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NDF Induces Expression of a Novel 46 kD Protein in Estrogen Receptor Positive Breast Cancer Cells

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Abstract Most human breast tumors start as estrogen-dependent, but during the course of the disease become refractory to hormone therapy. The transition of breast tumors from estrogen dependent to independent behavior may be regulated by autocrine and/or paracrine growth factor(s) that are independent of the estrogen receptor (ER). We have investigated the role(s) of NDF (neu-differentiation factor) in the biology of estrogen positive breast cancer cells by using MCF-7 cells as a model system. Treatment of MCF-7 cells with human recombinant NDF-β2 (NDF) inhibited the ER expression by 70% and this was associated with growth stimulation in an estrogen-independent manner. To explore the mechanism(s) of action of NDF in MCF-7 cells, we examined the expression of NDF-inducible gene products. We report here that NDF stimulated the levels of expression of a 46 kD protein (p46) (in addition to few minor proteins) in ER positive breast cancer cells including MCF-7, T-47-D, and ZR-75-R cells but not in ER negative breast cancer cells including MDA-231, SK-BR-3, and MDA-468 cells. This effect of NDF was due to induction in the rate of synthesis of new p46. The observed NDF-mediated induction of p46 expression was specific as there was no such effect by epidermal growth factor or 17-β-estradiol, and inclusion of actinomycin D partially inhibited the p46 induction elicited by NDF. NDF-inducible stimulation of p46 expression was an early event (2–6 h) which preceded the period of down-regulation of ER expression by NDF. These results support the existence of NDF-responsive specific cellular pathway(s) that may regulate ER, and these interactions could play a role(s) in hormone-independence of ER positive breast cancer cells.

Key words: NDF, estrogen receptor, breast cancer

Human breast cancer can be estrogen dependent or independent. A number of studies have demonstrated that about one-third of breast cancer patients respond to endocrine therapy, and this population of patients is usually estrogen receptor (ER) and/or progesterone receptor (PR) positive [Jordan, 1993]. Despite the presence of ER, a significant proportion of patients do not respond to hormone therapy and most who do respond will eventually develop acquired hormone-independence [Jordan, 1993]. One potential mechanism for transition of breast cancer cells from hormone dependence to independence is the influence of autocrine and/or paracrine growth factor pathways that may be dependent and/or independent of estrogen [Jordan, 1993]. In vitro and in vivo studies have shown the development of partial tamoxifen resistance in ER positive MCF-7 human breast cancer cells upon overexpression of HER2 gene product [Benz et al., 1992]. Clinical studies indicate that the presence of ER in breast cancer is closely associated with better responsiveness to tamoxifen treatment, and they suggest that co-overexpression of HER2 and the related receptor for epidermal growth factor (EGF receptor or c-*erb*B1) in ER positive breast cancer may be associated with tamoxifen resistance [Wright et al., 1992; Muss et al., 1994; Borg et al., 1994; Leitzel et al., 1995]. The underlying cellular mechanism leading to endocrine independence is not yet known.

Growth factors play a central role in controlling the proliferation of normal and malignant

Abbreviations used: ER, estrogen receptor; PR, progesterone receptor; E2, 17-β-estradiol; NDF, recombinant human neu-differentiation factor; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

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cells. Alterations in growth factor expression and action have been correlated with the malignant process: the products of many oncogenes are growth factors or receptors for growth factors. For example, c-*erb*B1 encodes the receptor for epidermal growth factor, and c-*erb*-B2 (commonly known as HER2), the human homolog of the rat proto-oncogene neu encodes a 185 kilodalton transmembrane glycoprotein with intrinsic tyrosine kinase activity [Stern et al., 1985; Connelly and Stern, 1990]. The specific ligand for this putative receptor has not been identified. HER2 has been shown to be over-expressed and/or amplified in a number of human malignancies including breast and ovarian cancer [Van de Vijver et al., 1987; Slamon et al., 1989]. In addition, HER2 is a potent oncogene capable of inducing transformation and tumorigenesis, when overexpressed in NIH3T3 cells [DiFiore et al., 1987; Hudziak et al., 1987]. Thus HER2 may have an important role in the development and progression of human breast cancer.

NDF, a 44 kD glycoprotein originally thought to be a specific ligand for HER2, was isolated from the medium of ras-transformed cells, and inhibits MDA-453 cell growth [Peles et al., 1992; Wen et al., 1992]. Heregulin (45 kD), another candidate ligand that was independently isolated from MDA-231 cells, has been shown to stimulate the growth of SK-BR-3 cells [Holmes et al., 1992]. NDF and heregulin have now been shown to be homologous molecules. These factors bind to and activate two other receptors in this family known as HER3 and HER4 [Plowman et al., 1993; Sliwkowski et al., 1994]. Interestingly, NDF and heregulin can also activate the HER2 receptor probably through the formation of receptor heterodimers with HER3 and/or HER4 [Plowman et al., 1993; Sliwkowski et al., 1994].

These observations suggest that the transition of breast tumors from estrogen-dependent to independent behavior might be related to the presence of an autocrine and/or paracrine polypeptide factor(s). The biochemical and molecular events that occur after receptor activation may contribute to hormonal independence need clarification. In the present study we investigated the role(s) of NDF in the biology of ER positive breast cancer cells by exploring the phosphorylation and expression of NDF-responsive cellular proteins using MCF-7 cells as a model system. We report here that human NDF-β2 (NDF) inhibits ER expression and also stimulates the growth of MCF-7 cells in an estrogen-independent manner. Furthermore, treatment with NDF stimulated the expression of a specific 46 kD protein (p46).

