Retinoic acid can change normal differentiation of rat egg-cylinders cultured *in vitro*

VESNA CRNEK, MAJA VLAHOVIĆ and NIKOLA ŠKREB*

Institute of Biology, Faculty of Medicine, University of Zagreb, Republic of Croatia, Yugoslavia

ABSTRACT The modified organ culture of rat egg cylinders provides favorable conditions for 2 weeks for the differentiation of main tissue types. To study the effect of retinoids on early rodent differentiation, retinoic acid (RA) was added in various concentrations to serum-supplemented or serum-free medium. Explant survival decreased when RA was added to serum-free medium. Although the cartilage was well differentiated even in cultures deprived of serum, RA inhibited chondrogenesis in all cultures without or with serum. The frequency of columnar epithelium was higher and its folds more often present when RA was added to the medium. Keratinization of squamous epithelium depended on the RA concentration added to the medium, and was almost absent when the concentration was high. Other tissues often present in serum-supplemented medium (such as neuroblasts and myotubes) were not affected by RA, a result that differs from those obtained in other experimental systems.

KEY WORDS: *rat embryo, organ culture, retinoic acid, differentiation*

Introduction

Vitamin A and its derivates, the retinoids, have very important functions *in vivo* at physiological concentrations, whereas low or high doses of most retinoids may be teratogenic in vertebrates (Hale, 1935; Giroud and Martinet, 1956). Retinoic acid embryopathy is characterized by severe limb and cranial defects (Kochhar, 1967; Abbott *et al.*, 1988). Recently, it was found that the endogenous RA (not retinol) forms concentration gradients with a high point in the posterior part of the chick limb bud, that also contains the zone of polarizing activity (Slack, 1987; Thaller and Eichele, 1987; Tamura *et al.*, 1990). After having discovered this fact it is now easier to understand the widespread occurrence of morphogenetic effects when RA has been applied in several model systems: during limb regeneration in Amphibians (Keeble and Maden, 1989; Ludolph *et al.*, 1990), and in developing chick and mouse limb buds (Tickle and Crawley, 1988; Eichele, 1989; Satre and Kochhar, 1989; Tickle *et al.*, 1989). It has also been shown that RA plays an important role in the development of the central nervous system of *Xenopus* (Durston *et al.*, 1989), chick (Maden *et al.*, 1989) and mammalian embryo (Maden *et al.*, 1990). Recently, it was found that the floor plate of the chicken neural tube has some polarizing activity when grafted to the limb bud (Wagner *et al.*, 1990). Furthermore, it is interesting to mention the influence of RA on the expression of latent feather placode forming properties instead of foot scale in the chick embryo (Cadi *et al.*, 1983). Much data has been published regarding the growth of cells in culture, which has shown the inhibition of growth of early chick embryonic cells (Mitrani and Shimoni, 1989) and melanoma cells (Lotan *et al.*, 1980).

Moreover retinoids are one of the few classes of naturally occurring compounds which are known to influence the process of differentiation of several cell types (for review see Silver *et al.*, 1983; Sherman, 1986). Long ago it was demonstrated that high doses of vitamin A produce mucus metaplasia in culture of chick embryonic skin (Fell and Mellanby, 1953; Fell, 1957), and this was confirmed more recently in cultures of various systems of cell or tissues (Peck *et al.*, 1977; Yaar *et al.*, 1981; Shapiro, 1986). Regarding chondrogenesis, many reports have stressed the reduction of cartilage matrix after RA application (Kochhar *et al.*, 1984; Horton *et al.*, 1987), whereas there are some other findings concerning selective stimulation of chondrogenesis, which depends on the dose and the type of cells used (Ide and Aono, 1988; Paulsen *et al.*, 1988; Langille *et al.*, 1989). The cell culture system has been used to induce differentiation in malignant cells such as some leukemia cell lines (Matzner *et al.*, 1987) and neuroblastoma cells (Haussler *et al.*, 1983). Teratocarcinoma stem cells may be induced to differentiate, although the effect depends also on cell line and the dosage used (Silver *et al.*, 1983; Jetten, 1986; Berg and McBurney, 1990). The important role of RA in normal chick development and the various experimentally provoked effects by retinoids on processes of embryonic development have caused us to carry out a series of

Abbreviations used in this paper: RA, retinoic acid.

