In vivo Therapeutic Synergism of Anti–Epidermal Growth Factor Receptor and Anti-HER2 Monoclonal Antibodies against Pancreatic Carcinomas

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Abstract

Purpose: Pancreatic carcinoma is highly resistant to therapy. Epidermal growth factor receptor (EGFR) and HER2 have been reported to be both dysregulated in this cancer. To evaluate the *in vivo* effect of binding both EGFR and HER2 with two therapeutic humanized monoclonal antibodies (mAb), we treated human pancreatic carcinoma xenografts, expressing high EGFR and low HER2 levels.

Experimental Design: Nude mice, bearing xenografts of BxPC-3 or MiaPaCa-2 human pancreatic carcinoma cell lines, were injected twice weekly for 4 weeks with different doses of anti-EGFR (matuzumab) and anti-HER2 (trastuzumab) mAbs either alone or in combination. The effect of the two mAbs, on HER receptor phosphorylation, was also studied *in vitro* by Western blot analysis.

Results: The combined mAb treatment significantly inhibited tumor progression of the BxPC-3 xenografts compared with single mAb injection ($P = 0.006$) or no treatment ($P = 0.0004$) and specifically induced some complete remissions. The two mAbs had more antitumor effect than 4-fold greater doses of each mAb. The significant synergistic effect of the two mAbs was confirmed on the MiaPaCa-2 xenograft and on another type of carcinoma, SK-OV-3 ovarian carcinoma xenografts. *In vitro*, the cooperative effect of the two mAbs was associated with a decrease in EGFR and HER2 receptor phosphorylation.

Conclusions: Anti-HER2 mAb has a synergistic therapeutic effect when combined with an anti-EGFR mAb on pancreatic carcinomas with low HER2 expression. These observations may open the way to the use of these two mAbs in a large panel of carcinomas expressing different levels of the two HER receptors.

The incidence of pancreatic cancer has steadily increased over the past four decades, and its prognosis is still dismal, despite all efforts in early diagnosis and therapy. Even with a complete surgical resection, the 5-year survival rate is <20% (1). Conventional therapies associating surgery and radiotherapy often in combination with chemotherapy show modest efficacy in local control and palliation and no real progress in patient survival (2–4). Thus, novel approaches to human pancreatic carcinoma therapy are urgently needed.

Among the numerous biochemical and genetic abnormalities that were found to be associated with this malignancy (3), we were interested by the fact that two receptors of the HER tyrosine kinase family epidermal growth factor receptor (EGFR; ErbB1 or HER1) and HER2 (ErbB2) are expressed in a significant percentage of cases ranging from 45% to 95% for EGFR (5, 6) and 43% to 69% for HER2 (7, 8). The levels of HER2 expression, however, were low in the majority of tumors. Furthermore, the overexpression of EGFR and HER2 receptors is often caused by encoding gene amplification (9).

EGFR and HER2 are known to play an essential role in regulating cell proliferation and differentiation. They have a strong tendency to assemble with other HER receptors into homodimers and/or heterodimers on extracellular growth factor binding, which results in various forms of signal transduction pathways activation, leading to either apoptosis, survival, or cell proliferation (9, 10). Mounting evidence suggests that not only receptor overexpression but also communication among HER receptors plays a crucial role in

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Note: B. Robert and I. Navarro-Teulon contributed equally to this work.

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tumor behavior (11, 12). In particular, binding of receptor-specific ligands to the ectodomain of EGFR often results in the recruitment of HER2, as the preferred heterodimerization partner (13–15). As HER2 is the only HER family member that does not bind a known specific ligand, its principal biological function, as a signal transducer, seems to result from its participation in heterodimeric receptor complexes with EGFR or other HER receptors (14–16).

The use of monoclonal antibody (mAb) binding with high affinity to these two members of the HER family, EGFR and HER2, thus seems to be rational for the development of new cancer therapy strategies, which would have the potential to inhibit the receptor dimerization. Until now, however, each of the two mAbs have been used in cancer therapy individually in conjunction with various chemotherapeutic drugs (17, 18) or more recently in association with different drugs having tyrosine kinase receptor inhibition properties (16, 19). The action of small tyrosine kinase inhibitor molecules, however, cannot be compared with the potential biological activity induced by the binding of a high molecular weight antibody molecule.

The mechanism of antitumor activity of individual anti-HER receptor mAbs is not entirely understood. A series of experimental results in mice knockout for the Fc receptor strongly suggested that most of the antitumor effect was due to the recruitment of effector natural killer cells by an antibody-dependent cell-mediated cytotoxic mechanism (20). However, the evidence of inhibition of receptor phosphorylation and receptor internalization induced on tumor cells by the anti-HER receptor mAbs argues in favor of an apoptotic or cytostatic signal transduced through the receptor (9, 21–24).

