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REVIEW ARTICLE

The Regulation of Cell Proliferation: Advances in the Biology and Mechanism of Action of Epidermal Growth Factor

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Epidermal growth factor (EGF), which has been isolated from the mouse submaxillary gland [1] and human urine [2], exerts pronounced hypertrophic and hyperplastic effects on epidermal tissues *in vivo* [1,3] and *in vitro* [4,5]. The importance of this polypeptide to biomedical research stems from (1) its role as one of the best characterized mitogens for the study of mammalian cell proliferation and/or differentiation and (2) its potential application to or role in human disease. EGF was last reviewed in this journal by Cohen in 1972 [6], therefore this article will concentrate on developments since that time. Additional aspects of this subject may be found in other recent review publications [7–9].

PHYSIO-CHEMICAL PROPERTIES OF MOUSE-DERIVED EGF

Studies by Taylor et al. [10] reported many of the basic chemical and physical properties of mouse-derived EGF (mEGF). The growth factor is a heat stable, single polypeptide chain of 53 amino acid residues and has a molecular weight of 6045. The iso-electric point of mEGF was reported at pH 4.60 and the extinction coefficient ($E^{1\%}_{1cm}$ at 280 nm) was determined to be 30.9. The complete amino acid sequence and location of 3 intramolecular disulfide bonds for this polypeptide growth factor are known [11,12]. Centrifugation studies of mEGF indicated a sedimentation constant of 1.25 S [1] and a frictional ratio (f/f_0) of 1.12 (L. Holladay, personal communication) suggesting the molecule has a compact and globular structure. Examination of mEGF by circular dichroism showed the absence of significant α-helical structure, the presence of approximately 25% β-helix, and a random coil content of 75% [10]. Circular dichroic studies of equilibrium unfolding and thermal stability in the presence of guanidinium hydrochloride demonstrated mEGF has a very stable tertiary structure and, in fact, is one of the most energetically stable proteins known [13].

HUMAN EPIDERMAL GROWTH FACTOR

A significant and recent development has been the identification [14] and isolation [2] of EGF from human urine. This molecule, i.e., human EGF (hEGF), has all of the biological activities previously ascribed to the mouse derived growth factor. The probable amino acid composition of hEGF has been determined and when compared to that of mEGF considerable similarities but definite differences are apparent. It is clear, however, that mouse and human EGF must contain some common amino acid sequences as they exhibit the same biological activities, cross-react antigenically, and cross-compete in radioreceptor assays. The evolutionary conservation of these sequences may suggest an important role for EGF in mammalian biology.

With the publication by Gregory [15] of the amino acid sequence of β-urogastrone, a gastric antisecretory hormone isolated from human urine, an intriguing aspect of the biology of EGF has emerged. The data show that the amino acid sequences of β-urogastrone and mEGF are 70% homologous and that the amino acid compositions of β-urogastrone and hEGF are nearly identical. The structural similarity of β-urogastrone and EGF is supported further by their capacities to elicit identical biological activities. EGF inhibits histamine-induced gastric acid secretion [16], β-urogastrone stimulates the proliferation of epidermal tissue [15], and the 2 molecules cross-compete in radio-receptor assays [17]. These facts indicate that human EGF and human β-urogastrone are probably the same molecule that possesses seemingly unrelated biological activities.

Since the detection of EGF in human urine, investigations have begun to measure EGF in human fluids and to correlate these data with normal or abnormal physiological states [18,19]. Radioimmunoassay of hEGF levels have shown that human urine contains 29–272 ng/ml (mean = 88 ng/ml), saliva contains 5–17 ng/ml, and the breast milk has approximately 80 ng/ml. Preliminary data indicate about 2–4 ng/ml in human plasma. No significant differences between adult males, females or young children have been detected in 24 hr urinary excretions of hEGF. These studies indicate no diurnal rhythm in hEGF excretion, but do show an excellent linear correlation between urinary hEGF and creatinine concentrations. Unusually low levels of urinary hEGF, 5–15 ng/ml (mean = 12 ng/ml), were reported for newborns and high levels were detected in 24 hr urinary excretions from females taking oral contraceptives—73.7 μg/total volume or 60.1 μg/gm creatinine compared to 52.0 μg/total volume or 39.8 μg/gm creatinine for women not taking contraceptives. Urinary concentrations of hEGF have been recorded for a relatively small sampling of patients with various disorders, particularly dermatological and gastrointestinal pathologies, but have not yielded significant deviations from the normal range of urinary hEGF. Of course, it would be more desirable and meaningful to measure plasma levels of hEGF; however, a sufficiently stable and sensitive assay is not available.

