Heregulin and Agonistic Anti-p185<sup>c-erbB2</sup> Antibodies Inhibit Proliferation But Increase Invasiveness of Breast Cancer Cells That Overexpress p185<sup>c-erbB2</sup>: Increased Invasiveness May Contribute to Poor Prognosis<sup>1</sup>

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ABSTRACT
Overexpression of p185<sup>c-erbB2</sup> (p185/NEU/HER2) by tumor cells is associated with a poor prognosis in many but not all studies of breast and ovarian cancer. The poor prognosis associated with overexpression of p185<sup>c-erbB2</sup> could result from an increased growth rate or increased invasive potential. The p185<sup>c-erbB2</sup> tyrosine kinase receptor can be activated with agonistic antibodies directed against p185<sup>c-erbB2</sup> or with the ligand heregulin through a combinatorial interaction with erbB3 or erbB4. Consequently, we have asked whether heregulin or agonistic antibodies increase anchorage-independent growth or invasiveness of the SKBr3 breast cancer cell line, which overexpresses p185<sup>c-erbB2</sup>. Incubation of SKBr3 breast cancer cells with heregulin inhibited anchorage-independent growth while enhancing tyrosine phosphorylation of p185<sup>c-erbB2</sup>. Heregulin treatment also increased adhesion of SKBr3 cells to plastic and increased invasiveness of tumor cells into Matrigel membranes while increasing expression of the CD44 (HCAM) and CD54 (ICAM-1) adhesion molecules. Tumor cell invasion of Matrigel membranes was partially blocked by either anti-CD44 or anti-CD54 antibodies, indicating a role for these adhesion molecules in the invasion process. Compatible with the increased invasiveness, heregulin increased expression of the matrix metalloproteinase 9. In contrast, the agonistic anti-p185<sup>c-erbB2</sup> antibody ID5 induced only a subset of the responses induced by heregulin. ID5 induced tyrosine phosphorylation of p185<sup>c-erbB2</sup>, increased invasiveness, and increased expression of CD44. Despite the similarity of effects of ID5 and heregulin on some outcomes, the ID5 antibody failed to increase adhesion to plastic, expression of CD54, or production of matrix metalloproteinase 9. Thus, the ID5 agonistic anti-p185<sup>c-erbB2</sup> antibody mimics rather than antagonizes some but not all of the actions of heregulin. Moreover, the poor prognosis of breast and ovarian cancers that overexpress p185<sup>c-erbB2</sup> could relate in part to enhanced invasiveness rather than to increased proliferative capacity.

INTRODUCTION
The c-erbB2 (Her-2/neu) proto-oncogene encodes a transmembrane tyrosine kinase, p185<sup>c-erbB2</sup>, that is structurally homologous to the receptor for epidermal growth factor receptor (1, 2). Although somewhat controversial (3, 4), overexpression of p185<sup>c-erbB2</sup> has been associated with a poor prognosis in several studies of patients with breast and ovarian cancer (5, 6). Monoclonal antibodies raised against the p185<sup>c-erbB2</sup> protein can inhibit growth of tumor cells that overexpress p185<sup>c-erbB2</sup>, a process that is associated with increased tyrosine phosphorylation of p185<sup>c-erbB2</sup> (7). Several different ligands have been described that can also stimulate tyrosine phosphorylation of p185<sup>c-erbB2</sup>, including the rat NDF<sup>3</sup> and human heregulin (8, 9). These ligands have been reported to stimulate growth of cells that express p185<sup>c-erbB2</sup> (10), but the impact of heregulin and NDF on cell growth may depend on the coexpression of other related receptors (c-erbB3 and c-erbB4) as well as on the relative levels of receptor expression (11–14). Furthermore, the response of cells that overexpress p185<sup>c-erbB2</sup> may differ from that of cells with normal p185<sup>c-erbB2</sup> levels. Monoclonal antibodies against the extracellular domain of the mutated rat neu membrane receptor have been demonstrated to suppress tumorigenesis by neu-transformed NIH3T3 cells (15, 16). In related studies, monoclonal antibodies against portions of the extracellular domain of the nonmutated human gene product can specifically inhibit the growth of human breast carcinoma cells that overexpress p185<sup>c-erbB2</sup> (17). These antibodies might accomplish their growth-inhibitory effects by blocking a putative autocrine/paracrine growth-stimulatory loop involving the

1 The abbreviations used are: NDF, neu differentiating factor; MMP, matrix metalloproteinase; PA, plasminogen activator; FBS, fetal bovine serum; TCM, tissue culture medium; VLKpNA, 6-Val-Leu-Lys-p-nitroanilide; ICAM, intercellular adhesion molecule.

