Differentiation and growth of rat egg-cylinders cultured *in vitro* in a serum-free and protein-free medium

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ABSTRACT Modified organ cultures of rat egg-cylinders were grown for 2 weeks in Eagle's minimal essential medium (MEM) without serum. Differentiation of epidermis and cartilage in the cultures deprived of serum was comparable to that in fully serum-supplemented medium, whereas other differentiated tissues were rare or absent. The purpose of the experiment was to determine whether terminal tissue differentiation is modified by various added factors. The factors used affected the growth and/or differentiation of explants as follows: bovine serum albumin and human transferrin had a positive permissive influence on the appearance of neuroblasts; human transferrin alone stimulated the formation of lentoids, a relatively rare tissue. Retinoic acid inhibited cartilage formation and stimulated the differentiation of cylindrical epithelium; neural growth factor inhibited the growth of explants; and 5-azacytidine impeded the survival of explants. One can conclude that these factors influenced the growth and differentiation of the early rat embryos cultured in a chemically defined medium.

KEY WORDS: *rat embryo, egg cylinder, serum-free medium, in vitro culture*

Introduction

The study of differentiation involves «attempts at specifying concretely how like becomes unlike and how the resulting differences are then perpetuated» (Weiss, 1973). We know that there is an informational equivalence of either undifferentiated or differentiated genomes. Therefore, some factors must act on the various genomes to produce different phenotypes by directly activating or repressing different genes in one genome or acting on the protein-synthesizing machinery in the cell.

First of all, we must recognize that the first condition for growth and especially differentiation of cells, tissues and whole embryos is the presence of certain general systemic factors. The specific factors acting on identical cells responsible for provoking «unlike» differentiated cells are numerous and partly unknown. In *Caenorhabtidis elegans* (Hall and Watt, 1989), there is an invariant asymmetric division producing both stem cells and cells that are committed to terminal differentiation. A major step towards understanding the genetic basis of this cell lineage has been the identification and characterization of mutations in the genes involved in causing sister, or mother and daughter cells to follow different fates (Horvitz, 1988).

After this relatively rare event, there are many data providing evidence that anisotropies of egg and various cytoplasmic factors bring about «unlike» cells after several normal divisions. The partition of cytoplasmic contents creates various environments for the nuclei (Davidson, 1990).

In recent years, there has been an explosion of information regarding the role of sequence-specific DNA binding proteins in the selective activation of eukaryotic promoters (Levine and Manley, 1989). Moreover, there is every reason to believe that selective repression is an important mechanism of transcriptional control which could conceivably prove to be a widely used strategy for selective activation in regulating eukaryotic promoters. Gene transcription can be controlled by positive or negative regulatory mechanisms: a combination of both is frequently responsible for the observed expression patterns (Renkawitz, 1990).

Eighteen distinct hemopoietic growth factors have now been cloned and characterized. More than one factor controls cells in any one lineage, and most factors are active on cells of more than one lineage (Nicola and Metcalf, 1991). However, although the major regulatory gene for myogenesis has been discovered, «we known little about the activation of myoD gene during development» (Weintraub *et al.*, 1991). The search for specific regulatory factors can be made in various model systems, including cell cultures *in vitro* or experiments carried out *in vivo*. We have chosen a modified organ culture of rat egg-cylinders with only three germ layers grown for 2 weeks in chemically defined protein-free medium, which is simpler than an *in vivo* system, but closer to the *in situ* situation.

Abbreviations used in this paper: MEM-minimal essential medium; NGF-neural growth factor; RA-retinoic acid.

As this article went to press, we learned with deep regret of the death of Nikola Skreb after a long illness. The Editors of the journal would like to express their sadness at this great loss to the scientific community.

