Neural growth factor inhibits the growth of rat egg-cylinders cultured *in vitro*

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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. Differentiation of the epidermis and cartilage was comparable to that in fully serum-supplemented medium, whereas neuroblasts were very scarce. In explants treated either with bovine serum albumin or transferrin, neuroblasts were observed, whereas the addition of NGF did not improve neuroblast differentiation. On the contrary, NGF impaired growth and tissue differentiation when compared with explants grown in serum-free medium.

KEY WORDS: *rat embryo, organ culture, NGF, growth, neuroblast differentiation*

Introduction

Mammalian embryonic ectoderm of the preprimitive streak stage after isolation and transfer to ectopic sites gives rise to tissue derivates of all three definitive germ layers: neural tissue, skin, «mesodermal tissues» and derivates of the primitive gut (Svajger *et al.*, 1986). Shortly after gastrulation and before neurons have differentiated, the cells of the neural ectoderm become committed to form different parts of the nervous system. In the developing mammalian nervous system substantially more neurons are generated than survive to maturity (Davies, 1988). The proposal that developing neurons compete for a supply of neurotrophic factor present in their target field in limiting amounts has received considerable support from work on nerve growth factor (NGF). This well-characterized protein promotes the survival of sensory and sympathetic neurons in culture (Levi-Montalcini, 1987).

Moreover, it was found that the innervating neurons possess specific cell surface receptors which mediate the uptake of NGF in the target field (Johnson *et al.*, 1986; Schatteman *et al.*, 1988). Two sorts of receptors have been analyzed: one with low affinity and the other with high affinity for NGF. Recently, receptors for NGF have been discovered even on nonneural cells such as thymocytes (Levi-Montalcini, 1987), Sertoli cells (Persson *et al.*, 1990) and during the development of teeth (Byers *et al.*, 1990). During embryonic chick development the receptors were found on the neural tube and the myotomes as well (Hallböök *et al.*, 1990; Heuer *et al.*, 1990). Besides these various cells, even the neurons of the central nervous system (Davies, 1988), cholinergic magnocellular neurons of the basal forebrain, respond to exogenous NGF by producing increased levels of enzyme choline acetyl transferase (Korsching, 1986). Therefore, the target of NGF action seems to be the neural-crest derivates, some CNS neurons and some cells of non-neuronal origin.

In *in vitro* culture it was discovered that NGF had both mitogenic and antimitogenic activity (Burnstein and Greene, 1982), and that it could stimulate differentiation of some cloned cells such as neuroblastoma (Reynolds and Perez-Polo, 1989) and pheochromocytoma cells (Burnstein and Greene, 1982). Moreover, nerve growth factor was recently investigated as a possible therapy for Alzheimer's disease (Marx, 1990).

Such findings also indicated that this growth factor has a wide range of activity.

In our laboratory we have developed a modified organ culture of rodent embryonic isolated and explanted at the primitive streak stage with only three germ layers. This technique has been shown to provide favorable conditions for the differentiation of the main tissues in teratoma-like explants after two weeks of culture (Skreb and Svajger, 1973). In serum-supplemented medium we obtained many well-differentiated tissues including the neuroblasts, whereas in a serum and protein-free medium only some rare tissues, but very few if any neuroblasts (Skreb and Bulic, 1987). The question arose, which factor or factors in the serum are responsible for neuroblast differentiation. The fact that the serum is a complex and largely undefined mixture of possible controlling factors confounds any precise analysis of this kind. Thus the original question of which serum ingredient is responsible for neuroblast differentiation can be re-posed as: which factor must be added to serum-free medium to obtain the same effect as with the whole serum.

Abbreviations used in this paper: NGF, nerve growth factor; MEM, Eagle's minimal essential medium with Hank's balanced salt solution.

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TABLE 1

TISSUE DIFFERENTIATION IN SERUM-FREE MEDIUM WITH OR WITHOUT THE ADDITION OF 100 ng/ml NGF

	EAGLE'S MEM	MEM with 100ng/ml NGF
analyzed explants	25	23
epidermis	24 / 96% / a /	12 / 52% / a /
keratin	24 / 96% /	4 / 17%
neuroblasts	2 / 8% /	0
cartilage	13 / 52% /	1 / 4%

a/X^2= 10, P<0.01

The first factor to be tested was chosen on the basis of findings concerning the role of NGF, which was found to perform many functions in neural development.

Here we report our results obtained with rat egg cylinder cultivated in chemically-defined or in serum-supplemented medium after addition of the nerve growth factor. Our data were surprising in that other proteins used stimulated neuroblast differentiation, whereas NGF did not. On the contrary, it inhibited the growth of explants.

