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FACTOR RECEPTOR GENE IN A HUMAN ASTROCYTOMA CELL LINE

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We report here that SK-MG-3, a human astrocytoma cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified and [redacted] detected inhibition of *in vitro* proliferation of the SK-MG-3 line by EGF.

The finding that the transforming gene of avian erythroblastosis virus, the *erb*-B oncogene, is homologous to the kinase portion of the epidermal [redacted] certain neoplasms.

[redacted] astrocytes and normal human glial cells in culture (3,4,5) and EGF concentrations comparable to those in human plasma have been reported in human cerebrospinal fluid (6). Recently Liberman et al. (7) reported that a significant proportion of primary human glioblastomas morphologically graded as glioblastoma multiforme (GM) (astrocytoma grade III or IV) exhibits an elevated EGF receptor level, and subsequently they demonstrated that 4 of 10 primary GM examined have an amplified EGFRG gene (8).

We have estimated EGF receptor number in 22 cell lines derived from grade III or IV astrocytomas. One cell line, SK-MG-3, exhibited an unusually high

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Abbreviations: EGF, epidermal growth factor; EGFRG, epidermal growth factor receptor gene; GM, glioblastoma multiforme; FCS, fetal calf serum; kb, kilobase.

number of specific EGF binding sites as estimated by [^{125}I]EGF binding studies. All cell lines were subsequently screened for EGFRG amplification, and SK-MG-3 was the only line with detectable amplification and rearrangement of the EGFRG. Despite the rearrangement, no abnormal EGFRG-related mRNA species were detected.

Since other cell lines which have an amplified EGFRG are growth-inhibited by EGF (9,10), we examined the effect of this growth factor on the [redacted]

MATERIALS AND METHODS

Cell Lines: The following cell lines originated from astrocytomas grade III or IV were studied: SK-MG-1,-2,-3,-4,-6,-7,-8,-10,-11,-12,-13-,-14,-15, SK-MS and SK-A02, derived at the Memorial Sloan-Kettering Cancer Center (11,12); U-87MG, U-138MG, U-178MG, U-251MG, U-343MG and U-373MG, derived at [redacted] provided by Dr. R. Phillips, were used as controls. MDA-468 cells were routinely cultured in L-15 medium, supplemented with 10% fetal calf serum (FCS). All the other cell lines were cultured in alpha medium also supplemented with 10% FCS.

[^{125}I]EGF-Binding Studies: 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 albumin and 5 mM HEPES, pH 6.8), and then incubated for two hours at 37^{o}C in binding [redacted] in 0.5N NaOH, and radioactivity determined. Specific binding was estimated by subtracting counts bound in the presence of excess unlabelled EGF (10^{-7}M) from total counts bound. The mean number of cells on two companion dishes as counted by hemocyometer following trypsinization was used to estimate the [redacted] varying concentrations of EGF between 5 x 10[redacted]M and 10[redacted]M, and Scatchard plots were derived.

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 (16). DNA was digested with HindIII or EcoRI, electrophoresed in 0.8% agarose and transferred to a Zetabind [redacted]

Isolation of RNA and RNA Blotting: Total RNA was isolated by guanidine isothiocyanate solubilization and centrifugation over a CsCl cushion (18). 3 µg of poly(A)$^{+}$ RNA, purified by passage over oligo(dT)-cellulose, was [redacted] filter (19) and hybridized using high stringent conditions.

Probes: The 2.4 kilobase (kb) c-DNA probe pE7 has been isolated by Merlino [redacted] its homology to the V-erb-B gene.

[redacted] cells were plated in triplicate in 35 mm multiwell plates containing alpha [redacted] alpha media supplemented with 0.1% FCS and varying concentrations of EGF at 37°C in 5% CO_2 for one week, with media changes every 48 hours, after which cells were trypsinized and counted with a coulter counter.