Concerning anti-EGFR mAbs, such as cetuximab (Erbitux) or matuzumab (EMD72000), they were shown to compete with the EGFR ligands and inhibit tumor cell proliferation *in vitro* and in xenograft mouse models. Both mAbs were shown in clinical trials to be well tolerated (18, 25) and active mostly in conjunction with various chemotherapeutic agents, against metastatic colorectal carcinomas (i.e., doxorubicin, Adriamycin, Taxol, and cisplatin; ref. 18), leading to approval by the Food and Drug Administration of cetuximab. Interestingly, clinical studies showed that the anti-EGFR mAb had antitumor activity even in patients with colorectal carcinomas expressing no detectable amounts of the receptor by immunohistochemistry (26). Thus, a phase II trial of pancreatic carcinoma treatment with the cetuximab anti-EGFR in combination with gemcitabine seemed justified, but the results of 12.2% partial responses were not very encouraging (6).

For anti-HER2 mAbs, their antitumor activity is unlikely to be due to an inhibition of ligand binding because no specific ligand for this receptor has been identified (9, 14). The therapeutic efficacy of the anti-HER2 mAb, trastuzumab, in breast carcinoma is well shown, but it is strictly limited and only approved for the 30% of tumors overexpressing HER2 (17). Thus, the present day consensus is that anti-HER2 mAb is inefficient in tumors with low HER2 expression, which is the case for most pancreatic carcinoma in the clinic (7, 8). Despite this limitation, in view of the known unique biological cooperation and dimerization properties of EGFR and HER2 and the numerous encouraging *in vitro* results (9–16), we decided to evaluate the antitumor effect of the combined injection of two high-affinity anti-EGFR and anti-HER2 mAbs, matuzumab and trastuzumab. The two-mAb treatment led to far greater tumor regression and mice survival than injection of each of the mAbs alone, suggesting that the two-mAb treatment could overcome some of the limitations encountered in the treatment with trastuzumab of low HER2-expressing tumors. Interestingly, the demonstration of therapeutic synergism of the two mAbs was also confirmed on xenografts from the human ovarian xenograft cell line, SK-OV-3, known to overexpress both EGFR and HER2, extending the potential broad application of this antitumor treatment by two anti-HER receptor mAbs.

Materials and Methods

mAbs and EGF. Anti-EGFR humanized antibody matuzumab was kindly obtained from Merck AG. The anti-HER2 humanized antibody trastuzumab (Herceptin) was purchased from Genentech, Inc. Recombinant EGF was obtained from Sigma-Aldrich.

Cell lines and culture conditions. Human pancreatic (BxPC-3 and MiaPaCa-2) and ovarian (SK-OV-3) carcinoma cell lines were obtained from the American Type Culture Collection. The BxPC-3 cell line was cultured in RPMI 1640 (Life Technologies); the MiaPaCa-2 and SK-OV-3 cell lines were cultured in DMEM (Life Technologies). The culture media were supplemented as recommended by American Type Culture Collection.

In vivo *tumor growth inhibition study.* All *in vivo* experiments were done in compliance with the French guidelines for experimental animal studies (Agreement no. B34-172-27). Nude mice, 6- to 8-week-old female athymic NMRI mice and BALB/c athymic mice were purchased from Janvier and Charles Rivers Laboratories, respectively.

BxPC-3 (3.5×10^6), MiaPaCa-2 (5×10^6), and SK-OV-3 (5×10^6) cells were injected s.c. into the right flank of athymic NMRI (BxPC-3 model) and BALB/c (MiaPaCa-2 and SK-OV-3) nude mice. MiaPaCa-2 cells were suspended in 50% culture medium and 50% Matrigel (BD Biosciences). Tumor-bearing mice were randomized in the different treatment groups when the tumors reached an approximate volume indicated in each experiment. For the BxPC-3 model, effects of antibody treatments were studied on small tumor (experiment S) and on large tumor (experiment L). The mice were treated by i.p. injections with 0.9% NaCl, trastuzumab, matuzumab, or both mAbs at a ratio of 1:1. The amounts of each injected mAb were 25, 50, or 200 μg/injection depending on the experiment, twice weekly for 4 weeks consecutively.

Tumor dimensions were measured twice weekly with a caliper and the volumes were calculated by the formula $D_1 \times D_2 \times D_3 / 2$. Tumor progression was calculated using the formula [(final volume) - (initial volume)] / (initial volume). The results were also expressed by an adapted Kaplan-Meier survival curve, using the time taken for the tumor to reach a determined volume of 1,000 mm^3 for SK-OV-3, 1,500 mm^3 for BxPC-3, and 2,000 mm^3 for MiaPaCa-2 xenografts, depending on the rapidity of growth of the tumors. A median delay was defined as the time at which 50% of the mice had a tumor reaching the determined volume. For experiment S in the BxPC-3 model, an increment factor during the treatment period was calculated by dividing the tumor volume at the end of treatment (day 55) by that at the beginning of the treatment (day 27).