MATERIALS AND METHODS

Cell Lines and Reagents

MCF-7 human breast cancer cells (sensitive to Tamoxifen) and MCF-7/HER2-18 cells (resistant to Tamoxifen) [Benz et al., 1992] were maintained in DMEM-F12 (1:1) supplemented with 10% fetal bovine serum (FBS). Other human breast cancer cells (T-47-D, ZR-75-R, MDA-231, SK-BR-3, and MDA-468) were obtained from the American Type Culture Collection. T-47-D, ZR-75-R, and MDA-231 cells were grown in L-15 medium, and SK-BR-3 and MDA-468 cells in DMEM-F12 (1:1) containing 10% FBS. Antiphosphotyrosine mAbs 4G10 were from the UBI [Kumar and Korutla, 1995]. Rabbit anti-mouse immunoglobulin (RAM) was purchased from Accurate Chemicals (Westbury, NY). Recombinant NDF β2 was obtained from the Amgen. ^{32}Pi (carrier free; 28.5 Ci/nmol), ^{35}S-labeled methionine (1,030 Ci/mmol), and ^{125}I-labeled protein A (10 μCi/μg) were purchased from New England Nuclear. Low-molecular-mass markers (Amersham Corp.) were used as standards.

Culture Treatments and Cell Extracts

All experiments were performed with cells in logarithmic phase by controlling the plating density. Viability of cells was assayed by trypan blue dye exclusion. Subconfluent (about 50% or less confluent) cells were cultured in phenol red-free DMEM-F12 (1:1) supplemented with 5% charcoal-dextran (DCC) treated FBS for 4 days before using them for experiments. Exponentially growing cells treated with the desired agents, were used to prepare cell extracts as described [Kumar and Atlas, 1992]. Briefly, cells were washed twice with cold PBS on ice and lysed in buffer (50 mM Tris-HCl, pH 8.0; 120 mM NaCl; 0.5% Nonidet P-40; 100 mM NaF; 200 μM $NaV0_5$; 1 mM PMSF; 10 μg/ml leupeptin) for 15 min on ice. The lysates were centrifuged in an Eppendorf centrifuge at 4°C for 15 min. Protein concentration was determined by using the Bio-Rad kit.

Immunoblotting and Immunoprecipitation

Cell lysates containing equal amounts of total protein were resolved on a 7% or 10% SDS-

PAGE followed by transfer onto nitrocellulose. Membranes were blocked in 2% BSA in TBS followed by probing with the desired Ab, and detection of immune complexes using ^{125}I-protein A or secondary antibody based alkaline phosphatase color reaction [Kumar and Korutla, 1995]. Expression of ER was performed by immunoblotting with either anti-ER mAb H222 (gift from Dr. Geoffery Green) and by anti-ER-mAb AER320 (Neomarker) [Abbondanza et al., 1993]. *HER2* was detected by using HER2 mAb 9G6 [Kumar et al., 1991], and PR by anti-PR-3mAb [Clarke et al., 1987]. For reprobing the blots, nitrocellulose filters were stripped in 0.1 M glycine buffer pH 2.5 for 1 h, and neutralized in 1 M Tris-Cl pH 8.0 [Kumar and Korutla, 1995]. Quantitation of specific protein bands was performed by using protein databases scanner (Molecular Dynamics).

Labeling of Proteins With [^{32}P]Orthophosphate and [^{35}S]Methionine

An equal number of cells from each experimental condition were incubated in methionine-free medium for 30 min, and labeled with 300 μCi/ml [^{35}S]methionine in methionine-free medium containing 2% DCC-treated FBS for desired periods of time. Cells were washed twice with PBS followed by preparation of lysates as described above. Aliquot of the cell lysates containing equal amount of protein (TCA precipitable counts) were resolved on a SDS-polyacrylamide gel. For labeling the cellular proteins with ^{32}P, cells were labeled with 400 μCi/ml of [^{32}P]orthophosphate in phosphate-free medium for 12 h, and treated with NDF (10–30 min) before harvesting [Kumar et al., 1991; Fan et al., 1993]. After autoradiography, labeled protein bands were excised for counting the radioactivity.

For studying the turnover of p46, MCF-7 cells were labeled with [^{35}S]methionine for 3 h. Cultures were washed five times and chased for different lengths of time (4 h or 24 h) in culture medium supplemented with 2% DCC-FCS in the presence or absence of NDF, and cell extracts were analyzed by SDS-PAGE.

Preparation of Cytosol and Nuclear Proteins

Nuclear extracts were prepared by a modification [Kumar and Korutla, 1995] of the procedure of Dignam et al. [1983]. Briefly, cells were washed with PBS, and pelleted by centrifugation. Cells were incubated on ice for 15 min in hypotonic buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM PMSF, 1 mM sodium vanadate, 1 mM sodium fluoride) and homogenized in a loose fitting glass homogenizer with 15 strokes. Nuclei were separated from the cytoplasm by centrifugation at 5,000 rpm at 4°C. Nuclei were suspended in buffer (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGDA, 25% glycerol, 1 mM dithiothreitol, 0.5 mM PMSF 1 mM $NaV0_5$, 1 mM NaF) and shaken for 20 min at 4°C, centrifuged at 15,000 rpm for 15 min at 4°C.

RESULTS

Expression of ER and PR in MCF-7 and MCF-7/HER2-18 cells. We first investigated the effect of 17-β-estradiol (E2) on the responsiveness of MCF-7 cells and MCF-7/HER2-18 cells (MCF-7 cells with 45 × increased expression of HER2) [Benz et al., 1992]. We observed that E2 treatment (48 h) resulted in 250% and 80% induction of PR expression (a target gene product marker for activation of the ER pathway) in MCF-7 and MCF-7/HER2-18 cells, respectively. These results suggest that overexpression of HER2 in MCF-7 cells was associated with a reduction in E2 responsiveness. In order to understand the basis of the difference in PR expression between MCF-7 cells and MCF-7/HER2-18 cells, we hypothesized that HER2 overexpression might be associated with reduced ER expression. To explore this possibility, we examined the effect of HER2 and NDF on the expression of ER and PR in MCF-7 and MCF-7/HER2-18 cells. Results in Figure 1 show that overexpression of HER2 in MCF-7/HER2-18 cells was associated with reduced expression of ER (by 65%, compare lanes 3 and 1 in panel B) and also PR (by 79%, compare lanes 3 and 1 in panel C) compared to the levels in parental MCF-7 cells. Interestingly, in MCF-7 cells treated with NDF (Fig. 1, panel B), there was also significant inhibition in the levels of ER (by 70%). There was only a modest effect of NDF on ER levels in MCF-7/HER2-18 cells (36%). Down-regulation of ER in NDF-treated cells was accompanied by reduced expression of PR (panel C), as expected. It is possible that the differences in NDF-mediated down-regulation of ER between MCF-7 and MCF-7/HER2-18 may be related to the difference in the baseline ER expression.

