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INDUCTION OF c-fos BY AN ANTI-EPIDERMAL GROWTH FACTOR RECEPTOR MONOCLONAL ANTIBODY

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The effect of epidermal growth factor (EGF) or a monoclonal antibody to the human EGF receptor on c-fos and c-myc gene expression was examined in KB epidermoid carcinoma cells. EGF increased c-fos transcripts within 30 min and c-myc transcripts within 6-24 hours. The antibody transiently increased c-fos expression within 30 minutes, but did not alter expression of c-myc. Cycloheximide potentiated the c-fos induction by both EGF and antibody. C-fos induction by antibody was less consistent, and was of briefer duration and lesser intensity than the induction by EGF. Nuclear transcription of c-fos decreased in cells treated with EGF or antibody, indicating that c-fos induction by EGF or antibody resulted from post-transcriptional mechanisms.

The EGF receptor is a transmembrane protein kinase regulating proliferation of normal and malignant cells (1). When the EGF receptor is bound by either EGF or α-transforming growth factor autophosphorylation and activation of the receptor tyrosine kinase occurs rapidly (1). EGF binding also induces expression of protooncogenes, such as c-fos and c-myc (1-4). These same genes are induced by other growth factors and may be critical to early events controlling normal cell growth (2, 5-7). In some cells, c-fos induction is required for DNA synthesis (8). However, in tumor cells, the significance of c-fos and c-myc gene induction is unclear, since tumor cells which are growth- inhibited or stimulated by EGF show similar increases in c-fos and c-myc mRNA after EGF treatment (4,9).

Monoclonal antibodies to polypeptide components of the EGF receptor have been described (10). One such antibody (MoAb 225; 10, 11) binds to the EGF receptor with an affinity similar to EGF, and like EGF, triggers EGF receptor internalization and "down-regulation" (12). In vitro, MoAb

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225 mildly inhibits (11, 13) and EGF completely arrests proliferation of malignant cell lines with amplified EGF receptor expression (13,14). Although MoAb 225 stimulates serine and threonine phosphorylation on the EGF receptor, it does not stimulate receptor tyrosine autophosphorylation nor does it activate the receptor tyrosine kinase (12). Further, MoAb 225 blocks both EGF binding to its receptor (11, 12) and EGF elicited effects on cellular transferrin receptor re-distribution (15). Because it mimics some, but not all actions of EGF, MoAb 225 has been a powerful tool for characterizing EGF receptor function.

In the present studies, we examined effects of MoAb 225 on c-fos and c-myc gene expression. Similar to the studies cited above, we find this MoAb partially mimics early effects of EGF on gene induction. However, important quantitative and qualitative differences in the cellular responses to EGF and to anti-EGF receptor MoAb were again noted.

MATERIALS AND METHODS

Cell Culture

Human KB carcinoma cells (ATCC, Rockville, MD) were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS) as previously described (13, 15). Exponentially growing cells were plated at several densities in 60 mm tissue cultures dishes and cultured overnight. The medium was removed and replaced with fresh medium/FBS, or with medium and FBS containing 10 nM EGF (Collaborative Research, Knoxville, TN) or 20 nM MoAb 225 (11). Cells were cultured for 15 min to 24 hours. Previous studies demonstrated that neither EGF nor MoAb 225 altered proliferation of these cells (13, 15). For some experiments, cells were grown overnight in medium and 10% FBS, followed by a reduction of FBS to 0.5 or 1% with or without MoAb 225. Because cell washing has been reported to induce c-fos expression (21) all media changes for control, antibody-treated and EGF-treated cultures were carried out in parallel.

