Biochemical and Biophysical Research Communications 388 (2009) 17–20

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Anchored and soluble gangliosides contribute to myelosupportivity of stromal cells

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ARTICLE INFO

Article history:
Received 2 July 2009
Available online 24 July 2009

Keywords:
Gangliosides
Myelopoiesis
Stromal cells
GM-CSF
GM3

ABSTRACT

Stroma-mediated myelopoiesis depends upon growth factors and an appropriate intercellular microenvironment. Previous studies have demonstrated that gangliosides, produced by hepatic stromal cell types, are required for optimal myelosupportive function. Here, we compared the mielossuportive functions of a bone marrow stroma (S17) and skin fibroblasts (SF) regarding their ganglioside pattern of synthesis and shedding. The survival and proliferation of a myeloid precursor cell (FDC-P1) were used as reporter. Although the ganglioside synthesis of the two stromal cells was similar, their relative content and shedding were distinct. The ganglioside requirement for mielossuportive function was confirmed by the decreased proliferation of FDC-P1 cells in ganglioside synthesis-inhibited cultures and in presence of an antibody to GM3 ganglioside. The distinct mielossuportive activities of the S17 and SF stromata may be related to differences on plasma membrane ganglioside concentrations or to differences on the gangliosides shed and their subsequent uptake by myeloid cells, specially, GM3 ganglioside.

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Introduction

Gangliosides have been associated with cell growth and differentiation in several studies of cell signaling [1,2]. In the hematopoietic system, leukemic and normal cells are influenced by gangliosides [3,4], while gangliosides shed by neuroblastoma inhibit hematopoiesis [5]. Moreover, it has been described adhesion, spreading, and motility of cells based on glycolipid–glycolipid interaction and interactions of gangliosides with the integrin $\alpha5\beta1$, EGF, FGF and the insulin receptors [1,6–10].

Granulocyte–macrophage-colony stimulating factor (GM-CSF) is one of the major cytokines involved in hematopoietic cell survival, proliferation and differentiation. Its biological effects are exerted by a high-affinity membrane-bound receptor (GMR) that has been reviewed elsewhere [11,12]. The biologically active GM-CSF could be recovered from several stromal cells, like the murine bone marrow-derived cell line S17, a liver inflammatory granuloma-derived stroma (GR) and normal skin fibroblasts. Besides this, the quality of the pericellular glycoconjugates of these stromata is determinant for its ability to sustain myelopoiesis [13]. The interaction between cell-surface proteoglycans and GM-CSF is shown to be modulated by the physicochemical conditions of the microenvironment between stromal and hematopoietic precursor cells occurring only at low pH [13–15]. The acidic pH induces a conformational change in GM-CSF, which would then interact with glycosaminoglycans [16]. Thus, negatively-charged glycolipids present on the plasma membrane of hemopoietic or/and stromal cells may be involved in the functional role of GM-CSF.

We showed that GM3 is the major ganglioside synthesized and shed by fetal liver-derived stromal cells and is required for the optimal stroma myelosupportive function. This ganglioside is released into the supernatant and selectively incorporated into the myeloid progenitor cells, where it segregates into rafts in which it co-localized with the GM-CSF receptor α chain [17]. Moreover, two other hepatic stromal cells (GR-WT and GR-IFNγ-R%) with a distinct quantitative pattern of gangliosides showed different myelopoiesis supportive capacity, which is apparently related to the GM3 content [18].

In the present study we investigate the role of gangliosides in myelopoiesis, using the bone marrow stromal cells (S17 cell line) and primary skin fibroblasts (SF). First, we analyze the pattern and synthesis of these lipids, and then we compared the ability

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0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.07.092

of these stromata to sustain the survival and proliferation of the myelopoietic progenitor cell line, FDC-P1.

Materials and methods

Cells and cell cultures. Permanent cell lines S17, WeHi-3B and FDC-P1 were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University, Rio de Janeiro, RJ, Brazil) and were maintained as described [14,17]. Primary cultures of skin fibroblasts (SF) that do not sustain myelopoiesis were obtained from newborn C3H/HeN mice as previously described [13] and were maintained in culture as described for S17 cells. For a typical co-culture experiment, FDC-P1 cells were washed with buffered saline solution (BSS) in order to remove IL-3, and were inoculated onto the semi-confluent monolayers of S17 or SF cells in 24-well plates (1×10^5 cells per well), and maintained in culture for 24 or 48 h. Cell proliferation was monitored under a phase-contrast-equipped inverted microscope.

