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Three-dimensional cultures of dissociated myenteric plexus as a model for the development of the enteric nervous system

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Abstract

Failures in the development of the enteric nervous system (ENS) can lead to severe diseases, such as Hirschsprung`s disease or any other dysganglionosis. Trying to imitate the development of the ENS, the myenteric plexus, in vitro might give us a better understanding of the underlying problems and the chance to develop new diagnostic and even therapeutic approaches.

Myenteric plexus of newborn rats was isolated by enzymatical digestion, dissociated and cultivated either on glass coverslips, or in a three-dimensional gel of extracellular-matrix-components. Supplementing the culture media with the neurotrophins Glial-cell-line-derived-neurotrophic-factor (GDNF) or brain-derived-neurotrophic-factor (BDNF), alone or in combination, lead to differences in the growth behaviour of the individual culture.

GDNF increased the survival, as well as the neurite outgrowth of the neurons, while BDNF had no obvious effect when administered alone. Only in combination with GDNF it increased the branching patterns of the neurites, indicating a higher degree of differentiation. These phenomena were seen on glass, as well as in the gel cultures. The only difference was, that cultures grown in the gels formed secondary ganglia which were similar to what is seen in more severe cases of dysganglionosis, while there was no specific reaggregation on glass. The degree of "artificial hypoganglionosis" varied, depending on the factors which were supplemented to the media.

Cultivating dissociated myenteric plexus in a three-dimensional environment seem to be a valuable model to imitate the development of the ENS, and so to investigate the effect respectively the lack of certain factors for the development of an intact and well working gastrointestinal innervation.

Introduction

During the last five years 132 cases of intestinal motility disorders of varying clinical expression were seen in the department of paediatric surgery of the Clinical Hospital Mannheim. The histological examination of suction biopsies did not really help in the classification and evaluation of severeness of the disease or necessity of surgical intervention. There was no correlation between the varying degrees of dysganglionosis and their clinical significance. Obviously the variety within this spectrum needs a much more intense investigation to understand the underlying principles of distinct pattern of the disease.

One subgroup of dysganglionic cases display a deficit of intrinsic innervation of the gastrointestinal tract at different locations, respectively segments. The etiology of this entity is still not fully understood, also there is strong evidence for neurotrophic deficit during development.

Whether this deficits are due to the lack of the necessary trophic factor receptor or its ligands is in the focus of interest [1]. Although histological examinations lead to a broader understanding [2], there is need for studies dealing with the dynamical aspects of development. The tissue culture technique, and especially the three-dimensional culture in a extracellular matrix gel [3] might help to elucidate the problems underlying a deficient development of the enteric nervous system (ENS).

We therefore investigated the growth behaviour of dissociated myenteric plexus in a three-dimensional matrix of extracellular matrix components, and compared the secondary aggregations with whole mount preparation of the gut wall of sick children.

Material and Methods

Myenteric plexus of newborn rats was dissected, dissociated and cultured as described previously [4]. Briefly, the complete small intestine was dissected and the muscle isolated by stripping the submucous and mucous layer from the muscle layer using watchmakers forceps. The muscle was incubated in Ca and Mg-free Hanks balanced salt solution, containing 1mg/ml Type II Collagenase (Worthington) at 37°C for 2 hrs, followed by mechanical agitation (Vortex) for about 20 sec. The combination of enzymatic digestion and mechanical agitation allowed the complete isolation of sufficient numbers of plexus pieces for further dissociation by trituration after a short period (10 minutes) of trypsin digestion (Trypsin-EDTA, Gibco BRL, 0,05%).

Cell numbers were counted using a hemocytometer. The cell suspension was diluted and plated either in 24 well-dishes (NUNC) at 100µl per well on 13 mm coverslips coated with poly-l-lysine (1mg/ml), or diluted in a commercially available extracellular matrix solution (ECM-gel, Sigma) and equally plated. After 30 minutes incubation the wells were topped with the serum free culture medium (DMEM/F12, Gibco) with N2-supplement, 3% bovine serum albumine and antibiotics) alone or supplemented with neurotrophic factors (GDNF, BDNF, 10 ng/ml, alomone labs, Israel). After cultivation for 48 hrs the cultures on glass were fixed with 4% formaldehyde for 10 min. Enteric neurons were stained with an antibody against the neurone-specific marker protein PGP-9.5 (5), and visualized through a peroxidase conjugated IgG and DAB (3‘3‘-diaminobenzidine-tetrahydrochloride) development. Neurite outgrowth and branching points were measured using the interactive DIGGER-program (MedVis, Homburg, Germany).

