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ORIGINAL ARTICLE

Cell migration and the ganglioside composition of the cell lines derived from Reuber hepatoma

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Summary
The migration potency of differentiated (H-4-II-E, H-4-II-E-C3) and dedifferentiated (H-5) cell lines originated from Reuber hepatoma H-35, and their connection to the morphology and expression of gangliosides were examined. The migration capacity tested by a Transwell assay was fifteen times higher in H-5 cells compared to H-4-II-E cells. The ganglioside pattern was assessed by thin-layer chromatography (TLC). H-4-II-E and H-4-II-E-C3 cell lines expressed Fuc-GM1, which was not found in H-5 cells, whereas H-5 expressed GM3, which was absent in differentiated cell lines. GM3 ganglioside is thought to be one of the key molecules involved in signal transduction of mammalian cells. We conclude that changes in the migration capacity of various hepatoma cell lines might relate to their ganglioside spectra.

Keywords: hepatoma – cell migration – ganglioside – GM3

INTRODUCTION

Adhesion and motility are crucial functions for successful tumour metastasis. Cell motility is a basic cell physiological process involving many factors and mechanisms which play a major role in defining pathobiological processes such as inflammatory response, wound healing, angiogenesis, etc. The ability of tumour cells to attach to the extracellular matrix proteins is necessary for migration. The cell migratory capacity is an important factor describing the power of cells to invade the surrounding environment (Ono et al. 2001, Wang et al. 2002). Ones of the many different molecules of cell surface that take part in those processes are the gangliosides.

Gangliosides are a family of sialic acid-containing glycolipids located in the outer lipid layer of the plasma membrane as a part of the glycosphingolipids-enriched domain. They are cell type-specific markers playing an important role in cellular interactions, signal transduction

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mechanisms, cell proliferation and differentiation of various cell types (Hakomori and Igarashi 1995). Gangliosides also participate in the regulation of cell adhesion, migration and cell motility; which are highly dependent on the organization and function of lipid membrane microdomains. However, the effects of gangliosides on cell motility and related signalling are scarcely known.

The ability of gangliosides to influence cell functions at various levels of differentiation relates to their role in tumorigenesis and tumour metastasis. The interaction between tumour cells and cells of the immune system appears to be critical for tumour growth and progression. Quantitative and qualitative changes in the expression of gangliosides are connected to the metastatic potential of cancer cells (Huyga et al. 1999). They may be produced by tumour cells and shed into the tumour microenvironment to suppress the antitumour immune response and modulate tumour angiogenesis (McKallip et al. 1999, Lang et al. 2001).

A number of tumours including neuroblastoma, melanoma, retinoblastoma and hepatoma are known to overexpress and shed gangliosides into the circulation (Shurin et. al. 2001). For example the analysis of sera obtained from patients with hepatoma showed increased levels of GD2 and GM3 gangliosides (Kawamura et al. 2001). In contrast, the metastatic capacity of fibrosarcoma cells is suppressed by ganglioside treatment (Facci et al. 1990). The present findings suggest that the particular roles of the gangliosides in metastasis may differ depending on cancer cell type and/or function of the ganglioside. Previous studies have established that the different composition of gangliosides in variable cell lines might affect their functions.

Differentiated rat cell lines H-4-II-E, H-4-II-E-C3 and spontaneously dedifferentiated cell line H-5 primarily derived from Reuber hepatoma H-35 were studied (Deschatrette and Weiss 1974). These models, which are frequently used in hepatology were tested on the cell migration capacity and ganglioside patterns for the first time.

MATERIALS AND METHODS

Cell lines

Hepatoma rat cell lines H-4-II-E, H-4-II-E-C3 and H5 were obtained from ECACC (European Collection of Cell Cultures, UK) and grown in 75 cm^2 tissue culture flasks in Ham´s F12 K medium with 2 mM glutamine (GibcoBRL, USA) supplemented with 5 % fetal calf serum (FCS) and 1 % of gentamicin (PAA Laboratories, Austria) at 37 ^oC in a humidified atmosphere of 5 % CO_2 and 95 % air.

