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Ganglioside composition of the rat choriocarcinoma cell line, Rcho-1

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The Rcho-1 cell line, originally established from a rat choriocarcinoma, shows differentiation into placental trophoblastic giant cell-like cells and has been used to study the mechanism of placental function control. In the present study, we analysed the ganglioside composition of Rcho-1 cells by HPTLC orcinol/H_2SO_4, TLC/immunostaining and immunohistochemistry. Rcho-1 cells expressed GM3 and GD3 as the major gangliosides and CTH as major neutral glycolipid when they were cultured in growth medium (20% FCS) or transplanted beneath the kidney capsule. The expression of these gangliosides was strong in the undifferentiated small cells, whereas the completely differentiated giant cells showed poor staining with antibodies against the gangliosides. Under culture conditions to induce cell differentiation using horse serum (1–20% HS), the expression of GD3 was suppressed and re-expressed when the medium was changed to growth medium, suggesting that a change of ganglioside components may trigger and define the direction of terminal differentiation. Thus the composition of glycolipids is conserved in Rcho-1 cells and is similar to that of the rat placenta, where GM3 is dominant in mid-pregnancy and decreased in late pregnancy, whereas GD3 is low in mid-pregnancy and increased in late pregnancy.

Keywords: ganglioside, glycosphingolipid, rat choriocarcinoma cell line

Introduction

The major gangliosides present in the rat placenta have been identified as GM3 and GD3, whereas the neutral glycosphingolipids are GlcCer, LacCer, Gb_3Cer and Gb_4Cer [1]. For the successful development and maturation of the embryo, the placenta expresses its various functions in a pregnancy stage-specific manner, including transport of nutrients, prevention of immune rejection and synthesis of placental hormones [2, 3]. The expression of placental gangliosides is similarly stage-specific during pregnancy. GM3 is dominant in mid-pregnancy and decreases in late pregnancy, whereas GD3 is low in mid-pregnancy and increases in late pregnancy [1]. Thus, the composition of the gangliosides changes markedly according to the stage of pregnancy. Interestingly, GM3 and GD3 are expressed in the placentas of human [4, 5], horse, ox, dolphin and seal (our unpublished data).

Several growth factors such as EGF, bFGF, TGF-β and their relevant receptors are expressed in the placenta, and data on autocrine/paracrine networks involving various growth factors or cytokines in the placenta relates to the growth and differentiation of trophoblasts have been accumulated [2, 3, 6–8]. Gangliosides are known to define the specificity of cell-cell or cell-substratum interaction and the control of transmembrane signalling via modulation of receptor kinase, protein kinase C, or other kinases [9, 10]. Therefore, the marked changes in ganglioside composition and their conserved expression in the placentas of these animal species may imply a specific biological role of gangliosides in the placenta for expression of pregnancy stage-specific functions [3, 11–14].

The Rcho-1 cell line was established from a rat choriocarcinoma [15, 16] and has been used for investi-

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gating the mechanism of placental function control. Rcho-1 cells differentiate into placental trophoblastic giant cell-like cells, which secrete members of the rat placental prolactin family under defined culture conditions or after transplantation *in vivo* [8, 15, 17, 18]. In the present study, we analysed the ganglioside composition of Rcho-1 cells to clarify whether the cell line expressed various gangliosides including GM3 and GD3.

Materials and methods

Materials

DEAE-Sephadex A-25 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), high-performance thin-layer chromatography plates (HPTLC, silica gel 60) were obtained from E. Merck (Darmstadt, Germany) and plastic TLC plates (POLYGRAM SIL G) from Machrey-Nagel (Postfach, Germany). NCTC-135 culture medium was purchased from GIBCO BRL Life Technology Inc. (New York, NY, USA), and penicillin, streptomycin and poly-L-lysine solution were obtained from Sigma Chemical Co. (St Louis, MO, USA). Fetal calf serum (FCS) was purchased from Causera (Ontario, Canada), horse serum (HS) from Starrate (Melbourne, Australia). All other reagents were of ultra-pure grade from commercial sources.