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p185\(^\text{erbB2}\) receptor (18, 19) or, alternatively, might mimic the activity of the ligand if heregulin or NDF inhibited, rather than stimulated, growth of cells that overexpress p185\(^\text{erbB2}\).

Most studies of p185\(^\text{erbB2}\) have focused on the potential growth-regulatory effects of the receptor. The prognostic impact of p185\(^\text{erbB2}\) overexpression could, however, relate to an increased potential for invasion and metastasis or to increased resistance to cytotoxic chemotherapy, rather than to an increased capacity for proliferation. The mechanism by which cancers spread and metastasize is still only partially understood. Tumor cells must adhere to endothelial cells and interact with components of the extracellular matrix to establish metastatic foci. The expression of cell surface adhesion molecules and their receptors such as CD54 (ICAM-1) and CD44 (HCAM) appear important for metastasis. Indeed, heregulin has been demonstrated to increase expression of ICAM-1 (20). Motility and protease expression are also required for invasion of the extracellular matrix and penetration of the lymphatic or blood vessel wall. Proteinases produced by invading tumor cells include PAs and MMPs.

MATERIALS AND METHODS

Cell Lines. SKBr3 (24), a human breast cancer cell line, was maintained in RPMI 1640 supplemented with 15% FBS and 2 mm l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. For all experiments, cells were detached with 0.25% trypsin-0.02% EDTA. Cells were washed once in TCM before use.

Monoclonal Antibodies. Murine monoclonal antibodies (TA1 and IDS) that react with the extracellular domain of p185\(^\text{erbB2}\) inhibited anchorage-independent growth of the SKBr3 breast cancer cell line that overexpressed p185\(^\text{erbB2}\) (7). In the present report, we have compared the effects of heregulin to the effects of agonistic anti-p185\(^\text{erbB2}\) antibodies on clonogenic growth of SKBr3, adherence to plastic, invasion of Matrigel membranes, expression of the CD44 and CD54 adhesion molecules, and production of MMP-9. In this p185\(^\text{erbB2}\)-overexpressing breast cancer cell line, heregulin inhibited anchorage-independent growth but increased adherence and invasiveness associated with enhanced expression of MMP-9, CD44, and CD54. Agonistic antibodies to p185\(^\text{erbB2}\) mimicked a subset of the responses induced by heregulin.
growth (7) and to activate p185c-erbB2 (27), decreased colony formation in semisolid agar of the SKBr3 breast cancer cell line, which overexpresses p185c-erbB2 (Fig. 1). In contrast, the TA1 antibody, which does not activate p185c-erbB2 (27), did not inhibit anchorage-independent growth of SKBr3 breast cancer cells. The ID5 and TA1 antibodies were used throughout to represent antibodies that did and did not inhibit anchorage-independent growth and activate p185c-erbB2, respectively. Heregulin, which in combination with erbB3 or erbB4 activates p185c-erbB2, induced a dose-dependent inhibition of anchorage-independent growth of SKBr3 breast cancer cells (Fig. 1). Indeed, from our assays, we were unable to identify a concentration of heregulin (between 10 ng/ml and 0.001 ng/ml) that would increase the anchorage-independent growth of SKBr3 cancer cells (data not shown). Thus, the ID5 antibody and heregulin both induce dose-dependent inhibition of anchorage-independent growth of SKBr3 cells, whereas the TA1 antibody did not alter anchorage-independent growth. Because antibodies to p185c-erbB2 and heregulin both decrease anchorage-independent growth of the SKBr3 breast cancer cell line, which overexpress p185c-erbB2, it is difficult to equate the poor prognosis associated with overexpression of p185c-erbB2 with alterations in cell proliferation rates, at least as indicated by anchorage-independent growth. To further explore the mechanisms potentially contributing to the poor prognosis associated with overexpression of p185c-erbB2, we examined whether overexpression of p185c-erbB2 may alter the ability of breast cancer cells that overexpress p185c-erbB2 to undergo in vitro correlates of adhesion and invasion, which could potentially contribute to patient outcome.

