EXPERIMENTAL MODELS TO STUDY GLYCOCONJUGATE MODIFICATIONS IN MALIGNANT TRANSFORMATION.

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Glycosphingolipids are assumed to play a crucial role in cell-cell and cell-substrate interactions, including cell adhesion, proliferation, differentiation and apoptosis. Furthermore cell surface glycolipid profile changes in the so called "social disorders" including malignant transformation. In order to better investigate these modifications, we have studied for many years the ganglioside composition in different solid brain tumors (B. Berra et al., *Int. J. Cancer* **47**, 329-331, 1991). The main results observed were the oversimplification of the ganglioside pattern, with the disappearance of some gangliosides which normally are present in very low amount. Altogether our data are in agreement with the review presented by Hakomori (S. Hakomori, *Cancer Res.* **45**, 2405-2414, 1985). Starting from results obtained in solid human brain tumors, a second approach was to investigate the glycosphingolipid profile in transformed cell lines, such as Sarcoma Galliera strain cells SGS/3A as compared to normal syngenic murine fibroblasts FG or 3T3 normal murine fibroblats versus SVT2 tranformed cells. In some of these studies we tried also to correlate the different glycolipid expression with the activities of the enzymes involved in their metabolism (for a review see "Glycolipid expression in solid tumors and transformed cell lines" by B. Berra et al.,. *Indian J. Biochem. Biophys.*, **34**, 170-177). More recently, we investigated three other models: 1 - mammary adenocarcinomas induced in transgenic mice by activated *neu* oncogene, kindly provided by Environmental Institute-Life Science,C.C.R. - Euratom, Ispra, Italy; 2 - C6 rat glioma cells (kindly provided by G. Finocchiaro, Neurological Institute "C. Besta", Milan) after stable transfection with β 1,4 N-acetylgalactosaminyl transferase (GalNacT-1) and α 2,8 sialyltransferase (SAT II); 3 - C6 rat glioma cells and HU 197 human glyoblastoma cells with or without treatment with Vitamin D3 derivative, kindly provided by L. Magrassi, Neurosurgery IRCCS Policlinico "S. Matteo", Pavia.

Lipids, including acidic and neutral glycolipids, and ceramides were extracted from the different biological materials, solid tumors or cell lines, with different procedures based on tetrahydrofuran or chloroform/methanol solvents, methods routinely used in our laboratory. Detection and identification of different glycolipid and ceramide species were performed by HPTLC analysis followed by scanning densitometry and/or by TLC immunostaining with specific monoclonal antibodies. Cell adhesion was evaluated in vitro on reconstituted extracellular matrix (Matrigel) and on some purified components of the ECM: fibronectin, laminin, type IV collagen and trombospondin. Cell motility and invasiveness were tested *in vitro* by the Boyden chamber, before and after chemiotactic stimulation with usual tecniques.

Mammary carcinomas developed in MMTV/*neu* transgenic mice: the tumors were induced *in vivo* by the *neu* oncogene, a gene encoding a 185 kDa transmembrane protein with close homology with EGF receptor. The model used consists of a transgenic mice line created by microinjection of a plasmid vector containing the activated form of the rat *neu* and MMTV-LTR sequences. According to the literature, in all MMTV/*neu* transgenic mice (females and males) the specific expression of the transgene in the mammary tissues results in the appearance of rapid and multifocal tumors. The ganglioside composition was analyzed in 10 mammary carcinomas developed in different MMTV/*neu* transgenic mice and in 4 normal mammary tissues obtained from control animals. Whereas control mammary tissues contain quite exclusively GM_3, all neoplastic samples show a substantial decrease of this ganglioside, an accumulation in variable amount of GM_3-derived species (GM_1, GD_3, GD_{1a}, GD_{1b}, GT and GQ) and the appearence of new, not yet identified, sialic acid containing molecules. Interestingly, three out of 10 tumors analyzed, even if histologically comparable to the others but with a larger dimension, show a significative difference as regard to the GM_1, GD_3 and GD_{1a} content.

subject	GM3	GM1*	GD3**	GD1a***	GD1b	GT	GQ
small tumor							
1232		0,63	0,88	1,14	1,01	1,39	2,53
1227	1,80	1,20	2,52	1,80	2,28	2,40	4,21
1227		1,82	2,54	1,82	2,67	1,21	3,63
1255		1,75	2,68	1,17	2,22	1,17	2,33
1224	1,13	1,70	2,60	1,7	2,83	1,70	3,40
1235	1,20	1,81	2,65	1,81	3,01	1,69	3,01
1248	2,91	1,46	2,19	2,48	2,48	1,02	2,19

subject	GM3	GM1*	GD3**	GD1a***	GD1b	GT	GQ
large tumor							
1247	1,20	4,19	4,19	9,57	1,2	1,32	2,99
1237	2,85	4,69	4,69	10,71		0,67	1,34
1240	5,09	5,74	5,74	12,64			

The values express the ng of each ganglioside-bound sialic acid/mg of wet tissue

Mean content ± standard deviation of:

