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MAMMALIAN EMBRYO IN ORGAN CULTURE *IN VITRO* AS A BASIC MODEL FOR PRECLINICAL STUDIES IN HUMAN NEUROGENESIS

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SUMMARY – Modified organ culture of mammalian postimplantation embryo developed in our laboratory provides favorable conditions for terminal differentiation of the main tissues of gastrulating embryo, including nerve tissue. Intracellular signals provided by growth and neurotrophic factors play a crucial role during neurogenesis. In the present study, the role of retinoic acid (RA), fibroblast growth factor (FGF) and nerve growth factor (NGF) in neural tissue differentiation was investigated in cultivated 9.5-day-old rat embryo in the critical period of mammalian development, gastrulation. The proper embryonic part of the whole embryo without extraembryonic membranes was cultivated in air-liquid interface for two weeks. To study the effect of neural tissue-inducing agents, various concentrations of RA, FGF, NGF and FGF/NGF combinations were added to the basic culture medium within the time frame of 9 days. The embryos developed into teratoma-like structures, then they were fixed and histologically examined for the presence of neural tissue. RA and NGF did not improve neural tissue differentiation. However, its proportion was significantly higher in FGF-treated embryos. FGF is less effective in the presence of NGF at the very beginning of neural tissue differentiation. It seems that NGF hinders the stimulating effect of FGF. In our experimental model, neural tissue differentiation is probably initially regulated by FGF signal, although there is evidence that RA and NGF signals are also needed later in development. Our model system has the advantage of being simpler than the embryo *in vivo* but closer to normal development than cell cultures. Therefore, our results are in accordance with the idea that FGF is a neural inducer in the vertebrates.

Key words: *Nervous system – embryology; Animal; Nerve growth factor; Fibroblast growth factors*

Introduction

In the vertebrate embryo, the normal process of neural tissue differentiation proceeds in two successive steps: determination of two domains in the presumptive ectoderm, a ventral domain fated to become epidermis, and a dorsal domain from which primordium of the nervous system will develop. In the second step through morphogenetic transformations the ectoderm is committed a neural fate (neural epithelium) in which neuronal and glial differentiation will take place. Briefly, the development of the vertebrate nervous system begins with the inductive differentiation of the neural plate from the dorsal side of ectoderm at the end of gastrulation. Many studies use *in vitro* cultures and ectopic transplantation of postimplantation mammalian embryo or its fragments to elucidate the mechanisms of neural tissue differentiation[1-3]. Such investigations provide deeper understanding of several aspects in both normal and abnormal processes of neurulation in mammals, and those findings may be applied as prevention of the pathogenesis of human birth defect[4]. To achieve experimental conditions for mammalian development, we designed a unique *in vitro* culture model of postimplantation mammalian embryo[5]. In this technique, the proper embryonic part of a whole gastrulating embryo cultivated

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in vitro develops during 14 days into a teratoma built of intermixed tissues, including nerve tissue.

During the last decade, considerable progress has been made in elucidating the molecular basis of neural tissue differentiation[6-9]. The aim of our study was to test various putative molecular signals which may play a role in early neurogenesis in mammals: retinoic acid (RA), fibroblast growth factor (FGF) and neural growth factor (NGF). They are present in different parts and at various times during development of human and vertebrate embryos[9-16], and also in control genes *via* specific receptor proteins[13,14,17,18]. RA has very important functions *in vivo* in physiologic concentrations but is also a powerful teratogen for developing embryos in low or high doses, inducing a wide spectrum of malformations in several species including human[19,20]. FGF receptor mutations cause a variety of skeletal disorders in humans[13]. Studies in animal and human models suggest a role of NGF in human disorders[21-24].

Our experimental model deals with gastrulating mammalian embryo, which is the most critical period in mammalian development, when embryo is extremely sensitive to various endogenous and exogenous molecular signals. All these facts provide a good support to our decision to investigate the role of the mentioned molecular signals in early neurogenesis.

Material and Methods

The experiments were carried out in Fisher inbred rats aged 3 to 4 months. They were kept in conventional cages, fed standard laboratory diet and water *ad libitum*. Males were used as donors of sera for supplementation of culture medium.

