Intercellular transfer of shed tumor cell gangliosides

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Abstract Three distinct steps underlie immunosuppression by tumor gangliosides: (i) their shedding by the tumor cell, (ii) binding to target leukocytes in the tumor microenvironment, and (iii) action upon the target cell. While shedding is well documented, cell to cell transfer of shed gangliosides is not. To address this, we employed a dual chamber culture system. In this system, metabolically radiolabeled lymphoma cells shed gangliosides into the conditioned medium of the contralateral chamber, which contained normal fibroblasts as the target cell. The shed lymphoma cell gangliosides bound avidly to the target fibroblasts in a trypsin-resistant manner ($1–2\times10^6$ and 7×10^6 molecules/fibroblast in 24 and 48 h). Significantly higher than binding rates of purified lymphoma gangliosides added exogenously, these binding rates in a system which models the in vivo microenvironment suggest that cell to cell ganglioside transfer is a highly efficient process.

Key words: Ganglioside; Ganglioside shedding; Ganglioside transfer; Intercellular signaling

1. Introduction

The shedding of endogenous gangliosides by tumor cells is a well-recognized process [1-4], and occurs at a rapid rate, i.e. up to 0.5% of the membrane ganglioside content per hour [5]. Less is known about the interaction of exogenous ganglioside molecules with cells. When highly purified gangliosides are added to cells in vitro, the ceramide moiety of the ganglioside molecule can intercalate into the membrane bilayer in a trypsin-resistant manner [6,7]. This is observed either when purified exogenous gangliosides, which exist in micellar form or as free molecular species, are used [8], or when gangliosides are incorporated in artificial membrane systems, such liposomes [9]. Both approaches have shown that gangliosides can be stably bound by target cells, but neither approach can address the question of whether the in vivo process of tumor cell ganglioside shedding can lead to suppression of the cellular immune response [10]. One aspect of this problem is to determine whether immunosuppressive gangliosides, as shed from tumor cells, bind to target cells in the tumor microenvironment.

The ideal in vitro conditions for studying this intercellular exchange of gangliosides would allow for both processes (shedding and cellular interaction) to occur as they do in vivo. Here, we describe a co-culture system which allows for the flow of shed gangliosides from tumor cells to target fibroblasts while physical contact between the two cell populations is prevented. The significant finding of this study is that the shed gangliosides, detected at a concentration significantly lower than that routinely used in ganglioside binding studies [7], were incorporated into the membrane of the target cells, and that this binding occurred to a degree which is likely to be physiologically relevant.

2. Experimental

2.1. Cell lines

YAC-1 murine lymphoma cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, and 100 units/ml penicillin and streptomycin. Normal human fibroblasts (line 42sk, ATCC), the target cells for ganglioside binding, were maintained in EMEM containing the same supplements and used between passages 4 and 12.

2.2. Metabolic radiolabeling of cellular gangliosides

YAC-1 lymphoma cells were metabolically radiolabeled by culture for 48 h in complete RPMI 1640 medium containing 10 μCi each of D-[1-^{14}C]glucosamine hydrochloride (specific activity 52.6 mCi/mmol) and D-[1-^{14}C]galactose (56.5 mCi/mmol; NEN). The cells were then washed six times by centrifugation in complete medium. Following the final wash, the cells were resuspended in EMEM at 3.5×10^6 cells/ml. Cell viability, assessed by trypan blue exclusion, was greater than 98%. Human fibroblasts were cultured to pre-confluency, radiolabeled for 48 h as above, washed six times with phosphate-buffered saline (PBS), and then harvested by trypsinization.

2.3. Ganglioside purification and autoradiography

Radiolabeled gangliosides were isolated from lyophilized cell pellets and conditioned medium, with 10 nmol purified human brain gangliosides added as a cold carrier, using previously described methods [11]. The ganglioside-associated radioactivity was quantified by β-scintillation counting, and confirmed by high-performance thin-layer chromatography (HPTLC) autoradiography [5]. Purified ^{14}C-labeled gangliosides were quantified by a modified resorcinol method [12] to determine specific activity.

