Early variations of the disialoganglioside GD3 in chicken embryonic brain support its role in cell migration

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Abstract

In the present study a primary culture system of chicken embryo brain neurons was used in the early period of chicken brain development from day 6 until day 8, which was shown to be a suitable model of neuritogenesis, cell migration and reaggregation. Dissociated chicken optic tectum cells from embryonic stage 31 were cultured on polylysine-coated dishes under serum-free conditions up to 3 days. Freshly dissociated neurons developed short processes, which contacted one another and formed fasciculated bundles. Cell somata migrated along the neurite bundles, similar to migrating neurons in vivo, forming three-dimensional tissue-like clusters. This system was used to study the possible functions of the disialoganglioside GD3 for these neuronal differentiation steps. GD3 represents the predominant ganglioside of embryonic neurons before neuritogenesis in vitro and in vivo. Its biosynthesis is followed during day 6 until day 8 of embryonic brain development. Incubation of dissociated neurons with the monoclonal antibody R-24, recognising the GD3 on the cell surface, led to a total blocking of neurite outgrowth. Accordingly, neither cell migration nor reaggregation could be found. These results indicate that the disialoganglioside GD3 plays a central role in neuronal differentiation and development in the embryonic chicken brain.

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1. Introduction

Spatially and temporally patterned differentiation processes are involved in the development of vertebrate nervous system, leading to constructions of highly ordered neuronal connections. The underlying basic events are cell proliferation, cell migration, reaggregation and the formation of specific neuronal connections and pathways. Specific membrane-bound surface molecules modulate the regulation of these events. One family of these components is the gangliosides, which are highly expressed in the vertebrate central nervous system (CNS). Gangliosides are neuraminic acid-containing glycosphingolipids, asymmetrically localised in the outer leaflet of the plasma membrane, with a hydrophilic carbohydrate moiety extended to the extracellular space and a hydrophobic ceramide portion inserted into the lipid layer. Gangliosides have been implicated in a variety of phenomena involving cell-cell recognition, synaptogenesis, transmembrane signalling, as well as cell growth and differentiation.

In embryonic chicken brain content and composition of gangliosides changes during the ontogenetic development of the CNS in dependency on the morphological differentiation of the neuronal cells. Therefore, gangliosides have been designated as cytochemical markers of neuronal development and maturation.

Abbreviations: BCA, bicin choninic acid; BSA, bovine serum albumin; CNS, central nervous system; ManNAc, N-acetylmannosamine; Mo9-O-Ac-GD3, mouse antibody against 9-O-Ac-GD3; MoSRBC, mouse antibody against sheep red blood cells; PBS, phosphate-buffered saline; The ganglioside nomenclature is that according to J. Neurochem. 10 (1963) 613–623.

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In this study the developmental profile of the disialoganglioside GD3 of chicken embryonic brain was followed from the 6th to 8th day of incubation and correlated to known morphological development. To get more information about the function of this disialoganglioside in neuronal development and differentiation, an established primary culture system of neuronal cells from embryonic chicken optic tectum was used to study the developmental processes of neurite growth and pattern formation of embryonic chicken neurons in vitro, with respect to the possible implication of GD3, which appears to be the major ganglioside of chicken immature neuronal cells. To elucidate the role of GD3, the monoclonal antibody R-24 was added to primary cultures of embryonic chicken optic tectum recognising GD3 on the cell surface.