***Address for reprints**: Institute of Biology, Medical Faculty, University of Zagreb, Šalata 3, P.O.Box 166, 41000 Zagreb, Croatia, Yugoslavia. FAX: 38-41-424.001

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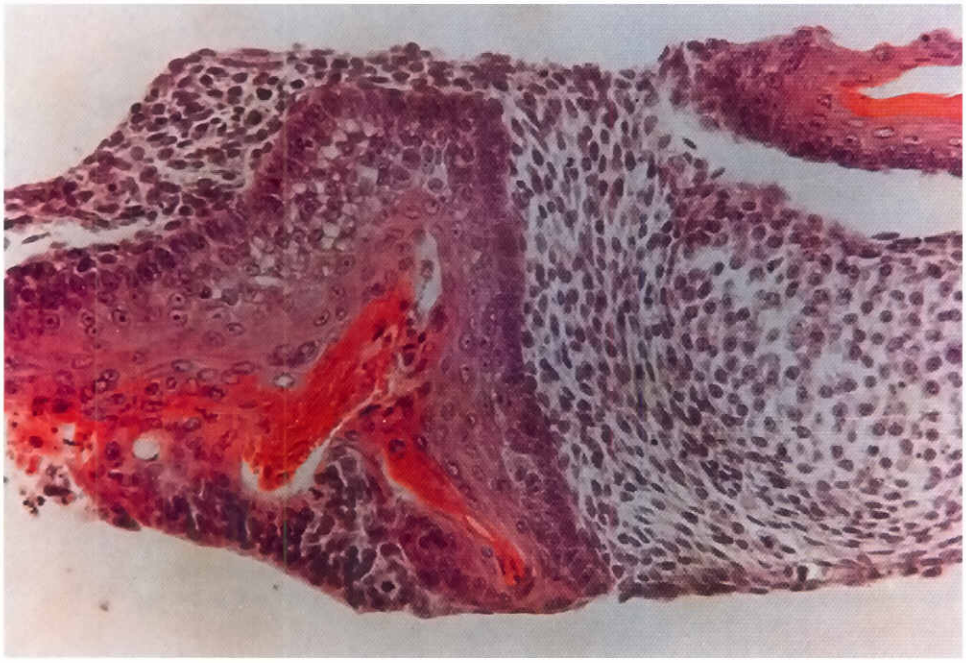

Fig. 1. Stratified squamous epithelium with keratin found in an explant cultivated in serum-free medium alone. x285.

experiments designed to establish the effect of different doses of RA on early rat development.

In our laboratory, we developed a modified organ culture of rodent egg-cylinders, isolated at the primitive streak stage with three germ layers, which has been shown to provide favorable conditions for the terminal differentiation of the main tissues in explants (Skreb and Svajger, 1973). Since the final results of our 2-week culture are teratoma-like explants without any morphogenesis and with a very small increase in volume, all the expected effects, including any potential morphogenetic effects will be visible only as putative changes of differentiation. Our model system has the advantage of being simpler than the embryo *in vivo*, but is nevertheless closer to normal development than the cell cultures used in the analysis of RA influence on differentiation. Moreover, we have succeeded in obtaining the survival and partial differentiation of this stage of rat development in serum-free and protein-free medium (Skreb and Bulic, 1987), which allowed deeper insight into the action of RA or any other factor on the cultivated rat embryo.

In brief, the present experiments were designed to find out whether RA will affect the formation of various terminally differentiated tissues in cultured rat egg-cylinders, either in serum supplemented or in serum-free and protein-free medium.

Results

After a two-week period of culture all explants were fixed and serially sectioned. Only the morphological criteria of differentiation have been used in this study so far. Each tissue type was identified as specifically as possible, so that the frequency of tissue types could be calculated. We also tried to assess the degree of differentiation. As terminal differentiation is a multiphasic process, several intermediate definite steps can be recognized in some tissues (e.g. intestine). The following tissues are frequent in our system: squamous stratified epithelium, columnar epithelium, neuroblasts, cartilage, and skeletal and cardiac muscle. On rare occasions we can see lentoids, glands and ganglions.

All our experimental series can be divided into two main groups:
1) The first group consists of three series treated with two different doses of RA (10^{-8} M and 10^{-5} M) in serum supplemented medium. This group may be envisaged as the basic experiment carried out with the purpose of being able to focus our further study on the findings relevant to our analysis. The results of the series treated with 10^{-8} M are not shown here because there was no difference between the control and treated explants (74 control and 68 treated explants). The only exception is the cartilage, which is impaired as in other series. Other results are presented in Table 1. Neuroblasts, epidermis and cardiac muscle are as frequent as in the control series, whereas the columnar epithelium seems to be more frequent in the treated series. In contrast, cartilage has a very low incidence, if any. As far as the myotubes are concerned they are more frequent only in one treated series, but the difference was not statistically significant.
2) As a consequence of the mentioned results (enhancement of columnar epithelium incidence) and due to the common knowledge that RA can affect all epithelia, we tried to analyze them in detail. Our attention was focused on the squamous stratified epithelium and its keratinization on the one hand and the columnar epithelium on the other. Particularly, goblet cells and the folds of this latter epithelium were analyzed. It is well known that the entire lining, including both mucosa and submucosa, may be formed into large folds (plicae).

