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THE INFLUENCE OF EPIDERMAL GROWTH FACTOR ON SURFACE MORPHOLOGY OF FETAL RAT HEPATOCYTES IN PRIMARY CULTURE

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Abstract

In an attempt to understand the hormonal regulation of somatomedin secretion in the fetus, we have confirmed that epidermal growth factor (EGF) stimulates fetal rat hepatocytes in primary culture to secrete somatomedin in a time and a dose-dependent fashion. Transmission electron microscopy (TEM) revealed that the cultured cells had ultrastructural features consistent with those of fetal hepatocytes. Scanning electron microscopy (SEM) showed that cells grown in either Medium 199 or EGF supplemented Medium 199 formed cellular aggregates within 6 h. The surface features of cells in control and experimental cultures were indistinguishable up until 24 h after exposure to EGF. At this point in time, morphological differences between treatment groups were first apparent with SEM. In the presence of EGF, cellular aggregates were thicker, cells were more rounded in contour, and the number of microvilli and cytoplasmic excrescences (blebs) was greater than in control cultures. These differences were further accentuated at 48 h after exposure to the growth factor. Since the appearance of microvilli and blebs coincides with increasing production of somatomedin, they may represent morphological evidence of secretory activity.

Key Words: Fetal rat hepatocytes, primary culture, epidermal growth factor, somatomedin secretion, surface morphology.

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Introduction

It has been suggested that somatomedins, a family of polypeptides with insulin-like and mitogenic activity, may play a role in both normal and abnormal growth (13). They can be detected in fetal plasma (1,12), and receptors specific for these factors are present in fetal tissues (12,25,26). It has been demonstrated recently that fetal liver explants (11,23) and cell cultures (14) synthesize and secrete somatomedin into their culture medium. However, the hormonal regulation of this process has not been determined. In children and adolescents somatomedin levels appear to be growth hormone dependent (29), but this does not seem to be the case in the fetus (16). Therefore, we have been attempting to determine which hormones regulate somatomedin secretion by fetal rat hepatocytes in primary culture and have shown that epidermal growth factor (EGF) stimulated the secretion of a fetal somatomedin in a time and dose-dependent manner in this system (24).

EGF is a low molecular weight peptide that acts as a mitogen for a variety of cell types, and most of the work done with this growth factor has been focused on this property. There have been relatively few studies involving the possible effect of EGF on the phenotypic expression of cells. Since there is increasing interest in the influence of hormones on the surface morphology of cells, the aim of the present investigation was to study the effect of EGF on the surface characteristics of fetal rat hepatocytes in primary culture. We attempted to correlate the morphological and physiological effects of EGF in this system.

Materials and Methods

Animals

Timed-pregnant Sprague-Dawley rats were purchased from Taconic Laboratories, Germantown, N.Y. They were housed for 1 week prior to delivery of the fetuses by Cesarean section on the 21st day of gestation.

Preparation of fetal rat hepatocytes

Hepatocytes were isolated from 21 day old fetal rats by a modification of the procedure of Leffert and Paul (20). Medium 199 (M-199) with 0.2% Hepes buffer was substituted for arginine-free medium. The collagenase concentration was reduced from 3 mg/ml to 2 mg/ml,and 2.5 mM calcium chloride was included in the digestion buffer. Prior to plating in Falcon film-lined tissue culture dishes (60×15 mm), ice-cold ammonium

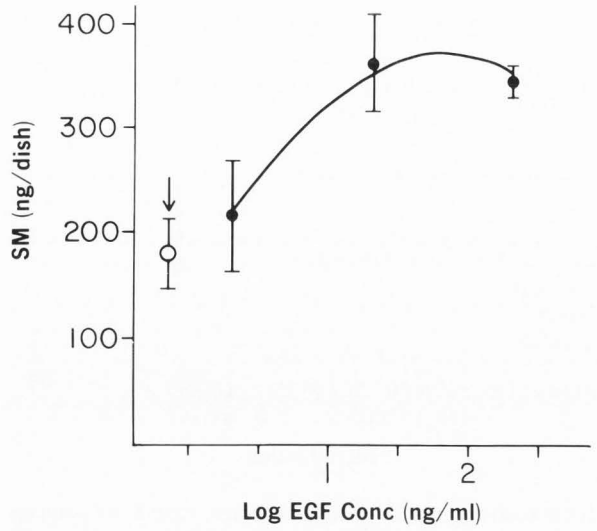


Fig. 1. Effect of EGF concentration on somatomedin secretion. Total somatomedin levels in the conditioned medium were determined by the competitive protein binding assay. Somatomedin secretion by hepatocytes cultured in control medium is indicated by the arrow. The means ± SEM for triplicate cultures, assayed in duplicate, are shown.

