Epidermal Growth Factor-mediated Apoptosis of MDA-MB-468 Human Breast Cancer Cells

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Abstract

MDA-MB-468 human breast cancer cells lack estrogen receptors, over-express epidermal growth factor (EGF) receptors, and are growth inhibited by EGF. We show that treatment of MDA-MB-468 cells with EGF leads to inhibition of cell proliferation, fragmentation of DNA into nucleosomal oligomers, and the development of apoptotic morphology. This treatment is associated with increased expression of c-myc, c-fos, jun family members, and transforming growth factor \( \beta_1 \) mRNA and with partial proteolytic cleavage of poly(ADP-ribose) polymerase and lamin B. The observation that EGF can mediate apoptosis in EGF receptor-over-expressing cells has important implications for clinical efforts directed at the EGF receptor.

Introduction

The EGFR\(^3\) is a \( M_r \) 170,000 transmembrane glycoprotein that is overexpressed in a number of human malignancies including cancers of the lung, head and neck, pancreas, brain, bladder, and breast (1). Most human breast cancer cell lines express EGFR at a level that is inversely proportional to the level of expression of ER (2, 3). MDA-MB-468 human breast cancer cells are ER negative and overexpress the EGFR as a result of gene amplification (4). While EGF and TGF-\( \alpha \), the natural ligands for the EGFR, have potent growth-promoting effects in many tissues including mammary epithelium (5, 6), MD-MB-468 cells, which express ~2 \( \times \) 10\(^6\) EGF binding sites per cell, are growth inhibited by EGF (4). There is generally an inverse relationship between EGFR number and EGF-induced mitogenesis such that relatively low expression of EGFR is associated with growth stimulation by EGF, while high EGFR expression, as in MDA-MB-468 cells, is associated with growth inhibition by EGF (7, 8). The mechanism of this EGF-induced growth inhibition is not clear.

Apoptosis, or programmed cell death, occurs in a variety of tissues in response to both physiological and pathological stimuli. It may be triggered by a specific signal which acts as a positive inducer of programmed cell death or it can follow the withdrawal of a trophic factor functioning to prevent apoptosis. Of particular interest is the observation that hormone-dependent tissues undergo apoptosis upon hormone withdrawal (9, 10). This observation has been extended to estrogen-dependent breast cancer cells where estrogen ablation activates apoptosis (11). In contrast, estrogen-independent breast cancer cells do not activate apoptosis in response to estrogen withdrawal. In previous studies, we have used the MDA-MB-468 human breast cancer cell line as a model system to study the mechanism of cell death in estrogen-independent breast cancer and have documented that these cells retain the capacity to undergo programmed cell death when treated with the fluorinated pyrimidines 5-FdUrd and trifluorothymidine (12). In this study, we investigate the hypothesis that growth-inhibitory concentrations of EGF induce apoptosis of EGF-signaling MDA-MB-468 human breast cancer cells.

Materials and Methods

Materials and Cell Culture. Murine natural EGF was purchased from Gibco-BRL (Gaithersburg, MD). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). MDA-MB-468 cells obtained from Dr. M. Lippman (Vincent T. Lombardi Cancer Center, Georgetown University, Washington, DC) were grown at 37°C in 5% CO\(_2\) in improved minimal essential media with 5% fetal calf serum (both from Biofluids, Rockville, MD), and passaged weekly.

Growth Curves. Cells were plated in triplicate in media containing 5% fetal calf serum. After 24 h, EGF was added to attain the noted concentrations. Cells treated for >48 h had EGF-containing media replaced every 48 h. At the indicated time points, adherent and nonadherent cells were pooled and counted by hemocytometer. For cell viability studies, an aliquot of this cell suspension was mixed with an equal volume of 0.08% trypan blue in Hanks’ balanced salt solution, and dye exclusion was assessed visually by microscopy. Viable cell number was calculated by multiplying total cell number by fraction of cells excluding trypan blue.

Analysis of DNA Fragmentation. For qualitative assessment of DNA fragmentation, cells were treated with 10 ng EGF/ml for the indicated period of time. After treatment, media were removed, cells were washed twice with 1X phosphate-buffered saline, and fresh medium was added. After 72 h, adherent and nonadherent cells were pooled, and DNA was isolated and quantitated as described previously (12). Ten \( \mu \)g DNA were loaded into each well and electrophoresed in a 1.6% agarose gel. Gels were stained with ethidium bromide, visualized by UV fluorescence, and photographed. For morphological examination, adherent and nonadherent cells were pooled 24 h after addition of 10 ng EGF/ml. Subsequent fixation and staining with bisbenzimide were performed as described (13).