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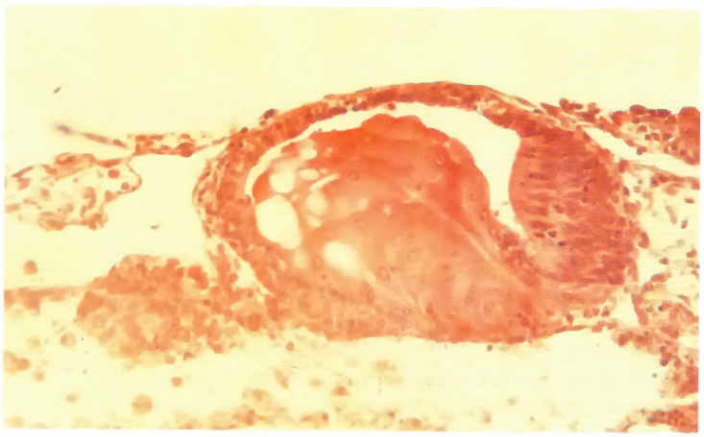

Fig. 1. Differentiation of lentoid cells in continuity with the retinal epithelium in a teratoma-like explant. The explant was grown in serum-free medium with 50 μg/ml human transferrin. *x470.*

This system permits the survival of explants with little growth, but the differentiation of epidermis and cartilage seems normal (Skreb and Bulic, 1987). Other differentiated tissues were rare or absent. Since serum-supplemented mediums promote the differentiation of main tissue types (Skreb *et al.*, 1983), and since serum is a complex and largely undefined mixture of possible controlling factors, we tried to single out some important factors with known activities in order to observe their effects. In this article, we are trying to compare 5 different exogenous factors affecting the growth and differentiation of the rat egg-cylinders. Albumin and transferrin were used because they are normal constituents of blood serum, and because they almost always appear necessary for cell culture in a chemically defined medium (Barnes and Sato, 1980). NGF was added to the medium since the neuroblasts were absent in our previous study using chemically defined medium alone (Skreb and Bulic, 1987), and it is well known that NGF is a neurotrophic factor for sensory and sympathetic neurons (Levi-Montalcini, 1987). Retinoic acid, a well recognized morphogen (Slack, 1987) acting during normal development (Tamura *et al.*, 1990), had never been used in a model system similar to ours. Finally, we used an antagonist to cytidine (5-azacytidine) presuming that it would block the methylation of cytosine and stimulate differentiation, as described in several other systems (Dynan, 1989; Tawa *et al.*, 1990; Boyes and Bird, 1991).

Briefly, the factors mentioned have been used to find out whether they could specifically modify the growth and/or differentiation of rat egg-cylinders cultivated in a chemically defined protein-free medium.

Results

Systemic general factors of differentiation in our study

As indicated by the methods used, the egg-cylinders were placed on a metal grid and the medium was changed every other day. We observed the appearance of fewer tissues than usual when the metal grid was omitted or the medium was changed only once during the cultivation period (Skreb and Crnek, 1980). In none of these cases did the cells remain at the same level of differentiation as at the moment of explantation, but they were never terminally differentiated.

Specific factors affecting the process of differentiation

In control series containing only the chemically defined medium, at the end of the culture, as already mentioned, we observed epidermis and cartilage, and very rarely some other tissues.

Albumin and transferrin stimulated the differentiation of neuroblasts in a similar percentage as when whole rat serum was added (Bulic-Jakus *et al.*, 1990).

Transferrin alone induced formation of neuroblasts and, surprisingly, the differentiation of lentoids (33.7%). Lentoids were very rare in serum-supplemented medium (2.1%) and even in embryos *in vivo* following the transfer of embryos under the kidney capsule (5.3%) (Bulic-Jakus *et al.*, 1990) (Fig. 1).

The effect of RA was consistent and very clear. We observed no cartilage and practically no squamous epithelium. All teratoma-like explants were surrounded by cylindrical epithelium with many folds (Crnek *et al.*, 1991) (Fig. 2).

Specific factors affecting only the survival and growth of explants

NGF did not stimulate the differentiation of neuroblasts as expected, but, on the contrary, inhibited the differentiation of other tissues and the growth of the explants. Neuroblasts were never observed (Stipic *et al.*, 1991) (Fig. 3).

5-azacytidine impeded the survival of explants and did not stimulate differentiation in a chemically defined medium (unpublished results).

Summarizing, we can conclude that the various factors specifically affected either the growth or the differentiation of the explants, but not always in a way similar to other data obtained in various model systems.

