FUNCTIONAL CORRELATES OF HORMONE RECEPTORS IN REPRODUCTION

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Virendra B. Mahesh, Thomas G. Muldoon, Brij B. Saxena and William A. Sadler

DEVELOPMENTS IN ENDOCRINOLOGY VOL. 12

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Proceedings of the Conference on Functional Correlates of Hormone Receptors in Reproduction held on October 13–15, 1980 in Augusta, Georgia, U.S.A.

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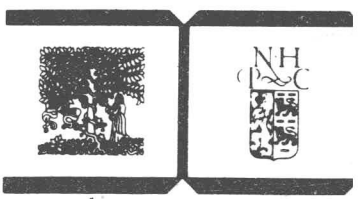

ELSEVIER/NORTH-HOLLAND
NEW YORK • AMSTERDAM • OXFORD

Published by:

Elsevier North Holland, Inc.
52 Vanderbilt Avenue, New York, New York 10017

Sole distributors outside USA and Canada:

Elsevier/North-Holland Biomedical Press
335 Jan van Galenstraat, P.O. Box 211
Amsterdam, The Netherlands

Library of Congress Cataloging in Publication Data

Conference on Functional Correlates of Hormone Receptors in Reproduction (1980: Augusta, Ga.)
Functional correlates of hormone receptors in reproduction.
(Developments in endocrinology; v. 12)
1. Hormones, Sex—Receptors—Congresses. 2. Reproduction—Congresses. I. Mahesh, Virendra B.
II. Title. III. Series. [DNLM: 1. Reproduction—Congresses. 2. Receptors, Hormone—Physiology—Congresses. 3. Gonadotropins—Physiology—Congresses.
W1 DE997VW v.12/WK 900 C743f 1980]
QP572.S4C63 1980 599.01'6 81-2544
ISBN 0-444-00604-4 AACR2
Series ISBN 0-444-80009-3

Manufactured in the United States of America

FUNCTIONAL CORRELATES OF HORMONE RECEPTORS IN REPRODUCTION

DEVELOPMENTS IN ENDOCRINOLOGY

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Foreword

The population of the world is currently approximately 4 billion, and birth rates are declining. To many individuals, this suggests that the population crisis is over. However, the world population will either double or triple by sometime after the year 2000, and the final number will depend upon when zero population growth is achieved. Between now and the year 2000 there will be a significant increase in public health measures which will markedly increase the number of children that live beyond one year of age. As a consequence, the need for family planning coupled with effective, convenient and safe contraceptives, continues to be a pressing problem. It is therefore appropriate that reproductive scientists continue to expand their knowledge on how the brain, pituitary and gonads interact to control the function of the reproductive tract. The ideas expressed at this meeting represent overviews of how hormones interact with cells in a variety of ways.

C. Wayne Bardin, M.D.

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Systems of Study and Functional Interrelationships

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A General Method for Cell Culture

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It is well established that cells *in vitro* require for growth a supplement of some type of biological fluids or extracts in addition to a basal nutrient medium containing salts, sugars, amino acids and vitamins. Investigators have at various times used embryo extract, spinal fluid, amnionic fluid, lymph, colostrum, plasma or serum to supplement the medium (Harrison, 1907; Carrel, 1913; Temin et al., 1972; Brooks, 1975; Klagsbrun, 1979) and the most popular supplement for routine cell culture has proved to be serum from the calf or fetal calf. Clearly the factors in serum are of critical importance to the maintenance and growth of cells *in vitro* and many attempts have been made to isolate and identify these factors (Temin et al., 1972; Brooks, 1975). However, purification of these factors for study or use as a replacement for serum in culture is a formidable problem for several reasons. Some of the stimulatory nutrients and hormones or hormone-like factors are present in serum in extremely small amounts, and the purification of these factors may be further complicated by their association with binding proteins (Fryklund et al., 1974; Antoniades et al., 1975; Sato, 1975; Kaufman, 1977; Crichton, 1975; Holley and Kiernan, 1971; Paul et al., 1971). The synergistic nature of some of the stimulatory activities in serum also can be a problem, causing the activity of all fractions to decrease as the stimulatory molecules are separated from each other (Holley and Kiernan, 1971; Paul et al., 1971; Stiles et al., 1979).

Studies in our laboratory and the laboratories of others in recent years led us to take a different approach to the replacement of serum in culture medium and the identification of the active factors in serum (Hayashi and Sato, 1976; Reid and Sato, 1978; Bottenstein et al., 1979; Rizzino et al.,

1979; Barnes and Sato, 1980). These studies were based on the following reasoning. In order to successfully maintain cells out of the body, it is reasonable to expect that one must provide for those cells the nutritional, hormonal and stromal influences on which they are dependent *in vivo*. In order to do this we must first eliminate or reduce the amount of serum to which the cell is exposed in culture and replace that serum with combinations of factors which are identical to or mimic the relevant extracellular effector molecules for the cell *in vivo*. That serum is successful at all in supporting the growth or maintenance of cells in culture is due to the fact that it contains nutrients, hormones, binding proteins which modulate the action of nutrients and hormones (Temin et al., 1972; Sato, 1975; Crichton, 1975, Kaufman et al., 1977) and attachment factors which mimic some of the stromal influences to which cells are exposed *in vivo* (Birdwell, 1978; Stenman and Vaheri, 1978; Yamada and Olden, 1978; Grinnell et al., 1977; Knox and Griffiths, 1979; Barnes et al., 1980a; Hewitt et al., 1980).

