often ambiguous and affected by widely differing levels of "probability".

In an attempt to introduce some standardised criteria, we proposed an operational method based on the present knowledge concerning diagnostic examinations currently used for liver cancers. The feasibility of the method was first tested in a hospital-based pilot study and second in a population-based study, i.e. in the same context in which cancer registries usually work. The proportion of PLC-defined cases was similar in the two studies, and no differences were observed with respect to the age, sex or hospital of admission of the patients (Table 3).

Our study shows that approximately 20% of routinely diagnosed liver cancer could not be defined as other than "unspecified" (ULC-cases). These ULCs represent a crucial point in cancer registration, since including or excluding them could lead, respectively, to an over or underestimation of the incidence.

Since a definite solution does not seem possible at present, we suggest that cancer registries put some effort into measuring and publishing the proportion of these cases, in order to improve comparability of international data. Further research addressing both validity of instrumental examinations and interobserver agreement would be particularly helpful in the clinical practice context.

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Acknowledgements—We are grateful to Marisa Coato and Vincenzo Tomasoni for their technical help and to Mr Suppi and Mr Uber from the Data Processing Centre of Local Health Unit No. 25 (Verona) who kindly provided hospital Discharge Codes. We are also indebted to Dr Franco Berrino for his helpful comments and suggestions.

Pergamon

European Journal of Cancer Vol. 31A, No. 1, pp. 79–84, 1995

0959–8049/95 $9.50+0.00

0959-8049(94)00436-6

Possible Involvement of Tumour Cell Membrane Gangliosides in Platelet–Tumour Cell Interactions

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The possible correlation(s) between platelet proaggregating activity, and sialic acid content and ganglioside expression of six human colorectal tumour cell lines (CBS, GEO, HT-29, WiDr, MIP and DLD-1) was evaluated. The three cell lines (HT-29, WiDr and DLD-1) capable of inducing remarkable *in vitro* platelet aggregation, had significantly higher amounts of lipid-bound sialic acid than those cell lines characterised by a lower platelet proaggregating activity (GEO, CBS and MIP). High performance thin-layer chromatography demonstrated the presence of one band comigrating with GM3 in all cell lines, while GD1a and GT1b comigrating gangliosides were present only in HT-29, WiDr and DLD-1 cells. Finally, an increased platelet pro-aggregating activity of GEO and CBS cell lines was observed after the incorporation of exogenous gangliosides. The present data support the hypothesis that lipid-bound sialic acid may be involved in platelet–tumour cell interactions.

Key words: platelets, platelet aggregation, metastases, sialic acid, gangliosides, colon cancer
Eur J Cancer, Vol. 31A, No. 1, pp. 79–84, 1995

INTRODUCTION

GANGLIOSIDES (Gs) ARE acidic glycosphingolipids characterised by the presence of one or more residues of sialic acid in the oligosaccharide head group. They may be possibly involved in basic cellular functions, such as regulation and maintenance of growth factor receptor functions, and cell adhesion and recognition. For example, it has been shown that GM3 and GM1 may regulate the activity of fibroblast growth factor (FGF) and the receptor function for platelet derived growth factor (PDGF), respectively [1]. Monoclonal antibodies directed against GD2 and GD3 inhibit and/or reverse the adhesion and spreading of cells onto fibronectin and related RGD-containing substrates [2]. Recently, the receptor for vitronectin has been shown to exist in a divalent cation-dependent complex with gangliosides [3].

A defective synthesis of these gangliosides, would, therefore, account for either loss or reduction of growth control and anchorage-dependent cell proliferation, the most common denominators of oncogenesis. Several studies with monoclonal antibodies have revealed unusual accumulation of certain glycolipids in human cancer, such as GD3 ganglioside in melanoma [4,5], Gb3 in Burkitt's lymphoma [6], Sialosyl-Le^a ganglioside in colorectal, gastric and pancreatic cancers [7], 6C-fucoganglioside in colorectal and hepatic cancer [8], and di- or trifucosyl type 2 chain with Le^x determinant in common human adenocarcinomas [9]. However, no definitive information is available on whether glycolipids play a functional role in defining tumorigenicity and metastatic properties, although a loss of GM3 crypticity and an enhanced level of GT or GD gangliosides seem to correlate to a higher metastatic potential in melanoma [1]. Moreover, the degree of sialylation in glycolipids and in glycoproteins has been claimed to be closely related to the degree of metastatic properties [10,11]. For example, the increased expression of sialyl dimeric Le^x antigen has been detected in the liver metastases of human colorectal carcinoma [12], and the surface expression of this P-selectin ligand was positively related to the metastatic potential of colorectal carcinoma cells in human samples [13]. In addition, it has been reported that a mixture of exogenous gangliosides stimulates the growth and the metastatic potential of 3LL cells either *in vitro* or *in vivo* [14]. More recently, Coulombe and associates [15], using the H59 murine tumour cell line, have suggested a possible relationship between GM2 and the establishment of metastases in the liver.

