Neuregulins and Cancer

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Abstract

The neuregulins represent the largest subclass of polypeptide factors of the epidermal growth factor family of ligands. These molecules are synthesized as membrane-bound, biologically active growth factors that act by binding to the HER/ErbB receptor tyrosine kinases. Preclinical data have indicated that increased expression and function of neuregulins may provoke cancer. Furthermore, neuregulin expression has been detected in several neoplasias, and their presence may correlate with response to treatments that target the HER receptors such as trastuzumab. In addition, the neuregulins have also been implicated in resistance to anti-HER therapies. Therefore, targeting of the neuregulins may be helpful in neoplastic diseases in which these polypeptide factors contribute to tumor generation and/or maintenance.

Background

The neuregulins represent the largest subclass of polypeptide growth factors of the epidermal growth factor (EGF) family (1–3). These factors were initially identified by searching for activators of HER2 receptors, and for this reason, the neuregulins are also known as heregulins (4). In parallel, other groups isolated the neuregulins as factors that up-regulated the amount of acetylcholine receptors in neuromuscular junctions (and therefore termed these factors as ARIA, for acetylcholine receptor–inducing activity; ref. 5). Other names for neuregulins include Neu differentiation factor (6), or glial growth factors (7), following some of their biological activities in breast and glial cells. Increasing evidence indicates that neuregulins may have a role in the development/progression of certain types of human cancer (3, 8). In vitro studies indicate that neuregulins act as strong mitogenic factors in cells that express HER receptors (4, 9). In vivo studies in mice have shown that overexpression of neuregulins in the mammary tissue results in the generation of adenocarcinomas (10), and favors the metastatic spread of breast cancer cells in vivo (11). In addition, reduction of neuregulin by the use of antisense oligonucleotides reduces tumorigenesis and metastasis of breast cancer cells (12). Furthermore, neuregulins are expressed in a significant subset of patients with breast cancer (13–15), and their presence correlates with clinical response to certain antitumoral treatments (14).

The neuregulins are the natural activators of HER receptors. The interest in the study of neuregulins in cancer started upon their identification as activators of HER2/ErbB2/neu (4), a transmembrane tyrosine kinase that is overexpressed in a subset of patients with breast cancer (16). HER2 belongs to the human EGF receptor (HER) family of transmembrane tyrosine kinases that also includes HER1/EGFR/ErbB1, HER3/ErbB3, and HER4/ErbB4 (17). Constitutively active forms of the HER/ErbB receptors have been reported in several tumors (18), and pathologic activation of these receptors may occur by several mechanisms, including increased amounts of the receptor present at the plasma membrane (overexpression), or structural alterations (point mutations or truncations; ref. 19). Treatments aimed at decreasing the amount/activity of these activated HER receptors have clinical benefit, demonstrating that targeting of these receptors is effective in cancer (19).

The natural mechanism of activation of the HER receptors is by ligand binding (20). Upon engagement of neuregulins to the HER3 or HER4 receptors, the conformation of these receptors is modified to expose a region of the extracellular part of the receptor called the dimerization arm (ref. 21; Fig. 1). This region is then able to interact with analogous dimerization arms of other activated HER receptors. When two HER receptors are in sufficient proximity, the kinase region of one of the receptors transphosphorylates tyrosine residues present in the intracellular region of the other receptor. Elucidation of this HER receptor activation mechanism has been important in the design of targeted therapies against HER receptors. These structural data led to the development of pertuzumab, a monoclonal antibody directed to the dimerization arm of HER2, which impedes neuregulin-induced HER receptor activation by inhibition of receptor-receptor interactions (22). The efficacy of pertuzumab in HER2-positive patients has been reported in a recent clinical trial (23).