2.4. Co-incubation studies

42sk fibroblasts were cultured in 6-well plates (Costar) and grown to confluence. At the beginning of the experiment, the culture medium was removed from four plates and 2.0 ml fresh medium was added to each of 24 wells. Upper chamber inserts containing a polycarbonate membrane with 0.4 μm pore size (Transwell, Costar) were then inserted in each well, and 7×10^6 radiolabeled YAC-1 cells in 2 ml medium were added to the top chamber. Following incubation for 24 h, the upper chamber was removed (and with it the YAC-1 cells) and the upper conditioned medium (CM) collected (1.5 ml/well). The YAC-1 cells were collected by washing the membrane three times with PBS. The PBS washes were added to the upper CM and the YAC-1 cells were pelleted by centrifugation at $300\times g$. The CM in the lower chamber (free of lymphoma cells) was removed (2.5 ml/well), and both the upper and lower CM were centrifuged at $1000\times g$ and the supernatants retained. Finally, the lower chamber was washed 5 times with PBS and the adherent fibroblast monolayer harvested by trypsinization. In a parallel study, the upper chamber containing the YAC-1 cells was removed after 24 h and the plates incubated for an additional 24 h to allow further binding of the already shed lymphoma gangliosides to the fibroblasts.

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2.5. Binding of purified exogenously added gangliosides

Fibroblasts were cultured in 6-well plates as described in the co-incubation studies. Metabolically radiolabeled purified YAC-1 gangliosides, briefly sonicated in basal medium, were added to each well at a final concentration of 1.0 μM. Following incubation for 24 and 48 h, the unincorporated gangliosides were removed, the fibroblast monolayer harvested, and the binding of the purified gangliosides to fibroblasts quantified by scintillation counting.

3. Results

3.1. Dual-chamber culture system

The dual-chamber culture system is illustrated in Fig. 1. When the upper chamber insert is in place, the total volume in the well is 4.0 ml and the volume in the upper chamber is 1.5 ml. A 1.0 mm distance separates the fibroblast monolayer in the lower chamber from the YAC-1 cells, seeded in the upper chamber at a concentration of 7×10^6 cells/1.5 ml. 6–8×10^6 YAC-1 cells (viability >95%) were recovered from the upper chamber at the end of the 24 h co-incubation period.

Several approaches established that no lymphoma cells were present on the contralateral side of the membrane, i.e. in the CM bathing the fibroblast monolayer, in this dual chamber system. First, visual inspection of the fibroblast monolayer by light microscopy showed no contamination by YAC-1 cells, which have a strikingly different morphology from that of normal fibroblasts. Secondly, when the lower CM was centrifuged at $1000\times g$ for 10 min to remove large membrane particles or intact cells, no pellet was visible, nor did the dpm of the CM decrease, indicating that no significant quantity of particulate or cell-associated gangliosides, which would have been pelleted, were present. Finally, as a very sensitive measure of viable cells, we observed no cell proliferation in fresh cultures which had been seeded with multiple aliquots of the lower CM removed following the 24 h incubation period.

3.2. Specific activity of radiolabeled YAC-1 gangliosides

The recovery of YAC-1 cellular gangliosides ranged from 13 to 15 nmol lipid-bound sialic acid (LBSA)/10^8 cells, in good agreement with our previous findings in YAC-1 cells proliferating in vitro [1,13]. The specific activity of the radiolabeled YAC-1 gangliosides from three separate experiments ranged between 9800 and 12400 dpm/nmol (Table 1). The presence of the two major gangliosides of YAC-1 cells, G_{M1b} and $GalNAcG_{M1b}$ [13], was confirmed in the present experiments by HPTLC (Fig. 2, lane 1). In contrast, the predominant ganglioside of human fibroblasts, the target cell, is the more rapidly migrating G_{M3} [14], which is easily distinguishable by HPTLC from that of the lymphoma cell gangliosides (Fig. 2, lane 2).