To compare approximately the same number of cases in all series we chose randomly about 50% of all explants treated only the second week of culture with 10^{-5} M in serum supplemented medium (Table 1). Consequently, in Table 2 the comparison is facilitated by the similar number of experimental cases. Out of five series (Table

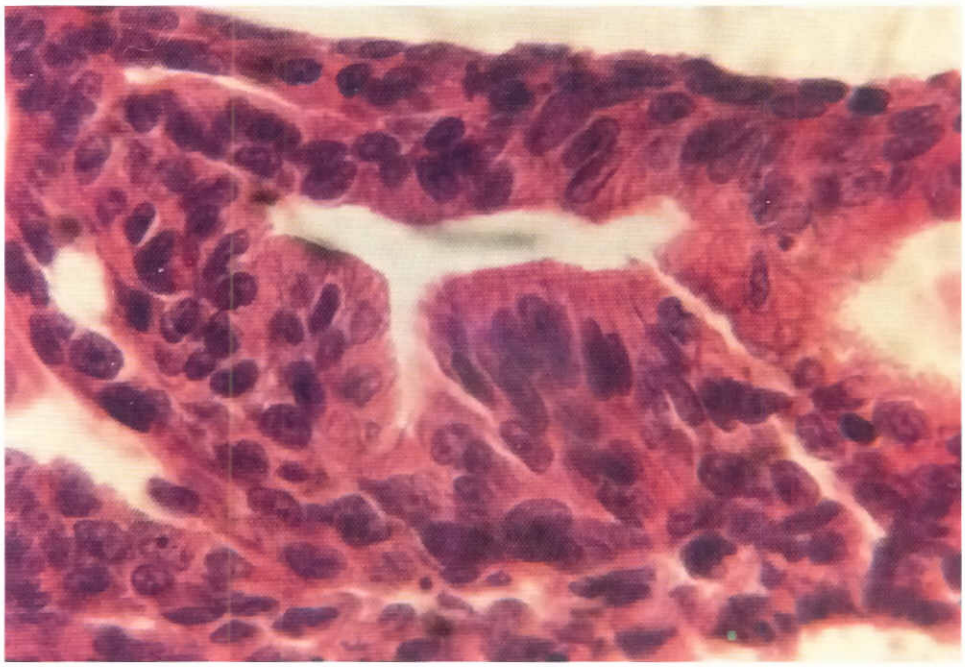

Fig. 2. Columnar epithelium found in an explant cultivated in serum-free medium with RA 10^{-5}M. x814.

2), one series in the serumless medium was the most conspicuous and presents a summary of all other results. In the control series in serum-free medium the squamous epithelium is very well formed with an obvious layer of keratin. The columnar epithelium is sometimes present but in less than 50% of all cases.

The dose of 10^{-8} M RA does not seem to affect the frequency of observed tissues. On the contrary, the dose of 10^{-5} M RA affects both epithelia very obviously in medium without serum. There are almost no squamous epithelia, whereas the incidence of the columnar epithelium is very high (100%) and its folds are more frequent than in the control series. The cartilage was never observed after RA treatment, whereas in control serum-free medium its frequency was 51.1% (23 out of 45).

Since it is reasonable to assume that in serum supplemented media there is at least a minute but variable concentration of retinoids, the results concerning the epithelia may vary. Only results obtained after the application of the concentration of 10^{-4} M RA resemble those obtained in serum-free medium. The lower doses do not contradict the previous findings but the results are rather less clear. All together the squamous epithelium is less frequent with little if any keratinization, and the folds of the columnar epithelium are more frequent in the treated series than in controls. The incidence of cartilage is always very rare (Table 1).

Discussion

After many attempts at determining the role of RA in different experimental systems the results obtained with our system have generally confirmed those obtained elsewhere. The rat egg-cylinder without any extraembryonic membranes cultivated for 2 weeks can be envisioned, on the one hand, as a whole embryo and on the other, as an organ culture. Lack of morphogenesis simplifies the analysis of the results, but this fact does not exclude the interactions between embryonic cells, as was the case in cell culture. Such a model system has not been tested so far in connection with RA function and influence. We may expect various effects of RA on an embryo especially in serum-free culture.