Results

Sequence of appearance of neuroblasts in vitro

Before experiments were carried out using the nerve growth factor, it was necessary to find out whether the lack of neuroblasts in explants cultivated in serum-free medium was due to the death of neuroblasts during the culture period or whether the neuroblasts were not differentiated at all. Therefore we carried out an experiment that enables us to analyze the sequence of events leading to the terminally differentiated tissues in explants. We made serial sections of explants every day during the 2-week culture period and did not find any neuroblasts in serum-free medium, whereas in serum-supplemented medium the first neuroblasts could be identified on the fifth day of culture. We cannot exclude the presence of neuroectoderm when the embryo was explanted at the stage with discernible germ layers. Nevertheless, morphologically, we could not identify any neuroblasts during days 1-11 of culture (36 explants). We found a different variety of immature epithelia and mesenchyme, and at the end of the culture period there were many well-differentiated epithelia with mesenchymal masses within them. Although necrosis was more often observed in serum-free medium than in medium with serum, the surviving cells seemed to be normal.

We can conclude that a putative action of NGF in serum-free medium may be on the germ layers and on various immature epithelia. In serum-supplemented medium NGF could possibly influence the already formed neuroblasts.

Tissue differentiation after the addition of NGF

Further experiments were carried out by adding NGF to the serum-free medium. To compare the action of NGF with other known proteins we added to the chemically-defined medium, transferrin (5µg/ml) or bovine serum albumin (40 mg/ml) as had previously been done in our laboratory (Bulic-Jakus *et al.*, 1990). In both repeated experiments the neuroblasts were as frequent as in serum-supplemented medium (data not shown). In our third experiment we added NGF to the chemically-defined medium and no neuroblasts were identified. Moreover, as far as cell differentiation is concerned, the frequency of differentiated tissues was very low in treated explants (Table 1).

Statistically, there was a great difference between the control and treated series concerning the epidermis. As far as other tissues are concerned the differences were even greater so that no statistical evaluation was necessary.

The growth of explants seems to be impaired in the treated series and will be analyzed in detail together with other data concerning the growth of explants.

We observed a difference in the number of analyzed explants between those which underwent histological analyses and those whose diameters were measured. This is due to the fact that the last diameter measurement was performed before the end of the usual culture period. Thus, some explants were partly necrotic at the end of the 2-week period and were lost during the histological procedure.

To determine whether NGF can affect the growth and differentiation in medium with serum we carried out three sets of experiments:

1. NGF was added to the liquid medium during the second week of culture in a concentration of 100 ng/ml.
2. The same dose of NGFc was applied during the whole period of the 2-week culture.
3. 200 ng/ml of NGFc was added during the whole period.

The frequency of tissues found in explants was the same in the controls and treated series of explants. Table 2 presents the results of the first set of experiments as an example, since other experiments did not differ from the first one.

Summarizing we can say that all tissues seem to be differentiated in the same manner in the treated and in the control series, regardless of the time and the concentration of the added NGF.

Growth of explants after the addition of NGF

Since the growth of explants seems to be impaired in all the examined series the results are presented together. Two sets of experiments were carried out in serum-free and three sets in serum-supplemented medium and despite the various media the results are quite similar.

Because the data are almost identical we present only part of the representative results in two Tables (3 and 4). However, the

TABLE 2

TISSUE DIFFERENTIATION IN SERUM-SUPPLEMENTED MEDIUM WITH OR WITHOUT 100 ng/ml NGF (FIRST SET)

	MEM+SERUM	MEM+SERUM+100ng/ml NGF
analyzed explants	21	20
epidermis	19	20
keratin	10	11
columnar epithelium	16	16
neuroblasts	18	17
cartilage	17	16
myotubes	13	10

TABLE 3

DIAMETER MEASUREMENTS OF EXPLANTS CULTURED IN SERUM-FREE MEDIUM

	MAJOR AXES					MINOR AXES				
	MEM		NGF 100 ng/ml[1]			MEM		NGF 100 ng/ml		
No. of explants	19		19			19		19		
day	mean	sd	mean	sd	dif P	mean	sd	mean	sd	dif P
2	1206[a]	122	1168[a]	133	38	1059	118	1033	107	26
5	1435	188	1288	186	147<	1267	138	1118	149	149<
7	1323	170	1258	182	65	1137	163	1106	181	31
9	1218	194	1240	120	+22	1131	189	1143	94	+12

1/ NGF added during the whole culture period
day= day of culture when the measurements were made
sd= standard deviation
dif= difference between means
P<=P is less than 0.05 (at least)
a/ control and treated explants measured on the same day of the culture period are called a group
Length of major and minor axes in μm

characteristics of all sets of experiments carried out either in serum-free or in serum-supplemented medium are also mentioned.