Evidence defining sites in the body where the growth factor is synthesized and/or stored is important for understanding the physiology of EGF. In the mosue it is clear that EGF is primarily stored, and probably synthesized, in the tubular duct cells of the submaxillary gland [20]. However, since plasma

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Abbreviations:
DDIG: density dependent inhibition of growth
EGF: epidermal growth factor
hEGF: human epidermal growth factor
mEGF: mouse-derived epidermal growth factor

levels of EGF in the mouse are not altered by ablation of the submaxillary gland, it is clear that there are other unidentified sites where the hormone is produced. Immunofluorescent staining of human tissues has demonstrated the localization of EGF in cells of the ducts of the submandibular glands and in cells of the glands of Brunner in the first part of the duodenum [21]. These results are interesting in regard to the homology of hEGF and urogastrone, the ability of EGF to inhibit gastric acid secretion, and the possible role of EGF in the rapid turnover of the cells lining the gut [22].

BIOLOGIC ACTIVITIES OF EGF ON SKIN

The most prominent activities ascribed to EGF are its distinct and direct effects upon the epidermis. Most of these studies were originally performed in animals or organ culture systems prior to 1972 and, unfortunately, have not been followed up in more detail in recent years. However, in view of the nature of this journal and the specialized interests of its readership these early studies will be reviewed briefly.

The initial observation which led to the recognition of epidermal growth factor was that when newborn mice were injected daily with extracts of the mouse submaxillary gland or the purified factor (1 μg EGF per gm body weight) the eruption of incisors and opening of the eyelids were accelerated: 6–7 days compared to the normal 12–14 days [1]. Similar results were demonstrated in newborn rats, rabbits, dogs and sheep. Histological studies showed that these gross anatomical changes were due primarily to an enhancement of both proliferation and keratinization of epidermal tissue [3]. However, the results do not allow a conclusion as to whether EGF directly stimulates keratinization or indirectly by promoting basal cell proliferation. Curiously, in older mice (12–20 days) the daily injection of EGF had little visible effect on dorsal skin but produced a marked thickening of tail and foot-pad epidermis of rats. The histological evidence of epidermal hypertrophy and hyperplasia following the administration of EGF to newborn rats has been augmented by cytological quantitation of increases in the number of mitotic epidermal cells [23], chemical measurements of increases in dry weight, DNA and RNA content of the epidermis [24], and biochemical assays of enhanced activity of epidermal enzymes such as ornithine decarboxylase and histidine decarboxylase [25,26]. Also an increase in the disulfide content of the epidermis concomitant with a decrease of free sulfhydryl groups has been reported [27]. Agents such as dibutyryl cyclic AMP, isoproterenol, or papaverine which elevate cyclic AMP levels block the ability of EGF to increase the number of mitotic cells in the epidermis of newborn rats [23]. These agents, however, also reduce the number of mitotic figures in control animals. Recently, evidence has been presented indicating EGF decreases both adenylcyclase activity and cyclic AMP concentrations in mouse epidermis by approximately 30%, but only after 3–5 days of treatment with the growth factor [28]. At this time no conclusions can be drawn concerning a possible relationship of EGF and cyclic nucleotides.

An affect of EGF on skin that is probably related to its mitogenicity, is the ability of EGF to enhance the carcinogenic potential of methylcholanthrene [29,30]. In these studies the growth factor by itself did not produce tumors, but decreased the latency period and increased the numbers of papillomas and carcinomas per animal when methylcholanthrene was topically applied to the skin. An early, persistent alopecia and a distinct pachyderma developed in the area of methylcholanthrene application in only those animals that also received EGF.

