

Epidermal growth factor-induced nuclear factor κ B activation: A major pathway of cell-cycle progression in estrogen-receptor negative breast cancer cells

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The epidermal growth factor (EGF) family of receptors (EGFR) is overproduced in estrogen receptor (ER) negative (–) breast cancer cells. An inverse correlation of the level of EGFR and ER is observed between ER– and ER positive (+) breast cancer cells. A comparative study with EGFR-overproducing ER– and low-level producing ER+ breast cancer cells suggests that EGF is a major growth-stimulating factor for ER– cells. An outline of the pathway for the EGF-induced enhanced proliferation of ER– human breast cancer cells is proposed. The transmission of mitogenic signal induced by EGF–EGFR interaction is mediated via activation of nuclear factor κ B (NF- κ B). The basal level of active NF- κ B in ER– cells is elevated by EGF and inhibited by anti-EGFR antibody (EGFR-Ab), thus qualifying EGF as a NF- κ B activation factor. NF- κ B transactivates the cell-cycle regulatory protein, cyclin D1, which causes increased phosphorylation of retinoblastoma protein, more strongly in ER– cells. An inhibitor of phosphatidylinositol 3 kinase, Ly294–002, blocked this event, suggesting a role of the former in the activation of NF- κ B by EGF. Go6976, a well-characterized NF- κ B inhibitor, blocked EGF-induced NF- κ B activation and up-regulation of cell-cycle regulatory proteins. This low molecular weight compound also caused apoptotic death, predominantly more in ER– cells. Thus Go6976 and similar NF- κ B inhibitors are potentially novel low molecular weight therapeutic agents for treatment of ER– breast cancer patients.

Steroid hormones 17 β -estradiol (E2) and progesterone, as well as growth factors, regulate growth of estrogen receptor (ER) positive (+) breast cancers (1). The pathway for the E2-induced cell proliferation is extensively studied and well defined (1–4). E2 interaction with its receptor (ER) initiates a sequence of events leading to the modulation of expression of genes presumably responsible for enhanced proliferation of mammary epithelial cells. In cells, ER exists in an inactive state as a complex with hsp90 (5). Binding of E2 releases the inhibitory protein, giving ER an active configuration that initiates downstream association with auxiliary proteins and interaction with its response element, ERE. This ERE–ER interaction leads to the expression of hormone-responsive genes (6–8). In some tissues, antihormones such as tamoxifen bind but cannot confer active configuration to ER, thereby blocking subsequent downstream events. Thus antihormones are suitably used for therapy of ER+ breast cancer patients (9–11), although only 60% of these patients respond to antihormones.

In contrast to ER+, the ER negative (ER–) breast cancers that constitute about 30% of the total lack the E2-ER-ERE-mediated hormone-dependent cell-proliferation pathway. An alternative regulatory pathway for the “acquired growth stimulation autonomy” (12) for ER– breast cancer cells is not clearly defined. Overexpression of the epidermal growth factor (EGF) family of receptors (EGFR) in ER– cells has been the basis for the implication of EGF-induced mitogenic signal for the enhanced proliferation of these cancer cells (13–16). An inverse correlation of ER and the EGFR levels between ER+ and ER– breast cancer cells has been demonstrated (17–20). However, events downstream of the EGF–EGFR interaction are not clearly defined in ER– breast cancer cells. The ras-signaling

pathway is implicated for mutated Neu-induced activation of transcription factors Ets, Ap-1, and NF- κ B in NIH 3T3 cells (14). EGF modulates the expression of many cell growth-related genes, some of which contain NF- κ B motifs (21–24). The phosphatidylinositol 3 (PI3) kinase pathway is involved in the transmission of the mitogenic signal of many growth factors (12, 25–28). NF- κ B controls cell-cycle progression by modulating action of cell-cycle regulatory proteins (22, 23, 29).

All these observations, supported by the established “gate-keeper” role of human retinoblastoma (Rb) (30) and p53 (31), are consistent with a hypothesis predicted several years back by Pardee (32, 33) defining the classical “restriction point” in cell-cycle progression of mammalian cells (34). Although EGF has been shown to induce activation of NF- κ B in human epidermal carcinoma cell line A431 (35), osteoblastic MC3T3-E1 cells (36), and rat aortic smooth muscle cells (37), its role in EGFR overexpressing ER– human breast cancer cells, as well as its significance and the molecular basis for the enhanced proliferation of these cells, has not been elucidated.

In this report, we propose the pathway of EGF–EGFR-initiated signal transduction by identifying and linking the intermediary molecules for the autonomous growth phenotype of EGFR overexpressing ER– cells (Fig. 1). Furthermore, a target-directed potential therapeutic approach for ER– human breast cancer patients with NF- κ B inhibitors is proposed.