Ganglioside standard GM3 was purified from dog erythrocytes. GD3 (bovine milk) was purchased from Wako Pure Chemical (Osaka, Japan). A neutral glycolipid mixture (porcine erythrocytes) was purchased from Diayatron (Tokyo, Japan).

A mouse monoclonal antibody, MECTOLONE-015 (IgM isotype), reactive with GD3 (NeuAc-NeuAc-) and moderately cross-reactive with GM3 (NeuAc-), was a gift from Mecto Co. (Tokyo, Japan). A mouse monoclonal antibody, R24, produced by immunization with SK-MEL-28 melanoma cells and reactive with GD3 (NeuAc-NeuAc- and NeuAc-NeuGc-) [19–21], was purchased from Signet Laboratories, Inc. (Dedham, MA, USA). FITC-conjugated $F(ab')^2$ goat anti-mouse immunoglobulin was purchased from Cappel Research Products (Cochranville, PA, USA). Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin was purchased from Jackson Immuno Research Labs. Inc. (West Grove, PA, USA).

Cell culture

The cells were cultured with NCTC-135 supplemented with 20% heat-inactivated FCS, 50 μM 2-mercaptoethanol, 110 mg l^{-1} sodium pyruvate, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin in a humidified atmosphere of 95% air-5% CO_2 at 37 °C until they became subconfluent before the start of the experiment according to the method published previously [17, 18]. According to the experiments for which they would be used, Rcho-1 cells were cultured in NCTC-135 culture medium containing FCS (20%) or HS (1% or 20%). The culture medium was changed every 2 days.

Transplantation of Rcho-1 cells

Rcho-1 cells (10^6) were transplanted under the kidney capsule of adult female Wistar rats (9–10 weeks old) under ether anesthesis. After 3–30 days, kidneys containing the transplanted tissue were collected (method of fixing) and subjected to immunohistochemistry. For biochemical analysis, the kidney and transplanted tissue were separated and stored at −80 °C until extraction of the glycolipids.

Extraction and separation of glycolipids from Rcho-1 cells

The glycolipid was extracted according to the method reported previously [1]. The transplanted tissue was homogenized with an ULTRA-TURRAX T25 blender (Ika- Labortechnik, Staufen, Germany). Crude glycolipids were extracted twice from the homogenized tissue successively with chloroform:methanol:water (1:1:0.2, by vol) and chlorofrom:methanol (2:1, by vol). Cultured Rcho-1 cells were sonicated in chloroform:methanol:water (1:1:0.2, by vol), and the crude glycolipids were extracted twice with the same solvent. The combined extract was then evaporated to dryness. The ganglioside fraction was separated according to the method reported previously [1, 22] using a DEAE-Sephadex A-25 column.

TLC orcinol/H_2SO_4 stain analysis

Ganglioside fractions were examined by high performance thin-layer chromatography (HPTLC) developed with chloroform:methanol:0.5% $CaCl_2$ (neutral glycolipid, 60:35:8, by vol) and chloroform:methanol:2.5 M NH_4OH (ganglioside, 55:45:10, by vol) and on plastic TLC plates with chloroform:methanol:0.25% $CaCl_2$, 1.25 M NH_4OH (ganglioside, 60:40:10, by vol). These glycolipids were visualized with orcinol/H_2SO_4 reagent.

TLC/immunostaining analysis

Enzyme immunostaining of gangliosides on TLC plates was performed according to the method reported previously [1, 23–25].

Immunohistochemistry

Rcho-1 cells were stained with either Hematoxylin and Eosin or immunohistochemically. The distribution of gangliosides in frozen sections of Rcho-1 cells was determined by immunofluorescence according to the method reported previously [1, 26] with slight modification. In Rcho-1 cells transplanted beneath the kidney capsule, serial sections (7 μm thick) were cut with a cryostat microtome and thaw-mounted on poly-L-lysine-coated glass slides. The mounted sections were air-dried for 2 h and fixed with acetone at −20 °C for 10 min.