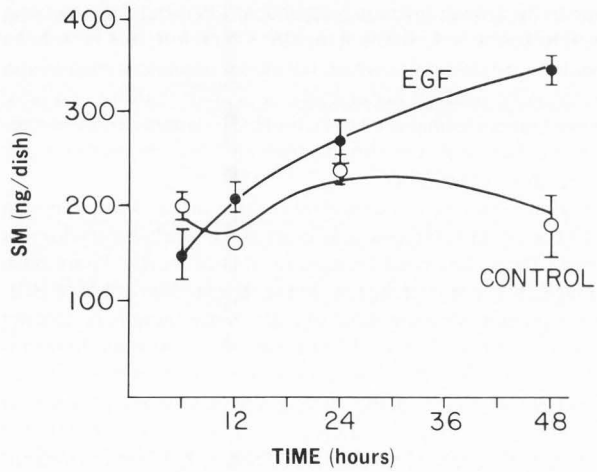


Fig. 2. Time course of EGF stimulation of somatomedin secretion. Fetal rat hepatocyte cultures were incubated in the presence or absence of EGF (200 ng/ml) for the indicated times. Total somatomedin levels in the conditioned medium were determined by the competitive protein binding assay. The means ± SEM for triplicate cultures, assayed in duplicate, are shown.

chloride (0.17 M) was added for 2 min (19). This treatment lysed the large number of erythrocytes present in fetal liver without damaging the hepatocytes. The hepatocytes were plated in triplicate at a density of 6×10^5 cells/ml in 5 ml of M-199 containing 5% fetal bovine serum and dexamethasone (1×10^{-7} M). The cultures were incubated at 37°C in an atmosphere of 5% CO_2-95% air. To take advantage of the more rapid rate of attachment of hepatocytes (22), the medium was aspirated off at

Fig. 3. Transmission electron micrograph of fetal hepatocytes cultured in control medium for 46 h. Note presence of desmosomes (arrow), bile canaliculus (C), accumulation of smooth endoplasmic reticulum (ser), and relative lack of modifications of the free surface (top of micrograph). Bar = 2.0 μm

Fig. 4. Transmission electron micrograph of hepatocytes that had been grown in the presence of EGF (200 ng/ml) for 46 h. Note increased thickness of aggregate and more conspicuous modifications of the free surface, especially cytoplasmic blebs (arrow). Nu, nucleus; n, nucleolus; L, lipid droplet. Bar = 5.0 μm.

Fig. 5. Low power scanning electron micrograph of an aggregate of hepatocytes cultured for 6 h in the absence of EGF. Note that it is difficult to distinguish intercellular boundaries between many of the cells. Bar = 10.0 μm.

Fig. 6. Scanning electron micrograph of several hepatocytes from a culture that had been grown in the absence of EGF for 6 h. These cells have irregular ridges and folds, as well as a few short microvilli. Bar = 5.0 μm.

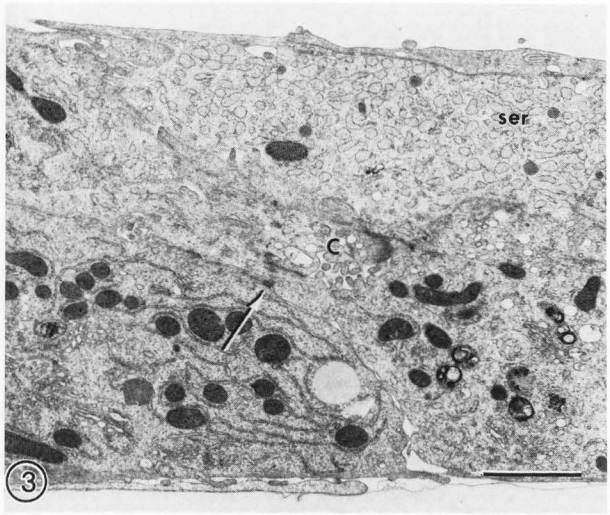
Fig. 7. Low power scanning electron micrograph of an aggregate of hepatocytes cultured for 12 h in the absence of EGF. Note increase in the number of cells with surface modifications. Bar = 10.0 μm.

Fig. 8. Scanning electron micrograph of several cells from a culture that had been grown in the absence of EGF for 12 h. These cells have more prominent ridges, folds and microvilli than do comparable cells at 6h. Cytoplasmic blebs (arrow) are present on a few of the cells. Bar = 5.0 μm.

