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FOREWORD

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INTRODUCTION

Breast cancer tissue consists of a mixture of autonomously proliferating tumor cells and supportive normal tissue recruited by them. Generally speaking, the primary carcinogenic events lead to uncontrolled growth of transformed cells. The secondary development into breast cancer then induces and requires a network of growth signals between the tumor cells and the normal surrounding host tissue [1,2]. These growth signals are primarily processed at the cell membrane by specific receptors and activation of the receptor triggers a cascade of downstream events. Aberrations in this signal transduction cascade can lead to deregulated cellular proliferation, differentiation, and ultimately to tumorigenesis. The most frequently implicated receptors and growth factors in human cancers are members of the EGF receptor family and their ligands [3]. In addition to the EGF receptor (HER-1), this family includes HER-2 (neu/erbB-2), HER-3 (erbB-3), and the more recently discovered HER-4 (erbB-4) receptor [4-6]. These receptors share a common molecular architecture and are able to form heterodimers with each other [7]. Heterodimeric formation is probably the mechanism that allows ligands to increase tyrosine phosphorylation of the different receptors of the HER family [8]. So far known ligands include EGF, TGF- α , cripto-1, amphiregulin (all binding to EGFR) and heregulin (NDF) which binds to HER-3 and HER-4. Until now no HER-2 specific ligand has been found, although there are at least three distinct mechanisms to activate the transforming potential of HER-2. This includes a point mutation within the transmembrane domain, truncations of catalytic sequences at both the cytoplasmic and the extracellular domains, or overexpression of the structurally normal gene [8].

HER-2 gene amplification and receptor overexpression has been detected in several human adenocarcinomas (including breast, ovarian, lung and gastric) at a frequency between 20-40% [9-11]. The role of HER-2 as prognostic marker in breast cancer has been the subject of numerous clinical studies. So far the results from these studies vary considerably, but there is a general consensus indicating a correlation between HER-2 overexpression, axillary lymph node involvement and worse patient survival [11-14]. The poor patient outcome in HER-2 positive patients is associated with a correlation between HER-2 expression and development of resistance to chemotherapy with alkylating agents like cisplatin and doxorubicin [15-19]. On the other hand data from the CALGB 8869 study [20] indicate an altered dose response relationship of doxorubicin in HER-2 overexpressing patients. These patients benefited dramatically by increasing doses of doxorubicin, whereas increasing doses of doxorubicin had no effect in patients expressing low levels of HER-2.

Overview of the goals pursued under this award:

Our studies are aimed at elucidating the contribution of HER-2 to breast cancer growth and progression to hormone independence as well as the development of resistance to treatment with cytotoxic drugs, anti-hormones and anti-HER-2 antibodies. Ultimately we wish to develop this understanding into novel therapeutic strategies. As a major tool we have used gene specific targeting of HER-2 with hammerhead-ribozyme expression constructs, a technology which we have applied successfully in the targeting of the growth factor pleiotrophin [21] and a number of other genes [22-25]. We have studied under **goal 1**: whether downregulation of endogenous HER-2 expression by targeting with HER-2 ribozymes will affect the *in vitro* growth and HER-2 signal transduction pathways of HER-2 overexpressing cancer cells, **under goal 2**: to what extent downregulation of HER-2 expression affects the sensitivity of breast cancer cells to treatment with steroid hormones, **under goal 3**: the role of HER-2 overexpression in the development of resistance to treatment with cytotoxic drugs, **under goal 4**: whether

downregulation in tumors *in vivo* of endogenous HER-2 by targeting with tetracycline regulated HER-2 ribozyme constructs will affect tumor growth in animals and **under goal 5:** whether expression of a truncated HER-2/ECD is involved in the development of resistance to therapy with HER-2 antibodies such as muAb4D5.

Summary of major new findings during the past report cycle:

We had found previously (see the report from 1998) -much to our surprise- that estradiol-induced growth of tumor cells (MCF-7) is dependent on the presence of HER-2. Downmodulation of HER-2 in MCF-7 cells by ribozymes had abolished the ability of the cells to form colonies in soft agar in response to estradiol. Due to the potential impact of this finding on the understanding of the cross-talk between the estrogen pathway and HER-2-directed growth factor pathway, we focussed our research on a detailed analysis of this interaction.

