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REVIEW

Complexity of Signal Transduction Mediated by ErbB2: Clues to the Potential of Receptor-Targeted Cancer Therapy

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The erbB2 oncogene belongs to the type I transmembrane tyrosine kinase family of receptors. Its medical importance stems from its widespread overexpression in breast cancer. This review will focus on the signal transduction through this protein, and explains how the overexpression of erbB2 may result in poor prognosis of breast cancer, and finally it will summerize our current understanding about the therapeutic potential of receptor-targeted therapy in breast cancer. ErbB2 does not have any known ligand which is able to bind to it with high affinity. However the kinase activity of erbB2 can be activated without any ligand, if it is overexpressed, and by heteroassociation with other members of the erbB family (erbB1 or epidermal growth factor receptor, erbB3 and erbB4). This interaction substantially increases the efficiency and diversity of signal transduction through these receptor complexes. In addition, erbB2 forms large scale receptor clusters containing hundreds of proteins. These receptor islands may take part in recruiting cytosolic factors which relay the signal towards the nucleus or the cytoplasm. Overexpression of erbB2 was linked to higher transforming activity, increased metastatic potential, angiogenesis and drug resistence of breast tumor in laboratory experiments. As a corollary of these properties, erbB2 amplification is generally thought to be associated with a poor prognosis in breast cancer patients. These early findings lead to the development of antibodies that down-regulate erbB2. Such a therapeutic approach has already been found effective in experimental tumor models and in clinical trials as well. Further understanding of the importance of erbB2 and growth factor receptors in the transformation of normal cells to malignant ones may once give us a chance to cure erbB2 overexpressing breast cancer. (Pathology Oncology Research Vol 5, No 4, 255–271, 1999)

Keywords: erbB proteins, erbB2, homoassociation, heteroassociation, breast cancer, Herceptin

Introduction

Tumors are thought to arise as a result of a series of mutations which alter the functioning of oncoproteins. A large number of these oncoproteins are transmembrane proteins that take part in signal transduction. In a lot of cases the protein product of an oncogene is overexpressed in cancers. As early as 1985 it was proven that down-modulation of an overexpressed oncoprotein can convert a malignant cell to a normal one.[41] It was achieved with an antibody against a protein which was barely known at that time: erbB2. Later the overexpression of this protein was verified in a number of human tumors, mainly breast, ovarian and gastric cancer, and gave hope for a purposeful approach to treatment.[61,71,109] For a better understanding of the therapeutic potential of receptor-targeted cancer therapy and of the controversial prognostic value of erbB2, a clear view of the function of this protein is needed.

This review attempts to summerize the diagnostic value of erbB2 in breast cancer, how it can be utilized in treat-

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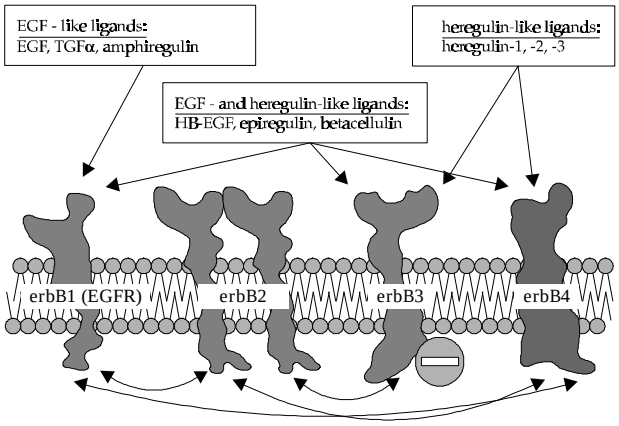

Figure 1. Distinct classes of peptide growth factors and erbB receptor homo- and heterodimers. EGF, transforming growth factor a (TGFa) and amphiregulin can only bind erbB1, heregulins can only bind erbB3 and erbB4, while HB-EGF, epiregulin and betacellulin can bind erbB1, erbB3 or erbB4. ErbB2 is an orphan receptor: currently there is no known ligand which can bind directly to erbB2, but erbB2 can associate with other members of the family which is shown by the arrows below the membrane. ErbB3 lacks intrinsic tyrosine kinase activity, so no transmembrane signal can enter the cytoplasm solely through this receptor.

ment, and how these practical applications are linked to the basic steps in erbB2-mediated signal transduction. We will only superficially talk about distinct ligand families and about the importance of diversity of possible receptor-ligand complexes in signal transduction, which are described in detail in the following references.[16,30,111,125] The role of erbB proteins in the development of the nervous and circulatory system is also beyond the scope of this review. The interested reader should consult one of the following papers.[52,80,93,119]

Ligand-dependent and -independent association of erbB proteins

Binding of a peptide growth factor to its receptor is usually accompanied by dimerization (or higher order complex formation) of its receptor.[64,82] The epidermal growth factor (EGF) receptor also known as erbB1 or HER1 is a paradigm for this association: EGF interacts with and induces homodimerization of its cell surface receptor.[50] Nowadays it is becoming accepted that homoassociation of the EGFR is induced by bivalent binding of the growth factor: EGF binds in a 1:1 ratio to EGF receptor, but a single EGF peptide interacts with two EGF receptor proteins, so a fully active receptor dimer contains two EGF receptors and two EGF molecules,[81] although complexes containing fewer components are also possible.[27]

In recent years more and more evidence was pointed to the fact that in the case of erbB family of receptors the situation is complicated by at least two additional considerations:

i. in addition to erbB1, at least three other erbB proteins exist (erbB2, erbB3 and erbB4, also known as HER2, HER3 and HER4, *Figure 1*), which can form different hetero- and homodimers,
ii. at least three classes of ligands exist which can stabilize different receptor dimers *(Figure 1).*[16] EGF-like ligands (EGF, transforming growth factor α [TGFα] and amphiregulin) can only bind erbB1 with high affinity, heregulins (also known as NDF [neu differentiation factor] or neuregulin) can bind with high affinity both erbB3 and erbB4, while heparin binding EGF (HB-EGF), epiregulin and betacellulin can efficiently interact with erbB1, erbB3 or erbB4. Therefore erbB1 is called the EGF receptor, while erbB3 and erbB4 are called heregulin receptors.

In spite of an early report about the identification of a peptide which is able to bind erbB2 directly,[90] it is widely accepted at this time that erbB2 is an orphan receptor without its own distinct ligand: it can only take part in signal transduction by forming heteroassocations with other members of the erbB family. ErbB3, the first heregulin receptor identified,[25] lacks an intrinsic tyrosine kinase activity, thus in order to take part in signal transduction it must associate with other members of the erbB family. ErbB2 (the receptor without a ligand) and erbB3 (the receptor without a tyrosine kinase activity) nicely complement each other's deficiency: it was found that erbB2 and erbB3 form a functional signal transduction complex. In the absence of erbB2, erbB3 has a low affinity for heregulin, but it is increased by the interaction with erbB2.[131] Both erbB2 and erbB3 become tyrosine phosphorylated as a result of the erbB2 tyrosine kinase.[74,131] Later all possible homo- and heteroassociations between members of the erbB family were identified in different systems. It has also been proven that heregulin receptors (erbB3 and erbB4) can take part in EGF-induced signal transduction. In a cell line expressing erbB1 and erbB3, EGF stimulated the tyrosine phosphorylation of erbB3.[74] A new expression, "secondary receptor dimerization" was coined:[51] after ligand-induced formation of a primary receptor dimer, the receptors become activated, and later they may dissociate from each other and associate with and activate other erbB proteins. It is not known if the activated receptor dimer must disassemble so that the liberated active monomers can reassociate with other non-activated erbB proteins or if secondarily activated receptors may associate with the primary receptor complex. In light of the identification of large receptor complexes (see in section "Large scale association of erbB proteins") the latter possibility cannot be excluded.[94,96] In accordance with the above considerations, the simple EGF-

induced EGF receptor homoassociation model was modified: EGF induces the formation of EGF receptor homodimers (as suggested previously) or EGF receptor-erbB2 heterodimers directly (by binding to erbB1 with its N-terminal tail and to erbB2 with its low-affinity C-terminal site[144]). Subsequently these primary receptor complexes activate other members of the erbB family. Indeed it was found that blocking signal transduction through erbB2 can severely inhibit EGF-induced signaling.[55,115]

