EMBRYO CULTURE IN TERATOLOGICAL SURVEILLANCE
AND SERUM PROTEINS IN DEVELOPMENT

Progress Report
for Period 1977 to 1978

MASTER

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INTRODUCTION

The present progress report covers the period from July 1977 to July 1978. In addition to this report the reader is referred to two papers that were accepted for publication last year, a paper recently submitted for publication in the Proceedings of the National Academy of Sciences U.S. and an abstract of a paper submitted for presentation at the American Society for Cell Biology, November, 1978.

This progress report will first consider our recent work on the development of a new and unique procedure for teratological surveillance. Next, our continuing efforts to understand the role of serum proteins in development will be discussed.

TERATOLOGICAL SURVEILLANCE

Our research on the role of proteins in development has led to the use of intact cultured rat embryos in our studies and to a consideration of serum proteins in development. In attempting to apply our experience and knowledge to the needs of society and the interests of the Department of Energy we are working on a new and unique approach to teratological surveillance. This approach, combining both rat embryo cultures and serum, involves the culture of standardized rat embryos on serum derived from animals exposed to a possible teratogen. In this way we hope to monitor the teratogenic potential of an animal. We hope this procedure will allow one to evaluate animals after long periods of exposure, to determine the amount of time following exposure that a substance remains in the serum and to determine whether a substance acts directly on the embryo or indirectly through maternal metabolism. Ultimately, we hope to be able to extend our evaluation to the teratological potential of human serum. To our knowledge, this is the first time this approach has been proposed.

Before considering the actual experiments it should be noted that in developing

a testing procedure it is important to standardize and simplify the technique. We have and are continuing to work on the achievement of this goal. Thus, we select as uniform a group of embryos as possible and analyze our results both morphologically (gross and histological) and quantitatively (embryo protein and DNA content). To date we simply "eyeball" to select embryos for experiments and extremes of size are easily omitted. We will now attempt selection with an ocular micrometer. We have also found that the serum used as culture medium should be kept frozen as much as possible, that the addition of 0.1 ml of water per 0.9 ml of serum for culture is beneficial, and that an extended period of pre-gassing with 5% CO₂ is essential to bring the serum pH down to 7.2. We have designed a unique culture vessel and have found that from 2 to 4 embryos can be cultured in this vessel for 48 hours in 2 ml of serum.

Our initial studies have involved two established teratogens as "model" substances, cadmium and cyclophosphamide, and four substances related to energy technology that were obtained through Dr. M. Guerin of the Oak Ridge National Laboratory, coal solid R, spent shale particulates S, shale oil S and coal oil C.

Most of our effort to date has been with cadmium. We have tested cadmium by direct addition to the culture medium and have cultured embryos on serum from animals injected with cadmium. The teratological range of cadmium added directly to the medium is quite narrow with 1.95 μ M cadmium chloride showing lethality and 1.80 μ M showing no effect. At 1.90 μ M embryos survived 48 hours of culture and showed reduction in size with hemorrhagic brains. Next, rats were injected with cadmium (2.13 mg Cd/kg, i.p.) and bled for serum after 1, 4, 8 and 16 hours. The serum after 1 and 4 hours was lethal to 10 day rat embryos while the 16 hour serum caused a slight reduction in embryo growth and development. The 8 hour group was of particular interest because the embryos survived 48 hours and showed a reduction in growth as well as a variety of abnormalities. The abnormalities

were distinct from what we had observed by direct addition of cadmium to the culture medium. Photographs of the embryos from this experiment are included with the Renewal Proposal 1978. We have tried a higher level of cadmium injection and the results were predictable in that the 8 hour group as well as the 1 and 4 hour groups died during the 48 hour culture period. We have also attempted to simulate long term chronic exposures. Rats were injected with cadmium chloride every other day for two weeks and then after an additional week they were sacrificed. The growth and development of embryos on the serum from these animals appeared only slightly reduced in comparison to controls. At present we are conducting comparable studies with cyclophosphamide and the energy related materials.