Dissection technique used for isolation of postimplantation embryos

Fisher inbred strain rats were mated overnight. Gestation was considered to have begun early in the morning when sperm was found in vaginal smear, and it was designated day 0.5 of pregnancy[25]. Immediately upon sacrificing animals on gestation day 9, embryos in the early headfold stage (E9.5) were isolated from the uteri. The isolated uteri from pregnant females were manipulated in sterile Tyrode's saline. The decidua in which the embryo was embedded was peeled off with watchmaker's forceps. The entire egg-cylinder (embryonic shield + extraembryonic part + ectoplacental cone) was isolated using a dissecting microscope. After separation of the embryo from the uterus, the ectoplacental cone and Reichert's membrane were removed, the extraembryonic part was cut using two fine needles as knife and fork at the level of the amnion, and discarded. The remaining embryonic part was cultivated.

Modified embryo culture model for investigation of neural tissue differentiation in vitro

Cultivation of the embryonic parts of egg-cylinders was performed at the air-liquid interface. Three embryos were put on lens paper supported by a stainless-steel grid placed in the center of an organ culture dish (Falcon No. 3037, Becton Dickinson, Oxford, UK). A certain amount of culture medium was put under the grid to keep the lens paper wet. Eagle's minimal essential medium (MEM) with Hank's balanced salt solution (MEM from Gibco BRL) supplemented with 50% of rat serum was used as basal medium. Serum was taken from the same strain as the embryos. The blood from rat aortas was immediately centrifuged, and the serum was inactivated at 56 °C for 30 minutes and sterilized through a 0.22 mm Millipore filter[5,26].

All agents were purchased from Sigma Chemical Co., St. Louis, MO, USA. NGF or basic FGF or both agents together were added to basal culture medium at a concentration of 100 ng/mL or 200 ng/mL, and three different doses of RA (all-trans retinoic acid, type xx) of 10^{-8}M, 10^{-5}M and 10^{-4}M were used. Embryos were exposed to RA, NGF, FGF or FGF/NGF signals from day 5 through day 14 of culture.

Embryos were grown in an incubator in humid atmosphere containing 5% CO_2 and 95% air at 37 °C. The medium was changed every second day during the culture period. Embryos were maintained *in vitro* for 14 days.

Evaluation of neural tissue differentiation in culture in vitro

After two weeks, all developed teratoma-like explants were fixed in Zenker's solution, washed in tap water, and processed by routine histologic methods. Serial histologic sections (5 mm) stained by hemalaun and eosin (HE) were made and checked for the presence of neural tissue by light microscopy. The proportions of differentiated neural tissue were compared between the groups of explants. c^2-test or Fisher's exact test was used to compare the proportion of nerve tissue developed. The level of statistical significance was set at $p<0.05$.

Results

After 14 days of cultivation, the postimplantation embryos at early head-fold (E9.5) stage developed into teratomas in which differentiation of neural tissue was analyzed (Table 1).

Teratoma-like explants developed from cultivated gastrulating embryos contained tissue derivatives of all three germ layers. We found the same tissues like myotubes and smooth muscle, gut and gland epithelium, and the proportion of cartilage was high (26/36) (data not shown in Table 1). The basal medium supplemented with serum, used in our experiments, provides favorable conditions for terminal differentiation of all tissues observed. In this medium, the proportion of neural tissue was about 60% of all cultivated explants (control, i.e. untreated explants) (Table 1). These results confirmed our previously published results[27], and here we used them for comparison with new experiments.

Neural cells contained typical large euchromatic nuclei with striking dark nucleoli and cells with smaller nuclei, irregular nuclear envelope and relatively irregular shape (glia cells) (Fig. 1).

We attempted to stimulate differentiation of neural tissue by the addition of the neural tissue-inducing agents RA, FGF or NGF to culture medium. Approximately the same number of control embryos were cultivated in basic culture medium simultaneously with each experimental series to compare the possible changes in the pattern of neural tissue differentiation (Table 1, Fig. 2).