Rcho-1 cells were cultured on cover glasses for several days. After removal of the culture medium, the cells were washed with phosphate buffer three times for 10 s at 4 °C, and then were fixed with acetone at −20 °C for 5 min. The slides were viewed and photographed with a Zeiss photo microscope (Germany).

Results

Neutral glycosphingolipids in Rcho-1 cells

The composition of gangliosides and neutral glycolipids in Rcho-1 cells, which were cultured or transplanted beneath the kidney capsule, was analysed by HPTLC (Fig. 1). Only ceramide trisaccharide (CTH: Gb_3Cer) was found in the Rcho-1 cells cultured in medium containing 20% HS for 4, 7 and 21 days, and other neutral glycolipids were not detected using orcinol/H_2SO_4 reagent (Fig. 1A). In contrast, several neutral glycolipids including ceramide monosaccharide (CMH: GlcCer), ceramide disaccharide (CDH: LacCer), CTH and ceramide tetrasaccharide (CQH: Gb_4Cer) were detected in the transplanted preparations of the Rcho-1 cells (Fig. 1B, lanes 7 and 8). Since these neutral glycolipids (CMH, CDH, CTH and CQH) were present in the rat kidney extract (Fig. 1B lane 6), the glycolipid pattern observed for the Rcho-1 cells transplanted for 3 days (lane 7) may have resulted from contamination with kidney tissue. The CDH and CTH contents were higher in the Rcho-1 cell preparation recovered 30 days after transplantation (Fig. 1B lane 8), and therefore may have been induced in the cells after transplantation. Alternatively, CTH and CDH may have reached detectable levels as the cells grew, while expression of these neutral glycolipids by the cells at an early time point (day 3) was not detected due to the difficulty of recovering the cells without contamination by kidney tissue. CTH was continuously expressed in the Rcho-1 cells during culture, and the composition of the neutral glycolipids in the cells appeared to be simple.

Gangliosides in the Rcho-1 cells

Gangliosides in the Rcho-1 cells were analysed by HPTLC after visualization with orcinol/H_2SO_4 reagent (Fig. 2) and resorcinol/HCl reagent (data not shown). GM3 was detected in the Rcho-1 cells cultured for 4 days in NCTC-135 medium containing 20% HS. Expression of GM3 in the cells was maintained by culturing for longer periods of 7 or 21 days. Glycolipid bands were revealed, by HPTLC with orcinol/H_2SO_4 reagent of the cultured

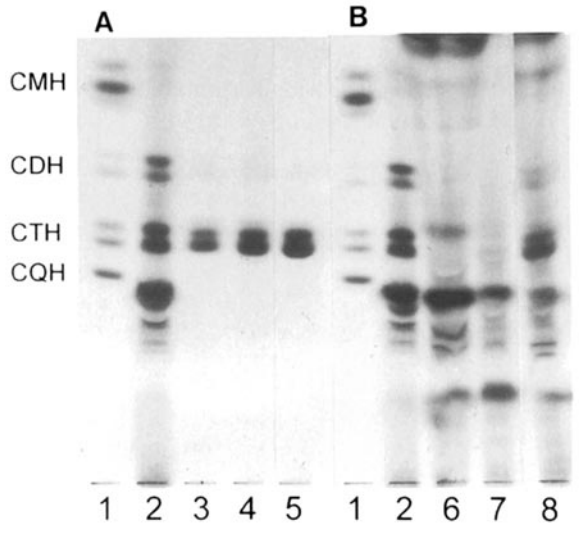

Figure 1. Thin-layer chromatogram of neutral glycosphingolipids isolated from Rcho-1 cells cultured or transplanted beneath the kidney capsule. (A and B) Lane 1, mixture of standard neutral glycosphingolipids (pig erythrocytes); lane 2, neutral glycosphingolipid fraction from rat placenta; (A) lanes 3, 4 and 5, neutral glycosphingolipid fractions from cells after 4 days, 7 days and 21 days of culture in medium containing 20% HS; (B) lane 6, neutral glycosphingolipid fraction from rat kidney; lanes 7 and 8, neutral glycosphingolipid fraction from 3-day and 30-day transplants. In each lane, extract from 1.5 mg (A) and 10 mg (B) wet weight cells and tissue was applied. The plate was developed with chloroform:methanol:0.5% $CaCl_2$ (60:35:8, by vol) and the spots were visualized with orcinol/H_2SO_4 reagent.

