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Special Article

Monoclonal Antibody Therapy of Human Cancer: Taking the HER2 Protooncogene to the Clinic

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The HER2 protooncogene encodes a 185-kDa transmembrane protein ($p185^{HER2}$) with extensive homology to the epidermal growth factor (EGF) receptor. Clinical and experimental evidence supports a role for overexpression of the HER2 protooncogene in the progression of human breast, ovarian, and non-small cell lung carcinoma. These data also support the hypothesis that $p185^{HER2}$ present on the surface of overexpressing tumor cells may be a good target for receptor-targeted therapeutics. The anti-$p185^{HER2}$ murine monoclonal antibody (muMAb) 4D5 is one of over 100 monoclonals that was derived following immunization of mice with cells overexpressing $p185^{HER2}$. The monoclonal antibody is directed at the extracellular (ligand binding) domain of this receptor tyrosine kinase and presumably has its effect as a result of modulating receptor function. *In vitro* assays have shown that muMAb 4D5 can specifically inhibit the growth of tumor cells only when they overexpress the HER2 protooncogene. MuMAb 4D5 has also been shown to enhance the TNF-α sensitivity of breast tumor cells that overexpress this protooncogene. Relevant to its clinical application, muMAb 4D5 may enhance the sensitivity of $p185^{HER2}$-overexpressing tumor cells to cisplatin, a chemotherapeutic drug often used in the treatment of ovarian cancer. *In vivo* assays with a nude mouse model have shown that the monoclonal antibody can localize at the tumor site and can inhibit the growth of human tumor xenografts which overexpress $p185^{HER2}$. Modulation of $p185^{HER2}$ activity by muMAb 4D5 can therefore reverse many of the properties associated with tumor progression mediated by this putative growth factor receptor. Together with the demonstrated activity of muMAb 4D5 in nude mouse models, these results support the clinical application of muMAb 4D5 for therapy of human cancers characterized by the overexpression of $p185^{HER2}$.

KEY WORDS: HER2; *neu*; TNF-α; monoclonal antibody therapy.

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BACKGROUND: THE HER2 PROTOONCOGENE AND HUMAN CANCER

Cellular protooncogenes encode proteins that are thought to regulate normal cellular proliferation and differentiation. Alterations in their structure or amplification of their expression lead to abnormal cellular growth and have been associated with carcinogenesis (1–4). Protooncogenes were first identified by either of two approaches. First, molecular characterization of the genomes of transforming retroviruses showed that the genes responsible for the transforming ability of the virus in many cases were altered versions of genes found in the genomes of normal cells. The normal version is the protooncogene, which is altered by mutation to give rise to the oncogene. An example of such a gene pair is represented by the EGF receptor and the v-erbB gene product. The virally encoded v-erbB gene product has suffered truncation and other alter-

ations that render it constitutively active and endow it with the ability to induce cellular transformation (5).

The second method for detecting cellular transforming genes that behave in a dominant fashion involves transfection of cellular DNA from tumor cells of various species into nontransformed target cells of a heterologous species. Most often this was done by transfection of human, avian, or rat DNAs into the murine NIH 3T3 cell line (1–5). Following several cycles of genomic DNA isolation and retransfection, the human or other species DNA was molecularly cloned from the murine background and subsequently characterized. In some cases, the same genes were isolated following transfection and cloning as those identified by the direct characterization of transforming viruses. In other cases, novel oncogenes were identified. An example of a novel oncogene identified by this transfection assay is the *neu* oncogene. It was discovered by Weinberg and colleagues in a transfection experiment in which the initial DNA was derived from a carcinogen-induced rat neuroblastoma (6,7). Characterization of the *neu* oncogene revealed that it had the structure of a growth factor receptor tyrosine kinase, had homology to the EGF receptor, and differed from its normal counterpart, the *neu* protooncogene, by an activating mutation in its transmembrane domain (8).