Our experiments can be divided into four main groups. The first group consisted of three series treated with three different doses of RA, 10^{-8}M, 10^{-5}M and 10^{-4}M, within the time frame of 9 days (Table 1). The results on neural tissue differentiation induction were always negative, i.e. in all series the differentiation of neural tissue was always similar to that recorded in the control group of embryos (Table 1). This is in agreement with our previous experi-

Table 1. Neural tissue differentiation in explants cultivated in vitro and treated with retinoic acid (RA), nerve growth factor (NGF), fibroblast growth factor (FGF), and FGF/NGF combination

Experiment	Treatment[a] agent exposure (days)	Time of analyzed	No. of explants neural tissue n (%)	Proportion of	p[c]
1	Control[b]	5-14	20	13 (0.65)	NS[d]
	RA 10^{-8}M		23	15 (0.65)	
2	Control	5-14	25	15 (0.60)	NS
	RA 10^{-5}M		22	12 (0.54)	
3	Control	5-14	25	16 (0.64)	NS
	RA 10^{-4}M		28	19 (0.67)	
4	Control	5-14	24	14 (0.58)	NS
	NGF 100		24	13 (0.54)	
5	Control	5-14	28	17 (0.60)	NS
	NGF 200		29	13 (0.45)	
6	Control	5-14	20	13 (0.65)	<0.05
	FGF 100		21	20 (0.95)	
7	Control	5-14	20	12 (0.60)	<0.01
	FGF 200		24	23 (0.95)	
8	Control	5-14	18	10 (0.55)	NS
	FGF 100 + NGF 100		14	10 (0.71)	
9	Control	5-7 + 7-14	19	11 (0.57)	NS
	FGF 100 + NGF 100		17	12 (0.70)	

[a]Explants treated with: RA 10^{-8}M, 10^{-5}M, 10^{-4}M; NGF 100 or 200 ng/mL; FGF 100 or 200 ng/mL; [b]untreated group grown in basic culture medium; [c]statistical difference from control (c^2-test); [d]non-significant

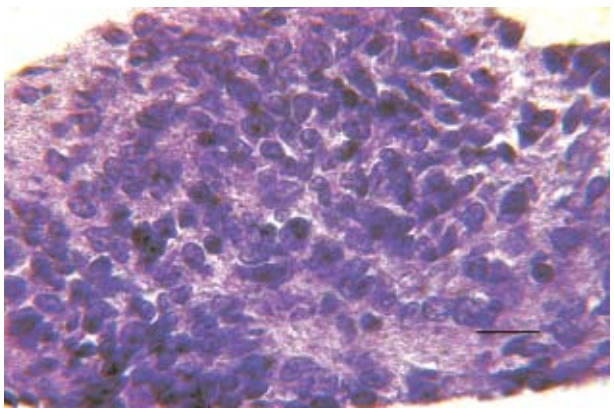

Fig. 1. Neural tissue developed in an explant treated with fibroblast growth factor (FGF) at a concentration of 200 ng/mL from day 5 through day 14 of culture; HE, scale=25 mm.

ments with 10^{-5}M RA in two medium types (medium supplemented with serum, and serum-free and protein-free medium)[28].

The second group consisted of two series treated with two different doses of NGF (100 and 200 ng/mL). This group may be envisaged as the basic experiment carried out in order to enable us to focus our further study on the findings relevant for analysis. The proportion of neural tissue in NGF-treated explants was approximately the same as in control untreated explants. Both NGF doses used failed to improve the rate of neural tissue differentiation (Table 1).

In the third group of experiments, embryos were treated with two different doses of FGF (100 and 200 ng/mL). Both of these doses were found to significantly improve the rate of neural tissue differentiation ($p<0.01$). Its proportion was highest in FGF-treated embryos, i.e. almost 100% (23/24) (Table 1). Comparison of the incidence of neural tissue differentiation among FGF-treated, RA-treated and NGF-treated explants showed it to be significantly higher in FGF-treated explants (at least at the level of $p<0.02$). In all cases, RA and NGF failed to stimulate neural tissue differentiation (Table 1, Fig. 2).