To further explore the effects of NDF on ER expression, immunoprecipitation studies were performed in MCF-7 cells. Results in Figure 2A

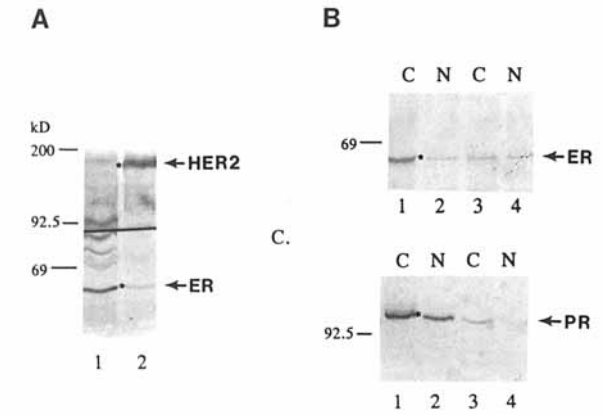

Fig. 1. Expression of ER and PR in MCF-7 and MCF-7/HER2-18 cells. **A:** Cell extracts from control MCF-7 (*lane 1*) and MCF-7/HER2 cells (*lane 2*) were made and aliquot containing equal amount of protein (40 μg) were resolved on a 7% SDS-PAGE, and transferred onto a nitrocellulose membrane. The blot was cut into two parts for immunoblotting with either a HER2 mAb (*upper part*) or with a ER mAb (clone AER320). Numbers on the left indicate the positions of colored rainbow molecular weight markers (Amersham). Positions of HER2 and ER are indicated on the right. **B, C:** E2-starved MCF-7 cells (*lanes 1 and 2*) and MCF-7/HER2-18 cells (*lanes 3 and 4*) were treated with or without NDFβ2 (25 ng/ml) for 16 h at 37°C. Cell lysates (40 μg) were resolved on a 7% SDS-PAGE, analyzed by immunoblotting with ER mAb **(B)** and PR mAb **(C)**. Relevant portions of blots are shown. C, control; N, NDF-treated.

indicated that treatment of MCF-7 cells with NDF for 24 h (lane 2) inhibited ER expression by 58% as compared with the levels in untreated cells. Kinetic experiments indicated that down-regulation (48% as compared to levels in control cells) of ER in NDF-treated MCF-7 cells could be detected by 6 h after NDF treatment (Fig. 2B). The observed inhibition in the steady-state levels of ER by NDF was also accompanied by inhibition (87% compared to control) of newly synthesized ^{35}S-labeled ER. Results in Figure 2C indicate that the observed inhibition of ER expression was a specific effect of NDF (compare lanes 2 and 1) as there was no significant effect of NDF on the expression of ^{35}S-labeled EGF receptors (lanes 3 and 4). Results of other experiments indicated that NDF also inhibits the steady-state levels of ER mRNA in MCF-7 cells, and ER protein in ZR-75R cells (data not shown). In brief, these results suggest that NDF down-regulates the expression of ER in MCF-7 cells, and this effect of NDF is consistent with the recently reported inhibitory effects of heregulin on ER expression [Pietras et al., 1995].

Effect of NDF on MCF-7 cell growth. Since we were interested in understanding the growth regulation of MCF-7 cells by NDF, we examined the effect of NDF on the growth of exopentially growing MCF-7 cells. Results in Table I show that treatment with NDF stimulated MCF-7 cell growth by 61% as compared to untreated cells. Treatment of MCF-7 cells with tamoxifen inhibited the growth by 95%, and co-addition of tamoxifen and NDF resulted in reduction of tamoxifen-mediated growth inhibition from 95% to 45%. These results suggested that NDF alone can stimulate the MCF-7 cell growth even in the presence of 5% FBS, and could partially reduce the inhibitory effects of tamoxifen. To understand the growth stimulatory property of NDF without any possible masking effect of estrogen present in serum, MCF-7 cells cultured in DCC-treated serum, were used for growth stimulation experiments (Table I). Results indicated that NDF is a potent *mitogen* as it stimulated the growth of E2-starved MCF-7 cells by 2.8 fold.

Effect of NDF on phosphorylation of cellular proteins. We next explored the possible mechanism(s) of NDF-mediated down regulation of ER by examining the phosphorylation of cellular proteins. As shown in Figure 3A, treatment (10 min) at 37°C of MCF-7 cells with NDF (N, lane 2), but not EGF (E, lane 3) rapidly induced tyrosine phosphorylation of a 185 kD protein. Results of immunoprecipitation with a HER2 specific mAb 9G6 indicated that this protein was HER2 (data not shown). There was no significant change in the levels of tyrosine phosphorylation of any other protein bands in the range from 46 kD to 92.5 kD. Additional experiments involving immunoprecipitation of ER (using ER mAb H222) followed by immunoblotting with antiphosphotyrosine mAb also indicated no effect of NDF on the levels of ER tyrosine phosphorylation over the control untreated MCF-7 cells (data not shown). Our results are not in agreement with those by Pietras et al. [1995] demonstrating an increase in the tyrosine phosphorylation of ER by heregulin. It is possible that these differences may be due to use of different MCF-7 cells and/or use of homologous but not identical polypeptides in two studies. Interestingly, treatment with NDF (and not EGF) also induced the tyrosine phosphorylation of another protein of approximate molecular weight 23 kD (±3000, marked by a arrow). To examine whether there are additional NDF-