Different lines of evidence demonstrate that EGF has a direct effect on the epidermis. Within 2 hr after the intraperitoneal injection of ^{125}I-labeled EGF into rats, the radioactivity was concentrated 3-fold in the epidermis relative to the blood [31]. The radioactivity was not concentrated in any other major organ with the exception of the cornea. Specific binding sites for ^{125}I-EGF in epidermal tissue *in vitro* have been demonstrated [32]. Cohen and co-workers have shown that EGF stimulates the proliferation of epidermal cells in organ cultures of chick embryo skin [4] and human fetal head skin [32]. A significant aspect of this work was the demonstration that EGF stimulated ornithine decarboxylase activity, RNA, protein and DNA synthesis, cell division and eventually keratinization either in the presence of "killed" dermis or in the absence of any dermis [4,5]. It appears, therefore, that EGF interacts directly with the epidermis and its action on this target tissue is not mediated by a second hormone.

Recently tissue culture systems have been devised to study human epidermal keratinocytes. Low concentrations of EGF, 0.3 to 30 ng per ml, affected the proliferation of cultured keratinocytes by enhancing the plating efficiency and expanding the lifetime or number of serial transfers [34]. The net result of EGF was to delay the onset of terminal differentiation in the cell population and increase the number of keratinocytes that can propagate *in vitro* by a factor of 10^{10}. Factors involved in the terminal differentiation of cultured keratinocytes *in vitro* have been studied and show that when a population of keratinocytes becomes confluent, the upper layer of cells is shed from the surface like stratum corneum cells *in vivo* and replaced by dividing cells in the basal layer. The cells shed into the medium are flattened, elongated squames and their rate of formation was increased 2–5 fold by the presence of EGF [25]. The development of cell culture systems to study the interactions between EGF and normal epidermal cells may provide an excellent experimental system to understand the biochemical actions of this hormone on skin *in vivo*.

BIOLOGICAL ACTIVITIES OF EGF ON EYE TISSUE

The corneal epithelium appears to be a second major target tissue for EGF *in vivo* and *in vitro*. Interestingly, both the corneal epithelium and epidermis are stratified squamous epithelia which embryologically are derived from related precursors. The basal cells in these epithelia are morphologically identical, but the superficial layers have differentiated in obviously different ways. When cultured *in vitro*, however, cells from the corneal and conjunctival epithelia and from the epidermis all produced keratins and other cellular proteins which were indistinguishable by polyacrylamide gel electrophoresis [36]. These results have been interpreted as evidence that the phenotypic differentiation of these epithelia *in vivo* is controlled by external factors and not totally determined by an intrinsic mechanism during development.

Two hours after ^{125}I-EGF is injected intraperitoneally into the rat, radioactivity is concentrated 800%, compared to blood, in the epithelial cells of cornea [31,37]. Specific binding of ^{125}I-EGF to isolated corneal epithelial cells has been demonstrated confirming the presence of specific receptors in these cells [31].

Studies by 2 groups [33,37] demonstrated that EGF is a potent mitogen for corneal epithelial tissue *in vivo*. The corneal epithelium of rabbits was experimentally wounded and EGF topically applied to one-half the wounded animals. Application of the growth factor to these wounds resulted in a marked hyperplasia of the corneal epithelium and a slight decrease in time required for wound closure. During regeneration of the epithelia in animals treated with EGF, the epithelium increased from its normal thickness of 4–6 layers to 10–15 cell layers after 6 days and then returned to the normal thickness by 14 days. Topical application of EGF to control corneas (non-wounded) had no discernable effect.

The hyperplastic effect of EGF on the corneal epithelium was demonstrated in organ cultures of chick or human corneas [33]. A similar stimulation of the proliferation of bovine corneal epithelium occurred in organ culture [38]. However, multiplication of these isolated bovine corneal epithelial cells in cell culture was not affected by EGF, although specific binding sites

for ^{125}I-EGF were present on these cells [38]. These authors have interpreted this observation to indicate that epithelial-mesenchyme interactions are necessary for EGF to have a mitogenic effect on the corneal epithelial. It is, of course, difficult to assess whether the technical manipulations or conditions used to isolate and culture these bovine corneal epithelial cells may have influenced their responsiveness to the hormone.

