主論文の要旨

St8sia1-deficiency in mice alters tumor environments of gliomas, leading to reduced disease severity

（マウスのSt8sia1欠損は神経膠腫の腫瘍環境を変化させ
疾患の重症度を低下させる）

名古屋大学大学院医学系研究科　総合医学専攻
生物化学講座　分子細胞化学分野
（指導：岡島 徹也　教授）
张 璞

INTRODUCTION

Gliomas account for approximately 50% of all primary brain neoplasms, and the most common type is highly malignant glioblastoma multiforme. Glioblastoma multiforme is an aggressive, rapidly progressive, infiltrative parenchymal neoplasm associated with a poor prognosis. Ganglioside GD3 and GD2 are over-expressed in many types of neuroectoderm-derived tumors, and are associated with tumor progression and development of the metastatic potential. Previous studies indicated that GD3/GD2 is involved in the enhancement of cancer properties such as rapid growth and increased invasiveness. However, little is known about the functions of GD3/GD2 in glioma cells and glioma microenvironments. To clarify the functions of GD3/GD2 in gliomas, we used a mouse glioma model based on the RCAS/Gtv-a system.

MATERIALS and METHODS

Mice used in this study

The Gtv-a mouse that expresses tv-a under the GFAP promoter, p53-decifient mice were from the RIKEN Bioresource Center. These mice have a mixed genetic background of C57BL/6, 129, BALB/c, and FVB/N. GD3 synthase gene (St8sia1)-deficient mice lacking all b- and c-series gangliosides (Fig. 1a) were generated as previously reported. After 1-2 weeks, tumors were generated and extracted.

Generation of tumor-bearing mice

To generate virus-producing cells, DF-1 cells were transfected with RCAS retroviral vectors that contain cDNA of PDGFB linked with GFP via the T2A sequence, using Lipofectamine 2000™. Approximately 1×10^4 virus-producing DF-1 cells were injected into the right cerebral cortex of newborn p53-deficient Gtv-a mice. After 2 weeks of injection of DF-1/RCAS containing cDNA of PDGFB, almost all mice generated brain tumors. Tumors were imaged by IVIS Spectrum™, and pathologically diagnosed as glioblastoma After one or two weeks after virus-producing DF-1 cells injection, we removed mouse brains to performed real-time qPCR, immunohistochemistry (frozen sections) and DNA microarray.

RT-qPCR

Total RNA was extracted from WT or GD3S-KO mouse-derived primary cultured glioma cells using TRIzol™ according to the manufacturer's protocol. The qPCR was carried out using DyNAmo SYBR Green™ qPCR Kit and CFX Connect™ Real-Time System.

Immunohistochemistry of frozen sections

Frozen sections were blocked with 10% BSA in PBS at room temperature for 1 h, and were stained with primary antibodies and appropriate secondary antibodies. Finally, sections were stained with DAPI and mounted on microscope slide with ProLong™ Gold antifade reagent.

DNA microarray

Pooled RNA samples of glioma tissues prepared from five each of GD3S-WT and GD3S-KO mice were analyzed by Agilent SurePrint G3 Mouse GE 8x60K Microarray. The analysis of

microarray results was performed by Takara Bio Inc.

RESULTS

- GD3S-KO mice exhibited smaller gliomas

Newborn Gtv-a mice were injected with DF1 cells carrying PDGFB cDNA linked with GFP via the T2A sequence (Fig. 1b)). After 1-2 weeks, tumors were generated. Hematoxylin and eosin staining of the brain tissues showed that tumors were generated in most mice after one week of DF-1 cell injection (Fig. 2a)). Compared with WT mice, GD3S-KO mice showed smaller tumors (Fig. 2b)) and slower progression.