4 h to remove hemopoietic cells. Fresh medium without dexamethasone was then added. After 24 h (considered zero time), the medium was replaced with serum-free M-199 with or without EGF. The conditioned medium was collected between 6 and 48 h later, centrifuged to remove cellular debris, and stored at −70°C until assayed. At 48 h cell viability, as determined by trypan blue exclusion, was approximately 80% in the presence or absence of EGF.

Competitive protein binding assay for somatomedin

Highly purified multiplication stimulating activity (MSA), similar to that described by Marquardt et al. (21), was radiolabeled with ^{125}I by a modification of the chloramine-T method using the iodo-bead reagent. The iodo-beads are chloramine-T derivatized polystyrene beads. One bead was added to a mixture of MSA, 2.5 μg, and 1.0 mCi carrier-free $Na^{125}I$ in 0.1 ml of 0.1 M phosphate buffer (pH 7.0). The reaction was terminated after 15 min at room temperature by removing the sample from the reaction vessel. Iodinated MSA was separated from unreacted ^{125}I by gel filtration using a 12 × 0.5 cm Sephadex G-25 column; the elution buffer was phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA). This procedure afforded rapid and complete separation with little dilution of the ^{125}I-MSA. The radiolabeled material was pooled, aliquoted, rapidly frozen in liquid N_2, and stored at −70°C in PBS plus 1% BSA. The ^{125}I-MSA was routinely repurified prior to use by Sephadex G-75 gel filtration in PBS.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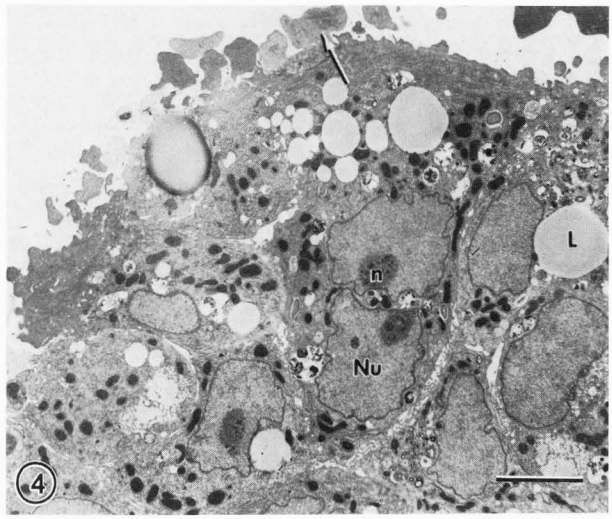