Discussion

In the Introduction we asked whether different applied factors can act specifically on the rat egg-cylinder cultured *in vitro*. First, it must be stressed that the embryo used in our study had only three germ layers without any well differentiated tissue. Second, the

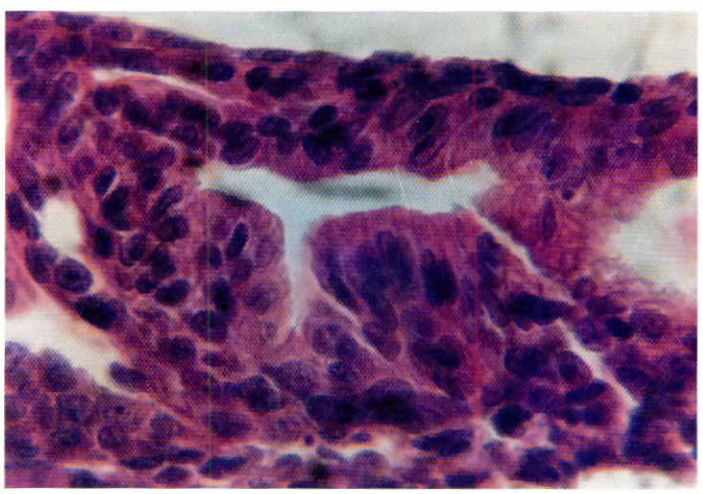

Fig. 2. Columnar epithelium found in a teratome-like explant cultivated in serum-free medium with RA 10^{-5}M. *x814.*

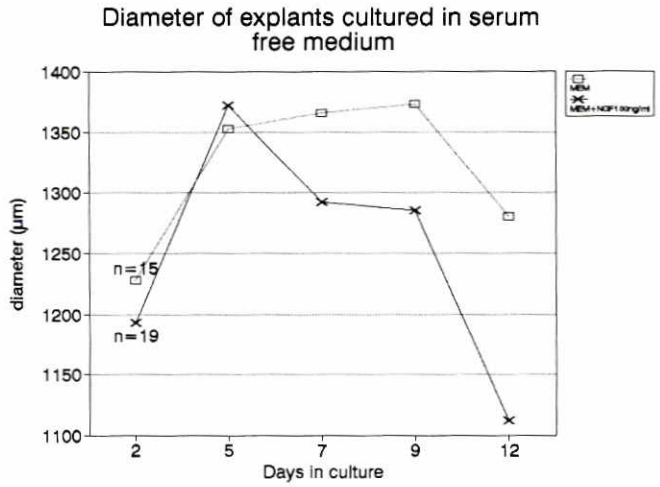

Fig. 3. To monitor growth the major and minor axes of an explant were measured using an eyepiece micrometer. *In this figure lengths of major axes of explants cultured in serum-free medium are presented as an example. 100 ng/ml of NGF was added during the whole culture period. The difference between lengths of control and treated explants measured on the 12th day of culture period was statistically significant.*

medium used for the culture consisted of Eagle's MEM with Hank's balanced salt solution. Therefore, every added factor acted in a chemically defined environment on undifferentiated embryonic cells. After 2-weeks of culture, control series had only two well differentiated tissues: epidermis and cartilage.

As we have previously carried out experiments in serum-supplemented medium, our discussion will try to compare the effects of each factor in the two media in order to find out its condition of action.

We used three proteins: albumin, transferrin and NGF. As far as the appearance of neuroblasts was concerned, two of them had a positive permissive action (Bulic-Jakus *et al.*, 1990), while NGF did not act in the same manner (Stipic *et al.*, 1991).

Furthermore, transferrin stimulated the formation of lentoids, a relatively rare tissue (Bulic-Jakus *et al.*, 1990). This effect disappeared when albumin was added to our medium with transferrin. We can speak of the antagonist action of these two proteins in spite of the fact that both had a permissive effect on the appearance of neuroblast (Jakus, 1989). However, the triggering action of transferrin, in the case of lentoid formation, for the time being, remains obscure. Nevertheless, the answer to our question about the specificity of action is affirmative. These three proteins act specifically on the early rat embryo. The effects of NGF, although different from the other two proteins, were the same in both media used. With or without serum, it never stimulated either differentiation or growth. On the contrary, its action was rather inhibitory, as was seen in some cell cultures (Burnstein and Greene, 1982; Lillien and Claude, 1985).

As far as RA is concerned, we also obtained the same results in both media, but the data were more impressive in the serum-free medium. Our data confirmed other previously published results (Sherman, 1986).

On the other hand, 5-azacytidine acted differently in two media. The differentiation of all tissues was stimulated in serum-supplemented medium, as was easily seen in the formation of myotubes (Fig. 4). In serum-free medium, this agent acted as an antimitotic, as had already been seen in other systems (Bartolucci *et al.*, 1989).