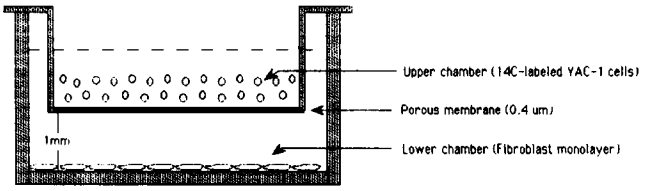

Fig. 1. A dual-chamber culture system for the study of cell to cell ganglioside transfer.

3.3. Shedding of YAC-1 gangliosides

Shed YAC-1 gangliosides were isolated from the upper and lower CM from four individual 6-well plates. The ganglioside concentration in the upper CM was 0.045 ± 0.009 μM (mean ± S.E.). In contrast, the ganglioside concentration in the CM on the contralateral side of the membrane from the YAC-1 cells was 0.0054 ± 0.0004 μM. This ten-fold lower concentration of shed YAC-1 gangliosides was in part due to slow diffusion through the membrane, as supported by the finding that trypan blue (MW=980) placed in the upper chamber of several control wells, even without YAC-1 cells, had not fully equilibrated in the lower chamber 24 h later.

3.4. Cell to cell transfer of tumor gangliosides

The fibroblasts in these studies were cultured to confluence, and approximately 1.2×10^7 cells were recovered from 4 plates at both the 24 and 48 h timepoints. When the total gangliosides were isolated from the fibroblasts, radiolabeled YAC lymphoma gangliosides equivalent to 1.3–1.8×10^6 and 7×10^6 ganglioside molecules per fibroblast, were recovered at 24 and 48 h, respectively (Table 1). As identified by HPTLC autoradiography (Fig. 2, lane 3) the two major YAC-1 gangliosides accounted for almost all of the total radiolabeled gangliosides isolated from the fibroblast monolayer. The relatively faint additional bands of higher mobility isolated from the fibroblasts and not seen in the YAC-1 cell ganglioside pattern (Fig. 2, lane 1) may represent either endogenous ganglioside synthesis in the fibroblasts (which may have metabolized trace amounts of radiolabeled precursor molecules) or catabolic products of YAC-1 cell gangliosides metabolized by the target cell.

3.5. Binding of purified exogenous gangliosides

To place the above results in perspective, we compared them with the binding of highly purified gangliosides added to the same target cell population. This was done to address the question of whether 'pure gangliosides' have binding characteristics different from those of gangliosides directly derived, by shedding, from tumor cells without any experimental ma-

Table 1
Binding of exogenous YAC-1 lymphoma cell gangliosides to human fibroblasts

Time of incubation	Shed gangliosides			Purified gangliosides	
	24 h (expt 1)	24 h (expt 2)	48 h (expt 3)	24 h (expt 4)	48 h (expt 4)
Specific activity (dpm/nmol)	9800	11500	12400	26700	26700
Ganglioside conc. in conditioned medium (μM)	ND[a]	0.005[b]	0.005[c]	1.0	1.0
Ganglioside binding (molecules/cell)	1.8×10^6	1.3×10^6	7.0×10^6	1.4×10^7	3.3×10^7

[a]Not determined.
[b]Mean of four determinations (S.E.<10%).

