Influence of extracellular matrices on ganglioside pattern of two hepatoma cell lines with different adhesive properties

Martin Fritsch*, Christoph C. Geilen**, Claudia Heidrich, Werner Reutter

Institut für Molekularbiologie und Biochemie der Freien Universität Berlin, D-14195 Berlin-Dahlem, Germany

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Abstract **A cell culture model was developed to investigate the involvement of gangliosides in cell–matrix adhesion. Two cell lines with different adhesive properties derived from solid Morris hepatoma 7777 were established. Cultured in horse serum-containing medium, the adhesive cell line (MH 7777_A) adheres and spreads on uncoated culture dishes, whereas the revertant cell line (MH $7777_{A>N}$) does not adhere and grows in suspension. The adhesiveness of both cell lines is dependent on the coating protein used (none, bovine serum albumin, fibronectin or collagen I) and the horse serum concentration in the culture medium. Both cell lines, although of the same origin, differed in their ganglioside composition. The most abundant ganglioside of both MH 7777_A and MH $7777_{A>N}$ cell lines was fucosyl-G_{M1}, 0.78 and 0.72 μg per mg cellular protein, respectively. The G_{M3} and G_{D1a} content of MH $7777_{A>N}$ cells was significantly higher than that of MH 7777_A cells. Furthermore, a matrix-dependency of the ganglioside pattern of both cell lines was demonstrated.**

Key words: Ganglioside; Cell-matrix adhesion; Extracellular matrix; Morris hepatoma 7777 cell line

1. Introduction

Gangliosides, sialic acid-containing glycosphingolipids, are found in nearly all mammalian tissues and cell lines. Despite their ubiquitous occurrence and their intensively studied metabolism [1–6], their function is not fully understood (for review see [7,8]). It has long been claimed that gangliosides and other glycosphingolipids are involved in cell adhesion. This includes cell–cell as well as cell–matrix interaction. Evidence that cell–cell adhesion may be mediated by glycosphingolipids was provided recently by the specific recognition of sialyl Lewis^x tetrasaccharides by E-selectin [9] and the adhesion and spreading of G_{M3}-expressing cells on various neutral glycosphingolipids [10]. In case of cell–matrix interactions, it was shown that gangliosides may be necessary to mediate the formation of fibrillar strands of fibronectin on the cell surface [11,12], but they seem not to be essential for cell spreading on fibronectin-coated plastic dishes [13].

In the present study, two cell lines derived from solid Morris hepatoma 7777 were established to investigate the role of gangliosides in adhesion to substrates of the extracellular matrix. One of these cell lines, called MH 7777_A, has the ability to adhere and spread on uncoated culture dishes in horse serum-containing medium. A revertant cell line, MH $7777_{A>N}$, has lost this property and grows in suspension. Furthermore, we analysed and characterized the ganglioside pattern of these cell lines and focused our interest on alterations of the ganglioside pattern induced by an altered adhesion state and by different adhesion matrices.

*Corresponding author.

***Present address:* Haut- und Poliklinik des Universitätsklinikums Benjamin Franklin, Hindenburgdamm 30, D-12200 Berlin, Germany.

2. Materials and methods

2.1. Establishment of the cell lines and culture conditions

The cultivation of an adherent cell line from Morris hepatoma 7777 by propagation of cells isolated directly from the solid carcinoma was performed as described [14]. The resulting cell line, called MH 7777_A, was cultured on plastic dishes (Becton/Dickinson, New Jersey, USA).

A revertant cell line, lacking the ability to adhere to uncoated cell culture plates, was established by the following procedure. In confluent cultures of the original adherent cell line some cells detached from the plastic surface and grew as floating aggregates. These aggregates were selected and dissociated using a glas pipette and again seeded. Repeating this procedure for about 20 times led to cells with stable, non-adhesive properties. Cloning of the cell line was performed once by limited dilution in a 96-well plate. The resulting non-adhesive cell line (named MH $7777_{A>N}$) was cultured in suspension in plastic flasks (Becton/Dickinson, New Jersey, USA).