Meanwhile, the ability of certain types of human tumour cells to induce *in vitro* platelet aggregation has been used as indirect evidence to explain the potential role of platelets in the development of haematogenous metastases [16]. Different mechanisms are now accepted to explain the nature of platelet–tumour cell interaction(s) including generation of thrombin by tumour cells [17–19]. Moreover, cell surface sialylation of two human tumour cell lines has been correlated with their platelet activating properties. These observations suggest that tumour cell surface sialylation could be one of the mediators in the phenomenon of platelet activation induced by tumour cell lines, although it cannot be considered the only mediator of tumour cell induced platelet aggregation [11,20,21].

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Revised 13 Aug. 1994; accepted 5 Sep. 1994.

Recently, it has been suggested that both glycoprotein (Gp)Ib and Gp IIb/IIIa complex, present on the platelet surface, are involved in the thrombin-dependent and -independent platelet aggregation induced by tumour cells [22–24]. Moreover, other authors have reported that the ligand binding activity of platelet glycoprotein IIb/IIIa complex may be modulated by gangliosides. In fact, exogenous gangliosides can inhibit platelet adhesion to fibronectin, fibrinogen and von Willebrand Factor with the following order of effectiveness: GT1b > GD1a > GM1 > asialo GM1 [25].

We have previously shown that human colorectal tumour cell lines induce platelet aggregation to various extents and in a dose-dependent manner, but without any correlation with the degree of differentiation [26]. Platelet proaggregating activity of these cell lines is not ADP-related, and is probably due to a thrombin-dependent mechanism. The present study was designed to evaluate whether lipid-bound sialic acid, the relative amount of tumour cell membrane gangliosides, or a particular class of gangliosides, may correlate to the tumour cell proaggregating activity of six human colorectal tumour cell lines (CBS, GEO, HT-29, WiDr, MIP and DLD-1).

MATERIALS AND METHODS

Cell lines and culture conditions

Six different human colorectal tumour cell lines were studied (GEO, CBS, HT-29, WiDr, MIP and DLD-1) (kindly provided by Dr Jeffrey Schlom, NIH, NCl, Bethesda, Maryland, U.S.A.). Cells were routinely grown in RPMI 1640 medium supplemented with glutamine (1×), 50 μg/ml gentamicin, and 10% heat-inactivated foetal bovine serum (FBS). All established cell lines were routinely subpassaged every 5 days. Tumour cells were harvested by decanting the culture medium, washing the monolayer twice with RPMI 1640 medium, and then treating them for 5 min with 1% trypsin EDTA at 37°C. The cell suspension was centrifuged at 300 *g* for 10 min, the supernatant solution was removed, and the cell pellets were washed twice with Phosphate Buffered Saline (PBS) without Ca^{++} and Mg^{++} (pH 7.2). Tumour cells were finally resuspended in PBS without Ca^{++} and Mg^{++} at a final concentration of 1×10^7 cells/ml. Viability was determined by trypan blue exclusion. Viable cells were always greater than 95%.

Incorporation of exogenous gangliosides

Monolayers of GEO, CBS, HT-29 and MIP cell lines were washed twice with RPMI 1640 medium without FBS. Purified GM3 and GD1a (Sigma Chemicals Co, St Louis, U.S.A.) (10 μg/10^6 cells), or a mixture of the two (5 μg/10^6 cells of each ganglioside) were then added to the cell monolayers. After 1 h incubation at 37°C, cells were washed twice with complete medium to remove the excess of gangliosides, and harvested as described before.