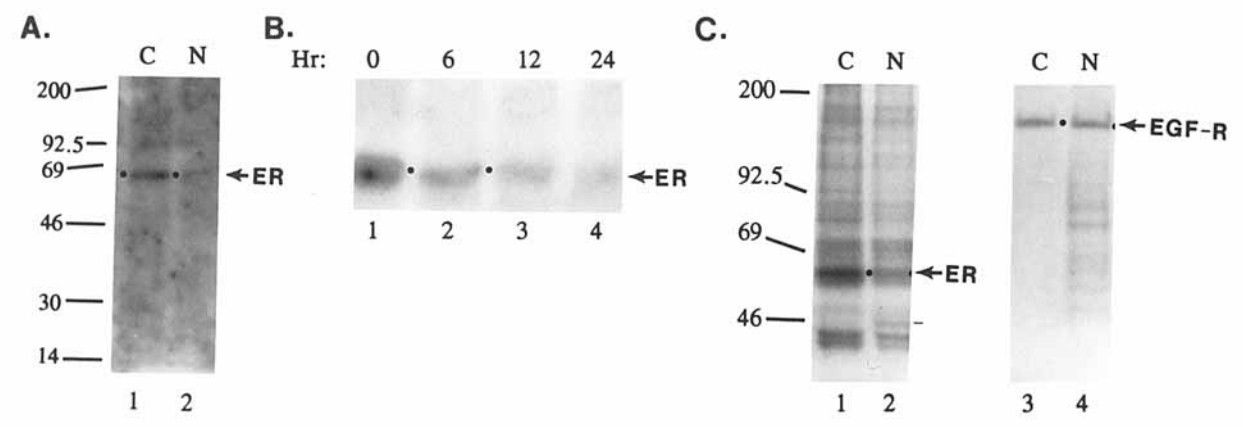

Fig. 2. Down-regulation of ER in NDF treated MCF-7 cells. **A:** E2-starved MCF-7 cells were treated with (N) or without (C) NDF (25 ng/ml) for 24 h. Cell lysates containing equal amount of protein (50 μg) were immunoprecipitated by using ER specific antibody H222 followed by 10% SDS-PAGE and immunoblotting with ER mAb H222. Position of ER is indicated by a arrow on the right. As a control, the same blot was also reprobed with an unrelated heat-shock protein (Hsp) 86 antibody and there was no change in the levels of Hsp 86 with or without NDF treatment (data not shown). Positions of molecular weight markers is indicated on the left. Experiment shown is representative of five experiments with similar results. **B:** E2-starved MCF-7 cells were treated with (*lanes 2–4*) or without (*lane 1*) NDF (25 ng/ml) for indicated period of time. Cell lysates (40 μg protein) were immunoblotted by using ER specific antibody H222. Position of ER is indicated by a arrow on the right. Relevant portions of blot is shown. As a control, the same blot was also reprobed with an unrelated Hsp 86 antibody and there was no effect of change in the levels of Hsp 86 with or without NDF treatment (data not shown). **C:** E2-starved MCF-7 cells were treated with (N; *lanes 2 and 4*) or without (C; *lanes 1 and 3*) NDF (25 ng/ml) for 15 h followed by labeling with ^{35}S-methionine for 3 h. Cell extracts containing equal amount of protein were immunoprecipitated with either anti-ER mAbs or anti-EGF receptor mAb 528. Metabolically labeled proteins were analyzed by 7% SDS-PAGE. Positions of ER and EGFR are indicated on the right. Mark under ER band shows a nonspecific protein in NDF-treated cells. These experiments are repeated two times.

TABLE I. Effect of NDF on the Growth of MCF-7 Cells*

Regular growth conditions (with 5% FBS)[a]		E-2 starved conditions[b]	
Treatment	Growth, % control	Treatment	Growth, % control
Control (0.1% ethanol)	100%	Control	100%
Tamoxifen, 0.5 μM	5%	NDF, 25 ng/ml	280%
NDF, 25 ng/ml	161%		
Tamoxifen + NDF	55%		

*Results are presented as percent of control after 72 h treatment with the indicated agents, by using MTT-method (21). Data shown is representative of five experiments with duplicate cultures.

[a]Cells were grown in regular DMEM-F12 (1:1) supplemented with 5% FBS.

[b]Cells were first grown in E-2 starved conditions for four days (similar to as in Fig. 1).

responsive cellular phosphoproteins, MCF-7 cells were labeled in vivo with ^{32}P-orthophosphoric acid followed by treatment with NDF for 10 min or 30 min (Fig. 3B). Results indicated that NDF rapidly stimulated the levels of phosphorylation of a 23 kD (±3000, marked by an arrow) protein within 10 min of treatment. Levels of phosphorylated 23 kD protein declined by 30 min after treatment. We did not, attempt to further characterize the role(s) of this phosphoprotein in the action of NDF in the present study.

Effect of NDF on the synthesis of cellular proteins in MCF-7 cells. Based on our previous experience with action of interferons which mediate direct effects via inducing the expression of interferon-inducible gene products [Kumar and Mendelsohn, 1990], we investigated the mechanism of action of NDF in MCF-7 cells by examining newly synthesized proteins in NDF-treated cells. Results in Figure 4A show that treatment (12 h) of MCF-7 cells with NDF enhances the expression of a 46 kD protein (±3000) (p46, indicated by "*", lanes 1 and 2) and also a few other proteins (indicated by "●"). Parallel ^{32}P-labeling experiments (Fig. 4A, lanes 3 and 4) indicated that p46 is not a phosphoprotein. Treatment of MCF-7 cells with NDF also enhances the levels of phosphorylation of a number of other proteins.

Since we were interested in protein(s) expression which is enhanced early after NDF treatment of MCF-7 cells, we performed experiments to analyze the time and dose course of stimula-