As far as growth and survival of our explants are concerned we did not observe any effect of RA in serum-supplemented medium. In contrast, in serum-free medium the survival was significantly lower in RA-treated series than in the control ones, and the survival in the serumless medium was not as good as in the serum-supplemented medium. It has been stressed that the effects of retinoids upon proliferation of epithelial cells are complex and in some cases apparently inconsistent and confusing. Besides the observation of survival we did not pay any special attention to the proliferation of cells.

On the contrary, we analyzed more systematically the process of differentiation. Since the assessment of tissue types may sometimes be biased by the expectations of the observer, all our histological sections were analyzed without knowledge of their origin and treatment.

In any attempt now to compare the reactions of certain tissues following the application of RA in other model systems, we would expect to find some changes in neuroblast formation. There were data from the cell culture and even from whole embryos that RA may induce neuron differentiation and have some impact on the central nervous system (Edwards and McBurney, 1983; Andrews, 1984; Thompson *et al.*, 1984; Andrews *et al.*, 1986; Sarma and Notter, 1988; Husmann *et al.*, 1989; Vaessen *et al.*, 1989). Contrary to these expectations we were unable to observe any visible change in neuroblast formation. In chemically defined medium without protein, the incidence of neuroblasts was very low. The addition of RA to this medium does not improve this low incidence, whereas the addition of either bovine serum albumin or transferrin, done in previous experiments (Bulic-Jakus *et al.*, 1990), greatly enhanced

TABLE 1

FREQUENCY OF VARIOUS TISSUES IN EXPLANTS EXPOSED TO RA CONCENTRATIONS OF 10^{-5}M IN SERUM SUPPLEMENTED MEDIUM (EXPRESSED AS THE PERCENTAGE OF EXPLANTS CONTAINING THE TISSUE)

Time of exposition (days)	1-14		5-14	
	Control	RA	Control	RA
Number of explants	20	30	107	120
Squamous epithelium	75	76.6	98.1	98.3
Neuroblasts	90	76.6	50.4	59.1
Columnar epithelium	70 A	96 A	64.5 B	93.3 B
Glands	10	3.3	10.2	11.6
Cartilage	80 C	3.3 C	61.6 D	10.8 D
Skeletal muscle	15	26.6	29.9 E	42.5 E

A) X^2= 6.612, P<0.02
B) X^2= 29.550, P<0.001
C) X^2= 20.746, P<0.001
D) X^2= 62.23, P<0.001
E) X^2= 3.343, P>0.05

neuroblast differentiation. The conclusion is clear the RA does not affect neuroblast formation in our model system.

The available data regarding cartilage are contradictory. Many findings suggest that RA can induce cartilage resorption in cultured fetal rat bones or inhibit chondrogenesis in limb mesenchymal cells *in vitro* (Lewis *et al.*, 1978; Kistler, 1982, 1984). In contrast, recently some data have been published concerning the enhancement of cartilage matrix formation after RA application, which depends on RA concentration and on the type of cells used (Ide and Aono, 1988; Paulsen *et al.*, 1988; Langille *et al.*, 1989).

In all our series the difference in the incidence of cartilage between RA-treated explants and the control ones was always highly significant statistically. We have never observed an enhancement but always the inhibition of chondrogenesis.

Although there have been reports that RA can induce some embryonal carcinoma cell lines into myoblast (Edwards and McBurney, 1983), our results could not confirm this observation. An exception is only one series in which the incidence of myotubes seems to be higher than in the control series (Table 1). Since in all the other series we could not confirm this finding and since even the difference in myotube incidence was not statistically significant, we believe that RA was not able to induce myotube formation in our system, as was the case with dbcAMP, shown in a previous paper (Skreb *et al.*, 1984).