Materials and Methods

Cell Lines and Growth Conditions. The ER– MDA-MB-231, MDA-MB435, and BT549, and ER+ MCF-7 and T47D breast cancer cell lines were obtained from American Type Culture Collection. Growth conditions of these cells have been described previously (38). The rich medium, designated by R, is DMEM supplemented with 10% FBS, and the basal medium, designated by B, is DMEM supplemented with 10% FBS stripped with dextran-coated charcoal (HyClone) (38, 39).

Materials. Anti-human ER- α antibody (SC543), anti-EGFR antibody (SC-03-G), anti-human Neu antibody (SC-284-G), and anti-human cyclin D1 (ccD1) antibody (Sc 8396) were obtained from Santa Cruz Biotechnology. Mouse monoclonal IgG raised against Rb protein (14001A) was obtained from PharMingen, and rabbit polyclonal IgG raised against the conserved region of actin was obtained from Sigma–Aldrich. The complementary strands of the oligonucleotide (5′-TCGACAGGGACTTTC-

Abbreviations: E2, 17 β -estradiol; ER, estrogen receptor; ER–, ER negative; ER+, ER positive; ERE, ER response element; EGF, epidermal growth factor; EGFR, EGF family of receptors; PI3, phosphatidylinositol 3; Rb, retinoblastoma; pRB, phosphorylated retinoblastoma; ccD1, cyclin D1; EMSA, electrophoretic mobility-shift assay; EGFR-Ab, EGFR antibody; IKK, I κ B kinase; IKK-M, IKK mutants.

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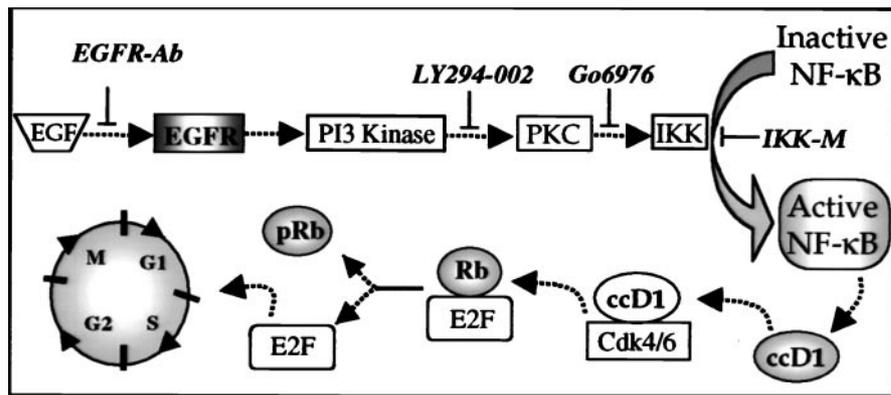


Fig. 1. A proposed pathway of EGF-induced cell proliferation of ER⁻ breast cancer cells. The interaction of EGF with EGFR and the proposed downstream events of NF- κ B activation and cell-cycle progression are schematically shown. The shaded molecules are monitored. The sites of action of agents used to block individual steps of this pathway, such as EGF-EGFR interaction by EGFR-Ab, PI3 kinase (phosphatidylinositol 3-kinase) activity by Ly294-002, PKC (protein kinase C) activity by G06976, and IKK activity by IKK-M (dominant-negative mutant of IKK) are shown. The role of G₁-specific cell-cycle regulatory proteins, ccD1 and phosphorylated retinoblastoma (pRb), and unphosphorylated Rb is illustrated.

CGAGAG-3') containing the NF- κ B motif (boldface) were custom synthesized by Integrated DNA Technologies (Coralville, IA). These strands were annealed to generate the double-stranded NF- κ B oligonucleotide, end labeled with ³²P-ATP (NEN) and T4 kinase (New England Biolabs), as described previously, and used for electrophoretic mobility-shift assay (EMSA) (38, 39). E2, hydrocortisone, insulin, DTT, DMSO, and phenylmethylsulfonyl fluoride were obtained from Sigma. The Hybond nitrocellulose membrane and enhanced chemiluminescence immunodetection kit were obtained from Amersham Pharmacia. Go6976 and Ly294-004 were purchased from Calbiochem-Novabiochem.

Methods: Preparation of Nuclear Extracts and Quantitation of NF- κ B

³²P-DNA-Binding Activity. Nuclear extracts from cells were prepared as described by Dignam *et al.* (40). The DNA-binding activity of NF- κ B was determined by EMSA as described previously (38-39, 41). The DNA-protein complex was detected as a retarded radioactive band by autoradiography of the dried gel (6, 7). This was characterized by competition experiments with nonradioactive wild-type or mutant double-stranded NF- κ B oligonucleotide and by interaction with anti-p65 and p50 antibodies that caused a supershift of the ³²P-DNA-NF- κ B complex. Results of this type of characterization of NF- κ B-DNA complex have been reported and are not included here (6, 38-39). The intensities of the autoradiographic signals were quantitated by scanning with an imaging densitometer (Bio-Rad, model GS-700), followed by integration of the signals with the program MULTI-ANALYST Ver. 1.0.2. (Molecular Dynamics). Integrated intensities of the autoradiographic signals of the complex (arbitrary numbers) are presented as DNA-binding activity.

