Abstract

The HER2/neu oncogene is overexpressed in a significant fraction of human tumors; such overexpression is thought to play a role in the aberrant proliferation of cancer cells. The effects of HER2/neu-specific phosphorothioate antisense oligodeoxyribonucleotides on HER2/neu expression, tumor cell proliferation, and activation of apoptotic cell death pathways have been examined. Antisense treatment down-regulates HER2/neu expression in a dose-dependent and sequence-specific manner. HER2/neu antisense treatment specifically inhibits the growth of tumor lines that overexpress HER2/neu, but it has little effect on the growth of tumor cells that express low levels of HER2/neu. Down-regulation of HER2/neu expression is not only cytostatic, but it also results in the activation of apoptotic cell death pathways in cells that overexpress HER2/neu. These results suggest that, in addition to stimulating tumor cell proliferation, HER2/neu overexpression in cancer cells acts as an antiapoptotic survival factor.

Introduction

The HER2/neu gene, also called erbB2, encodes a 185,000 glycoprotein with intrinsic tyrosine kinase activity, p185 (1–3). The HER2/neu-encoded p185 molecule occupies a critical position in the biochemical pathways responsible for the transduction of mitogenic signals from a variety of growth factor receptors (4, 5). In addition to its role in regulating normal cellular proliferation, overexpression of HER2/neu-encoded p185 molecule occupies a critical position in the biochemical pathways responsible for the transduction of mitogenic signals from a variety of growth factor receptors (4, 5). In addition to its role in regulating normal cellular proliferation, overexpression of its role in regulating normal cellular proliferation, overexpression of HER2/neu overexpression in cancer cells acts as an antiapoptotic survival factor.

Oligodeoxyribonucleotide Treatment. Phosphorothioate ODNs targeting the 5′ region of the HER2/neu mRNA molecule were obtained from Oligo, Inc. (Wilsonville, OR), bases in bold font indicate substitutions: Antisense: CTCCATGGTCGTCCAC Sense: GTGAGCACCATGGAG Scrambled antisense: CGCCCTATCCGTAAC One-mismatch: CTCCATGCTGCTCAC Four-mismatch: CTACCTGCTGCTGAC The lyophilized ODNs were reconstituted in sterile distilled water to 1 mM, filter-sterilized, and stored in aliquots at −20°C as stock solutions. For subsequent experiments, the stock solutions of ODNs were diluted to give final concentration of 1 μM. Diluted ODNs were mixed with 10 μg/ml Lipofectin (Life Technologies, Inc., Gaithersburg, MD), and cells were exposed to the mixture for 4 h, after which the mixture-containing medium was replaced with culture medium.

Cell Growth Assay. One-ml aliquots containing 2 × 10^4 cells were plated into the wells of 24-well culture plates and were allowed to adhere for 24 h. After cells were firmly attached, they were washed once with prewarmed OPTMEM-I (Life Technologies, Inc.), and 10 μg/ml Lipofectin mixture with or without 1 μM antisense, sense, or scrambled antisense ODNs were applied to each well for 4 h at 37°C. After the 4-h incubation, the ODN mixture was

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replaced with regular serum containing culture medium, and the cells were incubated for an additional 5 days. At the indicated times, cells were detached from the wells with trypsin-EDTA and counted with a hemocytometer. Only viable cells based on trypan blue dye exclusion were counted. Results are the means of triplicate samples.

Flow-Cytometric Detection of Apoptosis. BT474 cells (5 × 10^5) were treated with 1.5 ml of ODN mixture in 60-mm culture dishes as described. After an additional incubation of 72 h, cells were detached from the culture dish and collected via centrifugation. Floating cells from the supernatant were combined with the trypsinized cells. Pooled cells were washed once with the growth medium and stained with Annexin V-FITC and propidium iodide (ApoAlert Annexin V Apoptosis Kit; Clontech), according to the manufacturer’s protocol. After cells were stained in the dark for 10 min at 25°C, their fluorescence was analyzed via flow cytometry using a FACScan (Becton Dickinson).

CPP32 Assay. Cells (1 × 10^6) were treated in 100-mm culture dishes with 5 ml of ODN mixture as described previously. After incubating for 72 h from the initiation of the treatment, cells were detached from the culture dish and washed once with PBS. Activation of CPP32 protease was determined by the ApoAlert CPP32 Assay kit (Clontech), according to the manufacturer’s protocol. Absorbance of the DEVD-pNA cleavage product pNA at 405 nm from each sample was converted to CPP32 units using the standard curve generated by the absorbance of known amount of pNA.