Transwell assay

H-4-II-E and H-5 cells (grown confluently) were harvested, washed and poured at 10^6 cells into 200 µl of Ham´s F12 K medium containing 1 % FCS supplemented with 2 mM glutamine. Cells were plated into the upper compartment of a Transwell migration chamber (Corning Costar Corporation, United Kingdom). Membranes with two different pore sizes (3 µm and 5 µm) were used in two separate experiments. 500 µl of Ham´s F12 K medium containing 1 % FCS and glutamine (2 mM) were added to the lower well and migrating cells were counted after 24 h incubation.

Extraction of gangliosides

Cells were washed twice with phosphate-buffer saline (PBS, PAA Laboratories, Austria), harvested by gentle scraping of the cells into the buffer, counted and centrifuged at 650xg for 10 min. Pellets were resuspended in PBS and aliquots were used for protein determination. Cells were again centrifuged and homogenized in chloroform:methanol (2:1 by volume, 2 ml per $1x10^7$ cells). Glycolipids were extracted by the method of Ladish and Gillard (1985). Collected supernatant was evaporated under a nitrogen stream, dissolved in water with 0.5 % Tween 20 (Sigma-Aldrich, USA), dialyzed against distilled water for 48 hours and lyophilized.

Protein content determination

The cells homogenized and protein content in the homogenate was determined by the modified method of Lowry (Hartree 1972) with bovine serum albumin as a standard. All reagents were purchased from Sigma-Aldrich, USA.

Thin-layer chromatography of gangliosides

The extracted gangliosides were separated by thin-layer chromatography (TLC) on silicagel 60 HPTLC plates (Merck, Germany) using the solvent system: chloroform:methanol:0.2 % $CaCl_2$ in water (55:45:11 by volume). Gangliosides were visualized by a resorcinol-HCl reagent at 120 °C for 20 min with densitometrical measurement in reflected light at 560 nm (Šmíd and Reinišová 1973). Standards of gangliosides were purchased from Matreya, USA.

TLC Immunostaining with Cholera Toxin

TLC immunostaining with cholera toxin B-subunit after sialidase treatment was performed according to a modified method of Wu and Ledeen (1985). Briefly, after chromatography, TLC plates were incubated with sialidase from *Clostridium perfringens* (Sigma-Aldrich, USA) 0.1 U/ml in 0.05M acetate buffer pH 5.0 containing 4 mM $CaCl_2$ for 2 h to convert two or more sialic acids containing gangliotetraose to GM1. The detection of GM1 was performed by cholera toxin B-subunit

solution, anticholeragenoid (goat), anti-goat IgG-biotin labelled all from Sigma-Aldrich (USA) and Vectastain ABC kit (Vector Laboratories, USA) followed by colour reaction with α-chloronaphtol/H_2O_2 solution (Bohata et al. 1998).

RESULTS

Dedifferentiated H-5 cells in comparison with H-4-II-E and H-4-II-E-C3 differentiated cells showed a sufficient mitogenic ability to form foci (fusiform). H-4-II-E and H-4-II-E-C3 cells grew as monolayer and never formed foci.

Cell migratory capacity is an important factor describing the capacity of the cells to invade their surrounding environment. This parameter was measured by a Transwell assay. No difference in the migration ability between H-4-II-E and H-4-II-E-C3 cells was found (data not shown); therefore the migration only of H-4-II-E and H-5 cells was compared in the final experiments. As shown in Fig. 1, the number of H-5 cells leaving the upper well into the lower well was on 3 μm membrane about fifteen times higher and on 5 μm membrane about four times higher than the number of migrating H-4-II-E cells.