The neuregulins are synthesized as membrane-bound growth factors. Four neuregulin genes (NRG1–4) have been described. Alternative splicing of the mRNA products of these genes generates at least 20 different neuregulin isoforms (2, 3, 24).
Most of these isoforms are synthesized as transmembrane molecules that expose the biologically active EGF-like module to the extracellular space (Fig. 1B; ref. 25). This EGF module usually follows the NH₂ terminus that contains distinct domains such as hydrophobic, kringle-like, immunoglobulin-like, or cysteine-rich regions, depending on the isoform (2). An interesting aspect, from the biological and structural point of view, is the fact that many neuregulin isoforms do not contain an NH₂-terminal signal sequence (2, 25). The latter is known to target nascent polypeptide chains of proteins to the endoplasmic reticulum for export to the extracellular space or to the plasma membrane. This lack of NH₂-terminal signal sequence is unique to certain neuregulin isoforms, as the other EGF family membrane-anchored growth factors hold such a signal sequence (1). The presence of an internal hydrophobic sequence in the neuregulins has been indicated to act both as a substitute for the NH₂-terminal signal sequence, and a transmembrane anchor (25).

Once at the plasma membrane, the neuregulins may remain at this cellular location as anchored molecules, or may be solubilized (Fig. 1A). Solubilization allows these factors to travel to cells or tissues located at a distance from their site of production. As solubilization of membrane neuregulins may be therapeutically relevant (26), we will briefly describe such a mechanism.

Mechanisms of solubilization of transmembrane neuregulins. Release of soluble neuregulin from the plasma membrane precursor (termed pro-NRG) occurs by the action of cell surface proteases. Under resting conditions, most of the biosynthesized pro-NRG accumulates at the plasma membrane, and the proteolytic activities that act on these factors are quite

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Fig. 1. A, while in the membrane-bound situation, the neuregulins may only act on cells that are in physical contact with the cells that produce the neuregulins (juxtacrine stimulation), upon solubilization, these factors are able to act on cells that reside at a certain distance from the site of neuregulin synthesis (paracrine stimulation). In addition, cleavage of transmembrane neuregulins may favor interaction of the solubilized neuregulin with HER receptors present in the cells that produce the neuregulins (autocrine stimulation). At least two distinct transmembrane proteases, TACE and metalloprotease-β, have been implicated in this solubilization process. The activity of TACE may be up-regulated by agents that stimulate intracellular kinases such as Erk1/2, protein kinase C, or calcium-dependent kinases. The activity of these proteases may be blocked by specific hydroxamic acid – derived sheddase inhibitors. Binding of transmembrane or soluble neuregulins to the HER3 and HER4 receptors triggers their oligomerization through receptor-receptor interactions mediated by the dimerization arm, a subdomain present in the extracellular region of the HER receptors. In HER3 and HER4, the dimerization arm is hidden unless neuregulin binds to their extracellular domain. Interestingly, neuregulins do not directly bind to HER2, that however, presents a constitutively semiactive conformation exposing its activation arm. Pertuzumab is a monoclonal antibody designed to bind the activation arm of HER2, therefore impeding its interaction with other HER receptors. Trastuzumab is another anti-HER2 antibody that does not interfere with ligand-induced receptor activation. Engagement of HER receptors increases their tyrosine phosphorylation level, as the kinase domains of receptors in close proximity transphosphorylates the cognate HER receptor present in the oligomeric complexes. Agents that directly inhibit the HER receptor kinase activity (TKI) may be used to prevent receptor-mediated signaling. Stimulation of several intracellular signaling pathways such as the Erk1/2, Erk5, or Akt routes is the cellular responses to HER receptor activation. B, transmembrane neuregulin indicating the different structural domains.
silent (27). However, these proteolytic activities can be upregulated and provoke the shedding of transmembrane neuregulins (27–29). Studies on the mechanisms and molecular entities that participate in the solubilization of pro-NRGs have indicated that this solubilization process involves at least two different proteolytic activities. One of the proteases implicated is tumor necrosis factor-α–converting enzyme (TACE), also termed ADAM17. Experiments carried out in cells deficient of TACE activity indicated that stimulated release of the transmembrane neuregulin isoform pro-NRGα2c is blocked in these cells (27). Furthermore, introduction of wild-type TACE in these cells confers regulated cleavability of pro-NRGα2c. These studies have been complemented by others that support a role for another protease, ADAM19/meltrin-β (30), in the release of membrane-bound neuregulins.

