Epidermal Growth Factor: Receptors in Human Fibroblasts and Modulation of Action by Cholera Toxin

(cyclic AMP/insulin/DNA and RNA synthesis/cell surface)

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ABSTRACT Epidermal growth factor (EGF) stimulates both DNA and RNA synthesis in contact-inhibited human fibroblasts. Stimulation of DNA synthesis is observed at concentrations as low as 3 pM, is half-maximal at 70 pM, and is maximal at 300 pM EGF. The action of EGF is similar to that of fetal-calf serum, but is distinguished by the time-course of stimulation and by the ability of serum to stimulate further those cells maximally stimulated by EGF. Cells that synthesize DNA in response to physiological concentrations of EGF (10^{-11} to 10^{-10} M) are insensitive to physiological concentrations of insulin (10^{-11} to 10^{-10} M) and respond only minimally to very high concentrations of this hormone (10^{-6} M). The biological activity of EGF is paralleled by binding of this peptide to fibroblasts in a specific and saturable manner; the dissociation constant is about 800 pM. The binding of EGF is unaffected by either insulin or cholera toxin. Cholera toxin inhibits the action of both EGF and serum. Suppression of DNA synthesis is observed at 0.02 pM toxin, and is maximal at about 2 pM. Cells treated with cholera toxin at these concentrations appear to be otherwise viable by several criteria. The stimulatory effects of EGF are also inhibited by theophylline and dibutyl cyclic AMP separately or in combination. These observations indicate that fibroblasts possess receptors for EGF by biological and physicochemical criteria, and suggest that a similar if not identical peptide may be amongst those factors in sera which stimulate cell growth. The possibility is considered that EGF and cholera toxin modulate the ability of a cell to initiate polynucleotide synthesis by way of specific cell-surface interactions which in turn alter the levels of intracellular cyclic AMP.

Epidermal growth factor (EGF), a small (molecular weight = 6000) polypeptide purified from the submaxillary gland of mice, is a potent stimulator of proliferation and keratinization of epidermal tissue in several species (1). In some cells (HeLa, mouse mammary, and rabbit cornea) epidermal growth factor can stimulate the synthesis of both RNA and DNA, whereas in other cells (9-day chick-embryo epidermis and human KB) only RNA synthesis is stimulated (2). While the specific hormonal role of EGF has not been defined, the polypeptide may well serve as a model compound for those substances in serum which promote cell growth. In contrast to EGF, no natural macromolecule has been isolated in pure form which will act at physiological concentrations to inhibit rather than stimulate cell growth. The present studies demonstrate that the *Vibrio cholerae* exotoxin (3), whose actions in various tissues are presumably mediated by cAMP (4), can act at very low concentrations to inhibit both RNA and DNA synthesis. Cholera toxin thus behaves in some respects as a chalone (5), and it can be used as a tool to examine the effect of

specifically inhibiting the action of EGF on fibroblasts of presumably mesodermic origin.

We demonstrate here that very low concentrations of EGF stimulate both DNA and RNA synthesis in human fibroblasts, that the polypeptide interacts with specific cell-surface receptors, and that the effects of EGF on both DNA and RNA synthesis can be markedly affected by minute amounts of cholera toxin. The present observations provide a basis for the further study of how specific stimuli lead to the modulation of cellular growth.

MATERIALS AND METHODS

Human fibroblasts derived from newborn foreskin (HF), from female adult forearm punch-biopsy specimens (PB), and from fetal lung (WI-38 strain, purchased from Microbiological Assoc., Bethesda, Md.), were routinely cultivated in monolayer at 37° in a growth medium of Eagle's minimal essential medium with Earle's balanced salts, containing 2 mM glutamine, 100 U/ml each of penicillin and streptomycin, supplemented with 10% fetal-calf serum, and equilibrated with 5% CO₂ in air. Cells were subcultured with 0.25% trypsin, which was diluted 1:40 immediately after dispersion of the parent monolayer, and which was eliminated by subsequent feedings. No more than 12 passages of any cell line were used.