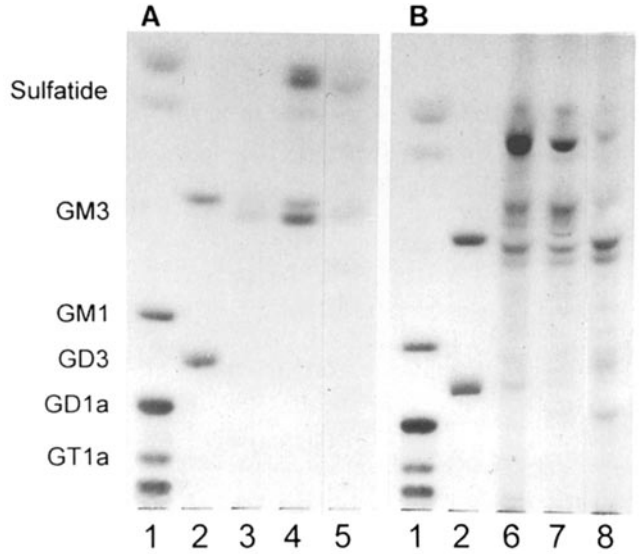

Figure 2. Thin-layer chromatogram of gangliosides isolated from Rcho-1 cells cultured or transplanted beneath the kidney capsule. (A and B) Lane 1, rat brain gangliosides; lane 2, mixture of gangliosides (GM3 from dog erythrocytes and GD3 from bovine milk); (A) lanes 3, 4 and 5, ganglioside fractions from cells after 4 days, 7 days, and 21 days of culture in medium containing 20% HS; (B) lane 6, ganglioside fraction from rat kidney; lanes 7 and 8, ganglioside fractions from 3-day and 30-day transplants. In each lane, extract from 15 mg (A) and 25 mg (B) wet weight cells and tissue was applied. The plate was developed with chloroform:methanol:0.5% $CaCl_2$ (55:45:10, by vol) and the spots were visualized with orcinol/H_2SO_4 reagent.

Rcho-1 cell extracts, which had the same R_f value as sulfatide.

The expression of GM3 was confirmed by TLC/immunostaining (Fig. 3). Only GM3 was identified in Rcho-1 cells cultured in the medium containing 20% HS throughout the culture period (Fig. 3A). The reactivity of the antibody, MECTOLONE-015, was stronger with GD3 (NeuAc-NeuAc-) than that with GM3 (NeuAc-). Nevertheless, this antibody recognized only GM3 under these culture conditions, indicating that the expression of GD3 was negligible.

In addition to GM3, GD3 was also detected in Rcho-1 cells transplanted beneath the kidney capsule (Fig. 2). Rcho-1 cells transplanted for 3 days showed a clear GM3 band. However, the expression of GD3 was not clear with orcinol/H_2SO_4 staining.

GM3 and GD3 were identified by TLC-immunostaining of Rcho-1 cells transplanted beneath rat kidney capsule (Fig. 3B). The MECTOLONE-015 antibody recognized GD3 (NeuAc-NeuAc-) and GM3 (NeuAc-), confirming that the acetyl types of GM3 and GD3 were expressed in the cells. Furthermore, it was observed that each of GM3 and GD3 had at least two bands. In transplanted Rcho-1 cells, the lower band of GM3 and GD3 was stronger than the upper band, whereas in normal rat kidney, the upper band was stronger than the lower band. Thus, the lower bands of GM3 and GD3 were dominant in Rcho-1 cells.

Induction of GD3 in Rcho-1 cells in vitro

The composition of the ganglioside appeared to change depending on the conditions under which Rcho-1 cells were maintained, as indicated above. Rcho-1 cells proliferate and grow rapidly in medium containing 20% FCS, whereas they stop growing and differentiate into trophoblastic giant cell-like cells, responsible for the secretion of placental lactogens, in medium containing lower (1%) HS. Therefore we next investigated the composition of gangliosides under these conditions.

