Hormones and Cell Culture

BOOK B

Sato/Ross

Cold Spring Harbor Conferences on Cell Proliferation
Volume 6

Hormones and Cell Culture

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COLD SPRING HARBOR CONFERENCES ON CELL PROLIFERATION
VOLUME 6

Cold Spring Harbor Laboratory
1979

Cold Spring Harbor Conferences on Cell Proliferation

Volume 1 Control of Proliferation in Animal Cells
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Volume 3 Cell Motility
Volume 4 Origins of Human Cancer
Volume 5 Differentiation of Normal and Neoplastic Hematopoietic Cells
Volume 6 Hormones and Cell Culture

Hormones and Cell Culture

Printed in the United States of America

Cover and book design by Emily Harste

Library of Congress Cataloging in Publication Data

Main entry under title:

Hormones and cell culture.

(Cold Spring Harbor conferences on cell proliferation ; v. 6)
Edited proceedings of the meeting held Aug. 1978.
Includes index.
1. Hormones—Physiological effect—Congresses. 2. Cell cultures—Congresses. 3. Cell differentiation—Congresses. 4. Growth promoting substances—Congresses. I. Sato, Gordon. II. Ross, Russell. III. Cold Spring Harbor, N.Y. Laboratory of Quantitative Biology. IV. Series. [DNLM: 1. Endocrine glands—Cytology—Congresses. 2. Hormones—Congresses. 3. Cells, Cultured—Congresses. W3 C162N v. 6 1978/ WK100.3 H812 1978]
QP571.H659 574.1'4 79-16328
ISBN 0-87969-125-5

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Growth of Mouse 3T3 Fibroblasts in Serum-free, Hormone-supplemented Media

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3T3 mouse fibroblasts have been used extensively in investigating the regulation of cell proliferation. The work of Todaro et al. (1965) and Holley and Kiernan (1974) has shown that the serum concentration in the medium controls 3T3 cell proliferation. Cells rendered quiescent by low serum concentration can proliferate again if high levels of serum are added back to the culture. Furthermore, it has been shown that trophic hormones and peptide factors purified from serum or tissue extracts could act like serum in stimulating DNA synthesis or cell division of quiescent cultures of 3T3 (for review, see Gospodarowicz and Moran 1976).

Ross et al. (1974) and Kohler and Lipton (1974) have found that the growth-promoting activity (GPA) of serum can be separated into two types, one contained in plasma and one lacking in plasma and synthesized by platelets. Recently, Pledger et al. (1977) postulated that these two types of factors controlled different phases of cell proliferation: platelet-derived growth factor (PDGF) commits the cells to enter the growth cycle, whereas plasma-derived growth factors (PGFs) maintain cell viability. PDGF has been purified and its mode of action studied (Vogel et al. 1978; Pledger et al. 1978), but very little is known about the nature of the PGFs.

Studies done by Sato and coworkers (Hayashi and Sato 1976; Hutchings and Sato 1978; Bottenstein et al. 1979) have demonstrated that various cell lines can be maintained in serum-free media that are supplemented with hormones and growth factors, and in which cells proliferate as well as in serum-rich media. Since such a defined medium would be a very useful feature of a system in which growth-control parameters are studied, we have attempted to develop a serum-free, defined medium that could support continuous growth and cell division of 3T3 fibroblasts. Experiments were done with three different clones of 3T3 mouse fibroblasts to compare their hormonal requirements in serum-free medium: two sublines of Swiss 3T3 mouse fibroblasts, 3T3-L_1 and 3T3-C_2, and a subline of BALB/c-3T3 mouse fibroblasts. 3T3-L_1 fibroblasts have the ability to differentiate into adipose cells in the presence of insulin after their growth is arrested at confluence (Green and Kehinde 1974); 3T3-C_2, however, does not undergo adipose conversion under the same conditions.

MATERIALS AND METHODS

Tissue Culture

3T3-L_1 and 3T3-C_2 were kindly provided by H. Green (Massachusetts Institute of Technology). BALB/c-3T3 fibroblasts were kindly given by R. W. Holley (Salk Institute). These three subclones were grown in Dulbecco's modified Eagle's medium (DEM) containing 10% fetal calf serum (FCS), 15 mM of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 192 units/ml of penicillin, 200 μg/ml of streptomycin, and 25 μg/ml of ampicillin. Stock cultures were kept at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed every other day and the cells were subcultured every 5–6 days.

Growth Experiments

Exponentially growing cells were harvested with 0.1% trypsin and 0.03% EDTA in phosphate-buffered saline (PBS), suspended in DEM with 10% FCS and plated at a density of 2×10^4 cells/35-mm dish containing 2 ml of DEM with 10% FCS. The cells were washed free of serum 12 hours later and incubated in serum-free DEM before adding the experimental media (details in Fig. 1). At the end of the experiment, the cells were detached from the plates by treatment with trypsin and EDTA (0.1% and 0.03%, respectively), suspended in PBS, and counted with a Coulter counter.