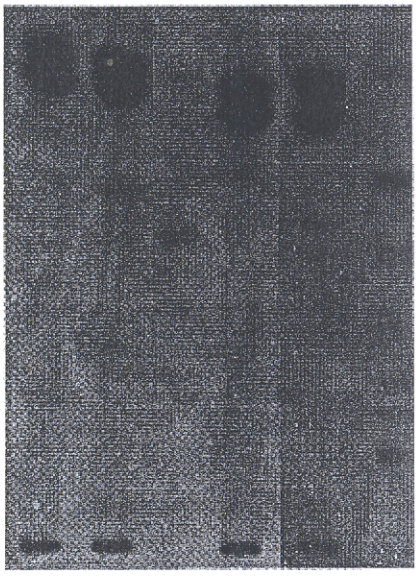
*GM1 large tumor = 4.87 ± 0.79 small tumor = 1.62 ± 0.25 P < 0.0001

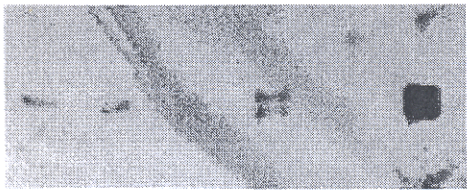
**GD3 large tumor = 4.87 ± 0.79 small tumor = 2.53 ± 0.18 P < 0.0002

***GD1a large tumor = 10.97 ± 1.55 small tumor = 1.8 ± 0.42 P < 0.0001

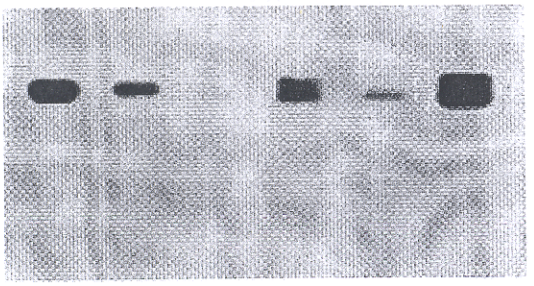
Our data suggest that an activated oncogene may induce also "in vivo" a specific and transmissible alteration in the ganglioside pattern, but this distribution could be susceptible to further modifications during the tumor progression.

C6 rat glioma cells, that show an extremely simplified ganglioside pattern (they contain exclusively GM_3, either N-acetyl or N-glycolyl), were stably transfected or with the empty pRC/CMV eukaryotic expression vector or with the same plasmid containing the GalNAcT-1 or SAT-II cDNA. Three different transfected clones were identified and characterized by Dot blot and PCR amplification of specific regions contained in the two cDNAs and in the gene encoding the neomicin resistance: p13 is the clone transfected with the empty vector; G1 is the clone transfected with the plasmid containing GalNAcT-1 cDNA; 5NF is the clone transfected with both constructs, the plasmid containing the GalNAcT-1 cDNA and that containing the SAT-II cDNA. Whereas the ganglioside profile of the clone p13 does not show any difference as regard to C6 glioma parental cells, in the clone G1, GM_3 and GM_2 were present; however, GM_1 was also found. One possibility to explain the expression of GM_1 could be the endogeneous presence in C6 cell of GalT-2. Transfected cells could express the substrate, GM_2, for GalT-2 activity. This hypothesis, if proved with further experiments, could justify the presence of GM_1 as above indicated. In the clone 5F only the expected products were present, i.e. GM_2 and GD_3. The presence of these new expressed gangliosides was ascertained using the specific antibodies.

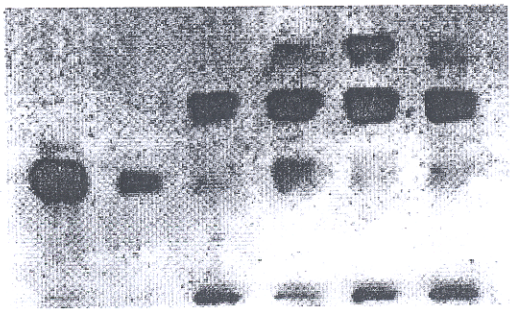




MAb R24 (anti-GD3)



Mab KM696 (anti-GM2)



Cholera toxin b-subunit

Between the different transfected clones, significant differences were also evident as regard to in vitro growth rate, adhesion, motility and invasiveness. In particular, whereas C6 glioma parental cells and clones p13 and G1 showed a similar behaviour, the clone 5F, which had an in vitro growth rate significantly higher than the other clones, resulted more adhesive to the ECM and to some purified components, such as fibronectin, laminin, type IV collagen and trombospondin, and increased motility and invasivness expecially after chemiotactic stimulation. All these differences of behaviour of the clone 5F could be related with the increased expression of GD3, whose presence has been observed only in this clone.

C6 rat glioma cells were treated with Cholecalcipherol: after extraction and analysis of lipids, we found an increase in the amount of ceramide parallel to a decrease of total gangliosides. Sphingomyelin content does not change.

	CERAMIDE mg/mg DNA	**SPHINGOMYELIN mg/mg DNA**	**GANGLIOSIDES mg/mg DNA**
C 6 control	0,060	0,039	0,022
C 6 Vit D3 treated	0,110	0,031	0,012
HU 197 control	0,045	0,014	0,09
HU 197 Vit D3 treated	0,124	0,006	0,07

This may suggest an activation of gangliosides catabolic pathway in contrast with our observations on HU197 cells (human glioblastoma cell line). In fact also after a brief treatment (6 hours) with Vitamin D3, we found in this cellular line an increase in ceramide concentration and a proportional decrease in sphingomyelin amount due to an activation of sphingomyelin pathway by Vitamin D3 metabolites.

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