Immunohistochemical analyses. Expression of receptors was analyzed in paraffin-embedded xenograft fixed in alcohol-formol-acetic acid. Analysis of EGFR expression was done by using the EGFR pharmDx kit (DakoCytomation) according to the manufacturer's recommendations. Diaminobenzidine (DakoCytomation) was used as the chromogen, and the sections were lightly counterstained with hematoxylin. The primary antibody used for the detection of HER2 was a rabbit polyclonal antibody (DakoCytomation).

Flow cytometry. Cell surface EGFR and HER2 expression was analyzed by flow cytometry (fluorescence-activated cell sorting) using

the murine anti-EGFR mAb (m225; HB8508; American Type Culture Collection) and trastuzumab, respectively. After washing, an antimouse or an antihuman FITC-conjugated mAb (Sigma-Aldrich) was added to detect the primary antibodies. Direct incubation of cells with the secondary antibody was used for background measurements. The samples were analyzed on a FACScan II (Becton Dickinson) by observing a minimun of 20,000 events.

Immunoblotting analysis. BxPC-3, MiaPaCa-2, and SK-OV-3 cells, plated at 10^6 cells for 24 h in Petri dishes, were starved for 2 days in a medium without growth factors (SM medium) and treated with 10 μg/mL trastuzumab, matuzumab, or both antibodies at a fixed 1:1 ratio (or controls without antibody). After a 48-h incubation, cells were incubated for 10 min in the SM medium with or without 100 ng/mL EGF, washed twice, and lysed with buffer (CliniSciences SA) containing 100 μmol/L phenylmethylsulfonyl fluoride, 100 mmol/L sodium fluorure, 1 mmol/L sodium orthovanadate, and one complete protease inhibitor mixture tablet (Sigma-Aldrich).

After electrophoresis on 8% SDS-PAGE under nonreducing conditions, the proteins were transferred to a polyvinylidene difluoride membranes (Millipore Co.), which were saturated in PBS containing 0.1% Tween 20 and 5% nonfat dry milk and then incubated with the antibodies against the phosphorylated forms of EGFR, HER2, extracellular signal-regulated kinase 1/2, and AKT obtained from Cell Signaling Technology. To ensure equal loading, immunoblots were also probed with anti–glyceraldehyde-3-phosphate dehydrogenase antibody (Chemicon International).

Statistical methods. Survival curves were drawn, and the log-rank test was done to assess differences between groups. All reported *P* values are two sided. For all statistical tests, differences were considered as significant at the 5% level.

Statistical analyses were done on an IBM PC-compatible personal computer using the Stata 9.0 software (Stata Corp.).

Results

EGFR and HER2 receptor cell surface expressions. Immunonohistochemical analyses on BxPC-3 xenografts showed a high level of EGFR (classified as +++) but no detectable levels of HER2 receptor expression compared with SK-OV-3 xenograft, classified as +++ for HER2 and ++ for EGFR, respectively (Fig. 1). MiaPaCa-2 xenografts were classified negative for HER2 and ++ for EGFR. In contrast, using the more sensitive flow cytometry technique (27), a moderate expression of HER2 was found on BxPC-3 and MiaPaCa-2 cells compared with SK-OV-3 cells. The overexpression of EGFR by BxPC-3 cells was confirmed. MiaPaCa-2 showed a moderate and equal expression of both EGFR and HER2 receptors. SK-OV-3 showed a moderate expression of EGFR and a high expression of HER2 (Fig. 1).

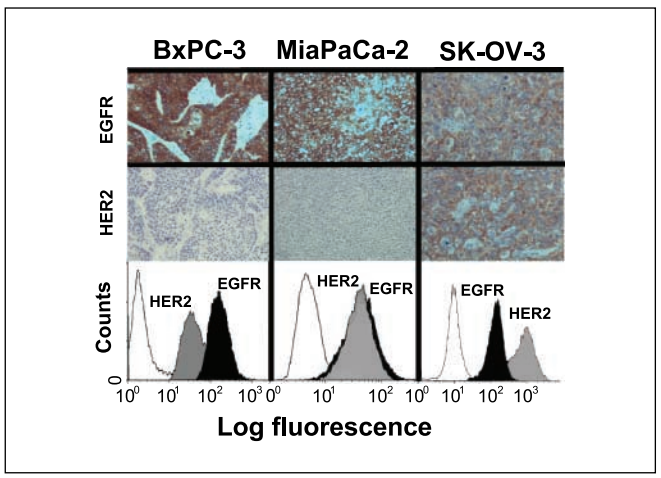

Fig. 1. Immunohistochemical and flow cytometry analyses of EGFR and HER2 expression on the two pancreatic carcinoma xenografts, BxPC-3 and MiaPaCa-2, and on an ovarian xenograft SK-OV-3. Negative controls: tissues incubated only with the immunoperoxidase conjugate. Flow cytometry analyses show cell surface staining with the anti-EGFR (*black peaks*) and the anti-HER2 (*gray peaks*) antibodies, respectively. White peaks, controls, obtained with cells incubated only with the FITC-labeled second antibody.