Western Blot Analysis. The level of ER, EGFR, Neu, ccD1, and phosphorylated and unphosphorylated Rb protein was determined by Western blot transfer analysis followed by immunoprobining with respective antibodies and was detected by the enhanced chemiluminescence system (42).

Cell Viability. Cell viability and sensitivity of the cells to different compounds were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (43). Cells (1×10^4) were plated in duplicate in 96-well cloning tissue culture dishes in specified media. After 48 h, the medium was changed, and Go6976 was added at the desired concentration every 2 days along with fresh medium. After the specified days of growth,

viability of cells was measured (43) and was expressed as percentage of the untreated control cells grown in the presence of the same concentration of the solvent (DMSO).

Results

Levels of ER and EGFR Family Receptors in Breast Cancer Cells. The level of ER protein in the extracts of ER⁻ and ER⁺ cells was determined by Western blot analysis by using an anti-ER antibody (Fig. 2A, row 1). As expected, ER was undetectable in ER⁻ MDA-MB231 (lanes 1-2) and MDA-MB435 (lanes 3-4) cells and was detected in ER⁺ cell lines, T47D (lanes 5-6), and MCF-7 (lanes 7-8). When the same blot was reprobed with anti-EGFR (Fig. 2A, row 2) and anti-Neu (Fig. 2A, row 3) antibodies, both of these receptor proteins were detected in ER⁻ MDA-MB231 (lanes 1-2) and MDA-MB435 (lanes 3 and 4) cells. Quantitation by densitometry showed that the EGFR and Neu protein levels in ER⁻ cells were 5- to 10-fold more than that in ER⁺, T47D (lanes 5 and 6), and MCF-7 (lanes 7 and 8) cells when normalized to actin (row 4). These results demonstrate an inverse relationship of ER and EGFR expression between ER⁺ and ER⁻ breast cancer cell lines, as reported previously with ER⁺ and ER⁻ human breast tumors (17, 18, 44).

EGF-Induced Activation of NF- κ B. The role of NF- κ B in the transmission of EGF-EGFR-induced mitogenic signal was established by determining the level of active NF- κ B in EGF-treated ER⁻ and ER⁺ breast cancer cells by EMSA (6, 38, 39). The active NF- κ B in cells grown in basal medium (B) was markedly reduced in comparison to cells grown in rich medium (R), suggesting the presence of NF- κ B activating factors in FBS-supplemented rich medium (Fig. 2B).

Although the basal level of NF- κ B activation was similar, the EGF-induced level of active NF- κ B was significantly greater in ER⁻, MDA-MB-435, and MDA-MB231 vs. ER⁺, MCF-7 cells (Fig. 2C). The selectively high induction level of NF- κ B in all three ER-negative cells, MDA-MB231, MDA-MB435 (Fig. 2C), and BT549 (Fig. 2D and E), correlates with the markedly elevated levels of EGFR and Neu in these cells (Fig. 2A). Quantitation revealed that stimulation was more than 10-fold for ER⁻ MDA-MB231 and MDA-MB435 cells vs. 2- to 3-fold in ER⁺ MCF-7 cells (Fig. 2C). These results qualify EGF as one of the NF- κ B-activating factors that are present in unstripped FBS (in rich medium) and establish the inducible nature of the activation of NF- κ B in EGFR-overexpressing ER⁻ breast cancer cells rather than constitutive, as reported by Nakshatri *et al.*