The mechanisms of activation of these proteases are still obscure. Stimulation of several intracellular kinases, such as protein kinase C, calcium-dependent kinases, or the mitogen-activated protein kinases Erk1/2 and p38, strongly up-regulates shedding of pro-NRGα2c and other EGF class membrane-anchored growth factors (27–29). Therefore, an increase in kinase activity within the cell may be a major determinant that causes the activation of these proteases. However, the final mechanism by which the proteases are sensitive to these changes in kinase activity has not been elucidated. Nevertheless, some reports have described phosphorylation of the cytosolic region of TACE in response to protein kinase C activation (31, 32). One of these phosphorylations, which occurs in the cytosolic tail of TACE at residue threonine 735, may be linked to the up-regulation of TACE activity (31). However, the fact that a mutated form of TACE that lacks the intracellular region reconstitutes regulated cleavage of membrane tumor necrosis factor-α, indicates that the cytosolic region of TACE may not be critical for the regulation of its activity (33).

**Biological properties of membrane-bound neuregulins.** Although there is no question that soluble forms of neuregulins are biologically active, one of the most debated properties of neuregulins and other membrane-bound EGF family polypeptides is whether they are active in their transmembrane form. Furthermore, the control of the solubilization of neuregulins or other membrane-bound growth factors of the EGF family may be therapeutically relevant (26, 34). Several reports have supported that membrane-bound neuregulins are biologically active (35–37). Thus, the transmembrane NRG1 isoform pro-NRGα2c has been shown to activate HER2, HER3, and HER4 receptors when expressed in the breast cancer cell line MCF7 (36, 37). Furthermore, dual phosphorylation of Erk1/2, Erk5, and Akt are constitutive in cells expressing pro-NRGα2c. Other reports, however, indicated that prevention of solubilization of membrane-bound neuregulins, or other membrane-anchored growth factors of the EGF family, impedes tumor generation in vitro and in nude mice (26, 34).

**Clinical-Translational Advances**

Although preclinical data supports the role of neuregulins in cancer generation/progression, the most relevant aspect to be addressed is whether neuregulins also play a role in human tumors. Several studies have investigated the expression of neuregulins in different tumoral cell lines (3, 8), and in human tumor samples (Table 1). Unfortunately, these studies have been less numerous than those done on the HER receptors, due to the complexity of neuregulin genes and splicing isoforms, as well as the lack of adequately characterized antibodies to probe tissue specimens for neuregulin expression. However, some groups have reported the expression of different neuregulin isoforms in breast cancer using antibodies that recognize isoforms derived from the four neuregulin genes (13). Interestingly, in that study, the expression of NRGα2a isoform correlated with node positivity. In addition to these pathologic studies, cytogenetic data have also shown alterations of the neuregulin genes in breast cancer. These analyses have evidenced a subgroup of patients with breast cancer whose tumors bear a break in the NRG1 gene, and this correlates with poor histopathologic grade (38).