Both cell lines were propagated in Dulbecco's modified Eagle's medium (DMEM) (Biochrom, Berlin, Germany) containing 10% heat-inactivated horse serum, 4 mmol/l L-glutamine (Merck, Darmstadt, Germany), 10 nmol/l insulin (Hoechst, Frankfurt/M., Germany), 1 μmol/l dexamethasone (Sigma, München, Germany), penicillin (100.000 U/l)/streptomycin (100 mg/l) (Boehringer, Mannheim, Germany). Cells were cultivated in 5% CO_2 in a humified incubator at 37°C. Cells in the stationary growth phase were used for all experiments.

Fibronectin was isolated from horse serum according to the method of Ruoslahti et al. [15] and collagen I was isolated from rat tail according to Strom and Michalopoulos [16].

2.2. Isolation, purification and separation of gangliosides

Gangliosides were extracted and purified according to a modification of the method published by Riboni et al. [17]. Briefly, cells were disrupted by an osmotic shock and the membranes were pelleted for 30 min at 100,000 × *g*. Total lipids were extracted twice with chloroform/methanol/water (4:8:3 by vol.) and once with chloroform/methanol (1:1, v/v) and (2:1 v/v), respectively. Gangliosides were separated from most phospho- and neutral lipids by phase partitioning according to Ladisch and Gillard [18]. Subsequently, the crude ganglioside fraction was submitted to alkaline methanolysis to remove the remaining glycerophospholipids [19]. The gangliosides were desalted by reversed-phase chromatography on Sep-Pak C18 cartridges (Waters Ass., Massachusetts, USA) [20].

For characterization, gangliosides were distinguished according to their neuraminic acid content in a mono-, di- and polysialated fraction using a DEAE-sepharose column according to the method of Fredman et al. [21]. Separation was performed on HPTLC plates (Merck, Darmstadt, Germany) using chloroform/methanol/0.2% aqueous $CaCl_2$ (50:45:11 by vol.) as solvent system. Gangliosides were stained by dipping the developed HPTLC-plates in a resorcinol/HCl solution, followed by heating the plate, face down, at 125°C for 30 min. Gangliosides were quantified using a video-densitometer (Biotec-Fischer, Reiskirchen, Germany) and ganglioside standards of known concentrations (Dr. Pallmann KG, München, Germany). Fucosyl-G_{M1} standards

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were a gift from Dr. A. Guiliani (University of Milan, Italy) and Dr. R. Ghidoni (University of Catania, Italy) and Dr. S.-i. Hakomori and Dr. F. Nudelman (The Biomembrane Institute, Seattle, WA, USA). Both fucosyl-G_{M1} standards used showed the same R_f-values on HPTLC. The detection was linear in the range 0.05 to 1.5 μg ganglioside per band.

2.3. Labelling of gangliosides

To characterize fucose-containing gangliosides, cells were labelled for 24 h with medium containing 1 μCi/ml L-[6-^{3}H]fucose (Amersham, Braunschweig, Germany). Gangliosides were isolated and purified as described above and the incorporation was visualized by autoradiography (Kodak XR-5, Rochester, USA). The radioactive ganglioside standards, G_{M1} and G_{M3}, were a gift from Dr. S. Sonnino (University of Milan, Milan, Italy).

2.4. Digestion of gangliosides by neuraminidase

About 3 nmol of gangliosides were dried in a reaction tube under N_2, then solubilized in distilled water. Sodium-acetate buffer (pH 4.8) was added to a final concentration of 0.02 mol/l. The sample was digested with 0.1 U neuraminidase from *Arthrobacter ureafaciens* in a volume of 50 μl for 48 h at 37°C. After desalting, the water was evaporated and the samples were redissolved in chloroform/methanol (1 : 1, v/v).