**Increased Adhesion to Plastic.** An increased ability of cells to adhere and invade could contribute to the poor prognosis associated with overexpression of p185c-erbB2. To test this possibility, we explored the ability of SKBr3 cells to adhere to plastic as a model for increased adhesiveness in vivo. Treatment of SKBr3 cells with heregulin (1–10 ng/ml) significantly in-
increased their ability to adhere to plastic (Fig. 2). In contrast, neither the ID5 nor the TA1 antibody increased the ability of SKBr3 cells to adhere to plastic. Inasmuch as incubation of SKBr3 cells with heRgulin is able to increase adhesion, activation of breast cancer cells that overexpress p185cerbB2 could potentially contribute to the poor prognosis associated with overexpression of p185cerbB2. Surprisingly, despite a similar effect on anchorage-independent growth, the ability of heRgulin and ID5 to increase adhesion to plastic was dissociated, with heRgulin increasing adhesion and ID5 having minimal effects on adhesion to plastic.

**Increased Expression of CD44 and CD54.** A number of different cell surface receptors have been demonstrated to increase adhesiveness both *in vivo* and *in vitro*. Both CD44 and CD54 are expressed by breast cancer cells and could contribute to increased adhesiveness of SKBr3 cells to plastic following incubation with heRgulin. Indeed, incubation of SKBr3 cells with 10 ng/ml heRgulin for 2 or 5 days significantly increased the expression of CD44 and CD54 both with regard to the percentage of fluorescent cells and the mean fluorescence intensity (Table 1). As little as 1 ng/ml heRgulin was sufficient to increase expression of both CD44 and CD54 (data not shown). Interestingly, although the ID5 antibody did not alter adhesion to plastic, incubation with the ID5 antibody (10 μg/ml) increased the expression of CD44 but failed to affect expression of CD54 (Table 2). Thus, increased expression of CD44 is not sufficient to mediate adhesion of SKBr3 cancer cells to plastic. Although the effects of the ID5 antibody and heRgulin on expression of CD44 were concordant, the effects of the ID5 antibody and heRgulin on expression of CD54 were discordant.

**Increased Invasion of Matrigel Membranes.** Invasion of cancer cells into artificial membranes is often an effective *in vitro* correlate of invasiveness *in vivo*. A 16-h incubation of SKBr3 cells with heRgulin (10 ng/ml) induced an approximately 5-fold increase in the number of cells invading into Matrigel membranes (Fig. 3). In a separate experiment, heRgulin induced a dose-dependent stimulation of invasiveness over the range of 10 ng/ml to 1 ng/ml (data not presented). The ID5 anti-p185cerbB2 antibody also significantly enhanced the ability of SKBr3 tumor cells to invade Matrigel membranes. Invasion of Matrigel by SKBr3 cells in the presence of ID5 was significantly greater than that observed in the presence of TA1 or MOPC21 (P < 0.01; Student's *t* test). Similar to the concordant effects on anchorage-independent cell growth, heRgulin and ID5 both increased invasiveness.

**Anti-CD44 or Anti-CD54 Antibodies Inhibit Invasion.** Because both heRgulin and ID5 induced invasion into Matrigel and invasion requires an initial adhesion step perhaps mediated by the adhesion molecules induced by heRgulin and ID5, we assessed whether heRgulin-induced or ID5-induced invasion required an interaction of CD44 or CD54 on the surface of SKBr3 cells with coreceptor molecules present in the Matrigel membranes. It is important to note that although both heRgulin and ID5 increased expression of the CD44 and heRgulin increased expression of CD54, untreated SKBr3 still expressed readily detectable levels of these adhesion molecules. HeRgulin-induced invasion of Matrigel membranes was inhibited approximately 50% by both anti-CD44 or anti-CD54 (μg/ml) antibodies, consistent with the hypothesis that heRgulin-induced expression of CD44 and CD54 plays a role in the increased invasiveness induced by heRgulin (Fig. 4).

**Increased Expression of MMP-9.** Increased expression of proteases capable of degrading the extracellular matrix is a critical component of the invasion and metastasis cascade. Compatible with the observation that heRgulin increased invasion into Matrigel membranes, incubation of SKBr3 breast cancer cells with heRgulin (1 or 10 ng/ml) markedly increased the expression of 92 kD MMP-9 (Fig. 5). Despite inducing increased invasiveness into Matrigel membranes, incubation of SKBr3 cells with ID5 failed to
increase MMP-9 levels (Fig. 5). ID5 may, however, induce expression of other proteinases not detected by gelatin zymography as performed herein. In addition, PA activity was not detectable in control or treated samples using either zymography or a spectrophotometric assay (data not shown).