2. Materials and methods

2.1. Cell culture

Fertilised eggs (White Leghorn) were incubated at 37 °C in a standard egg incubator. Embryos from 6 to 8 d, corresponding to Hamburger and Hamilton stages 29–35 [8] were used for the establishment of primary cultures under serum-free conditions. After decapitation, the heads were immediately transferred into sterile PBS. The optic lobes were dissected, freed from adhering tissue, transferred into tubes with 1 ml 0.1 M PBS containing 0.3 mg trypsin, 2.4 mg HEPES and 100 µl DNAse (Serva, Heidelberg, Germany) and incubated for 15 min at 37 °C for dissociation. All following steps were done under sterile conditions. The dissociating solution was removed and the cells were washed twice by centrifugation (9000 × g) and resuspension in 1 ml Dulbecco’s Modified Eagles Medium (DMEM, Sigma, Deisenhofen, Germany) and sieved through a nylon mesh (40 µm pore size). The cell suspension was plated at a density of 10^5/ml on polylysine-coated (Sigma) Petri dishes (35 mm i.d., Greiner, Frickenhausen, Germany). The culture medium consisted of a 1:1 (v/v) mixture of DMEM and Ham’s F-12 medium (Sigma), supplemented with 100 µg/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 0.5% Site Liquid Media Supplement (Sigma). The neuronal cultures were incubated at 37 °C in 5% CO₂ in humidified air.

For long-time video microscopy, cells were cultured in Leibovitz L-15 medium (Life Technologies, Eggenstein, Germany), supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mg/ml gentamycin and 1% Site Liquid Media Supplement and covered with 2 ml mineral oil in a sterile microscope heating chamber (Narishige, Charlotte, VT, USA).

2.2. In vitro assay with mAb R-24

Anti-GD3 antibody R-24 was purchased from Biotrend (Köln, Germany). Antibodies against 9-O-acetyl-GD3 (Mo9O-Ac-GD3) and sheep red blood cells were kind gifts of Dr. T. Suguri, Snow Brand, Tokyo, Japan and Dr. H. Lemke, Biochemical Institute CAU Kiel, Germany, respectively. Cells were seeded in supplemented DMEM or Leibovitz L-15 medium and allowed to adhere to the dish. Monoclonal antibody R-24, the control-antibodies Mo9O-Ac-GD3, and MoSRBC were dissolved in fresh medium in the final dilution 1:50. After 3 h, the culture medium was exchanged against antibody-containing medium.

To test the specificity of mAb R-24, the antibody was adsorbed with purified GD3 (kind gift of T. Suguri, Tokyo, Japan) for 1 h at room temperature and then used as described above. Excess of antibody was depleted by incubation with protein A-beads (Pharmacia Biotech, Freiburg, Germany) for 1 h. After centrifugation at 14 000 × g the supernatant was used for the in vitro assay as described above.

2.3. Metabolic labelling

Cells were seeded in normal culture medium and allowed to adhere to the ground. After 2 h, the culture medium was exchanged against 2 ml labelling-medium containing 1 µl N-acetyl-D-[14C]mannosamine (1850 kBq/ml, 1850 MBq/ mmol in ethanol/H₂O 1:1, v/v; Hartmann, Braunschweig, Germany). The cultures were incubated for 3, 5 or 48 h, respectively.

2.4. Extraction, purification and analysis of labelled gangliosides

 Cultures to be analysed were placed on ice. The medium was removed, and the cells were rinsed gently with PBS. Neurons were detached from the substrate with Trypsin-EDTA (PAA Laboratories, Cölbe, Germany) and pelleted by centrifugation (14 000 × g). The gangliosides were extracted thrice by resuspending and repelleting with 1 ml chloroform-methanol, 1:2 (v/v), 1:1 (v/v) and 2:1 (v/v), respectively. The supernatants were combined and dried by rotary evaporation. The dried material was redissolved in 2 ml solvent A (C/M/H₂O 30:60:8, v/v/v). Columns of DEAE-Sepharose (Sigma) were washed with 1 ml of the same solvent, the ganglioside-containing samples were applied and the columns were rinsed with 10 ml of solvent A. Gangliosides were eluted with 10 ml 0.8 M NH₄Ac in methanol. The eluate was concentrated by rotary evaporation and ammonium acetate was removed by lyophilisation.

