Recombinant humanised anti-HER2/neu antibody (Herceptin\textsuperscript{R}) induces cellular death of glioblastomas

J-F Mineo\textsuperscript{a,1,4}, A Bordron\textsuperscript{2,4}, I Quintin-Roué\textsuperscript{3}, S Loisel\textsuperscript{2}, KL Ster\textsuperscript{2}, V Buhe\textsuperscript{2}, N Lagarde\textsuperscript{3} and C Berthou\textsuperscript{2}

\textsuperscript{1}Department of Neurosurgery, University Medical School Hospital of Brest, BP 824, F29609 Brest Cedex, France; \textsuperscript{2}Laboratory of Hematology, University Medical School Hospital of Brest, BP 824, F29609 Brest Cedex, France; \textsuperscript{3}Laboratory of Pathoanatomy, University Medical School Hospital of Brest, BP 824, F29609 Brest Cedex, France

Glioblastoma multiforme (GBM) are the most common malignant tumours of the central nervous system. With an incidence of 0.4 to 2.8 per year per 100,000 persons (De Triboulet, 1996; Mineo et al., 2002), they are ranked fourth among the causes of death due to cancer in the middle-aged man (Rainov et al., 1997).

Glioblastoma multiforme are partially refractory to radiation and chemotherapy that are in standard use today (medial survival of 18 months despite radical surgery; Hiesiger et al., 1993; Puzzilli et al., 1998). The poor effectiveness of current therapy evokes development in areas, used for treatment of other cancers, such as immunotherapy. Human monoclonal antibody treatment against human epithelial receptor type 2 (HER2)/neu overexpressing breast cancer increases patient survival (Baselga et al., 1996). The effect of this antibody has led oncologists to prescribe immunotherapy in metastatic breast cancer. The HER2/neu is a 185 kDa transmembrane tyrosine/kinase receptor, which is a member of the tyrosine kinase receptor family. It is involved in the regulation of cell growth and in differentiation, especially during brain embryogenesis. The HER2/neu (also called c-erbB2) receptor is not found in the adult central nervous system (Press et al., 1990), but it appears in tumoral astrocytes. Its expression increases with the degree of astrocytoma anaplasia (Kristt and Yarden, 1996) and becomes frequent in glioblastomas (20–90%; Bian et al., 2000; Forseen et al., 2002; Koka et al., 2003). Overexpression of HER2/neu seems correlated to higher degree of glioma cells anaplasia (Kristt and Yarden, 1996). Activated HER2/neu can form homologous complexes, but the major action of HER2/neu results from heterodimerisation of HER2/neu with the other tyrosine kinase family-activated receptors (like epidermal growth factor receptor also called c-erbB1; Harwerth et al., 1993). Ligand–receptor complexes that include HER2/neu appear to be more potent that other receptor complexes and have a higher ligand affinity, a lower rate of internalisation and degradation and a higher tyrosine kinase activity (Sliwkowski et al., 1999). Human epithelial receptor type 2/neu inhibition could therefore not only decrease the activity of HER2/neu but also could affect the activity of other tyrosine kinase receptors. Owing to the association of HER2/neu proto-oncogene overexpression and human cancer, some monoclonal antibodies against a panel of epitopes from HER2/neu receptor were developed in animals. A more potent antibody for tumour inhibition was fully humanised for human therapeutic administration to create trastuzumab (Herceptin\textsuperscript{R}). The targeting of HER2/neu by its specific antibody is known to have antitumour effect in breast cancer (Pietras et al., 1998; Spiridon et al., 2002). The activated antibody containing human Fc region can generate two cytolytic pathways against the target cells by the activation of the complement cascade and antibody-dependent cellular cytotoxicity (ADCC). The ADCC is triggered by interaction between antibody-coated target cells and Fc receptor type III (CD16) on...
Efficacy of anti-HER2/neu antibody against glioblastoma