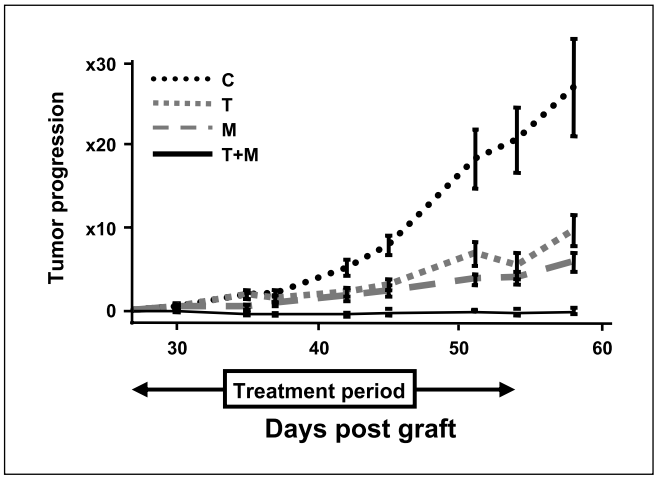

Fig. 2. Effects of trastuzumab and matuzumab alone or in combination on the growth of small size BxPC-3 xenografts in nude mice (experiment S). Mean pretreatment tumor volumes were 77 ± 49 mm^3. Mice (eight per group) received i.p. injections of 50 μg of each mAb twice weekly for 4 wks. Results are expressed as tumor progression: [(final volume) - (initial volume)] / (initial volume). C, control; T, trastuzumab; M, matuzumab; T+M, trastuzumab+matuzumab.

Antitumor activity of matuzumab and/or trastuzumab against two pancreatic and an ovarian carcinoma xenografts. The therapeutic efficacy of the anti-EGFR and anti-HER2 mAbs alone or in combination was first tested on BxPC-3 xenografts in nude mice. To evaluate the different forms of mAb therapy on tumor xenografts of various sizes, two series of representative experimental results, first on tumors of relatively small volume (77 ± 49 mm^3, experiment S) and second with larger

Table 1. Increment factor of BxPC-3 xenograft volume at the end of treatment in the small tumor (S) experiment

mAb dose	Treatment	Increment factor*	% Tumor-free mice
—	C	8.0 ± 4.0	0
	M	4.9 ± 2.1	0
50	T	6.4 ± 4.4	0
	T+M	0.7 ± 0.5	25 (2/8)
—	C	21.3 ± 7.0	0
	M	4.3 ± 2.8	0
200	T	5.5 ± 2.1	0
	T+M	0.8 ± 0.3	60 (3/5)

Abbreviations: C, control; M, matuzumab; T, trastuzumab; T+M, both mAbs.
*Increment factor: (tumor volume at the end of treatment) / (tumor volume at the beginning of treatment).

tumors (503 ± 205 mm^3, experiment L), were analyzed. Results from experiment S (Fig. 2), obtained in groups of eight mice treated with 50 μg of each antibody twice weekly for 4 weeks, showed a significantly higher inhibition of tumor growth in the combined antibody group compared with the groups treated with each antibody alone (P = 0.002). Further analysis of the same experimental results showed that the tumor increment factors at the end of treatment were markedly higher in the mice treated with anti-EGFR and anti-HER2 mAb alone, 4.9 ± 2.1 and 6.4 ± 4.4, compared with the combined mAb group 0.7 ± 0.5 (Table 1). Furthermore, only the combined mAb treatment induced two complete tumor remissions.

Results from an additional experiment on mice with similar size BxPC-3 xenografts treated with 4-fold greater doses of each mAb (200 μg) alone or in combination entirely confirmed the previous results, with tumor increment factors much higher for the single mAb treatment compared with the combined mAb therapy (Table 1). In addition, complete tumor remissions in three of five mice were observed only in the combined therapy group at the end of treatment.

Comparison of therapy with different antibody doses, reported in Table 1, showed that 50 μg of each mAb in combination induced markedly lower tumor increments (0.7 ± 0.5) than those obtained in mice treated with 4-fold greater doses of the single mAb (4.3 and 5.5). No detectable dose effect was observed between the 50 and 200 μg treatments, suggesting that the synergism of the two mAbs binding to the two HER receptors was more important than the absolute amounts of antibody. This observation is clearly in favor of a synergic effect rather than an additive one.

Results from larger xenografts from the BxPC-3 tumors (experiment L) obtained in four groups of six mice injected with 200 μg of each antibody showed in adapted Kaplan-Meier survival curves (Fig. 3A) that the median delay for tumors to reach 1,500 mm^3 volume was significantly longer (73 days) in mice treated with the mAb combination than in mice treated with only one mAb (anti-HER2, 30 days; anti-EGFR, 34 days; P = 0.001). The time tumor progression curves from the same experiment (Fig. 3B) confirmed that the time to reach a 3-fold larger tumor was significantly longer (P < 0.001) for the combined treatment group than for the two single mAb treatments. Interestingly, in an additional group of six mice with large tumors treated with only 50 μg of each mAb in combination, the time (70 days) for the tumor to reach a volume of 1,500 mm^3 was much longer compared with the tumors in the group treated with 200 μg of each mAb alone (30 and 34 days; Fig. 3A). This confirms that even with the larger tumors, the double specificity of the two mAbs was more effective than the absolute mAb doses and that the greater antitumor effect was due to a synergistic rather than an additive effect of the two mAbs.