The complexity of possible erbB homo- and heterodimers became disturbingly complicated. However, two recent findings have started to elucidate the rules that drive the formation of receptor associations. First and foremost Tzahar and coworkers[145] pointed out that erbB2 seems to be the preferential heteroassociation partner of all other erbB proteins. In addition they identified three possible heterodimers that are formed most frequently: erbB2-erbB3, erbB2-erbB4 and erbB1-erbB4. These findings were corroborated by others.[54,72] Secondly Chamberlin et al. indicated that in addition to the different propensity of distinct erbB proteins to heteroassociate, the expression level of these proteins has to be taken into account.[27] Even if the formation of a given heterodimer is not favored thermodynamically (according to the results of Tzahar et al.), it may become important, if the particular proteins are expressed at a high level. The calculations of Chamberlin et al. account for the reported dissociation constants of the EGF receptor-EGF complex, which are quite different depending on the experimental conditions, since association of the EGF receptor with other erbB proteins may alter its affinity for EGF,[145] similarly to the altered affinity of erbB3 for heregulin in the presence of erbB2.[131]

In addition to heterodimers, erbB2 also forms homodimers. ErbB2 homodimerization can be brought about in three ways:

i. erbB2 has a potent tyrosine kinase domain[38] which shows activity even in the absence of a ligand (that is in the absence of heterodimer formation with an EGF or heregulin receptor). The ligand-independent tyrosine kinase activity of erbB2 parallels the ligand-independent homodimer formation of the protein, and is especially important, if erbB2 is overexpressed in the plasma membrane.[153] Preformed erbB2 homoassociation is present in the membrane of unstimulated breast tumor cells. The distribution of erbB2 homoassociation was found to be heterogenous with some membrane areas showing anomalously high erbB2 homoassociation as compared to the rest of the plasma membrane. These areas may be the initiation sites of transmembrane signaling, although this statement has not been rigorously tested.[95] In the case of EGF receptor, neither ligand-independent tyrosine kinase activity nor ligand-independent homodimer formation seems to be important,[38,95] however preformed EGF homodimers were identified, albeit in low amounts.[50]
ii. A single mutation in the transmembrane domain of erbB2 converts it to a highly mitogenic protein, in which case ligand-independent homoassociation is very efficient.[10,122] However, no such mutation has been observed in human malignancies.
iii. Secondary homodimerization of erbB2, e.g. after EGF or heregulin stimulation[95] is also possible. Autocrine secretion of heregulin and tranforming growth factor-α (TGFα) and concomitant activation of erbB2 has been observed in rat mammary carcinoma cells.[45]

The association of members of the erbB family confers several advantages to the signal transducing mechanism. Involvement of erbB2 in a receptor complex leads to a high signaling activity due to the efficiency of the erbB2 tyrosine kinase domain.[38] In the case of erbB2 homoassociation the high tyrosine kinase activity of erbB2 may be amplified even more. The diversity of signaling is increased through the formation of receptor complexes, since a cell is able to respond in many different ways depending on the composition of the complex without expressing an equally high number of different receptors.[82] It was shown recently that erbB1 transmits different signals depending on its association partners and way of activation: it is not able to activate Grb2 if it is activated by heterodimerization with a heregulin receptor, while it avidly interacts with it, if activated directly by EGF.[100] Activation of the cbl proto-oncogene or the phospholipase-C pathway was only observed if erbB1 took part in the complex,[29,30,83] while phosphatidylinositol 3-kinase is the most efficiently activated if its SH2 domains associate with phosphotyrosine residues in erbB3.[65,132] Of course, since erbB3 lacks an intrinsic tyrosine kinase activity, these tyrosine residues have to be phosphorylated by other erbB proteins in a heterodimer containing erbB3.

Substantial effort has been exerted in order to understand the role of different domains of erbB2 in the dimerization process. Most authors agree that the extracellular domain of an erbB protein determines its association properties[116] due to bivalent binding of growth factors (see above). Although this finding identifies the extracellular domain as the major determinant of the dimerization partners of an erbB protein, other results point to the importance of the transmembrane part. A single Val→Glu amino acid substitution in the transmembrane domain of erbB2 increases its homoassociation,[10] which is attributed to hydrogen bonding involving the Glu residues in the membrane.[28] The fact that interference with the interaction between transmembrane domains of growth factor receptors inhibits signaling also implies the importance of transmembrane domains in the association process.[89] Burke et al. identified a dimerization surface on erbB2 in the transmembrane and juxtamembrane region of the protein,[22] which was already suggested previously.[28]

Amino acid substitutions that favor the hydrogen bonding between erbB2 proteins are only effective if they appear in the dimerization surface. One way to reconcile these seemingly contradictory findings is to suppose that normal, ligand-induced association is mainly driven by extracellular domains, while trans- and juxtamembrane domains mainly come into focus in the case of mutated receptors. However, as mentioned earlier, mutated erbB2 proteins have not been identified in human tumors.

So far we have only talked about association within the erbB family. However erbB proteins are able to associate with integrins and other adhesion molecules as well.[20,23,46]