Polynucleotide synthesis was measured in cells grown in 16-mm disposable multi-dish trays [Linbro Chemical Co. FB-16-24-TC (6)]. Each tray was seeded with about 5×10^4 trypsinized cells in 1 ml of growth medium containing 10% fetal-calf serum and a residual trypsin concentration of about 60 µg/ml. After 24-48 hr, the medium was changed to one entirely free of trypsin, containing 5% serum, and cells were allowed to grow to confluency. Experiments were initiated by changing the growth medium to one free from serum, but containing 0.1% crystallized bovine-serum albumin (Pentex, Miles Laboratories). Appropriate reagents were then added, and polynucleotide synthesis in triplicate samples was examined at timed intervals. The incorporation of either [methyl-³H]-thymidine (6.7 Ci/mmol) or [5-³H]uridine (25.7 Ci/mmol) was determined at 37° 1 hr after the addition of 1 µCi of nucleoside to each 1-ml culture. Monolayers were rinsed three times with successive 2-ml portions of Hanks' buffered saline, 5% trichloroacetic acid, and absolute methanol. The remaining nuclei and precipitated cell constituents, which adhered firmly to the tray, were solubilized at 70° for 15 min in 0.5 ml of 1 M Hyamine in methanol and transferred to vials with 3 ml of absolute ethanol. Radioactivity was measured by scintillation counting. The protein content (about 100 µg) of replicate monolayers, which varied less than 1% from tray to tray at confluency, was determined by the method of Lowry *et al.* (7)

Abbreviation: EGF, epidermal growth factor.

FIG. 1. Stimulation of human fibroblasts by epidermal growth factor and insulin. Confluent cells (about 100 μ g of protein per dish) were refed with serum-free growth medium containing 0.1% w/v bovine albumin, and either EGF or insulin. [^{3} H]Thymidine (1 μ Ci/ml) incorporation, measured by a 1-hr pulse at 23 hr, is expressed as cpm per 100 μ g of protein, corrected for the amount incorporated in the absence of stimulant (390 ± 100 cpm for insulin; 900 ± 180 cpm for EGF).

after solubilization of cells in 1 N NaOH at 70° for 15 min and neutralization with 1 N HCl.

Epidermal growth factor, a kind gift from Dr. S. Cohen, Vanderbilt University School of Medicine, was iodinated as described for insulin (8). The binding of [^{125} I]EGF (200 μ Ci/ μ g) to whole fibroblasts, harvested by scraping with a rubber spatula, and to a crude membrane preparation was determined as described (8). Membranes free from nuclei, but containing all other particulate components, were isolated by differential centrifugation in Hanks' buffer after brief homogenization (Polytron, 30 sec) and sonication (twice for 7 sec with a Savant model 500 sonicator at 30% output). The amount of [^{125} I]EGF specifically bound was determined by subtracting the amount of radioactivity bound in the presence of a saturating amount of native EGF (2 μ g/ml) from the amount bound in the absence of the native compound. Purified cholera toxin (lot 0572), prepared under contract for NIAID by Dr. R. A. Finkelstein, The University of Texas Southwestern Medical School, Dallas, and isolated by the method of Finkelstein and LoSpalluto (3), was obtained from Dr. R. S. Northrup, SEATO Cholera Research Program. The molecular weight of EGF was assumed to be 6000 (9), and that of cholera toxin, 84,000 (3). Crystalline porcine zinc-insulin (24.4 USP U/mg) was obtained from Eli Lilly & Co.

RESULTS

Stimulation of Polynucleotide Synthesis and Binding of EGF. EGF is a potent stimulant of DNA synthesis in HF fibroblasts (Fig. 1). The concentration required for half-maximal effect is about 70 pM. The maximal response, observed at about 300 pM, appears to be slightly diminished at higher concentrations. The other two cell lines (PB and WI-38) are less responsive to EGF (Table 1). The response of HF cells to EGF can be contrasted with the small response obtained with 5 μ g/ml (10^{-6} M) insulin; no response is observed at physiological concentrations (10^{-10} M) of insulin.