The samples were dissolved in 2.5 ml 0.1 M KCl and passed through SepPak C₁₈-columns (Waters, Eschborn, Germany), equilibrated with 10 ml C/M 2:1 (v/v), 5 ml methanol, 10 ml H₂O and 5 ml 0.1 M KCl. The columns were then rinsed with 7.5 ml H₂O and the lipids eluted with 10 ml C/M 2:1 (v/v). The eluates were dried by rotation evaporation. The samples were dissolved in 20 µl C/M 1:1 (v/v). Radioactivity of a 3 µl sample was measured by liquid scintillation counting. The ganglioside extracts were applied to silica HPTLC-plates (Merck, Darmstadt, Germany) and developed with C/M/0.2% CaCl₂ 50:40:10 (v/v/v). Standard ganglioside GD3 was run simultaneously and visualised by
spraying with the orcinol reagent for sialic acids \[^9\] and heating for 15–20 min at 120 °C. Detection and quantification of labelled ganglioside bands were carried out using a phospho-imager. Statistical significance of the corresponding values was proved by Student’s \(t\)-test.

Protein content of the neuron samples was determined with the BCA-Kit (Pierce, Rockford, USA) using BSA as standard.

2.5. Extraction and analysis of gangliosides from brain tissue

Whole brains of 6–8-d-old chicken embryos were removed, homogenised separately in 1 ml methanol, and sonicated. Samples were warmed up to 50 °C for 5 min. Gangliosides were extracted twice by resuspending and repelleting with 1 ml chloroform/methanol 1:1 (v/v). The supernatants were combined and dried by rotary evaporation. Pellets were saved for determining the protein-content.

The dried residue was sonicated with 2 ml hexane/methanol 1:0.1 (v/v) to remove phosphatides, centrifuged at 14 000 × g and the supernatant was discarded. The samples were freed from sphingomyelin according to Folch et al. \[^10\]. Gangliosides were separated by HPTLC as described above, and visualised by spraying with resorcinol and heating for 10 min at 150 °C. Quantification of gangliosides was carried out using a phospho-imager.

3. Results and discussion

3.1. Developmental changes of gangliosides in whole embryonic chicken brain

Prior to evaluate the possible functional role it was necessary to know the content of GD3 during different stages of development. Therefore, gangliosides purified from the embryonic chicken whole brain were chromatographed and visualised by spraying with resorcinol. GD3 was identified by comparison with an authentic standard.

Fig. 1 shows the GD3 content and composition after 6, 7 and 8 d of development. The data indicate that the early embryonic brain (day 6) contains predominantly the disialoganglioside GD3. During the next 2 d a remarkable decrease of GD3 occurred, from 65% of the total ganglioside at the 6th day of development to about 28% at days 7 and 8. This correlates with known developmental and morphological changes \[^11\]. During this phase, the relative amount to other gangliosides as well as the absolute content of GD3 in whole embryonic chicken brain decreased rapidly. The brain at day 6, which is the earliest developmental stage investigated, is characterised by maximal proliferative activity and enormous expansion of the surface of the optic tectum due to accumulation of undifferentiated or little differentiated neurons \[^12\]. In this period, we found the disialoganglioside GD3 as the predominant ganglioside. During the next days, the morphological development is characterised by a rapid decrease of mitotic activity. Most of the neurons migrate centrifugally, differentiate into different types and contact ingrowing optic nerve axons \[^12\].

These data indicate that proliferating neuroepithelial cells are characterised by a high content of GD3, which is the common precursor of multisialogangliosides. Furthermore, it is obvious that the transition from undifferentiated or little differentiated to the migratory and differentiating state of the young neurons is associated with an accelerated synthesis of other gangliosides, probably by glycosylation and sialylation of the pre-existing pool of GD3. Although it would be interesting to study the nature and changes of other gangliosides, especially of the oligo-sialylated species replacing GD3, this could not be carried out due to limited amounts of brain tissue.

The results of our experiments show that the decrease of the content of the disialoganglioside GD3 in embryonic chicken brain agree with previous experiments of Rössner \[^7\] and Hilbig et al. \[^13\] demonstrating GD3 as a predominant ganglioside of neurons before the start of neuritogenesis in chicken optic lobes and in mouse cerebral cortex, respectively.