Results

HER2/neu Antisense ODNs Inhibit p185 Expression and BT474 Tumor Growth in a Dose-dependent Manner. Antisense ODNs represent a potent approach to inhibiting the expression of specific genes. However, biological effects that result from antisense ODN exposure may also occur via a variety of non-antisense mechanisms (22, 23). We and others have demonstrated the biological activity of HER2/neu-specific antisense ODNs targeted to the 5' region of the HER2/neu mRNA molecule (18–21). To further characterize the specificity of HER2/neu antisense effects on BT474 breast carcinoma...
growth, we have examined the dose-dependence of ODN effects on p185 expression and tumor cell growth.

As shown in Fig. 1A, treatment of BT474 breast carcinoma cells with HER2/neu-specific antisense ODNs results in dose-dependent inhibition of p185 expression. There is no effect of control sense or scrambled antisense ODNs on p185 expression. The biological effects of HER2/neu antisense ODN treatment on BT474 growth parallel the biochemical effects on p185 expression. As shown in Fig. 1B, HER2/neu-specific ODNs have modest but reproducible tumor inhibitory effects at concentrations as low as 30 nM, with more potent effects (>80% inhibition of cell growth) at 1 μM. In contrast, control sense and scrambled antisense sequences have only minimal nonspecific effects on cell growth at concentrations ≤ 1 μM. To avoid such nonspecific toxic effects, all subsequent experiments have been performed using a 1-μM treatment dose.

**Antisense Effects on HER2/neu Expression and Tumor Cell Proliferation Are Sequence Specific.** To demonstrate conclusively that the antiproliferative properties of the HER2/neu-specific antisense sequences studied here are due to Watson-Crick base pairing between the antisense molecule and its target mRNA, we examined the sequence-specificity of antisense effects. The ability of antisense ODNs and of modified antisense molecules containing one or four mismatched bases to down-regulate HER2/neu mRNA, inhibit expression of the p185 protein, and inhibit BT474 tumor cell proliferation were studied and compared to the effects of control ODNs representing sense or scrambled antisense (nine base mismatches). HER2/neu antisense treatment significantly inhibits the expression of the p185 protein after 48 h of treatment (Fig. 1C). There is little effect of control sense and scrambled antisense sequences. Examination of the effects of mismatch sequences demonstrates the stringent sequence-specificity of the antisense effect. There is marked diminution of the activity of the one-mismatch sequence in terms of its effect on p185 protein levels, whereas the four-mismatch antisense sequence has essentially no effect on p185 expression.

The effects of these sequences on p185 protein levels correlate with their effects on BT474 tumor cell proliferation. As shown in Fig. 1D, antisense ODNs inhibit BT474 cell growth by >80%, whereas control ODNs have only a modest effect on cell growth, confirming earlier results. The one-base mismatch antisense ODN has a markedly less potent effect on BT474 cell growth, and the four-base mismatch sequence has an effect little different from those of sense and scramble control ODNs. Thus, the effects of antisense molecules on p185 expression and on tumor cell proliferation appear to be critically dependent on sequence complementarity with the HER2/neu mRNA molecule.

**HER2/neu Antisense Treatment Results in Cell Death and Cell Surface Changes Associated with Apoptosis.** It was observed that BT474 cell culture dishes treated with HER2/neu antisense ODNs not only contained fewer viable tumor cells, but also contained an increased fraction of dead floating cells compared with cultures treated with either lipofectin alone or with control ODNs. It was hypothesized that some of the HER2/neu antisense effect on tumor cell growth was not simply the result of inhibition of tumor cell proliferation, but might actually be due to an antisense-mediated increase in apoptotic cell death. To address this question, the fractions of cells in various stages of apoptotic death were assessed by simultaneous Annexin V and propidium iodide staining, using flow cytometry, 72 h after treatment with antisense or control ODNs. A significant fraction of BT474 tumor cells treated with HER2/neu antisense ODNs were in the early (Fig. 2B, lower right quadrant) or late (Fig. 2B, upper right quadrant) stages of apoptotic cell death. In contrast, there was little effect of sense (Fig. 2C) or scrambled antisense (Fig. 2D) ODNs on apoptotic cell death, which was comparable to that seen in cells treated with lipofectin alone (Fig. 2A). Cytometric analysis also revealed that HER2/neu antisense-treated cells showed evidence of nuclear condensation and an increased fraction of nuclei containing a sub-2N DNA content, an indicator of apoptotic cell death (Table 1).

