REGULATION OF FIBROBLAST GROWTH IN CULTURE

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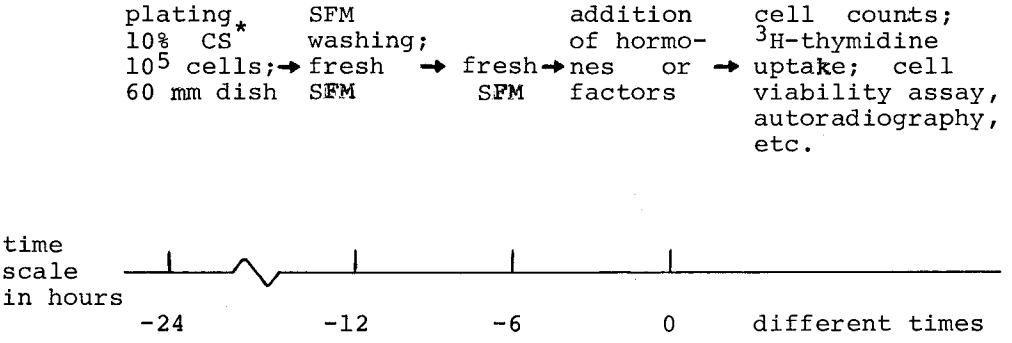
SUMMARY: A protein factor extracted from pituitary tissue stimulated cell growth of human and mouse 3T3 fibroblasts and its activity was enhanced by hydrocortisone and insulin at physiological concentrations; such synergistic effects were observed in a serum free medium. Cells stimulated by the hormones in serum-free medium traverse G1 and S but accumulate at G2, the "termination" of cell division being serum dependent.

INTRODUCTION: 3T3 cells (1) are mouse embryo fibroblasts, established in culture, which undergo the transition between a "resting state" and a proliferative phase (2 and 3) under very strict control of serum growth factors (4 and 5). Recently we showed (6) that pituitary gland contains a protein which stimulates 3T3 cell growth. This has been fully confirmed by the final purification of the active protein (7). The present paper presents results of further investigation showing that insulin, in addition to hydrocortisone, enhances the activity of the pituitary growth factor; this combination of hormones is effective in stimulating "resting cells" to traverse G1 and S and accumulate at the end of G2; however "termination" of cell division, seems to be dependent on another serum factor.

MATERIAL AND METHODS: Swiss 3T3 cells were of two different strains: one from Dr. Gordon Sato's laboratory, La Jolla, California (6) and the other from American Type Culture Collection. Human WI38 fibroblasts were used between the 27^{th} and 40^{th} passage. Cells were regularly monitored for mycoplasma contamination by autoradiography (8). Cell growth, medium prepara-

tion, cell counting and ^{3}H-thymidine uptake procedures have been previously described (6). In order to assay for DNA synthesis stimulation by hormones or hormone like-factors in serum free medium (SFM) the following protocol was designed:



Impure (NIH-LH-B8 and B9), as well as pure luteinizing hormone were provided by U.S. National Institutes of Health (NIAMD, Hormone Distribution Program). Dr. D. Gospodarowicz (Salk Institute) kindly provided pure LH and pure pituitary fibroblast growth factor (7). Epidermal growth factor (EGF) was a generous gift from Dr. H. Herschman's laboratory (UCLA). Porcine insulin was the commercially available product and hydrocortisone was the pure hormone from Nutritional Biochemical Corp. Extracts of fresh pituitary and NIH-LH-B8 or B9 which showed fibroblast growth factor activity, having approximately the same specific activity, will be refered to as crude factor (CF); whereas the Gospodarowicz's fibroblast growth factor will be refered to as pure factor (PF).

RESULTS: CF, insulin and hydrocortisone stimulated initiation of DNA synthesis in "resting" 3T3 cells in SFM (Figure 1). Figure 2

*ABBREVIATIONS: CS, calf serum; SFM, serum-free medium; LH, luteinizing hormone; EGF, epidermal growth factor; CF, crude pituitary factor; PF, pure pituitary factor. H, hydrocortisone; I, insulin.