In both serum-free sets (out of 34 control and 38 treated explants in 18 groups, only 19 control and 19 treated explants in 8 groups are shown in Table 3), the majority of lengths of major and minor axes of treated explants were smaller than in the controls, measured on various days during the culture period. A group consists of the measurements of one of the axes of all control and treated explants carried out on a particular day of the culture period. Consequently, the two mathematical means of a group can be compared (Table 3). In three groups the difference is statistically significant. Table 3 presents the results of only two of these groups.

The results obtained on the explants in the serum-supplemented medium were essentially the same, but nevertheless slightly different (Table 4). There were 72 control and 70 treated explants in 20 groups. As mentioned previously the table shows only some representative results (20 controls and 20 treated groups) because all results are almost identical. First, in the majority of cases the lengths of major and minor axes of explants are smaller in the treated series than in control ones, exactly as in previous cultures in serum-free medium. These differences are in 7 groups statistically significant. Second, we observed in 6 groups that the control explants are smaller than in the experimental one. Since the differences are not statistically significant, they can be disregarded. Table 4 shows only 3 groups where the difference is statistically significant. Other data are not shown because they are almost identical.

Discussion

The purpose of our investigation was to determine whether the addition of NGF to serum-free medium would stimulate neuroblast differentiation in rat egg-cylinders cultured *in vitro*. Without serum neuroblasts were usually rare in our model system (Skreb and Bulic, 1987). As previously shown, transferrin and bovine serum albumin were able to restore necessary conditions for neuroblast differentiation, which was confirmed by our repeated experiment (Bulic-Jakus *et al.*, 1990). In contrast, NGF does not stimulate the appearance of neuroblasts. Moreover, it inhibits the growth and impairs the already poor differentiation of other tissues in serum-free medium. The question now arises whether the absence of growth is what has impaired neuroblast differentiation or whether the neural factor does not provide the necessary stimulus for differentiation. Previously we showed in this organ culture system that growth could be dissociated from differentiation. Certain conditions of our culture system ensure good survival and growth, but only poor differentiation (Skreb and Crnek, 1980). On the other hand we found that in serum-free medium epidermis and cartilage appeared as frequently as in serum-supplemented medium although no growth was observed (Skreb and Bulic, 1987). Thus, the absence of growth does not always prevent differentiation and sometimes a good growth does not necessarily promote differentiation. Consequently, the problem in question cannot be resolved on the basis of our present data and therefore requires further study.

It has to be stressed that our analysis did not involve other markers of neural differentiation apart from tissue morphology. Therefore, the only conclusion that we can draw here is the fact that the morphologically identifiable group of neuroblasts does not appear after addition of NGF in serum-free medium.

As far as growth is concerned in both cultures, either in serum-free or in serum-supplemented medium, NGF impairs growth of explants if compared with controls. To monitor growth, the dimensions of explants were measured on various days of the culture period using an eyepiece micrometer. During the measurements, the length of major and minor axes of explants were taken into account because the flat explants have an oval surface. Although this method seems to be rough, previously we were able to show that the DNA, RNA and protein concentrations and wet weight measurements are superimposable on curves of diameter meas-

TABLE 4

DIAMETER MEASUREMENTS OF EXPLANTS CULTURED IN SERUM SUPPLEMENTED MEDIUM

	MAJOR AXES					MINOR AXES				
	Serum+MEM		NGF 100ng/ml[2]			Serum+MEM		NGF 100ng/ml		
No. of explants	20		20			20		20		
day	mean	sd	mean	sd	dif P	mean	sd	mean	sd	dif P
6	2314[a]	229	2243[a]	403	71	1926	204	1919	285	7
8	2316	270	2088	274	228<	1827	209	1751	171	76
11	2053	181	1889	300	164<	1726	254	1534	204	192<

2/ NGF added only the second week of culture
day= day of culture when the measurements were made
sd= standard deviation
dif= difference between means
P<=P is less than 0.05 (at least)
a/ control and treated explants measured on the same day of the culture period are called a group
Length of major and minor axes in μm

urements (Skreb and Crnek, 1980). All the curves showed an increase in growth of explants in the first week of culture, whereas in the second week we observed a decrease in volume. To analyze the values in our Table, we should first assess the difference between the experimental and control explants measured on a particular day of the culture period. All together we can analyze 38 groups of control and treated explants and see that in the majority of cases NGF seems to impair growth as the lengths of both axes are smaller in medium with NGF than in controls. In ten groups (treated and control explants measured the same day of the culture period) the differences were statistically significant. However, in some groups the control explants were smaller, but the difference was never statistically significant. Moreover, this apparently better growth in NGF appeared mostly in medium with serum, when the growth of explants is much better than in serum-free medium and where many ingredients of serum can interfere with NGF action.