The association of the HER2 protooncogene with cancer was established by yet a third approach, that is, its association with human breast cancer. The HER2 protooncogene was first discovered in cDNA libraries by virtue of its homology with the EGF receptor, with which it shares structural similarities throughout (5). When radioactive probes derived from the cDNA sequence encoding $p185^{HER2}$ were used to screen DNA samples derived from breast cancer patients, amplification of the HER2 protooncogene was observed in about 30% of patient samples (9). Further studies have confirmed this original observation and extended it to suggest an important correlation between HER2 protooncogene amplification and/or overexpression and worsened prognosis in ovarian cancer and non-small cell lung cancer (10–14).

The association of HER2 amplification/overexpression with aggressive malignancy, as described above, implies that it may have an important role in progression of human cancer; however, many tumor-related cell surface antigens have been described in the past, few of which appear to have a direct role in the genesis or progression of disease (15,16). The data which support a role of HER2 overexpression in the basic mechanisms of human cancer are summarized below.

Amplified expression of $p185^{HER2}$ can lead to cellular transformation as assessed by morphological alterations and growth of $p185^{HER2}$-overexpressing cells in soft agar and in nude mice (17,18). In addition, NIH 3T3 fibroblasts overexpressing $p185^{HER2}$ have an increased resistance to cytotoxicity mediated by activated macrophages or recombinant human TNF-α (19), the cytokine that appears to be mainly responsible for macrophage-mediated tumor cell cytotoxicity (20). This observation extends also to breast tumor cells, which overexpress $p185^{HER2}$ (19), and suggests that high levels of $p185^{HER2}$ expression may be related to tumor cell resistance to at least one component of the host's antitumor surveillance armamentarium, the activated macrophage. This work has been reviewed previously (21), and similar data have recently been reported for ovarian tumor cell lines which overexpress $p185^{HER2}$ (22). Further support for a role of $p185^{HER2}$ or the related *neu* oncogene-encoded tyrosine kinase in tumorigenesis comes from work with transgenic mice that have been manipulated to overexpress one or the other of these two related genes. Transgenic mice expressing the activated form of the rat *neu* protooncogene, under the control of a steroid inducible promoter, uniformly develop mammary carcinoma (23). In another transgenic mouse model the HER2 protooncogene product, "activated" by point mutation analogous to the rat *neu* oncogene product, or an unaltered form of the HER2 protooncogene, has been expressed in mice (24). The main malignancies induced in this model were either lung adenocarcinoma or lymphoma but not mammary carcinoma. While it is not known why the different transgenic mouse models give such distinct results, the latter model may be of particular significance given the recent report of an association between $p185^{HER2}$ overexpression and poor prognosis in nonsmall cell lung cancer (14). These differing results suggest some difference in the activity of activated *neu* and HER2-encoded tyrosine kianses, although effects due to mouse strain differences cannot be excluded.

The structural similarities between $p185^{HER2}$ and the EGF receptor suggest that function of $p185^{HER2}$ may be regulated similarly to the EGF receptor. In particular, one expects that the tyrosine kinase activity associated with the cytoplasmic domain of