Rcho-1 cells were cultured in medium supplemented with 20% FCS (growth medium). Then, they were split into one-fifth and cultured in 20% FCS medium or 1% HS medium (differentiation medium). TLC/immunostaining of the Rcho-1 cell culture is shown in Fig. 4. On day 0 culture in the growth medium (20% FCS), Rcho-1 cells expressed not only GM3 but also GD3. When the cells were cultured for 6 or 14 days, the expression of GD3 was lower than that on day 0. The cells reached confluence, when the culture period was increased by up to 4 days. When they start to differentiate (Fig. 5), there was a tendency for the expression of GD3 to be reduced. However, the expression of GM3 was constant throughout the culture period. Rcho-1 cells cultured in the 1% HS-containing medium for 4 days expressed only GM3, similar to the situation shown in Fig. 3. Thus, we have confirmed the results of a previous study, Rcho-1 cells differentiate when cultured with HS, regardless of its concentration, and proliferate in the presence of FCS until they reach confluence [17].

Immunocytochemical analysis

These results were confirmed by immunostaining of the cells (Fig. 5). Immunostaining with MECTOLONE-015,

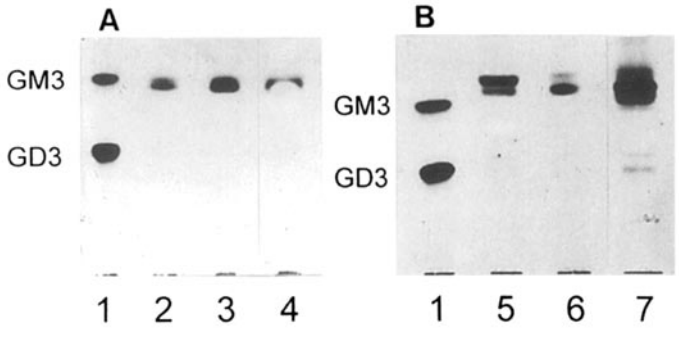

Figure 3. TLC/immunostaining with anti-GM3 and GD3 monoclonal antibody (MECTOLONE-015). (A and B) Lane 1, mixture of gangliosides (GM3(NeuAc) from dog erythrocytes and GD3(NeuAc-NeuAc) from bovine milk); (A) lanes 2, 3 and 4, ganglioside fractions from cells after 4 days, 7 days and 21 days of culture in medium containing 20% HS; (B) lane 5, ganglioside fraction from rat kidney; lanes 6 and 7, ganglioside fractions from 3-day and 30-day transplants. In each lane, extract from 5 mg (A) and 12.5 mg (B) wet weight cells and tissue was applied. The plate was developed with chloroform:methanol:0.5% $CaCl_2$, 1.25 M NH_4OH (60:40:10, by vol).

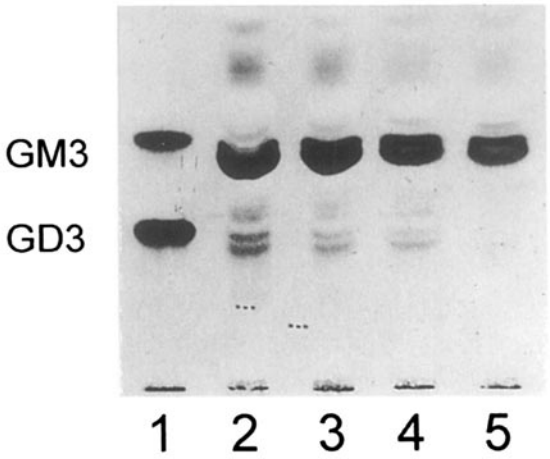

Figure 4. Changes in ganglioside composition under different medium conditions. Lane 1, mixture of gangliosides (GM3(NeuAc) from dog erythrocytes and GD3(NeuAc-NeuAc) from bovine milk); lanes 2, 3 and 4, ganglioside fractions from cells after 0 days, 6 days and 14 days of culture in medium containing 20% FCS; lane 5, ganglioside fraction from cells after 2 days in 20% FCS followed by 6 days in 1% HS. In each lane, extract from 10 mg wet weight cells was applied. The plate was developed with chloroform:methanol:0.5% $CaCl_2$, 1.25 M NH_4OH (60:40:10, by vol) and then immunostained with MECTOLONE-015 antibody.