RESULTS

Estimation of EGF Binding Sites in Astrocytoma Cell Lines We screened 22 astrocytoma cell lines for specific EGF binding sites using a $[^{125}I]$EGF [redacted] conditions (data not shown). Results from a typical astrocytoma line, (SK-MG-5), two EGFRG amplified cell lines (MDA-468 and A-431), normal human [redacted] lines. Although extrapolation of binding constants and receptor number from Scatchard plots is hazardous under conditions where there may be heterogeneity of receptors (22,23) and/or labelled ligands (24), and equilibrium is difficult to document due to receptor internalization, Kd was [redacted] receptor numbers were similar to those obtained by measuring specific binding at 5 x 10^{-9}M EGF.

Southern Blot Analysis Using a cDNA probe (pE7) all the cell lines were screened for amplification and rearrangements of the EGFRG.

DNA extracted from each cell line was digested with EcoRI or HindIII and Southern blot analysis was performed as described in Methods. Only SK-MG-3

Table 1. Estimates of EGF Binding Sites per Cell for various Cell Lines

Cell line	Origin	fmol EGF bound/10^5 cells	Estimated EGF receptors number per cell
SKMG-3	GM	47	2.8 x 10^5
SKMG-5	GM	16	9.3 x 10^4
468	Breast tumor	163	9.7 x 10^5
A-431	Epidermoid tumor	419	2.5 x 10^6
[redacted]			

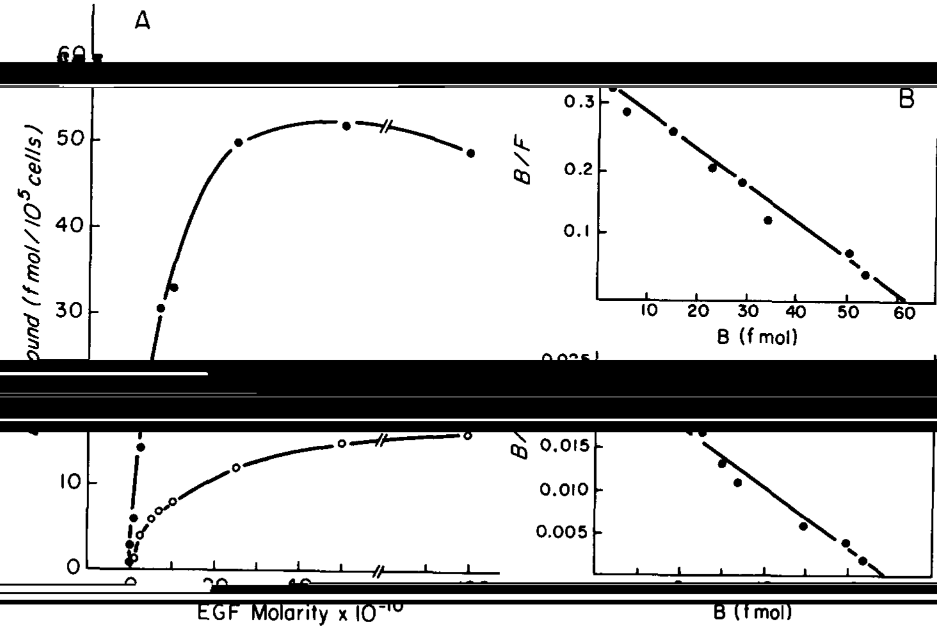


Figure 1. EGF biding to GM cell lines. (A) Specific binding of EGF to SK-MG-3 and SK-MG-5 cells as a function of EGF concentration. (B) and (C) Scatchard plots of binding data for SK-MG-3 and SK-MG-5, respectively.

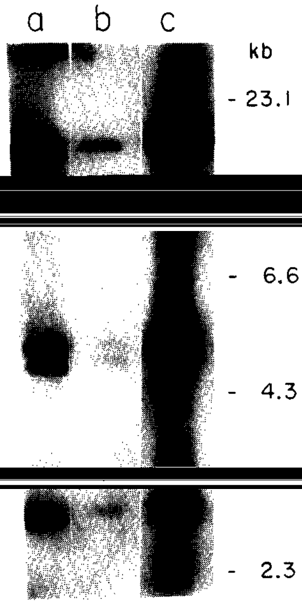
DNA showed an amplified EGFRG. Fig. 2 shows that the level of amplification of the SK-MG-3 cells is not as high as it is in MDA-468 cells, correlating with the number of EGF receptors found in each cell line (Table 1). Both the HindIII (Fig. 2) and the EcoRI (not shown) restriction patterns of the SK-MG-3 DNA allowed us to detect new bands (arrow) not

donors indicating that the EGFRG is rearranged. The EcoRI and HindIII restriction patterns of the other astrocytoma cell lines did not show any evidence of rearrangement of the EGFRG (data not shown).