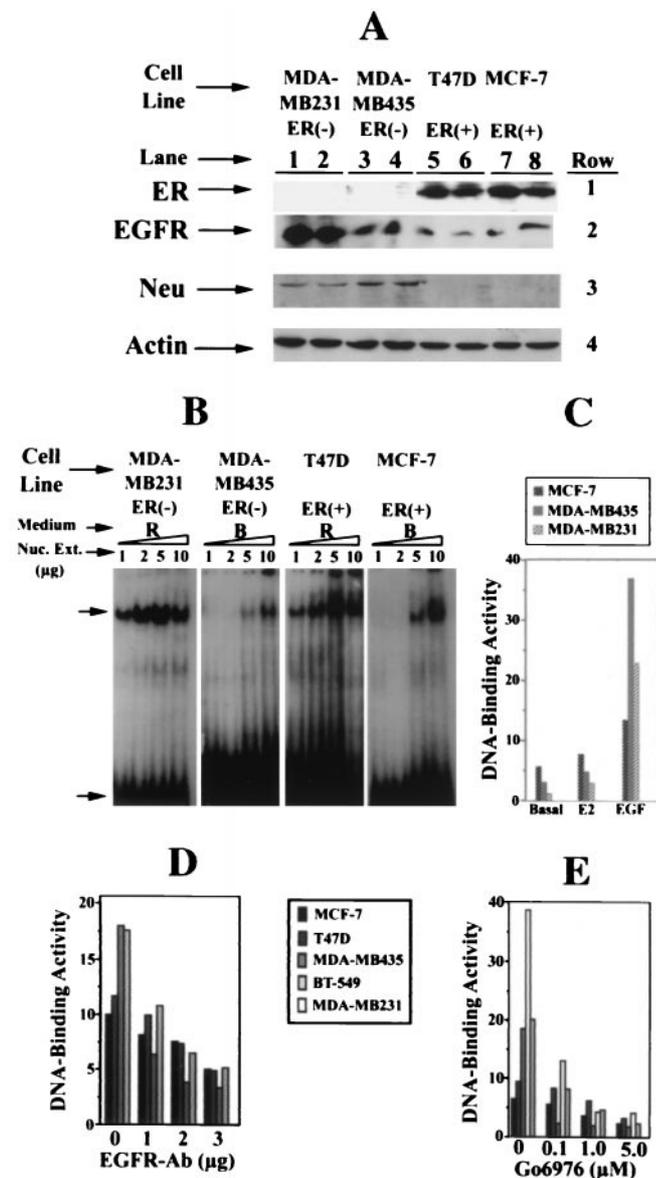


Fig. 2. Levels of ER and EGFR family receptors and activation and inhibition of NF- κ B in breast cancer cells. **A** shows the levels of ER, EGFR, Neu, and actin proteins in whole-cell extracts of ER- MDA-MB435 and MDA-MB231 and ER+ T47D and MCF-7 breast cancer cells in culture, as measured by Western blot analysis. Cells were grown in rich (R) medium to 90–95% confluency, whole-cell extracts were prepared (6), and 50 μ g of protein in samples (in duplicate, designated by numerals under each cell line) was subjected to Western blot analysis and immunodetected with anti-ER-antibody Sc-543 (row 1) (42). The same blot was stripped and reused for detection of EGFR with anti-EGFR antibody Sc-03-G (row 2). Row 3 shows the level of Neu detected similarly with the anti-Neu antibody (Sc 284 G), and row 4 shows the levels of actin in the same samples as determined by reprobing the same blot with antiactin antibody, which serves as a loading control. These determinations were made three times, and results of one experiment are shown here. **B** shows the level of 32 P-DNA-binding activity of NF- κ B in the indicated amounts (protein) of nuclear extracts from ER- MDA-MB231 and ER+ MCF-7 cells grown in rich medium (R) or basal medium (B), as measured by EMSA (38–39). The retarded specific NF- κ B- 32 P-DNA complex is indicated by the upper arrow, and the free 32 P-DNA (NF- κ B-oligonucleotide) is indicated by the bottom arrow. **C** shows stimulation of NF- κ B- 32 P-DNA-binding activity by E2 and EGF. The binding activity (numerals on the y axis) represents integrated intensity of the autoradiographic signals quantitated, as described in *Materials and Methods*. The ER- MDA-MB231 and MDA-MB435 and ER+ MCF-7 cells were plated in 25 ml of rich medium in 150-mm tissue culture dishes. Forty-eight hours later, the medium was removed, and cells were washed with basal medium (B) and replenished with 25 ml of the same medium. Seventy-two hours later, the

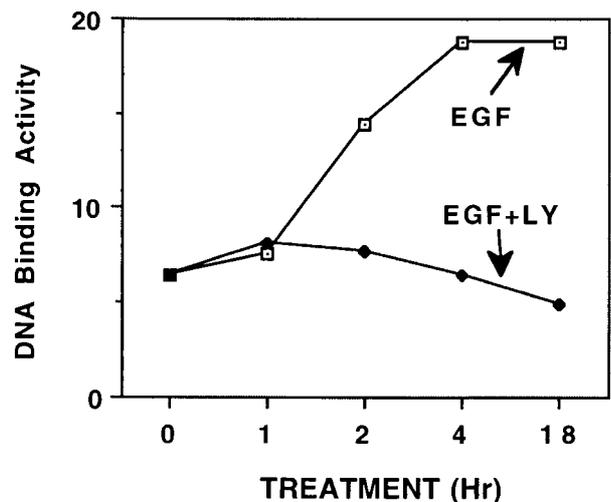


Fig. 3. Kinetics of EGF-induced activation of NF- κ B and inhibition by Ly294-002. The growth of ER- MDA-MB231, nuclear extract preparation, conditions for EGF treatment (12 ng/ml), and measurements of NF- κ B- 32 P-DNA-binding activity are the same as described in Fig. 2C. The nuclear extracts from cells treated with EGF alone (indicated by EGF) for the specified time period or EGF and Ly294-002 (100 nM) together (indicated by EGF + LY) were prepared and similarly analyzed.