Thiobarbituric acid assay for sialic acid. Lipids were extracted from cell pellets with chloroform/methanol (2:1 v/v) [17], and hydrolyzed in 0.05 M H_2S0_4 at 80 °C for 60 min. The sialic acid content was quantified as described by Skoza and Mohos [19]. Protein sediments obtained after lipid extractions were dissolved in 1.0 N NaOH and measured as previously described by Peterson [20] using bovine serum albumin as standard.

Metabolic labeling and lipid extraction. Cultures of stromal cells that reached confluence and cultures of FDC-P1 cells were incubated for 12 h with 0.5 μCi/mL [^{14}C] galactose (300 mCi/mmol, Amersham Life Science, UK). Radioactive sphingolipids were extracted with chloroform/methanol (2:1 v/v), purified, separated by chromatography and their relative contribution was determined as previously described [17].

PDMP and PPPP treatment. Inhibition of glycolipid synthesis with PDMP (DL-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol – Calbiochem, USA) and PPPP (DL-*threo*-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol-HCl – Matreya, USA) was carried out as follows: S17 cells were treated for 72 h with 10 μM PDMP added to the culture and FDC-P1 cells were treated with 2 μM of PPPP added to the culture for 48 h. Inhibition of glycolipid synthesis was monitored by pulsing PDMP-treated and untreated cells with 0.5 μCi/mL [^{14}C] galactose for the last 12 h of culture [6].

Shedding of gangliosides. The S17 cells and SF cells were metabolically labeled with 0.5 μCi/mL [^{14}C] galactose for 12 h, the cells were washed and cultured in fresh medium for 48 h. The culture supernatant was collected, dialyzed against distilled water. The lipids extraction and gangliosides analysis were performed as previously described [5].

Determination of FDC-P1 proliferation rate. FDC-P1 cells were inoculated onto 24 or 96 wells culture plates, at indicated densities and maintained in different medium preparations: (a) RPMI supplemented with 10% FBS and 50% medium conditioned by S17; (b) RPMI supplemented with 10% FBS and 50% medium conditioned by S17 pre-treated with 10 μM PDMP; (c) RPMI supplemented with 10% FBS and 50% of medium conditioned by S17 cells with addition of 10% DH2 hybridoma supernatant as a source of monoclonal anti-GM3 antibody (Gycotech, USA); (d) RPMI supplemented with 10% FBS, 50% of medium conditioned by S17 cells and 2 ηg/mL of murine recombinant GM-CSF (Peprotech, USA); (e) RPMI supplemented with 10% FBS and 2 ηg/mL of GM-CSF; (f) RPMI supplemented with 10% FBS and 20 μM of GM3 (Matreya, USA); (g) RPMI supplemented with 10% FBS, 20 μM of GM3 and 2 ηg/mL of GM-CSF and (h) RPMI supplemented with 10% FBS and 2 μM PPPP. RPMI supplemented only with 10% FBS was the negative control. RPMI supplemented with 10% FBS and 10% WeHi-3B cell-conditioned medium were the positive controls. After the indicated times, the viable cells were counted in a hemocytometer. Alternatively, 20 μl of Cell Titer One Aqueous Solution (Promega, USA) was added to medium and after 1 h the absorbance were read at 490 nm. Differences among the experimental groups were analyzed by one-way analysis of variance and means were compared by the SNK test. To exclude a non-specific effect of DH2 on FDC-P1 proliferation, cells were maintained in standard medium and 20% DH2. No effect of DH2 antibody was found (data not shown).

Results and discussion

Stroma-induced myelopoiesis was monitored using the FDC-P1 myeloid progenitor cell line, which is dependent upon GM-CSF or IL-3 for survival and proliferation. In accordance with previous results for co-culture of stroma and FDC-P1 cells [13,15], the bone marrow stroma-derived S17 cells induced a rapid and intense proliferation of FDC-P1 cells, while SF cells could sustain neither the proliferation nor the survival of the myelopoietic progenitors cells (Fig. 1).