The synthesis of RNA is also stimulated by EGF (Fig. 2). The time-course reveals that the enhancement of RNA synthesis slightly precedes that of DNA synthesis. In cells stimulated with EGF the synthesis of both RNA and DNA begins at a

FIG. 2. Time-course of polynucleotide synthesis in HF fibroblasts stimulated by fetal-calf serum and by EGF. Incorporation of either [^{3} H]thymidine (1 μ Ci/ml) or [^{3} H]uridine (1 μ Ci/ml) was measured by a 1-hr pulse at timed intervals after the addition of serum (10%, v/v) $\bullet$ — $\bullet$, or EGF (20 ng/ml) $\circ$ — $\circ$. (Left) [^{3} H]Uridine, cpm/100 μ g of cell protein. (Right) [^{3} H]Thymidine, cpm/100 μ g of cell protein.

slightly later time than in cells stimulated with 10% fetal-calf serum.

The binding of [^{125} I]EGF to fibroblasts (Fig. 3, upper) is saturable and is demonstrable at all concentrations of EGF that stimulate DNA synthesis. The effect of increasing concentrations of native EGF on the binding of [^{125} I]EGF (Fig. 3, lower) demonstrates the similarity between these two compounds. Neither insulin (5 μ g/ml) nor cholera toxin (0.75 μ g/ml) significantly affects the binding of [^{125} I]EGF to fibroblasts. The binding of [^{125} I]EGF to crude cell membranes is similar to the binding to whole cells. From the binding data, one can estimate an apparent dissociation constant of about 800 pM.

Additivity of Effects of EGF and Serum. The response of HF cells to the combined action of serum and EGF is greater than the response to either substance alone (Table 2). Even at EGF concentrations well above those required for maximal effects on DNA synthesis, the addition of serum produces a further stimulation of thymidine incorporation. This effect is observed with both 5% and 10% fetal-calf serum.

Effects of Cholera Toxin, Dibutyryl Adenosine 3':5' Cyclic Monophosphate, and Theophylline on Polynucleotide Synthesis.

TABLE 1. Stimulation of DNA synthesis in human fibroblasts and binding of EGF

Cell line	Stimulant	cpm		EGF binding
		Unstimulated	Stimulated	
HF	EGF	1,800 $\pm$ 500	13,700 $\pm$ 1,600	1.00
HF	Insulin	390 $\pm$ 100	900 $\pm$ 180	
HF	Serum	390 $\pm$ 100	9,900 $\pm$ 1,900	
PB	EGF	330 $\pm$ 160	1,760 $\pm$ 840	0.53
WI-38	EGF	340 $\pm$ 110	880 $\pm$ 500	0.38
WI-38	Serum	340 $\pm$ 110	9,400 $\pm$ 920	

[^{3} H]Thymidine incorporation, expressed as cpm/dish ($\pm$ SD, $n = 3$), was measured either at 23 hr (HF & PB) or 18 hr (WI-38) after the addition of EGF (0.2 μ g/ml), insulin (0.5 μ g/ml), or fetal-calf serum (10% v/v) to cells refed with fresh growth medium, containing bovine albumin (1 mg/ml). The binding of [^{125} I]EGF (4.8 ng/ml) to cells (1×10^6 /ml) is expressed relative to that of HF cells, which bind 0.35 ng/ 10^6 cells under these conditions.

FIG. 3. Binding of EGF to fibroblasts. (Upper) The binding of EGF to whole cells (1×10^6 per ml) and to crude cell membranes (0.31 mg of protein per ml) was measured in 0.2-ml suspensions in Hanks' buffer containing 0.1% w/v bovine albumin. $\circ$ — $\circ$, whole HF cells; $\bullet$ — $\bullet$, HF cell membranes; $\square$ — $\square$, whole PB cells. Binding is given both in ng and $10^{12} \times$ mol, either per 10^6 cells or per 0.8 mg of membrane protein. (Lower) The displacement from whole HF fibroblasts (6.5×10^6 per ml) of [^{125} I]EGF (46,000 cpm; about 2 ng/ml) by increasing amounts of native EGF was measured in triplicate 0.2-ml aliquots. $\blacksquare$ — $\blacksquare$, total cpm bound per sample.

