MDA-468, A HUMAN BREAST CANCER CELL LINE WITH A HIGH NUMBER OF EPIDERMAL GROWTH FACTOR (EGF) RECEPTORS, HAS AN AMPLIFIED EGF RECEPTOR GENE AND IS GROWTH INHIBITED BY EGF

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Epidermal growth factor (EGF) has been noted to stimulate proliferation of a variety of normal and malignant cells including those of human breast epithelium. We report here that MDA-468, a human breast cancer cell line with a very high number of EGF receptors, is growth-inhibited at EGF concentrations that stimulate most other cells. The basis for the elevated receptor level is EGF receptor gene amplification and over-expression. An MDA-468 clone selected for resistance to EGF-induced growth inhibition shows a number of receptors within the normal range. The results are discussed in relation to a threshold model for EGF-induced growth inhibition.

The epidermal growth factor (EGF) receptor molecule is a glycoprotein of molecular weight 170 kDa that is composed of an extracellular EGF binding domain linked by a transmembrane segment to an intracellular portion which has tyrosine kinase activity (1). Recently, several authors have reported that the transforming gene of avian erythroblastosis virus, the erb-B oncogene, almost certainly encodes the intracellular portion of the avian EGF receptor (2-4). This finding strongly suggests that rearrangements, amplification or abnormal expression of the epidermal growth factor receptor gene (EGFRG) may be related to the oncogenic process in certain neoplasms, and has reinforced the importance of studying the mechanisms by which EGF receptor interactions influence the proliferative behaviour of cells. A-431 cells have been extensively used for this purpose since they have an unusually high number of EGF receptors (30 to 100 times more than normal

Abbreviations

EGF, Epidermal growth factor; EGFRG, epidermal growth factor receptor gene; FCS, fetal calf serum; kb, kilobase

human fibroblasts) (5). The high number of EGF receptors in these cells has been related to the amplification and over-expression of the EGFRG (2,3,6,7).

EGF has shown a stimulatory effect on the growth of a wide variety of normal and malignant cells (8-11). The increased EGF binding capacity of A-431 cells does not lead, however, to an increased mitogenic response; rather A-431 cells are markedly inhibited by EGF (12,13). Kawamoto et al. (14), studying variant clones of A-431 cells with different numbers of EGF receptors, have concluded that there is a relationship between EGF receptors and growth response, and when an optimum amount of EGF receptors is exceeded, growth inhibition results on exposure to EGF.

Here we show that a human breast cancer cell line, MDA-468, which has a number of EGF receptors similar to A-431 and is also growth-inhibited by EGF, has an amplified and over-expressed EGFRG. The isolation of an MDA-468 clone which is not inhibited by EGF allows us to propose a relationship between the number of EGF receptors and the effect of EGF on the proliferative behavior of MDA-468 cells, suggesting that the relationship between EGF receptor number and proliferative response previously described for A-431 cells may in fact be a general phenomenon.

MATERIALS AND METHODS

Cell Cultures Cell lines MDA-468 and MDA-157 were derived from human breast tumors as previously described (15,16). The 427N human fibroblast line, derived from a skin biopsy of a normal individual, was kindly provided by Dr. R. Phillips. A-431 cells were kindly provided by Dr. G. Carpenter and MCF-7 by Dr. K. Osborne. MDA-468, MDA-157 and MDA-468/S1 lines were routinely cultured in L-15 medium, supplemented with 10% fetal calf serum (FCS) (Bocknek, Ont., Canada). A-431, MCF-7 and 427N cells were cultured in alpha medium, also supplemented with 10% FCS.

