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*Original Article*

# Transferrin enhances lentoid differentiation in rat egg cylinders cultivated in a chemically defined medium

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**ABSTRACT Rat egg cylinders at the primitive streak stage were grown in modified organ culture for 2 weeks using a chemically-defined medium. The purpose of the experiment was to determine whether the terminal tissue differentiation is modified by human transferrin. The control sets were grown in medium with or without rat serum. In explants treated with transferrin, groups of atypical cells of the ocular lens (lentoids) appeared more frequently than in both control sets; however neuroblasts were observed as often as in the serum-supplemented medium. Bovine serum albumin (BSA) stimulated the differentiation of neuroblasts but did not promote lentoid formation. We conclude that human transferrin does stimulate the differentiation of lentoids in rat embryonic explants, but the mechanism of its action remains unknown.**

KEY WORDS: *rat embryo, organ culture, chemically defined medium, transferrin, lentoids*

## Introduction

The differentiation of early postimplantation rodent embryos may be efficiently studied *in vitro* by culturing them on metal grids in various serum-supplemented or serum-free media. After 2 weeks of culture the resulting teratoma-like structures show no morphogenesis but contain several terminally differentiated tissues which can be evaluated histologically. The best results are obtained if the liquid medium is changed several times and when at least 20% of homologous serum is added to Eagle's MEM (Škreb and Švajger, 1973). Moreover we have described a serum-free and protein-free medium that allows the rat egg cylinders to survive the culture for an extended period and to give rise to terminally differentiated tissues such as keratinized epidermis and cartilage (Škreb and Bulić, 1987), whereas other tissues, especially neuroblasts, are scarce or even absent.

In order to investigate the possible effect of potential developmental factors on the growth and differentiation of the explanted rat egg cylinders we added various factors to the defined protein-free medium. First we tested transferrin, assuming its important role in early rodent development. It is, for example, well known that transferrin plays an important role in the transport and delivery of iron to cells and that it promotes cell growth. These two effects may be related, although there is also a possibility that transferrin stimulates growth independently of its iron transport activity (Mescher and Munaim, 1988).

It has been shown that transferrin passes from mother to embryo in the early stages of rat development (Huxham and Beck, 1985) and that it is synthesized in the embryo proper from the seventh day of development on. In later stages of development it is found in liver, brain and other organs of the embryo (Meek and Adamson, 1985).

In cell culture transferrin is almost always used when defined media are needed (Barnes and Sato, 1980). Many other findings point to its important role in stimulating differentiation in organ culture of metanephrogenic mesenchyme (Ekblom and Thesleff, 1985; Thesleff *et al.*, 1985) and tooth development (Partanen *et al.*, 1984; Partanen and Thesleff, 1989). There is also data supporting its role in the differentiation of embryonal carcinoma cells (Darmon *et al.*, 1981).

The present experiments were carried out to find out whether transferrin could specifically modify the growth and/or differentiation of rat egg cylinders cultivated in a chemically defined protein-free medium.

## Results

### Growth

The addition of either of the proteins used (transferrin or BSA) resulted in a similar increase in growth as compared to the serum-free medium even though the concentration of the two proteins was different. Moreover, the increase of growth was the same when the two proteins were added together or when insulin and selenium were in the medium. During the first week all values roughly increased, reached a plateau and then decreased during the

*Abbreviations used in this paper*: BSA, bovine serum albumin; RS, rat serum; MEM, Eagle's minimal essential medium; Tr, transferrin; In, insulin; SE, standard error; EGF, epidermal growth factor.

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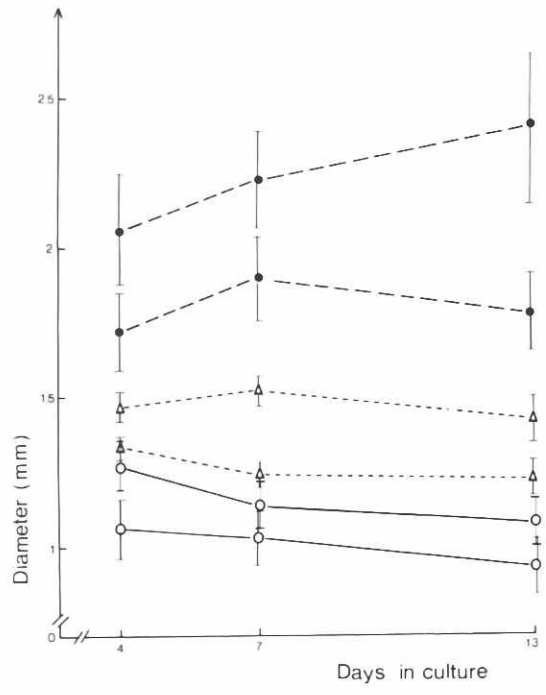