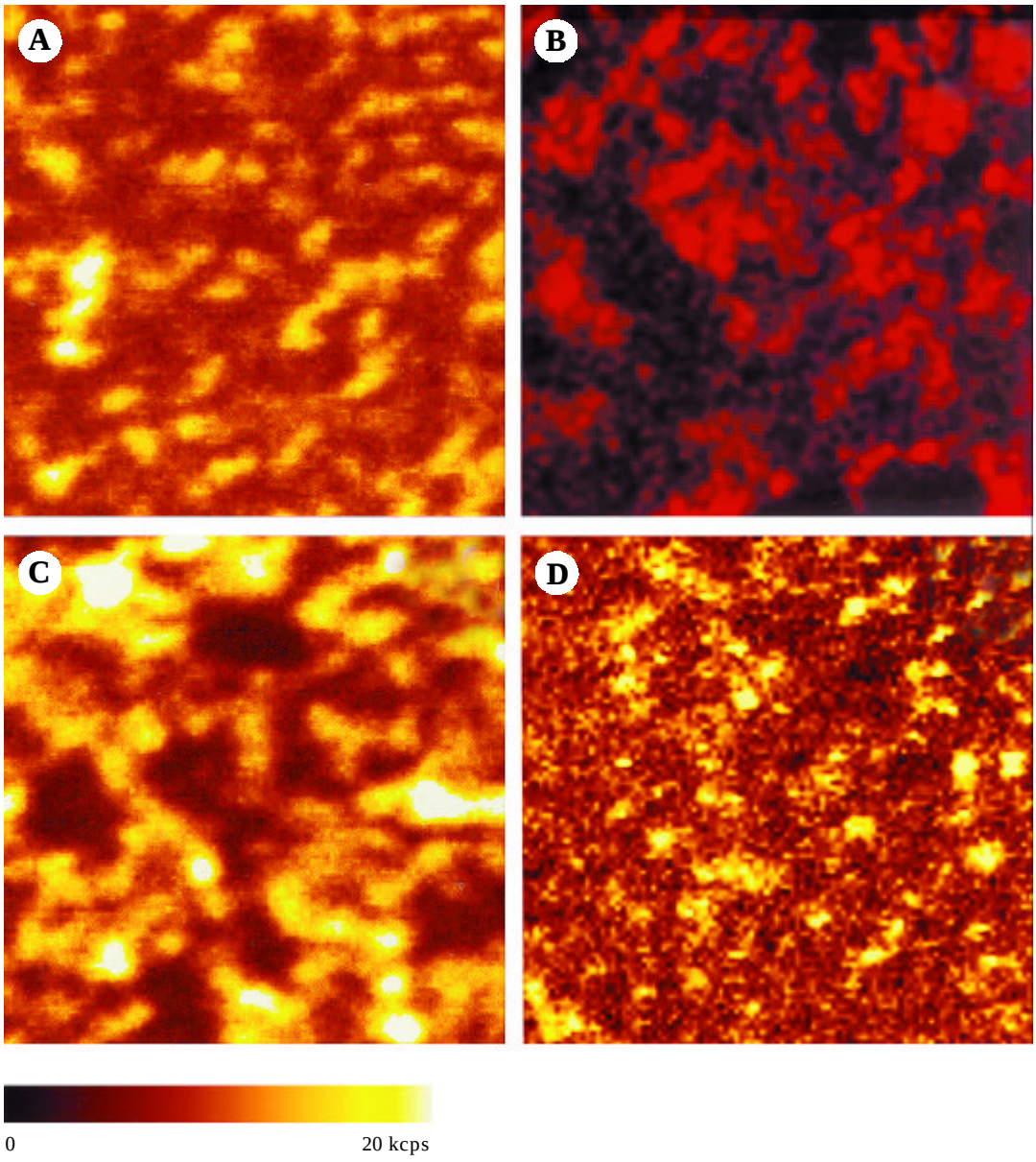

Figure 2. Scanning near-field and confocal microscopical images of SKBR3 breast tumor cells labeled with fluorescent anti-erbB2 antibodies (each image is 8x8:m). A,B: Quiescent SKBR3 breast tumor cells were labeled with rhodamine-labeled primary anti-erbB2 monoclonal antibodies on ice on the surface of glass coverslips. Cells were imaged with a scanning near-field (A) or a confocal microscope (B). Both image show the existence of apparent receptor clusters with a mean diameter of 400–500 nm. In the case of this and other near-field microscopical images, yellow color refers to high photon count, i.e. high fluorescence intensity. The scale at the bottom of the figure indicates the number of photons (kcps=kilo count per second). C: SKBR3 breast tumor cells were stimulated with 50 ng/ml EGF for 30 minutes at 37 °C, and then labeled as mentioned above. The average diameter of erbB2 clusters increased to about 700 nm. D: SKBR3 breast tumor cells were stimulated with EGF in the presence of an EGF receptor specific receptor tyrosine kinase inhibitor, and then labeled and imaged as above. The diameter of erbB2 clusters did not increase upon EGF challenge, if the inhibitor was present.

This interaction was found to increase tumor cell activation.[20] In this case they may take part in altering integrin-mediated responses. It was shown that erbB2 overexpression can interfere with collagen-induced morphogenesis,[31] and this fact also have implications for the role of erbB2 in tumor cell attachment and metastasis.[37] An $\alpha 2b\beta 3$ integrin which is in a constitutively high affinity form has been found to take part in the metastatic process of melanoma cells.[142] Association of integrins and erbB2 may be a plausible explanation for the increased affinity of these integrins.

Large scale association of erbB proteins

When biochemists speak about receptor homo- or heteroassociation, they usually mean dimerization. The reason for neglecting higher order complexes is that it is almost impossible to isolate them from membranes. Since the first step in biochemical methods in receptor association studies (e.g. gel electrophoresis) is always the disruption of the plasma membrane, these methods are inherently almost unable to detect receptor trimers, tetramers, etc. Physical methods, like fluorescence resonance energy transfer, are also mainly sensitive to direct receptor-receptor interactions or dimer formation; however, there are indirect ways to infer a greater degree of association of membrane proteins under consideration.[73]

Considering the advantages of higher order receptor association it would appear insufficient for the system to limit the number of directly interacting receptors at two. Indeed, protein modeling studies suggested the possible formation of erbB1 and erbB2 tetramers.[94] But even larger complexes of receptor proteins (clusters) may exist containing hundreds of proteins. The existence of lipid domains[43] and cholesterol-enriched "rafts" (or detergent-insoluble glycolipid-enriched membrane = DIG) containing a special collection of proteins[62,127] is well known. With the introduction of atomic force microscopy (AFM) and scanning near-field optical microscopy (SNOM) these receptor complexes have become amenable to investigation. Large scale association of the major histocompatibility complex I (MHC-I) protein was found by both of these and other approaches: MHC-I clusters with a mean diameter of about 500 nm were identified.[26,35,68] The existence of a second hierarchical level of receptor association was suggested which may take part in signal transduction: the first level is receptor dimerization, while the second level involves association of receptor dimers and the formation of receptor clusters containing at least tens of proteins.[35] More specifically, erbB2 clusters with an average diameter of 400–500 nm containing 1000–2000 erbB2 proteins were identified on the surface of unstimulated breast tumor cells.[96] The diameter of these clusters increased upon stimulation of erbB2, and the EGF-induced increase in erbB2 cluster size could be blocked by an EGF receptor specific tyrosine kinase inhibitor (*Figure 2*) supporting the role of these complexes in signal transduction.

The exact role, composition, dynamics of these clusters and the forces which assemble them are currently under intense investigation. It is almost certain that direct protein-protein interactions (like the ones responsible for the formation of dimers) are not the only forces responsible for maintaining an association of tens or hundreds of proteins. Early results suggested that interaction between the EGF receptor and the cytoskeleton leads to EGF receptor aggregation[159,160] and an increased receptor tyrosine kinase activity.[56] Association of EGF receptor with the cytoskeleton was established on the basis of detergent insolubility. The identification of detergent insoluble membrane fractions, DIGs, and methodological problems with detergent extraction techniques emphasize the need for a re-evaluation of the exact role of EGF receptor-cytoskeleton interactions. Partitioning of transmembrane proteins into distinct domains may be driven by forces similar to ones active in DIGs. Proteins found in erbB2 clusters may have similar preferences for lipid molecules or for other proteins; therefore, their optimal distribution may be reflected by accumulation in small membrane domains. The relationship between the clusters of transmembrane proteins (like that of erbB2) and rafts (DIGs) is not known. Although signal transduction through erbB2 has been linked to caveolae (which are closely related to DIGs[44]), a rigorous test of colocalization between erbB2 and other DIG components is missing. We can, however, envision that accumulation of a large number of proteins in a small membrane domain substantially increases the local concentration of the proteins (*Figure 3*). In this way interaction between activated and non-activated receptors and other proteins becomes easier, because the time of diffusion decreases. As a consequence, the local concentration of proteins associating with the intracellular domains of receptor proteins will also be high, making interaction between them easier. According to a generalized model of receptor association, ligand binding directly induces small-scale receptor association, which in turn increases the large-scale association of proteins by recruiting more proteins into preformed clusters. These receptor complexes may be the integration sites of transmembrane signaling, because they probably contain not only erbB proteins, but other receptors, integrins and signal integrating molecules, e.g. tetraspan proteins,[136] as well. This may have implications in the metastasis of tumor cells: overexpression of erbB2 is linked to increased metastatic potential and motility of tumor cells.[70,139,147] The association and cross-activation of erbB2 and integrins in receptor clusters may take part in this process.