J-F Mineo et al

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The peripheral large granular lymphocytes (LGL, including natural killer lymphocytes), used as human effector cells, were separated from the blood of healthy donors in density gradients (first on Ficoll–Hypaque density gradients and then on Percoll (Pharmacia) density gradients). A total of 2 × 10⁴ GBM cells (targets) and LGL (effectors) were mixed in 1 ml culture medium (ratio targets/effectors 1/75 or 1/100) and then incubated for 6 h at 37°C with 1 ml of antibodies. To stain effectors, we incubated the washed cells with 5 µg of FITC-conjugated anti-human CD45 antibodies for 30 min at room temperature (CD45 is not present on glioblastomas cells). Two further rinses in PBS were carried out before cytometric analysis, cells were then incubated for 10 min at 4°C with 10 µg of PI to determine the percentages of dead cells.

Statistical analysis

The data were analysed for significance by Student’s t-test.

RESULTS

GBM cell lines express HER2/neu

Human epithelial receptor type 2/neu membranous density was evaluated by immunohistochemistry (IHC) and flow cytometry. Histopathologists classified immunostaining as follows (as used for breast cancer): 0 = no staining; 1+ = faint, incomplete...
membranous pattern; 2+ = moderate, complete membranous pattern; and 3+ = strong membranous pattern (Forseen et al., 2002). For the cytometric assay, the density was estimated by comparative method between the MFI of isotype control and the MFI after HER2/neu-FITC staining. BT474 is an overexpressing 3+ breast cancer cell line used as reference in most studies. Its MFI was 20. By cytometry, the MFI of A172 cell line was 4; this cell line was classified + by IHC. The MFI of U251 cell line was 2, indicating a less important intensity of HER2/neu molecule per cell than for A172; U251MG was classified 0 by IHC. Based on these two different techniques, we noted that U87MG cell line showed no membranous HER2/neu positivity (Table 1). The effect of Herceptin® was tested on the U251MG and A172 cell lines. The U87MG cell line was used as a negative cell line for the expression of HER2/neu.

Anti-HER2/neu antibody induces glioblastomas cells apoptosis

Herceptin® induces the apoptosis of GBM cell lines expressing HER2/neu, as shown by staining with Annexin V of A172 cells incubated with anti-HER2/neu antibody and nearly no staining by PI (Figure 1). Best results were obtained at 24-h cell incubation with antibodies (6 h tests showed less activity and 48 h tests did not show higher apoptosis; Figure 1C). When mixed with A172 cell line, the efficiency of the antibody increased with the antibody concentration until 25 µg ml⁻¹ and then decreased (probably corresponding to the saturation of antibody recognition sites). The spontaneous apoptosis without antibody of 10 tests were compared by Student’s t-test to the apoptosis with 25 µg ml⁻¹ of antibody, the test was significant with P<0.01. With U251MG cell line, the highest activity was observed with 50 µg ml⁻¹ (comparison between the apoptosis without or with 50 µg ml⁻¹ of antibody of five tests was significant with P<0.05, Figure 2). Moreover, the higher apoptosis induction was obtained in the A172 cell line than in the U251MG cell line. No apoptosis induction was found for U87MG cell line. The apoptosis induction decreases in accordance with the density of HER2/neu receptors.

Complement-dependent cytotoxicity assay

We wanted to evaluate the ability of Herceptin® to induce complement-mediated cytotoxicity on HER2/neu-expressing GBM cell lines. For this purpose, human serum AB (used as source of complement) was added to the medium with antibodies, but it did not increase cell death induction. The inefficiency of complement-mediated cytotoxicity was explained by a strong expression of complement inhibitory factors (CD55 and CD59 on A172 and U251MG cell lines, data not shown).