The tumor growth inhibition property of the combined anti-EGFR and anti-HER2 mAbs was further tested on xenografts from a second human pancreatic carcinoma line MiaPaCa-2, which also expresses low HER2 levels. Injection twice weekly of 50 μg of each mAb alone or in combination was initiated in groups of six mice when the mean tumor volume had reached 64 ± 5 mm^3 and continued for 4 weeks. Animals were euthanized when the tumor reached a volume of 2,000 mm^3. The adapted survival curves for tumors to reach this volume show (Fig. 4A) that at day 120, no tumor >2,000 mm^3 was observed in the combined antibody group, whereas four of six mice from both groups treated with a single mAb had tumors reaching that volume (P = 0.0072). Furthermore, a complete tumor remission was observed only in the combined mAb therapy group. Interestingly, in a separate experiment, treatment of MiaPaCa-2 xenografts of similar size (65 ± 6 mm^3) with only 25 μg of each antibody in combination had an efficient antitumor effect (Fig. 4B) similar to the results obtained with 50 μg antibodies, confirming, for this tumor also, the important role of the attack by two mAbs directed against different HER receptors.

To verify whether the therapeutic advantage of the combined action of the two anti-HER receptor mAbs observed against the two pancreatic carcinoma cell lines can also function on another type of carcinoma, the same comparison between single and combined mAb injections was tested on the reference ovarian carcinoma line SK-OV-3, known to overexpress both EGF and HER2 receptors. The therapy consisting of 200 μg of either anti-EGFR or anti-HER2 mAb, or the two mAbs in combination, was initiated in four groups of six mice (including an untreated control) when the SK-OV-3 xenograft had a median volume of 42 ± 4 mm^3. As expected, in view of the overexpression of both HER receptors on these tumor cells, a therapeutic activity of each single mAb was observed with one and two complete remissions in the group of mice injected with anti-EGFR and anti-HER2, respectively. However, the injection of both mAbs gave a clearly superior tumor growth inhibition

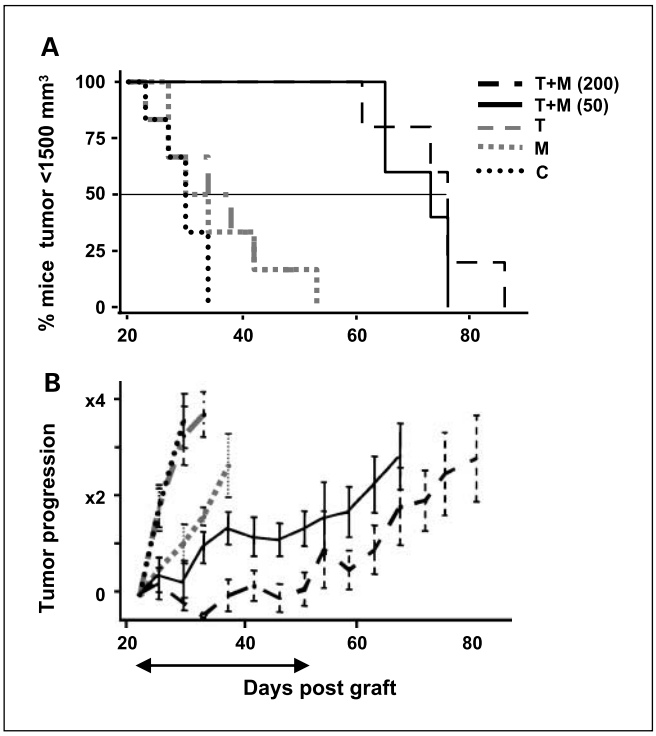

Fig. 3. Effects of trastuzumab and matuzumab alone or in combination on the growth of large-size BxPC-3 xenografts in nude mice (experiment L). Mean pretreatment tumor volumes were 502 ± 205 mm^3. Mice (five per group) received i.p. injections of 50 or 200 μg of each mAb twice weekly for 4 wks. *A,* Kaplan-Meier survival curves obtained as a function of time adapted for primary tumor to reach a volume of 1,500 mm^3. C, control; T, trastuzumab (200 μg/injection); M, matuzumab (200 μg/injection); T+M, trastuzumab+matuzumab (50 or 200 μg of each mAb/ injection). *B,* results from the same experiments, expressed as tumor progression curves. Double head arrows, treatment period.