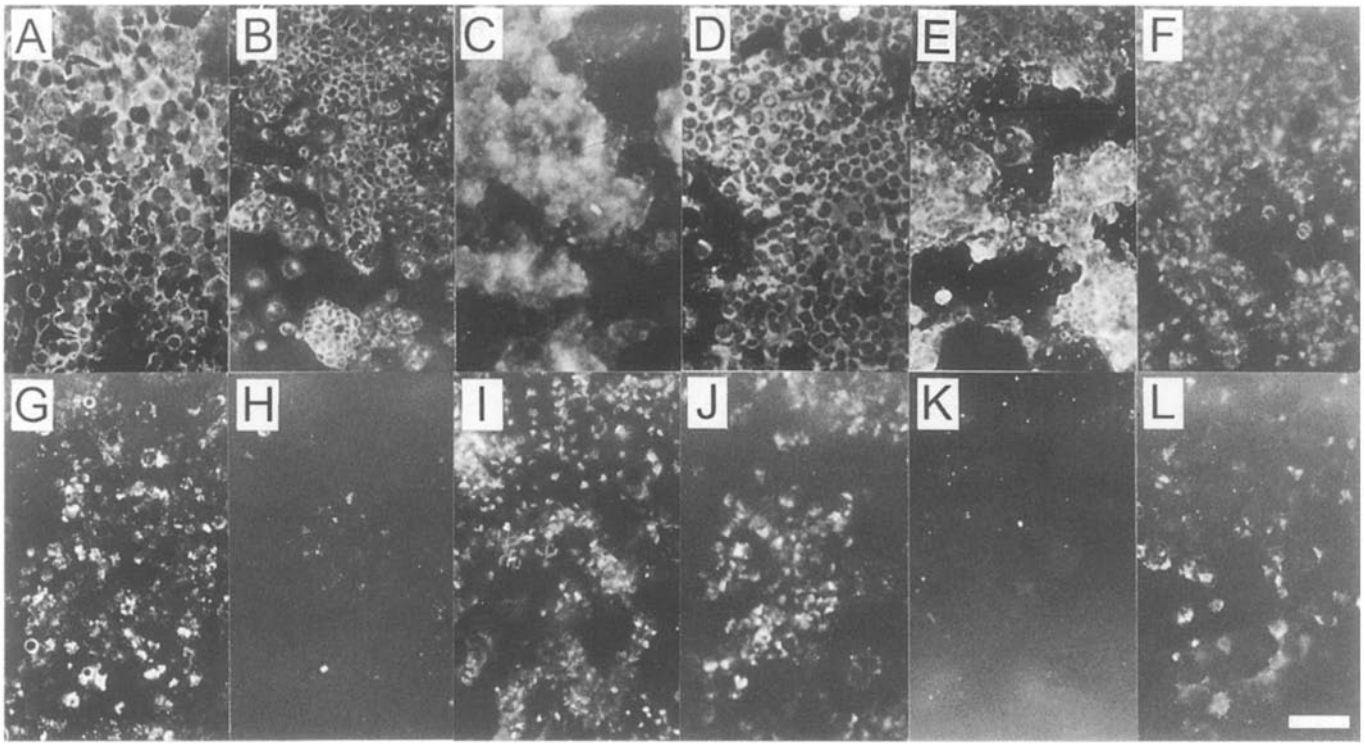

Figure 5. Immunofluorescence analysis of ganglioside GM3 and GD3 expression in Rcho-1 cells. Rcho-1 cells were immunostained with monoclonal antibodies using FITC-labeled goat anti-mouse immunoglobulin antibody. A-F, MECTOLONE-015 antibody (anti-GM3 (NeuAc) and anti-GD3 (NeuAc-NeuAc)); G-L, R24 antibody (anti-GD3 (NeuAc-NeuAc)). A and G, 2 days culture in 20% FCS; B and H, 2 days in 1% HS; C and I, 2 days in 20% FCS and then 2 days in 1% HS; D and J, 4 days culture in 20% FCS; E and K, 4 days in 1% HS; F and L, 4 days in 1% HS and then 2 days in 20% FCS. Bar (L) represents 25 μm.