(45). This discrepancy may be explained by the growth conditions used by Nakshatri *et al.* (45), i.e., the use of growth medium with unstripped FBS that presumably contains NF- κ B-activating factors. The DNA-binding activity of NF- κ B was not significantly affected by E2 treatment in either ER- or ER+ cells grown in basal medium, suggesting that E2-ER-mediated actions do not involve NF- κ B activation (Fig. 2C).

Inhibition of EGF-Induced NF- κ B Activation. The EGF-induced activation of NF- κ B was reduced in a concentration-dependent manner by pretreatment of the cells with anti-EGFR-Ab (Fig. 2D). Three micrograms decreased the stimulation to the basal level (Fig. 2D). Inhibition by EGFR-Ab was greater, like the more prominent stimulation by EGF, in ER- than in ER+ cells (Fig. 2D). These results demonstrate that NF- κ B activation is a consequence and downstream event of EGF-EGFR interaction, and that it occurs to a greater extent in ER- breast cancer cells, probably because of the large amount of EGFR in these cells.

The intermediary role of NF- κ B in the EGF-mediated signal transduction pathway was further illustrated by blocking these

medium was removed and replenished with 25 ml of basal medium, and cells were grown for an additional 12 h in the presence of either E2 (10^{-6} M) or EGF (12 ng/ml). Nuclear extracts from the treated and control cells were prepared (40), and NF- κ B- 32 P-DNA-binding activities in 5 μ g of nuclear extracts of these samples were measured by EMSA. One of four such experiments is reported here. **D** shows the NF- κ B- 32 P-DNA-binding activity in nuclear extracts (5 μ g) from the four breast cancer cell lines grown in basal medium plus EGF (12 ng/ml) and indicated amounts of anti-EGFR-antibody per 10 ml of basal medium for 12 h. Growth and treatment conditions of the cells were the same as described in **C**. NF- κ B- 32 P-DNA-binding activity was determined in nuclear extracts from two ER- MDA-MB-435 and MDA-MB231 and two ER+, MCF-7, T47D cells by EMSA and quantitated, as described above. **E** shows similar analysis for the determination of NF- κ B- 32 P-DNA-binding activity in nuclear extracts of cells treated with indicated concentrations of G6976. Growth and treatment conditions of cells are the same as described in **C**. Nuclear extracts from three ER- and two ER+ cells were prepared and subjected to EMSA. Quantitation of the autoradiographic signals of the NF- κ B- 32 P-DNA complex was the same as described above.

events with a well-characterized NF- κ B inhibitor (38, 46–50), which should block downstream events, specifically in ER– cells (Fig. 2E). Go6976 inhibits protein kinase C activity (46–50) and thereby inactivates NF- κ B (Fig. 1). Treatment with Go6976 in the presence of EGF reduced the EGF-stimulated NF- κ B DNA-binding activity more in the three ER– cells than in the two ER+ cells and depended on the concentration of the drug (Fig. 2E).

Kinetics of EGF-Induced NF- κ B Activation. Kinetic studies revealed that EGF-induced elevated NF- κ B DNA-binding activity could be detected after about 1 h, was significantly elevated after 2 h, and reached a maximum at about 4 h, persisting at this level after 18 h of treatment with 12 ng/ml (Fig. 3). Treatment of the ER– MDA-MB231 cells simultaneously with an established PI3 kinase inhibitor (Ly294–002) blocked EGF-induced activation of NF- κ B, suggesting the involvement of PI3 kinase in this signal transduction pathway (Figs. 1 and 3).

We chose to use a comparatively lower concentration of EGF (12 ng/ml) for a treatment period longer than that of others (26), which enabled us to study distant downstream events such as NF- κ B activation, modulation of cell-cycle regulatory proteins, and cell-cycle progression. Different experimental conditions and also the use of a different working system, such as breast cancer cells vs. NIH 3T3 cells, may explain the delay in the observed EGF-induced signals. NF- κ B activation and the subsequent downstream events all were detected between 2–4 h of EGF treatment (data not shown).

The Role of Cell-Cycle Regulatory Proteins in EGF Signal Transduction.