Cholera toxin inhibits polynucleotide synthesis stimulated either by serum or by EGF (Table 3). The effects of cholera toxin on DNA synthesis can be detected at concentrations as low as 10^{-14} M (Fig. 4). While the response to EGF can be completely suppressed by cholera toxin, the response to fetal-calf serum cannot be inhibited more than about 80%, even at

TABLE 2. Combined effects of serum and EGF on DNA synthesis in HF fibroblasts

Stimulant	[^{3} H]Thymidine incorporation (cpm)	Sum of separate effects
EGF	$4,400 \pm 1,200$	
10% Serum	$11,700 \pm 1,300$	
EGF and 10% serum	$19,000 \pm 1,900$	$16,100 \pm 2,500$
5% Serum	$6,600 \pm 800$	
EGF and 5% serum	$12,800 \pm 1,800$	$11,000 \pm 2,000$

Confluent cells (about 100 μ g) were refed with growth medium containing bovine albumin (1 mg/ml) and either fetal-calf serum or EGF (0.1 μ g/ml). Incorporation of [^{3} H]thymidine, measured 23 hr after the addition of stimulant, is expressed as cpm/tray and is corrected for the radioactivity incorporated in the absence of stimulant (1350 ± 600 cpm).

FIG. 4. (left). Inhibition by cholera toxin of DNA synthesis in HF fibroblasts stimulated by serum and EGF. HF cells at confluency (100 μ g of protein) were refed with fresh growth medium containing 0.1% bovine albumin and either 10% fetal-calf serum or 10 ng/ml of EGF in the presence of increasing amounts of cholera toxin. [^{3} H]Thymidine incorporation, measured at 23 hr, is expressed as a percent of the maximum incorporation in the absence of either EGF or serum (about 2000 cpm). Maximum thymidine incorporation was $12,900 \pm 155$ cpm for serum and $20,900 \pm 1,862$ cpm for EGF. Serum, $\circ$ — $\circ$; EGF, $\bullet$ — $\bullet$.

FIG. 5 (right). Kinetics of cholera toxin inhibition of EGF-stimulated DNA synthesis in HF fibroblasts. Confluent cells were refed with growth medium containing 0.1% bovine albumin and 10 ng/ml of EGF. Cholera toxin (0.15 ng/ml) was added to triplicate samples at the indicated times after stimulation, and [^{3} H]thymidine incorporation was measured at 23 hr. Results are expressed as a percent of the incorporation observed in the absence of toxin. $\circ$ — $\circ$, effect of cholera toxin; $\bullet$ — $\bullet$, effect of 0.25 mM dibutyryl cAMP on serum-stimulated DNA synthesis (replotted from ref. 19).

concentrations of toxin three orders of magnitude greater than those shown in Fig. 4.

At concentrations of cholera toxin sufficient for maximal inhibition of DNA synthesis, cells appear to be viable by several criteria: (i) by phase contrast microscopy, (ii) by Nigrosine-dye exclusion, where more than 96% of both toxin-treated and control cells exclude dye 24 hr after the addition of toxin, and (iii) by the ability of toxin-treated cells, which are completely refractory to EGF, to respond (DNA synthesis) to the subsequent addition of fetal-calf serum. EGF-treated cells also appear viable by the same criteria.

The time-period during which cells stimulated by EGF are susceptible to the inhibitory action of cholera toxin, and beyond which cells are committed to DNA synthesis, is indicated in Fig. 5. Cholera toxin added as long as 7 hr after the initial stimulus of EGF can still inhibit 80% of the subsequent DNA synthesis observed in control cells.

The effects of cholera toxin are similar in time-course and magnitude to the effects of dibutyryl cAMP, theophylline, or combinations of the two (Fig. 5 and Table 4). These compounds inhibit EGF-stimulated DNA synthesis in both HF and PB fibroblasts.

DISCUSSION

The present studies show that human cells respond to EGF by initiating both DNA and RNA synthesis. Effects on DNA synthesis are seen at concentrations (10^{-10} to 10^{-11} M)

TABLE 3. *Effects of cholera toxin on polynucleotide synthesis*

Cell	Stimulant	[³ H]Nucleoside	Addition			
			None (cpm)	Stimulant alone (cpm)	Stimulant plus toxin	
					(ng/ml)	(cpm)
HF	Serum	Thymidine	1,700 ± 500	8,300 ± 560	10	3,200 ± 240
HF	EGF	Thymidine	1,900 ± 290	6,200 ± 630	0.015	1,400 ± 150
HF	EGF	Uridine	N.D.	2,000 ± 250	0.15	730 ± 80
WI-38	Serum	Thymidine	1,300 ± 110	8,400 ± 100	15	2,300 ± 170