In view of the described role of negatively-charged sialic-acid-containing molecules at the membrane interface between the sup-

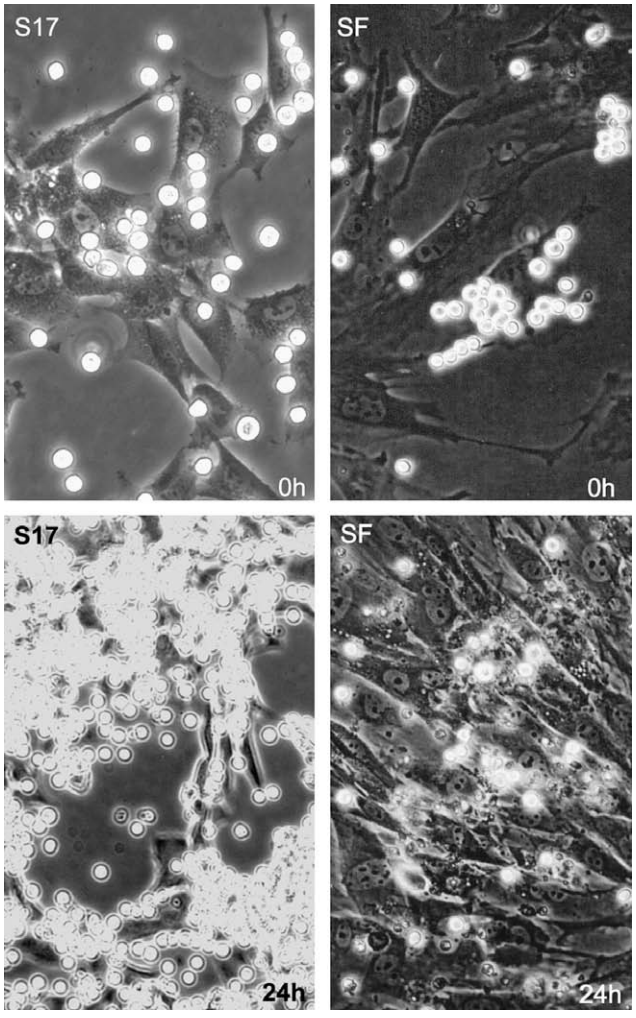

Fig. 1. FDC-P1 cell survival and proliferation in co-culture with S17 and SF cells. FDC-P1 cells previously washed with CMF BSS were inoculated onto the semi-confluent monolayers of S17 or SF cells at 1×10^5 cells per well (above) and maintained by 24 h (below). Phase-contrast microscopy, magnification 200 x.

porting stroma and the hematopoietic cells [15], we quantified the total sialic (*N*-acetylneuraminic) acid in the two studied stromal cells. The values obtained were 10.0 ± 0.23 ηmol/mg protein to S17 cells and 0.95 ± 0.16 ηmol/mg protein to SF cells. These results indicate a high concentration of sialic acid in the bone marrow stroma cells and a very low level in skin fibroblasts. The increased amount of sialic acid could be related to local decrease in pH necessary for the interaction of heparan-sulfate and GM-CSF [16].

We previously demonstrated that the ganglioside pool of FDC-P1 cells could be provided, at least in part, by the myelosupportive stroma [17,18]. Therefore, we analyzed the ganglioside synthesis and shedding from S17 and SF cells. S17 cells synthesizes essentially GM3, to a lesser extent a neutral ceramide trihexoside, and a small amount of GD1a, while the SF cells synthesized GM3, GD1a in similar quantities and a small quantity of GM1 (Table 1). These patterns are similar to those obtained for AFT-024 [17], GR-WT and GR-IFNγ-R% cells [18], showing that myelosupportive stromata accumulated the GM3 ganglioside.

The spatial organization of gangliosides in the cell membranes and/or their association with other membrane components may also be required in high cell–cell myelosupportive activity of S17 cells. The presence of GM3 apparently generates optimal conditions for sequestering GM-CSF on the cell membranes, putatively on heparan-sulfate proteoglycans, as indicated by previous studies [14,15]. On the other hand, skin fibroblasts have a relatively high amount of GM3, but their capacity to sustain myeloid cells is poor. Then, we hypothesized that in the case of these cells the membrane context of molecular complexes is not optimal or may even be inhibitory.

Gangliosides' shedding into the supernatant releases them from the molecular context of cell membranes and, in their soluble form they can be uptaked by FDC-P1 myeloid progenitors and modify the biological response of these cells to GM-CSF [21]. The shedding of both stromal cells was similar to the ganglioside synthesis pattern. The only ganglioside shed by S17 was GM3, while SF cells shed GM3, GD1a and GM1 (Table 1). The conditioned media of mielossuportive stromal cells was also reported to promote FDC-P1 cell proliferation [13,17,18]. In accordance with previous results [13,15], we found that S17-conditioned medium had a very low capacity to sustain FDC-P1 cell survival and/or proliferation (Fig. 2A). However, the survival of FDC-P1 cells was even lower in S17-conditioned medium in which GM3 was depleted (Fig. 2A). Notably, the supplementation of S17 supernatant with GM-CSF restored its myelosupportive capacity (Fig. 2B). Hence, we can assume that the supernatant of S17 cells contains sufficient GM3, but a suboptimal quantity of GM-CSF, having a poor myelosupportive capacity that can be enhanced by supplementation with GM-CSF.