The studies reported here mostly contribute to **goal 2** (Steroid hormones and HER-2) and set the stage for studies under **goal 4** (tumor growth *in vivo*).

Significance of the findings under goal 2:

We are currently preparing the data and conclusions in the present report on HER-2 / estrogen interdependence as a manuscript for publication. We discussed the data and obtained the opinion of a number of investigators established in the HER-2 / hormone field to extend into reasonable and informative experiments and can present a relatively complete analysis in this report.

[We discussed the data with Drs. Stuart Aaronson (NYU), Chris Benz (UCSF), Benita Katzenellenbogen (UIL), Neal Rosen (Sloan-Kettering), Jossi Yarden (Weizmann, Israel).]

It is planned to submit the manuscript containing these data in the fall of 1999 to one of the top-rated journals (Cell or Nature / Nature Medicine).

Findings under the other goals of this award (1,3 and 5) were discussed in the 1998 report.

BODY

Goal (2):

Background:

Hormones and growth factors define the capacity of human breast cancer to grow and metastasize. One of the essential requirements for the development of breast cancer are circulating steroid hormones and one of the most widely used drug therapies of breast cancer with the anti-estrogen tamoxifen is based on this fact. Furthermore, growth factor gene expression can supplement for hormone stimulation and thus contribute to hormone-independent cancer growth as well as to resistance to anti-hormone therapy (reviewed e.g. in [26]).

Work reported earlier:

We had demonstrated that HER-2 can be downregulated in MCF-7 cells using tetracycline-regulated HER-2-targeted ribozymes (see Fig. 3, page 17 of the 1998 report). In this particular system, we used isogenic MCF-7 cells in which the HER-2-targeted ribozymes can be turned on or off by addition of tetracycline to the growth media.

As reported also, estrogen receptor levels and functionality was not affected by the HER-2 downmodulation. Surprisingly, upon downregulation of HER-2, estrogen-mediated colony formation in soft agar of these cells is lost.

Work accomplished during the most recent award cycle:

1. Dependence of estrogen-induced colony formation and proliferation on HER-2.

In an initial series of studies, we tested whether the dependence of estrogen-induced colony formation of MCF-7 cells on the presence of HER-2 was affected by steroid hormones present in fetal-calf **serum**. The rationale for these experiments was that serum contains a number of (unknown) factors that may affect the sensitivity to hormones and growth factors and we thus repeated the experiments with charcoal treated ("**stripped**") **serum** from which steroid hormones have been removed. Figure 1 (top) shows that estradiol (10 nM) induces colony formation in soft agar of MCF-7 cells even if charcoal-stripped serum is used. Earlier studies were done with full serum and the effect of estrogen present in the serum were blocked by adding a hormone receptor antagonist (see earlier report 1998). Upon downmodulation of HER-2 by removal of tetracycline and thus turning the HER-2-targeted ribozyme on, estrogen loses its effect (Fig. 1, top).

A similar effect was observed in proliferation assays with the same cells: The data depicted in Figure 1 (bottom) show that estradiol (10 nM) stimulates proliferation of the cells. Upon reduction of the endogenous HER-2 by the tetracycline-regulated ribozyme, the estrogen effect is reduced significantly.

For both assays in Fig. 1, ANOVA (= analysis of variance) showed that there was no significant difference between the baseline values (no estradiol; $p > 0.05$). A significant effect of estradiol versus baseline was found under control levels of HER-2 (the ribozyme-off condition; $p < 0.01$). No significant effect of estradiol was found under the reduced levels of HER-2 (ribozyme-on condition; $p > 0.05$).

We conclude that the growth effects of estrogen in this model cell line of hormone-dependent breast cancer depend on an intact HER-2 pathway.