Extraction of Total Cellular mRNA and Northern Blotting

At varying times after culture with medium/FBS, medium/FBS with EGF, or medium/FBS with MoAb 225, cells were removed by trypsinization and immediately placed on ice. Total cellular RNA was extracted using guanidium isothiocyanate and analyzed by Northern blotting as previously described (16). In some experiments, cells were lysed directly on the plates using guanidium isothiocyanate and RNA extracted. Control cells, or cells exposed to EGF or MoAb 225 were also cultured with 10 ug/ml cycloheximide and RNA extracted. Northern blots were probed with nick translated plasmid DNA containing c-fos or c-myc inserts as described (15). The probes used were a 3 kb XHOI - NCOI fragment of the c-fos cDNA clone (Kindly provided by Dr. Inder Verma, Salk Institute for Biologic Studies, La Jolla, CA) (17); a SST-I exon 2 fragment of the c-myc gene subcloned from clone pHSR-1 (18) and kindly provided by Dr. Alice Barrieux, U.C.S.D. Cancer Center; and a 1.5 kb EcoRI fragment of the N-ras gene (19).

Nuclear Transcription Assays

Nuclear transcription of nascent mRNA was assessed as previously described (16). Equal TCA precipitable counts from cells incubated for 30 min under control conditions, with EGF, or with MoAb 225 were hybridized to linearized pBR plasmid, c-myc exon 2, or c-fos DNA.

RESULTS AND DISCUSSION

Consistent with reports by others (4.9), exposure of KB carcinoma cells to EGF caused a time-dependent increase in c-fos and c-myc mRNA as assessed by Northern analysis (Figure 1A), but did not alter expression of the ras gene. In this and all subsequent studies, the dominant c-fos transcript induced by EGF was the smaller 2.2 kb form, although variable amounts of the larger 3.7 kb transcript were seen in some experiments. EGF induced c-fos and c-myc mRNA accumulation in every experiment. The 2.2 kb c-fos transcript increased 80 fold (mean of 4 experiments; Range 40-100x) and induction of the 3.7 kb transcript increased 2.2 fold (Range 1.5 - 3x) over levels measured in control cells. The induction of c-fos by EGF was potentiated by incubation with cycloheximide (Figure 2).

When KB cells were incubated with MoAb 225, c-fos mRNA was also induced, but there was no effect on c-myc or ras gene transcripts (Figure 1A,B). In contrast to the response to EGF, in some experiments, the

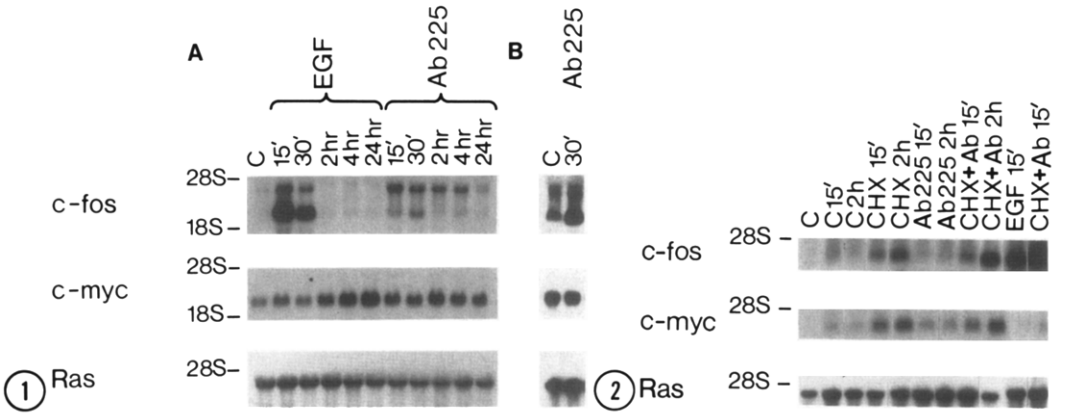

Figure 1. Northern analysis of gene expression by KB carcinoma cells after treatment with EGF or anti-EGF receptor monoclonal antibody 225.

Figure 2. Northern analysis of gene expression by KB carcinoma cells after treatment with EGF or anti-EGF receptor monoclonal antibody alone or with 10 ug/ml cycloheximide (CHX).

dominant transcript induced by the MoAb was the larger 3.7 kb species (Figure 1A). Induction of the 3.7 kb c-fos transcript averaged 7 fold (mean of 5 experiments, range 1 to 17x) and induction of the 2.2 kb form averaged 9 fold (Range 1 - 14x).