which reacts with GM3 and GD3, revealed that Rcho-1 cells cultured for 2 or 4 days in 20% FCS were positive (Fig. 5A and D). The R24 antibody specifically recognizes GD3 (NeuAc-NeuAc- and NeuAc-NeuGc-) [21]. The cells were also positively stained with R24 (Fig. 5G and J), which reacts only with GD3, whereas the cells cultured for the same period in 1% HS were negative (Fig. 5H and K). Thus, GD3 was shown to be induced *in vitro* by FCS-containing medium. In contrast, changing the growth medium to differentiation medium reduced the expression of GD3.

Rcho-1 cells consist of trophoblastic giant cell-like cells and small cells morphologically, and the proportion of these cell types changes according to culture conditions. These cell types were easily identified by morphological observation using phase-contrast microscopy or DAPI staining. Interestingly, expression of the gangliosides, GM3 and GD3, was observed only on the surface of the small cells and not on the differentiated giant cell-like cells, irrespective of culture conditions (Fig. 5). Similarly, the surface of the giant cell-like cells was not stained by the antibodies in specimens that were implanted beneath the kidney capsule (Fig. 6). Therefore, the appearance or disappearance of GD3 and the expression of GM3 were demonstrated on the surface of the small undifferentiated cells.

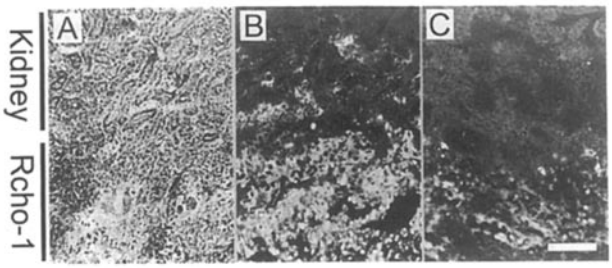

Figure 6. Immunohistochemical analysis of ganglioside in Rcho-1 cell transplanted beneath the kidney capsule. A, Hematoxylin-Eosin stain; B, MECTOLONE-015 antibody; C, R24 antibody stain. 6 days after cell transplantation. Bar (C) represents 50 μm.

Reversible expression of GD3 in Rcho-1 cells

Rcho-1 cells cultured for 2 days in 20% FCS expressed both GM3 and GD3, whereas GD3 was not detected in cells cultured for only 2 days in 1% HS (Fig. 5H). When the Rcho-1 cells were first cultured in 1% HS for 2 days, followed by culture in 20% FCS for 2 days (Fig. 5I), re-expression of GD3 occurred. Also when the period of the first culture in 1% HS medium was prolonged to 4 days, after which the cells were cultured for 2 days in 20% FCS medium (Fig. 5L), GD3 was still expressed, but to a reduced extent.

Discussion

GM3 and GD3 are expressed in the placenta of the human [4, 5], horse, ox, dolphin and seal (our unpublished data) and CTH and CQH are also expressed in human and mouse placenta [27–30]. Their conserved expression in the placenta of these animal species implies a specific biological role of neutral glycolipids and gangliosides in placental function. Although it is well documented that the glycolipid composition changes after transformation of the cells or transfer to *in vitro* culture conditions [31], the major gangliosides expressed in Rcho-1 cells were GM3 and GD3, and neutral glycosphingolipids were CDH, CTH and CQH. Thus, the composition of glycolipids is conserved in Rcho-1 cells and is very similar to that in the rat placenta.