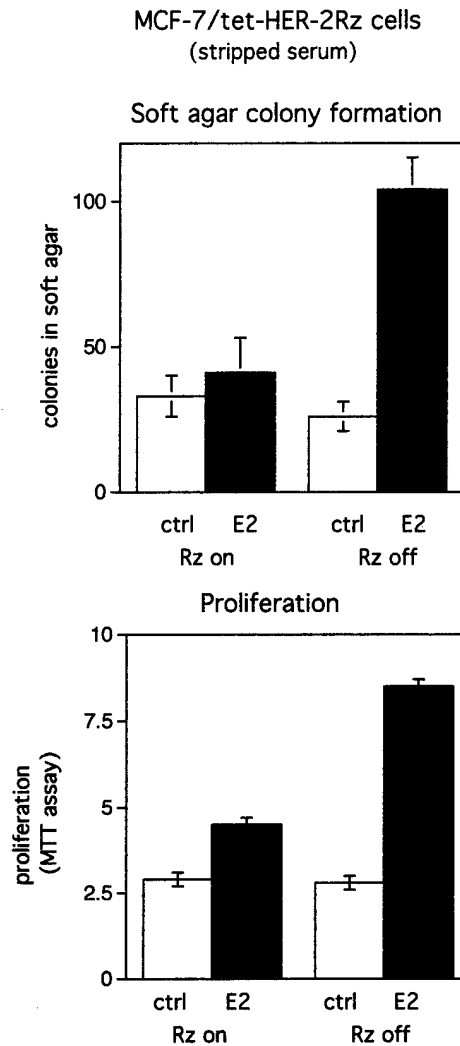


Fig. 1. Effect of HER-2 reduction by tetracycline-regulated ribozymes on estradiol-induced soft agar colony formation (*top*) and proliferation (*bottom*)

MCF-7/tetHER-2ribozyme cells as described in previous reports were used for the experiments. Soft agar colony formation and proliferation was measured after 12 days. The cells were grown in the absence or presence of tetracycline as described and without or with 10 nM estradiol. 10% charcoal-stripped fetal calf serum was included in the growth media. ANOVA analysis was performed to analyze differences between the groups and the results are given in the text. Mean $\pm$ SEM values are shown for each data point which was run in triplicate (*top*) or in sixuplicate (*bottom*).

2. Lack of cell cycle effects (FACS-analysis) of HER-2 regulation on estrogen-stimulated MCF-7 cells.

Next we tested whether the dependence of estrogen-induced growth of MCF-7 cells on the presence of HER-2 (see Fig. 1) was due to a block in cell cycle progression of the cells. For this we ran FACS analysis of the cells at different time points after estrogen addition (10 nM) in the presence or absence of tetracycline to keep the HER-2-targeted ribozyme off or on. Figure 2 compiles the data from this experimental series. Inspection of the FACS profiles showed no striking difference between the HER-2-reduced and non-reduced MCF-7 cells. A closer analysis by computer modelling gave us the different portion of cells being in different phases of the cell cycle. This data is shown in Figure 2. No significant effect of the HER-2 reduction was seen.

We conclude that reduction of HER-2 does NOT affect the progression of MCF-7 cells through the cell cycle and that blockade of cell cycle progression can not explain the effect on soft agar colony formation and cell proliferation.