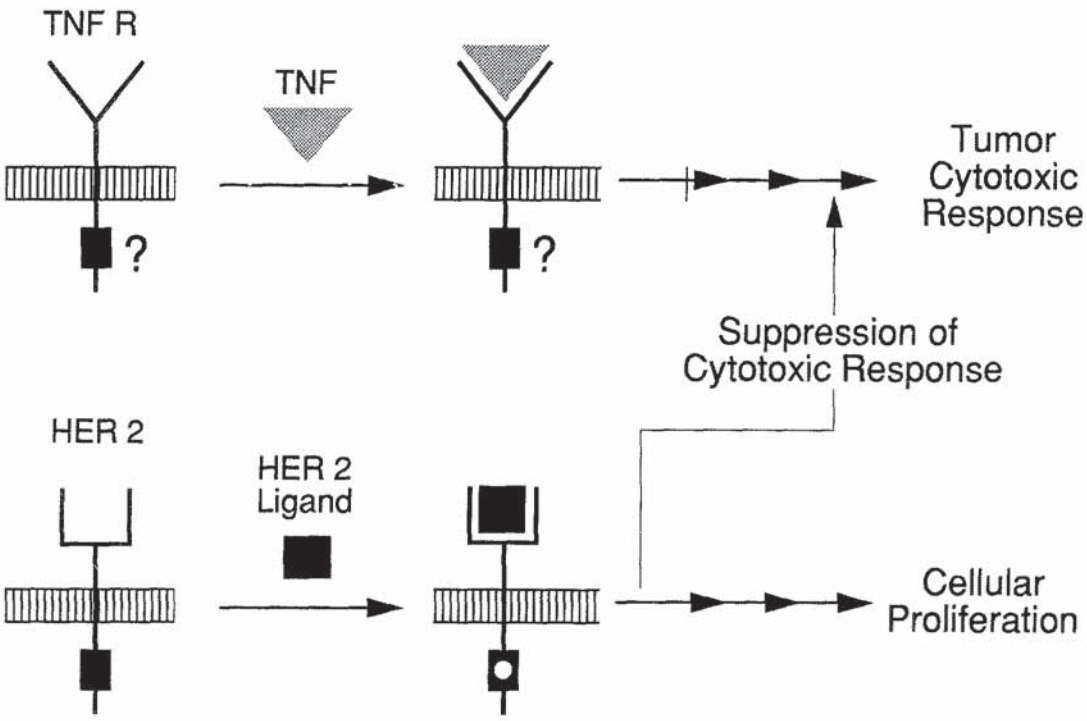

Fig. 1. Suppression of the TNF cytotoxic response by activation of $p185^{HER2}$. The schematic shows both the TNF cytotoxic pathway (top) and the $p185^{HER2}$-stimulated cell proliferation/transformation pathway (bottom). Signaling from the TNF receptor following interaction with TNF has not been characterized. Binding of ligand to $p185^{HER2}$ is shown to activate the receptor-associated tyrosine kinase activity, resulting in stimulation of cellular proliferation and suppression of the tumor cell cytotoxic response to TNF.

the receptor would be ligand activated. This proposal receives support from recent work describing a ligand for $p185^{HER2}$ (25). These data lead to a model (Fig. 1) wherein antagonists that downregulate the function of $p185^{HER2}$ should have the effect of inhibiting growth of tumor cells dependent upon $p185^{HER2}$ function and of increasing the sensitivity of such tumor cells to TNF-α. By analogy with previous work done with two related tyrosine kinases, the EGF receptor (26) and the activated *neu* protooncogene product (27), we hypothesized that monoclonal antibodies targeted to the extracellular domain of $p185^{HER2}$ may have the desired properties.

DERIVATION OF muMAb 4D5

A family of monoclonal antibodies focused against the extracellular domain of $p185^{HER2}$ were prepared (28). To do this, an NIH 3T3 fibroblast cell line that overexpresses $p185^{HER2}$ [NIH 3T3/HER2-3-400 (18)] was used to immunize BALB/c mice. The mice were subsequently boosted with NIH 3T3/HER2-3-400 and, finally, with a preparation enriched for $p185^{HER2}$ by wheat germ agglutinin chromatography of membrane extracts of this cell line. Following splenocyte fusion with a mouse myeloma partner, the hybridomas were cultured in 96-well microtiter plates. Hybridomas positive for anti-$p185^{HER2}$ activity, but with little or no anti-EGFR activity, were detected by ELISA (Fig. 2). A critical property of an anti-$p185^{HER2}$ monoclonal antibody with potential for therapy would be its lack of cross-reactivity with the closely related EGF receptor, which is expressed at elevated levels in multiple tissues (29). To select further monoclonal antibodies with this characteristic, a number of assays were performed, including immunoprecipitation assays utilizing *in vivo* labeled EGF receptor and $p185^{HER2}$ (Fig. 3A) and FACS analysis of antibody binding to tumor cells overexpressing either $p185^{HER2}$ or the EGFR (Fig. 3B). The screening results are summarized in Table I. Based upon these results, nine of the $p185^{HER2}$ monoclonal antibodies were chosen for further characterization, including a cell growth inhibition assay utilizing the SK-BR3 human breast adenocarcinoma cell line, which greatly overexpress $p185^{HER2}$. The monoclo-