Since it is known that 9-\(\text{O}\)-acyetylated gangliosides are present in chicken brains at day 10 and later \[^14,15\] we checked the ganglioside extracts from chicken brains of day 6–8 by the use of a monoclonal antibody directed towards 9-\(\text{O}\)-acyetylated GD3 and also by influenza C viruses in TLC overlay assays, but no positive reaction was detectable in these experiments. \(\text{O}\)-acyetylation of gangliosides, therefore, might be a phenomenon, which is significant for later stages of development in the chicken brain, probably from day 10 onwards. Also in chicken erythrocytes, \(\text{O}\)-acyetylation of sialic acids appears only after hatching \[^16\].

3.2. Labelling of gangliosides in the primary cell culture system

Optic tectum cells from 7-d-old embryonic chicken were kept in primary culture for several times and metabolically
labelled by [14C]-N-acetylmannosamine. ManNAc is the metabolic precursor for N-acetylneuraminic acid, which is readily taken up by the cultured neurons and converted to sialic acid. To investigate possible correlations between the biosynthesis of the ganglioside GD3 and the process of morphological differentiation, we analysed the content of radioactively labelled GD3 before the development of neuronal processes, and with the onset and completion of neurite outgrowth. In the primary culture, dissociated neuronal cells started forming processes after 5 h of incubation. After 48 h in culture, most of the neurons completed differentiation and typically formed into large reaggregates connected by thick bundles of neurites used as migration routes. As described by Thampy et al. [18] these cultures are free of glial cells, astrocytes and fibroblast, which was controlled by immunostaining glial fibrillary acidic protein (GFAP) and the astrocyte marker protein S100 (data not shown). These results show that under the used serum-free conditions the typical formation of reaggregation patterns followed the same steps of in vitro development as described for serum-containing cultures [19].

Incorporation of the radioactively labelled precursor into the disialoganglioside GD3 found in the cultivated, 6-d-old embryonic chicken neurons are shown graphically for different cultivation times in Fig. 2, indicating that the pattern of labelling was modified from 3 to 48 h of incubation. The total content of labelled GD3 decreased about 40-fold in neuronal cultures with the differentiation of neurons during 48 h and also the relative amount of GD3 declined with progressing time in culture. While GD3 accounted for about 85% of the labelling after 3 h of incubation, its contribution decreased progressively to reach values lower than 8% after 48 h. Conversely, the contribution of other larger and higher sialylated gangliosides, which was very low after 3 h of incubation (about 15%), increased to a value of about 90%. These metabolic changes leading to the shift in the synthesis of GD3 to other gangliosides during the critical phase from day 6 until day 8 of chicken brain differentiation, reflects the changes taking place during comparable developmental periods in vivo. Therefore, the decrease of the disialoganglioside GD3 in favour of other gangliosides seems to reflect a general principle of brain development in higher vertebrates, indicating the transition from the undifferentiated to the differentiation state of development of young neurons.

3.3. Effect of anti-GD3 mAb R-24 on neurite outgrowth

Since immature chicken neurons highly express the disialoganglioside GD3 in vivo and in vitro, we used dissociated cells from optic tectum of 7-d-old chicken embryo to elucidate a possible involvement of this surface molecule by incubating the developing neurons with monoclonal antibody R-24, which binds to GD3 on the cell surface and, is therefore, able to mask it. After a few minutes on polylysine-precoated petri dishes, the dissociated cells adhered to the plate and were homogeneously distributed. With progressing time they developed neuronal processes, which contacted each other and fasciculated. On these bundles of neurites the neurons were migrating in both direction, changing from one reaggregation centre to another, predominantly from the smaller to the greater ones. This led to the formation of large reaggregates, which were connected by thick bundles forming a regular triangularly shaped pattern.