Briefly, factors used in this study acted specifically on the early rat embryo. Moreover, their action sometimes depended on the other factors present in the culture medium.

To discuss the mechanisms of action of the above-mentioned agents is for the time being somewhat premature. Our present study was focused mainly on their probable influence on the terminal differentiation recognizable on tissue sections. Our second step, in the near future, will be oriented more towards possible causes of visible effects and cellular targets of their actions. There is evidence so far that NGF (Escandon and Chao, 1990; Marchetti *et al.*, 1991) and RA (Giguere, 1990; Dolle *et al.*, 1990) affect cells through specific receptors, which at least for RA act as transcription factors (Green and Chambon, 1988).

Finally, it is impossible to say whether all these factors play a role during normal embryonic development. The presence of RA in the developing chick limb leaves no doubt that it acts as a morphogen (Thaller and Eichele, 1987; Tamura *et al.*, 1990). NGF is also found in various parts of an embryo, but its specific action seems different from that found in our study (Davies, 1988). The two other proteins, albumin and transferrin, although surely present in embryos, probably affect normal development in a different way than in our study because they act together with many other factors.

The antagonist of cytidine is an artificial product and apparently cannot participate in normal embryonic development.

Materials and Methods

Female rats of inbred Fischer strain were killed after 9 days of pregnancy. Egg-cylinders at the primitive streak stage were isolated from surrounding decidua using watchmaker's forceps. After removal of Reichert's mem-

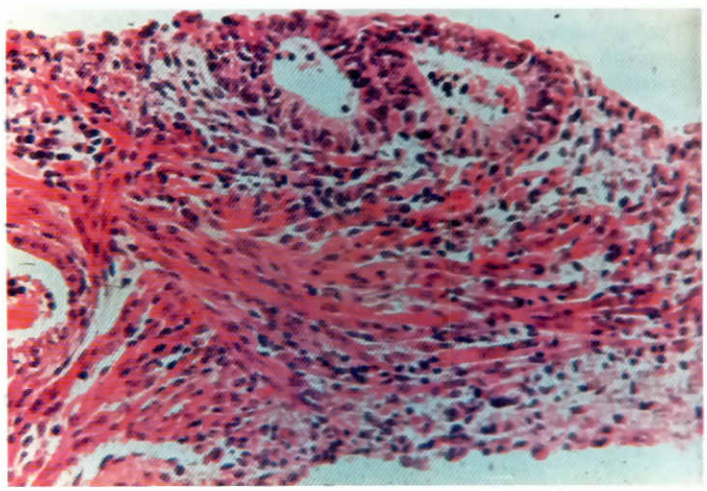

Fig. 4. Developing myotubes in an explant cultivated in serum-supplemented medium with 30 µM 5-azacytidine (5th day of culture period). *Without added factor myotubes differentiated in about 20% of explants, whereas after its addition in almost 100%. x450.*

brane, the extraembryonic part was cut off at the level of the amnion. Three egg-cylinders were placed on lens paper supported by stainless-steel grid placed in the center of an organ tissue culture dish (Falcon No. 3037) (Skreb and Svajger, 1973; Skreb *et al.*, 1983). Medium was poured under the grid to wet the lens paper. Eagle's minimal essential medium with Hank's balanced salt solution was used, with or without some added factor. All agents used were purchased from Sigma (St. Louis, MO). The concentrations of factors applied were as follows: Human transferrin 50 μg/ml, bovine serum albumin 40 mg/ml, neural growth factor 100-200 ng/ml, retinoic acid 10^{-4} to 10^{-8}M, and 5-azacytidine 30 μM. Usually they were added to the medium from the 5th through the 14th day of culture period. RA and NGF were added also in special series during the whole period of 2-week culture; 5-azacytidine was added only once on various days.

Differences in time of exposure had no consequences as far as the development of explants was concerned.

All culture dishes were incubated in 5% CO_2, 95% air at 37°C. Culture medium was replaced every other day. After 2 weeks, the explants were fixed in Zenker's fluid, washed in tap water and processed by routine histological methods. Serial sections were made and care was taken not to lose any sections. Every section was checked for the presence of the various tissue types. Data were statistically evaluated using chi-square analysis.

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