The gels were kept for periods up to ten days in vitro and than equally stained for PGP.

Cultures used for electron microscopy were fixed in 2% glutaraldehyde in cacodylate buffer (0,12 M) overnight. After embedding in epoxy resin (Araldite 502) ultrathin sections were cut and contrasted with lead.

Specimen from children being operated due to their motility disorders were treated as whole-mount preparations [2] and also stained for PGP.

Neurite length and branching points were compared using student-t-and Wilcoxan ranking tests.

Results

Cultures on glass

Already 1 hour after seeding enteric neurons and glial cells can be distinguished by their morphological characteristics. While the glial cells spread on the substrate, the neurons keep their phase-bright appearance, which is correlated with a still rounded morphology. Depending on the culture conditions the neurons start to develop neurite like processes. After 48 hours in vitro these processes and the number of their branching points were measured. Interestingly while GDNF promoted neurite outgrowth, BDNF supplemented cultures did not show any significant difference compared to the control medium (DM), either supplemented alone or in combination with GDNF (Fig.1).

In contrast, looking at the number of branching points, BDNF alone did not stimulate a more differentiated neuritic tree. But combined with GDNF, there was an increase in sprouting which was higher as the effect seen with GDNF alone (Fig. 1).

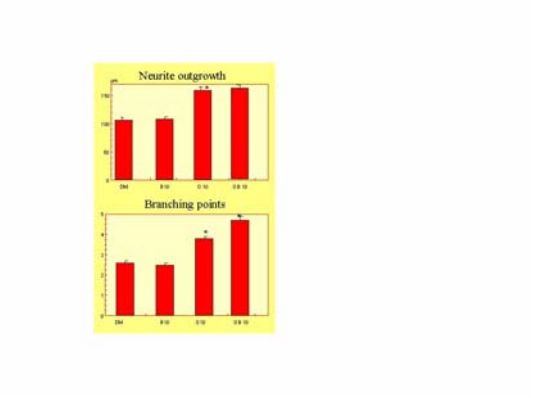

Fig 1: *Neurite outgrowth in µm and number of branching points after 48 hours in vitro.DM: serum-free, chemically defined medium (control), B10: 10 ng/ml BDNF, G10: 10 ng/ml BDNF, GB10 10 ng/ml BDNF and GDNF.*

Fig. 2: *Phase-contrast pictures of GDNF supplemented cultures after 2 (A) and 10 (B) days in vitro. While after 2 days there are still numerous isolated cells to be seen, after 10 days a closed network of neurons and glial cells has developed (400x).*

ECM-cultures

At the beginning of the cultivation, the cells of the dissociated myenteric plexus were homogenously dispersed in the ECM-solution. In contrast to the cultures on glass, there was much less glial growth. During a ten-day period there was a continous formation of secondary ganglia and an interconnecting network of fibres and glial cells (Fig. 2). These ganglia, as well as the network, developed depending on the supply of BDNF or GDNF. Similarily to the results seen on glass, BDNF did not alter significantly the formation of the secondary network. Only GDNF, as well as GDNF and BDNF together influenced the growth pattern of the reaggregating plexus. GDNF enhanced the survival, respectively reduced the amount of apoptotic neurons. GDNF and BDNF together increased the density of the netwok, reflecting the increase of branching points seen on glass.

Comparing the reaggregating secondary plexuses with whole mount preparation from children with severe hypoganglionosis, there was a striking similarity to be seen (Fig. 3). Also looking at the ultrastructural level, we found corresponding structures in both the in vivo and in vitro specimen (Fig. 4). Especially cultures which were not supplemented formed ganglia with only few neurons per ganglia, as was seen in one case of severe hypoganglionosis of the small intestine.