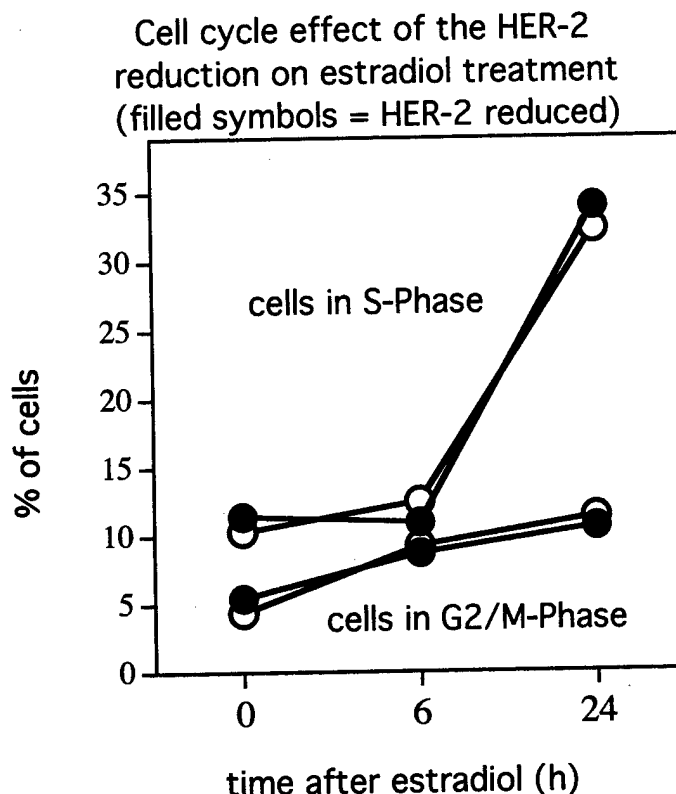


Fig. 2. Effect of HER-2 reduction by tetracycline-regulated ribozymes on cell cycle progression of MCF-7 cells induced by estradiol

MCF-7/tetHER-2ribozyme cells as described in previous reports were stimulated by 10 nM estradiol and approximately 1 million cells at different time points submitted to FACS analysis. The portion of cells in the S-phase and at the G2/M boundary is shown. No significant differences were observed in a comparison of HER-2 reduced (filled symbols) and non-reduced (open symbols) cells.

3. Increased apoptosis after estrogen treatment of HER-2-reduced MCF-7 cells.

Since we did not detect a significant effect in cell cycle progression after HER-2 reduction, we next tested whether the dependence of estrogen-induced growth on the presence of HER-2 was due to a change in the portion of cells undergoing apoptosis. For this we used a commercially available ELISA assay that quantitates apoptotic DNA fragmentation in cells by detecting DNA/histone complexes [27]. The data from this experiment revealed that after estrogen stimulation there was a significantly increased extent of apoptosis when HER-2 was reduced ($p < 0.001$). Figure 3 shows the data from the 24 hour time point. Virtually the same relationship was also found at 72 hours after initiation of estradiol treatment (not shown).

We conclude that this increased apoptosis can sufficiently explain the overall effects seen on estrogen-induced colony formation and proliferation after HER-2 reduction (see Fig. 1).

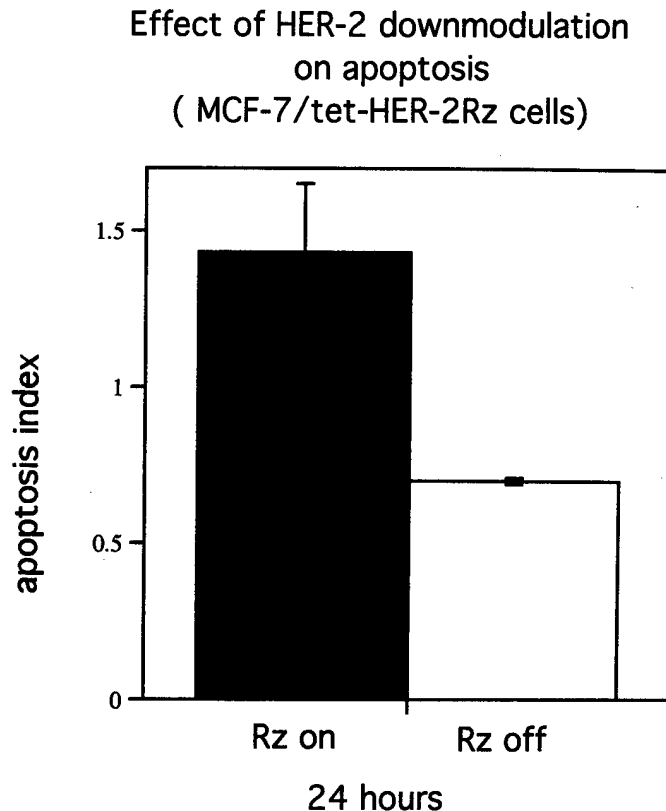


Fig. 3. Effect of HER-2 reduction by tetracycline-regulated ribozymes on apoptosis of MCF-7 cells after estradiol stimulation

MCF-7/tetHER-2ribozyme cells as described in previous reports were stimulated by 10 nM estradiol and assayed for apoptosis as described under "Methods". A significant difference ($p < 0.001$) between the two groups was observed.