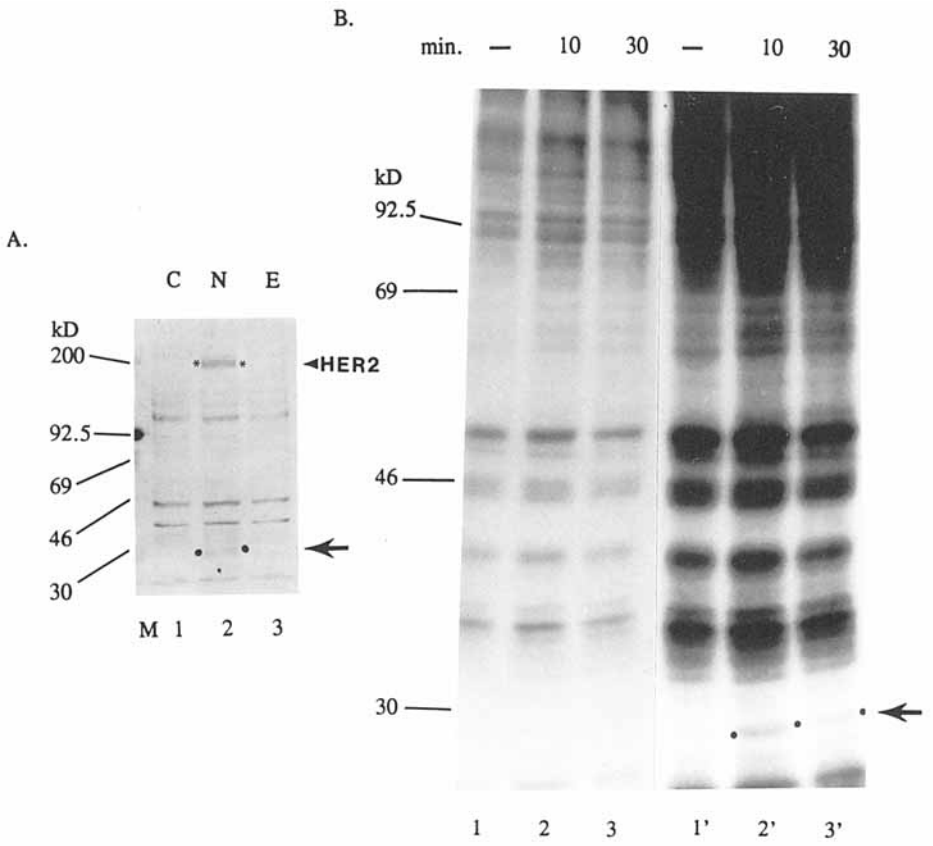

Fig. 3. Effect of NDF on phosphorylation of cellular proteins in MCF-7 cells. Cells were grown in E2-free condition as in Figure 1. **A:** Phosphotyrosine blot. Cells were treated with NDF (25 ng/ml, *lane 2,* N) and EGF (30 nM, *lane 3,* E) for 10 min at 37°C. Cell lysates were separated on a 7% SDS-PAGE, and immunoblotted with anti-phosphotyrosine mAb (clone 4G10, UBI). Positions of HER2 and another specific 23 kD protein is shown on the right. *Lane M* indicates the positions of markers. **B:** In vivo ^{32}P-labeling. Cells were labeled with 400 μCi/ml of ^{32}P-orthophosphoric acid for 16 h. Some cultures were treated with NDF (25 ng/ml) during the last 30 min (*lanes 3 and 3'*) or last 10 min (*lanes 2 and 2'*) before harvesting. Equal amounts of labeled proteins (TCA ppt) were analyzes on a long 10% SDS-PAGE. *Lanes 1–3* (5 min exposure) and *lanes 1'–3'* (20 min exposure) represent two different exposures of the same gel. Position of a 23 kD is shown by an *arrow* on the right. Positions of molecular weight markers are indicated on the left. These experiments are repeated three times.

tion of expression of cellular proteins in NDF-treated MCF-7 cells (Fig. 4, panels B and C). Results indicated that NDF predominantly induces the expression of p46 as a early response in a time-dependent manner, and 5 ng/ml dose of NDF was as potent as a dose of 50 ng/ml. Induction of other proteins in NDF-treated cells required 12 h treatment. As little as 2 h treatment was sufficient to stimulate the p46 expression by 21% (panel B, lane 3) with a maximum induction of 3.2 fold at 6 h after treatment. These results in the context of kinetics of ER down-regulation in NDF-treated cells (Fig. 2B) suggested that NDF-induced stimulation of p46 expression was an early event which precedes the down-regulation of ER.

Specificity of induction of p46 expression by NDF. Data in Figure 5A show that expression of p46 was specifically enhanced by NDF and not by either EGF or E2 in MCF-7 cells under identical experimental conditions (6 h). This suggests that p46 could represent an NDF-inducible gene product in MCF-7 cells. To further establish the inducibility of p46 expression by NDF in MCF-7 cells, we examined the effect of Actinomycin D (Act. D, an inhibitor of transcription) on the levels of NDF-induced p46 expression. Results in Figure 5B indicated that addition of Act. D to the cultures inhibited the NDF-mediated induction of p46 expression from 98% (Fig. 5B, without Act. D, lanes 3 and 4) to 33% (with Act. D, lanes 1 and 2) over the p46 levels in respective untreated MCF-7 cells. This result suggests that inhibition of transcription by Act. D may reduce the expression of NDF-inducible p46 to a significant extent. We also examined the effect of NDF on p46 expression in SK-BR-3 cells (ER-negative cells), and results in