**DISCUSSION**

In other studies, we have shown that both heregulin and the ID5 antibody, but not the TA1 antibody, increase autophosphorylation of p185\(^{c-erbB2}\), concomitant with growth inhibition of cells that overexpress p185\(^{c-erbB2}\) (23). The ID5 and TA1 antibodies bind to rodent cells that express human p185\(^{c-erbB2}\) but not to cells engineered to overexpress human epidermal growth factor receptor, c-erbB3 or c-erbB4, demonstrating the specificity of ID5 and TA1 for p185\(^{c-erbB2}\) (data not shown). Thus, ID5 but not TA1 represents an agonistic antibody able to stimulate directly p185\(^{c-erbB2}\). Heregulin, although cloned initially as a ligand for p185\(^{c-erbB2}\), likely does not bind to p185\(^{c-erbB2}\) alone but rather activates p185\(^{c-erbB2}\) through formation of a ternary complex with erbB3 or erbB4, both of which are expressed on SKBr3 cells (28, 29). Strikingly, both heregulin and agonistic antibodies to p185\(^{c-erbB2}\) inhibited the anchorage-independent growth of SKBr3 cells. Indeed, we were unable to identify a concentration of heregulin or ID5 that increased the growth of SKBr3 in semisolid agar. Thus, it is difficult to equate the poor prognosis associated with overexpression of p185\(^{c-erbB2}\) observed in several different studies of breast and ovarian cancer with increased proliferative rates.

Strikingly, heregulin increased a number of in vitro correlates of increased invasiveness and metastatic potential, including adhesion to plastic, increased expression of the CD44 and CD54 adhesion molecules, and increased production of the protease MMP-9. ID5, despite inducing similar decreases in anchorage-independent growth, invasion into Matrigel membranes, and expression of CD44 as did heregulin, failed to induce increases in adhesion to plastic, expression of CD54, and production of MMP-9. Thus, the agonistic ID5 antibody mimics some but not all aspects of activation of SKBr3 cells with heregulin.

Overexpression of p185\(^{c-erbB2}\) by cancer cells has been associated with a poor prognosis in some but not all studies of breast and ovarian cancer (5, 6). Prognosis, however, may not relate simply to the proliferative capacity of cancer cells but could also depend on the ability of cancer cells to invade, metastasize, or resist cytotoxic chemotherapy. Data presented in this communication suggest that heregulin can, in fact, enhance the adhesive and invasive potential of tumor cells that overexpress p185\(^{c-erbB2}\). Molecular correlates of this increased adhesive and invasive potential included up-regulation of the adhesion molecules CD44 and CD54 as well as increased secretion of the 92 Kd collagenase MMP-9. The functional importance of CD44 and CD54 in this setting was suggested by inhibition of heregulin-stimulated invasion by anti-CD44 and anti-CD54 antibodies. The anti-p185\(^{c-erbB2}\) antibody ID5 also increased invasive potential and up-regulated CD44 but failed to augment MMP-9 secretion, consistent with the potential role of other proteases in the invasion of Matrigel membranes.
The ID5 anti-p185<sub>erbB2</sub> antibody stimulated invasion of Ma- 
trigel by SKBr3 breast cancer cells. The TA1 anti-
p185<sub>erbB2</sub> antibody and the MOPC21 control antibody were sig-
nificantly less active. As p185<sub>erbB2</sub> is currently being assessed as 
a target for serotherapy with monoclonal antibodies (27), serother-
apy with antibodies that have little impact on invasiveness and 
potentially lack other agonistic activities may maximize their ther-
apeutic activity. In this regard, the TA1 antibody that fails to affect 
tumor growth when unconjugated serves as an optimal carrier for 
the A chain of ricin when used to prepare immunotoxins (27). The 
TA1-RTA immunotoxin can eliminate more than 99.99% of clo-
nogenic SKBr3 cells that overexpress p185<sub>erbB2</sub>, but have little 
activity against normal cells with lower levels of p185<sub>erbB2</sub> (27). 
Consequently, such immunotoxins may have a favorable therapeu-
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