Reduction of erbB2 gene product in mamma carcinoma cell lines by erbB2 mRNA-specific and tyrosine kinase consensus phosphorothioate antisense oligonucleotides

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A considerable reduction of up to 75% at the protein level of the erbB2 gene product was observed using phosphorothioate antisense oligonucleotides directed against specific sequences of the erbB2 mRNA. Antisense oligonucleotides used were the 14-mer (translation start region) and the 17-mer (3' translated region) all-phosphorothioate oligonucleotides (S-ODNs). Sense or random sequences were used as a control. The greatest reduction at the erbB2 protein level was obtained after a 24h incubation with a single dose of 2 µM antisense S-ODN. The erbB2-mRNA-specific and the tyrosine kinase consensus antisense S-ODNs were comparably effective in inhibiting erbB2 expression. Addition of the transfection reagent DOTAP diminished the efficiency of erbB2 protein reduction by antisense S-ODNs after an incubation period of 24h but was more effective after 48h compared to the application of antisense S-ODNs alone.

The erbB2 gene product is a receptor type tyrosine kinase (1, 2). Its amplification and overexpression in several types of human adenocarcinomas, especially in tumors of the breast and the ovary is correlated with a short time to relapse and a poor prognosis of cancer patients (3). In human cancer patients there is predominantly an amplification and overexpression of the erbB2 gene (3, 4) contributing to the uncontrolled proliferation of these tumor cells. An amplification of the human neu oncogene was reported in 16 of 95 samples (17%) or 63 of 189 samples (30%), respectively (5, 3), resulting in an overexpression of the erbB2 mRNA and the erbB2 gene product (p185). These findings were confirmed by recent investigations (6, 7). Elevated expression of the erbB2 gene product in mamma and ovarian carcinomas can be correlated to most known risk factors (large tumor size, lymph node positivity, absence of steroid receptors) and to a high rate of proliferation (3, 6). Therefore it is a tempting approach to inhibit specifically erbB2 expression or function to reduce the erbB2 related cellular malfunctions. Specific inhibition of erbB2 function could be achieved by the administration of monoclonal antibodies (8) either alone or conjugated with drugs or toxins (4). Another, potentially more specific approach was the aim of this study. As a target for the reduction of erbB2 expression we have chosen the erbB2 mRNA using antisense technology (9); preliminary results have been published (10).

MATERIALS AND METHODS

S-ODN1: 5` CAT GGT GCT CAC TG 3`; control S-ODN1: 5` GTG CCT GTA CGT AC 3`; S-ODN2: 5` CCA (AT)AG GAC CA(GC) AC(AG) TC 3`; control S-ODN2: 5` ACT ACG AAC GAG CCA CA 3`
S-ODN2 contains three wobble-positions (X/Y) according to the tyrosine kinase consensus sequence published by Wilks (11).

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Human mamma carcinoma cell lines SKBR-3 and MCF-7 (ATCC HTB 30 and HTB 22, respectively) were used throughout this study. Cells were grown in CLICKS/RPMI (Gibco), 100U/ml penicillin, 100 µg/ml streptomycin, supplemented with 10% fetal calf serum (FCS). For the antisense experiments cells were harvested and washed 3 times with the above mentioned medium without FCS to remove residual serum, adjusted to 5 x 10^4/ml and seeded to microtiter plates at a final volume of 100 µl/well. S-ODNs (synthezized either by two of us - W. and K.-H. - or by BIOMETRA GmbH, Göttingen) were added to the desired final concentration (0,2; 2 or 5 µM) in a total volume of 10 µl of medium without FCS. Only a single dose of antisense S-ODN or the respective control S-ODN was added immediately after cells had been seeded to the microtiter plates. After a 12h incubation period in the absence of FCS, serum was added to a final concentration of 10%.

At the end of the incubation ranging from 8 to 72h after the addition of FCS, cells from 6 wells (containing antisense or control S-ODN) were harvested, combined and the total protein concentration was measured using the LOWRY protocol (Sigma) as recommended by the manufacturer.

For the quantitative determination of the erbB2 protein a commercially available ELISA system (DIANOVA, FRG) was used following the instructions of the manufacturer with the following modification. The protein amount used in the ELISA system was adapted to the content of erbB2 in relation to the total protein amount extracted from SKBR-3 or MCF-7 cells, respectively. Thus 5-20 ng total protein from cell line SKBR-3 and 5 µg total protein from cell line MCF-7 were used for each ELISA. At least 3 independent assays were run for each modification.