**HER2/neu Antisense Treatment Results in Activation of CPP32 and Degradation of Caspase Substrates.** In addition to changes in cell surface phosphatidyserine expression, which is the basis for Annexin V staining, cells undergoing apoptotic death typically activate proteases (caspases) that degrade specific intracellular proteins. As shown in Fig. 3A, BT474 cells treated with HER2/neu-specific antisense ODNs demonstrated activation of the caspase CPP32, also known as caspase 3, whereas cells treated with control ODNs did not. Moreover, antisense-treated BT474 cells showed evidence of degradation of the proteins PARP and cyclin B, which are substrates of CPP32 and other proteases activated in apoptotic cells (Fig. 3B). Thus, by multiple criteria, it was demonstrated that HER2/neu antisense ODN treatment resulted in activation of apoptotic cell death pathways in BT474 breast carcinoma cell lines.

**HER2/neu Antisense Treatment Inhibits Cancer Cell Growth and Activates CPP32 in Cancer Cells of Varied Histological Origin That Overexpress p185.** Prior studies of HER2/neu down-regulation, using HER2/neu-specific monoclonal antibodies, have demonstrated that the growth of some tumor cell lines that over-expression of HER2/neu.

| Table 1 Effects of HER2/neu antisense ODNs on nuclear DNA content |
|---------------------------------|---|---|---|---|
| BT474 cell treatment<sup>a</sup> | Lipofectin | Antisense | Sense | Scrambled antisense |
| Dead cells with DNA content < 2N | 2% | 18% | 2% | 2% |

<sup>a</sup> DNA content determined 72 h after ODN treatment by flow cytometry.
express p185 can be inhibited by antibody exposure, whereas there is little effect of such treatment on tumors that express more modest levels of p185 (9, 24). To further define the effects of HER2/neu-specific antisense ODNs, we examined the effects of antisense treatment on tumor cell growth and activation of CPP32 in a panel of tumor cell lines of distinct histological origins that express varying levels of p185 (Fig. 4A).

The growth of several different tumor lines that overexpress p185 was inhibited by antisense treatment, whereas there was little effect on the growth of tumor cell lines, as well as a nontransformed diploid fibroblast line, that express more modest levels of p185 (Fig. 4B). The effects on cell growth mirrored the effects of antisense treatment in triggering apoptosis as demonstrated by CPP32 activation (Fig. 4C). These findings demonstrate that a variety of tumor cell types are sensitive to the pro-apoptotic effects resulting from antisense-mediated down-regulation of HER2/neu expression. These results also suggest that the growth inhibitory effects of HER2/neu-specific antisense ODNs result in part from triggering apoptotic cell death in cells that overexpress HER2/neu.

It is of interest that the CFPAC cell line, which only moderately overexpresses p185, was quite sensitive to the effects of HER2/neu down-regulation, whereas the MCF-7 cell line, which expresses somewhat lower levels of p185, was not affected by antisense ODN exposure. Examination of the sensitivity of these cell lines to growth inhibition after p185 down-regulation by a distinct mechanism, using p185-specific monoclonal antibodies, confirmed their relative sensitivity and resistance to HER2/neu-directed therapy.4 This may reflect a threshold level of p185 overexpression required for sensitivity to HER2/neu down-regulation.

Discussion

The effects of a 15-base antisense ODN molecule, targeted against the 5’ region of the HER2/neu ODN, on HER2/neu expression, tumor cell proliferation, and apoptotic tumor cell death were examined. It was demonstrated that such HER2/neu-directed antisense ODNs exert sequence-specific effects on HER2/neu expression and tumor cell growth. Mismatched antisense ODNs altered at a single base are markedly less effective at down-regulating HER2/neu expression and inhibiting BT474 tumor cell growth; sequences containing four or more mismatches have minimal effects on either HER2/neu expression or tumor cell growth. The biological effects of HER2/neu-specific antisense ODNs are dose-dependent, occurring 2–3 logs below the concentrations at which nonspecific effects occur. Collectively, these results strongly support the view that antisense-mediated down-regulation of HER2/neu expression results in inhibition of BT474 tumor cell proliferation.

A number of reports of antisense effects on tumor cell proliferation have been published; in several cases it was subsequently demonstrated that non-antisense mechanisms accounted for the tumor inhibitory effects (22, 23, 25, 26). ODNs can bind growth factors (23) and their receptors in sequence non-specific fashion (25). They may also bind other cellular proteins, resulting in aptameric effects (26). The results presented here are unlikely to be due to such non-antisense mechanisms for a number of reasons, including avoidance of sequences known to cause non-antisense effects (23, 25, 26); the use of relatively short (15 base) ODNs at concentrations of 1 μM and below; demonstration of parallel effects of antisense compounds on protein expression as well as tumor cell growth; and the use of multiple control ODNs and tumor targets that define the effect in terms of antisense sequence and target cell susceptibility.