Platelet aggregation studies

Platelet rich plasma (PRP) was obtained as previously described [26]. In all cases, PRP was adjusted to a final count of 3.5×10^5/μl by dilution with autologous PPP (Platelet Counter PL100 Sysmex TOA). Platelet proaggregating activity was evaluated in a four channel aggregometer, at 37°C and under continuous stirring at 1000 rpm. Tumour cell suspensions, 150 μl (1.5×10^6 cells) were added to 300 μl of PRP; as a control the same PRP (300 μl plus 150 μl PBS w/o Ca^{++} and Mg^{++}, pH 7.2) was stimulated by the addition of adenosine diphosphate (ADP) at a final concentration of 2 μM.

Ganglioside extraction and sialic acid determination

Gangliosides were extracted as previously described by Svennerholm and Fredman [27]. Briefly, homogenised cells (3×10^7) were extracted twice with chloroform : methanol : water (4 : 8 : 3 v/v/v). After centrifugation, the resulting supernatants were pooled, and distilled water was added to give a final chloroform/methanol/water ratio of 1 : 2 : 1.4. The solvents were carefully mixed and then centrifuged for 30 min at 2000 *g* at room temperature to yield two phases. The upper phase was removed and the volume of the lower phase measured. Additional methanol (half of the lower phase volume) and 0.01 M KCl (20% of the total volume of the lower phase plus added methanol) were added to the lower phase, which was mixed and then centrifuged for 30 min at 2000 *g* at room temperature. The two upper phases were combined and evaporated to dryness in a rotary evaporator (Buchi RE 121, Brinkman, Westbury, New York, U.S.A.). The dried residue was redissolved in 2 ml of 0.1 M KCl, and recycled three times over a Supelclean LC18 tube (Supelco, Bellefonte, Pennsylvania, U.S.A.), previously activated three times with 20 ml chloroform/methanol (2 : 1) and 10 ml methanol, and then with 20 ml 0.1 M KCl. The bound material was eluted with 15 ml of methanol and 5 ml of chloroform : methanol (2 : 1 v/v).

In some experiments, gangliosides were also extracted according to the method of Ladisch and associates [28]. The overall procedure involved three main steps. The first step consisted of total lipid extraction, using chloroform/methanol (1 : 1), that markedly reduced the high mucin concentrations interfering in Gs extraction. Next, the dried Gs were separated by partitioning in disopropylether-1-butanol-water (6 : 4 : 5). Finally, the aqueous lower phase containing the gangliosides was desalted and chromatographed as described below. Lipid-bound sialic acid was measured according to the method of Jourdian and colleagues [29]. The absorbances were determined at 630 nm in a Perkin-Elmer Lambda 4B spectrophotometer.

High-performance thin-layer chromatography

Analytical precoated silica gel 60 HPTLC plates (Merck, Darmstadt, Germany) were activated at 100°C for 30 min. Extracts equivalent to 3×10^7 cells were spotted onto plates, and chromatography was performed in chloroform/methanol/0.25% aqueous KCl (5 : 4 : 1). Plates were air-dried and the gangliosides visualised with resorcinol spray reagent. Ganglioside markers (GM3, GM2, GM1, GD1a, GD1b, GT1b) (Sigma Chemicals Co) were used as standards. In some experiments, purified Gs were added to cell extracts as internal standards.

A densitometric analysis of gangliosides, visualised by resorcinol staining on HPTLC plates, was performed using the computer software NIH Image 5.1 (National Institutes of Health, Bethesda, Maryland, U.S.A.). A semiquantitative determination of ganglioside content was obtained referring the peak area of the bands present in the different cell extracts to the peak area of known amounts of the correspondent ganglioside standards. Values are calculated as ng/10^6 cells.

Immunostaining

Extracts from colon cancer cells were separated by thin-layer chromatography, using HPTLC aluminum-backed silica gel 60 (20 × 20) plates (Merck). GD3 and GD2 purified standards (Sigma Chemicals Co) were used as positive controls. Plates were soaked in a 1% solution of polyisobutylmethacrylate in hexane for 90 s, air-dried, and incubated in blocking solution (1% bovine serum albumine (BSA) in PBS with 0.05% polyoxyethylenesorbitanmonolaurate (PBS-Tween 20)) for 30 min at room temperature, and then washed with PBS-Tween 20. The plates were then incubated for 1 h at room temperature with 70 μg/ml of antiGD3 or antiGD2 monoclonal antibodies (MAbs) (Behring, Scoppito, l'Aquila, Italy) diluted in 1% BSA-PBS-Tween 20. MAbs were then removed, and plates were washed twice for 5 min with PBS-Tween 20. Horseradish (HRP)-conjugated goat antimouse immunoglobulins (Sigma Chemicals Co), diluted 1 : 200 in 1% BSA-PBS-Tween 20, was added and incubated for 45 min at room temperature. The enzymatic reaction was initiated by the addition of sodium nitroprusside (2 mg/ml) and O-dianisidine (0.8 mg/ml) (Sigma Chemicals Co) dissolved in distilled water containing 0.01% H_2O_2.