The transfection reagent used was DOTAP (N-[1-(2,3-Dioleoyloxy) propyl] -N,N,N trimethylammoniummethylsulfate) obtained from Boehringer Mannheim. DOTAP (10 µl/ml medium) was incubated with the appropriate amount of oligonucleotide to achieve the respective final concentration of the oligonucleotide in the cell suspension (e.g. 0,2; 2 or 5 µM) for 10 min at room temperature. Then the mixture was added directly to the cells on the microtiter plates and incubated together for the desired time period (8, 24, 48 or 72h). There was no cytotoxicity of the transfection reagent as controlled by trypan blue dye exclusion assay.

Immunohistological staining was performed using a monoclonal antibody against the internal domain of the erbB2 gene product p185 (MEDAC, FRG) as primary antibody. Rabbit anti mouse antibody and the APAAP complex (DAKO) were used according to the manufactorers instructions with the following modifications: Cells transfered to glass slides by cytocentrifugation were fixed in 90% acetone/10% phosphate-buffered saline for 5 min, then by 100% acetone for 5 min at 4 °C. A blocking buffer of heat-aggregated human immunoglobulin (1mg/ml, 0,01% Na-azid) was used after heat inactivation at 63 °C for 20 min without a prior centrifugation step. Experiments with the same immunoglobulin subtype as detector antibody were run as controls.

RESULTS

Two different regions of the erbB2 mRNA were chosen as targets; the translation start region (S-ODN1, 14mer all-phophorothioate oligonucleotide-PTO, GC content 60%) which has proven to be a good object for antisense strategy in different systems (12) and a region (nucleotides 2884-3000) at the 3' translated part of the erbB2 mRNA (S-ODN2, 17mer PTO, GC content 65%) which was chosen because of its high homology to a tyrosine kinase consensus sequence (11). A S-ODN with a randomized sequence of the same GC content as S-ODN1 or S-ODN2, respectively were designated as control-S-ODN1 and control S-ODN2.

It was the aim of this study to compare the efficiency of antisense oligonucleotides directed against these two target regions (translation start versus 3' translated region) in the inhibition of erbB2 expression. The 3' translated sequence was considered to be an inferior target for the antisense S-ODNs because a ribosome running along the message just before finishing its "job" may spill off the ODN without any effect. On the other hand the translation initiation complex might represent a more sensitive region for inhibtion by mRNA/ODN hybrids.

Secondly, the efficiency of a consensus ODN directed against a highly conserved region of the catalytic domain of the tyrosine kinase protein family in inhibiting the expression of a specific gene product, e.g. the erbB2 protein was investigated. Experiments were performed as depicted in MATERIALS AND METHODS using two mamma carcinoma cell lines (SKBR-3 and MCF-7). The effect of S-ODNs was monitored at the protein level using an ELISA test system.

Different incubation times (8, 24, 48 or 72h, in addition to the serum-free incubation period) and varying concentrations (0,2; 2 and 5 µM) of oligonucleotides were used. The most efficient reduction (75%, Figure1A) of erbB2 expression was obtained after a 24h incubation of SKBR-3 cells in the presence of S-ODN1 or S-ODN2 at