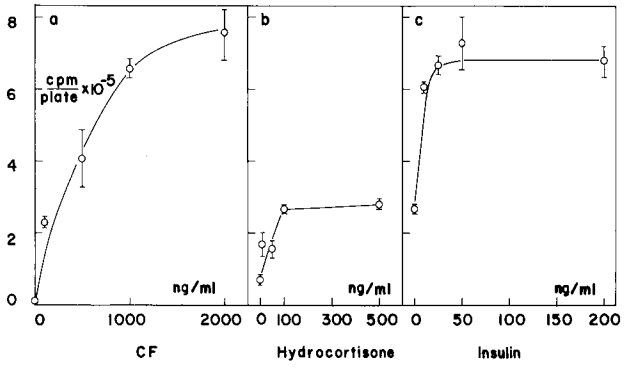


FIGURE 1 - Dose response curves for crude pituitary factor, hydrocortisone and insulin in SFM. Hormones added together at zero time. ^{3}H-thymidine: 10^{-8}M, 1 µC/ml; incorporation for 12 hours (from time 12 to 24 h). Additions to medium: a) SFM plus 100 ng/ml hydrocortisone and 50 ng/ml insulin; b) SFM plus 1,000 ng/ml CF; c) SFM plus 1,000 ng/ml CF and 100 ng/ml hydrocortisone. Each point in the figures and tables is an average of two independent measurements (uptake of thymidine in absence of hormones was not subtracted).

presents results for the same kind of experiment with PF. The epidermal growth factor (9) has small DNA synthesis stimulating activity for mouse (6) and human fibroblasts (10), but we have not observed any synergistic effect with insulin and hydrocortisone. The frequency of cells synthetising DNA has been quantitated by autoradiography, (Table I and Figure 3a). The enhancement effect due to insulin and hydrocortisone is a consequence of the increasing number of cells initiating DNA synthesis. These results are in contradiction to the suggestion of Gospodarowicz (7), whereas they agree with recent results of Holley and Kiernan (11).

CF, insulin and hydrocortisone also proved effective in stimulating DNA synthesis in human fibroblasts in SFM as Table II indicates. G1 duration for 3T3 was the same (10 to 11 hours) for cells stimulated with hormones alone or supplemented with low serum level (0.25% CS). Completion of cell division, however,

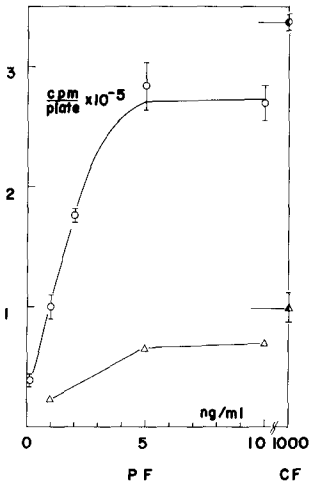


FIGURE 2 - Dose response curves for pure pituitary factor in SFM. Procedures as in Figure 1. Δ PF; O PF plus 100 ng/ml hydrocortisone and 50 ng/ml insulin; ▲ CF and ● CF plus 100 ng/ml hydrocortisone and 50 ng/ml insulin.

TABLE I

Additions to SFM (at zero time)	% of labeled nuclei
None	zero
Hydrocortisone (H)	zero
Insulin (I)	3
Crude pituitary factor (CF)	9
I + H	0,4
CF + H	31
CF + I	26
CF + I + H	49
CF + I + H + 0.25% serum	98
0.25% calf serum	8
10% calf serum	98

Frequency of cells stimulated to initiate DNA synthesis estimated by autoradiography. ^{3}H-thymidine incorporated between 12 and 24 hours. Concentrations: hydrocortisone 100 ng/ml; insulin 50 ng/ml and CF 1,000 ng/ml. Nuclei countings by phase contrast microscope. Two plates per point. 200 to 300 nuclei counted per plate.