It follows from our reasoning that some cell types cannot be cultured in serum-containing medium because serum is an unsuitable substitute for the *in vivo* environment in some cases. One should not expect the components of serum to be adequate, qualitatively or quantitatively, at providing all of the necessary hormonal, nutritional and stromal elements for cell types such as those found in the thyroid follicle, anterior pituitary, adrenal medulla, seminiferous tubule or pancreatic islets, all of which exist in unique environments with regard to hypothalamic releasing factors, glucocorticoids, androgens and peptide hormones, as well as with regard to their nutritional and stromal environments. Furthermore, serum might be expected to be toxic at some concentration for most cell types, since serum contains substances which never come in contact with most cells (Gospodarowicz et al., 1979).

The approach in which the extracellular environmental influences on the cell *in vivo* are replaced by combinations of hormones, nutrients, binding proteins and attachment factors in culture has allowed several advances in tissue culture methodology. For instance, we and others have found it possible to replace serum in culture medium for a large number of widely divergent established cell lines by adding to the medium combinations of factors which carry out the functions of serum for these cells (Reid and Sato, 1978; Rizzino et al., 1979; Barnes and Sato, 1980). These lines include lines derived from rat pituitary carcinoma (GH3), human cervical carcinoma (Hela), canine kidney (MDCK) rat neuroblastoma (B104), mouse melanoma (M2R), human breast carcinoma (MCF-7 and Zr-75-1), mouse embryonal carcinoma (F9), mouse testes (TM4), rat ovary (RF1) and mouse embryo (SV-3T3). In addition to those lines which may be grown continually in the total absence of serum, other lines may be grown in medium supplemented with factors from the four classes listed above if a small amount of serum is included, or if the culture dishes on which the cells are grown are first pretreated with

serum-containing medium. In the case of cell lines whose residual serum requirements may be satisfied by pretreating the dishes with serum in the absence of the cells, such a procedure is probably providing attachment factors which adhere to the plastic surface. Included in this group of cell types are lines derived from human prostatic carcinoma, lung adenocarcinoma, astrocytoma and pheochromocytoma, rat thyroid and mouse embryo and mammary carcinoma (Reid and Sato, 1978; Serrero et al., 1979; Ambesi-Impiombato et al., 1980; Barnes et al., 1960b; Barnes and Masui, 1980).

Approximately thirty hormones, binding proteins and attachment factors have been found to be useful in serum-free medium for various cell lines (Reid and Sato, 1978; Rizzino et al., 1979; Barnes and Sato, 1980). Some lines also have been found to be stimulated by specific nutrients in serum-free medium which are not in the usual basal nutrient or are not present in the media at concentrations sufficient to support continuous exponential growth. The B104 rat neuroblastoma line, for instance, is stimulated to grow better in serum-free medium supplemented with insulin, transferrin and progesterone if trace amounts of selenium and relatively high concentrations of putrescine are added (Bottenstein and Sato, 1979), and growth of SV40 virus-transformed 3T3 cells is stimulated in serum-free medium by the addition of relatively high concentrations of linoleic acid complexed with bovine serum albumin (Rockwell et al., 1980).

We include in the group of factors which we call hormones the hormone-like growth factors such as epidermal growth factor (EGF) (Cohen, 1962) and fibroblast growth factor (FGF) (Gospodarowicz, 1975), as well as molecules recognized as hormones in the classical sense such as insulin, follicle stimulating hormone or hydrocortisone. Insulin has been found to be stimulatory for the growth of almost every cell type we have examined in serum-free medium. Often the concentration of insulin required is much higher than could be considered indicative of a physiologically relevant insulin effect, and it may be that insulin in these cases is acting to mimic the activity of an insulin-like factor such as a somatomedin. Insulin also is rapidly degraded in serum-free culture medium (Hayashi et al., 1978) and this may partially explain the high insulin concentrations required. Insulin at reasonably low concentrations is stimulatory for two lines of human mammary tumor cells in serum-free medium, however (Allegra and Lippman, 1978; Barnes and Sato, 1979).

The binding proteins which we have found to be useful in serum-free medium are the iron-binding serum protein transferrin (Crichton, 1975) and serum albumin, which is used in serum-free medium as a fatty acid carrier (Rockwell et al. 1980; Wolfe et al., 1980a). Lembach (1976) has reported that in short-term culture the binding protein for EGF found in the mouse submaxillary gland is stimulatory for fibroblast growth in serum-free medium in the presence of EGF. Although it is likely that such hormone-binding pro-

teins may be quite important in modulating the action of their ligands, the limited availability of such factors has made it difficult for us to test this subclass of binding proteins on a large scale in serum-free medium. Virtually every cell type examined has been found to respond to transferrin in serum-free medium. Addition of extra ferrous sulfate to the medium can replace the transferrin requirement for optimal growth for some cell lines (Mather and Sato, 1979; Wu and Sato, 1978). It is also possible that transferrin may be acting as a detoxifying agent in some serum-free media, removing toxic metals which may be present in trace amounts as contaminants of the salts used to make the nutrient medium (Barnes and Sato, 1980).