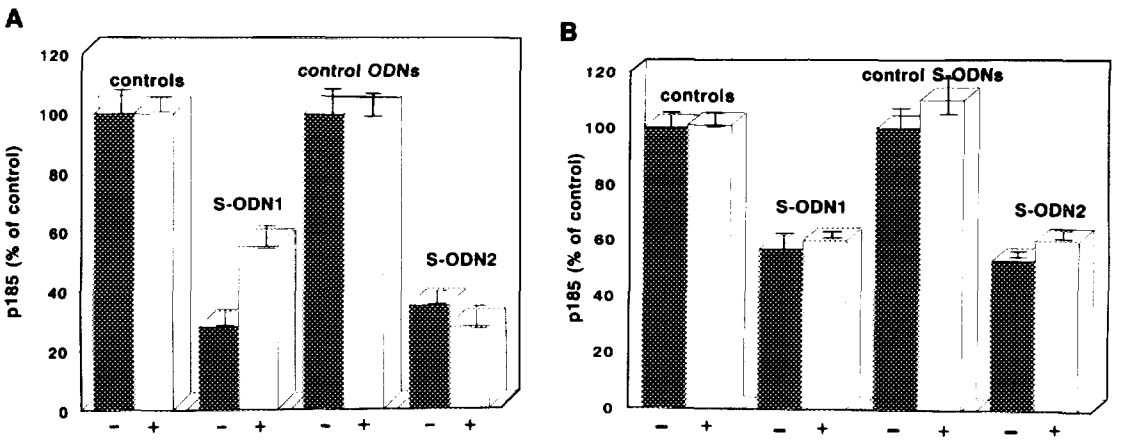

FIGURE 1. Modulation of p185 protein (erbB2 gene product) level by S-ODNs. **A.** Mamma carcinoma cell line SKBR-3. **B.** Mamma carcinoma cell line MCF-7. Cells were incubated for 24h in the presence of 2 µM S-ODN (antisense [S-ODN-1 or S-ODN2] or control sequences, see "Materials and Methods") in the presence or absence of the transfection reagent DOTAP (+/-). Control experiments were run in the absence of phosphorothioate oligonucleotides. Protein extracts were subjected to an ELISA-based quantitation of the p185 protein as described in "Materials and Methods". Error bars indicate percentage of control +/- SD (n=3).

a final concentration of 2 µM. For cell line MCF-7, with a low level of erbB2 mRNA expression compared to SKBR-3, the reduction was in the range of 50% (Figure 1B). S-ODN1 and S-ODN2 were almost equally efficient in inhibiting erbB2 expression. Specificity of antisense oligonucleotides was proved by randomized control sequences. Addition of the transfection reagent DOTAP reduced the inhibition of erbB2 expression after 24h in the highly overexpressing cell line SKBR-3, whereas the effect in cell line MCF-7 was negligible. After an incubation period of 48h (Figure 2A and 2B) however, a reduction in the amount of erbB2 protein of around 20% was observed in both cell lines (SKBR-3 and MCF-7) only in the presence of the transfection reagent (in addition

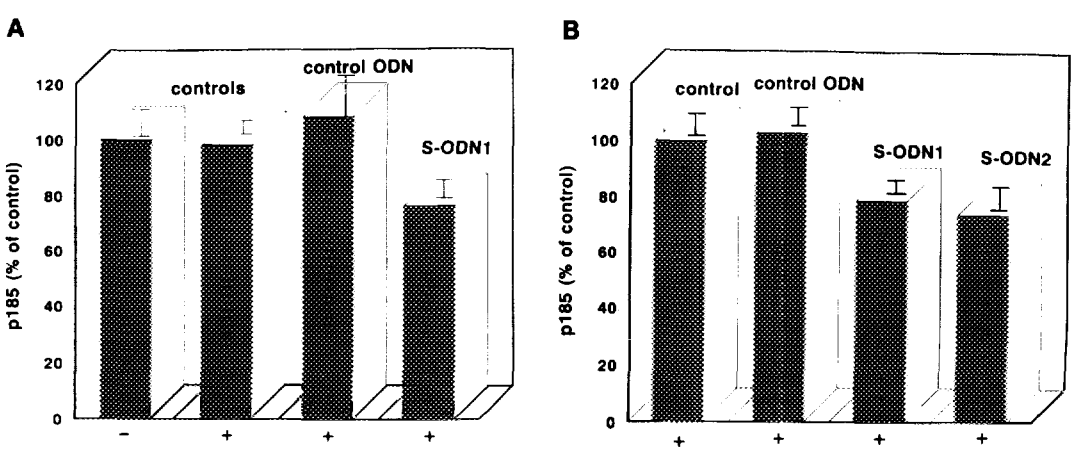

FIGURE 2. Effect of incubation time on S-ODN mediated modulation of p185 protein levels. **A.** Mamma carcinoma cell line SKBR-3. **B.** Mamma carcinoma cell line MCF-7. After a single dose of S-ODN (2 µM) cells were grown for 48h before quantitation of p185 protein level by an ELISA. Assays were run in the presence of DOTAP; in the absence of DOTAP there was no reduction in erbB2 gene product after 48h (data not shown). Data are expressed as mean +/- SD of percentage of control (n=3).

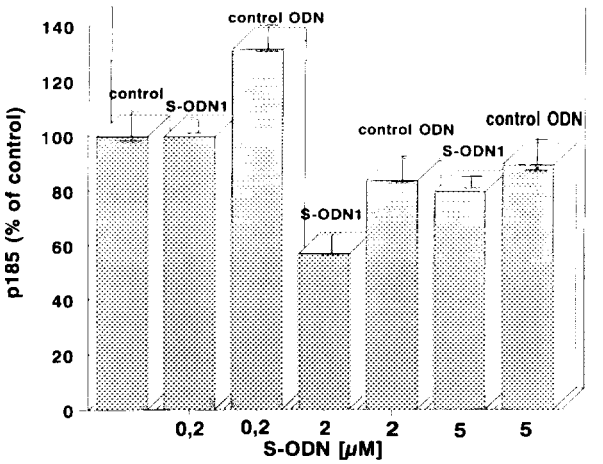

FIGURE 3. S-ODN concentration dependence of p185 protein expression. MCF-7 cells were incubated for 8h (after the addition of FCS [see "Materials and Methods"]) in the presence of varying concentrations of S-ODNs. p185 protein level was determined using an ELISA. The resulting values are expressed as the percentages +/- SD (n=3).

to the antisense S-ODN). Even after 72h this reduction in the amount of erbB2 protein (around 20%) was still present (data not shown).