The level of ccD1, a key regulatory protein of G₁/S progression, is modulated by the interaction of NF- κ B with its motif in the ccD1 promoter (22, 23, 51, 52). Treatment of ER+ MCF-7 and ER– MDA-MB231 and MDA-MB435 cells with EGF elevated the level of ccD1 5-fold in ER– (Fig. 4A, row 1) and about 1.5-fold in ER+ cells vs. untreated cells (designated by 0). Elevations of ccD1 by EGF correlated with the increased levels of EGFR family receptors and stimulation of NF- κ B-DNA-binding activity in these cells. Treatment of ER+ MCF-7 cells with E2 stimulated ccD1 level by 2- to 3-fold over the basal level and, as expected, ccD1 level in ER– MDA-MB231 and MDA-MB435 cells was unaffected by E2 (Fig. 4A, row 1).

Pretreatment of the cells with EGFR-Ab blocked EGF-induced up-regulation of ccD1 in ER– MDA-MB231 (Fig. 4C, row 1 Left) and MDA-MB435 (Fig. 4D Left). A much smaller effect was seen in ER+ MCF-7 cells (Fig. 4B, row 1 Left). Greater levels of overexpression and inhibition of ccD1 occurred in ER– cells, which can be correlated with the overexpression of EGFR and stimulation and inhibitory pattern of NF- κ B by EGF and EGFR-Ab, respectively.

Treatment of the EGF-stimulated cells with the NF- κ B inhibitor Go6976 also reduced the levels of ccD1 in ER– MDA-MB231 (Fig. 4C, row 1 Right), MDA-MB435 (Fig. 4D, row 1 Right) and to a lesser degree in ER+ MCF-7 cells (Fig. 4B, row 1 Right), supporting the role of NF- κ B activation in up-regulation of this cell-cycle regulatory protein.

Results with one ER– cell line (MDA-MB435) demonstrated complete inhibition of EGF-induced Rb phosphorylation (a target of ccD1) even at the lowest concentration of 0.1 μ M Go6976 (Fig. 5, row designated by pRb). Pretreatment of the cells with anti-EGFR-Ab along with EGF also blocked Rb phosphorylation (Fig. 4, row designated by pRb). These results confirm that NF- κ B activation is an intermediary step, and activation of ccD1 and Rb phosphorylation are downstream consequences of NF- κ B activation in the EGF-EGFR signal transduction pathway for enhanced cell proliferation. These observations were further substantiated by results obtained with dominant-negative I κ B kinase (IKK) mutants (IKK-M) (Fig.

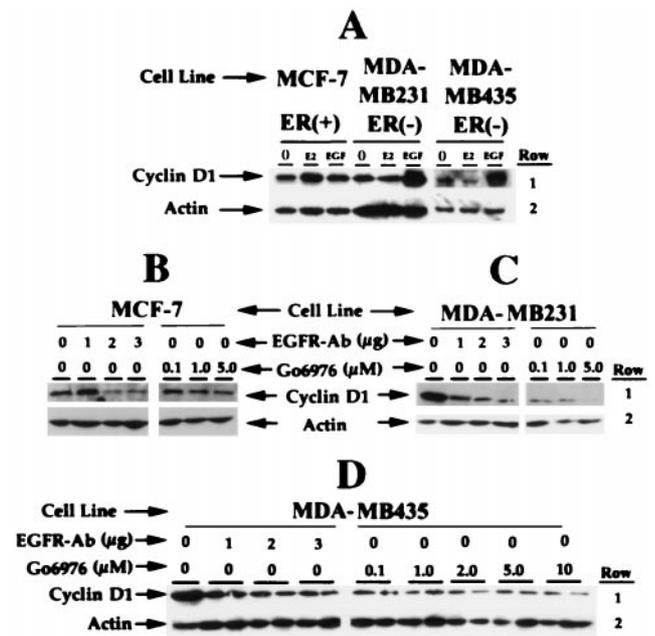


Fig. 4. Level of ccD1 in EGF-treated cells and inhibition by EGFR-Ab and Go6976. A shows the levels of ccD1 in EGF (12 ng/ml) and E2 (10^{-6} M)-treated ER– and ER+ breast cancer cells. Growth conditions of the cells were the same as described in the legend to Fig. 2. The ER– MDA-MB231 and MDA-MB435 and ER+ MCF-7 cells were grown in basal medium in the presence of EGF (12 ng/ml) or E2 (10^{-6} M) for 12 h. Whole-cell extracts were prepared, and their level of ccD1 protein in 50 μ g was determined by Western blot analysis. The membranes with blot-transferred fractionated proteins were then subjected to detection with anti-ccD1-antibody (Sc 8396) and enhanced chemiluminescence immunodetection system, as described (42). The same blot was stripped and reprobed with antiactin antibody, which serves as loading control. These experiments were repeated three times. The levels of ccD1 (row 1) in EGF-stimulated ER+ MCF-7 (B), ER– MDA-MB-231 (C), and ER– MDA-MB-435 (D) cells, simultaneously treated with indicated amounts of EGFR-Ab (micrograms per 10 ml) or Go6976 (μ M) are shown. Fifty micrograms of whole cell extract proteins is used in each lane. Duplicate samples were analyzed in the case ER– MDA-MB-435 cells (D). Lanes designated by Actin (row 2) (A–D) represent the results of reprobings of each of these membranes with antiactin-Ab, which served as loading controls.