Adipose Differentiation of 3T3-L_1 Fibroblasts

When the cultures of 3T3-L_1 fibroblasts reached confluence, 1–10 μg/ml of insulin was added to various dishes. Cultures were kept 4 weeks for differentiation experiments, the medium being changed every 2–3 days. In these conditions, the cells rounded up and accumulated lipid droplets (mainly triglycerides), which were stained with Oil Red O (Green and Kehinde 1974).

Hormones and Growth-factor Preparations

Bovine crystalline insulin and human iron-free transferrin were purchased from Sigma. Bovine luteinizing hormone (LH) B10 and growth hormone (GH) B18 were gifts from the National Institutes of Health Hormone Distribution Program. Epidermal growth factor (EGF) and fibroblast growth factor (FGF) were obtained from Collaborative Research. Pure human growth hormone was kindly given by J. Lewis (Scripps Foundation for Medical Research).

Preparation of Rat Submaxillary Gland Factor

Female rat submaxillary gland extract was prepared as described by Bottenstein et al. (1979). The fraction used in the experiments reported was a 60% ammonium sulfate precipitate of crude submaxillary gland extracts.

RESULTS AND DISCUSSION

The results presented below were obtained using 3T3-L_1. We have carried out similar experiments with BALB/c-3T3 mouse fibroblasts and have obtained similar results.

Table 1
Medium Supporting the Growth of Serum-deprived 3T3 Fibroblasts

Factors	*Optimal concentrations (μg/ml)*
Insulin	0.5
Transferrin	2
LH B10	2
GH B18	0.05
Female rat submaxillary gland extract	2.5

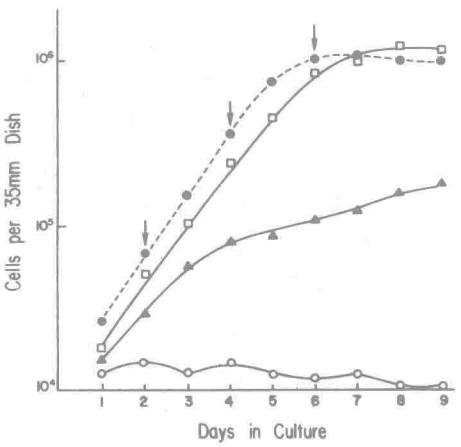
Composition of the serum-free, hormone-supplemented medium that supports the growth of 3T3 fibroblasts, 3T3-L_1, 3T3-C_2, and BALB/c-3T3. The concentrations indicated for each component are those giving maximum growth.

Growth of 3T3 Cells in Serum-free Medium

The growth rate and final density of 3T3 cells decrease with decreasing serum concentration, until, when serum is absent, no growth occurs (as first described by Holley and Kiernan 1974). We found, however, that growth in serum-free media could be restored to the rate and final density seen in serum-containing media upon addition of a mixture of partially purified growth factors and hormones (Table 1) as a replacement for the serum (Fig. 1). In the experiment shown, replicate cultures, 24 hours after subculturing, were washed free of serum and incubated with a 3:1 mixture of DEM and F-12 medium supplemented with the hormones and factors listed in Table 1.

The growth curve for cells in serum-free medium seen in this experiment is typical. The cells grow exponentially with a generation time of approximately 18 hours before reaching a plateau at high density (10^5 cells/cm^2). The plateau, seen in this case 5 days after plating, coincides with the attainment of a confluent monolayer (see Fig. 2B). The cells can be maintained at this density for at least 1 month provided the medium is changed every 3–4 days (results not shown).

Figure 1
Growth curve of 3T3-L_1. Cells were inoculated into DEM containing 10% FCS. After 15 hr medium was removed, and cells washed three times with 3 ml of DEM. They were grown subsequently in DEM:F-12 (3:1) containing 8 μg/ml biotin and 8 μg/ml calcium pantothenate and no supplement (○) or supplemented with 10% FCS (●) and 5F-DEM/F-12 with (□) and without (▲) rat submaxillary gland extract. Times of medium changes are indicated (→).



