DENSITY DEPENDENT PROLIFERATION OF HUMAN GLIA CELLS STIMULATED BY EPIDERMAL GROWTH FACTOR

Bengt Westermark, The Wallenberg Laboratory, University of Uppsala, UPPSALA, Sweden.

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Summary

Epidermal growth factor (EGF) isolated from mouse salivary glands, enhanced the multiplication and [^{3}H]TdR incorporation of human normal glia cells in serum-free medium supplemented with human serum albumin. Optimal dose was 2 ng/ml for both dense and sparse cultures but dense cultures were stimulated by EGF to a much less extent than sparse cultures. Data are presented that make the possibility unlikely that the density dependent inhibition of the EGF response is due to depletion of EGF in the medium or a local, juxtacellular starvation for the factor.

Introduction

Normal anchorage dependent cells cease to multiply in crowded cultures ("contact" - or "density dependent inhibition of growth") (16). The details of the underlying mechanism are essentially unknown. A widely adopted hypothesis is that crowded cells have an unsaturated demand for serum growth factors. The finding that the addition of fresh serum to a density inhibited culture leads to a wave of mitoses (19) and that the terminal cell density increases with the serum concentration (9, 20) supports this concept.

The possible regulatory role of a growth factor makes it desirable to study the molecular basis for its interaction with the cell. This requires a pure factor. The purification of small amounts of partially characterized serum growth factors has been described (4, 7). The present communication will show that epidermal growth factor, isolated from mouse salivary glands (1), is a potent growth factor for human glia cells and that the response of the cells to the factor is density dependent.

Material and Methods

Cell line and routine culture conditions. A normal glial line (787 CG) was initiated from juvenile brain tissue as described (11). A stock of this line was maintained in 50 mm plastic dishes (Nunclone, Roskilde, Denmark) in Eagle's MEM (3)

containing 10% calf serum and antibiotics (100 IE penicillin; 50 µg streptomycin; 1.25 µg amphorotericin B/ml). Cultures were kept at 37°C in humidified air, containing 5% CO_2.

Experimental culture conditions. All experiments were performed on cells at passage level 10-20 (phase II according to Hayflick (5)).

Sparse cultures. Cells were trypsinized, pooled in Eagle's MEM, 10% calf serum, and distributed at 100,000 - 200,000 cells/dish. After 24 h of incubation, the medium was changed to serum-free Ham's nutrient medium F-12 (5). After 24 h of serum-starvation, the serum-free F-12 was renewed and additives were given as described in the text.

Dense, stationary cultures. Cells were trypsinized, pooled and seeded sparsely in Eagle's MEM 10% calf serum and incubated. Medium (MEM 10% calf serum) was changed twice a week. After two weeks, when confluent, stationary monolayers had formed (12), the cultures were deprived of serum as described above.

Cell counts. The cells were detached by 0.01% versene, 0.25% trypsin in phosphate buffered saline (PBS) and counted in an electronic cell counter (Celloscope, Lars Ljungberg, Stockholm, Sweden). Values are given as means of duplicate dishes.

Liquid scintillation. [^{3}H]TdR labelled cultures were washed twice in PBS, extracted 3 x 5 min with cold trichloroacetic acid at 4°C, washed once with PBS and air dried. The cells were then dissolved in 0.3 M NaOH at room temperature and an aliquote was counted in Instagel® (Packard Instr. Company, Downer's Grove, Ill., USA) at 10°C in an Intertechnique liquid scintillator. Values are given as means of duplicate dishes.

Autoradiography. [^{3}H]TdR labelled cultures were washed twice in PBS and fixed for 60 min in methanol-acetic acid (3:1), washed in methanol and air dried, and processed for autoradiography as described (12, 20). On duplicate dishes, 500 cells were analysed. Nuclei with more than 10 grains were regarded as labelled.

Epidermal growth factor was obtained from Dr. Stanley Cohen (Dept. of Biochemistry, Vanderbilt University, School of Medicine, Nashville, Tennessee, USA).

Tritiated thymidine ([^{3}H]TdR), 2 Ci/mM, was obtained from Radiochemical Centre, Amersham, England. It was added to the cultures at 0.02 µCi/ml, final concentration.

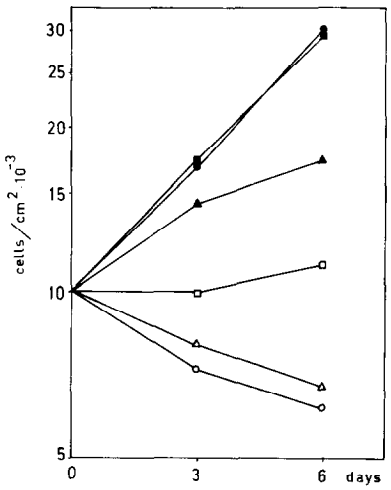

Figure 1

Multiplication of glia cells in F-12 medium with no addition (open circles), 1 mg/ml of HSA (open triangles), 1 mg/ml of HSA + 1 ng/ml of EGF (filled triangles), 0.5% calf serum (open squares), 0.5% calf serum + 1 ng/ml of EGF (filled squares) and 5% calf serum (filled circles).

Human serum albumin (HSA) was from Kabi, Stockholm, Sweden.