**Modulation of anticancer treatments by neuregulins.** Preclinical and translational studies have indicated that neuregulins may modulate the response to certain agents used in the treatment of breast cancer. One study, a side by side comparison of the effects of transmembrane neuregulin expression or HER2 overexpression, using MCF7 breast cancer cells as a model, not only showed the capability of transmembrane neuregulin to activate HER receptors, but also showed that stimulation of these cells by the transmembrane factor was higher, in terms of proliferation, than the overexpression of HER2 (37). These in vitro studies also showed that sensitivity to trastuzumab (a monoclonal antibody used in the treatment of breast cancer patients with tumors overexpressing HER2) was high in cells expressing pro-NRGα2c, and this sensitivity was superior to that of cells overexpressing HER2 (37). In line with these findings, Menendez et al. showed that sensitivity to cisplatin was lower in MCF7 cells expressing transmembrane neuregulin than the sensitivity of wild-type MCF7 cells (15). Interestingly, as MCF7 cells do not overexpress HER2, these data indicated that perhaps patients not overexpressing HER2 but having transmembrane neuregulin, could also respond to trastuzumab-based therapies. This idea has been tested in a translational study that showed a correlation between the expression of transmembrane neuregulin and clinical response to trastuzumab-based therapies (14). In this study, the expression of membrane forms of neuregulins was studied in 124 patients with breast cancer using antibodies that recognized transmembrane NRG1, NRG2, or NRG3 forms. Patients with tumors expressing neuregulin, but without HER2 overexpression, responded better to trastuzumab-based therapies than patients with low neuregulin. These data somehow reproduce the exquisite sensitivity to trastuzumab of MCF7 cells expressing neuregulins (15, 37), as we mentioned above. Therefore, the expression of transmembrane neuregulins may help in identifying an additional subset of patients that could benefit from treatments that target HER2. These findings could partially explain the unexpected results observed in the analysis of the NSABP-B31 clinical trial. In this trial, patients with HER2-negative tumors benefited from trastuzumab similar to HER2-positive patients (39). Although conceptually and clinically interesting, these exploratory studies should be confirmed in retrospective studies in larger series of patients, as well as in well-designed clinical trials. One of the main questions to be defined is the exact proportion of patients in which neuregulin expression is observed, because in different studies, neuregulin expression has been reported to range from 25% to >50% (13–15, 40). In this context, the development of a well-validated assay for neuregulin testing is a main goal.
As neuregulin expression may modulate the clinical response to trastuzumab, a relevant question to be studied is whether neuregulin expression may influence the response to other anti-HER2 strategies, such as treatments based on the monoclonal antibody pertuzumab or the dual small tyrosine kinase inhibitor lapatinib. Unpublished results from our laboratory show that HER2-negative breast cancer cell lines expressing neuregulin are sensitive to pertuzumab or lapatinib in the same range as trastuzumab. Another interesting question to be addressed is whether or not neuregulin expression is associated with a specific breast cancer subtype.

**Targeting neuregulins in cancer.** From the therapeutic point of view, targeting neuregulins will be useful in cases in which activation of the HER receptors is due to the availability of ligands, and not to other mechanisms. Two clinically interesting strategies have been used to target neuregulins. One is based on the use of blocking antineuregulin antibodies that prevent the interaction of neuregulins with their receptors. Some preclinical data have shown the effectiveness of this approach in breast cancer cell lines (41). This strategy may be more efficient than anti-HER antibodies or even small molecule tyrosine kinase inhibitors that target EGF receptor and HER2, as it eliminates the possibility of neuregulin signaling through all HER receptors.

An alternative way of attacking neuregulin signaling is based on the inhibition of shedding of membrane-bound neuregulins (26). In lung cancer, treatment with the anti-EGF receptor agent gefitinib results in clinical benefit in patients with increased functioning of the EGF receptor. However, tumors frequently progress due to resistance to gefitinib. Resistance to this drug may occur by mutation of the EGF receptor, or other mechanisms that involve the solubilization of EGF family ligands with activation of HER3 (26, 42). Inhibition of shedding of neuregulins and other EGF transmembrane growth factors by an inhibitor of TACE resulted in antitumoral activity in preclinical models (26).

The targeting of HER receptors in human cancer is of unquestionable clinical benefit. Usually, in neoplastic pathologies in which HER receptors may play a pathophysiological role, study of the expression of the receptors precedes the decision of whether to treat with an antireceptor strategy or not. However, as activation of the receptors may occur by availability of the ligands, adequate pathologic analyses of the role of these receptors should include a study of the expression of the ligands and the amount of activated receptors, rather than just the total amount of the receptor. In fact, as some patients respond to antireceptor therapeutics even without amplification of HER receptors (39, 43, 44), it is possible that such activation may be due to the presence of ligands such as neuregulins. To assess the activation status of HER receptors in tumoral samples, validated antibodies that recognize tyrosine phosphorylated forms of these receptors in paraffin-embedded tissues or frozen samples could be used (15). At present, several commercial sources of such antibodies already exist. It will be insightful to analyze whether patients that respond better to anti-HER therapies correspond to those with active HER receptors in the tumoral pathologies in which these receptors are used as therapeutic targets.