Ligand-dependent or -independent association of erbB proteins is followed by the activation of intracellular signaling proteins. The details of these processes will not be discussed in this review, but are described elsewhere.[69]

Effect of erbB2 on the proliferation of cells

It became clear from the study of erbB2 that the efficiency of transmembrane signaling is greatly enhanced if this member of the erbB receptor family forms part of the signaling complex. At least two components were identified that could make erbB2 such an efficient signal transducer. Firstly, the intracellular kinase domain was found to be more active than that of the EGF receptor,[38] and it is necessary for erbB2-mediated signal enhancement.[116] Secondly ligand-induced down-regulation of erbB2 is absent or it is slow.[15] This is in contrast to other erbB receptors, especially erbB1, which are effectively down-regulated after ligand binding, although this process depends on the stimulating ligand as well.[49,101,149] In addition, it was found that in the presence of erbB2 overexpression, down-regulation of erbB1 is also inhibited, making repeated stimulation through erbB1 possible.[153]

The above findings raised the possibility that erbB2 overexpression increases the efficiency of transmembrane signaling by the entire erbB family, and supported the notion that enhanced expression of erbB2 decreases growth factor dependency of tumors. At the same time these claims identified erbB2 as a possible target for anti-tumor therapy, since interference with erbB2-mediated signaling can inhibit other erbB protein-mediated events, as well.[55,115] On the other hand it became clear that both the signaling efficiency and down-regulation properties of erbB2 depend on the expression of other erbB proteins. As mentioned previously erbB2 was reported to be down-regulation deficient,[15] but in these experiments erbB2 was expressed without other members of the erbB family. In contrast to this, Daly et al found that erbB2 was down-regulated after heregulin stimulation, but the cells expressed erbB1 and erbB3 in addition to erbB2.[34] The same study proved that erbB2/erbB3 heterodimers are able to promote apoptosis of erbB2 overexpressing cell lines, while the erbB1/erbB2 heterodimer was unable to achieve this effect in the same type of cells. At the same time other authors also found indirect evidence for the down-regulation of erbB2,[85] and it became generally accepted that some anti-erbB2 monoclonal antibodies are very efficient in down-regulating erbB2,[121,137] serving as possible therapeutical tools in cancer treatment. These findings emphasize the importance of a clear understanding of erbB2-mediated signaling before we can utilize its possible clinical potential to the full extent.

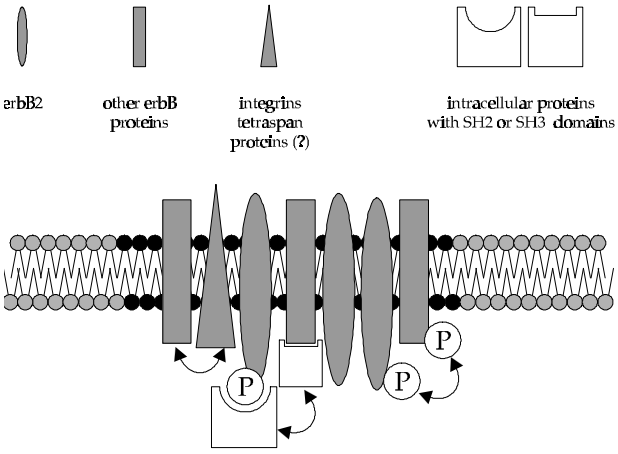

Figure 3. Model for the role of receptor clusters in transmembrane signaling. Large-scale erbB receptor clusters contain a collection of different erbB and non-erbB proteins. These proteins are held together either by protein-protein interactions or by a special lipid composition which is indicated by the different color of lipid molecules around the cluster. Interaction between members of the complex and other proteins attached to them on the intracellular side is marked by arrows. These interactions are made easier by the more frequent encounter between these proteins due to high local concentration of proteins. The involvement of other proteins (e.g. integrins and tetraspan molecules) could make these clusters an integration site of transmembrane signaling.

An important and fairly straightforward way to characterize the tumorigenic potential of an oncoprotein is to measure the colony and tumor forming capability of transfected cell lines in soft agar and nude mice, respectively. These studies found that expression of erbB2 or any other erbB protein is not sufficient by itself to induce colony or tumor formation, and that transformation and tumor forming ability do not necessarily occur simultaneously.[29,99] Alimandi et al also found that co-expression of erbB2 and erbB3 was necessary for neoplastic transformation of cells.[2] It can be concluded from these studies that erbB2 is almost always part of a highly tumorigenic signaling complex. Even if erbB2 takes part in growth inhibitory signaling in some cases,[7,9,34] the fact that it is indispensable for tumor formation (especially when it is overexpressed) again strengthened hopes for its clinical exploitation.

Interaction between erbB2 and estrogen receptors

Tumor formation is a multi-step process. In addition to increased cell proliferation and colony formation, growth factor and hormone independence, increased invasiveness, metastatic potential and drug resistance are other important aspects of malignancy. In the case of breast cancer estrogen dependence has paramount importance. Both estrogen and EGF-related peptide growth factors[97,123] are necessary for the normal physiology of breast epithelial cells, and loss of hormone and growth factor dependence is one important step in tumorigenesis. Early studies suggested that these two pathways are interrelated: erbB2-mediated signals were found to suppress estrogen receptor function, and estrogen inhibited erbB2 expression.[57,69,108,148] These findings raised concerns about the possibility that anti-estrogen

therapy in breast cancer may have unwanted side-effects by enhancing erbB2-mediated signaling, as was later confirmed by a clinical investigation[19].

The effects of erbB2 stimulation are dependent on the hormone receptor status of cells. ErbB2 stimulation induces differentiation in estrogen-dependent cells[53]; however the effects of stimulation depend on the concentration of heregulin and the relative expression level of estrogen receptor and erbB2.[57] On the other hand it was clearly shown that erbB2 overexpression promotes estrogen-independent growth.[108] Benz et al found evidence of estrogen-dependent, but tamoxifen-resistant growth of erbB2-transfected estrogen receptor-positive breast tumor cells.[17] In addition, estrogen-induced changes in erbB2 expression are much weaker in erbB2 overexpressing cells than in low expressors.[57] In conclusion we can state that erbB2-estrogen receptor interactions appear to become less important in the presence of erbB2 overexpression, and erbB2 overexpression clearly promotes progression towards hormone independence.