**Fig. 1. Growth curves of explants.** *Dimensions of experimental explants (cultivated in Eagle's MEM with 50 µg/ml transferrin) and those of two control groups of explants (1. cultivated in 50% rat serum and 50% MEM, and 2. in MEM) were measured. Length of major and minor axes respectively, of experimental (Δ——Δ) and control explants (with serum ●——● and without serum ○——○). Data points are means -+ SE (error bars)*

second week. The sets of explants grown in serum-supplemented medium showed the highest increase, whereas those grown in Eagle's MEM alone did not increase in size at all. The curves of the dimensions of experimental sets of explants showed an intermediary increase between the curves of the two control sets as shown in Fig. 1 (other data not shown).

### *Differentiation*

Three parallel sets of experiments were carried out in each series. The first set of explants was cultivated in Eagle's MEM supplemented with one or more additional factors.

The results of various combinations of added factors are presented in 7 series as follows (Table 1): 1. Human transferrin in a concentration of 50 µg/ml. 2. Human transferrin 50 µg/ml + insulin 10 µg/ml. 3. Human transferrin 50 µg/ml + selenium 5 ng/ml. 4. Human transferrin + insulin + selenium (same concentration as above). 5. Bovine serum albumin 40 mg/ml (BSA). 6. Bovine serum albumin + human transferrin + insulin + selenium (same concentration as above).

After having found lentoids in explants cultivated with transferrin a special series of experiments was carried out using three forms of transferrin. In these experiments only the development of lentoids was assessed in histological sections. 7. a) transferrin 98%; substantially iron-free (60 µg/ml). b) transferrin iron-saturated and ulteriorly depleted of iron surplus with magnesium carbonate. c) transferrin iron-saturated without any ulterior depletion.

Two small additional series of experiments were carried out with smaller concentrations of BSA (4 mg/ml, 66 µg/ml).

Although several tissues differentiated in various amounts in particular experimental series (cartilage, myotubes, stratified squamous or columnar epithelium), we restricted our analysis to the differentiation of lentoids and the developmentally related nervous tissue (neuroblasts) as the most prominent finding.

### *Neuroblasts*

We denote as neuroblasts a diffuse mass of immature nerve tissue which showed no cell stratification, cell type diversification or axonal outgrowth. Only exceptionally very large neurons with abundant basophilic cytoplasm (reminiscent of the neural crest-derived spinal ganglionic neurons) were observed. Groups of undifferentiated neural retina cells, which are common in teratomas derived from early embryos grafted to ectopic sites (Švajger *et al.*, 1987), were not present in our cultures.

TABLE 1

**DIFFERENTIATION OF NEUROBLASTS AND LENTOIDS IN VARIOUS SERUM-FREE MEDIA IN COMPARISON WITH TWO PARALLEL CONTROL SERIES**

| Series | No. of explants | Survival(%) | Neuroblasts(%)* | Lentoids(%)* |
|---|---|---|---|---|
| 1. | | | | |
| Tr | 41 | 85.4 | 80a | 72.8 |
| RS | 36 | 100 | 77.8 | 0 |
| MEM | 37 | 81.1 | 13.3a | 3.3 |
| 2. | | | | |
| Tr+In | 21 | 61.9 | 61.5b | 30.8 |
| RS | 20 | 95 | 84.2 | 5.3 |
| MEM | 20 | 85 | 3b | 0 |
| 3. | | | | |
| Tr+Se | 10 | 100 | 50 | 20 |
| RS | 13 | 92.3 | 75 | 0 |
| MEM | 13 | 61.5 | 0 | 0 |
| 4. | | | | |
| Tr+In+Se | 28 | 100 | 78.6c | 53.6d |
| RS | 28 | 92.8 | 73.1 | 3.8d |
| MEM | 28 | 75 | 4.8c | 0 |
| 5. | | | | |
| BSA | 29 | 96.5 | 57.1 | 0 |
| RS | 28 | 96.4 | 66.7 | 0 |
| MEM | 28 | 17.8 | 0 | 0 |
| 6. | | | | |
| Tr+In+Se+BSA | 17 | 94.1 | 56.2 | 6.2 |
| RS | 17 | 94.1 | 75 | 6.2 |
| MEM | 17 | 82.3 | 0 | 0 |
| 1+2+3+4 | 100 | 86 | | 33.7ef |
| RS | 97 | 95.9 | | 2.1e |
| MEM | 98 | 77.5 | | 1f |