From the known variety of activities of NGF mentioned in the Introduction there are few data which point to the mitogenic or antimitogenic action of NGF, which depends in the majority of cases on the type of cell involved. Consequently, any data in connection with the role of NGF in proliferation would be interesting, especially if embryonic cells are used.

A mitogenic effect of NGF was studied using adrenal chromaffin cells from young rats, which are endocrine cells derived from neural crest and are closely related to sympathetic neurons. When these cells were cultured for 6 days in a liquid medium with 100 ng/ml NGF, a mitotic response could be seen (Lillien and Claude, 1985).

In another experiment, a noradrenergic clonal line of rat adrenal pheochromocytoma cells (PC 12) was established and after 1 week of exposure to NGF, cells ceased to multiply and began to expand, branching varicose processes similar to those produced by sympathetic neurons (Greene and Tischler, 1976).

If two variant sublines from clonal PC 12 rat pheochromocytoma cells were obtained, the results were slightly different (Burnstein and Greene, 1982). The original PC 12 cells ceased to divide after addition of NGF to the culture medium either with 1% or 15% serum. U 7 and U 2 variants of the same clonal line PC 12 reacted differently. The addition of NGF stimulated proliferation in both medium with low or high serum concentrations. The dose-response relationship between NGF concentration and cell proliferation is very interesting. The mitogenic effect of NGF was detectable at concentrations as low as 0.1 ng/ml, and was maximal at about 3 ng/ml. The higher doses up to 1000 ng/ml do not change the mitogenic effect, which was similar to that obtained after only 3 ng/ml. There was no reversal of NGF action. Since we used relatively high doses of NGF, the above data support the idea that the concentrations in our experiments were probably not noxious in themselves. It seems that higher doses do not change the original specific action of NGF even if much smaller doses can have the same effect.

The neuroblastoma cells clone C 1300 were also used in studying NGF action and almost the same results were obtained. Two variants of the mentioned clone NB 1R and NB 6R reacted rather differently to the NGF. Cultured NB 1R cells are stimulated to proliferate, whereas NB 6R cells are stimulated to cell differentiation (Revoltella and Butler, 1980).

Furthermore, NGF can inhibit the synthesis of RNA and proliferation melanocytes cultured *in vitro* (Rakowicz-Szulczynska *et al.*, 1988), whereas the lymphocytes from rat spleen can be stimulated in the uptake of thymidine (Thorpe and Perez-Polo, 1987). The culture of cells from a carcinoma of the human thyroid gland has shown that NGF can stimulate DNA synthesis (Goretzki *et al.*, 1987).

All the above-mentioned data were obtained on cells in culture, so that our model system, when whole rat egg-cylinders were cultivated and treated with NGF, is rather different from all experiments carried out in culture *in vitro* so far. Consequently, this fact increases the value of our results and confirms the general idea that NGF can act on cell proliferation not only on isolated cells, but also on embryos in culture.

Our efforts to observe a qualitative improvement of neuroblast differentiation after NGF application was always negative. On the other hand, the impairment of growth was easily observed not only after diameter measurements, but even upon histological analysis as well.

Materials and Methods

Female rats of the inbred Fischer strain were killed after 9 days of pregnancy and the entire egg cylinder was isolated. After the removal of Reichert's membrane and the ectoplacental cone, the egg cylinder was cut out transversely at the level of the amnion. The extraembryonic part was discarded, and the isolated embryonic shields were placed on a small piece of lens paper supported by a stainless steel grid at the center of an organ tissue culture dish (Falcon No. 3037). A sufficient amount of liquid medium was poured under the grid to reach and wet the lens paper. Eagle's minimal essential medium with Hank's balanced salt solution (MEM) 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. The culture dishes were placed in an incubator with 5% CO_2 and 95% air at 37°C. Antibiotics were added in a standard concentration and the medium was changed every second day. Explants were maintained *in vitro* for 14 days.

Details of this modified organ culture technique have been published elsewhere (Skreb and Svajger, 1973; Skreb and Bulic, 1987). Human transferrin, bovine serum albumin and neural growth factor were purchased from Sigma. Concentrations used were: human transferrin 5 µg/ml, bovine serum albumin 40 mg/ml, and neural growth factor (NGF) 100 and 200 ng/ml.

To monitor growth, dimensions of explants were measured on various days during the culture period using an eyepiece micrometer. The major and minor axes of an explant were measured. For practical purposes the control and treated explants were measured the same day of the culture period to form a group, so that comparison between the two arithmetical means of one of the axes could be assessed.

We have previously shown that DNA, RNA, protein concentrations and wet weight measurements are superimposable on curves of diameter measurements (Skreb and Crnek, 1980). 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 and Student's t test.

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