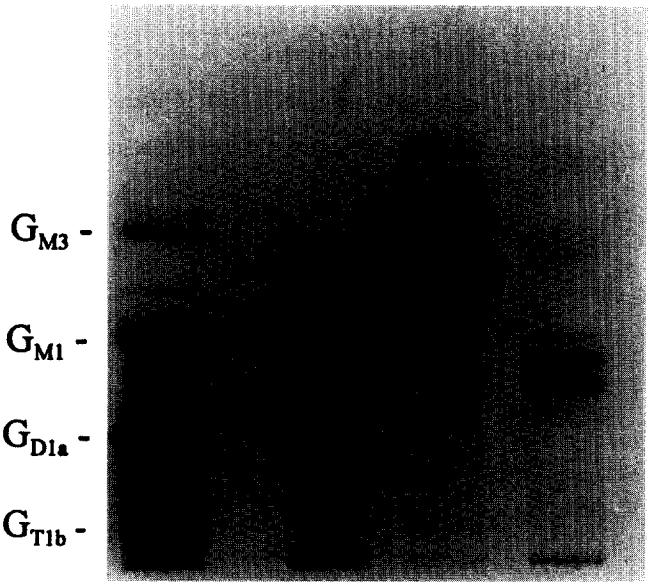

Fig. 2. Autoradiogram of shed tumor cell gangliosides which have become tightly bound to target fibroblast cells. RBG, ^{14}C-labeled rat brain gangliosides; lane 1, radiolabeled cellular gangliosides of YAC-1 lymphoma cells; lane 2, radiolabeled cellular gangliosides of normal human fibroblasts (target cell); lane 3, radiolabeled gangliosides recovered from the fibroblast monolayer following 24 h co-incubation with ^{14}C-labeled YAC-1 lymphoma cells. Visualized by exposure of the HPTLC plate to X-ray film for 6 weeks. Lanes 1, 2, and 3 contain approximately 1000, 1000, and 400 dpm ^{14}C-gangliosides, respectively.

nipulation. In this experiment, we measured the binding of exogenously added ^{14}C-labeled purified YAC-1 gangliosides under the same culture conditions used in the co-incubation studies, except that a higher concentration of 1.0 μM was used to optimize the ability to detect bound gangliosides on the target cells. Under these conditions, 1.4 and 3.3×10^7 molecules were bound per fibroblast at 24 and 48 h, respectively (Table 1).

4. Discussion

In a system which closely mirrors the in vivo microenvironment of a tumor, i.e. in which normal 'target' cells coexist with tumor cells which shed gangliosides, we have shown that uptake and stable binding of shed gangliosides occurs, and occurs to a significant degree. The fact that the transfer of gangliosides from the lymphoma cells to the fibroblasts occurred at a relatively low concentration of shed gangliosides (0.005 μM surrounding the target cell) underscores the potential biologic importance of the interaction of these bioactive molecules with target cells in the tumor microenvironment.

It should be noted that there are a number of factors that could artificially reduce the binding of gangliosides to the target cell in vitro, as compared to the circumstances existing in vivo. The most significant of these is the prevention, by the membrane, of contact between the target cell and ganglioside-containing large particles which are released by tumor cells in vivo [15,16], since gangliosides shed as large membrane vesicles of high molecular weight ($>40\times10^6$ Daltons) [15] may not readily pass through the membrane separating the two cell populations. This is suggested by our findings in the dual-chamber system that the ganglioside concentration in the upper CM is ten-fold higher than that found in the lower CM, and that even trypan blue only slowly equilibrates in the two chambers. Therefore, the shed lymphoma cell gangliosides in contact with the fibroblasts will likely be in small vesicles or exist in micelles or as individual molecules. While the latter two also make up the composition of purified gangliosides when added directly to culture medium, the direct comparison of exogenous binding efficiency between shed and purified ganglioside molecules suggests that shed molecules bind to target cells with higher efficiency.

Other factors which may influence ganglioside binding include the presence of serum proteins [17] and lipoproteins [18,19], present in the tissue culture medium but absent in the extravascular tumor microenvironment [20]. These proteins will compete with the target cell for ganglioside binding in vitro [21], thus reducing effective binding to the target cells. Finally, the thousand-fold lower tumor cell density in this in vitro system ($\sim10^6$ cells/ml) than in the in vivo microenvironment ($\sim10^9$ cells/ml) markedly reduces the quantity of shed gangliosides subsequently available for binding to the target cell. It therefore seems highly likely that the process of transfer we have now documented in vitro is quantitatively much greater in vivo. This is extremely interesting, since even the level of binding we show here (approaching 1×10^7 molecules per cell at 48 h) is, in the case of human peripheral blood mononuclear cells, a degree of binding associated with 70–90% inhibition of their immunologic responsiveness in an assay of antigen-induced lymphoproliferation [22]. Such paralysis of host immune function by tumor gangliosides can be a significant factor in tumor escape from host immune destruction.

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