*Differentiation of tissues was expressed as a percentage of tissue frequency in viable explants

a, c, d, e, f= $\chi^2$, $P < 0,01$

b= Fisher's test, $P < 0,02$

Tr= transferrin iron-saturated (50 µg/ml), In= insulin (10 µg/ml), Se= selenium (5 ng/ml), BSA= bovine serum albumin (40 mg/ml), RS= Eagle's MEM + 50% rat serum, MEM= Eagle's MEM with Hank's balanced salt solution

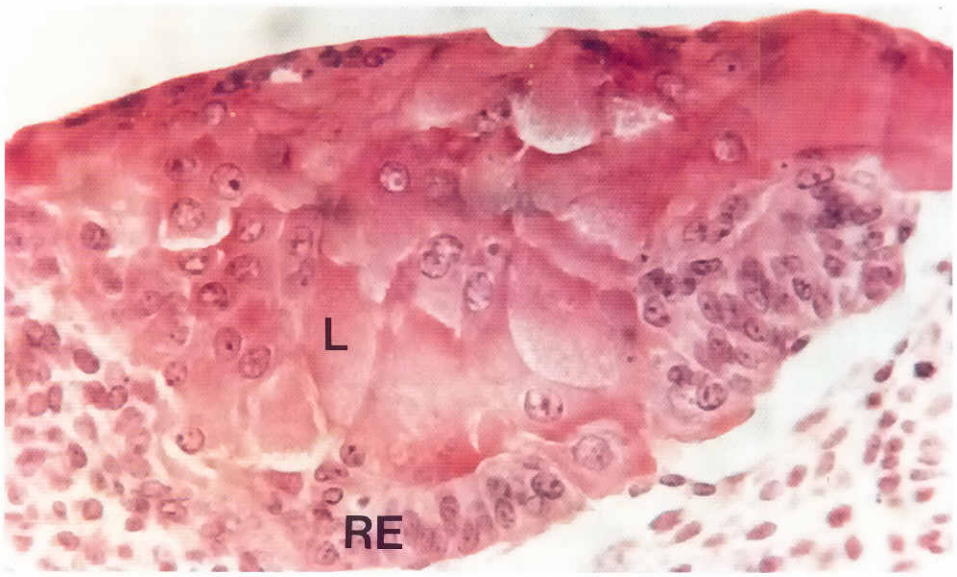


**Fig. 2. Lentoid found in an explant cultivated in Eagle's MEM with transferrin** *(50 μg/ml. RE, retinal epithelium; L, lentoid, magnification= x472). RE, retinal epithelium; L, lentoid.*

Neuroblasts were almost absent in the serum-free medium. Out of 95 explants we found only 8 explants containing neuroblasts (8.4%). All combinations of used factors had the same effect as the whole serum: the neural tissue was easily identifiable in more than 60% of explants.

If the concentration of BSA was only 4 mg/ml, the percentage of neuroblasts was different from that in serum-supplemented medium (out of 23 explants only 7, or 30.4%, contained neuroblasts, $P < 0,01$).