Table 1  Evaluation of HER2/neu expression on glioblastoma cell lines by IHC and by flow cytometry

| Cell lines   | Species     | HER2/neu expression |
|--------------|-------------|---------------------|
| GL15         | Human       | –                   |
| U87          | Human       | –                   |
| U87MG        | Human       | –                   |
| LN18         | Human       | –                   |
| T98G         | Human       | –                   |
| U251MG       | Human       | ++                  |
| A172         | Human       | ++                  |
| BT474        | Human/breast| +++                 |

HER2 = human epithelial receptor type 2; IHC = immunohistochemistry.

ADCC of anti-HER2/neu antibody

During ADCC assay establishment, we measured the density of membranous CD45. We observed that this molecule was highly expressed by effector cytotoxic cells. This allowed us to differentiate the two cellular types (GBM and effectors; Figure 3) and to analyse ADCC (Figure 4). In order to determine the level of cytotoxicity induced by the antibody and by the effectors alone, control tests were performed using Herceptin® alone without effectors and effectors alone without antibody. Herceptin® did not induce apoptosis in U87MG cell line and ADCC was not tested for this cell line. During this assay, we observed that ADCC was already presented using 1 µg ml⁻¹ of antibody (comparison between the cellular death without or with 1 µg ml⁻¹ of antibody of five tests was significant with P<0.05) and was still moderately increased with higher concentrations (Figure 4C). Moreover, as apoptosis was mediated by Herceptin®, more cytotoxicity was induced in A172 cell line than in U251MG.

Figure 1  Apoptosis induction: The A172 cell line expressing HER2/neu undergo apoptosis, following incubation with Herceptin®. Cells were incubated for 24 h with medium (B) or with 25 µg of Herceptin® (B). The cells staining with FITC-conjugated Annexin V and PI. Apoptotic cells were identified as Annexin V positive and PI negative. (A) Time course analysis of PS exposure after incubation of A172 cell line Herceptin® 25 µg ml⁻¹. Only 24 h results are statistically significant (P<0.01).

Figure 2  Increasing apoptosis in glioblastomas cell lines expressing HER2/neu. Cells were incubated for 24 h with different concentrations of Herceptin®.
This study demonstrates, for the first time, the ability of Herceptin\textsuperscript{\textregistered} to induce \textit{in vitro} apoptosis of HER2/neu-expressing GBM.

Of the cases studied (results from tumour biopsy), the range in results observed for HER2/neu \textit{in vivo} positivity is wide (20 – 90\%). Some authors detect HER2/neu positivity without density analysis of receptors, so they report more than 70\% positivity (Dietzmann and Von Bossanyi, 1994; Kristt and Yarden, 1996; Westphal et al., 1997; Bian et al., 2000). Some authors consider only the 2+ and 3+ tumours as positive, so they report only 20\% positivity (Forseen et al., 2002; Koka et al., 2003).

The low number of cell lines overexpressing HER2/neu \textit{in vitro} was unexpected (more than 70\% \textit{in vivo} positivity and only 2 positivity from seven different human cell lines, Table 1). Random selection may explain the difference but it seems that very few cell lines \textit{in vitro} overexpress tyrosine kinase receptors I (EGFR) or II (HER2/neu (Thomas et al., 2003). Some authors report decreasing of tyrosine kinase receptor cellular density during long \textit{in vitro} culture and increasing receptor density on \textit{in vivo} implantation (Fischel, 2003). Paracrine stimulation of the receptor by its ligand \textit{in vivo} could explain this difference.

The effect of Herceptin\textsuperscript{\textregistered} was first demonstrated for breast cancer (Peress et al., 1999), then apoptotic effects were reported in different cancers (Büchner et al., 2001; Fujimura et al., 2002; Kono et al., 2002). We report similar results with GBM cell lines: Herceptin\textsuperscript{\textregistered} induces a significant apoptosis induction at 24h. Most studies reported significant results after 72h except one on ovarian adenocarcinoma, which was significant after 24h (Fujimura et al., 2002). Two factors could explain this faster effect on GBM cell lines. First, GBM is a very aggressive tumour with a short doubling time. These particular high kinetics could explain an earlier effect. Second, we used Annexin V – IP assay that could be positive earlier than thymidine incorporation assay often describes in other studies.