In regard to the epithelia, our results are very similar to those obtained in other experimental systems. The experiments with human embryonic skin fibroblasts are very interesting and together with already mentioned data in the Introduction are in good agreement with our results. Human skin fibroblasts were cultivated by French authors as confluent monolayer and were raised at the liquid-air interface on stainless steel grids during one week (Asselineau *et al.*, 1989). The technique resembles that used in our laboratory with a difference in that they used a monolayer of fibroblast in delipidized serum, whereas we cultivated rat embryonic shields in serum-free or serum-supplemented medium. Different doses of RA give rise to various results as far as the keratinization of stratified squamous epithelium is concerned. The dose of 10^{-10} M RA gave rise to hyperkeratosis, which we obtained in serum-free medium. Orthokeratosis was obtained with doses of 10^{-9} and 10^{-8} M RA, which resembled our results obtained in serum-supplemented medium without an RA addition. As already stated, there is no doubt that the rat serum used in our culture medium contained some small doses of retinoids. Finally, the dose of 10^{-6} M RA prevented keratinization and these results are similar to the effect of either 10^{-4} M RA in serum-supplemented medium or 10^{-5} M in serum-free medium when rat embryos were used. Although the doses are not the same, both experiments have the same conclusions. Lack of retinoids or very small doses of them permits a massive keratinization which disappears with the increased doses.

The intestinal cells normally synthesize and secrete mucus material, and the absence of retinoids results in a decrease in the number of goblet cells (Shapiro, 1986; Obinata *et al.*, 1987). The latter finding was not confirmed by our results. First we observed the columnar epithelium even in serum-free medium when no retinoids were present, and second the incidence of goblet cells was approximately the same in the treated and in the control series. Nevertheless, we observed in our series that the frequency of columnar epithelium is higher when RA was added to the medium and that it was better differentiated, which was easily identified by the presence of many folds in the lining of the columnar epithelium.

Of course, it is impossible to hypothesize about how RA works on the basis of these results. However, it is necessary to point out that the new findings of diversity of RA receptors with their isoforms may possibly explain the diversity of RA effects (Brockes, 1990; Mangelsdorf *et al.*, 1990). Finally, the sequential activation of

TABLE 2

TISSUES FOUND IN EXPLANTS CULTURED IN SERUM-FREE AND SERUM SUPPLEMENTED MEDIUM (EXPRESSED AS THE PERCENTAGE OF EXPLANTS CONTAINING THE TISSUE)

Series	Time of expos. (days)	No. of expl.	No. of fixed expl.	Surv. (%)	Squam. epith.	Ker-atin	Col. epith.	Folds
MEM RA		47	31	65	93	77	35	6
10^{-8}M	5-14	45	19	42	84	73	57	20
MEM RA		69	45	65	100	95	44	8
10^{-5}M	5-14	70	23	32 A	0	0	100 B	86 C
Serum + MEM RA		36	36	100	66	30.5	83.3	41.6
10^{-5}M	5-14	41	41	100	60	26.8	97	63.4
Serum + MEM RA		20	20	100	75	40	70	5
10^{-5}M	1-14	30	30	100	76.6	6 D	96	63.3 E
Serum + MEM RA		38	38	100	89	10	78	10
10^{-4}M	5-14	40	40	100	15 F	0	95	72 G

A) X^2= 13.7, P<0.001
B) X^2= 17.887, P<0.001
C) X^2= 37.272, P<0.001
D) X^2= 6.3802, P<0.02
E) X^2= 14.6701, P<0.001
F) X^2= 40.405, P<0.001
G) X^2= 28.239, P<0.001

homeobox genes by RA in human embryonal carcinoma cells confirms the hypothesis that the RA acts through the gene regulator activity of the above-mentioned receptors (Simeone *et al.*, 1990). We would like to pursue the question further in future studies.

Materials and Methods

Female rats of inbred Fischer strain were killed after 9 days of pregnancy and the egg cylinders, at the primitive streak stage, were isolated. The extraembryonic part was cut off at the level of amnion and the shields were put on lens paper. The lens paper carrying three shields was supported by a stainless-steel grid placed in the center of an organ tissue culture dish (Falcon No. 3037). Eagle's minimal essential medium with Hank's balanced salt solution was used with or without 50% serum from male rats of the same strain. Rat blood was immediately centrifuged and the serum was inactivated at 56°C for 30 minutes and sterilized through a Millipore filter.

Details of this modified organ culture technique have been published elsewhere (Skreb and Svajger, 1973; Skreb *et al.*, 1983; Skreb and Bulic, 1987).

RA was purchased from Sigma (Trans retinoic acid, type xx) and was added to Eagle's MEM in concentrations of 10^{-5} and 10^{-8} M, and to MEM with serum in three various concentrations: 10^{-4}, 10^{-5} and 10^{-8} M.

After two weeks, the explants were fixed in Zenker's fluid, washed in tap water and processed for routine histology (haematoxylin and eosin). Uninterrupted serial sections were made and checked for the presence of various tissues. Data were statistically evaluated using chi-square analysis.

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