***Lentoids***

An especially interesting feature in our explants was the appearance of lentoids. These appeared as masses of variously shaped, very large cells with typical cytoplasmic characteristics of ocular lens fibers. In all cases observed these cells were in continuity with a cuboidal epithelium characterized by pale cytoplasm (Fig. 2), identified as retinal epithelium in ectopic grafts of rat egg cylinders (Švajger *et al.*, 1987). The frequency of lentoid formation in explants showed a clear-cut dependence on the presence of transferrin in medium. While lentoids developed in only 2 explants in the serum-supplemented medium (1.4%) and in only one explant in the serum-free medium, they were much more common in cultures supplemented with transferrin (Table 1, series 1-4). The difference was not always statistically significant because of a relatively small number of explants in each series. However, when the data from the first four series were taken together, the difference between explants in transferrin-enriched medium and other media was statistically significant. Other additives used together with transferrin showed no impact on lentoid differentiation (data not shown). Moreover the frequency of lentoid formation was high in all three sets of explants in the seventh series, i.e. regardless of the form in which transferrin was added to the culture medium (115 explants, 54 survived, 18 lentoids = 33%).

Summarizing all the data, a total of 47 lentoids were obtained in cultures with transferrin, giving a yield of 33%. In contrast the incidence of lentoids was very low when transferrin was combined with the BSA and was comparable to the results obtained in the whole rat serum (Table 1, series 6).

To check the influence of a low concentration of BSA, identical to that of transferrin, a small additional series of experiments was carried out. Out of 17 explants treated with BSA (66 μg/ml), none contained either neuroblasts or lentoids, whereas from only 8 explants cultured in medium with transferrin (66 μg/ml) 2 explants with neuroblasts and 2 lentoids were obtained.

## Discussion

Our main goal was to study the effect of transferrin on the development of rat embryonic shields in a modified organ culture. For this reason we cultivated five series in a chemically-defined medium and treated them with transferrin alone or in combination with other factors known to promote proliferation and/or differentiation. To compare the action of transferrin with another protein we treated some series with BSA.

Our results related to the growth process correlate with previously published data. Transferrin seems to be indispensable for proliferation of various cell and organ cultures (Barnes and Sato, 1980; Mescher and Munaim, 1988; Partanen and Thesleff, 1989).

Moreover, our results confirm the findings of *in vitro* cultures of postimplantation rat embryos (Pratten *et al.*, 1988). In the latter study the explanted 9.5-day embryos were cultured in homologous sera in glass bottles in a roller incubator. After cultivation for 50 hours the serum was frozen and later on sterilized and reused for culture of further embryos. The growth of embryos in the recycled serum was greatly retarded. Only whole rat serum was able to restore completely the exhausted and reused rat serum. The addition of a single growth-promoting factor (EGF, insulin or transferrin) did not restore the growth of rat embryos to the level observed when whole rat serum was used. Although our techniques differ from the above-mentioned culture method, the results were the same: transferrin was not able to replace the whole serum as far as growth is concerned.

The effects of transferrin on the histological differentiation of embryonic explants deserves special attention because there is

very little data in the literature on this field of transferrin action.

The immature neural tissue is a common constituent of both ectopic grafts and *in vitro* explants of early postimplantation rat embryos (Škreb and Švajger, 1973; Švajger *et al.*, 1981; Švajger *et al.*, 1987). The neuroepithelium and not the surface ectoderm has been shown to be a source of atypical lens cells (lentoids) in ectopic grafts (Švajger *et al.*, 1987). This is the reason for considering neural tissue differentiation in explants in which lentoids appear in a regular dependence on the composition of the medium. In ectopic grafts the immature neural retina appears as a particular population of densely packed cells with the tendency to form rosettes. However, this feature cannot be observed in *in vitro* explants. The same holds true for any other distinctive traits of cells of neuroectodermal origin, which we therefore denote as neuroblasts in this communication.

In explants grown in the serum-free medium the neuroblasts were present at low frequency or even absent. Transferrin restored the percentage of neuroblasts close to the level obtained in the serum-supplemented medium. This effect cannot be considered as specific because BSA showed a similar effect. Although the concentrations of these two proteins in the medium were different, the results were approximately the same. In contrast, if the concentration of BSA in the medium becomes lower neuroblasts appear rarely or not at all. There are some other data confirming the need of transferrin for neural cell proliferation and differentiation in cell culture (Bottenstein and Sato, 1979; Darmon *et al.*, 1981; Aizenman *et al.*, 1986; Weiss *et al.*, 1986).

However, some data are partly at variance with our results with respect to the action of the whole serum. In Le Douarin's laboratory it was found that in the medium containing serum, quail and mouse neural crest cells did not exhibit neuronal phenotype (Ziller *et al.*, 1987; Boisseau and Simonneau, 1989). Only in their own defined cell culture medium containing BSA, transferrin and certain hormones, were numerous neurons visible. Their results, however, confirm our own results concerning the effect of transferrin and BSA on neuroblast differentiation.