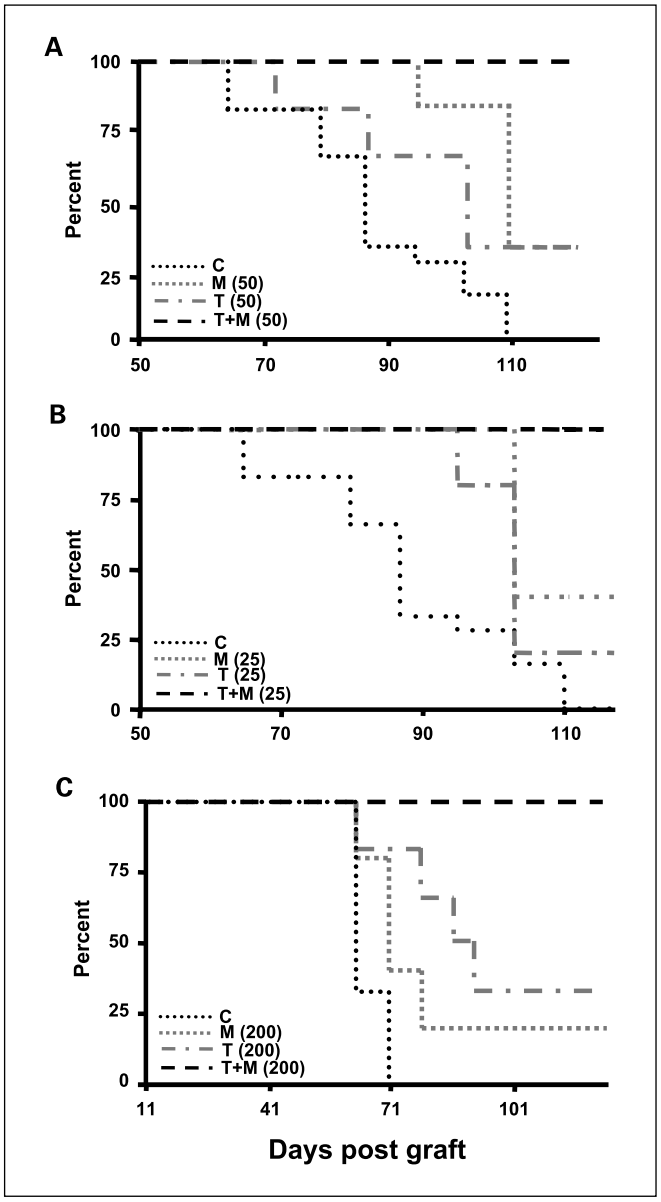

Fig. 4. Effects of trastuzumab and matuzumab alone or in combination on the growth of MiaPaCa-2 xenografts in nude mice. Mean (six per group) pretreatment tumor volumes were 64 ± 5 mm^3. Mice received i.p. injections of 50 μg (*A*) or 25 μg (*B*) of each mAb twice weekly for 4 wks from day 15 to 43. Kaplan-Meier survival curves obtained as a function of time adapted for primary tumor to reach a volume of 2,000 mm^3. *C,* effects of trastuzumab and matuzumab alone or in combination on the growth of SK-OV-3 xenografts in nude mice. Mean (six per group) pretreatment tumor volumes were 42 ± 4 mm^3. Mice received i.p. injections of 200 μg of each mAb twice weekly for 4 wks from day 11 to 40. Kaplan-Meier survival curves for primary tumor to reach a volume of 1,000 mm^3.

in all mice, including three complete remissions. The results of the adapted survival curve for the tumor to reach a volume of 1,000 mm^3 (Fig. 4C) showed that the tumors of all six mice treated with the mAb combination did not reach 1,000 mm^3 during the period of observation of 120 days, whereas the delay for 50% of the mice to reach this tumor volume was 69 and 85 days for the group of mice treated with anti-EGFR and HER2, respectively (P = 0.0001). These results confirmed the therapeutic synergism of the two anti-HER mice mAbs against a human ovarian carcinoma xenograft and suggests that the combined injection of these two mAbs could be useful in the treatment of many types of carcinomas expressing EGF and HER2 receptors.

Inhibition of receptor autophosphorylation. The ability of matuzumab or trastuzumab used alone or in combination to inhibit the tyrosine kinase activity of EGFR and HER2 on BxPC-3, MiaPaCa-2, and SK-OV-3 cells *in vitro* was assessed by Western blot with quantification of the phosphorylated proteins by densitometry and analysis using the NIH Imager 6.3 (Fig. 5). Analysis on the carcinoma cell lines was compared after a 48-h incubation with the two mAbs, either alone or in combination, followed by a 10-min activation with EGF. It should be noted first that both pancreatic cell lines showed a relatively high phosphorylation baseline for the EGFR and the HER2 receptor and SK-OV-3 cell line a high phosphorylation baseline for HER2 receptor. As expected, treatment with exogenous EGF induced an increased level of tyrosine autophosphorylation of EGFR and HER2 in both pancreatic carcinoma cell lines, BxPC-3 and MiaPaCa-2. Interestingly, incubation with the two mAbs in combination resulted in a much higher inhibition of phosphorylation of HER2 than that obtained with anti-HER2 mAb alone in both pancreatic carcinoma cell lines. In SK-OV-3 cell line, however, treatment with trastuzumab alone or in combination with matuzumab reduced the HER2 phosphorylation to a similar extent.