2.5. Cell adhesion assay

The adhesion assays were performed in 96-well ELISA plates using a staining procedure described by Gillies et al. [22]. The plates were used uncoated or coated for 1 h as described in Results. Prior to use, the coated plates were washed thoroughly with D-PBS. Cells were washed twice with ice-cold D-PBS and incubated with 0.1% trypsin in D-PBS/EDTA for 1 min. Subsequently, cells were seeded at a density of 20,000 cells/well in 200 μl of medium. At given time points, cells were washed once with D-PBS followed by fixation for 15 min with 100 μl 1% glutaraldehyde solution in D-PBS. Subsequently, DNA was stained for 30 min with 0.1% Crystal violet solution in D-PBS. After intense washing with distilled water, the dye bound to DNA was solubilized in 125 μl of 0.2% Triton X-100 in D-PBS. Absorption was measured at 570 nm against 410 nm as the reference wave length using a microplate reader (Dynatech MR600, Alexandria, USA). The determined absorption was used as an index of cell adhesion.

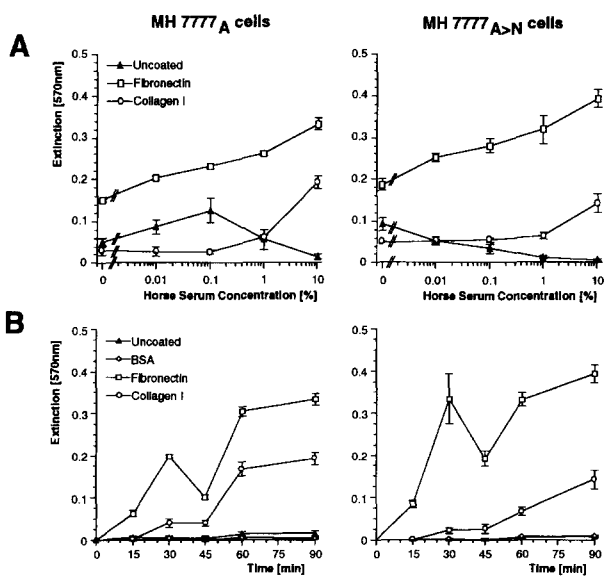

Fig. 1. Horse serum and time dependent adhesion of MH 7777_A and MH $7777_{A>N}$ cell lines on different substrates. (A) Adhesion dependency on horse serum concentrations. Plates were coated with 50 μg/ml of fibronectin or collagen I for 1 h and washed thoroughly with D-PBS, or used uncoated. Cells were seeded in medium supplemented with horse serum as indicated at a density of 20,000 cells/well and incubated for 90 min. Cells were fixed and stained as described. (B) Time-dependent adhesion. Plates were coated as described in A. In addition, plates coated with 1% BSA for 1 h were used as negative control. Cells were seeded in a density of 20,000 cells/well in medium containing 10% horse serum. At the time points indicated, cells were fixed and stained as described. Values represent the mean of triplicate experiments; the standard deviation is shown as a vertical bar, which may be within the symbol.