Statistical analysis

Results were statistically analysed using the linear regression analysis and the Spearman Rank correlation test. Percentages of tumour cell-induced platelet aggregation are reported as median values. Only results with $P < 0.05$ were regarded as significant. All calculations were made using a personal computer software (Stat View II, Abacus Concepts, Berkeley, U.S.A.).

RESULTS

An example of the platelet proaggregating activity of the six human colorectal tumour cell lines is reported in Figure 1, where the aggregation waves obtained with the addition of 150 μl of a suspension containing 10^7 cells to 300 μl of PRP (approximately 3.5×10^5 platelets/μl) are shown. The data presented are in agreement with our previous findings [26], indicating that the GEO and CBS cells are capable of inducing only slight aggregation, occasionally represented by a monophasic wave. In contrast, the HT-29, WiDr and DLD-1 cells always induce remarkable *in vitro* platelet aggregation. A moderate degree of platelet aggregation is observed with the MIP cell line.

Table 1 summarises the lipid-bound sialic acid content, as well as the ganglioside expression and platelet proaggregating activity of the six cell lines under investigation. As shown, using the method of Svennerholm and colleagues, high levels of sialic acid (expressed as μg/mg of protein) were present in extracts obtained from the GEO (1.48 μg/mg of protein), HT-29 (0.95 μg/mg of protein), WiDr (1.49 μg/mg of protein), and DLD-1 (0.67 μg/mg of protein) cells, while lower levels were observed in extracts obtained from the CBS (0.26 μg/mg of protein) and MIP (0.28 μg/mg of protein) cells. A statistical analysis, performed using both linear regression analysis and Spearman Rank test, showed a lack of correlation ($r = 0.12$, $P > 0.05$)

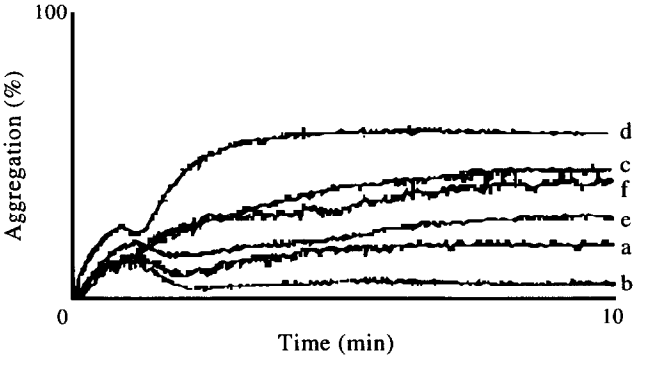

Figure 1. Platelet proaggregating activity of GEO (a), CBS (b), HT-29 (c), WiDr (d), MIP (e) and DLD-1 (f) human colorectal tumour cell lines (1.5×10^6 cells).

Table 1. *Lipid-bound sialic acid content, ganglioside expression and platelet proaggregating activity in human colorectal tumour cell lines*

Cell line	Sialic acid (μg/mg of protein)*		Ganglioside content (ng/10^6 cells)					
	Svennerholm *et al.* method	Ladisch *et al.* method	GM1	GM3	GD3	GD1a	GT1b	% Platelet aggregation†
GEO	1.48	0.13	64.9	46.8	+/−	0.0	0.0	13
CBS	0.26	0.10	66.0	37.2	0.0	0.0	0.0	16
HT-29	0.95	0.60	44.9	39.6	0.0	65.8	35.8	47
WiDr	1.49	0.85	0.0	71.0	0.0	126.5	56.6	42
DLD-1	0.67	0.52	61.1	78.1	0.0	76.7	0.0	31
MIP	0.28	0.28	64.4	57.3	0.0	0.0	0.0	27

*Values represent the mean of triplicate experiments; †median values calculated on the basis of 20 different experiments.