^{125}-I-EGF Binding Assay Binding studies were carried out in triplicate on subconfluent cells grown on 35 mm dishes. Cells were washed twice with binding buffer (serum-free alpha medium with 1 mg/ml bovine serum albumim and 5 mM HEPES, pH 6.8), and then incubated for two hours at 37°C in 500 µl of binding buffer containing 5 x 10^{-9}M [^{125}I]-EGF (150 µCi/µg, New England Nuclear), a concentration previously noted to saturate available EGF receptors under these conditions. Cells were then washed 6 times with binding buffer, solubilized in 0.5N NaOH, and radioactivity determined. Specific binding was estimated by subtracting counts bound in the presence of excess unlabelled EGF from total counts bound. The mean number of cells on three companion dishes, as counted by hemacytometer following trypsinization, was used to estimate the number of binding sites per cell.

Effect of EGF Concentration in the Proliferation of Cell Lines 5×10^3 cells were plated in multiwell plates of surface area 2 cm^2 containing appropriate serum-suplemented media and allowed to attach overnight, after which varying concentrations of EGF were added. Cells were incubated at 37°C in 5% CO_2 for 8 days, and then trypsinized and counted by hemacytometer.

Isolation of Variant MDA-468 Clone Approximately 10^7 MDA-468 cells were plated in 10 cm culture dishes containing L-15 media supplemented with 10% FCS and allowed to attach overnight. The media was then changed to L15

supplemented with 10% FCS and 10^{-7}M EGF (Collaborative Research). This media was replaced twice weekly, with removal of floating cells and debris. After 14 days, cloning rings were used to isolate colonies. These cells were then transfered to microwells, and subsequently propagated in L-15 supplemented with 10% FCS and 10^{-7}M EGF.

Isolation of DNA and Southern Blotting High molecular weight genomic DNA was isolated by using $NaDodSO_4$/proteinase-K lysis, organic extraction and NaCl/ethanol precipitation (17). DNA was digested with HindIII, electrophoresed on 0.8% agarose and transfered to a Zetabind membrane (AMF CUNO). Hybridizations and washings were performed using high stringency conditions. Probe-related nonspecific background was reduced as described (18).

Isolation of RNA and RNA Blotting Total RNA was isolated by guanidine isothiocyanate solubilization and centrifugation over a CsCl cushion (19). Poly(A)$^+$ RNA, purified by passage over oligo(dT)-cellulose, was denatured with glyoxal and dimethylsulfoxide and electrophoresis was performed in a 1.1% agarose gel. The RNA was then transferred to a Zetabind filter (20) and hybridized using high stringency conditions.

Probes The 2.4 kilobase (kb) c-DNA probe pE7 has been isolated by Merlino et al. (6). It encodes a portion of the EGFRG and is highly homologous to a portion of the V-erb-B oncogene. λhe-B is a 15 kb c-erb-B probe isolated by Spurr et al. (21). Probes were ^{32}P nick-translated as described (22).

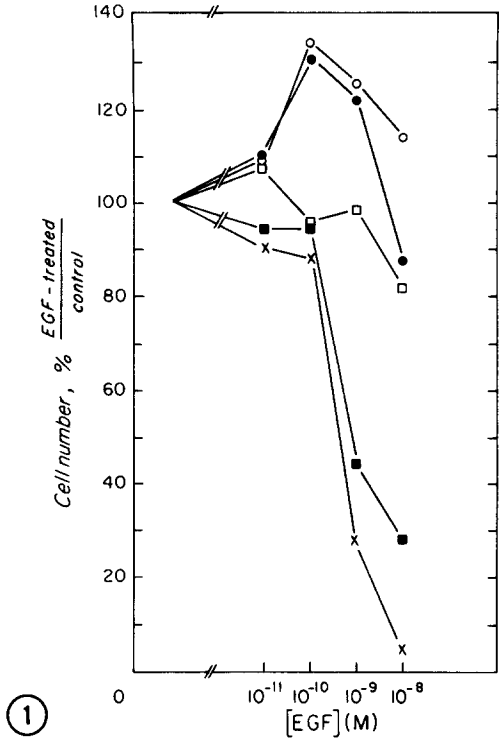
RESULTS

Table 1 shows that different human breast cancer cell lines express various EGF receptor levels. This is in agreement with previous reports (23,27). MDA-468 cells exhibit a level of receptors similar to those seen in A-431 cells while MCF-7 and MDA-157 have receptor levels in the range of normal human fibroblasts.