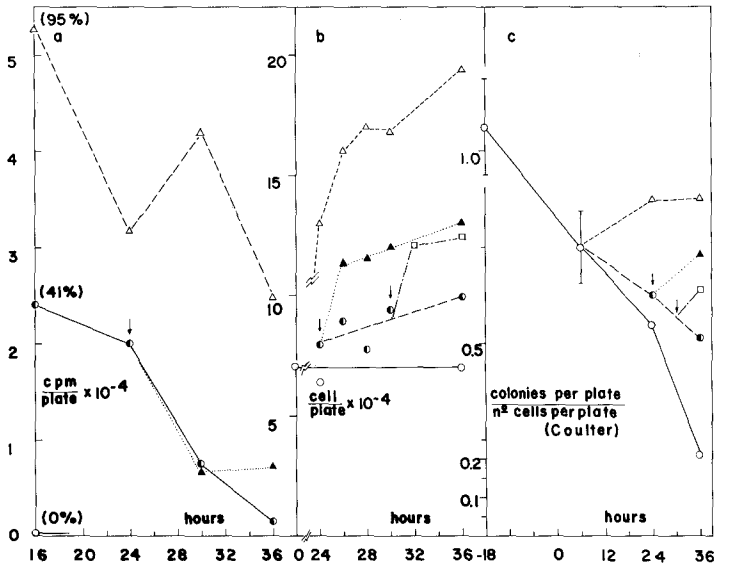


FIGURE 3 - Initiation of DNA synthesis, termination of cell division and cell survival in SFM: effect of hormones and unknown serum factors. 3a: ^{3}H-thymidine uptake into DNA, 20 minutes pulse; (95%), (41%) and (0%) represent percent of labeled nuclei measured by autoradiography after ^{3}H-thymidine pulse at 16 hours. 3b: Coulter counts. 3c: Cell survival measured by colony development ability (plating efficiency); at indicated times cells were trypsinized and aliquots were taken under sterile conditions; cell suspensions were immediately counted in a Coulter Counter; after appropriate dilutions the aliquots (saved before countings) were plated in rich medium (12.5% horse serum, 2.5% fetal calf serum and 85% DME) at 250 to 400 cells/plate, in 100 mm plates; 8 days of growth were allowed for colonies to develop and then cells were fixed and stained with crystal violet. The number of colonies developed per plate was determined and the ratio of colonies per plate over number of cells plated (from Coulter countings)per plate were plotted in the graph. O——O Control, no additions; ●——● hormones added at zero time; Δ---Δ 0.25% calf serum plus hormones added at zero time; ▲---▲ hormones at zero time and 0.25% serum at 24 hours; ▫---▫ hormones at zero time and 0.25% serum at 30 hours. Arrows indicate time of 0.25% serum addition.

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only occured efficiently if the medium was supplemented with at least 0.2% CS. A detailed kinetics of cell division is presented in Figure 3 (experiment performed four times). 95% of the cells which received hormones plus serum at zero time, initiated DNA synthesis (Figure 3a) and started cell division at 23 hours (Figure 3b); at 27 hours the population had doubled. 41% of the

TABLE II

Additions to SFM (at zero time)	Uptake in $\frac{cpm}{plate} \times 10^{-3}$	% of labeled nuclei
None	16	13%
H + I	20	-
CF	45	-
CF + H + I	83	48%
10% calf serum	120	82%

WI38 cells: ^{3}H-thymidine uptake into DNA and frequencies of DNA synthetising nuclei. WI38 cells plated according to the protocol used for 3T3 cells. Procedures like described for Figure 1 and Table I. ^{3}H-thymidine incorporation from 12 to 36 hours.

cells treated with only hormones at zero time were synthetising DNA at 16 hours (Figure 3a); under these conditions the cell number increased very slowly but a burst of cell division occurred in 2 hours after serum addition, either at 24 or at 30 hours (Figure 3b); such increase in cell number did not occur for serum addition before 24 hours (not shown). The increments in cell number were the ones expected considering the amount of cells which initiated DNA synthesis (41% Figure 3a) and provided they had been arrested at the end of G2 or mitosis (probably end of G2, because we have not observed accumulation of mitotic cells).