For EGFR, the phosphorylation inhibition by the two mAbs was almost identical or only slightly superior to that obtained with the anti-EGFR mAb alone on the two pancreatic carcinoma cell lines. In contrast, in the SK-OV-3 cell line, incubation with the two mAbs in combination resulted in a nearly complete inhibition of EGFR, whereas incubation with each mAb alone induced only minor inhibition.

Analysis of the inhibition of AKT and extracellular signal-regulated kinase 1/2 phosphorylation was done on the MiaPaCa-2 and SK-OV-3 cell lines. On the pancreatic carcinoma cell line, similar inhibitions of phosphorylation of the two intracellular signaling molecules were observed with anti-EGFR mAb alone, as with the two mAbs in combination, whereas, in the ovarian carcinoma line, similar phosphorylation inhibitions were observed with the anti-HER2 mAb as with the two mAbs in combination.

These results, as well as more extensive *in vitro* studies from the literature (22–24), only partially explain the striking *in vivo* therapeutic synergism of the two anti-HER receptor mAbs against human carcinoma xenografts described here.

Discussion

In view of the present demonstration of the synergistic therapeutic effect of two anti-EGFR and anti-HER2 mAbs on two pancreatic carcinoma lines with moderate expression of HER2 and one ovarian carcinoma line overexpressing both HER receptors, we will consider the extent to which these experimental results may have an effect on the management of pancreatic and other types of carcinomas.

In pancreatic carcinoma, both EGFR and HER2 are expressed in a significant percentage of the cases (5–8) and expression of these receptors is involved in the initiation and progression of this tumor (5, 7, 28). Thus, in view of the toxicity and very modest results of the most advanced and intensive regimens of chemotherapy and radiotherapy in this type of cancer (4), it

would seem logical to consider a combined treatment with the two anti-EGFR and anti-HER2 mAbs in patients with pancreatic carcinomas expressing even low levels of the two HER receptors.

In favor of the treatment of other types of carcinomas by the two anti-EGFR and anti-HER2 mAbs, the following arguments can be given. (*a*) Treatment with each of the two anti-HER receptor mAbs has shown clinical benefits but rarely as a single modality therapy and mostly in conjunction with different forms of chemotherapy. The coinjection of a second anti-HER receptor mAb, which has the potential synergistic antitumor effect experimentally shown here, seems more rational than the addition of a relatively nonspecific chemotherapy. (*b*) For the anti-HER2 mAb, it has been well established that the clinical benefits are limited to tumors with marked overexpression of the receptor (29), whereas, in our experimental results, the synergism of anti-HER2 with anti-EGFR was shown on two pancreatic carcinomas with low to moderate HER2 expression. (*c*) It has been shown in various carcinoma cell lines that treatment with small molecules inhibiting tyrosine kinase receptors, such as Iressa for EGFR (19) or the dual kinase inhibitor lapatinib for both EGFR and HER2 (16), can have a synergistic effect with anti-HER2 mAb treatment. However, the synergistic effect was mainly shown *in vitro* and exclusively on target carcinoma lines overexpressing HER2. Furthermore, the effect of a small molecule with tyrosine kinase inhibitory property cannot be considered as identical to that of a large antibody molecule binding to the external domain of the receptors. (*d*) For many years, several groups presented *in vitro* studies on tumor cell suspensions showing that simultaneous incubation with the two anti-EGFR and anti-HER2 mAbs induced more efficient inhibition of receptor phosphorylation and/or specific internalization of the receptors than incubation with one mAb alone (21–24). However, only one of the groups (23) tried a limited *in vivo* study with the two mAbs on one carcinoma cell line, which responded well to the anti-EGFR mAb alone, and thus failed to show any synergism between the two mAbs, although the other authors concluded that their *in vitro* studies may have important implications in cancer therapy (21, 22, 24).

Overexpression of HER2 is an established criteria tool to evaluate breast cancer patients for trastuzumab therapy (29, 30), and accordingly, only patients with immunohistochemical 3+ HER2 tumor expression benefit from this mAb therapy. That is why it is so interesting that in the two pancreatic carcinoma xenografts tested here, expressing a very low level of HER2, the synergy of anti-HER2 with the anti-EGFR mAb gave a significant therapeutic advantage. For the EGFR, it is known that the correlation between the receptor expression and curative efficacy of anti-EGFR antibodies is not as clear as for HER2. The clinical response does not seem to correlate with the level of EGFR expression by cancer cell, but preferentially with the receptor mutation, particularly in NSCLC (31). In this context, it should be noted that efficacy of the anti-HER2 mAb treatment alone was superior to that of the anti-EGFR mAb alone in the HER2-overexpressing SK-OV-3 model and that in the MiaPaCa-2 xenograft expressing moderate amounts of EGFR, the situation was reversed. However, the novelty is that the two-mAb combination therapy gave significantly superior therapeutic results in the three human carcinoma xenograft models.