The effect of increasing EGF concentrations on growth of these cell lines is shown in Fig. 1. Both MDA-468 and A-431 were significantly inhibited at EGF concentrations greater than 10^{-10}M while, under the serum-containing conditions employed, MDA-157, MCF-7 and normal human fibroblasts showed no effect or a mild stimulation.

Table 1. Estimates of [^{125}-I]-EGF binding sites per cell for various cell lines

Cell line	Origin	[^{125}I]-EGF bound specifically (fmol/10^6 cells)	Binding sites/cell
A-431	Epidermoid carcinoma	4169	2.5×10^6
MDA-468	Breast cancer	3160	1.9×10^6
MDA-157	Breast cancer	17	1.0×10^4
MCF-7	Breast cancer	20	1.2×10^4
427N	Human fibroblast	86	5.1×10^4
MDA-468/S1	MDA-468 clone resistant to EGF-induced growth inhibition	27	1.6×10^4



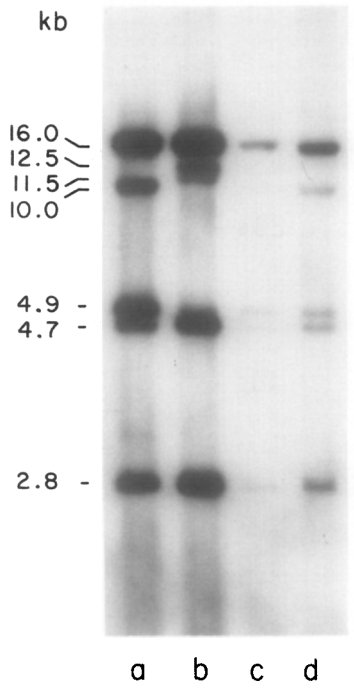
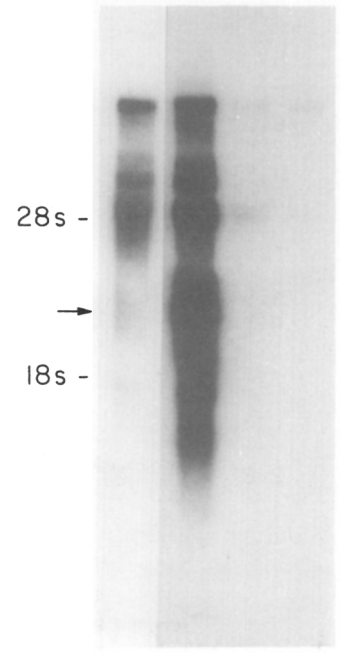


Figure 1 Effect of EGF concentration on proliferation of various cell lines. Growth studies were carried out by incubating 5 x 10^3 cells in media containing various concentrations of EGF. Points represent percentage change in cell number for cells grown in various EGF-supplemented medium as compared to cells grown in control media. □—□427N; ●—●MCF-7; ■—■MDA-468; ○—○MDA-157; X—X A-431.