In the fourth group of experiments, we explored the ability of embryos to respond to combined FGF-NGF signals. Therefore, we simultaneously applied NGF and FGF at concentrations of 100 ng/mL from day 5 through day 14 of culture. In another experiment, FGF was applied from day 5 through day 7, whereafter the embryos were exposed to NGF until the end of the culture period (Table 1, Fig. 2). In both FGF/NGF combination experiments, the incidence of neural tissue differentiation was stimulated although the difference from the control group was not significant. There was no statistical signficance between FGF/NGF-treated and FGF-treated embryos either.

Discussion

In the present study, our attention was focused on the question whether differentiation-inducing agents were responsible for stimulation of neural tissue differentiation. Our technique of cultivation of gastrulating embryos confirmed that differentiation of neural tissue in the neurons and glia was possible in the conditions described. The embryos used in the study were built of three germ layers and had no differentiated tissues. Therefore, the added differentiation-inducing signals acted upon undifferentiated embryonic cells. The putative neural tissue inducing

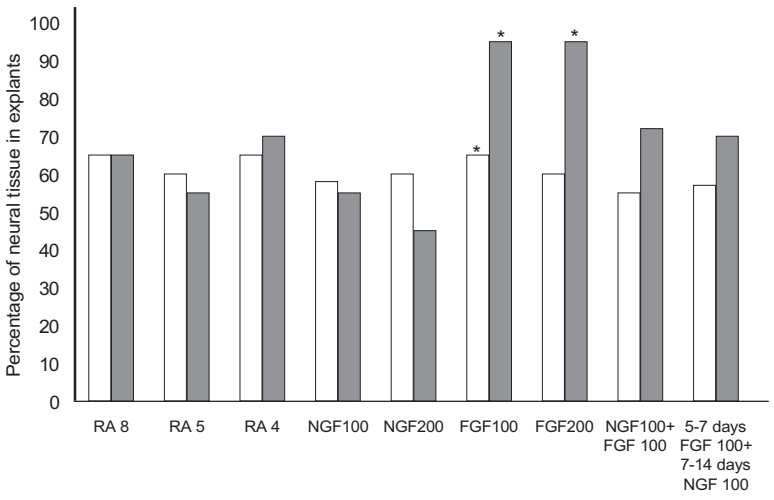

*Fig. 2. Effect of retinoic acid (RA), fibroblast growth factor (FGF), nerve growth factor (NGF), and FGF/NGF combination on neural tissue differentiation pattern in developing explants in vitro. Control groups of explants were untreated. RA 8=10^{-8}M RA; RA 5=10^{-5}M RA; RA 4=10^{-4}M RA; NGF 100=100 ng/mL NGF; NGF 200=200 ng/mL NGF; FGF 100=100 ng/mL FGF; FGF 200=200 ng/mL FGF; *significant difference from control groups at least at the level of $p<0.05$.*

agents were added on day 5 of culture, when overt differentiation began[27]. When we investigated the sequence of appearance of various tissues in the serum-supplemented medium, the first neuroblasts could be identified on day 5 of culture[27].

An important point in our analysis of neural tissue differentiation should be emphasized. In early development, inductive interactions between embryonic cells are very important, and this is impossible to achieve in isolated cells cultivated in cell cultures. Therefore, our experimental model provides better conditions than cell cultures, because it allows for interaction between cells in the whole embryo. It is important because the vertebrate neural development is initiated during gastrulation by the inductive action of dorsal mesoderm on the neighboring ectoderm.

Although experiments with RA in human[29-32] and rodent[33-35] cell cultures as well as those with whole cultured embryos[32,36] suggest that RA could induce neural differentiation, our experimental model could not confirm these findings.

Studies with NGF on human neuroblastoma cell cultures were performed to analyze NGF action as a model of differentiation of cholinergic neurons[36] and pheochromocytoma PC12 cells in which NGF can also induce neuronal differentiation[37,38], however, NGF could not induce neuronal differentiation in our model system. When we used NGF, it produced no effect on neural tissue differentiation. Obviously, RA and NGF did not produce the necessary stimulus for neural differentiation in gastrulating embryo.