Confluent fibroblasts were stimulated either by fetal-calf serum (10%, v/v) or by EGF (10 ng/ml), in the presence or absence of cholera toxin at concentrations higher than those required for maximal inhibition (Fig. 4). Incorporation (cpm/dish) of [³H]nucleoside was measured at 18 hr for WI-38 and 23 hr for HF cells. *N.D.*, not determined.

equivalent to those normally present in mouse serum (10) and at concentrations which are lower than those that stimulate mouse-mammary gland cells *in vitro* (11). It is of interest that EGF, like insulin, is a polypeptide with molecular weight of about 6000 which has three disulfide bridges in its structure. While insulin has multiplication-stimulating activity in a number of cell-culture systems (reviewed in ref. 12), these effects are observed only at concentrations several orders of magnitude above those which obtain in serum. In the present studies insulin at concentrations as high as 5 µg/ml (10⁻⁶ M) has only a very small effect on HF fibroblasts when compared to EGF (Fig. 1), and no observable effects on DNA synthesis occur with physiological concentrations of the hormone. It appears likely, therefore, that in these culture systems insulin may not normally behave as a multiplication factor, but that it may at very high concentrations somehow mimic the action of a peptide present in serum. The possibility that these effects of insulin result from a contaminating natural peptide, perhaps present in minute quantities relative to insulin, cannot be excluded.

It is intriguing that Pierson and Temin (13) have identified in calf serum a trypsin-sensitive multiplication-stimulating factor which is slightly smaller than insulin and which probably contains disulfide bonds. The compound is 3- to 8-times as active as insulin in stimulating chicken-embryo fibroblasts, but it has only 1/5000 of the activity of insulin in the isolated fat-cell assay (14). Studies in this laboratory indicate that purified EGF does not have insulin-like activity in isolated fat cells, it does not bind to these cells, and it does not interfere with the binding of insulin to its receptors (manuscript in preparation). In view of some similarities between EGF, insulin, and the aforementioned serum factors in size and composition, and in view of the sensitivity of human fibroblasts to EGF and the specificity with which EGF binds to both fibroblasts and other mammalian tissue membranes (manuscript in preparation), it is tempting to speculate that a peptide similar to if not identical with EGF is indeed among those factors in sera which stimulate cell growth. EGF is clearly distinct from somatomedin ("sulfation factor," "thyridine factor"), a small polypeptide present in serum, since this peptide has potent insulin-like effects in isolated fat cells and it can effectively compete with insulin for binding to receptors in several tissues (15).

Such an EGF-like peptide need not be the only serum factor affecting polynucleotide synthesis. The present studies show that the time-course of stimulation by serum, and the

ability of serum to stimulate further those cells already maximally stimulated by EGF, clearly differentiate the effects of whole serum from those of EGF alone. It can be concluded that factors in serum other than an EGF-like peptide must also affect polynucleotide synthesis. It is possible that different serum factors stimulate confluent cells (presumably in G₀) to enter S phase at different times, as is suggested in Fig. 2 by an earlier increase in nucleoside incorporation in serum-stimulated compared with EGF-stimulated cells. While 23-24 hr is the time of maximal thymidine incorporation in cells stimulated both by EGF and by serum, this time need not be optimal for each of the factors present in serum. Part of the effect of serum may be due to the presence of cortisol, which, like EGF, can stimulate contact-inhibited fibroblasts at micromolar concentrations (16), but which, unlike EGF, does not appear to be markedly inhibited in its action by dibutyryl cAMP. Also, other compounds such as calcium, folic acid, and putrescine can, under certain conditions, act as growth-promoting substances (17). It will be of interest in future experiments to study the relation between the actions of the several compounds in serum which affect cell growth.