In this case, the child displayed a permanent subileus condition from birth on, and had to be operated several times. Three ileostomas had to be performed and left con tinuously. Intraoperatively the small intestine was found to be maximally distended. The child therefore suffered from various bacterial translocation and had to be parenterally fed all the time. A severe reduction of gastrointestinal motility was proven by radiological transport studies (Fig. 5) and an 22 h intraluminal manometry of the small bowel (Fig. 6).

Interestingly, the symptoms of the motility disorder decreased with increasing age. The marker x-ray study, when the child was two years old demonstrated an increased transport capacity of the GI-tract. (Fig. 7)

Discussion

The colonization of the gut wall by neural crest cells leads to a fully developed and well working enteric nervous system [1,6]. The migration is very sensitive to disturbances during the early periods of migration. A premature differentiation or a deficit in neurotrophic support can end in the complete lack of the ENS (Hirschsprung´s disease) or to a reduced or immature innervation [2,7]. Clinical and histological observations suggest that development of the ENS continues postpartally at least during the first 18 month of life. In rats, a similar plasticiy could be demonstrated [8,9]. These observation correspond to the development of the child with the severe dysganglionosis, which is still getting better.

GDNF seems to be one of the essential factors. This neurotrophin is a potent neuronal survival factor [8,9], and can also reduce apoptosis in dopaminergic neurons [10]. Mice lacking the GDNF gene fail to develop an enteric nervous system [11,12,13]. BDNF as a sprouting factor might also influence the ENS development. Its receptor, Trk B, is expressed in the human enteric nervous system [14]. Isolating neuronal tissue and keeping it in tissue culture allows to study influences neurotrophic factors in vitro. Expanding the culture technique to the three-dimensional condition, might increase the insight into the plexus organization , especially because components of the extra cellular matrix can play an important role in neural crest cell migration [15], and the ECM-proteins are the major components of the basal membrane surrounding the enteric ganglia [16].

Looking at the electron microscopical level the often striking similarities of the structures found in the gels, compared to their equivalents in vivo underlines the importance of the three-dimensional culture approach. Morevoer, the comparison of whole mount preparations of the gut muscle layer from children with hypoganglionosis with the ECM-cultures gives a first insight into the possibilities of this technique. For the future it does not seem impossible to mimick the appropriate microenvironment, to establish

***Fig.3**: Dissociated myenteric plexus in ECM-Gel after 10 days in vitro (B, C), stained for PGP 9.5, compared with a whole-mount preparation from a hypoganglionic child (A). B: control. C: supplemented with GDNF (300x).*

an adequate counterpart of the ENS in vivo. Especially if the ingredients of the extracellular-matrix-gel can be adapted to the in vivo situation, and also the neurotrophic support corresponds to what we see in the natural envi

ronment of the ENS. A first step toward this goal might be reached by supplementation the culture media with proteins from the microenvironmentof the myenteric or submucous plexus [17,18,19].

Fig. 4: *Varicosities from the enteric nervous system of a newborn rat are very similar to varicosities found in the secondary ganglia of the ECM-cultures. In both preparations dense core and granular vesicles, as well as typical microtubular structures are to be found (10000x).*

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Fig. 5: *Modified Hinton Test in 4 months old girl with severe motility disorder: The marker were given 2 days before the x- ray was done. No transport activity, but enormous dilatation of small bowel despite three existing stomata, can be shown*

Fig.6: Two characteristic episodes out of the 22 hour small bowel manometry.

A: Rare moments of segmental phase II tonic activity. No propulsive activity is to be seen. (channel 2 : heart frequency as artefact but no migrating motor compelexes (MMC = Phase III)

B: Further on there was nearly no activity detected.

Fig. 7: The same girl, now two years old, shows a transport of the markers to the rectum and the small bowel dilatations are decreased.

Still, we do not know how much ENS is necessary to establish a well working GI-tract. That means that quantification and objectivation in the methodological approach have to be improved [20], and more sophisticated methods, like three-dimensional culture systems, alone or as co-cultures for example with interstitial cells, must be used.

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