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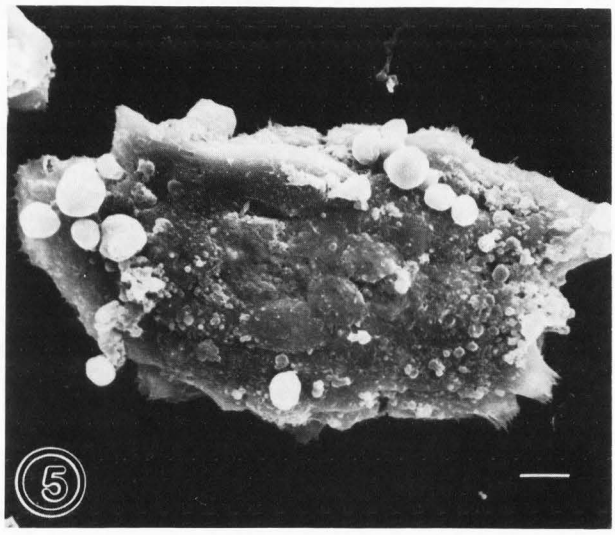
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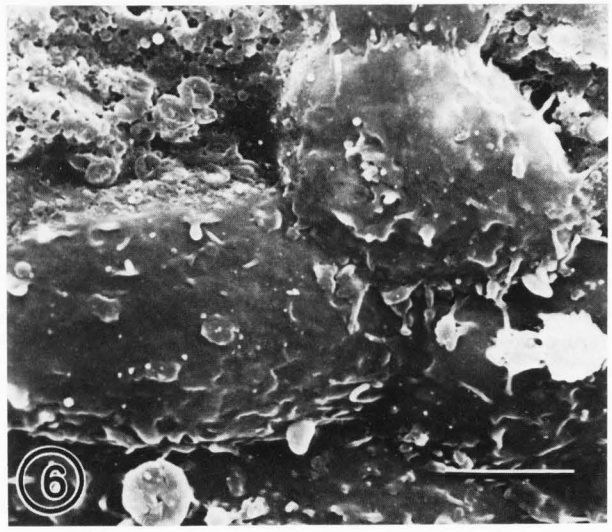
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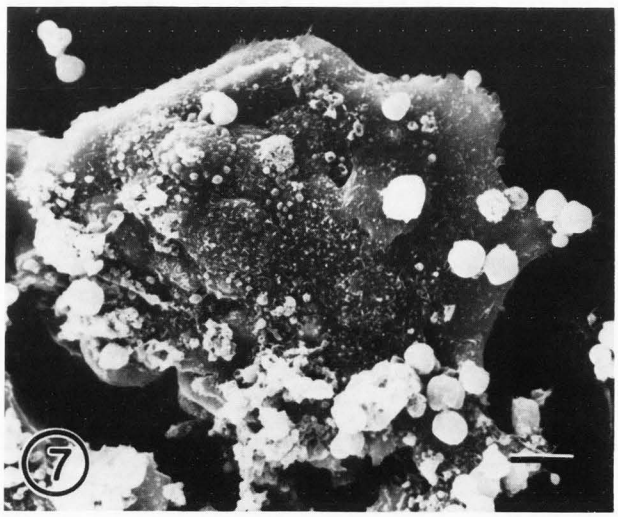
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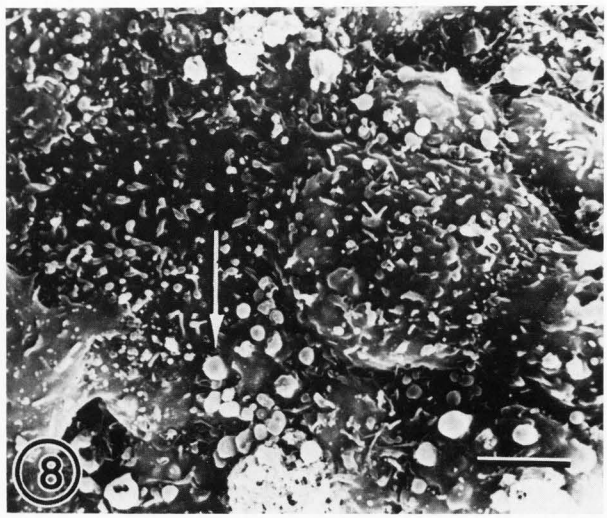
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Somatomedin carrier protein was prepared from normal rat serum by chromatography on Sephadex G-50 in 1 M acetic acid (30). The excluded protein peak was dialized extensively against 0.1 M PBS, aliquoted, and stored at −70°C. Both the MSA standards and the conditioned medium samples (usually 0.05 ml) were acidified to a final concentration of 1% acetic acid, centrifuged to remove any acid-stripped carrier protein in conditioned medium, and resuspended in 0.2 ml binding buffer (0.1 M PBS, 0.25% BSA, 0.01% sodium azide, pH 7.2). Then 0.2 ml of partially purified rat somatomedin carrier protein and 0.1 ml of ^{125}I-MSA (10,000 cpm) were added. After incubation at room temperature for 2 h, the reaction was terminated by the addition of ice-cold 2.5% activated charcoal in binding buffer. Under these conditions, unbound ^{125}I-MSA was adsorbed to the charcoal which was removed immediately by centrifugation. An aliquot (0.5 ml) of the supernatant fraction (containing the bound ^{125}I-MSA) was counted in a Beckman Biogamma 2000.