It is interesting to compare the results presented here with prior studies of HER2/neu down-regulation using monoclonal antibodies or antisense ODNs. Monoclonal antibodies specific for p185 have been demonstrated to have inhibitory effects on the growth of some tumor lines in cell culture (9), including several of the lines tested here. In general, these antibodies appear to be exclusively active in inhibiting the growth of cancer cells that strongly overexpress HER2/neu, sug-

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*Rh et al., unpublished results.*
Antisense compounds directed against the HER2/neu oncogene have also been shown to inhibit the growth of some breast carcinoma and ovarian carcinoma cell lines (18–21). These agents also appear to be most effective against tumor cells that overexpress HER2/neu. We have demonstrated that other tumor cell types, including lung and pancreatic adenocarcinoma cell lines, can be inhibited by HER2/neu-specific antisense ODNs. Preliminary data from our laboratory suggest that antisense ODNs directed against HER2/neu sequences are superior to monoclonal antibodies specific for p185 at both down-regulating HER2/neu expression and inhibiting the in vitro growth of tumors that overexpress HER2/neu. Whether HER2/neu antisense ODNs are more potent than p185-specific monoclonal antibodies in vivo, and whether the combination of such agents can result in enhanced antitumor effects will require further study.

Perhaps the most intriguing finding presented here is the observation that antisense-mediated down-regulation of HER2/neu expression in tumor cell lines that overexpress HER2/neu is not simply cytostatic, but actually results in activation of apoptotic cell death mechanisms. Standard monoclonal antibodies reactive with p185 have not been shown to activate cell death pathways in tumor cells that overexpress HER2/neu (9, 12). However, multimeric antibodies directed against p185, as well as intracellular expression of a single-chain p185-specific antibody, have been shown to activate apoptotic cell death pathways in tumor cells that overexpress HER2/neu (16, 27). These prior reports suggested that factors specific to the altered anti-p185 antibodies, rather than the result of interfering with p185 expression, were critical in inducing apoptosis (16, 27). The studies presented here demonstrate that down-regulation of p185 by a non-antibody mechanism, using antisense ODNs, can also trigger cell death in cancer cells that overexpress the p185 protein. Thus, cancer cells that overexpress HER2/neu are dependent on p185 for cell survival as well as proliferation, and strategies that interfere with p185 expression or function can induce apoptotic cell death.

The molecular pathways by which HER2/neu overexpression interacts with cell death/survival signaling have not been defined. Several of the cell lines studied here, including BT474, have known p53 mutations (28). Thus, the effects of HER2/neu-specific antisense ODNs inactivating apoptosis must be p53-independent. It has been demonstrated previously that increased HER2/neu expression resulting from gene transfection can increase Bcl-2 levels (29). Furthermore, p185 physically associates with FAS ligand on tumor cell surfaces and may interfere with FAS signaling by this mechanism (30). Thus, it is possible that modulation of Bcl-2 and/or FAS ligand function links HER2/neu overexpression to cell survival signaling. Regardless of the underlying molecular pathways involved, the triggering of apoptotic cell death following p185 down-regulation may prove a useful approach to the therapy of tumors that overexpress HER2/neu.

HER2/neu overexpression has also been shown to play a role in resistance to the lethal effects of tumor necrosis factor (24) and chemotherapy (31, 32). Because these both are activators of apoptotic cell death, this may provide yet another example in which the level of HER2/neu expression contributes to altering the balance between tumor cell survival and cell death signals. We have demonstrated that antisense-mediated down-regulation of HER2/neu expression can, by itself, shift this balance in favor of tumor destruction. Antisense compounds are beginning to enter clinical trials in patients with cancer and other diseases (33–35). The ability of HER2/neu antisense ODNs to inhibit tumor growth, using in vivo xenograft models, and to potentiate the cytotoxic effects of other modalities, such as chemotherapy, currently is under investigation (36).

Fig. 4. HER2/neu antisense ODNs inhibit tumor growth and activate apoptosis in tumor cells of various histological origins which overexpress p185. A, Western blot demonstrating relative p185 expression in diverse cell lines (20 μg protein/lane). B, HER2/neu antisense ODNs (filled columns) inhibit the growth of tumor cells that overexpress p185. Sense (open columns) and scrambled antisense (stippled columns) ODNs have little or no effect. C, HER2/neu antisense ODNs induce apoptosis in tumor cells that overexpress p185. Cells were treated with Lipofectin alone (striped columns), HER2/neu antisense ODNs (filled columns), sense ODNs (open columns), and scrambled antisense ODNs (stippled columns), and assayed for CPP32 activation after 72 h.

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Down-Regulation of HER2/neu Expression Induces Apoptosis in Human Cancer Cells That Overexpress HER2/neu

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