between the platelet proaggregating activity and the sialic acid content of the six cell lines (Table 1). This lack of correlation was mainly due to the elevated sialic acid content found in the GEO cell line, which was in contrast with its low proaggregating activity. It is worthwhile noting that GEO is a well differentiated cell line, characterised by prominent production of mucin contained in intracellular vacuoles at the apical portion of the cells. Similar characteristics, although to a lesser extent, are also seen for the well differentiated CBS cell line and the two moderately differentiated HT-29 and WiDr cell lines. Therefore, the analysis of sialic acid content was also performed using a modification of the extraction procedure (see Materials and Methods the method of Ladisch and colleagues) capable of removing contaminating mucins, without affecting the recovery of gangliosides (data not shown). Using this method lipid-bound sialic acid in the GEO cells was equal to 0.134 μg/mg of protein, instead of 1.48 μg/mg of protein in the presence of mucins. Similar results were obtained with the CBS (0.1 versus 0.26 μg/mg of protein) and the DLD-1 (0.52 versus 0.67 μg/mg of protein) cell lines, while no differences were observed using the MIP cell line. The results obtained are summarised in Table 1. Linear regression analysis of the values obtained showed a significant correlation ($r = 0.9$, $P < 0.02$) between the lipid-bound sialic acid content and the platelet proaggregating activity of the six cell lines. The analysis of Spearman Rank correlation was not significant, although a tendency could be observed ($r = 0.9$, $P = 0.058$).

The content of lipid-bound sialic acid and platelet proaggregating activity were also compared with the expression of gangliosides. An example of HPTLC analysis of the gangliosides present in the six cell lines is reported in Figure 2. As shown, one resorcinol positive band comigrating with GM3 was constantly found in all cell extracts, while a GM1 comigrating band was present in all the cell lines with the exception of WiDr. GM2-like gangliosides were constantly negative in all cell lines. It is worth noticing that the presence of disialo-or trisialo-gangliosides was always found in extracts obtained from those cells characterised by a high proaggregating activity. In fact, a GD1a comigrating band was present in HT-29, WiDr and DLD-1 cells, while a GT1b comigrating band was found in both HT-29 and WiDr. Conversely, GEO, CBS and MIP cells, which all induce a slight or moderate platelet activation, did not express GD1a or GT1b comigrating gangliosides. Trace amounts of GD3 were detectable only in the GEO cells, although it was not possible to demonstrate its presence by resorcinol staining on HPTLC plates. In fact, only immunostaining with specific MAb anti-GD3 was capable of detecting the presence of this ganglioside on extracts obtained from this cell line.

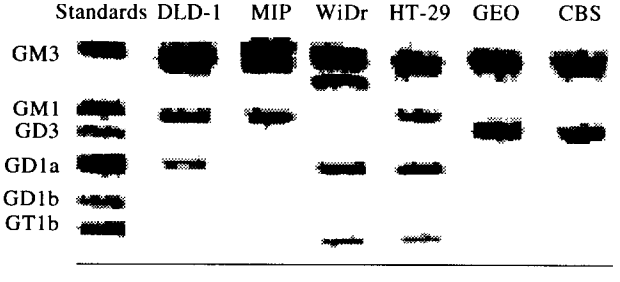

Figure 2. HPTLC analyses of ganglioside expression in extracts of various human colorectal tumour cell lines. DLD-1, MIP, WiDr, HT-29, GEO and CBS were harvested from subconfluent flasks. Extracts were prepared as outlined in Materials and Methods and acidic glycolipids obtained from approximately 3 × 10^7 cells were spotted on to plates. Lane 1 received purified standards.

A semiquantitative analysis of the amount of Gs found in the different cell lines was performed using a computer software. Peak areas were calculated for each of the bands observed, and gangliosides were measured by reference to known quantities of Gs standards. The results obtained are summarised in Table 1. The linear regression analysis showed a direct correlation between GD1a ($r = 0.97$, $P < 0.002$) or GT1b ($r = 0.86$, $P < 0.03$) content and lipid-bound sialic acid content. Moreover, a strict correlation was observed, although not fully significant, between the amount of these gangliosides and platelet proaggregating activity ($r = 0.80$, $P = 0.056$). Conversely, no correlation was found between either GM1 ($r = -0.68$) or GM3 ($r = 0.29$) content and platelet proaggregating activity.