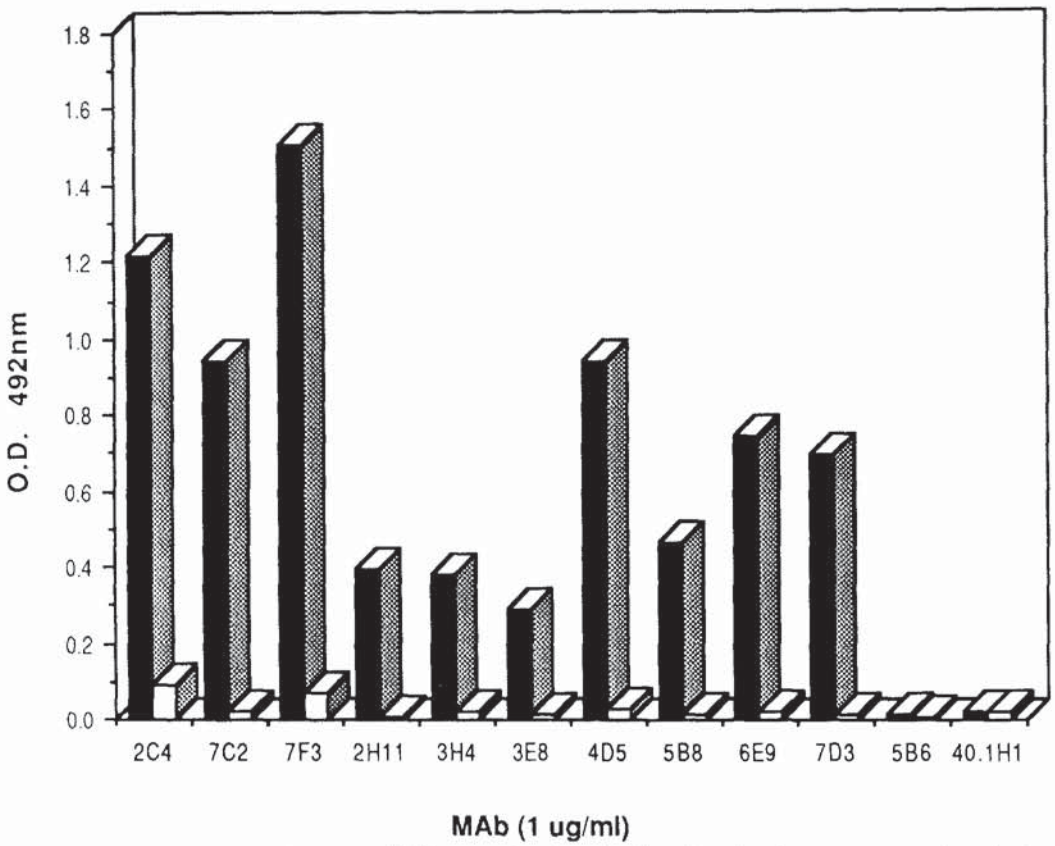

Fig. 2. ELISA screening of anti-p185^{HER2} monoclonal antibodies. Results shown measure the relative reactivities of the purified anti-p185^{HER2} monoclonal antibodies (added to 1 μg/ml) with membrane extracts enriched in EGF receptor (open bars; from A431 squamous carcinoma cells) or enriched in p185^{HER2} (filled bars; from NIH 3T3/HER2-3-400).

nal antibody, muMAb 4D5, was clearly the most effective of the group in this assay (Table II).