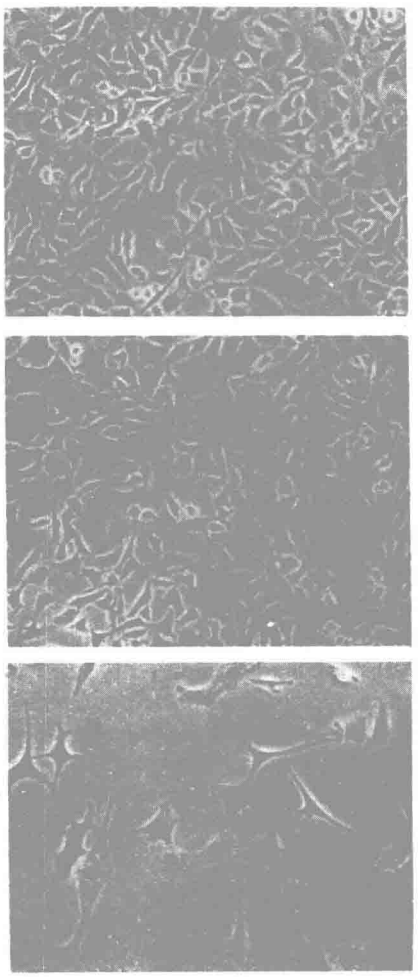


Figure 2
Photomicrographs of 3T3-L_1 fibroblasts grown 4 days in DEM:F-12 (3:1) supplemented with 10% FCS (*A*); 5F-DEM/F-12 (*B*); and no addition (*C*).

Not only are the growth curves in the hormone-supplemented and serum-containing media remarkably similar, but the appearance of the cells under the two conditions is nearly indistinguishable. This can be seen by comparing Figure 2 A and B, in which cells after 4 days in the two conditions demonstrate the same "cobblestone" morphology.

Growth of cells in serum-free medium is not stimulated by the individual addition of any of the five components in Table 1. The requirement for these components can be seen, however, when any single one is omitted from the mixture. Figure 3 shows the effect on growth of omitting each factor from the mixture of the five, as compared to growth in medium with 10% serum, serum-free medium, and serum-free medium supplemented with the five factors. With the exception of GH B18, the omission of any one of the components significantly reduces the growth response produced when all five factors are added simultaneously. GH B18 had a slight effect on growth in the experiment shown here. The response to this factor is usually but not always seen, and only with the 3T3-L_1 clone.

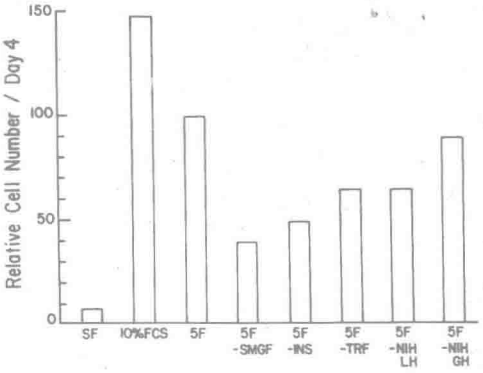


Figure 3
Effect on the growth of 3T3-L_1 of removing individual growth factors from 5F-DEM/F-12. Cells were inoculated, washed free of serum, and grown in DEM:F-12 (3:1) in the following conditions: no serum (SF); 10% FCS; 5F-DEM/F-12 (5F); or 5F-DEM/F-12 lacking submaxillary gland factor (−SMGF), insulin (−INS), transferrin (−TRF), LH B10 (−NIH LH), and GH B18 (−NIH GH). Cells were counted after 4 days of growth. Cell number is presented relative to the number of cells grown in 5F-DEM/F-12.

Role of LH B10

In addition to LH, LH B10 has been shown to contain FGF, which stimulates DNA synthesis in quiescent 3T3 cells (Armelin 1973; Gospodarowicz 1975). To determine which of these factors is required for serum-free growth, highly purified LH was tested for its ability to replace LH B10 and was found to have no activity. In contrast, when purified FGF was assayed in the absence of added LH B10, full growth activity was found. These results are consistent with the explanation that FGF and not LH is responsible for the LH B10 growth-promoting effect.

Role of Submaxillary Gland Extract

The least defined of the components listed in Table 1 and perhaps the most crucial one, as shown by the data in Figures 1 and 3, is the extract prepared from rat submaxillary glands. In the experiment described in Figure 1, if instead of all five components only insulin, LH B10, GH B18, and transferrin were added to the culture medium, the cells grew exponentially for only 2 days, ceased dividing, and reached a plateau, seen in this case 4 days after plating, at a density of 10^4 cells/cm^2. Since in this particular experiment the medium was replaced at least twice subsequent to the attainment and maintenance of stationary phase with little or no change in the number of cells/dish, it appears doubtful that the depletion of medium components is responsible for limiting the multiplication of these cells under the above conditions. Moreover, in experiments not shown, if rat submaxillary gland extract or serum was added to cultures that had stopped dividing, the cells resumed growth until they had reached the same saturation density as cultures provided with the extract throughout the growth period.

The chemical and biological properties of the active components present in rat submaxillary gland extracts are currently under investigation. Purified EGF, a peptide that is contained in the submaxillary gland (Cohen 1962) and has specific GPA for several cell types including 3T3 cells, does not replace crude submaxillary gland extract in supporting continuing cell