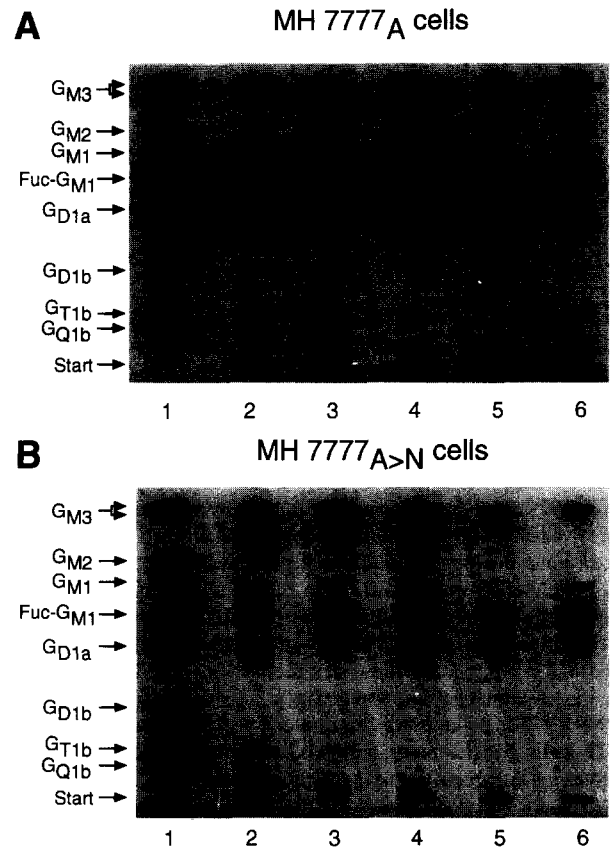

Fig. 2. Alteration of the ganglioside content and pattern of MH 7777_A and MH $7777_{A>N}$ cells incubated on different substrates. Gangliosides from confluent MH 7777_A cells (7×10^6 cells/dish 10 cm ⌀) and dense aggregates of MH $7777_{A>N}$ cells (8×10^6 cells/flask) grown as described in section 2 were used as controls (lanes 2). Cell culture dishes were coated as described in Fig. 1. Cells were incubated for 30 min on the different substrates at the same density as for the adhesion assay (52,000 cells/cm^2). Gangliosides were isolated, purified, separated and stained on a HPTLC-plate as described. (A) MH 7777_A cells, gangliosides in each line corresponding to 2.8 mg cell protein. (B) MH $7777_{A>N}$ cells, gangliosides in each line corresponding to 1.5 mg cell protein. Lanes 1: standard gangliosides (1.5 μg each) as indicated on the left side of the figure; lanes 2: gangliosides of control cells as described above; lanes 3: gangliosides of cells incubated on untreated dishes; lanes 4: gangliosides of cells incubated on BSA coated dishes; lanes 5: gangliosides of cells incubated on collagen I coated dishes; lanes 6: gangliosides of cells incubated on fibronectin coated dishes.

3. Results

3.1. Adhesive properties of MH 7777 $_A$ and MH $7777_{A>N}$ cells

The revertant, non-adhesive cell line MH $7777_{A>N}$ was selected and cloned in DMEM culture medium containing 10% horse serum using uncoated cell culture dishes. To test the adhesion on substrates other than plastic, culture dishes were coated with fibronectin and collagen I. First, the concentration of each protein giving maximal cell adhesion was determined. Coating of cell culture dishes with 50 μg protein/ml D-PBS for