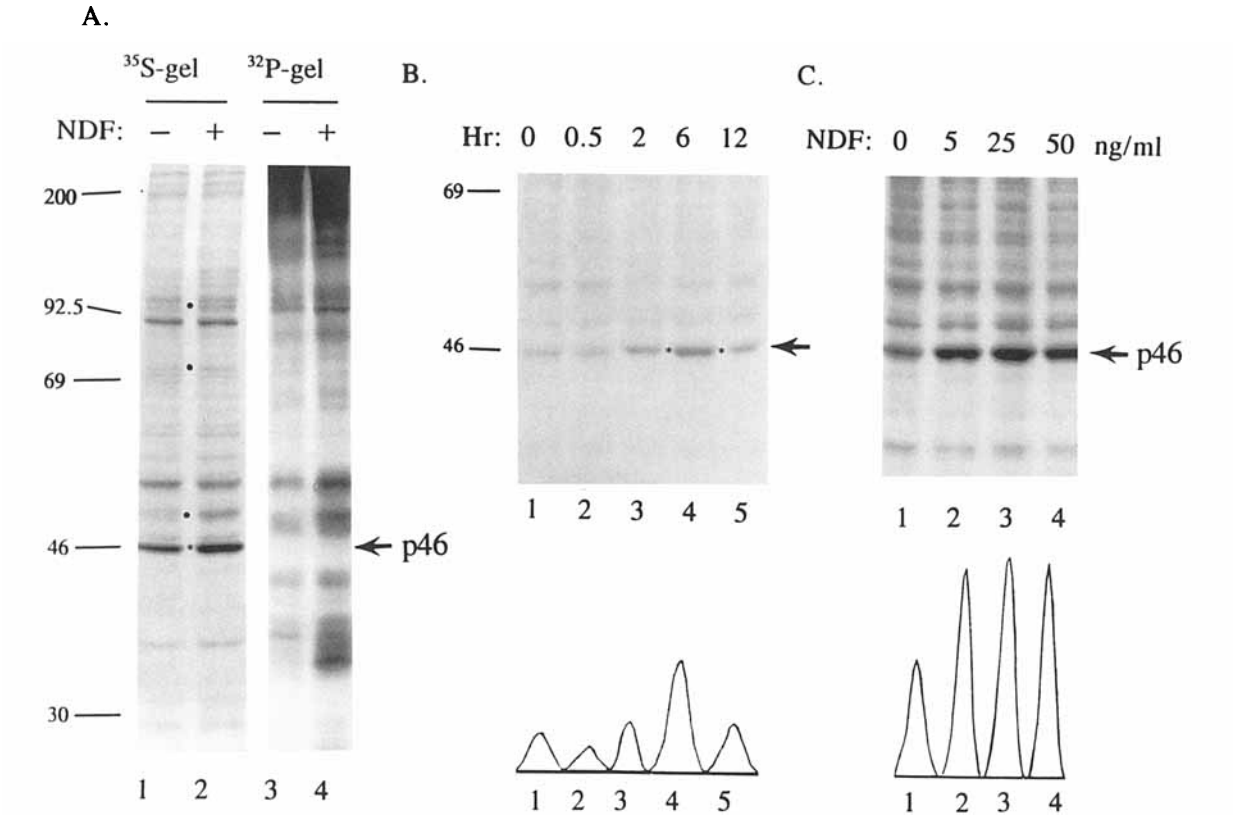

Fig. 4. Effect of NDF on the synthesize of cellular proteins in MCF-7 cells. **A:** MCF-7 cells were labeled in vivo with either ^{35}S-methionine (*lanes 1 and 2,* 100 μCi/ml) or ^{32}P-orthophosphoric acid (*lanes 3 and 4,* 400 μCi/ml) in the continuous presence (*lanes 2 and 4*) or absence (*lanes 1 and 3*) of NDF (25 ng/ml) for 12 h. Cell extracts from ^{35}S- and ^{32}P-samples were analyzed by resolving on the same 10% SDS-PAGE. Position of p46 (marked as "*") is indicated by an *arrow.* "●" indicates positions of other minor inducible proteins. **B:** MCF-7 cells were treated with NDF (25 ng/ml) for indicated time periods. All cultures were pulse-labeled with ^{35}S-methionine during the last 30 min. Note that cells in lane 2 were treated with NDF during the labeling period. **C:** MCF-7 cells were treated with the indicated doses of NDF for 6 h and labeled with ^{35}S-methionine during the last 3 h of treatment. Metabolically labeled proteins were analyzed by 10% SDS-PAGE. Relevant portions of gels are shown. Position of p46 is indicated on the right. Numbers on the left indicate positions of 69 kD and 46 kD molecular weight markers in both B and C. Quantitation of p46 bands in B and C are shown under the respective gels. The NDF-inducible stimulation of p46 expression was observed in more than fifteen separate experiments.

Figure 5B, lanes 5 and 6 indicated that there was no effect of NDF on p46 in SKBR-3 cells. In brief, these results suggested that NDF-induced stimulation of p46 expression may be due to both transcription and/or post-transcription regulation of a putative gene for p46 by NDF.

Effect of NDF on the degradation and synthesis of p46. Since expression of a given protein is influenced by the rate of its degradation (half-life) as well as its synthesis, experiments were also performed to test whether NDF induces the p46 expression by enhancing the synthesis or affecting degradation of pre-exiting p46. In these experiments, MCF-7 cells were either metabolically labeled for 3 h and chased in the presence or absence of NDF or first treated with or without NDF beforing labeling with ^{35}S-methionine. As shown in Figure 6, the observed induction of p46 in MCF-7 cells was due to the induction of new p46 synthesis as NDF treatment (6 h) stimulated p46 expression by 2.9 fold, and there was no effect of NDF on the levels of p46 in pulse-chase experiment (lanes 1–5).

Localization of NDF-induced p46. Next, we examined the localization of NDF-inducible p46 in MCF-7 cells before and after treatment with NDF. As shown in Figure 7, treatment of MCF-7 cells with NDF stimulated the levels of newly synthesized p46 predominantly in the cytosolic fraction. Interestingly, this analysis also revealed the enhanced expression of a number of minor NDF-inducible proteins which could not be detected so distinctly by using the whole