- Glioma tissues over-expressed ganglioside GD3 and GD2

After 1 or 2 weeks of DF-1 cell injection, mice were sacrificed and glioma tissues were extirpated. Using these tissues, frozen sections were prepared for immunohistochemistry (IHC-F). We stained those sections with anti-GD3 and anti-GD2 mAbs (Fig. 2c)). GD3/GD2 were highly expressed in glioma tissues of WT mice. However, normal brain tissues scarcely expressed those gangliosides (Fig. 2c), right). In GD3S-KO mice, neither GD3 nor GD2 was detected in glioma or normal tissues (Fig. 2c)). HE staining of glioma tissues revealed that GD3S-KO mice showed a less aggressive histology with necrosis (Fig. 2d) right, indicated by an arrow), and a clearer tumor border than WT mice (see black arrows in Fig. 2d)).

- Glioma sections from GD3S-KO mice expressed higher levels of inducible nitric oxide synthase-positive (iNOS$^+$) CD68$^+$ MI/MΦ

At one week after injection of DF-1 cells, the numbers of CD68$^+$ cells increased in gliomas compared with those in normal tissues in both WT and GD3S-KO mice (Fig. 3a)). At 2 weeks after injection, the numbers of CD68$^+$ cells as well as Ibal$^+$ cells significantly increased in tumor tissues of GD3S-KO mice compared with those of WT mice (Fig. 3a)).

To observe MI/MΦ localization, we used Iba1 as a marker for MI/MΦ, and CD68 as a marker for activated cells for staining the glioma frozen sections. The results showed that Ibal$^+$ cells broadly existed inside glioma tissues (Fig. 3b), *top*) in the sections at 1 week after injection. CD68$^+$/Ibal$^+$ cells showed different localization patterns between WT and GD3S-KO mice (*2nd row* and *bottom* in Fig. 3b), and Fig. 3c)). CD68$^+$/Ibal$^+$ cells formed a dense band surrounding the glioma mass of WT tumors. Furthermore, WT mouse sections showed highly activated (CD68$^+$) cells with retracted processes (round shape) around gliomas (Fig. 3c), *middle*).

- WT mouse-derived glioma cells expressed higher levels of pro-inflammatory cytokines

Although the IHC-F data suggested that MI/MΦ were reacting with glioma as shown by iNOS staining (Fig. 4a)). Then, we investigated expression levels of cytokine genes in gliomas. We extracted RNA from primary cultured glioma cells, and performed RT-qPCR. Consequently, IL-6, TGF-β1, PGE-2, and M-CSF mRNA levels were significantly elevated in WT glioma-derived cells (Fig. 4b). IL-6 was 4-fold higher than in GD3S-KO mice. TGF-β1 and M-CSF also increased 50% more in WT than GD3S-KO mice.

- DNA microarray showed different expression patterns of chemokine genes

Between WT and GD3S KO mice, there are marked differences in the expression profiles of chemokines in glioma tissues in the results of DNA microarray performed to screen and raise candidate genes for further analysis as shown in Fig. 5.

DISCUSSION

We previously reported increased malignant properties of human glioma cells and also enhanced malignant cell signals based on the expression of GD3/GD2 in mouse glioma models. In addition to the alteration in phenotypes of tumor cells themselves, effects of GD3S on the tumor environments were also assumed. We actually observed that the density of vessels was higher in gliomas of WT mice than of GD3S-KO mice in the tumor tissues. We eventually elucidated here that GD3S deficiency altered not only glioma phenotypes, but also the tumor microenvironment including the nature of MI/MΦ and their distribution.

Glioma cells secrete a wide variety of factors that suppress immune cells, such as IL-4, IL-6, IL-10, M-CSF, MIF, TGF-beta1, and prostaglandin E2, and many other molecules. These cytokines are known to change the polarization of M1 macrophages towards the M2 phenotype. Tumor-associated MI/MΦ secret chemo-attractants toward tumor cells in turn. Accordingly, DNA microarray and RT-qPCR on chemokine genes revealed distinct expression profiles between WT- and GD3S-KO mouse-derived gliomas.

CONCLUSION

All these observations suggest that the expression of GD3S causes activation of glioma cells, and makes glioma cells secrete cytokines to promote MI/MΦ polarizations from M1-like towards M2-like phenotypes. Consequently, these results suggest that growth factors and chemokines that enhance tumor progression/invasion via M2-like MI/MΦ tumor might be targets of glioma therapy, and GD3S might also be one of such target molecules (Fig. 6b)).