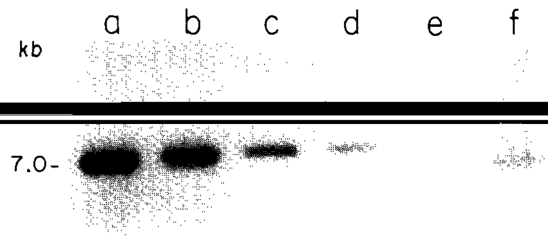
We estimated the degree of amplification comparing the signal intensity of receptor DNA fragments in successive dilutions of DNA extracted from SK-MG-3 cells with the signal intensity in DNA obtained from normal human fibroblasts (427-N). For this purpose we used a genomic c-erb-B probe which codes for a part of the kinase portion of the EGFRG. Fig. 3 shows that the gene is amplified approximately 8 times.

Northern blot analysis Since the EGFRG is rearranged in the SK-MG-3 cells,

species. Such a situation has been found in A-431 cells, which also have a rearranged EGFRG (20). mRNA was extracted and Northern blot analysis was



(2)



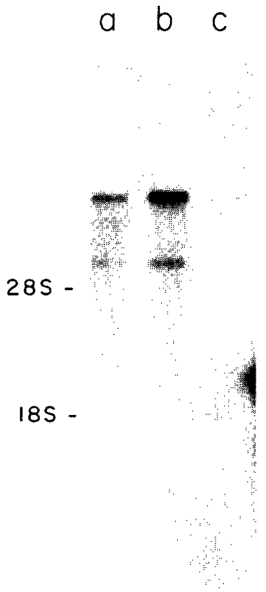
(3)

Figure 2. Southern blot analysis of the EGFRG. 10 μg of DNA were digested with *Hind*III and hybridized with a ^{32}P-labelled pE7 probe. a) SK-MG-3; b) 427-N; c) MDA-468.

Figure 3. Quantification of the EGFRG amplification in SK-MG-3 cells. Serial dilutions of *Eco*RI-restricted DNA from SK-MG-3 were performed and hybridized with a ^{32}P-labelled c-*erb*-B probe. Small differences in the position of the bands are due to different salt concentrations after the dilution of the DNA. a) 20 μg; b) 10 μg; c) 5 μg; d) 2.5 μg; e) 1.25 μg; f) 20 μg from normal human fibroblasts (427-N)

[illegible] that the EGFRG is overexpressed in SK-MG-3 cells (Fig. 4) although the [illegible] transcripts.

Effect of EGF in the Growth of SKMG-3 Cells It has been reported that A-431 and MDA-468, two cell lines that have very high number of EGF receptors as a consequence of gene amplification, are growth-inhibited by EGF concentrations that stimulate most other cells (9,10). Fig. 5 shows that EGF stimulates the growth of the SK-MG-3 cell line at the same concentrations that strongly inhibit the growth of A-431 and MDA-468 cells. The degree of stimulation found in the astrocytoma cells (50%) is similar to [illegible]