The results presented herein represent the first *in vivo* experimental demonstration of a long expected new improvement of cancer therapy by the synergistic action of two anti-EGFR and HER2 mAbs. Furthermore, our results suggest that a patient having breast carcinoma with moderate expression of HER2, who could not be treated with anti-HER2 mAb therapy, might benefit from the synergic treatment of the two anti-HER mAbs, provided that the tumor also expresses EGFR.

Three other cancer treatments based on the synergistic effect of two mAbs of different specificities are presently under investigation. First, a phase I clinical trial is under way for the treatment of non-Hodgkin's lymphoma with a combination of anti-CD20 and CD22 mAbs (32). The antigens recognized by these two mAbs are very different, however, and do not have the heterodimerization property, characteristic of the two HER receptors antibody targeted in our study (9, 24). A second, entirely experimental, study involves an anti–insulin-like growth factor receptor mAb, which is reported to have a synergistic antitumor effect, either with a chemotherapeutic agent (vinorelbine) or with an anti-EGFR mAb, in a breast and a lung carcinoma xenograft model (33). This study is closer to ours because it involves two tyrosine kinase receptors and may implicate a similar synergism of two transducing factor

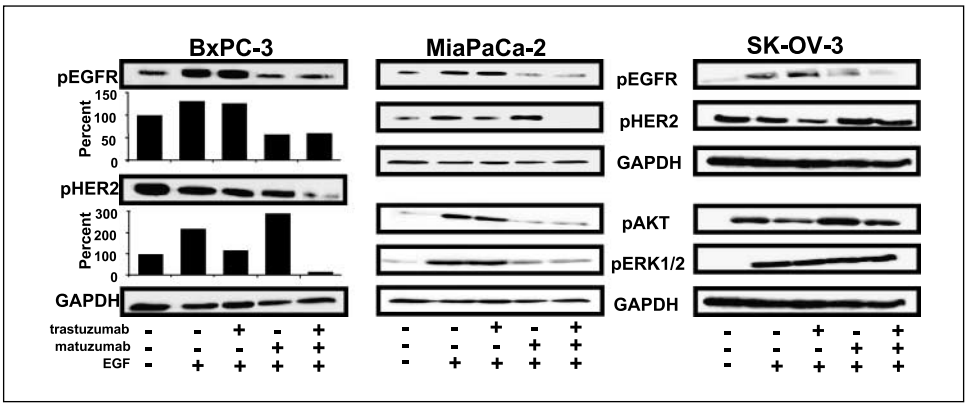

Fig. 5. *In vitro* effect of trastuzumab and matuzumab alone or in combination on EGFR, HER2, extracellular signal-regulated kinase (*ERK*) 1/2, and AKT phosphorylation by Western blot analysis. Cells were incubated or not with 10 μg/mL of each mAb for 48 h followed by 10 min with 100 ng/mL EGF or no EGF. For the BxPC-3 cell line, quantification of receptor phosphorylation, correlated to the glyceraldehyde-3-phosphate dehydrogenase antibody (*GAPDH*) spot density and expressed in percentage of control (cells without EGF treatment) is plotted as a bar graph and shows the inhibition induced by the indicated treatment conditions. For the MiaPaCa-2 and SK-OV-3 cell lines, because the glyceraldehyde-3-phosphate dehydrogenase antibody spot densities were constant in each experiment, no correlation with this control was necessary.

pathways. However, the anti–insulin-like growth factor receptor does not belong to the HER receptor family and in terms of potential treatment, application anti–insulin-like growth factor receptor is less tumor restricted than is the HER2 receptor. A third experimental study involves an anti-EGFR and an anti–vascular endothelial growth factor receptor mAb in a pancreatic carcinoma xenograft model (34). This study is markedly different from ours because the two mAbs used are not directed against the same tumor target cells. Indeed, anti–vascular endothelial growth factor receptor mAb binds to a receptor, which is expressed on endothelial cells, but not on pancreatic carcinoma cells (35). Thus, the mechanism of action of the two mAbs in that study seems to be related to tumor vascularization variables and is distinct from the binding of the two mAbs on two HER receptors located on the same target carcinoma cells, which seems responsible for the synergistic antitumor effect presented here. In addition, two mAbs directed against different epitopes from either HER2 (9, 24) or the EGF receptor (36) have been reported to induce additive or synergistic antitumor effect, paying the way to the efficient therapeutic synergy of the coinjection of anti-HER2 and anti-EGFR mAbs demonstrated here against three different human carcinoma cell lines.

Finally, a word of caution should be given before considering the combination of anti-EGFR and anti-HER2 mAbs for clinical application. In principle, the fact that the two mAbs react with two HER receptors known to be associated with malignant transformation should increase the tumor specificity of the treatment. However, because the combined mAb treatment presented here is active against tumor cells with a moderate expression of HER2, one could expect some new side effects, due to the fact that some normal tissues with a low HER2 expression, not sensitive to single anti-HER2 mAb, may become sensitive to the proposed combined mAb treatment.

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