Scanning electron microscopy

Cells were grown in Falcon film-lined dishes. At the appropriate times, 6 to 48 h after the addition of epidermal growth factor or control medium, the cultures were washed twice with serum-free M-199 and fixed *in situ* at 4°C overnight in 2% collidine-buffered glutaraldehyde (300 mOsm). Prior to three 5 min washes with collidine buffer, film liners were cut into squares for further processing. Cells were postfixed for 1 h with 2% aqueous osmium tetroxide, washed 3 times in distilled water, dehydrated in a graded series of cold ethanol, and critical point dried in a Sorvall critical point drying system using carbon dioxide. Squares of film liner were mounted on aluminum stubs with silver paint, sputter coated with gold-palladium in a Hummer II apparatus, and observed in a Coates and Welter field emission or Hitachi Model S-520 scanning electron microscope.

Transmission electron microscopy

Cells were grown in Falcon film-lined or unlined dishes. Forty-six h after the addition of EGF (200 ng/ml) or control medium, the cultures were washed twice with serum-free M-199 and fixed *in situ* at 4°C overnight in 2% collidine-buffered glutaraldehyde (300 mOsm). The cells were then rinsed several times with 0.2 M phosphate buffer and post-fixed with veronal-buffered 1% osmium tetroxide for 1 h. Dehydration through a graded series of ethanol and propylene oxide was followed by embedding in Araldite 502. Cells grown on film liners were embedded *in situ* with small strips of the liner material. Dehydration of the cells grown directly on plastic culture dishes was followed by the addition of propylene oxide and gentle mixing. This resulted in the release of the cells as a layer from the substrate. The layers of cells were collected, rinsed with propylene oxide, centrifuged in conical BEEM capsules, and embedded in Araldite. Sections were cut perpendicular to the substrate on which the cells had been grown with an LKB Ultrotome III. They were placed on copper grids, stained with uranyl acetate and lead citrate, and examined in a Philips EM 300 transmission electron microscope.

Results

Since the experiments in which we first demonstrated that EGF stimulated the secretion of somatomedin in our system were performed in unlined polystyrene dishes (24), it was necessary to determine whether this effect could also be shown with fetal hepatocytes grown in dishes lined with polyester. To this end, total somatomedin levels in conditioned medium were measured by the competitive protein binding assay. Once again, EGF was a potent stimulator of somatomedin secretion. As was the case in the earlier experiments, the stimulatory effect was both dose (Fig. 1) and time dependent (Fig. 2). The optimum concentration of EGF was approximately 20 ng/ml, and the level of somatomedin in the conditioned medium rose until 48 h after exposure to the growth factor.

Transmission electron microscopy revealed the cultured cells to have ultrastructural features consistent with those of fetal hepatocytes (Figs. 3, 4). They were characterized by the presence of eccentrically placed nuclei containing finely dispersed chromatin and usually a single, prominent nucleolus. Their cytoplasm was abundant and contained a well developed Golgi apparatus, numerous mitochondria, many scattered profiles of rough endoplasmic reticulum, focal accumulations of smooth endoplasmic reticulum, many lipid droplets, and occasional lysosomes. The cells were closely applied, and numerous desmosomes were present. It was not unusual to observe fairly well developed bile canaliculi between adjacent cells. In control cultures, the hepatocytes tended to be elongated, and they grew on the substrate in a partially overlapping manner, at most two to three cell layers in thickness. In the EGF treated cultures, the cell clusters were larger and were most often four or five cells thick. The cells were more rounded, and there appeared

Fig. 9. Scanning electron micrograph of several cells from the center of an aggregate of hepatocytes that had been grown in the presence of EGF (200 ng/ml) for 24 h. Note that intercellular boundaries are discernible and that some cells are characterized by the presence of large blebs (arrow). Bar = 5.0 μm.

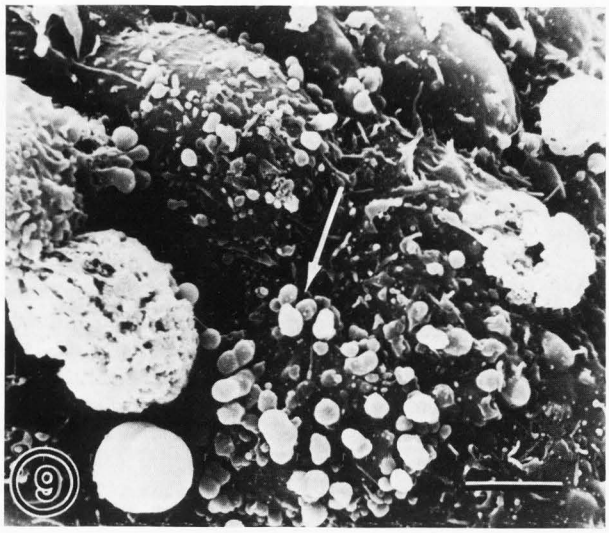
Fig. 10. Scanning electron micrograph of several cells at the periphery of an aggregate of hepatocytes that had been grown in the presence of EGF (200 ng/ml) for 24 h. These cells are relatively flat and bear numerous short, irregular microvilli on their free surface. Bar = 5.0 μm.

Fig. 11. Scanning electron micrograph of parts of several hepatocytes from a culture that had been grown in the absence of EGF for 48 h. Note that the cells are almost devoid of surface modifications. Bar = 5.0 μm.

Fig. 12. Scanning electron micrograph of several cells from an aggregate of hepatocytes that had been grown in the presence of EGF (200 ng/ml) for 48 h. These cells have numerous blebs and short microvilli. Bar = 5.0 μm.

Fig. 13. Scanning electron micrograph of several cells at the periphery of an aggregate of hepatocytes that had been grown in the presence of EGF (200 ng/ml) for 48 h. Note that these cells are flat, display numerous microvilli, and form structures resembling bile canaliculi (arrow). Bar = 5.0 μm.