Concentration dependent (0,2; 2 and 5 μM) inhibition of erbB2 expression was studied after a 24h exposure to the S-ODNs because a max. effect was observed after this time. As shown in Figure 3 there is a concentration dependent effect in the inhibition of erbB2 expression. In our hands maximal reduction of erbB2 protein levels was observed at an S-ODN concentration of 2 μM. 5 μM S-ODN was less effective and there was only a little (after 24h) to no effect (8h) using 0,2 μM S-ODN.

We immunocytologically stained S-ODN treated SKBR-3 cells for p185 protein, the erbB2 gene product, to evaluate if the antisense S-ODN effects are also visible at the single-cell level. SKBR-3 cells were treated with S-ODN1 under conditions of maximal erbB2 protein reduction as determined in the ELISA experiments (24h, 2 μM S-ODN1). The result is shown in Figure 4 demonstrating a considerable reduction in protein p185 by S-ODN1 compared to control-S-ODN1. At the moment we are establishing a FACS protocol for an in situ more rapid quantitative measurement of erbB2 gene product modulation using antisense technology.

DISCUSSION

Successful antisense approaches to specifically inhibit very different cancer related genes such as IGF1 (13), c-myc (14), bcl2 (15) or c-myb (16) have recently been reported.

The results of the present investigation clearly demonstrate the capability of antisense phosphorothioate oligonucleotides to reduce the expression of the erbB2 gene product p185. Moreover, the effects of the S-ODN were concentration and sequence dependent (Figure 1A, 1B and Figure 3). in the case of S-ODN1; specificity for the targeted gene was demonstrated in comparison to control sequences. The effect of S-ODN2 (consensus sequence against tyrosine kinases) on other tyrosine kinases beside the erbB2 gene will be the subject of further investigations.