In SFM viability decreases progressively (Figure 3c); hormones diminish the rate of cell "decay" but they are not sufficient to support cell survival. The ability to synthetize DNA (detected by autoradiography), however, is maintained for a longer period of time than colony-developing ability: even after 48 hours in SFM up to 80% of the cells are able to incorporate ^{3}H-thymidine, if stimulated by the combination of serum and hormones. Therefore the relatively low number of labeled cells (41%) in SFM plus hormones (indicated in Figure 3a) is not due to inability of

60% of the cells to synthetize DNA. Rather, it seems that a factor, other than the hormones, is necessary to achieve 100% stimulation.

In spite of the decreasing viability we have been able to rescue viable cells in SFM plus hormones for at least 12 days. However attempts to select cells able to grow in SFM plus hormone have been unsuccessful so far. The results strongly suggest that "termination" of cell division requires a special serum factor. Dr. R.W. Holley has kindly checked our results by flow microfluorometric analysis using his strain of 3T3 cells (11); his results indicated that cells, stimulated by the hormones in SFM, do accumulate in G2 or M (about 25% at 27 hours), these results are in close agreement with ours.

DISCUSSION: The extent of cell response to a) pituitary factor; b) pituitary factor plus hydrocortisone and insulin and c) hormones combination plus 0.25% serum; has varied. This variation was determined by the number of cells which responded in each case but the duration of G1 for the stimulated cells was the same in all cases. On the other hand the duration of the whole "cycle" (G1 + S + G2) seems to be independent of serum (as results in Figure 3 show), though cell division "termination" was dependent on serum. These results imply that the "extracellular growth regulators" only determine the initiation of cellular growth. Moreover; in the present results, the pituitary protein seems to be the primary factor. We have hypothesized that normal growth is exclusively dependent on "extracellular regulators", whose role is to control the probability of a "resting cell" to take the transition to the "proliferative phase" (3). This would be accomplished by triggering "initiation" reactions (G1 "initiation"), which commit the cell to the growth process. As far as mechanisms of

action are concerned a few effects of the pituitary growth factor are worth mentioning: increase in cGMP intracellular levels (12), stimulation of membrane bound guanyl cyclase (13) and stimulation of 2-deoxiglicose uptake (14) in resting cells. Further studies of the stimulation of the fibroblast system by hormones should unravel basic mechanisms of growth control in mammalian cells.

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REFERENCES

1. Todaro, G. and Green, H. (1963) - J. Cell Biol., 17: 299.
2. Smith, J. and Martin, L. (1973) - Proc. Nat. Acad. Sci. USA, 70: 1263.
3. Armelin, H.A. (1974) - Biochemical Actions of Hormones, Vol. 3, Ed. G. Litwack, Academic Press, New York (in press).
4. Todaro, G.; Lazar, G. and Green, H. (1965) - J. Cell Comp. Physiol., 66: 325.
5. Holley, R.W. and Kiernan, J. (1968) - Proc. Nat. Acad. Sci. USA, 60: 300.
6. Armelin, H.A. (1973) - Proc. Nat. Acad. Sci. USA, 70: 2702.
7. Gospodarowicz, D. (1974) - Nature, 249: 123.
8. Culp, L.A. and Black, P.H. (1972) - J. Virology, 9: 611.
9. Cohen, S. (1962) - J. Biol. Chem., 237: 1555.
10. Hollenberg, M. and Cuatrecasas, P. (1973) - Proc. Natl. Acad. Sci. USA, 70: 2964.
11. Holley, R.W. and Kiernan, J. (1974) - Proc. Nat. Acad. Sci. USA, 71: 2908.
12. Armelin, H.A.; Nishikawa, K. and Sato, G. (1974) - In: Control of Proliferation in Animal Cells (B. Clarkson and R. Baserga, eds.) Vol. I, 97. Cold Spring Harbor Press, New York.
13. Rudland, P.S.; Gospodarowicz, D. and Seifert, W. (1974) - Nature, 250: 741.
14. Holley, R.W. and Kiernan, J. (1974) - Proc. Natl. Acad. Sci. USA, 71: 2942.