Placental expression of GM3 and GD3 changes markedly according to the stage of pregnancy [1]. GM3 is dominant on days 12–16 and then decreases, whereas GD3, which is low on day 12, increases slightly on day 16 and maintains the same level thereafter [1]. In the present study, the expression of GD3 changed markedly in Rcho-1 cells under the experimental conditions employed. GD3 was expressed only when the cells were cultured in the medium containing 20% FCS or transplanted beneath the kidney capsule in the rat. Under these conditions, the cells grow rapidly [2, 15, 17, 18]. In contrast, GD3 could not be detected in Rcho-1 cells cultured under conditions necessary to induce differentiation, i.e., in medium containing HS [2, 15, 17, 18] or for a longer period in medium containing FCS. Since GD3 is known to be often expressed by proliferating cells [32, 33], proliferation potential may be related to the amount of GD3 in Rcho-1 cells. Monitoring the presence of the gangliosides in the FCS and HS revealed that the amounts of GM3 (NeuAc and NeuGc) and GD3 (NeuAc-NeuAc- and NeuAc-NeuGc-) were lower in the FCS than in the HS (data not shown). Therefore, the observed change in the cells' ganglioside composition with different culture conditions was not simply the result of incorporation of gangliosides into the cells. Indeed, trophoblastic giant cell-like Rcho-1 cells, the finally differentiated type, possessed only a small amount of GM3 and GD3. This phenomenon resembles that in the placenta shown in the previous study [1], where trophoblastic giant cells did not express GM3 and GD3.

There were two bands for each of GM3 and GD3 extracted from Rcho-1 cells. The antibody used in this study recognizes only the acetyl type of sialic acid, and not the glycolyl type. Therefore, the difference in the bands does not depend on the difference in sialic acid but on a different part of the gangliosides molecule including the ceramide moiety.

With regard to the action of various cell growth factors and cytokines, evidence for modification of the intracellular signal pathway by ganglioside has been reported [9, 34]. For example, tyrosine-phosphorylation of the EGF receptor in A431 cells is inhibited by the exogenous addition of GM3 but not by other gangliosides or neutral glycolipids [35]. Furthermore, exogenous administration of NeuAc type GM3 promotes cyclic AMP-dependent protein kinase activity and inhibits protein kinase C activity in brain-derived microvascular endothelial cells *in vitro* [36]. Data on autocrine/paracrine networks involving various growth factors or cytokines in the placenta have accumulated in relation to the growth and differentiation of trophoblasts [2, 7], and thus the way in which expression of GM3 may change the response of trophoblasts to growth factors may be related to the pregnancy stage-specific functions of the placenta [3, 11–14].

Hattori *et al.* described high expression of GM3 on the surface of ovarian granulosa cells during differentiation and suggested that such differentiation involves follicle-stimulating hormone-stimulated transformation of immature into mature cells [37, 38]. It is also reported that addition of antibody against GD3 (R24) stimulates the proliferation, cytotoxicity, and expression of IL-2R alpha- and beta chains, HLA-DR, CD11a, and CD11c in T cells *in vitro* [39], suggesting that cell surface GD3 also functions in cellular differentiation or growth. Interestingly, in the present study expression of GD3 was reversible in the small cells we studied. The decrease in GD3 expression was not the result of final differentiation of Rcho-1 cells, because the expression of GM3 and GD3 was limited to the small undifferentiated cells, and GD3 appeared on the cell surface before morphological differentiation. Expression of GD3 in Rcho-1 cells may be required during the process of cell proliferation, and the disappearance of GD3 may be one of the steps involved. Changes in the components of gangliosides may trigger and define the direction of terminal differentiation in the placenta, and in this context, Rcho-1 cells should prove a useful model.

Glycolipid composition depends on the balance between glycosyltransferase and glycosidase activities. For example, an increase in LacCer in cultured human neuroblastoma cells (SK-N-MC) is accompanied by an increase in plasma membrane sialidase activity [40]. In human hematopoietic cells (HL-60), α2-3 sialyltransferase (GM3 synthase) activity increases during monocyte differentiation [41]. Whether the disappearance of GD3 in Rcho-1 cells is related to an increase in α2-8 sialidase activity and/or a decrease in α2-8 sialyltransferase activity, is not yet known.

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