4. Changes in cell cycle versus apoptosis pathway gene expression patterns after estrogen treatment of HER-2-reduced MCF-7 cells.

Our above data show that reduction of apoptosis appears to be the rate-limiting parameter for the estrogen-mediated growth of MCF-7 cells and that HER-2 is the conduit for this anti-apoptotic effect. We next wished to assess, which changes in expression of cell cycle- and apoptosis-related genes reflect alterations of either pathway. Furthermore, we hypothesized that these changes could be useful in the further understanding of drug effects, since such effects may be reflected in different patterns of effects.

For our analysis we utilized one of the commercially available cDNA arrays (CLONTECH) that contains 588 different genes and allows to analyze effects on the expression of the major (known) genes along the cell cycle and apoptosis pathways.

Figure 4 (next page) shows the comparison of gene expression levels at different time points after stimulation with estradiol in the MCF-7/tetHER-2Rz cells that were used throughout this study. Circles were used to indicate genes regulating the cell cycle and crosses to indicate genes regulating apoptosis. The comparison is between cells in which HER-2 was NOT reduced (with tetracycline = abscissa) and cells in which HER-2 was reduced (no tetracycline = ordinate).

The graphs in Fig. 4 represent mRNA levels of 125 different genes that we were able to detect simultaneously by the cDNA display array by phosphorimager analysis. An initial inspection of these graphs shows the following:

a. At time point 0 and 24 hours after estradiol cell cycle and apoptosis-related gene expression levels are scattered around the identity line. A simple statistical analysis (Chi-square test) looking at the frequency of distribution above and below the identity line gave no significant difference amongst the two groups at either of the two time points.

b. At time point 6 hours after estradiol, no significant difference for the expression levels of cell cycle-related genes is apparent. A statistical analysis by chi-square also confirms that. However, it is obvious from an inspection of the panel that expression levels of apoptosis-related genes are significantly shifted from the identity line. Expression levels of 26 of the apoptosis-related genes were below the 1.4-fold cut-off, 23 were within the identity range (less than 1.4-fold outside the identity line) and no gene was above this cutoff. This data set is shown in Figure 5. A Chi-square analysis gave a $p < 0.0001$ value.

As a **CAVEAT** it should be noted that this is only an initial analysis assessing overall effects. We are currently running a further series of experiments at an earlier time point (1 hour after estradiol). Furthermore, we plan to confirm regulation of some of the the genes by Northern analysis, although the extent of that analysis will be limited by the amount of effort required. We estimate that we will be able to confirm by Northern analysis expression levels of approximately one dozen of the genes that showed differential regulation in the cDNA array analysis.

We conclude from this initial analysis that the interaction of estrogen and HER-2 seen at the cell function levels (cell growth, cell cycle and apoptosis) can be traced to the gene expression level along the apoptosis pathway. Obviously, expression of genes regulating apoptosis is affected significantly when the estrogen effect on cell growth is blocked by reduction of HER-2. In contrast, the expression of genes regulating the cell cycle does not appear to be affected to a measurable extent.