Figure 3 shows the effects of the incorporation of exogenous GD1a and/or GM3 in the GEO (Panel A) and the CBS (panel B) cells. Treatment of monolayers for 1 h at 37°C led, not only to the prompt incorporation of the exogenous gangliosides in the cell membranes (lanes 2–4), but also to the appearance of a second band, migrating with an Rf similar to that of GD3. Immunostaining with anti-GD3 MAb resulted in a lack of reactivity, suggesting the different nature of this neosynthesised ganglioside. Moreover, the incorporation of exogenous asialo-GM1 or GD1b had no effect on the appearance of this band in both cell lines (data not shown). From these data, it may be speculated that GEO and CBS cells may have a strong sialidase

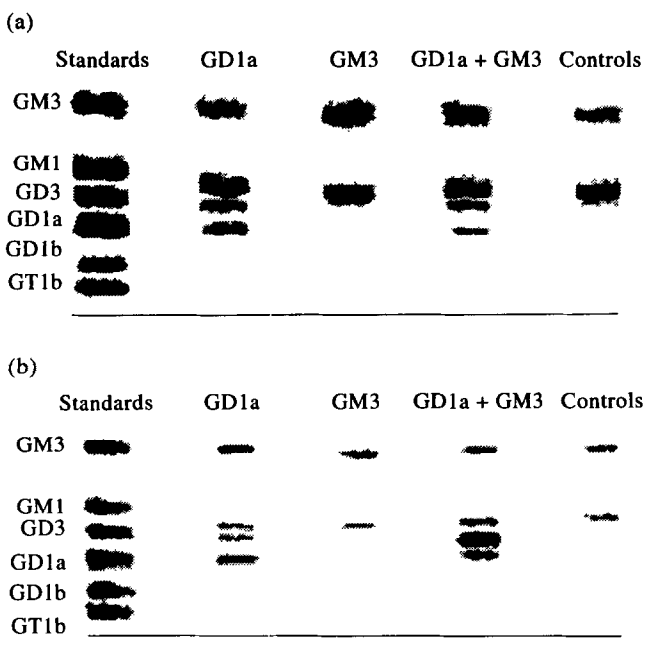

Figure 3. HPTLC analysis of ganglioside expression after incorporation of exogenous GD1a (lane 2), GM3 (lane 3), or a mixture of the two (lane 4) in GEO Fig. (a) and CBS Fig. (b) cell lines. Lane 5 represents control cells. Lane 1 received purified standards.

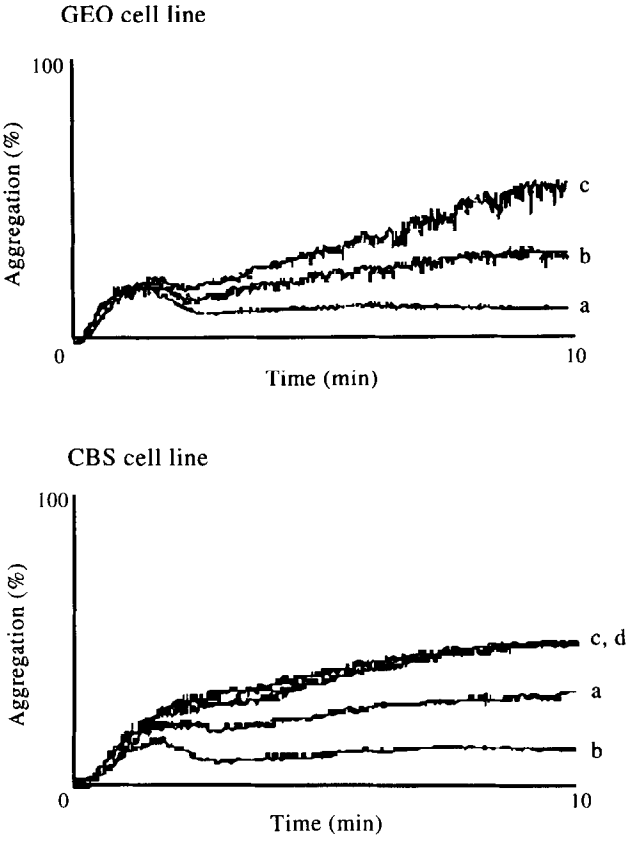

Figure 4. Effects of ganglioside incorporation on platelet proaggregating activity. (a) Control aggregation obtained with 1.5 × 10^6 cells; (b) platelet proaggregating activity after incorporation of exogenous GD1a (10 μg/10^6 cells); (c) platelet proaggregating activity after incorporation of a mixture of exogenous GD1a (5 μg/10^6 cells) and GM3 (5 μg/10^6 cells); (d) platelet proaggregating activity after incorporation of exogenous GM3 (10 μg/10^6 cells).

activity, responsible for the rapid conversion of added GD1a into a GM1b-like ganglioside. Treatment of monolayers of HT-29 and MIP resulted in the incorporation of exogenous Gs, without expression of any newly synthesised ganglioside (data not shown).