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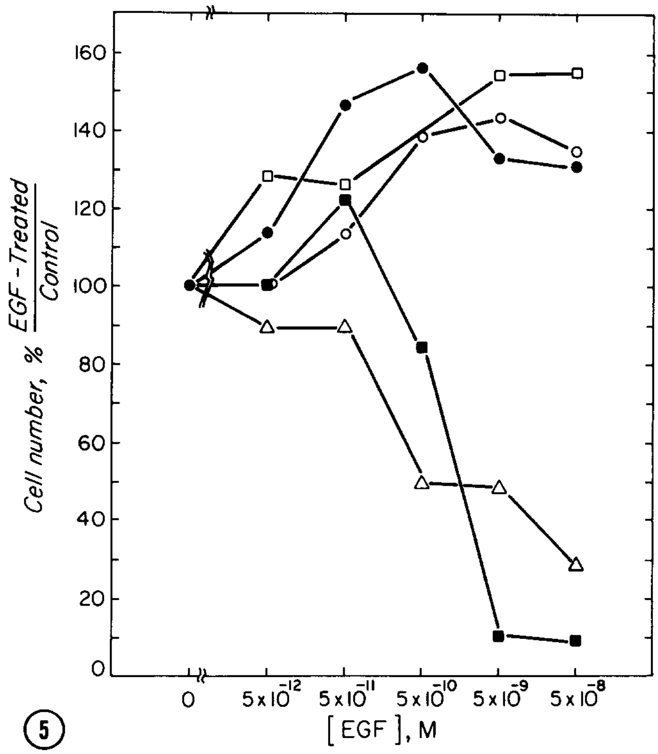

(5)

Figure 4. Northern blot analysis of the EGF receptor. ^{32}P-labelled pE7 was used as probe a) SK-MG-3; b) MDA-468; c) 427-N.

Figure 5. Effect of EGF concentration on proliferation of cell lines. Points represent percentage change in cell numbers for cell grown in various EGF-supplemented media as compared to cells grown in control media. □-□, SK-MG-3; O-O, SK-MG-5; ●-●, 427-N; Δ-Δ, MDA-468; ■-■, A-431.

DISCUSSION

[redacted]

Karyotypic analysis of SK-MG-3 cells has revealed the presence of 2 to 3 [redacted] demonstrated (data not shown). As EGFRG amplifications seem to occur in a significant proportion of primary GM (7), the SK-MG-3 line should be useful as a model for further studies investigating the relationship between EGF receptor abnormalities and the neoplastic behavior of certain glioblastomas.

Our results indicate that EGFRG amplifications are less common in cell lines derived from GM than in primary GMs. It has been clearly shown that primary GM tumors are comprised of karyotypically heterogeneous cellular [redacted] amplification.

The selective pressures imposed during cell line propagation are not [illegible] confer a proliferative advantage to neoplastic cells under certain *in vivo* conditions, by rendering cells super-sensitive to exogenous or autocrine-produced mitogens (26). Indeed, the frequency of EGFRG amplification in less differentiated, more aggresive primary astrocytomas [illegible] mechanism of neoplastic progression *in vivo*. However, the rarity of EGFRG amplifications in astrocytoma cell lines selected and propagated in monolayer suggests that the amplification does not represent an advantage, and may even lead to counter-selection, at least under certain culture conditions.

In squamous cell carcinomas, significant overexpression of EGFR has been reported in a very high proportion of cell lines (27) and primary tumors (28), but extensive molecular genetic studies have not yet been reported. Recently, Merlino et al. have reported a four-fold amplification of the EGFRG in a squamous carcinoma cell line derived from the human tongue (29). A-431, another squamous carcinoma cell line, has a ≃ 30 fold amplification of this gene (20).

SK-MG-3 cells are not growth-inhibited by EGF concentrations that paradoxically inhibit A-431 and MDA-468, cell lines with about 30 and 20-fold, respectively, amplification of the EGFRG. [illegible] presence of high concentrations of EGF. All the variants have shown fewer EGF receptors than the parental cell lines (8,30). Kawamoto et al. (31) have proposed that there is a relationship between the number of EGF receptors and growth response to EGF, and that when a given amount of EGF receptors is exceeded, growth inhibition results on exposure to EGF. There is no evidence that there is an absolute threshold concentration for inhibition common to all cells, and the molecular mechanism underlying such a threshold phenomenon remains unclear. [illegible] may be a consequence of the fact that the number of EGF receptors present in these cells, while elevated, is below a critical threshold. Alternatively, it can be proposed that the EGF receptors are not functional in this astrocytoma cell line, but this does not seem to be the case since SK-MG-3 are growth stimulated by EGF at concentrations similar to other cell lines with functional receptors (Fig. 5). Also, the K_d for EGF binding in SK-MG-3 cells is in the same range as other Kd's calculated for other cell lines known to have normal EGF receptors (33).

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