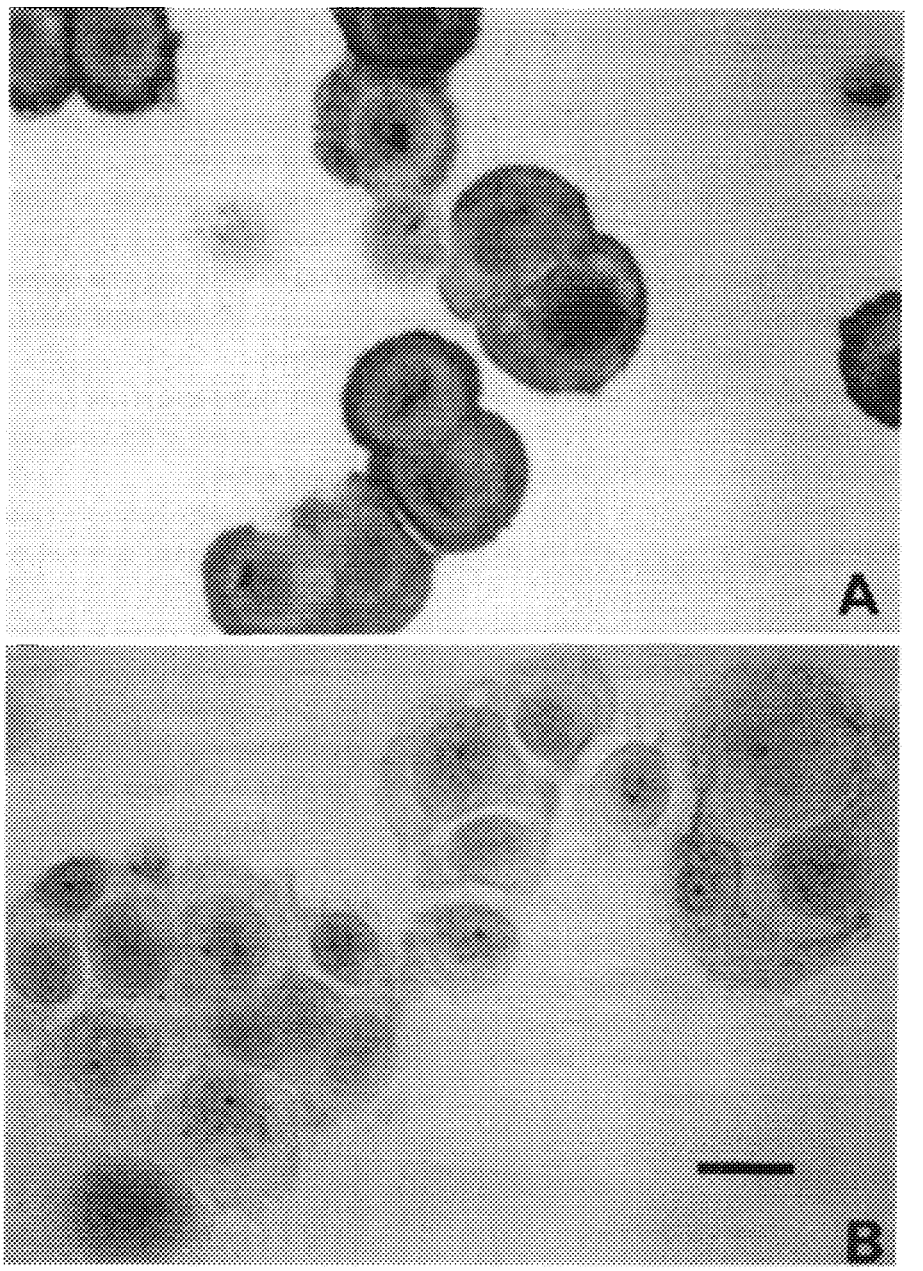

FIGURE 4. Immunocytological staining (for further details refer to "Materials and Methods") of p185 protein (monoclonal antibody directed against the cytoplasmic region of the protein; MEDAC, FRG). Cells were incubated for 24h in the presence of 2 μM S-ODNs. Bar indicates 10 μM.
A. Control-S-ODN1.
B. Antisense S-ODN1.

Although there are several potential mechanisms that could account for the reduction in erbB2 expression, the most likely is that the antisense ODN specifically binds to the targeted complementary sequences of the erbB2 message and that the resulting duplex formation leads to a specific decrease of erbB2 mRNA translation.

Whether or not there is also an RNase H dependent reduction of the stady state level of the erbB2 mRNA, as shown for some systems (17), has not yet been investigated.

Our results demonstrate, that the tyrosine kinase consensus sequence we selected according to the paper of Wilks (11) is as effective as the translation start antisense sequence. In preliminary experiments, this consensus sequence S-ODN has also been applied to the bcr/abl system (cell line K562) and a PDGF receptor overexpressing primary cell culture leading to a reduction in the proliferation (data not shown) as monitored by proliferation assays (^{3}H-thymidine incorporation or MTT dye reduction). The combination of a gene specific antisense oligonucleotide with the type of tyrosine kinase consensus oligonucleotide used in this study could be a promissing approach for studying synergistic effects of antisense oligonucleotides which might result in a lower effective antisense oligonucleotide concentration to obtain the same efficiency.

This tyrosine kinase consensus oligonucleotide could eventually facilitate the screening of cancer cell lines for the role of known or uncharacterized tyrosine kinases in the proliferative capacity of these cells and/or the appearance of new phosphorylated proteins using an anti-phosphotyrosine antibody.

It should be interesting to look more carefully at the effects of erbB2 down regulation on known or postulated erbB2 related signal transduction pathways, not only at the expression of the erbB2 gene product or on proliferation alone.

After a short incubation time (24h) the effects of antisense oligonucleotides were diminished by the addition of DOTAP compared to antisense oligonucleotide alone. However, after a longer incubation period (48-72h) there is only a measurable erbB2 protein reduction in the presence of DOTAP. These observations could be explained as follows: for a short incubation period it is important that the oligonucleotide is present in a free form instead of transfection micelles to reach and interact specifically with the target mRNA. After a longer incubation period, however, the oligonucleotide micelles could act as an extracellular and also as an intracellular reservoir allowing for a prolonged protection and delivery of the S-ODN to the target.

A further improvement of this transfection reagent approach (18) is the incorporation of antisense oligonucleotides into liposomes (19). By encapsulation into liposomes the oligonucleotides can be further protected from degradation and liposomes with selected lipids or in combination with antibody driven drug targeting may result in enhanced effectivity and specificity of antisense oligonucleotides. Especially in locally recurrent and exulcerating mamma carcinoma a topical application of liposomal erbB2 antisense oligonucleotides can be envisaged.

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