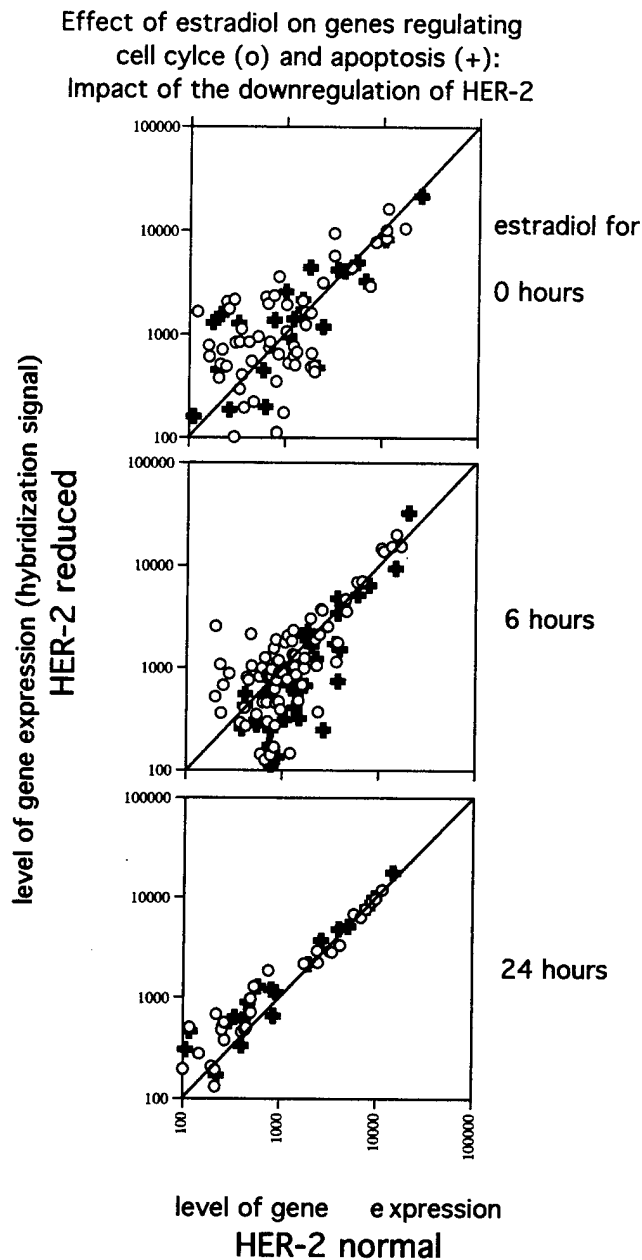


Fig. 4. Effect of HER-2 reduction by tetracycline-regulated ribozymes on gene expression levels of MCF-7 cells at different times after estradiol stimulation

MCF-7/tetHER-2Rz cells were stimulated for different time intervals by 10 nM of estradiol. mRNA expression levels in cells with the ribozyme off (**HER-2 normal = Abscissa**) compared to ribozyme on (**HER-2 reduced = Ordinate**). Circles: cell cycle regulating genes. Crosses: Apoptosis regulating genes. CLONTECH Atlas array