The results obtained in preclinical and clinical studies on neuregulins indicate that these factors may be therapeutic targets. The situation in which their targeting may be of clinical benefit involves the activation of HER receptors due to neuregulin expression in the tumoral cells, or in cells of surrounding tissues that feed the tumor. Future studies on the pathophysiological role of neuregulins in human cancer, together with the development of pharmacologically relevant antineuregulin strategies and adequate clinical trials, will define whether the targeting of these molecules should be incorporated into the therapeutic armamentarium to fight cancer.

**Table 1. Neuregulin expression in human tumors**

<table>
<thead>
<tr>
<th>Type of tumor</th>
<th>References</th>
<th>Assay</th>
<th>Estimate of expression (sample size)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer</td>
<td>(14)</td>
<td>Immunohistochemistry</td>
<td>50% (n = 151)</td>
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<tr>
<td></td>
<td>(45)</td>
<td>Immunohistochemistry</td>
<td>60-95% (n = 60)</td>
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<tr>
<td></td>
<td>(13)</td>
<td>Immunohistochemistry</td>
<td>35-45% (n = 45)</td>
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<td></td>
<td>(46)</td>
<td>Immunohistochemistry</td>
<td>84% (n = 115)</td>
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<tr>
<td></td>
<td>(47)</td>
<td>Immunohistochemistry</td>
<td>48% (n = 35)</td>
</tr>
<tr>
<td></td>
<td>(48)</td>
<td>Western blotting</td>
<td>25% (n = 60)</td>
</tr>
<tr>
<td></td>
<td>(49)</td>
<td>Immunohistochemistry</td>
<td>38% (n = 34) and 50% (n = 34)*</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>(24)</td>
<td>Immunohistochemistry</td>
<td>100% (n = 40)</td>
</tr>
<tr>
<td></td>
<td>(50)</td>
<td>Immunohistochemistry</td>
<td>100% (n = 24)*</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>(52)</td>
<td>RNA microarrays</td>
<td>72% (n = 50)</td>
</tr>
<tr>
<td></td>
<td>(53)</td>
<td>Immunohistochemistry</td>
<td>53% (n = 45)</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>(54)</td>
<td>Immunohistochemistry and RT-PCR</td>
<td>77-87% (n = 53) and 83% (n = 24)</td>
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<tr>
<td>Pancreatic cancer</td>
<td>(55)</td>
<td>Immunohistochemistry</td>
<td>85% (n = 14)</td>
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<tr>
<td>Papillary thyroid cancer</td>
<td>(56)</td>
<td>Immunohistochemistry</td>
<td>78-83% (n = 134)</td>
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<td>Vestibular schwannoma</td>
<td>(57)</td>
<td>Immunohistochemistry</td>
<td>100% (n = 8)</td>
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<tr>
<td>Endometrial cancer</td>
<td>(58)</td>
<td>Immunohistochemistry</td>
<td>Not described</td>
</tr>
<tr>
<td>Bladder cancer</td>
<td>(59)</td>
<td>RT-PCR</td>
<td>Not described (n = 41)</td>
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<tr>
<td></td>
<td>(60)</td>
<td>RT-PCR</td>
<td>Not described (n = 88)</td>
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<tr>
<td>Medulloblastoma</td>
<td>(61)</td>
<td>RT-PCR</td>
<td>87% (n = 48)</td>
</tr>
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</table>

*Expression in stromal tissue surrounding the tumor.
1In bladder cancer, neuregulin is down-regulated.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
Agus D, Akita R, Fox W, et al. Targeting ligand-
18.

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