The formation of lentoids and the significant dependence of their appearance on the presence of transferrin in the defined culture medium deserves special attention. Here we are dealing with the differentiation of lens cells from an atypical embryonic source, i.e. from the retinal epithelium (an outgrowth of the proencephalic neuroepithelium *in situ*) rather than from the surface ectoderm as their natural tissue of origin during normal embryogenesis *in situ*. Since the early discovery of the capacity of the retinal epithelium of the iris in adult salamanders to regenerate the whole functional lens (Wolffian lens regeneration), this peculiar feature of "transdifferentiation" has attracted the attention of many investigators (see Reyer, 1954 and Yamada, 1977, for review). More recently cells giving a positive reaction with antibodies to lens-specific proteins were found in spreading cultures of chick embryonic cells of various origins (Yamada, 1977; Okada, 1983, 1986; Eguchi, 1986).

True lentoids or lens-like bodies (pleiomorphic aggregates of cells characterized by their giant size and the abundant, diffusely fine granular eosinophilic cytoplasm in which no other structural details can be visualized by light microscopy) were observed by Moscona in aggregates of dissociated chick embryo retinal cells and believed to originate from Muellerian glial cells (Moscona, 1957; Moscona and Degenstein, 1981, 1982) and in cultures of dissociated cells of quail embryonic pineal bodies (Watanabe *et al.*, 1985). The first finding of lentoids arising from mammalian tissues was that of Yasuda *et al.* (1978). Later on it became evident that lentoids appear in ectopic grafts (experimental embryonic teratomas) of early postimplantation mouse and rat embryos (Diwan and Stevens, 1976; Bennett *et al.*, 1977; Švajger *et al.*, 1981, 1987) and in renal grafts of lentectomized rat fetal eyes (Jurić-Lekić and Švajger, 1989).

In all the numerous experiments (including those with Wolffian lens regeneration in adult salamanders) it was not possible to draw any clear cut conclusion on the factor(s) which trigger the atypical transformation of retinal cells into lentoids. On the basis of experiments with ectopic transfer of the rat embryonic retina one can only presume in a most general sense that in experimental conditions that include the absence of a competent surface ectoderm the epithelial cells of retina and some other cells of diencephalic neuroectodermal origin can assume (or reassume a phylogenetically extinct) potency to differentiate into atypical lens cells. The present results with the *in vitro* culture of rat embryonic shields differ from those obtained in ectopic grafts (Švajger *et al.*, 1987) in: 1) the absence of neural retinal cells and presence of only epithelial retinal cells in explants, and 2) the high incidence of lentoid formation when transferrin is added to the chemically defined medium (33% in comparison with 5.3% in ectopic grafts). The true nature of the triggering action of transferrin, however, remains obscure. The same holds true for the probable inhibiting influence of proteins contained in the serum (BSA in rat serum). It is also not clear whether the adverse action of BSA is due to the properties to the albumen itself or to possible contaminants of serum origin.

## Materials and Methods

Female rats of the 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 (RS). 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 (Škreb and Švajger, 1973; Škreb *et al.*, 1983; Škreb and Bulić, 1987). Human transferrin, bovine serum albumin (BSA) and the selenium salt were purchased from Sigma. Insulin was a gift from Pliva, Zagreb. The concentration of applied factors was similar to that used in other model systems of culture *in vitro* and BSA was added in a concentration similar to that circulating in the blood serum.

Each series of experiments consisted of three sets of explants: one cultivated in Eagle's MEM alone, another in Eagle's MEM supplemented with rat serum and finally in Eagle's MEM supplemented with one or more of the additional factors mentioned above.

To monitor growth, dimensions of explants were measured on various days of culture using an eyepiece micrometer. We have previously shown that DNA, RNA, protein concentrations and wet weight measurements are superimposable on curves of diameter measurements (Škreb and Crnek, 1980). After two weeks, the explants were fixed in Zenker's fluid, washed in tap water and processed for routine histology (haematoxylin + eosin). Uninterrupted serial sections were made and checked for the presence of various tissues. Data were statistically evaluated using chi-square analysis or Fisher's exact probability test.

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