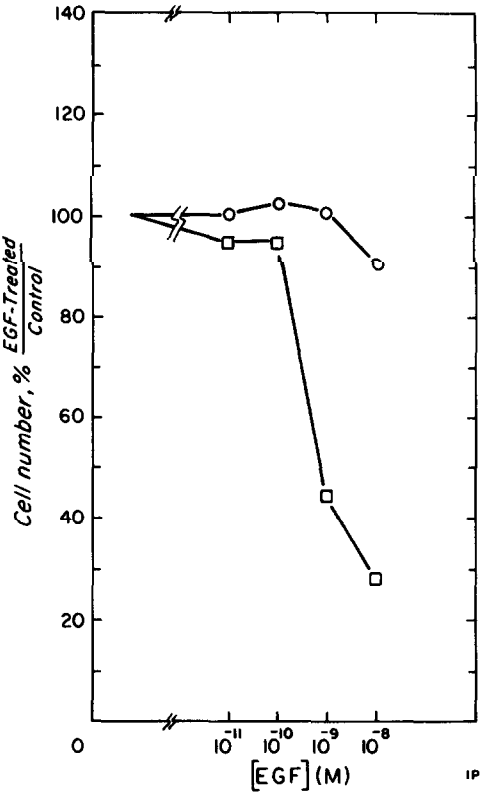
Figure 2 Southern blot analysis of the EGFRG. 10 μg (a,b,c) or 30 μg (d), of DNA were digested with HindIII before being fractionated on agarose and transfered to a Zetabind filter. The blot was hybridized and washed using high stringent conditions. pE7 cDNA nick translated with ^{32}P was used as probe. λDNA digested with HindIII was used as a size marker. a) MDA-468; b) A-431; c) 427N; d) normal fibroblasts of the patient from whom the MDA-468 cell line was derived.

The similarities between A-431 and MDA-468 cells prompted us to compare the EGFRG in these cell lines. Southern blot analysis using a c-DNA probe (pE7) for the EGFRG shows that MDA-468 cells have approximately the same degree of amplification of the EGFRG as A-431 cells (Fig. 2, lanes a and b). On re-hybridization with λhe-B, a c-erb-B probe, two bands of 16 kb and 4.7 kb with the same degree of amplification were detected in MDA-468 and A-431 cells (data not shown).

DNA of cultured normal fibroblasts obtained from a skin biopsy of the patient from whom the MDA-468 cell line was derived was analyzed using the pE7 probe. 30 μg of this DNA were loaded to allow all the bands to be clearly seen. It can be observed that the EGFRG is not amplified in the germ line (Fig. 2, lane d) and it is also apparent that the same bands are



(3)



(4)

Figure 3 Analysis of the EGF receptor RNA by blotting. 3 μg of poly(A)$^+$ RNA was denatured, fractionated on 1.1% agarose gel, transfered to a Zetabind filter and hybridized with ^{32}P-labelled pE7 probe. Hybridization and washings were performed using high stringent conditions. Arrow shows the prominent 2.9 kb band which is only present in A-431 cells. Human 28S and 18S rRNAs were used as markers. a) MDA-468; b) A-431; c) MCF-7; d) 427N.

Figure 4 Effect of EGF concentration on proliferation of the MDA-468 parental line and clone MDA-468/S1. Growth studies were carried out by incubating 5 x 10^3 cells in media containing various concentrations of EGF. Points represent percentage change in cell number for cells grown in various EGF-supplemented medium as compared to cells grown in control media. □—□MDA-468; ○—○MDA-468/S1.

detected in both the fibroblasts and the MDA-468 breast tumor cells (Fig. 2, lanes a and d). Moreover, when *EcoRI* or *BamHI* were used as restriction enzymes no rearrangements could be detected in the MDA-468 cell line (data not shown). Some differences can be detected, on the other hand, between the *HindIII* restriction patterns of A-431, MDA-468 and normal human fibroblasts (Fig. 2). These differences may be due to polymorphisms or to rearrangements. In particular, several authors have suggested that the EGFRG is rearranged in A-431 cells (2,3,6).

Northern blot analysis of A-431 cells using the pE7 probe has shown not only an over-expression of the EGFRG but also the presence of a prominent 2.9 kb band which has not been found in other normal or tumor cells (3,6).

It has been shown (2,3) that the 2.9 kb mRNA codes for the binding part of the EGF receptor which is actively produced and secreted by A-431 cells (24,25). Fig. 3 shows that the EGFRG is also over-expressed in MDA-468 cells in comparison with MCF-7 cells and normal fibroblasts. The mRNA species found in MDA-468 are the same as those described for other cells using the pE7 probe (6,7,18). The 2.9 kb mRNA and other mRNA species reported to be present so far only in A-431 cells (and also shown in Fig. 3, lane b) are not detected in the MDA-468 cells.