Recent experiments have indicated that the slow release of EGF from a polyacrylamide pellet inserted in the rabbit cornea induces neovascularization of this tissue [39]. Also, corneal and human vascular endothelial cells cultured *in vitro* are stimulated to proliferate by EGF [39,40].

No effects of EGF on lens tissue *in vivo* have been reported. Cultured bovine and rabbit lens epithelial cells do possess receptors for ^{125}I-EGF and the growth factor did stimulate DNA synthesis in cultured rabbit cells [38,41]. However, enhanced DNA synthesis, by itself, is not necessarily a sufficient measure of mitogenicity which requires increased cytokinesis, as measured by increases in cell numbers.

BIOLOGIC EFFECTS OF EGF ON OTHER TISSUES AND CELLS

Infusion of fetal lambs *in utero* with EGF for 3–5 days markedly stimulated the development of epithelial tissue in lungs, trachea and esophagus [42]. In addition, these authors present preliminary data suggesting that EGF may protect the premature fetal lamb from the development of hyaline membrane disease. Comparison of lung deflation pressure-volume curves from fetal rabbits, showed that fetuses injected with EGF had increased total lung capacity consistent with an increase in aveolar surface active material [43]. EGF treatment did not alter wet lung weight to body weight ratios.

Studies of cleft palate fusion in rodents indicates the epithelium of the palatal shelves is sensitive to EGF. Normal fusion of the secondary palate in rodents requires movement of the palatal shelves into apposition, adhesion, and fusion of the epithelial and mesenchymal layers of the joining shelves. During the fusion process, epithelial layers of the adhering shelves degenerate and fusion occurs between layers of mesenchymal cells. Hassell has demonstrated that when EGF is added to organ cultures of apposed palatal shelves from rat embryos, the epithelia thickens and keratinizes and the adhesion and fusion processes are inhibited [44]. An effect on EGF on palate development has been observed *in vivo* [45]. Cortisone was injected into pregnant mice at 11 and 14 days post-conception and a high proportion (61%) of the fetuses developed cleft palates. When EGF was administered with cortisone, the incidence of cleft palate rose to 100%. Administration of EGF alone did not effect palatal development.

Butcher et al reported that intraperitoneal infusion of EGF increased DNA synthesis in hepatocytes in the intact liver of normal adult rats [46,47]. This action of EGF was markedly enhanced by the simultaneous administration of glucagon or insulin. Neither glucagon nor insulin alone or in combination induced DNA synthesis. Treatment with EGF and insulin also produced hepatic enlargement, with considerable hypertrophy and hyperplasia. Similar effects of EGF in combination with glucagon or insulin on DNA synthesis have been demonstrated in cultured hepatocytes [48].

Mitogenic effects of EGF have been demonstrated on various cell types in cell culture and/or organ culture systems. In addition to those mentioned previously these include; mammary epithelial cells [49,50], chondrocytes [51], glia cells [52], vascular smooth muscle cells [53], human umbilical vein endothelial cells [54], granulosa cells [55], and various fibroblasts [56,57] which have been studied in greatest detail due to their relative ease of culturing *in vitro*. Details of the action of EGF on cultured fibroblasts are presented in the next section.

An interesting report of the effect of EGF on cultured cells that apparently is not directly related to mitogenesis is stimulation of the secretion of chorionic gonadotropin by human choriocarcinoma cells [57]. These results suggest that EGF may have a function in some cell types as a modulator of the secretion of other hormones. Although these data cannot be extrapolated to the situation *in vivo*, it is interesting to note that human placental tissue contains high levels of a specific binding protein for EGF [32].