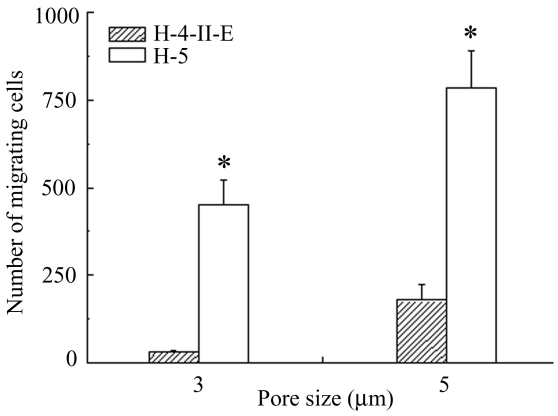

Fig. 1. **Migration of differentiated (H-4-II-E) and dedifferentiated (H-5) cell lines.** Each assay was performed in duplicate and repeated twice. Data are presented as means, each error bar represents SD, * statistically significant as compared with differentiated cells (2α=0.05).

The glycolipids were extracted from H-4-II-E, H-4-II-E-C3 and H-5 cell lines and gangliosides were detected by TLC and identified by comparison of their retention factor (Rf) with standards separated on the same TLC plate (Fig. 2). The presence of gangliosides derived from gangliotetraose (i.e. GM1, Fuc-GM1, GD1a, GD1b, GT1a and GT1b) was confirmed by cholera-toxin detection after neuraminidase treatment (Fig. 3). As shown in Fig. 2 (detection with resorcinol), the major ganglioside in all cell lines was GD1a. Gangliosides GM3 and GM2 were presented only in the dedifferentiated cell line H-5. In contrast, Fuc-GM1 ganglioside was highly expressed in H-4-II-E and in trace amounts also in H-4-II-E-C3 (differentiated cell lines). The quantification of gangliosides was determined using densitometry whilst the results are shown in Table 1.

Table 1: **Percentage distribution of sialic acid in individual gangliosides** (resorcinol detection, data from densitometry of TLC on Fig. 2).

	H-5	H-4-II-E	H-4-II-E-C3
GD1a	43	60	63
GM1	21	10	20
Fuc-GM1	-	30	18
GM2	8	-	-
GM3	28	-	-

DISCUSSION

The hepatoma cell lines derived from Reuber H-35 are the common, well-characterised experimental models used in many different studies. The original differentiated cell line established in the culture was H-4-II-E. H-4-II-E-C3 is the differentiated clone derived from H-4-II-E and H-5 is one of the spontaneously dedifferentiated clones originated from H-4-II-E-C3 (Reuber 1961, Pitot et al. 1964). The phenotype and the characterization of these cell lines have been studied extensively (Deschatrette and Weiss 1974, Zvibel et al. 1998). Other studies include the expression and function of different enzymes, receptors and signalling pathways in both differentiated and dedifferentiated clones of these cells (Wiebel et al. 1984, Garcia-Pelayo et al. 2004). In the present study we focused on the relationship between the level of differentiation of the hepatoma cells derived from Reuber H-35 and their migration potency.

Differentiated H-4-II-E and H-4-II-E-C3 cells differed from H-5 cells in terms of cellular morphology. H-5 cells formed foci (fusiform), but H-4-II-E and H-4-II-E-C3 cells grew as monolayers. Similar changes in morphology have been described on fibroblasts obtained from sialyltransferase-I knockout mice (Hashiramoto et al. 2006). As shown in Fig. 1, dedifferentiated H-5 cells had a significantly higher capacity of

migration compared with H-4-II-E, which is in line with the results of Hashiramoto et al. (2006).

The ability of tumour cells to adhere to extracellular matrix proteins is critical for migration and invasion. Many recent studies indicate that the gangliosides play essential roles in cell adhesion and modulation of signal transduction. This suggested an examination of the correlation between the expression of gangliosides and the migratory capacity of differentiated and dedifferentiated cell lines derived from Reuber H-35. The ganglioside patterns of H-4-II-E, H-4-II-E-C3 cells differed from H-5 cells, which highly expressed GM3 (Fig. 2). This ganglioside was not detectable in H-4-II-E and H-4-II-E-C3 cells lines. Recent studies have shown that GM3, a glycosphingolipid containing monosialic acids, is thought to be one of the key molecules involved in signal transduction of mammalian cells. For instance, embryonic fibroblast cells obtained from sialyltransferase-I knockout mice (which do not express GM3) show unique characteristics in mitogenic signalling (Hashiramoto et al. 2006). Our results are also in line with the report of Saha et al. (2005) who showed that GM3 ganglioside enhanced the migratory capacity of mouse B-16 melanoma cells.