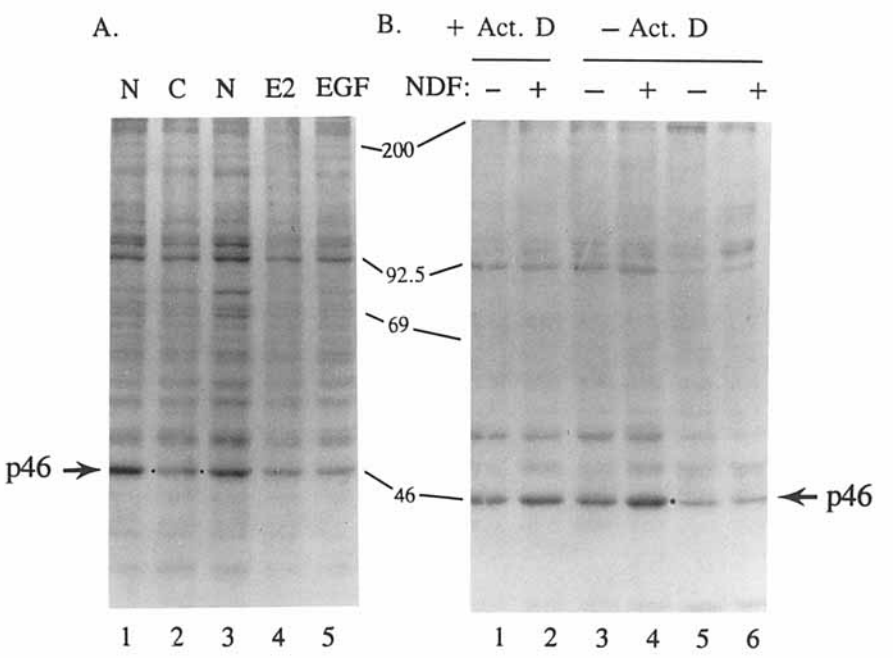

Fig. 5. Specificity of induction of p46 expression by NDF. **A:** MCF-7 cells were treated with either NDF (*lane 1,* N, 25 ng/ml; *lane 3,* N, 50 ng/ml) or 17-β-estradiol (*lane 4,* E2, 10^{-8} M) or EGF (30 nM) for 6 h followed by labeling with ^{35}S-methionine during the last 3 h of treatment. Metabolically labeled proteins were analyzed by 10% SDS-PAGE. **B:** MCF-7 cells (*lanes 1–4*) and SKBR-3 (*lanes 5 and 6,* also grown in E2-free conditions like MCF-7 cells) were treated with or without NDF (25 ng/ml), as indicated, for 6 h. Some cultures (*lanes 1 and 2*) were also treated with Actinomycin D (Act. D, 1 μg/ml). Cells were labeled with ^{35}S-methionine during the last 3 h of treatment. Metabolically labeled proteins were analyzed by 10% SDS-PAGE. Panels A and B are from two different gels. Relevant portions of gels are shown. *Arrow* indicates the position of p46. Positions of molecular weight markers is shown between panels A and B. These experiment are repeated three times.

cell lysate, in addition to the major p46. To quantitate the differences between the background radioactivity and specific induction of p46 by NDF, we also measured the radioactivity associated with the p46 band and a dintinct protein band (shown by •, this may or may not be related with p46) in NDF-treated and untreated cells. Results indicated that the ratio of NDF-inducible specific p46 band was 4.9 as opposed to 2.4 fold for an unrelated protein band. In brief, p46 was the major NDF inducible protein in MCF-7 cells.

Observations with other breast cancer cells. In order to determine whether induction of p46 expression by NDF is a phenomenon restricted to MCF-7 cells or a general mechanism of action of NDF in ER positive breast cancer cells, we extended our investigation to two other ER positive cell lines: T-47D and ZR-75-R. The results in Figure 8 indicated that exposure to NDF enhanced the expression of p46 in both of these ER positive breast cancer cells (45–80% compared to untreated cells) but not in ER negative MDA-231 cells (and also MDA-468, not shown). Furthermore, treatment of T-47D and ZR-75-R cells with NDF (25 ng/ml) was also associated with growth stimulation and down-regulated of ER expression (data not shown).

DISCUSSION

One-third of breast cancer patients respond to endocrine therapy, and this population is usually ER and PR positive [Jordon, 1993]. It is generally recognized that the presence of ER is closely associated with responsiveness to Tamoxifen, and co-overexpression of HER2 in ER positive breast cancer patients and also MCF-7 leads to development of resistance to hormone therapy [Wright et al., 1992; Muss et al., 1994; Borg et al., 1994; Leitzel et al., 1995; Benz et al., 1992]. Therefore, transition of breast cancer cells from hormone dependence to independence may be regulated by interactions with HER2 and/or NDF. To explore this hypothesis, we investigated the possible role(s) of NDF in ER positive breast cancer MCF-7 cells as a model system.

The results presented here indicate that treatment of MCF-7 cells with NDF significantly

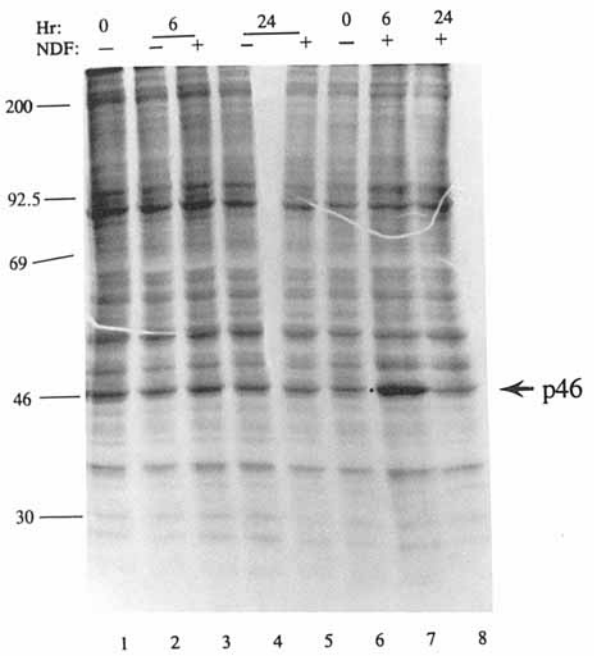

Fig. 6. Effect of NDF on the degradation and synthesis of p46 in MCF-7 cells. *Lanes 1–5,* degradation rate of p46. Cells were labeled with ^{35}S-methionine (3 h) and chased in the presence of NDF (25 ng/ml) or none for indicated length of time. *Lanes 6–8,* synthesis of p46. Cells were treated with (lanes 7 and 8) or without NDF (25 ng/ml) for indicated lengths of time, and labeled with ^{35}S-methionine during the last 3 h of treatment. Cell extracts were analyzed by 10% SDS-PAGE. Numbers on the left indicate positions of molecular weight markers.