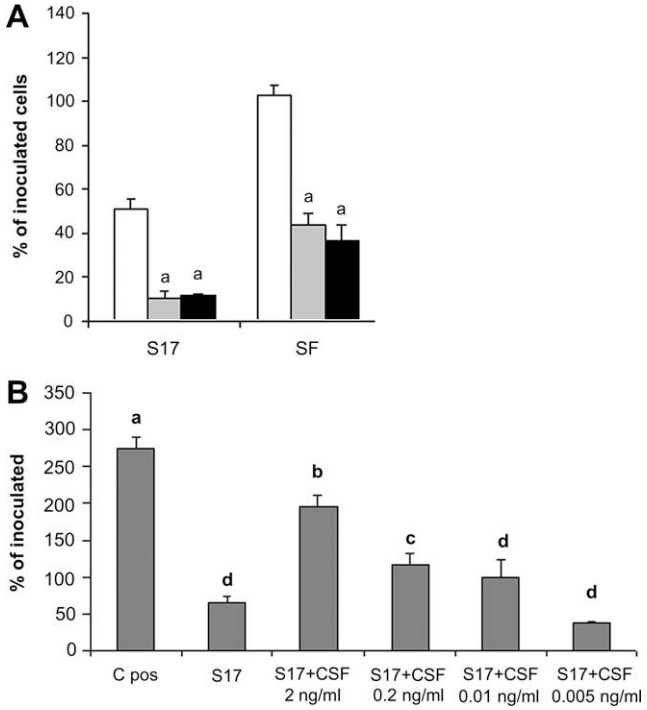

Fig. 2. FDC-P1 cells proliferation in conditioned medium of S17 and SF cells. (A) FDC-P1 cell proliferation in the presence of the supernatant of S17 or SF cells (white bars), of the supernatant of S17 or SF cells treated with 10 μM PDMP (gray bars) and supernatant of S17 or SF cells in the presence of DH2 (a monoclonal antibody to GM3) (black bars). FDC-P1 cells cultivated in medium with supernatant of WeHi cells were used as positive control (not shown). (B) FDC-P1 cell proliferation in the presence of supernatant of S17 or in the presence of this supernatant supplemented with 2 ηg/mL GM-CSF. The positive control was RPMI with 2 ηg/mL GM-CSF (C pos). Data are from two separated experiments, each done in triplicate. Values are mean ± SEM. (a), (b) and (c), significant difference to $p < 0.05$.

Despite the fact that SF cells are unable to maintain myelopoiesis in co-cultures through a cell–cell contact (Fig. 1) the supernatants of these cells sustain the FDC-P1 proliferation, as previously described by Carvalho et al. [13]. Actually, the inhibition of the total ganglioside synthesis in SF cells, as well as neutralization of GM3 by the monoclonal antibody in the conditioned medium, decreased both the FDC-P1 cell proliferation and survival (Fig. 2A).

The apparent requirement of gangliosides for survival and proliferation triggered by GM-CSF was assessed by culturing FDC-P1 cells under conditions of ganglioside depletion (PPPP) and murine recombinant GM-CSF stimuli (Fig. 3A). Cells were treated with PPPP in order to inhibit ganglioside synthesis and stimulated with of GM-CSF during 48 h. GM-CSF-treated cells undergone proliferation, reaching an eightfold increase during 48 h and PPPP-treated cells were unable to respond to the cytokine. As expected, GM-CSF-treated cells which received GM3 had an increased proliferation when compared to the stimuli of the GM-CSF-treated cells (Fig. 3B). GM3 alone had no proliferative effect on FDC-P1 cells. These data suggest that gangliosides are involved in GM-CSF-mediated cell survival and proliferation.