The effects of ganglioside incorporation on platelet proaggregating activity of tumour colorectal cell lines were investigated in GEO and CBS cells, which exhibit the lowest activity and express neither GD1a or GT1b, and in HT-29 cells, which, in turn, exhibit a high platelet proaggregating activity and express both gangliosides. Incorporation of exogenous GD1a and/or GM3 resulted often (Figure 4) but not always, in an increased platelet proaggregating activity in both GEO and CBS cells, while no further increase could be observed with the HT-29 cells (data not shown). A noticeable increase of the *in vitro* platelet activation could be observed not only after the incorporation of GD1a alone (10 μg/10^6 cells), but also, and to a greater extent, with either GM3 alone (10 μg/10^6 cells) or a mixture of the two (5 μg/10^6 cells of each ganglioside).

DISCUSSION

The present findings demonstrate that the cell content of lipid-bound sialic acid significantly correlates to the degree of platelet proaggregating activity exhibited by six different human colorectal tumour cell lines. High levels of lipid-bound sialic acid were present in extracts obtained from HT-29, WiDr, and DLD-1 cells, which always induced a remarkable *in vitro* platelet aggregation, whereas low levels of lipid-bound sialic acid were present in GEO, CBS and MIP cells, characterised by a lower proaggregating activity. These findings support the hypothesis that the portion of sialic acid bound to lipid may be preferentially involved in the interactions between platelets and tumour cells.

This hypothesis is further supported by the HPTLC findings, showing a strong association between the presence of different gangliosides and the degree of platelet proaggregating activity. The analysis of extracts obtained from the six tumour cell lines showed that GD1a and/or GT1b comigrating bands were present only in those cell lines characterised by a higher content of lipid-bound sialic acid, and by a stronger pro-aggregating activity. However, the presence of a band comigrating with GM3 was observed in all cell lines, which is in agreement with previously published studies, reporting the presence of GM3 on villous cells of rat intestinal epithelium [1].

Therefore, it can be postulated that sialic acid residues on Gs are possibly involved in platelet–tumour cell interaction. This hypothesis is substantiated by the experimental observation that the ligand binding activity of platelet glycoprotein IIb/IIIa complex, involved in the thrombin-dependent and independent platelet aggregation induced by tumour cells [22–24], may be modulated by gangliosides, mainly GT1b, and GD1a [25].

The implication of lipid-bound sialic acid in platelet–tumour cell interactions can also be speculated from the observation that the incorporation of exogenous gangliosides is capable of enhancing platelet proaggregating activity of certain cell lines. In fact, the incorporation of exogenous GD1a and/or GM3 resulted often, but not always, in increased platelet proaggregating activity of both GEO and CBS cells (expressing only GM1 and GM3 comigrating bands), while no further increase in tumour cell-induced platelet aggregation could be observed using the HT-29 cells (expressing a more complex pattern of membrane Gs). The occasional finding of a lack of effect following the incorporation may be explained by several considerations. According to previously published results, the

addition of exogenous gangliosides to a cell preparation may produce, depending on the site of incorporation, a misleading effect if the site of action is already occupied by endogenous gangliosides [30]. In our opinion, the presence of a strong sialidase activity, in both GEO and CBS cell lines, as suggested by the immunostaining and incorporation studies, may be reponsible for a different rate of conversion of GD1a from one experiment to another. Therefore, depending on either the rate of GD1a effectively incorporated by the cells, or the qualities of the newly synthesised ganglioside, unexpected results can be explained.

Finally, the observation that the incorporation of exogenous GM3 or a mixture of GD1a/GM3 by tumour cells is capable of enhancing tumour cell induced platelet aggregation to a greater extent than that observed with GD1a alone, suggests that the presence of a certain number of lipid-bound sialic acid residues on the cell membrane, more than a specific type of ganglioside, is implicated in this kind of interaction.

In conclusion, the present data support the hypothesis that lipid-bound sialic acid may be involved in the multifaceted interactions between platelets and tumour cells. However, the frequent association between GD1a and/or GT1b comigrating bands in those cell lines with a stronger platelet proaggregating activity, suggests that these gangliosides could probably act as modulating molecules of so far unidentified recognition sites.

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