A modification (14) of the pulse field gel electrophoresis method of Stamatovic and Denko (15) was used for quantitative analysis of double-stranded DNA fragmentation. Briefly, \( \text{[}^{3} \text{H}]\)thymidine-labeled MDA-MB-468 cells were treated with 10 ng EGF/ml. After treatment, adherent and nonadherent cells were washed and suspended in a plug of low melting point agarose. Cells were then lysed and treated in situ with RNase A. The plugs were subjected to inverted pulse field gel electrophoresis to separate fragmented (\( F_1 \)) and unfragmented (\( F_2 \)) DNA. The media (\( S \)) and these gel fragments were counted to determine \( ^{3} \text{H} \) radioactivity, and the percentage of DNA fragmentation was calculated by the formula:

\[
\text{% DNA fragmentation} = \frac{S + F_1}{S + F_1 + F_2} \times 100
\]

RNA Isolation and Northern Analysis. Cells in logarithmic growth phase were treated with 10 ng EGF/ml. At the indicated time points, total cellular RNA was isolated from pooled adherent and nonadherent cells by the guanidinium isothiocyanate method of Chomczynski and Sacchi (16). For Northern analysis, 30 \( \mu \)g total RNA were denatured in formaldehyde, size-fractionated...
in 1.1% agarose-2.2% formaldehyde gels, and transferred to membranes by the method of Thomas (17). Probe preparation, hybridization conditions, autoradiography, and densitometry were performed as described previously (12, 18).

**Protein Isolation and Western Blotting**. After reduction, alkylation, and preparation for electrophoresis, 50 µg protein samples from separated adherent and nonadherent EGFrreated cells were resolved on sodium dodecyl sulfate gels and transferred to nitrocellulose. Western blotting was performed as described previously using C-2-10, a mouse monoclonal antibody that recognizes an epitope in the DNA binding domain of pADPRp (13, 19) and a chicken polyclonal antibody that recognizes mammalian lamin B (20, 21).

**Results**

**Effect of EGF on MDA-MB-468 Cell Proliferation and Viability**. We analyzed the effect of EGF treatment on MDA-MB-468 cells and found that the lowest dose of EGF tested (1 ng/ml) had no significant effect on MDA-MB-468 proliferation as compared to untreated control cells (Fig. 1). In contrast, EGF concentrations of 10 and 100 ng/ml resulted in sustained inhibition of overall cellular proliferation followed by as much as a 75% decline in viable cell number after 6 days of treatment. Of note, >90% of treated cells continued to exclude trypan blue at 72 h, indicating that the early effects of these doses of EGF were not associated with loss of membrane integrity. These findings indicate that treatment with ≥10 ng EGF/ml is not simply cytostatic for MDA-MB-468 cells but is ultimately cytotoxic. For all subsequent studies, 10 ng/ml EGF was used.

**Evidence of Apoptosis in EGF-treated MDA-MB-468 Cells**. To gain insight into the mechanism of EGF-associated cytotoxicity, we analyzed cellular DNA for the pattern of fragmentation following exposure of MDA-MB-468 cells to 10 ng/ml EGF. As can be seen in Fig. 2A, a ladder of DNA nucleosomal oligomers, indicating cleavage of chromosomal DNA at internucleosomal loci, was detected 48 h after initiation of EGF treatment and continued to be detectable at 72 h. Control cells in culture treated for an equivalent length of time without EGF failed to demonstrate DNA fragmentation. In order to obtain a quantitative comparison of DNA fragmentation after treatment of MDA-MB-468 cells with EGF, a pulse field gel electrophoresis method that exploits differences in electrophoretic mobility between intact and fragmented DNA was used. As demonstrated in Fig. 2B, control MDA-MB-468 cells spontaneously fragment less than 10% of their DNA in the first 48 h after plating. In contrast, EGF-tREATED cells manifest an increase in DNA fragmentation at 24 h, and this fragmentation progresses over time. This DNA fragmentation is associated with morphological changes of apoptosis as shown in Fig. 2D. These results demonstrate that the growth-inhibitory/cytotoxic effects of EGF in MDA-MB-468 cells are associated with changes in cell morphology, nucleosomal DNA ladder formation, and a quantitative increase in double-stranded DNA fragmentation that are all hallmarks of apoptosis.