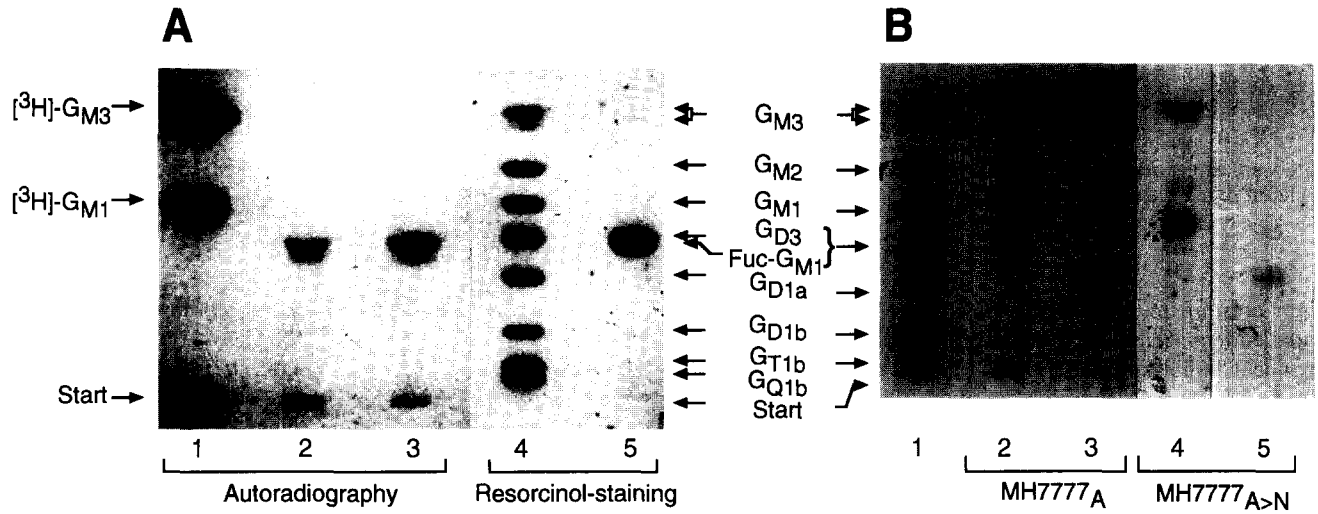

Fig. 3. Characterization of Morris Hepatoma 7777 cell line gangliosides. (A) Labelling of MH 7777_A and MH $7777_{A>N}$ cells with L-[6-^{3}H]fucose. $3 \cdot 10^6$ cells of each cell line were labelled for 24 h in 10 ml cell culture medium containing 10 μCi L-[6-^{3}H]fucose. Gangliosides were extracted, purified and separated as described in section 2. The part of the HPTLC-plate which contains the labelled gangliosides (lane 1, 2 and 3) was visualized by autoradiography for 2 months as described. Standard gangliosides separated on the same plate in lane 4 and 5 were stained with resorcinol as described. Lane 1: standard [^{3}H]G_{M1} and [^{3}H]G_{M3} (10.000 dpm each); lane 2: gangliosides from MH $7777_{A>N}$ cells corresponding to 1 mg cell protein; lane 3: gangliosides from MH 7777_A cells corresponding to 1 mg cell protein; lane 4: standard gangliosides (1 μg each) as indicated on the right side of the figure; lane 5: standard Fuc-G_{M1}. (B) Separation of MH 7777_A and MH $7777_{A>N}$ cell gangliosides according to their charge. Total cell gangliosides equivalent to 1 mg cell protein were poured on a DEAE-sepharose anion exchange column and were eluted stepwise with increasing concentrations of methanolic ammonium acetate as described [21]. Lane 1: standard gangliosides (1 μg each) as indicated on the right side of the figure; lane 2: monosialylated; and lane 3: disialylated fraction of MH 7777_A cells; lane 4: monosialylated; and lane 5: disialylated fraction of MH $7777_{A>N}$ cells. The fractions eluting with higher ammonium acetate concentrations did not show any resorcinol-stainable bands.

1 h was sufficient for both matrix proteins. We then determined the maximal number of cells that still gave a linear correlation between the cell number and the measured extinction; this number was 20,000 cells per well of a 96-well plate. Using these data, the adhesion for both cell lines on the different substrates was assayed (Fig. 1A).

The results show that MH 7777_A and MH $7777_{A>N}$ cell lines adhere to collagen I and to fibronectin. In the case of fibronectin, for both cell lines increasing amounts of horse serum resulted in increasing adhesion. On collagen I, adhesion of both cell lines was enhanced by horse serum too, but total adhesion always remained less than on fibronectin. Furthermore, concentrations higher then 0.1% (MH $7777_{A>N}$) and 1% (MH 7777_A) horse serum were necessary for clear stimulation. Taken together, both cell lines showed similar adhesion characteristics on fibronectin and collagen I with different concentrations of horse serum. On uncoated dishes, MH 7777_A cells showed increased adhesion with increasing concentrations of horse