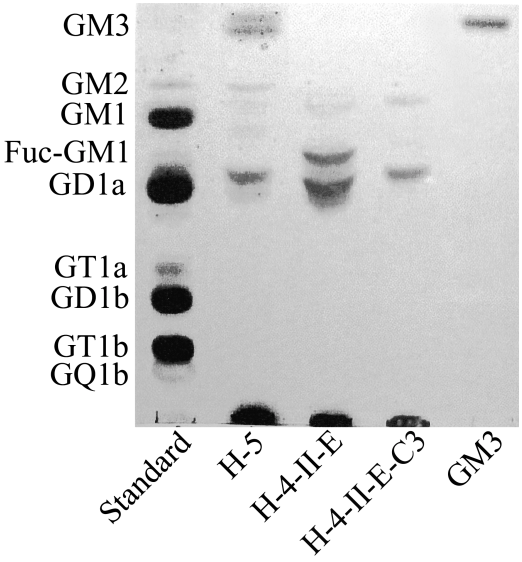

Fig. 2. **TLC of gangliosides extracted from H-5 (dedifferentiated), H-4-II-E and H-4-II-E-C3 (both differentiated) cell lines with resorcinol-HCL detection.** Gangliosides extracts equal to 7 mg of protein content were chromatographed on a HPTLC plate in chloroform:methanol:0.2% $CaCl_2$ in water (55:45:11 v/v) and visualized by resorcinol-HCL reagent.

Although a correlation between a high level of GM3 and the cell motility of different cell types has been recently reported, the relationship between GM3 and cell migration is still a controversial issue. Mitsuzuka et al. (2005) has recently shown a correlation between a high level of GM3 and a low motility of cells in urinary bladder cancer cells. The role of gangliosides in the regulation of cell adhesion and signalling pathways has been poorly characterized and is still under investigation. Our study is the first to demonstrate an association between the ganglioside GM3 and cell motility of hepatoma cell lines. Further studies are needed to explore the precise mechanism of this association.

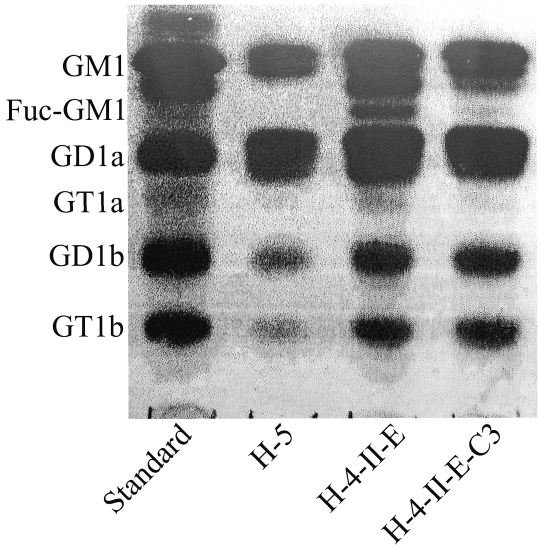

Fig. 3. **TLC of gangliosides extracted from H-5 (dedifferentiated), H-4-II-E and H-4-II-E-C3 (both differentiated) cell lines with immunostaining detection.** Gangliosides extracts equal to 50 μg of protein content were chromatographed on a HPTLC plate in chloroform:methanol:0.2% $CaCl_2$ in water (55:45:11 v/v) and visualized by immunostaining with cholera toxin followed with ABC kit and colour reaction.

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