**Expression of Immediate Early Response Genes after Treatment of MDA-MB-468 Cells with EGF or 5-FlUrd**. Since increased expression of the early response genes has been associated with growth stimulation by EGF (18, 22, 23), we examined EGF-treated MDA-MB-468 cells for expression of the early response genes. Fig. 3A demonstrates that EGF treatment is associated with 18-fold induction of c-fox, 16-fold induction of c-jun, 8-fold induction of jun B, and 6-fold induction of jun D. Peak expression of these immediate early nuclear genes was seen after 15 min of EGF treatment and returned to baseline or near-baseline levels of expression by 2 h of EGF treatment. Since expression of c-myc has been associated with programmed cell death in several models (11, 24), we examined MDA-MB-468 cells for expression of this transcript and found that EGF treatment was associated with greater than an 11-fold increase in expression of c-myc mRNA (Fig. 3B). These results demonstrate that EGF-associated gene induction in these apoptotic cells is comparable to that seen in cells stimulated to grow by EGF (17). Increased expression of the TGFβ1 gene has also been associated with programmed cell death in a variety of systems (11, 12, 25–27). Thus, expression of TGFβ1 gene was examined at various time points after...
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Discussion

The present study demonstrates that MDA-MB-468 human breast cancer cells, which overexpress EGFR and are growth inhibited by EGF, undergo programmed cell death upon exposure to EGF. The characteristic loss of cellular proliferative capacity, morphological changes, fragmentation of DNA into nucleosomal oligomers, and enhanced TGFβ mRNA expression are analogous to the changes seen following exposure of these same cells to the fluorinated pyrimidines, 5-FdUrd and trifluorothymidine (4). However, the response to EGF is characterized by a number of differences when compared with the cytotoxic response to these chemotherapeutic agents (12): (a) EGF treatment results in a greater than 6-fold induction of c-fos, c-jun, jun B, and jun D, whereas fluoropyrimidine treatment does not; (b) EGF treatment is accompanied by greater than an 11-fold increase in c-myc expression, whereas no such increase is observed following fluoropyrimidine treatment (12); and (c) EGF fails to increase the proteolytic cleavage of pADPRp, whereas 5-FdUrd treatment is associated with a greater than 3-fold increase in a Mr, 85,000 cleavage product of pADPRp (13). These differences suggest that a single cell type may use distinct signaling pathways to activate a final common pathway characterized by enhanced TGFβ expression and programmed cell death.

Our results bring into question the role of immediate early gene expression in apoptosis. Previous reports from our laboratory and others (18, 22) have documented that human breast cancer cells which are mitogenically stimulated by EGF show a distinct pattern of immediate early gene induction which is similar to that reported in the current study for apoptosis induced by EGF. The observation that cells exhibiting a mitogenic response to EGF and those exhibiting an apoptotic response to EGF have similar patterns of immediate early gene induction suggests that these genes initiate a signal whose specificity is regulated by some downstream determinant. In a similar fashion, the role of c-myc in the programmed death of MDA-MB-468 cells can be questioned. We show that EGF-mediated apoptosis is associated with an 11-fold induction of c-myc, whereas no change in basal c-myc expression is seen with fluoropyrimidine-induced apoptosis (12). In an elegant study of activation-induced death of T cells, Shi et al. (24) used antisense oligonucleotides to c-myc to demonstrate that c-myc expression is a necessary component of the apoptotic