THE RESPONSE OF EMBRYOS TO SERUM PROTEINS

The research on serum proteins in the remaining sections of this report is based on our research which has led to the working hypothesis that serum proteins serve as developmental regulatory signals and through changes in their relative synthesis, the serum proteins mediate the response of an embryo to its environment. The research based on this hypothesis follows along two lines, namely, attempts to demonstrate the direct response of developing systems to serum proteins and attempts to determine the mechanism regulating the synthesis of serum proteins.

A. The "depletion" proteins. We originally turned to the culture of rat embryos because the proteolytic activity of the chick embryo yolk-sac made it impossible to study the direct effects of proteins on chick embryos. We became intrigued by the fact that it was essential to frequently change the serum culture medium for the rat embryos although it was obvious that the embryo could not be depleting a significant amount of total protein from the serum. We reasoned that a minor constituent of serum might be depleted and so analyzed serum before and after embryo culture by acrylamide gels. We found a single protein banding at a molecular weight of 125,000 to be depleted during embryo culture. We also found

that this same protein was present at a reduced concentration in regular serum as compared to immediately centrifuged (true) serum. Now, what is the difference between regular and immediately centrifuged serum? In 1972, Steele (*Nature-New Biology* 237:150, 1972) reported that rat embryos developed abnormal "double hearts" and grew poorly on standard serum derived from a clot of rat blood. However, if blood was immediately centrifuged prior to clot formation, the serum gave improved embryo growth and normal heart development. Thus, the 125,000 protein may be important not only for embryo growth but also for heart development. A publication concerned with this study appeared this year and a copy is included (Klein, et al *J. Exp. Zool.* 203:313, 1978). It should be noted that a group at NIH recently informed us that they were able to confirm this observation.

A major effort is now being directed at the isolation of the 125,000 molecular weight "depletion" protein in order to evaluate its developmental activity and to show that it is an essential protein. We have attempted without success to isolate the protein by Sephadex and DNA-affinity column chromatography. The use of these procedures was based on the isolation of a 126,000 α -2-globulin DNA binding protein by Hoch and McVey (*J. Biol. Chem.* 252:1881, 1977). We next considered the possibility of isolating the protein directly from acrylamide gels. The protein was first located on non-detergent gels because previously we had used SDS gels. However, before proceeding we tested an extract from a blank gel on the cultured 10-day rat embryos. The extract was detrimental to embryos causing abnormal development of the nervous system. Now, we have turned to the use of rabbit antiserum in conjunction with Bio-Rad Affi gel and hope to separate the protein by immunosorption affinity chromatography. Immediately centrifuged serum is first passed through Sephadex G-100 to remove most of the serum albumin, isolated on acrylamide gels and the 125,000 protein band is extracted. This has been done and we will start rabbit injections in the very near future.

B. Conalbumin. Conalbumin is a transferrin similar to serum transferrin but found in egg white. We have observed that the cultured chick embryo requires a transferrin for growth and development. The protein itself appears essential because the removal of iron does not decrease the activity of this protein with the cultured chick embryo. We have attempted to determine the possible basis for the conalbumin requirement in the chick embryo. Our study was concerned with the possible role of transferrin (conalbumin) in the regulation of protein turnover. In brief, working with yolk-sacs made radioactive with ^{14}C -valine, transferrin greatly reduced the breakdown of intracellular proteins in comparison to ovalbumin. In addition, we showed that transferrin was a preferred substrate for yolk-sac proteolytic enzymes when compared to either ovalbumin or yolk-sac intracellular proteins. A manuscript based on these studies is presently being prepared for publication.

C. Embryo specific serum proteins. Another group of proteins that we are isolating are the serum proteins from the chick embryo. Similar to the rat "depletion" protein and transferrin we are interested in the effects of these proteins on developing systems. The serum proteins of the chick embryo are of particular significance because several of these proteins are embryo specific. We have devised a method of obtaining large quantities of serum from the 8-day chick embryo and with a variety of chromatographic procedures we have been able to isolate with reasonable purity transferrin, α -globulin-a, α -globulin-b, albumin and prealbumin. To date, we have observed a growth stimulation when α -globulin-a and prealbumin were added as serum supplements in the culture of 10-day rat embryos. In addition, we found that 11-13 somite chick embryos without yolk-sacs could be cultured on 8-day chick embryo serum. This may sound confusing but chick embryos with intact yolk-sacs could not be cultured successfully on serum. This work on embryo-specific serum proteins has been greatly reduced in order to devote more time to the teratological surveillance studies.