5B). Transfection of ER– MDA-MB-231 cells with IKK α -M or IKK β -M expression vectors (53) not only blocked NF- κ B activation but also decreased EGF-induced elevated levels of ccD1 (Fig. 5B).

Differential Sensitivity of ER– and ER+ Cells to the NF- κ B Inhibitor Go6976.

A differential sensitivity to Go6976 by ER– and ER+ breast cancer cells was observed (Fig. 6). The ER– cells were more sensitive and less viable after treatment with this NF- κ B inhibitor. The IC₅₀ (50% killing) by Go6976 was 1 μ M for ER– MDA-MB435 and MDA-MB231 breast cancer cells, whereas it was greater than 10 μ M for ER+ MCF-7 and T47D or the normal mammary epithelial H16N (54) cells (Fig. 6). At 10 μ M Go6976, about 80% of the ER– cells were killed, whereas only 15–30% of ER+ and normal H16N cells were sensitive to this compound. The relative resistance of the H16N normal human mammary cells indicates a possible high therapeutic index of Go6976 against ER– cancer cells.

This observation is consistent with the previously observed role of NF- κ B as an antiapoptotic agent. FACS analysis demonstrated accumulation of sub-G₁ population (67%) in Go6976-treated (48 h at 1 μ M) ER– vs. only 10–15% in ER+ cells, indicating enhanced apoptotic cell death preferentially of ER– cells caused by this low molecular weight compound.

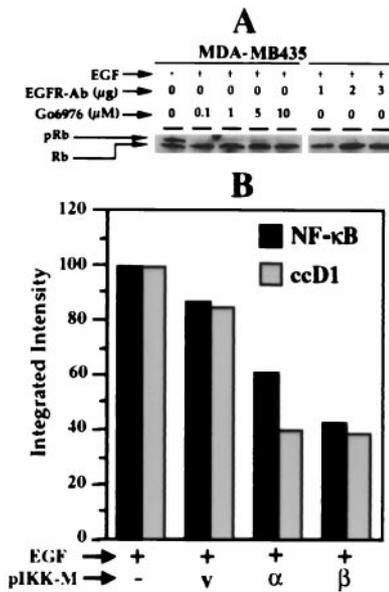


Fig. 5. (A) Level of pRb protein in EGF, EGFR-Ab, and Go6976-treated ER- MDA-MB435 cells. Cells were grown in basal medium in the presence of EGF (12 ng/ml) and EGFR-Ab or Go6976 at the indicated concentrations for 12 h. The level of pRb and underphosphorylated Rb (Rb) in 50 μg of whole-cell extracts was detected by Western blot analysis and immunodetection with anti-Rb-antibody, as described above. (B) NF-κB DNA-binding activity and ccD1 level in IKK-M-transfected ER- MDA-MB435 cells. Results presented demonstrate the levels of NF-κB activation and ccD1 in EGF-treated ER- MDA-MB435 cells transiently transfected with dominant-negative IKK-α (α), IKK-β (β) mutants (IKK-M), and vector control (v) (53). Growth conditions and EGF treatment are as described in A. Cells were transfected with indicated plasmids (10 μg) by using Superfect Transfection Reagent (Qiagen, Chatsworth, CA) following the protocol of the supplier. Cells in fresh basal medium (B) were grown for an additional 48 h after transfection. EGF was added 4 h before harvesting, nuclear extracts were prepared, and the level of NF-κB-binding activity and ccD1 was measured as described above.

Discussion

The mode of action of E2 in ER+ breast cancer cells has been extensively studied and is well characterized (2, 3, 7, 8). In contrast, the pathway of autonomous cell growth of ER- breast cancer cells is not clearly defined. The goals of this investigation are based on the EGFR-overexpression phenotype of ER- cells. These goals are: (i) to establish the downstream events of EGF-EGFR interaction-induced mitogenic signal for the highly aggressive growth phenotype of ER- breast cancer cells, and (ii) to identify novel targets in the signal transduction pathway for intervention with a low molecular weight substance, which could potentially be used therapeutically for ER- breast cancer patients.