Results

Stimulation of glia cell multiplication by EGF

Sparse, serum-deprived cultures of glia cells were given either of the following additives (final concentrations): 0.5% calf serum; 0.5% calf serum + 1 ng/ml of EGF; 1 mg/ml of human serum albumin; 1 mg/ml of HSA + 1 ng/ml of EGF; 5% calf serum. Cell counts were made at intervals (fig. 1). EGF, though added at a very low concentration, had a remarkable stimulatory effect on the multiplication of the human glia cells, even in serum-free medium, provided HSA was added. With no HSA, the effect of EGF was reduced by some 90% (not illustrated). In combination with 0.5% calf serum, which itself just gave a flat growth curve, EGF sustained growth equally to 5% calf serum.

Attempts to cultivate cells in HSA+EGF for an extended period of time were fruitless, since the cultures deteriorated after 1-2 weeks, unless serum was added.

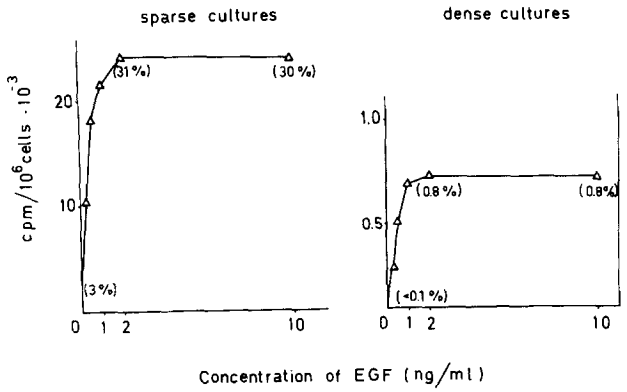

Figure 2

Stimulation of serum deprived glia cells with different doses of EGF in combination with HSA, 1 mg/ml. The left panel represents sparse cultures and the right panel dense cultures. The figures in brackets represent the labelling index of autoradiograms.

Density dependence of the EGF response

Sparse or confluent glia cultures were serum deprived and incubated in HSA--fortified F-12 containing different concentrations of EGF, ranging from 0 to 10ng/ml. After 18 h, when DNA synthesis was expected to commence (22), [^{3}H]TdR was added and after further 24 h cultures were harvested for liquid scintillation and, in some cases, for autoradiography. Dose-response curves were drawn as depicted in fig. 2. The background [^{3}H]TdR incorporation in serum-free, non-stimulated cultures was very low, both in dense and sparse cultures, demonstrating an efficient cell cycle block. The principal course of the dose response curves of dense and sparse cultures were quite similar with a rapid increase in [^{3}H]TdR incorporation from 0 to 1 ng/ml of EGF and a levelling off at higher concentrations of EGF. However, the level of stimulation was density dependent, since the maximal [^{3}H]TdR incorporation obtained with sparse cells was some 30 times higher than that with dense cells. The corresponding labelling indices were 30% for sparse cells stimulated by 10 ng/ml of EGF and 0.8% for dense cells with the same concentration of EGF.

The low [^{3}H]TdR incorporation by the crowded cultures was not due to ge-

neral medium exhaustion as shown in an experiment in which a wound was created in the cell layer (20) before incubation in serum-free F-12, supplemented with HSA and 2 ng/ml of EGF. These cultures were labelled and processed for autoradiography as described above. The cells lining the wound were labelled to 51% whereas the labelling index of the cells in the undisturbed layer was 0.9%, though bathed in the same medium.

Discussion

The properties of the mouse epidermal growth factor should make it an excellent tool for further studies on the possible role of a growth factor in the density dependent growth control. Sufficient amounts of EGF can rather easily be purified (13) and the factor is well characterized physically and chemically (17), including the amino acid sequence (14). Furthermore, EGF enhances the proliferation of human glia cells, as the present results show, as well as human skin fibroblasts (8) at very low, "hormone like" concentrations. This is in contrast to insulin, which works as a growth factor for certain fibroblasts at unphysiologically high concentrations (18). Insulin causes very little glia cell stimulation (23). EGF is also more potent glia cell factor than somatomedin B(4) and fibroblast growth factor (22).

Though capable of maintaining glia cell multiplication in a serum-free and thus well defined medium (only HSA was added), EGF could not sustain cell viability for an extended period of time. This is in accord with findings by others (10), that serum, apart from factors that stimulate DNA synthesis ("growth factors") contains "survival factors", necessary for long term culture. Thus, in order to make it possible to grow cells in an entirely defined medium, these factors must also be purified and characterized.

An important finding was that the response of the glia cells to EGF was density dependent. In this respect, the pure polypeptide behaves as whole serum. The apparent insensibility of the crowded cells to EGF was not due to exhaustion of growth factor or other depletion of the medium as shown by the wound/layer experiment in accordance to what has been found for serum treated cultures (2, 21). Stoker (15) has proposed that crowded cells have a reduced access to the growth factor due to a juxtacellular diffusion boundary layer. The growth limiting event would then be the rate of diffusion across the boundary layer. Since the boundary layer is more pronounced among crowded than sparse cells, the former will proliferate more slowly. However, the

present dose response curves (fig. 2) do not make it likely that this mechanism operates for EGF on glia cells. Since the diffusion across the boundary layer should be proportional to the concentration of the factor in the medium, the access to EGF, and hence the [^{3}H]TdR incorporation, of the packed cells should increase corresponding to the EGF concentration. The finding that the dose response curve of the dense cultures levelled off at the same EGF concentration as that of the sparse cells rather implies that the access of the cell to EGF is density independent. If so, it may be that the ability of the crowded cell to respond to a given amount of factor is density dependent. Increased density may lead to a modification of the cell membrane making it resistant to EGF perhaps by reducing the number of available receptor molecules. Further studies are needed to clarify on this point, preferably including experiments with other purified growth factors.

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