The initial results characterizing the growth inhibitory activity of these monoclonal antibodies were extended by comparing them for activity against a battery of human breast and ovarian tumor cell lines that expressed varying levels of p185^{HER2}. These results reveal that the monoclonal antibodies can be growth inhibitory, they may have no affect on cell proliferation, or they may stimulate the proliferation of breast tumor cells. Growth inhibition appears to depend upon overexpression (Table III). This property, in particular, is shared by the monoclonal antibodies 4D5 and 3H4. These monoclonal antibodies may exert their effects on cell growth by similar mechanisms since they compete for binding to the receptor (Tables I and III) (28) and, therefore, may recognize the same or overlapping epitopes. The other monoclonal antibodies vary in their ability to inhibit proliferation, but 7C2 and 6E9 are consistently less active in this respect. The potent growth inhibitory activity of 2C4 for MDA-MB-175 breast tumor cells is not understood at present but may represent cross-reactivity with another receptor expressed on these cells. Similarly, the properties that distinguish 7C2 from the other antibodies with regard to its ability to stimulate the proliferation of several of the tumor cell lines shown in Table III has not been determined. The 6E9 monoclonal antibody has been shown to bind to the extracellular domain of p185^{HER2}, although only to a subset of receptors on the surface of SK-BR-3 tumor cells (30). The functional significance of this subset of receptors is unclear. In addition to its activity on breast tumor cells, which overexpress p185^{HER2}, muMAb 4D5 is also clearly the most active of the monoclonal antibodies with respect to its ability to inhibit growth of SKOV-3, a human ovarian adenocarcinoma cell line that overexpresses p185^{HER2} (Table III). Experiments are currently planned to try to understand in more detail how these monoclonal antibodies may exert