MECHANISM OF EGF ACTION— BIOLOGICAL RESPONSES

The regulation of cell proliferation by EGF has been most extensively studied using cultured human fibroblasts as a model system [57,59–62]. These cells are considered "normal" as they maintain a diploid chromosome number *in vitro* and their growth is well regulated by mechanisms such as density dependent inhibition of growth (DDIG), a high serum requirement, and anchorage dependent growth. When human fibroblasts are cultured in the continuous presence of EGF (4 ng/ml), two of these growth controlling mechanisms are no longer evident [61,62]. Cells grown in the presence of EGF continue to proliferate when the culture becomes confluent, forming a multilayered cell population that is not restricted by density dependent inhibition of growth and reaching population densities 4–6 fold higher than the controls. Also, when human fibroblasts are incubated in low or deficient serum (1% calf serum or 10% γ-globulin free serum or plasma) cell proliferation is normally restricted, but supplementation of these media with EGF yields cell growth equal to that obtained in the presence of optimal serum concentrations (10% calf serum). The apparent loss of sensitivity to these growth regulating mechanisms of cells cultured in the presence of EGF bears some resemblance to the behavior of transformed cells *in vitro*. Transformed cells are frequently characterized by their insensitivity to the normal growth controlling mechanisms mentioned above. Thus, EGF, while not transforming cells, does have the effect under cell culture conditions of creating an imbalance of homeostatic signals that favors cells proliferation.

The effects of EGF on the growth of individual cells and cell populations is, of course, the end result of a complex series of biochemical and morphological events. When quiescent cells are exposed to a mitogenic stimulus, a temporal sequence of alterations in cell biochemistry occurs which appears to be similar regardless of the cell type or the mitogenic agent. These cellular reactions to mitogens have been collectively termed the pleiotropic response [63]. For cells stimulated *in vitro* by EGF, the following biochemical changes have been described and are listed according to their approximate temporal sequence in intact cells: increases in K^+ [64], sugar [65], amino acid [59] and uridine [57] uptake during the first 10 min to 2 hr following EGF addition; increased glycolysis with the activation of phosphofructokinase at 1–2 hr [66,67]; stimulation of hyaluronic acid synthesis [68] and increased membrane activity, such as macropinocytosis and ruffling [69], within 6 hr; enhanced protein [70] and RNA [57] synthesis by 12 hr; and entry into the S phase of the cell cycle with maximally increased DNA synthesis 20 hr after addition of EGF [59,61]. Although some points on this temporal "map" have been established, it is clear that many other biochemical alterations have not been uncovered. In regard to the mechanism by which EGF elicits this complex series of biochemical changes, there is little information from which to propose a model. Tata has suggested that polypeptide mitogens may enhance different parts of the pleiotropic responses by different mechanisms [71]. He proposed separate mechanisms by which "early" changes such as the transport of nutrients and "late" changes such as macromolecular synthesis are enhanced by mitogens. There is some evidence for this in the case of EGF. Experiments in which antibody to EGF is added to remove the growth factor from media, indicate that stimulation of DNA synthesis requires the continual presence of EGF in the media for at least 3–5 hr. Since many of the "early" changes induced by EGF are maximally stimulated

within 1–2 hr or less, the EGF mechanism responsible for enhanced DNA synthesis may be slightly or substantially different from that required for "early" events.

MECHANISM OF ACTION—BIOCHEMICAL INTERACTIONS

A more biochemical approach to the mechanism of EGF action has been initiated by studies of the binding and subsequent metabolism of ^{125}I-labeled EGF to cultured human fibroblasts [60,72,73]. Iodination of EGF, at the level of 1 or 2 atoms of ^{125}I-per mole of growth factor, does not reduce the biological potency of EGF and yields low levels of nonspecific binding—less than 2% [72].

The binding reaction of ^{125}I-EGF with fibroblast surface receptors is rapid, specific and saturable as are most hormone: receptor interactions. Interestingly, at 37° the binding of ^{125}I-EGF to receptors in the plasma membrane of intact cells is essentially irreversible, although at low temperature with intact cells or at 37° with isolated membrane fractions the binding is reversible [73]. The time course of ^{125}I-EGF binding to fibroblasts at 37° shows that maximal binding is reached in 45 min and that the amount of cell-bound radioactivity decreases thereafter. The decrease in cell-bound radioactivity amounts to 80% of the total when the incubation is carried out in the continuous presence of unbound labeled EGF. If the unbound hormone is removed from the incubation media after 45 min, then 100% of the cell-bound radioactivity is subsequently lost from the cells into the medium. The loss of cell-bound radioactivity at 37° is accompanied by the coordinate appearance of ^{125}I-monoiodotyrosine in the medium indicating extensive degradation of the hormone. Only cell-bound ^{125}I-EGF and not the free hormone in the medium is degraded. With intact cells at 5° or isolated membrane fractions at 37° no degradation can be detected.