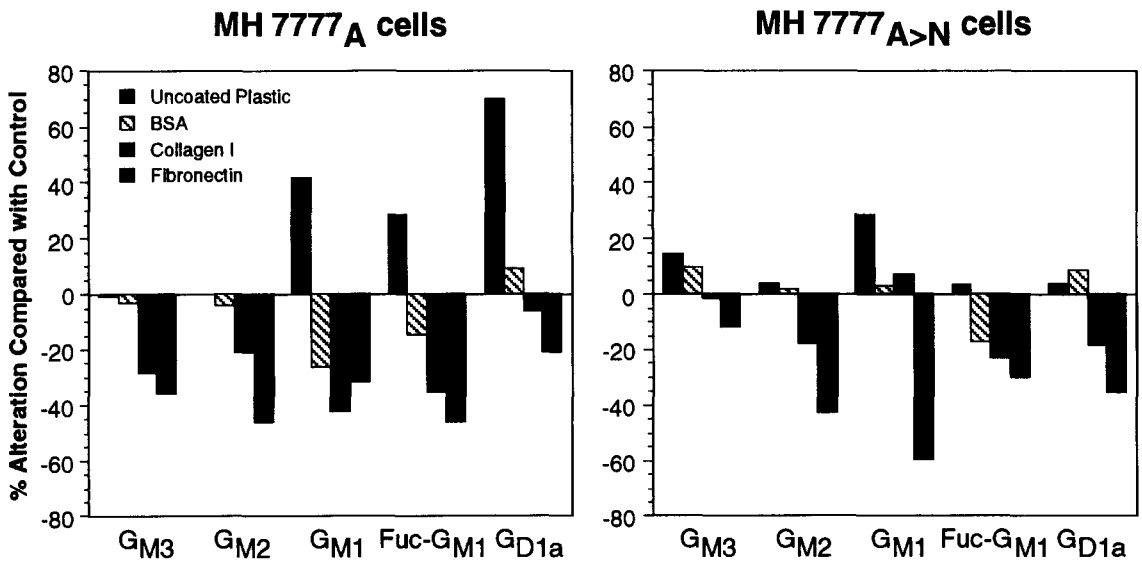

Fig. 4. The amount of gangliosides from MH 7777_A and MH $7777_{A>N}$ cells incubated on different substrates as shown in Fig. 2 were evaluated with a video-densitometer. Alterations in the contents of specified gangliosides are given as a percentage of the values for control cells.

Table 1
Ganglioside distribution and content of MH 7777_A and MH $7777_{A>N}$ cells

	G_{M3}[1]	G_{M2}	G_{M1}	Fuc-G_{M1}	G_{D1a}[2]	total[3]
MH 7777_A	0.39 ± 0.03[a]	0.03 ± 0.01	0.17 ± 0.01	0.78 ± 0.04	0.25 ± 0.03	1.62 ± 0.11
MH $7777_{A>N}$	0.59 ± 0.06	0.11 ± 0.03	0.19 ± 0.03	0.72 ± 0.03	0.67 ± 0.12	2.27 ± 0.25

[a] Values are expressed in μg ganglioside per mg cellular protein. Results are the mean ± S.D. of three independent experiments. Values showing a significant difference using Student's *t* test were indicated by [1]: $P < 0.02$, [2]: $P < 0.01$ and [3]: $P < 0.05$.

serum only up to 0.1%, but higher concentrations of horse serum led to a decrease in adhesion. In contrast, a horse serum dependent inhibition of MH $7777_{A>N}$ cell adhesion was observed. In both cases, the adhesion of the cell lines on uncoated dishes without horse serum was higher than on collagen I under the same conditions.

In further experiments, the time course of adhesion for both cell lines on different substrates was determined (Fig. 1B). Similar results were obtained for both cell lines. Surprisingly, adhesion to fibronectin showed a first maximum 30 min after seeding, followed by a decrease after 45 min and then a continously increase. A biphasic adhesion was also observed on collagen I; after a first increase the adhesion remained constant for a certain time, which was followed by a continous increase, as also found for fibronectin. This time course was confirmed by three independent experiments, and it also occurred in the absence of horse serum. After 90 min, the adhesion of the adherent cell type to uncoated dishes was still small. In the presence of 10% horse serum, the cells begin to spread on uncoated dishes after 3 h.

3.2. Characterization of the ganglioside pattern

Next, the ganglioside pattern of the original cell line and its revertant were examined. The gangliosides were isolated from cells in the stationary growth phase. The main glycolipid bands of both cell lines, which give a specific ganglioside staining reaction with resorcinol, comigrate with G_{M3}, Fuc-G_{M1} or G_{D3}, and G_{D1a} of the standard ganglioside mixture (Fig. 2A/B, lanes 1 and 2).

For further characterization, the gangliosides from both cell lines were digested with neuraminidase from *Arthrobacter ureafaciens*. In both cases, the band comigrating with G_{D1a} was replaced by a band comigrating with G_{M1}, and the band comigrating with G_{M3} vanished. The bands in the region of G_{M2} and G_{D3}/Fuc-G_{M1} could not be digested. This suggests that the second of these bands may be identical with Fuc-G_{M1} rather than with G_{D3}. To test this possibility, cells were incubated with L-[6-^{3}H]fucose as described. The band in question became labelled (Fig. 3A), confirming that it is probably identical to Fuc-G_{M1}. For quantification of the cellular gangliosides, it was necessary get further hints that the band in the G_{D3}/Fuc-G_{M1} region was not a mixture of both gangliosides. Therefore, the gangliosides of both cell lines were separated on a DEAE-Sepharose anion exchange column according to their charge in a mono-, di- and polysialylated ganglioside fraction (Fig. 3B). For both cell lines, only the band comigrating with the G_{D1a} standard appears in the disialylated fraction, whereas the band in the G_{D3}/Fuc-G_{M1} region was eluted in the monosialylated fraction. These results further confirm the identity of these bands with authentic Fuc-G_{M1} and G_{D1a}. The total ganglioside content of the cells was given in Table 1.