Effect of erbB2 on motility, adhesion and drug resistance of tumor cells and on angiogenesis

Tumor cell motility is very important in the metastatic process which ultimately leads to incurable metastatic tumor.[126] Early reports suggested that erbB2 may be related to tumor cell mobility; it was found to be present in membrane areas involved in cell motility.[37] Later the association of erbB2 with integrins, e.g. $\alpha 6\beta 4$ (laminin receptor) was demonstrated, and functional coupling between these two proteins was proven: laminin induced erbB2 tyrosine phosphorylation and the integrin and erbB2 were co-capped with an anti-integrin antibody.[23] A 50 kDa protein was able to stimulate cell spreading and motility in SKBR3 breast tumor cells by increasing erbB2 tyrosine phosphorylation.[36] Falcioni et al demonstrated that expression of both $\alpha 6$ integrin and erbB2 was necessary for increased invasiveness of tumor cells.[46] All these findings emphasize that in addition to conferring a higher proliferation activity, erbB2 (over)-expression may result in increased motility of tumor cells. In addition to motility, altered expression and activity of cell surface adhesion receptors are also important in the metastatic process. Decreased expression of adhesion molecules contributes to loss of cell-cell or cell-matrix contact which is important when cancer cells detach from their original tissue. Indeed, overexpression of erbB2 was found to decrease E-cadherin gene transcription.[32] In addition, heregulin-induced erbB2/erbB3 heterodimer activation was found to increase homophilic cell adhesion, which is also important in the metastatic process.[38]

Angiogenesis is also indispensable for the survival of both the primary tumor and metastatic cells. Overexpression of erbB2 was found to increase the production of vascular endothelial growth factor (VEGF), thereby contributing to angiogenesis. More importantly, an anti-erbB2 antibody (the same as the one in clinical trials, see later) was able to decrease the production of VEGF in erbB2 overexpressing cells.[107] All the above findings suggest that besides promoting hormone-independent cell proliferation, erbB2 expression can enhance the survival of tumor cells by increased generation of blood vessels and increased metastatic ability. Last but not least, survival of cancer patients also depends on the ability of currently used chemotherapeutic agents to block progression and proliferation of the tumor. Even though an erbB2-induced increased rate of proliferation would be expected to enhance sensitivity to these drugs, overexpression of erbB2 generally correlates with resistance to chemotherapeutic agents (described in detail in the next section). p53 is thought to play a key role in correcting damaged DNA and in maintaining cellular integrity after DNA damage. Both p53 dependent and independent induction of p21/WAF1 by peptide growth factors (following MAP kinase induction) have been observed.[9,24] In addition p21/WAF1 down-regulation has been linked to enhanced sensitivity to DNA damage.[88] These findings suggest that erbB2 dependent p21/WAF1 induction may be responsible for the drug resistance of these cells. However, other experiments with erbB2 overexpressing cell lines showed that rapid regrowth, rather than intrinsic drug resistance may be responsible for the chemoresistance of tumor cells overexpressing erbB2.[104] As practical consequence of these experiments it was found that an anti-erbB2 antibody, which down-regulates erbB2, significantly increased the sensitivity of tumor cells to cisplatin, paclitaxel (Taxol®) and doxorubicin,[12,109,110] an effect exploited in ongoing clinical trials in breast cancer patients as described in section "Therapeutic results with Herceptin in experimental animals and in human patients".

Prognostic significance of erbB2 overexpression in breast tumor

All the previously described effects of erbB2 demonstrated that overexpression of this protein may confer selective growth advantage. Therefore, a number of studies were aimed at determining its value in predicting disease outcome and resistance of cancer to hormone- and chemotherapy. Two recently published studies deal with the prognostic value of erbB2 in breast cancer; we will only briefly address this issue.[118,120]

Most authors agree that erbB2 overexpression is mainly the result of erbB2 gene amplification.[4,11,33] This correlation has been corroborated on a cell-by-cell basis using fluorescence in situ hybridization (FISH, *Figure 4*). It was shown both in cell lines and clinical specimens that cells

with very high erbB2 expression have more erbB2 gene copies, and also a higher number of chromosome 17 (which harbors the gene for erbB2).[135] It is also generally accepted that erbB2 malfunction in human cancers is the consequence of overproduction of the protein, rather than mutations. ErbB2 overexpression is more common in ductal breast carcinomas than in lobular carcinomas.[118] It is worth mentioning that *in vitro* reconstruction of mammary gland development showed that c-met tyrosine kinase activation was important for branched tubule formation, while erbB2-mediated signaling was responsible for the formation of lobular/alveolar structures and promoted the production of casein.[97] It is interesting to speculate that preferential overexpression of erbB2 in ductal cancers may be related to ectopic expression of erbB2 in ductal cells.

Early results suggested that erbB2 overexpression correlates with estrogen receptor negativity in breast tumors.[70] This report was confirmed in later studies.[118] In addition erbB2 overexpression seems to predict resistance to anti-estrogen therapy.[17,66,128]

Although there is agreement that erbB2 takes part in the pathogenesis of breast cancer,[124] reports about its value in predicting overall and disease free survival are contradictory. Some studies found that erbB2 overexpression indicates a poor prognosis.[117,130] Other studies either found no prognostic value for erbB2,[60] or its value was limited to either node positive[141] or node negative[5] cases. After reviewing several analyses Revillion et al concluded that erbB2 retains its prognostic value mainly in node positive cancer. Several reasons could explain this.[118] In the case of node negative tumors, the number of recurrences and relapses is relatively low, and demonstration of a significant effect of erbB2 overexpression would need a higher number of cases and longer follow-up period. Another possible explanation is the involvement of erbB2 in drug and anti-estrogen resistance. Node positive patients usually receive systemic chemo- and hormone therapy. If their tumor overexpresses erbB2, they do not benefit from this treatment, since erbB2 overexpressing tumors are usually resistant to these agents, while tumors without erbB2 overexpression are inhibited.

Another possible source of contradiction is the association of erbB2 overexpression with other common predictors of survival (aneuploidity, bad histological grade, high rate of proliferation, estrogen receptor negativity). There-