We obtained greatest apoptosis induction in the A172 cell line than in U251 cell line and no apoptosis in the U87MG cell line. In the same way, the HER2/neu receptor density decreases from the A172 cell line to negative for U87MG. In our study, the effect of Herceptin\textsuperscript{\textregistered} was correlated with the HER2/neu receptors density. This correlation was previously reported for breast, gastric and ovarian tumours (Büchner et al., 2001; Fujimura et al., 2002).

Moreover, we report less apoptosis or cytotoxicity induction by antibodies with GBM cell lines than in most other publications about Herceptin\textsuperscript{\textregistered} in other cancer cell lines. The cell lines we used, however, exhibited less HER2/neu overexpression than most other cell lines usually tested with Herceptin\textsuperscript{\textregistered}. The MFI of BT474 breast cancer cell lines was with our technical conditions of 20. The MFI of A172 cell line was 4 and the MFI of U251MG cell line was 2. The ability of Herceptin\textsuperscript{\textregistered} to induce apoptosis or cytotoxicity seems to be correlated with HER2/neu receptor density in cancer cell lines from the same organ, so the lower receptor density of our cancer cell lines could explain the lower activity of Herceptin\textsuperscript{\textregistered}.

Furthermore, Herceptin\textsuperscript{\textregistered} is known to induce ADCC in different organ cancer cell lines (Carter et al., 1992; Sliwkowski et al., 1999). We report the ability of Herceptin\textsuperscript{\textregistered} to induce ADCC with higher cytotoxicity in the A172 cell line than the U251MG cell line. The antibody inducing ADCC is again higher in the cell line with the highest HER2/neu density. This result suggests a correlation between the HER2/neu receptors density and the effect of ADCC. Similar correlation was reported for gastric adenocarcinomas (Pegram et al., 1999). Microglia was found to make up 7.5 – 9\% of the total glial population in white matter (Akiyama and Mc Geer, 1990). These cells have a leucocyte origin (Flugel et al., 2001), phagocytic function and can produce a cytotoxic action when triggered by antibody coated through Fc gamma receptors (Peress et al., 1999; Vedeler et al., 1994). Proteins (150 kDa) such as IgG are supposed to cross the abnormal GBM blood–brain barrier (Vajkoczy et al., 1998). The ability of IgG against EGFR to cross the blood brain barrier was confirmed by a phase I study (Faillot et al., 1996). Herceptin\textsuperscript{\textregistered} could interact with microglia to produce ADCC \textit{in vivo}.

Adding human serum did not induce cytotoxicity in our GBM cell lines. However, the ability of Herceptin\textsuperscript{\textregistered} to induce cytotoxicity with complement is well established (Niculescu et al., 1992). We explain the lack of effect to the overexpression by A172 and U251MG cell lines of the two complement inhibitors: CD55 and CD59.

We have shown the efficacy of Herceptin\textsuperscript{\textregistered} to induce apoptosis against relative low HER2/neu-overexpressing GBM cell lines by...
classical IHC. No induction of apoptosis was observed for breast cell lines with such level of HER2/neu expression. It is known that one of the differences between GBM and other cancers is the absence of expression of HER2/neu on the surface of normal glial tissue. Cells from other normal organ (breast or ovarian) express a weak density of HER2/neu. This difference could explain the greater importance of HER2/neu activity on GBM physiology and the greater effect of its blockage. Moreover, the effect of Herceptin® could be increased by synergistic effects with several chemotherapies (as etoposide or cisplatin; Pegram et al., 1999).

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Finally, the evidence of Herceptin’s ability to induce apoptosis or cytotoxicity in GBM cell lines overexpressing HER2/neu made us commence animal experiments in the following months.

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