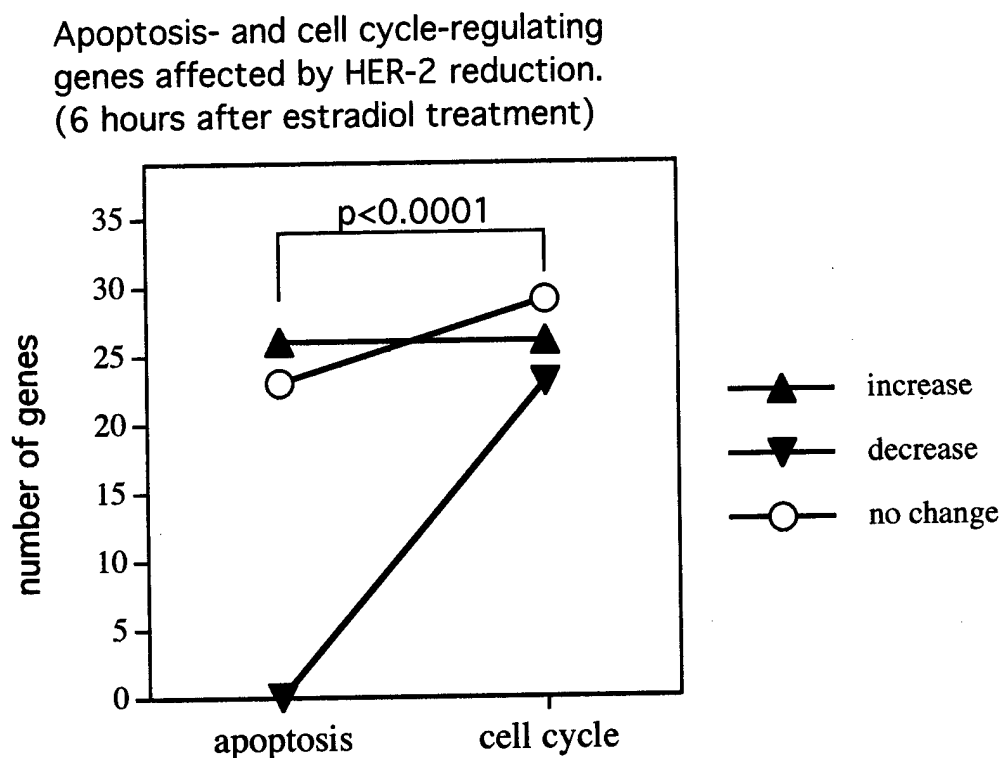


Fig. 5. Effect of HER-2 reduction by tetracycline-regulated ribozymes on gene expression patterns of MCF-7 cells 6 hours after estradiol stimulation

Apoptosis-regulating genes and cell cycle-regulating genes from Fig. 3 were included. The number of genes that showed expression levels above or below the identity range (± 1.4 -fold of the identity line) were counted and the data compared by Chi-square test.

Methods

Serum preparation

Charcoal-stripped serum is prepared routinely by the Lombardi Cancer Center CORE facility and was obtained from that facility.

Proliferation and colony formation assays

The soft agar colony formation assay was described in the previous reports. As a read-out cell colonies greater than 60 microns formed in suspension are counted by image analysis after 12 days of incubation.

For cell growth assays, cells are plated in 12-well dishes and cell numbers estimated at two-day intervals after initiation of treatments. The 12-day-time-point is shown here for a direct comparison with the soft agar assay. For the measurements a commercially available dye assay (MTT; Promega) was used according to the instructions of the manufacturer. The read-out of this assay is proportionate to the number of cells in a dish.

FACS analysis and evaluation of apoptosis.

FACS analysis was described in previous reports. The extent of apoptosis was assessed by an ELISA ("cell death detection"; Boehringer Mannheim) that quantitates apoptotic DNA fragmentation in cells by detecting DNA/histone complexes [27]. The assay was applied according to the manufacturer's specifications.

cDNA array analysis of gene expression levels

The CLONTECH Atlas array with 588 different cDNAs spotted in duplicate each was used according to the instructions of the manufacturer. We prepared mRNAs from cells under all six treatment conditions in Figure 4 simultaneously, reverse transcription labeled the mRNAs in parallel and hybridized all sets of mRNAs with the cDNA arrays at the same time. Six separate membranes of arrayed cDNAs were used for this purpose. The membranes were then stripped for two repeat experiments in which the membranes were rotated to different time points and treatment conditions. Levels of gene expression were read from phosphoimager analysis of the cDNA arrays. Here we present the data of the first round of analysis that was completed at the time of this report.

Statistical analysis

Unless stated otherwise data points were run in triplicate and experiments repeated at least twice. Typically the mean $\pm$ SE from a representative experiment is presented. As appropriate, Chi-square, Student's t-test or ANOVA (=analysis of variance) was used to assess the statistical significance of differences between measurements. The Statview 4.02 program (Abacus Concepts Inc.; Berkeley, CA) as well as the Prism program (GraphPad Inc., San Diego, CA) were used on PowerMacs. The respective p-values are given in the text. $p < 0.05$ was considered significant.

CONCLUSIONS

In the past cycle under this award, we analyzed the interaction of estrogen and HER-2 with respect to MCF-7 human breast cancer cell growth. We had observed that upon reduction of HER-2 in MCF-7 cells by ribozyme-targeting estradiol lost its ability to induce colony formation of the tumor cells and cell growth on dish surface. We now found that this was due to the failure of estradiol to inhibit apoptosis when HER-2 is reduced and not due to increased progression into the cell cycle. By cDNA array analysis we traced this effect to a series of genes involved in the regulation of apoptosis. Expression of genes regulating apoptosis was affected significantly when the estrogen effect on cell growth was blocked by the reduction of HER-2. In contrast, the expression of genes regulating the cell cycle did not appear to be affected to a measurable extent.

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