Although the EGF-EGFR-induced cell proliferation pathway (Fig. 1) was detected in both ER+ and ER- cells, this is secondary to the E2-ER-ERE pathway in the former and operates at a much lower level than in the latter type of cells. Correlative observations led us to propose that EGF is the driving force for the autonomous growth of ER- breast cancer cells via the EGF-EGFR-initiated signal transduction pathway, a proposed scheme of which is outlined in Fig. 1. The levels of the shaded molecules were monitored, and their role in the EGF-EGFR-induced cell proliferation pathway was elucidated. The mitogenic effect of EGF is initiated by interaction with the overexpressed specific receptors, transmitted via activation of PI3 kinase and protein kinase C-mediated and IKK-dependent activation of NF-κB. The activated NF-κB then up-regulates the expression of the cell cycle regulatory ccD1 gene that induces

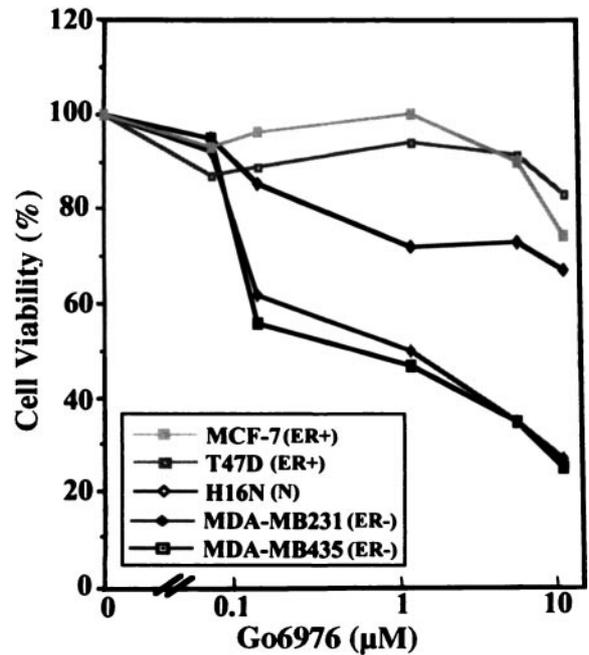


Fig. 6. Sensitivity of ER- and ER+ breast cancer cell lines to Go6976. Sensitivity to Go6976 of ER- MDA-MB435 and MDA-MB231 and ER+ MCF-7 and T47D breast cancer cells and the immortalized normal human mammary epithelial cells H16N (54) grown in rich medium was determined by measurement of the number of viable cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay after treatment with different concentrations of the drug for 7 days (43). Details of growth conditions are described in *Materials and Methods*. Results of the average of duplicate samples of one of the three experiments are shown here. The number of cells in drug-treated samples was expressed as percent of the untreated control, designated as 100%.

phosphorylation of Rb and cell-cycle progression. This hypothesis was experimentally tested by blocking EGF action with anti-EGFR-Ab and with well-characterized inhibitors of PI3 kinase and NF-κB, as shown in Fig. 1.

That NF-κB is an intermediary in the transmission of the EGF-EGFR interaction-induced signal is supported by using a well-characterized NF-κB inhibitor, Go6976, whose action is mediated by inhibition of protein kinase C (38, 46-50). This synthetic compound inhibited NF-κB activation and also blocked the downstream events of NF-κB-dependent up-regulation of cell-cycle regulatory proteins.

Involvement of PI3 kinase in the mitogenic effect of other growth factors in different cell systems has been reported (26-27, 55-57). The pathway linking all of the downstream events of PI3 kinase activation, such as NF-κB activation leading to modulation of the cell-cycle regulatory proteins in EGFR-overexpressing ER- cells, has not been demonstrated previously and is illustrated in this study.

Sixty percent of ER+ breast cancers are hormone responsive, antihormone sensitive, and successfully treated with antihormones (4, 10). On the basis of the essential role of the calcium-binding protein calmodulin (CaM) in a downstream event of the E2-ER-ERE pathway, we proposed that hormone-resistant ER+ breast cancer patients may be responsive to the CaM-antagonists (6). Currently a target-directed therapy for ER- breast cancer patients with an antibody to the EGFR family receptors is being used, but this approach showed limited success because of its high molecular size (16, 58).

The practical outcome of this study is the identification of NF-κB as a target for therapy of ER- breast cancer patients with low molecular weight compounds. A compound like Go6976

showed differential lethal effects on ER⁻ vs. ER⁺ and immortalized normal cells, being more lethal to the former than the latter classes of mammary epithelial cells. Our results suggest that Go6976 caused apoptotic death of EGFR-overexpressing ER⁻ breast cancer cells. Although Go6976 showed differential toxicity for ER⁻ vs. ER⁺ breast cancer and normal epithelial cells in culture, its therapeutic efficacy needs to be further examined in an experimental animal-model system. However, results presented in this investigation demonstrate an encouraging greater sensitivity to this compound of ER⁻ breast cancer cells than the ER⁺ breast cancer cells or immortalized normal mammary epithelial cells (H16N). We propose that NF- κ B is a

suitable target, and Go6976 or similar compounds with NF- κ B-inhibitory activity are potentially novel therapeutic agents for ER⁻ breast cancer patients.

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