Fig. 14. Scanning electron micrograph of several cells from an aggregate of hepatocytes that had been grown in the presence of EGF (2 ng/ml) for 48 h. The surface morphology of these cells is similar to that of hepatocytes exposed to higher concentrations of the growth factor. Bar = 5.0 μm.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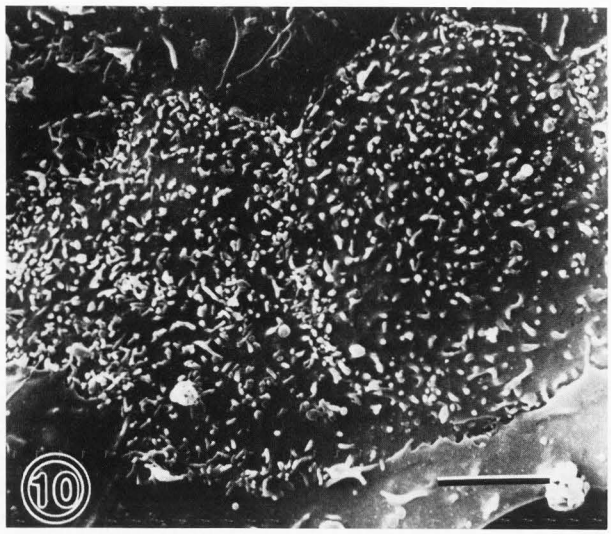
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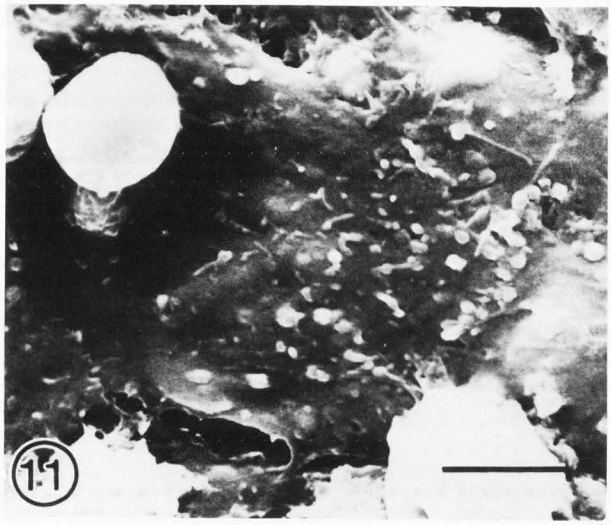
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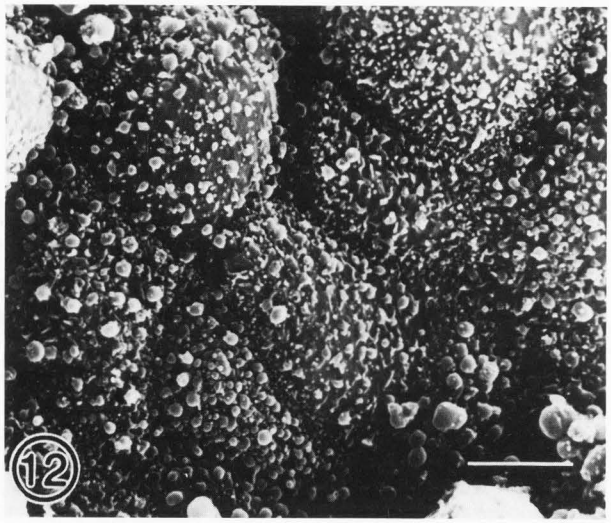
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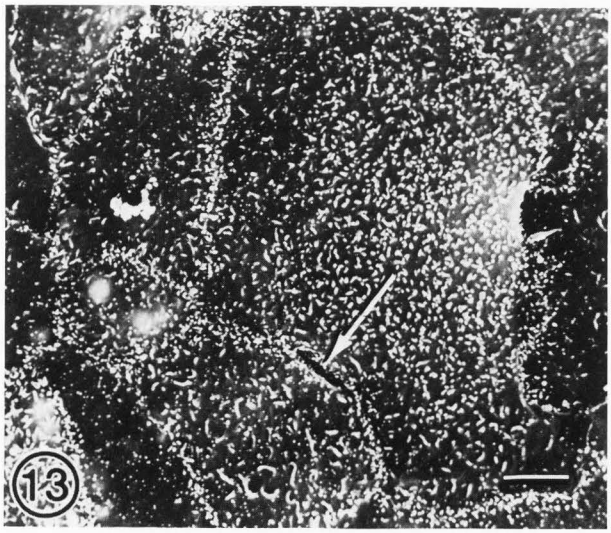
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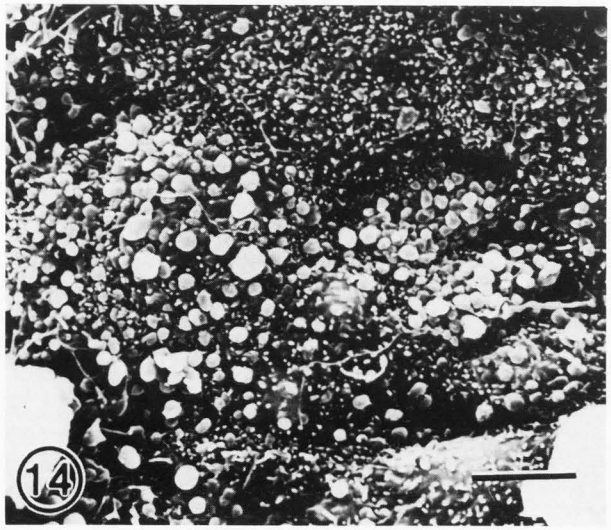
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to be more blebs and microvilli. However, the ultrastructure of cytoplasmic organelles of cells grown with or without EGF was similar.