The mechanism responsible for the loss of cell-bound radioactivity has been extensively studied and determined to be internalization and intracellular degradation of ^{125}I-EGF [73]. The authors suggest that shortly after binding of the growth factor to the cell surface at 37°, EGF: receptor complexes are internalized by an endocytotic mechanism that may be preceded by microclustering of these complexes on the cell surface. They also indicated that the most likely subsequent course of events taking place within the cell are fusion of endocytotic vesicles containing EGF:receptor complexes with primary lysosomes and proteolytic degradation of EGF to free amino acids. The degradation of EGF via lysosomes is indicated by the detection of ^{125}I-tyrosine as the only degradation product (only free amino acid can diffuse through the lysosomal membrane) and the inhibition of degradation by a known lysosomotropic agent, i.e., chloroquine. Recent and more direct investigations of the metabolic fate of cell-bound EGF have been undertaken by fluorescence microscopy of fluorescein conjugated EGF [74] and quantitative electron microscopic autoradiography of cell-bound ^{125}I-EGF [75]. Both of these studies confirm that cell-bound EGF is internalized within endocytotic vesicles which subsequently fuse with lysosomes.

However, the most important question, i.e., are internalization and/or degradation necessary for the biological activity of EGF?—remains unanswered. These metabolic processes may be part of the hormonal signal or they may be a cellular mechanism for stopping the signal.

During the course of the metabolism of cell-bound EGF it is likely that the occupied EGF receptor also is internalized. Cells incubated with EGF for 1 hr, washed to remove unbound hormone, and reincubated at 37° to allow EGF degradation show a reduced level (20% of controls) of receptor activity [73]. This regulation of the receptor by the homologous hormone is referred to as desensitization or "down regulation" and is observed with many polypeptide hormones. In the case of EGF and cultured fibroblasts the hormone induced loss of receptor activity can be recovered when serum is added [73]. Since the addition of either cycloheximide or Actinomycin D with serum prevents recovery of receptor activity, it appears that *de novo* synthesis of EGF receptors takes place under these conditions. The mechanisms which control receptor synthesis and determine receptor concentration is the membrane (fibroblasts have approximately 90,000 receptors per cell) are of obvious importance, but are completely unknown.

An interesting correlation has been observed between the capacity of cells to bind ^{125}I-EGF and transformation by sarcoma viruses. For example, normal rat kidney cells bound ^{125}I-EGF, but no binding was detected following transformation by the Kirsten sarcoma virus [72]. This observation has been extended by Todaro, DeLarco, and Cohen to include a variety of cell types and the Maloney, Kirsten, or Gardner sarcoma viruses [76]. This effect of sarcoma virus transformation on EGF receptors is specific in that transformation by other agents, such as DNA viruses, did not alter ^{125}I-EGF binding and sarcoma virus transformation did not effect the binding of other polypeptide hormones [77].

Clearly it will be necessary to delineate information concerning the chemical and physical properties of the EGF receptor before a biochemical understanding of the mechanism of action of this growth factor can be achieved. Based on the ability of lectins to inhibit ^{125}I-EGF binding it has been proposed that receptor is a glycoprotein [78] and binding studies utilizing a photosensitive cross-linking derivative of EGF have indicated a putative molecular weight of 190,000 for the receptor [79]. Little progress has been made in purifying the EGF receptor as solubilization of the membrane by standard detergent techniques results in no detectable binding activity. These results, although disappointing, may indicate that the active site of the receptor is strongly dependent on a hydrophobic environment which in natural membranes is provided by the interaction of proteins with specific lipids.

Although considerable progress has been made in the last decade, significant advances in the biology and mechanism of action of EGF undoubtedly will occur in the near future. Current research activities are progressing toward an understanding of the role of EGF in human biology and possibly some disease states. This work is being paralleled by efforts to define in biochemical terms the mechanism by which EGF regulates cell proliferation.

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Correction

In the August 1978 (vol. 71, No. 2) issue of the *Journal* the cover index listing for an article by Louis J. Rusin appeared with two errors. Dr. Rusin's name appeared incorrectly as Ruskin and the compound Ro 20-1774 should be Ro 20-1724.