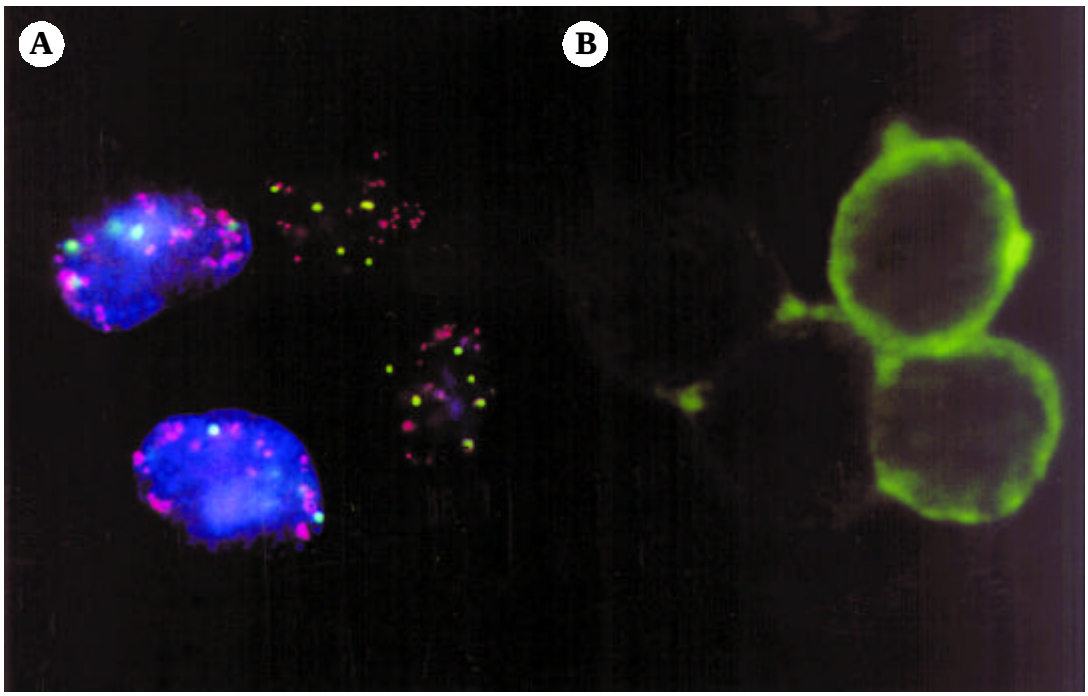

Figure 4. Simultaneous investigation of DNA synthesis, chromosome 17 number, erbB2 gene copy number and erbB2 expression in breast cancer cells. Breast tumor cells were first pulse-labeled with bromodeoxyuridine (BrdUrd) then fixed in 0.5% formaldehyde. Afterwards expression of erbB2 protein was determined with unlabeled primary anti-erbB2 antibody and a secondary fluoresceinated secondary antibody (part B). After immunofluorescence analysis cells were refixed in methanol:acetic acid (3:1) to make the nucleus accessible for oligonucleotide probes. Antibodies bound to cell surface erbB2 were removed by the incubation in methanol:acetic acid and by the harsh conditions which the cells went through before hybridization. Then chromosome 17 number was determined with fluoresceinated chromosome 17 probes (green dots in part A) and erbB2 gene copy number was determined with rhodaminated erbB2 probe (red dots in part A). BrdUrd incorporation was visualized using unlabeled primary anti-BrdUrd antibody and secondary Cascade Blue-labeled antibody (blue color in part A).

fore erbB2 may be a prognostic factor in univariate analyses, but it loses its prognostic significance in multivariate analyses,[118] although according to some studies erbB2 overexpression retains its prognostic value even in multivariate analyses.[70,117]

Another interesting and clinically useful correlation was found between the expression of erbB2 in ductal carcinoma in situ (DCIS) and high cell proliferation rates, aneuplodity and invasive disease,[3,21,120,158] thus erbB2 abnormalities seem to identify a particularly virulent form of DCIS.

Since erbB2 has been experimentally found to take part in tumor cell motility, it is not surprising that several studies attempted to find a correlation between the metastatic capability of cells and erbB2 expression. ErbB2 protein overexpression was found to positively correlate with increased risk of early[143] and visceral metastasis as opposed to bone metastasis.[70] Another study found a correlation between erbB2 overexpression and lymph node involvement, but only in patients whose tumor did not express ICAM-1.[6] Several other studies also found a correlation between lymph node status and erbB2 expression, although according to the review of Revillion et al., more investigations were unable to find a correlation.[59,118] An increased risk of recurrence has generally been associated with erbB2 positivity.[86,114]

The final outcome of breast cancer depends not only on the pathological characteristics of a tumor, but also on its sensitivity to anti-estrogen and chemotherapy. As mentioned earlier erbB2 expression is linked to resistance to anti-estrogen therapy.[17,66,128] In addition, several studies found that erbB2 overexpressing breast tumors are less sensitive to chemotherapeutic agents containing mitoxantrone,[154] methotrexate, mitomycin,[151] taxol[157] or the CMF (cytoxan, methotrexate, 5-fluorouracil) combination,[18] although other authors found superior sensitivity of erbB2 overexpressing tumors to taxol[13] and doxorubicin.[42] These reports support the notion that the effect of erbB2 on chemoresistance is agent specific: in the case of taxol and cisplatin it may confer resistance, while in the case of doxorubicin it does not confer resistance, or quite the contrary it gives rise to higher sensitivity of tumor cells[120]. The mechanisms leading to these effects may be linked to DNA repair,[110] quick recovery of tumor cells after chemotherapy[104] and p53-mediated pathways (see above), but they were not correlated with multidrug resistance.[157]

The contradictory conclusions in the studies about the importance of erbB2 as a prognostic marker in breast cancer may have their roots in the method for determining erbB2 overexpression. The methods used include immunocytochemistry and immunofluorescence. These methods are able to detect the localization of erbB2 expression, while in the case of blotting techniques, the localization of proteins is not straightforward. In this respect it is worth mentioning that cytoplasmic staining of erbB2, as opposed to membrane staining, was found not to indicate erbB2 expression, and must be the result of non-specific antibody binding.[140] A thorough study of the sensitivity of antibodies used in clinical laboratory practice showed that their ability to detect the presence of erbB2 in archived, fixed tissues was markedly different. If erbB2 overexpression rates were normalized to this sensitivity to archival tissue samples, most antibodies yielded an overexpression rate of 30%, compared to the wide range between 9–38 % without correction.[113] In addition, Northern blotting to detect mRNA overproduction, Southern blotting and fluorescence in situ hybridization (FISH) to detect gene amplification have also been used with varying degree of success. According to the review of Ross et al.,[120] FISH and immunohistochemistry on fresh or frozen sections produce more consistent results than immunohistochemistry on formalin-fixed, paraffin-embedded material.

Since the evolution of cancer is a multistep process, the simultaneous analysis of several parameters is important. In this respect it is worth mentioning that overexpression of erbB1,[58] erbB3[78] and erbB4[112] were all suggested as factors involved in breast cancer. However, another study found that expression of erbB3 and erbB4 in breast cancer is a feature of endocrine responsive tumors.[77] In addition, lower than normal expression of erbB4 was found in adenocarcinomas and squamous cell carcinomas suggesting that erbB4 expression is linked to the differentiated phenotype.[133] Most of these studies focused on a single parameter. Simultaneous flow cytometric analysis of erbB1 and erbB2 expression identified two distinct evolutionary pathways in breast cancer: one predominantly overexpressing erbB1, and the other mainly overexpressing erbB2.[124] On a population basis erbB1 and erbB2 expression were found to correlate with each other,[124] emphasizing the importance of single cell analysis.

The results of studies on the prognostic significance of erbB2 are sometimes contradictory. The limited value of this parameter may stem from the association of erbB2 overexpression with several other well established prognostic markers. In many cases erbB2 overexpression proper is the causative agent resulting in the appearance of these phenotypes, e.g. hormone independence, chemoresistance, high proliferation rate. However, the level of expression of other oncogenes, e.g. other members of the erbB family, is also important, and has been largely neglected so far. However, erbB2 was found to play a central role in mediating signaling through the erbB family,[55,115] so inhibition of erbB2-mediated signaling could efficiently subvert transmembrane signaling in breast cancer independently of other factors. This is unquestionably true if erbB2 is overexpressed, since once a cell overexpresses erbB2, it seems to rely on it heavily. This reasoning lead to the introduction of erbB2-targeted tumor therapy which will be discussed in the next sections.