In all experiments where direct comparisons were made, c-fos induction by EGF was more prolonged and of greater magnitude than the induction elicited by MoAb 225 (Figure 1). The induction of c-fos expression by the MoAb was inconsistent, and in some experiments was detectable only in the presence of cycloheximide (Figure 2).

In vitro, the c-fos gene can be induced by a variety of stimuli, including physical wounding of a monolayer culture (20) or even cell washing (21). Induction under these conditions may be explained in part by alterations in membrane polarization or ion flux (22). To define differing culture conditions allowing c-fos gene induction by MoAb 225, we varied the KB cell plating densities over a 10 fold range; the FBS concentration from 0 to 10%; or increased the MoAb 225 concentration increased to 100nM. None of these parameters consistently affected MoAb induction of c-fos mRNA. Similarly, direct lysis of cells on tissue culture plates using guanidium isothicyanate buffer had no effect. When cells were cultured with 2.5 mM EGTA to chelate extracellular calcium or 250 uM verapamil as a Ca^{++} channel blocker, no decrease in c-fos induction by MoAb 225 was observed. Thus, the increase in c-fos mRNA elicited by MoAb 225 differed from the effect of EGF in the consistency with which it was observed, the magnitude of gene induction, the ratio between the two transcripts, and the duration of the response. Although EGF induced c-fos mRNA consistently in these studies, conditions allowing consistent induction of c-fos expression by MoAb 225 were not defined.

Cycloheximide increases expression of rapidly induced transcripts such as c-myc and c-fos by inhibiting production of labile proteins which degrade these transcripts or prevent their expression (23). Cycloheximide potentiated c-fos induction by both EGF and MoAb (Figure 2) suggesting that

c-fos gene expression was altered by post-transcriptional mechanisms and did not require the synthesis of new proteins. Therefore, we examined nuclear transcription of c-fos under control conditions and 30 min after treatment with EGF or MoAb 225 (Figure 3). After treatment with either EGF or MoAb 225, c-fos transcription rates decreased by 40-60% (Average of 3 experiments). In contrast, the rate of c-myc transcription was 90% of control. These studies indicate that EGF and MoAb 225 induce c-fos mRNA accumulation in KB cells by a post-transcriptional mechanism(s).

EGF receptor - EGF binding is accompanied by complex processes including membrane ruffling, receptor internalization, receptor autophosphorylation and tyrosine protein kinase activation, and mobilization of potential second messenger molecules (1,24). These events alter cellular gene expression and cell behavior. The complexity and number of these events makes dissection of their individual roles in gene expression difficult. Alternate EGF receptor ligands, such as MoAbs, provide tools for examining components of these processes. Previous studies showed that MoAb 225 binding to the EGF receptor mimics some events associated with EGF receptor activation, including receptor internalization and degradation, receptor down-regulation, and increased receptor serine/threonine phosphorylation (10-12). However, this MoAb does not stimulate cell growth (13, 15).

The present studies indicate that EGF receptor - MoAb 225 binding also partially mimics EGF effects on c-fos induction. Although details remain to be elucidated, the present studies indicate both EGF and MoAb 225 induce c-

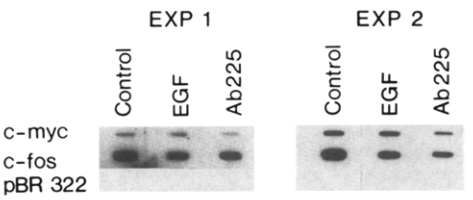

Figure 3. Nuclear transcription assays under control conditions or 30 min after exposure to EGF or monoclonal antibody 225. Equal TCA precipitable RNA counts were hybridized to c-fos, c-myc exon 2, or pBR control DNA.

fos mRNA by post-transcriptional mechanisms which do not require new protein synthesis. These early events in response to EGF may contribute to more extensive changes in gene expression which alter later cell behavior. However, important differences between the response to MoAb and response to EGF were again noted, indicating that EGF binding and events associated with receptor kinase activation are required for full effects of the EGF - EGF receptor interaction on early gene expression.

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