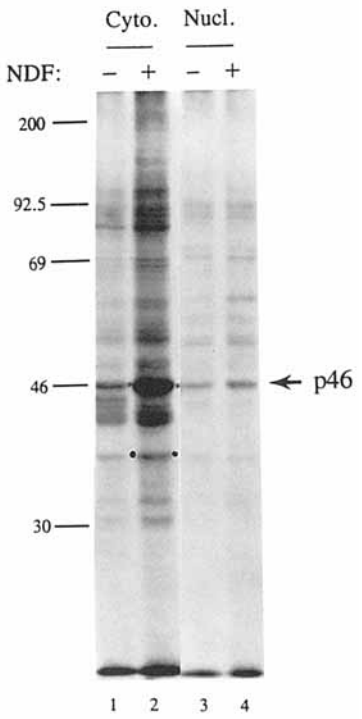

Fig. 7. Localization of NDF-induced p46. MCF-7 cells were treated with or without NDF (25 ng/ml) for 6 h, and labeled with ^{35}S-methionine during the last 3 h of treatment. Cytosolic (Cyto) and nuclear (Nucl) cell extract were made by using hypertonic buffer (without detergent). Fractionated cell extracts were analyzed by 10% SDS-PAGE. *Arrow* indicates the position of p46. Position of NDF-induced major p46 is indicated on the right. Numbers on the left indicate positions of molecular weight markers. The experiment shown here is representative of three experiments with similar results. Quantitation of p46 was also performed by measuring the radioactivity associated with specific protein bands. Note that the free radioactivity in *lane 2* was 1.5 fold that of *lane 1.*

reduced the levels of ER and PR (a functional marker for ER expression), and this was accompanied by specific early induction of expression of a 46 kD protein p46. In addition, overexpression of HER2 in MCF-7 cells was also associated with the inhibition of ER and PR expression. The observed inhibition of ER expression by NDF and/or HER2 may provide the biochemical basis of previously known reduced sensitivity of HER2 overexpressing MCF-7 cells to Tamoxifen [Benz et al., 1992]. The finding that NDF inhibited the expression of ER in MCF-7 cells which has normal levels of HER2 is important as it suggests that a growth factor such as NDF, in addition of HER2, can also contribute to development of Tamoxifen resistance (Table I). This implies that down-regulation of ER may, at least, in part, may account for earlier observed reduced sensitivity of MCF-7 cells under condition of HER2 overexpression, and NDF treatment (this study). In this context, it is important to note that recently heregulin which is homologous to NDF has been shown to stimulate the growth of breast cancer cells in an estrogen-independent manner and also down-regulate the ER expression [Tang et al., 1995; Pietras et al., 1995]. Moreover, data from the literature also suggest that the development of hormone-independence of breast cancer cells could result from the loss of ER [Murphy et al., 1990], and alterations in the expression of autocrine growth factors such as transforming growth factor [Herman and Katzenellenbogen, 1994]. The mechanism of observed down-regulation of ER by NDF remains to be delineated. This may occur at the transcription level and/or post-transcription level and may also involve reduced ER mRNA stability, leading to an decrease in ER expression.

To explore the mechanism of action of NDF in MCF-7 cells, we investigated the effect of NDF on the phosphorylation and expression of cellular proteins. Our results indicated that NDF-

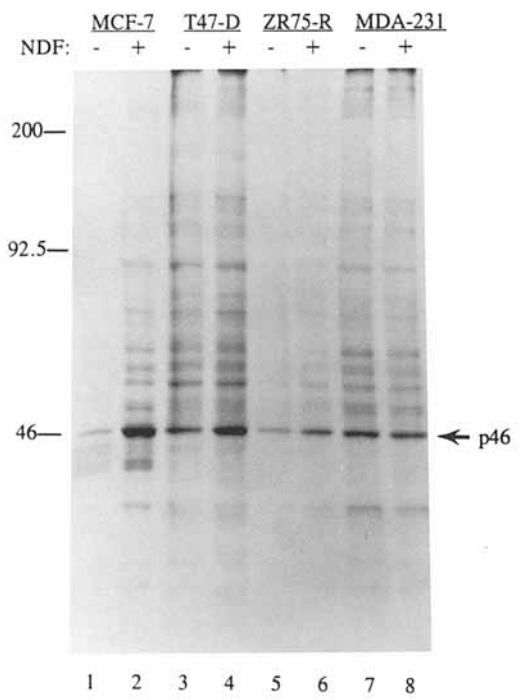

Fig. 8. Effect of NDF in other breast cancer cells. E-2 starved cells were treated with or without NDF (25 ng/ml) for 6 h, and labeled with ^{35}S-methionine during the last 3 h of treatment. Cell extracts were analyzed by 10% SDS-PAGE. Position of NDF-induced major p46 is indicated on the right. Numbers on the left indicate positions of molecular weight markers.

mediated stimulation of tyrosine phosphorylation of HER2 was associated with the transient activation of tyrosine phosphorylation of a 23 kD (p23) protein. We do not know the precise role(s) of p23 in the action of NDF. It is possible that the transient activation of p23 may activate some unidentified pathway(s) including the p46 that may in-turn be responsible for the observed down-regulation of ER in NDF-treated cells.

NDF but not EGF or E2 induces the stimulation of expression of p46 primarily by enhancing its synthesis. This effect could be blocked (to a significant extent, 65%) by an inhibitor of transcription suggesting that p46 is a NDF-inducible gene product. The precise role(s) of NDF-inducible p46 remains to be delineated. Since NDF (or homologous heregulin) is known to also induce the growth of ER negative cells such as SKBR-3 [Holmes et al., 1992], and the fact that NDF does not enhance p46 expression in ER negative cell lines, it is possible that NDF-inducible p46 may have a role(s) in the biology of ER positive breast cancer cells and could be closely related with the down-regulation of ER. This view is further supported by the observed temporal relationship between the induction of p46 induction and down-regulation of ER.

Our finding of enhanced expression p46 by NDF in ER positive breast cancer cells raises a number of new issues regarding the mechanism(s) of regulation of p46 and its implication in modifying the biology of ER positive breast cancer cells: Why is NDF-mediated induction of p46 restricted to ER positive breast cancer cells? What is the mechanism(s) of p46 induction by NDF? Is p46 related to already known NDF-responsive proteins? Is there any role(s) of transient activation of tyrosine phosphorylation of p23 in the regulation of p46 in NDF-treated cells? What is the mechanism of ER down-regulation by NDF and participation of p46? Is p46 is physically associated with ER? Further investigation is needed to explore these possibilities, and such efforts are underway.

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