It is recognized that the effects of gangliosides may be dependent upon its concentration, location and/or type of cytokine receptor. Gangliosides can be determinant for the inclusion of receptors into glycolipid-enriched membrane regions (GEM) like rafts, which control the activation, the turnover and the subcellular localization of the signaling compounds [1,2]. GM3-enriched rafts have been related to several signaling events triggered by cyto-

Table 1
Densitometric analysis of lipids synthesized and shed from S17 and SF cells. Cells were cultured for 48 h and incubated for the last 12 h with 0.5 μCi/mL [^{14}C]galactose. Lipids were extracted, purified, analyzed by HPTLC, visualized by fluorography and their relative contribution was determined by densitometric scanning of the X-ray film. Results are expressed as percentage of total radioactivity incorporated. GD1a, GM1, GM2, GD3 and GM3: gangliosides; CTH: ceramide trihexoside; CDH: ceramide dihexoside; CMH: ceramide monohexoside; PC: phosphatidylcholine; Nd: non-detected.

	% of total incorporation			
	S17 cells		SF cells	
	Cells	Shed	Cells	Shed
GD1a	5.3	Nd	40.4	33.1
GM1	1.3	Nd	6.7	10.2
GM2	1.4	Nd	Nd	Nd
GM3	63.9	70.7	52.2	35.9
PC	1.3	Nd	Nd	12.9
CTH	24.4	11.7	Nd	3.1
CDH	0.7	6.1	Nd	4.7
CMH	1.1	Nd	Nd	Nd

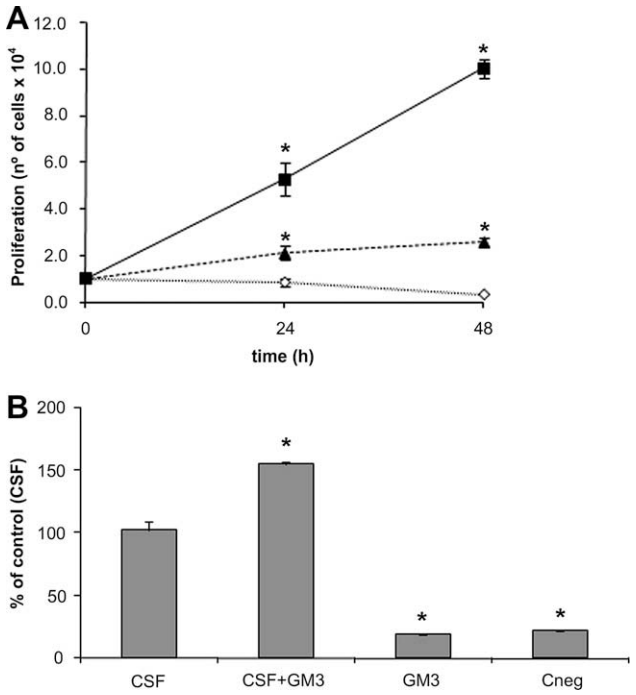

Fig. 3. Effect of PPPP and GM3 on FDC-P1 cell proliferation. (A) 3×10^4 cells per well were cultivated for 48 h in RPMI 10% FBS (◇), RPMI 10% FBS with GM-CSF (2 ηg/mL) (■), RPMI 10% FBS with GM-CSF (2 ηg/mL) and PPPP (2.5 μM) (▲). Cells were counted in hemocytometer. (B) 3×10^4 cells per well were cultivated for 24 h in RPMI 10% FBS with GM-CSF (2 ηg/mL) and/or GM3 (20 μM). Negative control was RPMI 10% SFB only (Cneg). Proliferation was monitored by Cell Titer Proliferation assay read at 490 nm. Data are from triplicates. Values are mean ± -SEM. (∗) Significant difference to $p < 0.05$.

kines [8,9,22]. The capping of proteoglycans and gangliosides at the interface between hemopoietic and stromal cells suggest that GM-CSF receptors might be sequestered in lipid rafts and modulated by macromolecular complexes, placing the growth factor message in the appropriate required microambient.

Conclusions

The present study indicates that gangliosides contribute for myeloid survival and proliferation mediated by stromal cells, the GM3 being apparently the major ganglioside involved. The differences in the capacities of stromal cells to promote myeloid cell proliferation could be related to differences on ganglioside distribution in plasma membrane of the studied stromas (S17 and SF) and/or to differences on the amount of ganglioside shed to the extracellular medium and its subsequent uptake by myeloid cells.

Acknowledgments

This research was supported by Grants from CNPq and CAPES of the Brazilian Federal Government, FAPERGS of the Rio Grande do Sul State Government/Brazil, FAPERJ of the Rio de Janeiro State Government/Brazil and SECyT-UNC; Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Agencia Nacional de Promoción Científica y Tecnológica, Argentina.

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