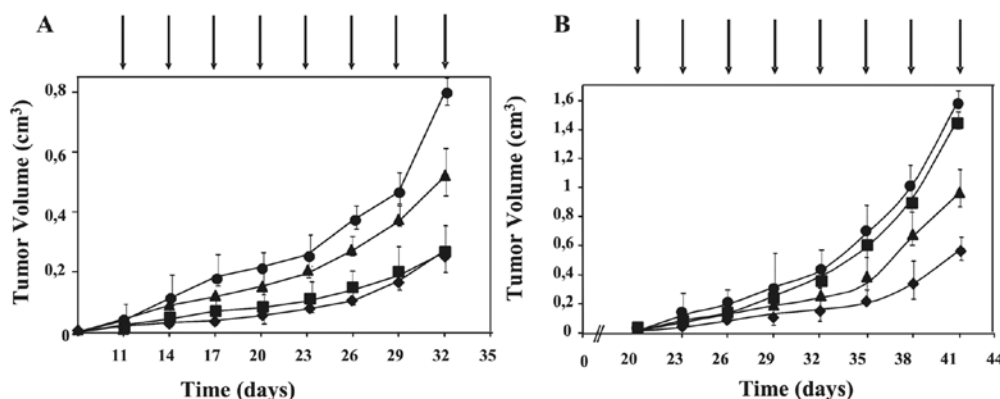

Figure 4. *In vivo* effects of Erb-hcAb on tumor growth. Mice inoculated with 2x10^6 (A) PC-3 or (B) LNCaP prostate cancer cells were untreated (circles) or treated with 2C4 (squares), Herceptin (triangles) or Erb-hcAb (diamonds).
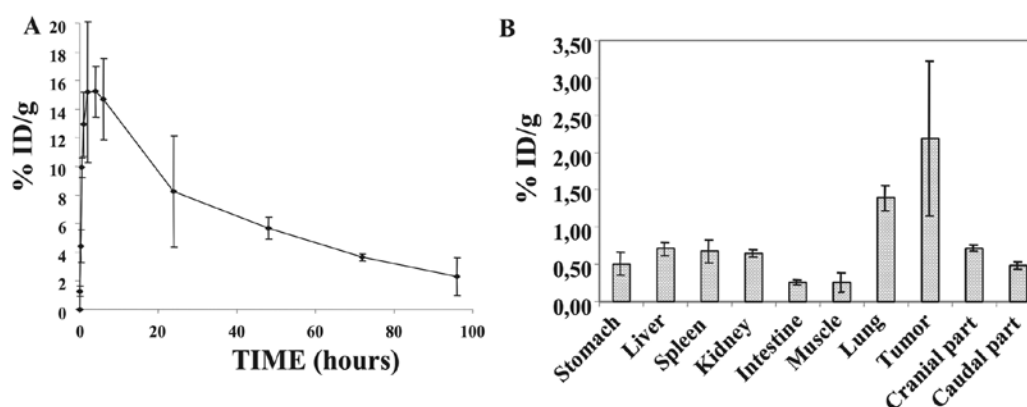
Figure 5. Pharmacokinetics and biodistribution of Erb-hcAb. (A) Blood clearance profile of ^{125}I-Erb-hcAb. The estimated α and β rate parameters corresponding respectively to the rapid early and the slower late clearance phases are as expected for radiolabeled proteins of the given molecular weight. (B) Tissue and tumor biodistribution of radiolabeled Erb-hcAb at 96 h post-injection. The LNCaP xenografts retain higher amounts of the radiolabeled immunoconjugate compared to all the other organs measured.

hormones, growth factors regulate cellular responses through binding to membrane receptors. Growth factor or cytokine binding initiates a phosphorylation cascade that ultimately results in phosphorylation of transcription factors or transcription factor-interacting proteins (31,32). A number of recent reports suggests that activation of ErbB kinase axis results in AR activation (30-32).

The overexpression of the ErbB family members, particularly ErbB2, has been observed clinically in a number of cancer types and it is associated with poor prognosis in breast cancer patients (4,5). It is possible that increased expression of ErbB2 in prostate carcinomas is related to the development of hormone resistance (28). This is likely due to the protection by ErbB2 of prostate cancer cells from the androgen ablation therapy, as it allows AR transcription under conditions of extremely low levels of circulating hormone. ErbB2 activation of AR through Akt has been proposed as a major pathway that promotes androgen independent prostate cancer growth (28,33). The mechanisms by which ErbB2 is activated in prostate cancer are still unclear, but the mutual regulation between androgens and the ErbB2 network could shed light on the coincidence of onset of ErbB2 overexpression with the emergence of androgen independence. Indeed, androgen ablation could promote the survival of some cells by stimulating ErbB2 expression that is negatively regulated by androgens.

Thus, the use of antibodies capable of inhibiting the ErbB2 signaling could provide clinical benefits to prostate cancer patients. Despite the advances made by the discovery and characterization of Herceptin, the only humanized antibody currently used for the treatment of breast cancer (11), most of the prostate cancer cells are less sensitive than breast tumor cells to Herceptin treatment (13) and all responding patients eventually relapse. Consequently, there is a need for additional therapeutics directed to the ErbB2 signaling pathway.

In previous studies it has been shown that Erb-hcAb, a fully human compact anti-ErbB2 antibody developed in our laboratory, is able to inhibit both *in vitro* and *in vivo* the growth of breast cancer cells either sensitive (19,21) or resistant to the treatment with Herceptin (23).

Thus, we tested whether Erb-hcAb could be useful for the treatment of prostate cancer by determining its effects on prostate cancer cells *in vitro* and *in vivo* in comparison with those of Herceptin and pertuzumab (2C4), another anti-ErbB2 antibody recently tested in clinical trials for prostate cancer (25,34). To this aim, we firstly examined the levels of ErbB2 expression on a panel of prostate cancer cells by western blotting and measured the binding abilities of the antibodies to the receptor by ELISA assays; then we evaluated their effects on the cells *in vitro* and in a mouse model *in vivo*.

Here, we show that Erb-hcAb is able to efficiently inhibit the growth of both androgen-dependent and androgen-independent tumor cells, with antitumor effects more potent than those observed with either 2C4 (pertuzumab), which had no activity on the androgen-dependent tumors, or Herceptin, that showed only slight antitumor effects on both types of tumor cells.

These differences could be explained by the recent interesting finding (35,36) that Erb-hcAb recognizes an epitope different from those recognized by either Herceptin or pertuzumab. This has been shown to be responsible for different effects on signaling in both tumor and cardiac cells (23). On the basis of these observations, we can speculate that the combination of Erb-hcAb with the other antibodies targeting different epitopes of ErbB2 could lead to synergistic or additive effects on prostate tumor cells as previously shown for breast tumor cells (37,38).

These results therefore suggest a promising potential of Erb-hcAb in the immunotherapy of prostate cancer for which no obvious treatment has been reported so far.

Acknowledgements

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