FGF, previously studied as a mesoderm inducer[39], has more recently been proposed as a neural inducer[40,41]. Both aFGF (acidid) and bFGF (basic) induce neural markers from ectodermal cells dissociated at the gastrula stage[40]. The expression pattern of FGFs supports a possible role in neural induction or patterning[42]. Some studies found FGF in vertebrates to have a neural inducing activity at the appropriate time[43], and it seems that other signaling factors cooperate to produce complete pattern.

It is interesting that in our experimental model, the FGF/NGF combined action was less effective than FGF action alone (Table 1, Fig. 2), although there was evidence for cooperative action of FGF and NGF[9,44]. FGF is less effective in the presence of NGF at the very beginning of neural tissue differentiation (Table 1, Fig. 2). In our experiments we did not observe any kind of cooperative action of FGF and NGF. Rather, it seems that NGF hinders the stimulating effect of FGF, however, it requires additional studies.

In conclusion, in our embryo culture model none of the RA or NGF signals used stimulated neuronal differentiation. Neuronal differentiation is probably initially regulated by FGF signal in rat gastrulation embryo. It can be speculated that the other two signals were not applied at the appropriate time for their action. This approach to the study of differentiation of embryonic cells provides conditions for the investigation of various molecular signals that could direct or prevent neuronal differentiation, and resembles *in vivo* situation more closely than cell cultures.

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Sažetak

EMBRIJI SISAVACA U KULTURI ORGANA *IN VITRO* KAO TEMELJNI MODEL ZA PREDKLINIČKA ISTRAŽIVANJA NEUROGENEZE U LJUDI

V. Crnek-Kunstelj, J. Stipić i T. Zeljko

U našem laboratoriju je razvijena modificirana kultura organa za kultiviranje embrija sisavaca, koja osigurava najbolje uvjete za terminalnu diferencijaciju osnovnih tkiva zametka u stadiju gastrulacije, a između ostalog i živčanog tkiva. Unutarstanični signali, kao što su čimbenici rasta i neurotrofični čimbenici, imaju ključnu ulogu u procesu neurogeneze. Istraživali smo ulogu retinoične kiseline (RA), faktora rasta fibroblasta (FGF) i faktora rasta živaca (NGF) u diferencijaciji neuralnog tkiva u kultiviranim 9,5 dana starim embrijima sisavaca u kritičnom razdoblju razvitka sisavaca, gastrulaciji. *In vitro* su bili tijekom dva tjedna kultivirani samo embrionalni dijelovi zametaka, bez izvanembrionalnih membrana na granici zračne i tekuće faze. Kako bismo istražili učinak potencijalnih uzročnika koji induciraju neuralnu diferencijaciju, u temeljni medij za kultiviranje su tijekom 9 dana bile dodavane različite koncentracije RA, FGF, NGF i kombinacija FGF/NGF. Embriji su se razvili u teratome, tada su bili fiksirani i histološki ispitani na prisutnost neuralnog tkiva. RA i NGF nisu poboljšali diferencijaciju neuralnog tkiva. Međutim, zastupljenost neuralnog tkiva bila je značajno češća u embrijima tretiranim pomoću FGF. FGF je na samom početku neurogeneze manje učinkovit u prisutnosti NGF. Stoga se čini da NGF smanjuje stimulacijski učinak FGF. U našem eksperimentalnom modelu je diferencijacija neuralnog tkiva vjerojatno inicijalno regulirana signalom FGF, iako postoje podaci da su RA i NGF također, ali vjerojatno kasnije, potrebni za razvitak. Prednost našega eksperimentalnog modela je u tome što je to jednostavniji sustav nego embrij *in vivo*, ali ipak bliži normalnom embrionalnom razvitku od staničnih kultura. Prema tome, rezultati su u suglasju s idejom da je FGF induktor živčanog tkiva u kralješnjaka.

Ključne riječi: *Živčani sustav – embriologija; Životinja; Faktor rasta živaca; Faktori rasta fibroblasta*