THE REGULATION OF SERUM PROTEIN SYNTHESIS

Two approaches are being used to study the regulation of serum protein synthesis. First, the endoderm cells of the 4-day chick embryo yolk-sac are being cultured so that the direct effect of various environmental factors on this single cell type can be studied. Second, the functional state of the various messenger RNA molecules for serum proteins in endoderm cells is being studied in an attempt to determine if the regulation of serum protein synthesis is under transcriptional and/or translational control.

A. Endoderm cell cultures. Our previous studies (Kram and Klein, Devel. Biol. 52:300, 1976) showed that the yolk-sac of the early chick embryo was the exclusive site of serum protein synthesis. This past year we completed a study to determine which of the cell types constituting the yolk-sac was involved in the synthesis of serum proteins. The ectoderm, mesoderm and endoderm cell layers were manually separated and incubated in the presence of ^{14}C -valine. The incubation media were then analyzed by double labeled gel electrophoresis with ^3H -labeled serum proteins derived from intact yolk-sacs. This analysis showed that only endoderm synthesized serum proteins and all the serum proteins synthesized by the intact yolk-sac were also synthesized by endoderm. This was then confirmed with antiserum prepared against chick embryo serum proteins. A manuscript based on this work has been submitted for publication to the Proc. Natl. Acad. Sci. U.S. and a copy is included with this progress report.

During the course of the above study, we developed procedures to obtain sufficient quantities of endoderm for cell culture experiments. Endoderm from 4-day chick embryo yolk-sacs is dissociated in Tris buffered saline with gentle stirring at 37.5°C and after rinsing they are dispensed into Falcon dishes at a concentration of 10^6 cells/ml. We have tried a variety of tissue culture media and have settled tentatively on Dulbecco's medium with 15% serum. With this medium we have been able to maintain viable cultures for periods up to two weeks. The

crucial question has now been answered. Endoderm in single cell culture will synthesize serum proteins. In our first experiment, endoderm cells after 2 days in culture were exposed for 2 days to ^{14}C -valine. The medium was then analyzed by acrylamide gel electrophoresis as well as by antibody precipitation. The acrylamide gel permits the analysis of the relative synthesis of the individual serum proteins. We found that transferrin, albumin and prealbumin had been synthesized but not the globulins. The antibody precipitation showed that 4,600 cpm of ^{14}C -valine were incorporated into serum proteins per μg of DNA. In this case monolayer cultures were used but with suspension cultures only 200 cpm/ μg of DNA were synthesized. It is clear from this preliminary study that endoderm cells in culture can be used to study the factors involved in regulating the synthesis of these cell specific products.

B. Cell-free synthesis of serum proteins. The rationale for these studies is to determine if the "functional" levels of messenger RNA's for the various serum proteins are related to the actual synthesis of serum proteins by the intact endoderm cells. If they are related, transcriptional control would be implicated and alternative if a relationship is not found translational control would be suggested. We plan to look at this relationship in two situations where changes in the synthesis of serum proteins have been previously observed. The first condition involves culturing chick embryos on various media while the second involves changes during development.

To briefly summarize our results to date, the synthesis of transferrin, α -globulins and prealbumin has been achieved with pure endoderm derived poly-ribosomes in a "run-off" protein synthesizing system. In this system, 62% of the protein synthesized was precipitated with antibody to serum proteins. Poly-A containing messenger RNA has been isolated from endoderm cells. When this messenger RNA was added to a protein synthesizing system derived from wheat germ, the high molecular weight serum proteins were not synthesized. For this reason we prepared

the rabbit reticulocyte lysate system and we are currently testing the ability of this system to synthesize serum proteins with the poly-A containing messenger RNA from endoderm. We recently found with the reticulocyte lysate that 70% of the total protein radioactivity was precipitated by antiserum to chick embryo serum proteins. This was most encouraging and we will now analyze the products by acrylamide gels to determine if all the serum proteins were synthesized.

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