![Fig. 3. A, levels of immediate early gene mRNA in MDA-MB-468 cells exposed to 10 ng/ml EGF. Total cellular RNA was isolated from MDA-MB-468 cells after treatment with EGF for 0, 0.25, 0.5, 1, or 2 h. After electrophoresis and transfer, membranes were hybridized with 32P-labeled complementary DNA probes for c-fos, c-jun, jun-B, jun-D, and -y-actin. Hybridization with -y-actin is shown as a control for the amount of RNA loaded in each lane (30 µg/lane). Typical cellular yield of RNA was not discernibly different between EGF-treated and control cells (data not shown). B, levels of c-myc and TGFβ mRNA in MDA-MB-468 cells exposed to 10 ng/ml EGF. Total cellular RNA was isolated from MDA-MB-468 cells after treatment with EGF for 0, 0.25, 0.5, 1, 2, 4, or 6 h. After electrophoresis and transfer, membranes were hybridized with 32P-labeled complementary DNA probes for c-myc, TGFβ, and -y-actin. Hybridization with -y-actin is shown as a control for the amount of RNA loaded in each lane (30 µg/lane). C, level of TGFβ mRNA in MDA-MB-468 cells exposed to 10 ng/ml EGF. Total cellular RNA (30 µg/lane) was isolated from MDA-MB-468 cells after treatment with EGF for 0, 24, or 48 h. After electrophoresis and transfer, membrane was hybridized with 32P-labeled complementary DNA probes for TGFβ and -y-actin. Hybridization with -y-actin is shown as a control for the amount of RNA loaded in each lane (30 µg/lane).](image)

![Fig. 4. Assessment of pADPRp (PARP) and lamin B cleavage in MDA-MB-468 cells after treatment with EGF. Adherent cells (Lanes 1–4, 6, 8, 10, 12, 14, and 16) and nonadherent cells (Lanes 5, 7, 9, 11, 13, 15, and 17) were collected separately after treatment with 10 ng/ml EGF for 0–72 h as indicated. Samples containing 50 µg of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blotting with monoclonal anti-pADPRp (A) or polyclonal anti-lamin B (B). Lanes 2–4 are serial dilutions of the Lane 1 sample and contain 25, 12.5, and 5 µg protein, respectively. Numbers at left, molecular weights of standard proteins in kilodaltons (Arrow heads, cleavage product of pADPRp (A), Lamin B (B).](image)
process seen with T-cell receptor activation. In the same study, however, they report that c-myc antisense had no effect on dexamethasone-induced apoptosis in the same cell line, suggesting that glucocorticoid-induced and activation-induced apoptosis may proceed through different pathways. Our findings support an analogous hypothesis in this breast cancer cell line since EGF-mediated apoptosis and fluoropyrimidine-mediated apoptosis are associated with distinctly different patterns of early gene induction.

It has been suggested previously that proteolysis of certain nuclear polypeptides might also be an important event in apoptosis (13, 20, 21, 26). Proteolytic cleavage of pADPRp has been observed in cells spontaneously undergoing apoptosis (e.g., Fig. 4, Lane 5) and in diverse cell types treated with a variety of cytotoxic chemotherapeutic agents (13). In addition, it has been observed that certain protease inhibitors prevent DNA fragmentation and morphological changes of apoptosis in addition to cleavage of pADPRp (13, 28). This observation has led to the suggestion that limited proteolysis might play a role in initiating or controlling the apoptotic cascade. Although this might be true in cells that undergo apoptosis after treatment with cytotoxic chemotherapeutics, EGF-treated MDA-MB-468 cells appear to undergo apoptosis with relatively little proteolysis of the two major protease substrates identified to date.

A common phenotype of EGFR-overexpressing cells is growth inhibition by high levels of EGF but maintenance of a proliferative response to low concentrations of EGF (4, 8). Based on the findings reported here, we postulate that EGF can drive two coupled functions, proliferation and programmed cell death. Overexpression of EGFR could allow for a selective growth advantage for tumor cells in the presence of normal or decreased ligand availability. However, excessive ligand binding would result in deregulated growth signaling, particularly in the absence of other requisite growth factors and/or nutrients. Studies from Ennis et al. (8) using MDA-MB-468 breast cancer cells have shown that treatment with monoclonal anti-EGFR antibodies or with EGF will inhibit cell growth, but simultaneous treatment with antibody and EGF can stimulate cell growth (8). Similar results have been noted in A431 human epidermoid carcinoma cells that overexpress EGFR (7). In breast cancer, EGFR expression is inversely proportional to the level of expression of ER (2, 3), suggesting that the loss of hormone responsiveness may be associated with altered responses to ligand binding of the EGFR. Our results may thus have implications for current clinical efforts to use anti-EGFR antibodies therapeutically. Based on our data, it is possible that attempts to block ligand binding to the EGFR may not only interrupt the proliferative response to EGF but may also interfere with apoptotic pathways.

Acknowledgments

Wendy Berry, Laura Prestigiacomo, and Chris Buckwalter are recognized for their expert technical assistance. C-2—10 antibody was kindly provided by Dr. Guy Poirer.

References

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