To examine further the relationship between number of EGF receptors per cell and EGF-induced growth inhibition, we isolated, by selection in EGF-containing culture media, an MDA-468 clone (MDA-468/S1). As shown in Fig. 4, this clone does not exhibit the growth inhibition seen in the parental line. The number of EGF receptors per cell on this clone was estimated to be approximately two orders of magnitude lower than that present on the parental MDA-468 cells (Table 1).

DISCUSSION

We have shown here that MDA-468, a human breast cancer cell line with a very high number of EGF receptors, has an amplified and over-expressed EGFRG. Although EGFRG amplification does not appear to be a common phenomenon in primary breast tumors (unpublished observations), some tumors that exhibit high receptor levels may utilize regulatory mechanisms other than gene amplification to over-produce EGF receptor RNA and protein (18). The degree of amplification seen in MDA-468 is comparable to that seen in the A-431 line, but, unlike A-431, MDA-468 expresses the same EGFRG-related mRNA species as normal cells. Since the biological responses to EGF may be mediated by tyrosine phosphorylations, we are presently measuring the tyrosine kinase activity in the MDA-468 cell line. Cytogenetic analysis and immunoprecipitation of the EGF receptor are also being performed. It is possible that this cell line will be a useful model for EGF receptor studies.

No EGFRG amplification was found in normal fibroblasts of the patient from whom MDA-468 cell line was derived, implying that the gene amplification is not present in the patient's germ line. Unfortunately, primary cells from the original tumor are unavailable, making it impossible for us to ascertain whether the amplification was present in the original biopsied tumor or arose during the establishment of the line.

The results presented here relating the number of EGF receptors seen in breast cancer cell lines to the modulation of proliferative behavior induced by EGF are consistent with the threshold model proposed by Kawamoto et al. (14). According to this model, the presence of a number of receptors above

a critical threshold may be the cause of the marked EGF-induced growth induction in MDA-468 cells. The low receptor number of the MDA-468/S1 clone is consistent with this hypothesis. We are presently studying additional MDA-468 clones that are resistant to the EGF-induced growth inhibition; this analysis will allow a detailed examination of the validity of the threshold model in describing the proliferative responses of MDA-468 cells to EGF.

Certain rat pituitary cells (26) and human breast cell lines (27) with a relatively low number of EGF receptors have been shown to exhibit a moderate EGF-induced growth inhibition in serum-supplemented media but have shown growth stimulation under serum-free conditions (10,28). Although the growth inhibitory effects described in Fig. 1 were obtained in serum-containing media, we have found that MDA-468 cells, like A-431 cells (13), are also markedly growth-inhibited by EGF under serum-free conditions (data not shown).

EGF plays an important role in the proliferation and differentiation of breast epithelium (28-33). Recent studies (34,35) have provided evidence that some steroid-receptor negative human breast tumors have very high levels of EGF receptors, suggesting that the behaviour of these neoplasms may be regulated predominantly by EGF rather than estrogens. Since MDA-468 can be grown in immune-suppressed mice (unpublished observations), these cells may provide a very useful model for *in vivo* and *in vitro* studies of this subpopulation of breast cancers.

ACKNOWLEDGEMENTS

This work was supported by grants from the Medical Research Council of Canada and The National Cancer Institute of Canada. Michael N. Pollak is a recipient of a Terry Fox Postdoctoral Fellowship from the National Cancer Institute of Canada. We are indebted to Drs. I. Pastan and G. Merlino for providing the pE7 cDNA probe and to I. Robson for the c-*erb*-B probe. Our thanks also for the excellent technical assistance of R. Pullano and B. Choo.

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