Different ways to interfere with the tumor promoting function of erbB2

ErbB2-targeted tumor therapy has several options to achieve its final goal, interference with erbB2-mediated signal transduction:

i. direct blocking of erbB2 activity with tyrosine kinase inhibitors. Specific inhibitors of receptor and non-receptor tyrosine kinases were found to be effective in reducing erbB2 tyrosine kinase activity and protein expression.[63] The effect of these agents is usually considered to be reversible and cytostatic, since termination of inhibitor therapy in nude mice results in regrowth of tumor.[39] Clinical experience with these agents is scarce.

ii. coupling of toxins to anti-erbB2 antibodies (immunotoxins). We classify this as a distinct group since in this case the effect of treatment is dependent on the toxin, and the antibody is mainly used for targeting of the toxin to the tumor.[79]

iii. utilization of erbB2 as a tumor-associated antigen in immunotherapy. It was found that tumor-associated lymphocytes recognize erbB2-bearing tumor cells in an HLA-restricted fashion.[106] As a continuation of this approach, peptide vaccination with an erbB2 peptide was found to elicit T cell immunity to erbB2 in patients with breast or ovarian cancer.[40]

iv. inhibition of erbB2 protein production with antisense oligonucleotides.[87,146] To our knowledge no clinical data are available in this field.

v. cleavage of erbB2 mRNA with a ribozyme. Wiechen et al.[150] demonstrated that their ribozyme construct efficiently cleaved erbB2 mRNA both in a cell-free system and in living ovarian cancer cells leading to a decrease in erbB2 protein production.

vi. erbB2-targeted antibody therapy, which will be discussed in detail in the coming sections.

Mechanisms of anti-erbB2 antibody-mediated anti-tumor effects

More laboratory and clinical experience is available with erbB2-targeted antibody therapy than with any other approach mentioned above. It has long been known that heregulin treatment of mammary epithelial cell lines or breast cancer cells may promote differentiation (e.g. induce production of milk proteins), and sensitize breast tumor cells to the action of other lactogenic hormones.[7,91] Heregulin takes part in normal maturation of the breast.[123] In addition, one of the first antibodies raised against erbB2 was also shown to inhibit tumor cell growth.[121] This was the mouse monoclonal antibody, 4D5, produced against an extracellular epitope in erbB2, which is not present in the EGF receptor.[48] The humanized version of this antibody is now in clinical use under the patented name Herceptin® In spite of substantial efforts, it is still not known completely how 4D5 achieves its growth inhibitory effects.

i. One of the first effects attributed to 4D5 was efficient down-regulation of erbB2.[121] As mentioned earlier, down-modulation of erbB2 causes reversion of the transformed phenotype in tumor cells.[41] It was discussed above that cells overexpressing erbB2 are largely dependent on this oncoprotein; thus it may be argued that down-regulation of erbB2 showed growth inhibitory effects on its own by decreasing the signaling efficiency of the entire erbB signaling network. In accordance with this it was established that the growth inhibitory effect of erbB2 is dependent on the extent of erbB2 overexpression.[84,137]

ii. Down-regulation of a protein is generally preceded by activation of the protein in question. In most cases antibody-induced activation of a receptor is thought to require bivalent binding of the antibody; monovalent Fab fragments of the 4D5 antibody are without tumor inhibitory effects.[103] This suggests that crosslinking of erbB2 is indispensable for the therapeutic effect of 4D5. One problem with the down-regulation concept is that erbB2 was shown to be at least partly down-regulation deficient.[15] However it is known that down-regulation of a receptor caused by a bivalent antibody or a natural ligand may follow distinct pathways. In addition the study demonstrating the down-regulation deficiency of erbB2 used a construct consisting of the extracellular domain of the EGF receptor and the intracellular domain of erbB2, and expressed this protein in the absence of other erbB proteins. Thus, the conclusion of the authors are based on an artificial system. Indeed it was found that erbB2 can be down-modulated in some cases.[34] Even if the ligand-induced down-regulation of erbB2 is slower than that of the EGF receptor, a bivalent antibody might still be able to achieve efficient erbB2 down-regulation. It follows that the need for bivalent binding of 4D5 may be necessary either because it efficiently down-regulates erbB2 (as an antibody) or because it activates and down-regulates erbB2 mimicking a “natural” ligand. In support of an agonistic function of 4D5 it was found that the antibody stimulates tyrosine phosphorylation of erbB2,[121] but the real significance of either the activation or down-regulation effect for the therapeutic potential of 4D5 is obscure.[85] Stancovski et al. reported that the anti-proliferative activity of erbB2 antibodies does not always correlate with antibody-induced erbB2 down-regulation. In the same study it was found that an anti-erbB2 antibody, which efficiently stimulates cell growth, induces tyrosine phosphorylation of erbB2.[134] Thus, a mechanistic linking of antibody-

induced effects to its therapeutic potential is difficult to establish without thorough investigation.

iii. The problem of down-regulation of erbB2 is further complicated by the fact that activated growth factor receptors may keep on signaling from within endosomes.[129]

iv. Both homoassociation and large-scale clustering of erbB2 may be important in its signal transducing activity as discussed in previous sections. So the finding that the extent of erbB2 homoassociation decreases after treatment of breast tumor cells with 4D5 may be relevant.[95] Upon closer inspection, it was found that the decrease in erbB2 homoassociation was brought about by the disappearance of membrane areas with anomalously high erbB2 homoassociation. Although not tested directly, the supposition that preferential removal of membrane areas with high erbB2 homoassociation (and presumably high signaling efficiency) is part of the therapeutic potential of anti-erbB2 antibodies seems to be reasonable.

v. Klapper et al identified two possible mechanisms for the blocking by anti-erbB2 antibodies of transmembrane signaling and proliferation: 1. increased internalization and degradation of erbB2 and consequent inhibition of erbB2 homodimerization, 2. inhibition of heteroassociation of erbB2 with other erbB proteins thereby effectively blocking growth factor-mediated signal transduction.[76] These effects are also dependent on the bivalent nature of antibodies. A 4D5-mediated block of signal transduction is supported by the findings of Kumar et al,[134] who found that 4D5 antibody inhibits erbB2 tyrosine phosphorylation in the long run, an effect which could not be completely accounted for by erbB2 down-regulation. It is possible that disruption of small and large scale association of erbB2 interferes with the signaling capacity of non-internalized erbB2 proteins, and could block autocrine signaling loops as well.[45] The anti-proliferative mechanisms identified by Klapper can be linked to different epitopes. In this respect it is interesting to note that using fluorescence energy transfer measurements, the epitope of the 4D5 antibody (which is with one of the highest anti-proliferative effects) was found to be closest to the plane of the membrane, while epitopes of other, less efficient antibodies were farther away.[95] This finding lends support to the motifs of epitope-specific effects of erbB2 antibodies. It is worth repeating that juxtamembrane domains of erbB2 are important in mediating receptor association[22] antibody binding close to the membrane may efficiently interfere with this process.

vi. In addition to inhibiting proliferation, anti-erbB2 antibodies induce differentiation of breast tumor cells.[8] Even if differentiation may not be terminal/irreversible, recent kinetic approaches to cancer therapy indicate that mere inhibition of proliferation, even when combined with increased rate of apoptosis, may not be effective enough to cure cancer. A therapy must also change the tendency of cancer cells to grow in solid clumps, which is achieved by inducing differentiation.[98]

vii. Even if the anti-tumor effect of anti-erbB2 antibodies is usually proportional to the extent of erbB2 overexpression,[84,137] their effect is not predictable based solely on this parameter: large tumor-to-tumor variation has been found in the anti-proliferative effect of 4D5, even if erbB2 overexpression was considered.[84] It can be concluded that some tumors are more dependent on erbB2 than others.