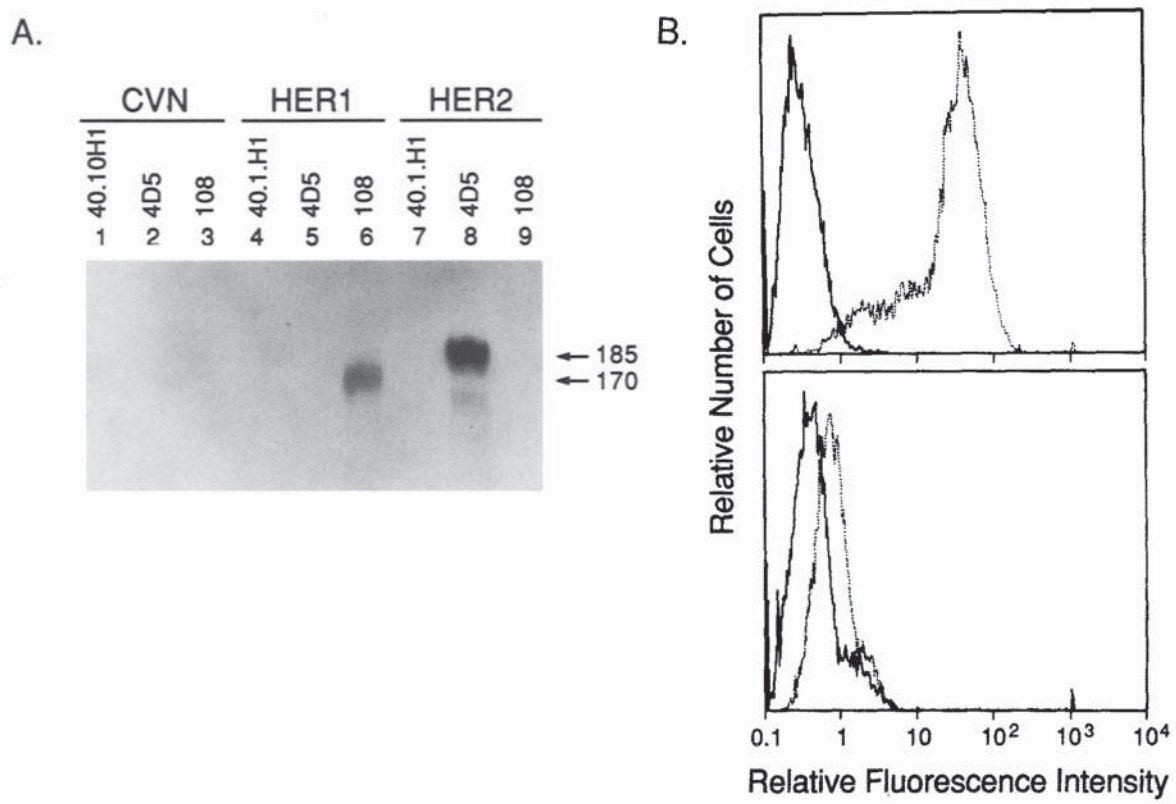

Fig. 3. MuMAb 4D5 does not cross-react with the EGFR. (A) Immunoprecipitation of metabolically labelelled NIH 3T3 cells transfected by control plasmid (CVN), by a plasmid encoding the EGFR (HER1) or a plasmid encoding $p185^{HER2}$ (HER2). MuMAb 40.1.H1 is directed against hepatitis B surface antigen (lanes 1, 4, 7); muMAb 4D5 is directed against $p185^{HER2}$ (lanes 2, 5, 8); muMAb 108 is directed against the EGFR (lanes 3, 6, 9). (B) Fluorescence-activated cell sorter histograms of muMAb 40.1.H1 (solid lines) or muMAb 4D5 (dotted lines) reacted with SK-BR-3 tumor cells (approx. 2×10^6 receptors per cell; upper panel) or the same antibodies reacted with the A431 squamous carcinoma cell line (approx. 2×10^6 EGFR per cell; lower panel).

distinct effects on tumor cell proliferation. The *in vitro* results summarized in Table III clearly show that when the monoclonal antibodies are compared for efficacy, as measured by their abilities to inhibit growth of breast and ovarian tumor cells overexpressing $p185^{HER2}$, muMAb 4D5 is usually the most potent and is therefore a good candidate for further characterization in other models that may be predictive of its efficacy in human clinical trials. Interestingly, the most dramatic activity of the antibody is seen in cell lines that overexpress greater than fivefold the level observed in MCF-7 breast tumor cell lines [a low expressor control cell line; Table III (19)]. Patients who overexpress greater than fivefold the normal level of $p185^{HER2}$ have been shown to have a very poor prognosis (10). These results will aid in choosing patients who are most likely to respond in clinical trials.

The model depicted in Fig. 1 predicts that down-regulation of $p185^{HER2}$ by a monoclonal antibody or other reagent should result in decreased cellular proliferation, as shown in Table III, but also increased sensitivity to TNF-α. Results of experiments in which tumor cells overexpressing $p185^{HER2}$ were treated with muMAb 4D5 or a control monoclonal antibody, alone and in combination with TNF-α, suggest the validity of this model (Fig. 4) (31). MuMAb 4D5 treatment of breast tumor cells overexpressing $p185^{HER2}$ resulted in enhanced sensitivity of these cells to TNF-α. The growth and the TNF-α sensitivity of normal cells or tumor cells that do not overexpress the receptor were unaltered.

In addition to the relationship between TNF-α resistance and $p185^{HER2}$ overexpression, a possible relationship between protoncogene expression and resistance to the chemotherapeutic drug cisplatin has been investigated. A correlation between HER2 protooncogene overexpression and resistance to chemotherapeutic drugs rests on the grounds that

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