The binding curve for EGF (Fig. 3) indicates that the capacity of the cells to bind EGF exceeds by 5-fold the amount of EGF required for maximal DNA synthesis. This result contrasts with that obtained in fat cells, where insulin binding closely parallels the biological response to the hormone (8). The relationship between EGF binding and DNA synthesis is

TABLE 4. *Effects of theophylline and dibutyryl cyclic AMP on EGF-stimulated DNA synthesis*

Cell line	Addition (cpm)			
	None	Bt ₂ cAMP	Theophylline	Bt ₂ cAMP plus theophylline
HF	15,300 ± 1,300	N.D.	5,800 ± 200	2,700 ± 600
PB	1,800 ± 850	1,200 ± 65	700 ± 37	390 ± 2

Fibroblasts were refed with serum-free medium containing 1 mg/ml of bovine albumin and EGF (10 ng/ml for HF, 200 ng/ml for PB). Dibutyryl cAMP (Bt₂ cAMP, 50 µM) and theophylline (1 mM for HF, 0.5 mM for PB) were added separately or in combination. [³H]Thymidine incorporation (cpm/dish) was measured at 23 hr. *N.D.*, not determined.

undoubtedly complicated, with both events widely separated in both space and time. Further work will be required to elucidate the amplification process whereby occupancy of only 20% of the cell-surface receptors leads to a maximal stimulus of DNA synthesis. It may be of significance in this respect that those cells which respond best to EGF have a substantially greater ability to bind EGF compared with cells only minimally responsive to the polypeptide (Table 1).

It is striking that cholera toxin can completely inhibit DNA synthesis at concentrations two orders of magnitude lower than those at which EGF is maximally active; toxin effects are clearly demonstrable even at 2×10^{-14} M. These results are consistent with the essentially irreversible nature of the binding of ^{125}I -labeled cholera toxin to cell membranes (18). Although cholera toxin also inhibits serum-induced DNA synthesis, no more than 80% inhibition is observed in this case. The presence in fetal-calf serum of substances known to affect the binding of cholera toxin to cells (18) may, in part, be responsible for this result. It has recently been found that membrane-bound gangliosides (especially GM_1) are the chemical determinants which bind cholera toxin in various tissues, and these glycolipids appear to be the biological receptors for the toxin (18). It will be of interest in the future to determine whether cell-surface gangliosides are also involved in the interaction of cholera toxin with cells.

Data from many studies indicate that cholera toxin probably acts by way of cAMP to mediate its biological effects in a variety of tissues (4). In virtually every tissue examined, including fibroblasts (unpublished), cholera toxin can stimulate the adenylate cyclase activity of plasma membranes (4). In the present studies the time-course of toxin inhibition of DNA synthesis parallels closely that which Froehlich and Rachmeler (19) described for inhibition of serum-stimulated DNA synthesis in human fibroblasts by dibutyryl cAMP and theophylline (Fig. 5). Moreover, the present experiments indicate that theophylline or theophylline plus dibutyryl cAMP inhibit EGF-stimulated DNA synthesis as effectively as does cholera toxin (Table 4). It is therefore reasonable to suppose that cholera toxin blocks DNA synthesis by raising intracellular levels of cAMP.

Such a proposal for the action of toxin is in agreement with many other recent studies demonstrating inverse correlations between the level of cAMP and the level of DNA synthetic activity (20). Contact inhibition of growth, decreased rates of cell growth, and inhibition of transformation of lymphocytes by plant lectins are associated with increased levels of intracellular cAMP or with the addition of substances (e.g., prostaglandins and catecholamines) which are known to stimulate adenylate cyclase activity. Reversal of contact inhibition and rapid rates of cell growth appear to be associated with a fall in cAMP levels.

It is pertinent that certain growth-promoting and anabolic substances, such as insulin (21), concanavalin A (22), and somatomedin (23), which all initiate similar kinds of metabolic events in cells (a "pleiotypic response," ref. 24), can directly inhibit the activity of adenylate cyclase in subcellular systems. Is it possible that EGF may belong with this group of compounds? It will thus be important in future work not only to confirm the proposed mechanism of action of toxin in blocking DNA synthesis by raising levels of cAMP, but also to determine whether EGF stimulates DNA synthesis by just the opposite effect, namely by lowering levels of cAMP. Such

studies, based on the techniques and observations described here, will increase our understanding of the manner in which specific cell-surface interactions modulate the ability of cells to initiate polynucleotide synthesis.

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