Therapeutic results with Herceptin in experimental animals and in human patients

After all the effort exerted in order to understand the molecular mechanisms of erbB2-targeted immunotherapy, anti-erbB2 antibodies were found to have anti-proliferative effects on erbB2 overexpressing tumors not only *in vitro,*[67,121] but also in experimental animals[103] and in human subjects.[14] The 4D5 anti-erbB2 antibody was able to eradicate erbB2 overexpressing tumor xenografts in athymic nude mice.[103] The effect was reversible and cytostatic, since tumor growth resumed on termination of antibody therapy.[110] However mouse monoclonal antibodies induce the production of anti-mouse antibodies in human patients. In order to facilitate application of the antibody in human cancer therapy, a humanized version of 4D5 was constructed which contains the complementarity determining region of the original mouse monoclonal antibody inserted into a human IgG_1 framework.[14] This recombinant humanized anti-erbB2 antibody is currently marketed under the name Herceptin® In a phase II study of the antibody including 46 patients with metastatic, erbB2 overexpressing breast cancer, the toxicity of Herceptin was minimal as expected, since Herceptin exerted its effects only on erbB2 overexpressing tumor cells. No antibodies against Herceptin were detected in any patients. The overall response rate to Herceptin was 11.6%. One should note, however, that most patients enrolled into the trial had very advanced disease, and had already received chemotherapy before Herceptin. Ongoing phase II trials corroborate the above mentioned results. Good news was also presented at the 1998 annual meeting of the American Society of Clinical Oncology. A study found a 16% response rate when Herceptin was used as a single agent in metastatic breast cancer patients who had not responded to prior chemotherapy regimens. Researchers now call for the application of the antibody in less advanced stages of breast cancer.[92]

Since the effects of the antibody as a single agent are far from being perfect, new ways to improve its efficiency were looked for. It has been known for some time that the anti-tumor efficacy of a monoclonal antibody can be increased by concomitant chemotherapy.[47] A similar approach has been undertaken by two groups.[12,105,110] One of these studies was a clinical trial to which patients with very advanced breast cancer have been enrolled who had progressive disease during prior chemotherapy.[105] In this study Herceptin was used in combination with cisplatin. About 40% overall response rate and a median response duration of 5.3 months were reported. These results are better than those achievable with either of the treatments alone. In addition, Herceptin did not increase the toxicity of cisplatin.

In addition to clinical trials, experiments with tumor xenografts are still underway. In one of the studies Herceptin was combined with either paclitaxel (Taxol®) or doxorubicin. The combination of Herceptin with paclitaxel resulted in significantly better results in tumor growth inhibition and complete tumor regression than any of the agents alone. The combination of doxorubicin and Herceptin was only slightly superior to any of the agents used alone.[12] Another study corroborated the above mentioned results. These authors also found that the combination including Herceptin and doxorubicin was less synergistic than Herceptin plus cisplatin. The synergistic action of these agents is dependent on close temporal administration of the antibody and the drug. The authors also demonstrated that DNA repair and p21/WAF1 induction after cisplatin treatment is disrupted in Herceptin-treated cells.[110]

The above results are important in two ways. First, it is interesting that erbB2 overexpression was found to be associated with higher sensitivity to doxorubicin (see in previous sections), and cotreatment with Herceptin and doxorubicin is not significantly better than any of the agents alone. Secondly, the enhanced efficiency of combination of chemotherapy and Herceptin implies that in addition to all the above mentioned mechanisms that could explain the therapeutic efficiency of Herceptin, the antibody is also able to increase the chemosensitivity of breast tumor cells, albeit not to all agents. This phenomenon has been termed "receptor enhanced chemosensitivity".[109] This action of Herceptin may be dependent on the ras signaling pathway: a dominant negative ras mutant prevented DNA repair modulation by anti-erbB2 antibody.[156] In addition, as already mentioned in previous sections also, Herceptin-mediated enhanced chemosensitivity may be related to the p53-p21/WAF1 pathway.

The results of larger clinical trials reported at the annual meeting of the American Society of Clinical Oncology in 1998 were also promising. Genentech, the company producing Herceptin, reported that patients taking a combination of Herceptin and paclitaxel or Herceptin, cyclophosphamide and doxorubicin did much better than those taking either of the therapies alone. A 53% better response rate, 57% improvement in median duration of response and a 65% improvement in time to progression were achieved with the Herceptin combinations compared to chemotherapy alone.[92] Encouraged by the good results, trials with a combination including Herceptin, taxol, doxorubicin and cyclophosphamide are underway. Generally the toxicity of these combinations is not higher than that observed in usual chemotherapy. Only in one of the combinations was it observed that Herceptin enhanced the cardiac toxicity of doxorubicin (and cyclophosphamide).[92]

Ways to achieve even better anti-tumor effect with Herceptin

One possibility for better delivery of chemotherapeutic drugs to tumor cells is to pack them into liposomes. Therapy with drugs entrapped in conventional liposomes has two disadvantages:

i. conventional liposomes are taken up by macrophage cells in the liver or spleen, so their plasma half life is short,
ii. conventional liposomes are not taken up by cancer cells, but they deposit their drug load in the extracellular space of the cancerous tissue.

Attachment of polyethylene glycol (PEG) to liposomes substantially increases their stability and plasma half life. These liposomes are called sterically stabilized liposomes.[102] In addition, blind liposomes can be targeted to tumor cells by attaching tumor specific antibodies to them: in this way doxorubicin entrapped in sterically stabilized liposomes coated with tumor specific antibodies were able to eradicate lung cancer in mice.[1] A similar approach was undertaken with doxorubicin loaded immunoliposomes coated with the Fab fragments of Herceptin. Free Fab fragment of Herceptin was completely ineffective in inhibiting growth of breast cancer xenografts in mice. Immunoliposomes were approximately as effective as free Herceptin bivalent antibodies. In addition, it was shown that doxorubicin was taken up by erbB2 overexpressing breast cancer cells, but not by surrounding muscle cells.[103] A later study reinforced the above findings, and proved that uptake of anti-erbB2 Fab fragment-coated immunoliposomes correlates with the cell surface density and not the total number of erbB2 expressed by a cell.[75]

One study pointed out that combination of anti-erbB2 antibody therapy with tamoxifen has superior anti-tumor effect to the one seen with any agent alone. Blocking of both erbB2 and estrogen pathways seems to be most useful to patients with erbB2 overexpressing, but estrogen receptor positive tumors in future clinical trials.[152] A group reported additive anti-proliferative effects of Herceptin and an anti-EGF receptor antibody on human ovarian car-

cinoma cells.[155] It is also known that Herceptin sensitizes breast tumor cells to tumor necrosis factor.[67] This finding may also find application in the management of human breast cancer patients.

Conclusions

Great progress has been made from the identification of the EGF receptor, the first member of the erbB family of growth factor receptors, to our current understanding of the complex, hierarchical signaling mediated by these proteins. The multitude of possible small and large scale interactions between the erbB proteins makes them especially efficient in transmembrane signaling. ErbB2 seems to be a key player, both from an experimental and clinical point of view; it was therefore chosen as a target for current clinical trials. The introduction of Herceptin signals the appearance of a new modality in tumor treatment: interference with a key component in the maintenance of the cancerous phenotype. Highly promising results achieved with Herceptin treatment have been reported, and even better ones are foreseen with the application of this drug in combination with conventional chemotherapeutical agents applied during less advanced stages of the disease. With improved selection of drug combinations including Herceptin breast cancer may once become a sustainable disease or condition or may be cured, even in advanced stages.

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