In the absence of serum it is important that the cells be provided with the proper substrate upon which to attach. Polylysine, which promotes attachment and spreading because it is a highly, positively charged molecule attached to the plastic surface of the culture dish, has been used by several laboratories to promote attachment or growth of some cell types in serum-free or low-serum medium (McKeehan and Ham, 1976; Bottenstein and Sato, 1980). A specific attachment protein, cold-insoluble globulin or fibronectin, is effective on many cell types in culture under serum-free conditions (Hook et al., 1977; Orly and Sato, 1979; Rizzino and Crowley, 1980; Rockwell et al., 1980). We also have used successfully a partially purified preparation of a protein which we have termed serum spreading factor, which was first reported to exist in human serum by Holmes (1967) (Barnes and Sato, 1979; Barnes, 1980; Barnes et al., 1980a; Barnes et al., 1980b). The active factor in these preparations appears to be a glycoprotein immunologically distinct from cold-insoluble globulin with a molecular weight between 70,000 and 80,000 as determined by SDS gel electrophoresis (Barnes et al., 1980a). Other effective ways to deal with the problem of providing a proper substrate for cells in serum-free medium include the use of collagen (Murakami and Masui, 1980; Wu et al., 1980) or dishes coated with factors synthesized *in vitro* by cultured endothelial cells and deposited directly on the plate (Birdwell et al., 1978; Gospodarowicz et al., 1980).

Another advantage to the use of serum-free medium containing hormones, binding proteins, supplemental nutrients and attachment factors is that it is possible to find sets of medium components which support the growth or maintenance of some cell types better than that seen in serum-mammary medium. Examples of such situations include the ZR-75-1 human mammary tumor cell line, the HC84S human colon tumor cell line and the HLE222 tumor epidermoid carcinoma cell line (Allegra and Limmpan, 1978; Murakami and Masui, 1980; Barnes et al., 1980b). The human colon tumor cell line maintains a more differentiated state in the serum-free medium, forming villus-like secretory structures only under serum-free conditions (Murakami and Masui, 1980). Similarly, the human lung epidermoid carcinoma cell line becomes extensively keratinized in serum-free, but not serum-containing medium (Barnes et al., 1980b).

Some differentiated cell types can be maintained in serum-free medium which cannot be maintained at all in a functional state in serum-supplemented medium. For example, rat ovarian granulosa cells in serum-free medium supplemented with insulin, transferrin, hydrocortisone and cold-insoluble globulin maintain responsiveness to follicle stimulating hormone which is not seen in serum-containing medium (Orly et al. 1980). Another example is rat thyroid cells in culture. Diploid, differentiated cells which synthesize thyroglobulin, concentrate iodide and show a growth response to thyroid stimulating hormone can be established in hormone-supplemented medium containing a low serum concentration, but not in the usual serum-containing media (Ambesi-Impiombato et al., 1980).

Since in serum-free medium we can control completely the environment of the cell, it is possible in some cases to select for a specific cell type from a mixture of cell types in culture, such as exist in primary tissue explants, by manipulating the composition of the culture medium. Thus is it possible to avoid fibroblast overgrowth of primary cultures. Such techniques have been applied to a number of cell types in primary culture. For instance, the medium designed for the serum-free growth of the MDCK line of dog kidney cells (Taub et al., 1979) also supports growth in primary culture of kidney cells from other species (Taub and Sato, 1979), and a modification of the serum-free medium designed for the growth of the B104 line of rat neuroblastoma cells allows the selective survival of neurons from embryonic chick dorsal root ganglia in the absence of growth of fibroblasts or Schwann cells (Bottenstein and Sato, 1979; Bottenstein et al., 1980).

The replacement of serum in culture medium has many practical advantages for studies of the interaction of hormones or drugs with cells, new approaches to cell selection and somatic cell genetics, and studies of nutrition and secretion at the cellular and molecular level. Through the use of serum-free medium it has been established that the drug tamoxifen, an antiestrogen, is capable of inhibition of growth of the ZR-75-1 human mammary tumor cell line in the absence of estrogen, suggesting that the mechanism of tamoxifen action may be more complex than the simple inhibition of stimulation of proliferation by estrogen (Allegra and Lippman, 1978). It has also been shown through the use of serum-free medium that the differentiation of F9 embryonal carcinoma cells induced by retinoic acid is independent of serum components (Rizzino and Crowley, 1980). In studies of hormone action in serum-free medium it has been reported that the time course of binding of radiolabeled EGF and subsequent loss of radioactivity is different for Hela cells grown in serum-free medium compared with cells grown in serum-containing medium (Wolfe et al., 1980b), suggesting that serum components exert previously undetected effects on cell-hormone interactions.

Exact nutritional studies can be carried out in serum-free medium without the complication of the nutritional contribution of the serum component to