Scanning electron microscopy (SEM) permits the study of large areas of cell surface with great depth of field. At 6 h after the addition of growth factor there were essentially no differences in surface morphology between cells in control and experimental cultures. In both cases cellular aggregates one to two cells in thickness were present (Fig. 5). These were attached to the substrate by means of lamellipodia and microspikes. The free surface of most cells was smooth, and it was difficult to distinguish intercellular boundaries. A few cells in each cluster were somewhat rougher in surface texture (Fig. 6). They were characterized by the presence of irregular ridges and folds of different width, length and shape, as well as a few short microvilli.

Control and experimental cultures were also virtually indistinguishable 12 h after exposure to EGF. In comparison to the cultures at 6 h, the cell clusters were not as flat, and more cells bore surface modifications (Fig. 7). These cells tended to be rounded in contour, and they had more prominent ridges, folds and microvilli. Small cytoplasmic excrescences or blebs were now observable on a few cells within each aggregate (Fig. 8).

Striking morphological differences between treatment groups were first apparent 24 h after the addition of EGF. The surface morphology of cells in control cultures was similar to that observed in the previous sample. In the presence of EGF, however, regions of cellular aggregates were often three to four cells thick. In such areas the cells were rounded in contour, and intercellular boundaries were easily discernible (Fig. 9). Some of these cells were similar to those noted in the 12 h sample, but others displayed numerous, large blebs. At the periphery of experimental cultures, the cells were flat, and numerous short, irregular microvilli protruded from the free surface of many of them (Fig. 10).

The final sampling for SEM was done at 48 h after exposure to the growth factor. At this point in time, cell aggregates were flat in the presence of M-199 alone, and cell surfaces were almost devoid of modifications (Fig. 11). In the presence of EGF, on the other hand, rounded cells were more numerous than at 24 h (Fig. 12). In addition, more of these cells had blebs, and there were many more blebs per cell than was the case in the earlier sample. Once again, cells at the periphery of each aggregate were flat and displayed numerous microvilli that tended to cluster at the periphery of the cells. Structures resembling bile canaliculi were observed between the flattened cells (Fig. 13).

These results with the SEM were obtained with cultures that had been grown in the presence of EGF at a final concentration of 200 ng/ml. Similar findings were noted with cells that had been exposed to the growth factor at 2 and 20 ng/ml (Fig. 14).

Discussion

Epidermal growth factor is a potent mitogen for a variety of cultured cells (4), and this effect of the peptide has attracted the most attention. Recently interest has been shifting to the possible influence of EGF on the phenotype of cells. There are indications in the literature that it can influence the expression of cell type-specific gene products. Chen et al. (5) showed that EGF is capable of inducing cell surface fibronectin in mouse 3T3 cells. Johnson et al. (18) demonstrated that EGF increased the synthesis of prolactin and repressed the production of growth hormone by cultured rat pituitary cells. These effects were concomitant with alterations in cell shape. More recently, Chikuma et al. (6) reported that the factor increased dipeptidyl-aminopeptidase and collagenase-like peptidase activities in clonal osteoblastic cells.