3.3. Altered ganglioside pattern in relation to the adhesion substrates

The main difference in the ganglioside pattern between MH 7777_A and MH $7777_{A>N}$ cells is the content of G_{M3} and G_{D1a}. It is not clear whether the altered ganglioside pattern depends directly on the different adhesion properties of the cell lines. It is still possible that the selecting and cloning procedure has produced two cell lines with genetically different ganglioside metabolism. To rule out this possibility, the relationship between ganglioside pattern and cell–matrix interaction was measured in a more direct way.

Therefore, the ganglioside pattern of both cell lines on different substrates was determined after 30 min. This time point was selected because data from the literature indicate that gangliosides are probably involved in early events of cell adhesion. Furthermore, our own data on the adhesion of both cell lines revealed a first adhesion maximum after 30 min, and this might be connected in some way with the expression of gangliosides. The results are shown in Figs. 3 and 4.

There was a clear correlation between the ganglioside pattern and the adhesion-state of the cells. In mH 7777_A cells, the amount of the more complex gangliosides, G_{M1}, Fuc-G_{M1} and G_{D1a}, increased dramatically on uncoated dishes. On fibronectin, and to a lesser extent on collagen I, all gangliosides showed a similar rate of decrease. The smallest decrease is seen in cells incubated on BSA. In contrast, the increase of adhesion to uncoated dishes is much less for MH $7777_{A>N}$ cells, and changes were only seen in the levels of G_{M1} and G_{M3}. As for MH 7777_A cells, the total gangliosides decreased on fibronectin and collagen I. On BSA, no clear tendency was seen (Fig. 4).

4. Discussion

The involvement of glycosphingolipids in cellular adhesion processes is well known, but their role in adhesion and spreading of cells, and the interaction of glycosphingolipids with adhesion receptor proteins at the cell surface is not fully understood [11–13,23–25]. In the present study we developed a cell culture model to determine serum-dependent cell–matrix adhesion and to investigate the involvement of gangliosides in this process.

It was shown that the adhesiveness of both cell lines, MH 7777_A and MH $7777_{A>N}$, was matrix- and serum-dependent and that both cell lines showed matrix-dependent changes in their ganglioside pattern. These matrix-dependent alterations of gangliosides are in agreement with results of Augustin-Voss et al. [25]. Using lectin-binding studies they showed that the expression of glycoconjugates on the surface of an epithelial cell line can be modulated by adhesion on different extracellular matrices.

In a study on the relationship between gangliosides and integrins in the adhesion of a melanoma cell line to vitronectin it

was shown that in the presence of Ca^{2+} the ganglioside G_{D2} co-localizes with the vitronectin receptor and is able to enhance the affinity of the receptor to its substrate. Furthermore, a synergistic effect on the delay of cell adhesion was detected with both the monoclonal antibodies against G_{D2} and G_{D3} and a synthetic peptide containing the RGD-sequence [26]. These results as well as those presented in the present paper, which suggest a regulatory role of gangliosides in the early phase of cell adhesion, agree well with investigations on the ganglioside pattern of cell adhesion plaques. A redistribution of complex gangliosides into the adhesion-involved domains of the plasma membrane was demonstrated after 3 h, but was no longer detectable 24 h after the cells had been plated [27].

In conclusion we suggest that gangliosides play an important role as a docking promotor in the first phase of cell–matrix adhesion. Subsequently this docking seemed to be followed by protein-dependent adhesion processes. Both cell lines, MH 7777_A and MH $7777_{A>N}$, will be useful tools to obtain new insights into the mechanisms of adhesion and the role of glycosphingolipids in this process.

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