In contrast to control cultures, which were growing like the above described untreated cultures, neuronal cells, incubated with the GD3-specific antibody R-24, showed a total different formation of somata reaggregates.

Fig. 3 shows typical cultures, grown without (a–c) or with (d–f) addition of the GD3-specific antibody R-24. No development of neuronal processes took place and neither migration nor formation of reaggregates could be seen during the observation period of 3 d in the antibody-supplemented cultures. Incubation with R-24 blocked neurite outgrowth and the cells remained homogeneously distributed, although the neurons tried to generate neurites, visible in numerous evaginations of the plasma membrane. During the whole period of observation a lasting protrusion and retraction of pseudopodia-like membrane cones is visible, but no elongation to normal neurites occurred. This suggests that it is not the initiation of neuritogenesis, but the stabilisation of neurite outgrowth and elongation that is inhibited by masking GD3 on the cell surface. Furthermore, primary cultures incubated with antibody-depleted medium or supplemented with antibodies, which were blocked with GD3 prior to cell culture addition as controls, developed neuronal processes and showed typical migration and reaggregation, indicating the GD3-specific inhibition of neuronal outgrowth by R-24.

Cells cultured with a 9-O-acetyl-GD3-specific antibody or with a polyclonal antibody against sheep red blood cells, as an unspecific control, showed a well-developed differentiation. Since in later development O-acetyl sialic acid-containing gangliosides occur in chicken brain [15] a hy-
hypothesis may be that after neuritogenesis and formation of neuronal contacts a further growth is prohibited by masking gangliosides like GD3 with $O$-acetyl groups.

Our data, suggesting an important role of GD3 in the differentiation processes of neuronal cells, are in accordance with previous findings of Dippold et al. [20], who demonstrated an inhibition of cell growth of human melanoma cells in the presence of the antibody R-24. In addition, Schauer [9] assumed that the GD3-specific antibody R-24 is a potent inhibitor of epithelial morphogenesis in the mouse embryonic kidney.

Various reports indicate associations of gangliosides and different adhesion-mediating receptors on the cell surface (for review see [3]). Besides the direct participation of GD3 in cell adhesion processes cooperating with such receptors, there is another possible explanation of the R-24-dependent inhibition of neuritogenesis in influencing receptors in a more indirect way. Analyses on artificial membranes have demonstrated a colocalisation of gangliosides and cholesterol in lateral assemblies termed “rafts”, which have been proposed to form platforms for numerous cellular events including membrane trafficking, signalling and also cell adhesion [21]. The fundamental principle by which rafts exert their function is described as a separation or concentration of signalling molecules like specific membrane proteins and lipids in membrane microdomains [22]. The GD3-specific antibody R-24 is known to form multivalent lattices on the cell surface due to its ability to simultaneously bind to GD3 antigen, to other molecules of R-24, and furthermore unspecifically to the membrane surface through the variable domain of the light chain [23]. Therefore, it appears possible that GD3, if it is present in the rafts, influences cell-matrix

Fig. 3. Influence of the monoclonal antibody R-24 on the differentiation of 7-d-old chicken optic tectum cells in primary culture; bar: 50 µm. a–c: The control cultures incubated without the GD3-specific antibody R-24 show a well-developed differentiation. The cells form neuronal processes contacting each other and the neurons begin to migrate and assemble to large reaggregates forming a regular triangularly shaped pattern. d–f: Cells were cultured on polylysine-precoated petri dishes for 3 d in the presence of the antibody R-24. The cells attached to the dishes, but no neuronal sprouting took place. The neurons remained homogeneously distributed, and did not migrate or reaggregate during the observed time.
adhesion by preventing accessibility of adhesion-mediating receptors, located in these rafts, through the R-24-lattices.

4. Conclusion

Although the exact molecular mechanism of the involvement of GD3 in these cell-matrix interactions remains to be investigated further, these data suggest that R-24 binding the disialoganglioside GD3 plays an important role in the interactions between neuronal cells and the extracellular matrix underlying neuronal migration and neurite fasciculation.

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