The results of the present investigation, obtained with a slightly different culture system than we used before, confirm our observation that EGF stimulates fetal rat hepatocytes to secrete somatomedin in a time and dose-dependent fashion. Although plasma somatomedin levels in children appear to be regulated by growth hormone (29), this does not seem to be the case in the fetus (16). Our findings suggest that EGF may play a significant role in the fetal regulation of somatomedin secretion and offer a possible explanation for the observations that children with growth hormone deficiency (28) or fetal rabbits decapitated *in utero* (17) are of normal size at birth. It is interesting to note that the optimum concentration of EGF required for the stimulation of somatomedin production in fetal rat liver cells (20 ng/ml) is similar to the concentration found in human plasma, urine, and saliva (3,27).

A considerable amount of work has been done on the influence of hormones on the morphology of cell surfaces. Much of this has been stimulated by the desire to understand how changes in surface morphology may be involved in hormone-triggered responses. The changes in surface architecture of PC12 pheochromocytoma cells that occur after exposure to nerve growth factor (NGF) and EGF have been well documented (9,10). Both factors induced a rapidly initiated series of surface changes including ruffling, loss of microvilli, and blebbing. Similar effects have been reported for NGF on sympathetic neurons (8) and for EGF on glial cells (2), carcinoma A-431 cells (7), and MMC-E mouse epithelial cells (15). These changes occurred within seconds of exposure and appeared to be correlated in time with the binding of the growth factors to their target cells. In view of this earlier work, our observation that EGF has an effect on the surface morphology of fetal rat hepatocytes in primary culture is interesting, but not surprising. It must be pointed out, however, that the changes in surface architecture reported in our system occurred according to an entirely different time frame.

At present the significance of the changes in surface topography brought about in fetal liver cells by exposure *in vitro* to EGF has not been determined. These alterations coincide with increasing production of somatomedin. Thus, it is possible that the appearance of microvilli and/or blebs may represent morphological evidence of secretory activity.

Acknowledgements

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Discussion with Reviewers

U. Brunk: You suggest some relationship between formation of blebs and production of somatomedin. Your illustrations of the blebbing phenomenon are low magnification micrographs. Could you describe whether cellular organelles are found in these blebs or if high resolution TEM would provide some other findings to support your hypothetical relationship between somatomedin production and cellular blebbing?

Authors: No cellular organelles other than clusters of free ribosomes were observed in the blebs even at higher magnification and resolution. Therefore, at this point in time, no other structural correlates of increased secretory activity have been noted, and the suggestion that the appearance of microvilli and/or blebs may represent morphological evidence of secretory activity is speculative.

U.I. Heine: Have the authors tried to remove EGF from the somatomedin-producing cultures to see if the effects of the growth factor are reversible?

Authors: This is an interesting idea; however, because of the limitations of our culture system, we do not feel that it would be feasible to evaluate the reversibility of EGF's effects on cell morphology and somatomedin secretion. Our studies are performed with primary cultures of fetal hepatocytes in the absence of serum. Under such conditions, the number of viable cells decreases dramatically sometime after 48 h in serum-free medium. Since we do not observe a significant response until 48 h of exposure to EGF, we would have difficulty interpreting the results of removing the growth factor at this time under conditions in which cell number begins to decrease.

U.I. Heine: In Fig. 4 the numerous vacuoles and lipid bodies are outstanding. How do they relate to the treatment?
Authors: Lipid droplets and vacuoles were present in both EGF-treated and control cells. Although no attempt was made to quantitate these observations, we do not feel that the presence of these inclusions is related to the treatment.

U.I. Heine: A comparison of Fig. 4 with Fig. 3 shows clearly that the EGF-treated cells are not as tightly connected to their neighboring cells as is the case in the controls. Did the authors observe changes in intercellular junctions?
Authors: In the EGF-treated cultures the cell clusters were thicker and the cells were more rounded than in control cultures. With the SEM the intercellular boundaries were more easily discernible, and with the TEM the intercellular spaces appeared to be wider in the presence of the growth factor. The desmosomes, however, were morphologically the same under both culture conditions. We did not study serial sections, so that it is not possible for us to comment with respect to changes in the number of desmosomes.

Reviewer 4: The observation that epidermal growth factor stimulates fetal rat hepatocytes to secrete somatomedin in primary culture is interesting. The effect of EGF on the surface morphology of these cells could be better documented, however, by quantitating the observations.
Authors: Thank you for your insightful comments. We realize that objective measurements would strengthen our data, and we plan to investigate this aspect of the work in the future.

Reviewer 4: It would be very important to know how reproducible these results are.
Authors: The physiological experiments have been done on numerous occasions, and the results are quite reproducible. The morphological experiments utilizing both scanning and transmission electron microscopy were performed twice. The results were essentially the same both times.