主論文の要旨

Expression analysis of 0-series gangliosides in human cancer cell lines with monoclonal antibodies generated using knockout mice of ganglioside synthase genes

（ガングリオシド合成酵素遺伝子のノックアウトマウスを用いて
作成した単クローン抗体によるヒト癌細胞株における
0-シリーズガングリオシドの発現解析）

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Abstract

Some acidic glycosphingolipids, gangliosides have been considered as tumor-associated antigens. Ganglioside GD1α or GD1α synthase *ST6GalNAc5* was reported to be involved in the metastasis of murine lymphomas or human breast cancers, respectively. But expression patterns of 0-series gangliosides GD1α and its precursor GM1b in human cancers have not been investigated mainly due to lack of specific antibodies. We have established monoclonal antibodies (mAbs) specifically reactive with GM1b or GD1α by immunizing GM2/GD2 synthase (*B4galnt1*)-knockout (KO) mice lacking all complex gangliosides including 0-series gangliosides with GD1α or acidic brain extracts from GM3 synthase (*St3gal5)*-KO mice as immunogens. Specificities of established mAbs as analyzed by enzyme-linked immunosorbent assay and thin-layer chromatography-immunostaining. Expression analysis of GD1α and GM1b using generated antibodies, and of relevant glycosyltransferase genes showed a few human cancer cell lines significantly expressed of these gangliosides with reasonable expression of relevant glycosyltransferase genes.

Materials and Methods

Glycosphingolipids (GSLs) from the brain of Knockout mice were separated by thin layer chromatography (TLC). To generate mAbs, *B4galnt1*-KO mice were immunized intravenously with liposomes containing GD1α (10 μg) or GSLs from *St3gal5*-KO mouse brain (10 μg) as immunogens. Screening and specificity of generated mAbs were examined by enzyme-linked immunosorbent assay (ELISA) as well as thin layer chromatography (TLC)-immunostaining. Reactivity of mAbs towards glycoproteins were analyzed by flow cytometry, and immunoprecipitation as well as western immunoblotting. Detection limits of GD1α and GM1b by mAbs as analyzed by TLC-immunostaining and ELISA. Expression analysis of cell membrane gangliosides GD1α and GM1b was analyzed by flow cytometry. Glycosyltransferase genes expression level involved in the synthesis of GD1α and GM1b were analyzed by RT-qPCR. Antitumor activity of mAbs for Y79 and HS cell were examined by complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC). Reactivity of mAbs to glycolipids expressed on Y79 and HS cells were analyzed TLC-immunostaining as well as flow cytometry.

Results

As a strategy of immunization, brain gangliosides from *St3gal5*-KO mice accumulated GD1α or 0-series gangliosides were used for immunogens to immunize *B4galnt1*-KO mice lacking all complex gangliosides with accumulation of GM3 and GD3 (Fig. 1A and 1B). Gangliosides from mouse brains were analyzed by TLC, showing that *B4galnt1*-KO mouse brains lacked all 0-, a- and b-series gangliosides except GM3 and GD3, while *St3gal5*-KO mouse brains accumulated GD1α and GM1b, and lacked all a- and b-series gangliosides. (Fig. 1C).

B4galnt1-KO mice were immunized with liposomes embedded with GD1α or brain gangliosides from *St3gal5*-KO mice. After immunization, sera were examined for antibody reactivity to immunized gangliosides by ELISA (data not shown). We generated mAbs according to a standard method. Specificity and reactivity of the selected clones were analyzed by ELISA, showing that only clone 122 specifically reacted with GD1α up to 640 fold dilution and brain gangliosides from *St3gal5* (-/-) mice (Table I and Table II), and clones MR155A-7 and MR30-3 generated from *St3gal5*(-/-) brain ganglioside-immunized mouse, specifically reacted with GM1b up to 1280 and 320 fold dilution, respectively (Table III). Further specificity analysis of obtained mAbs by TLC-immunostaining showed that mAb 122 specifically bound to purified GD1α and brain gangliosides from *St3gal5* KO mice, whereas mAbs MR155A-7 and MR30-3 specifically bound to purified GM1b and also brain gangliosides from *St3gal5* KO mice (Fig. 2A and 2B). Binding of obtained mAbs by flow cytometry showed increased levels of GD1α and decreased levels of GM1b after transfection of the GD1α synthase cDNA into RAW117, mouse lymphosarcoma (Fig. 3). Reactivity of mAbs towards glycoproteins by flow cytometry and immunoblotting showed no reactivity of anti-GD1α mAb 122 or anti-GM1b mAb MR155A-7 to LN319 cells as possess O-linked glycans such as sialyl-T and disialyl-T antigens in podoplanin (Fig. 4A and Table IV), while MAL II, a lectin bound to sialyl-T and disialyl-T structure (Fig. 4B-left). Immunoprecipitation of podoplanin from cell lysates of LN319 cells and subsequent immunoblotting of the immunoprecipitates showed binding of anti-podoplanin mAb to precipitated podoplanin, but not of anti-GD1α mAb 122 or anti-GM1b mAbs MR155A-7 and MR30-3 (Fig, 4B-right and Fig.4C). Detection limit of amounts of GD1α and GM1b by mAbs 122, MR155A-7 and MR30-3 were performed by TLC-immunostaining as well as ELISA. TLC-immunostaining showed GD1α could be detected to 8.75 ng by 500 fold-diluted ascites of mAb 122, and GM1b could be detected to 17.5 ng by both anti-GM1b mAbs MR155A-7 and MR30-3 with diluted ascites and supernatants at 500 and 20 folds, respectively. (Fig. 5A). ELISA showed that GD1α was detected to 4.375 ng by ascites of mAb 122 at 500 fold dilution, and GM1b was detected to 8.75 ng or 17.5 ng by anti-GM1b mAbs MR155A-7 or MR30-3 3 with dilution of ascites and supernatants at 500 and 20 folds, respectively (Fig. 5B). Expression analysis of GD1α and GM1b by flow cytometry using 71 human cancer cell lines derived from various tissues showed GD1α positive cells in about 2% of HS and in 58% of Y79, and GM1b positive cells at about 56% in Y79 cells, but all other cell lines showed very low expression at about 1% or undetectable levels of GD1α and GM1b. (Table IV). Anti-tumor activities of these mAbs showed that mAbs 122 (IgM), MR155A-7 (IgM) and MR30-3 (IgG) mediated CDC activity ranging from 12 to 23% in Y79 cells, and mAb 122 mediated CDC from 1 to 2% in HS cells when used as five fold-diluted complement serum with various dilution of ascites or supernatants. Results of CDC were 1 to 4% for Y79 cells, and 0.5 to 1% for HS cells when twenty or fifty-fold diluted rabbit serum was used as a complement source (Figure 6 Aa and Ab). Mab MR30-3 showed dose dependent ADCC activity for Y79 cells ranging from 9 to 21% and 3 to 14% by the diluted mAb (supernatant, at twenty and forty folds) with effector/target ratios of 10:1 and 5:1, respectively (Fig. 6 B). mRNA

expression levels of relevant glycosyltransferase genes by quantitative (q)RT-PCR analysis showed GD1α synthase *ST6GalNAc5* gene was highly expressed in HS and Y79 cells (Fig. 7B). Examination on the correlation of the expression of GD1α and GM1b with glycosyltransferase genes showed that high expression levels of *ST6GalNAc5* gene in Y79 and HS cell lines well correlated with those of GD1α (Fig. 7). Reactivity of anti-GD1α mAb to Y79 and HS, and anti-GM1b mAb to Y79 were also confirmed by TLC-immunostaining as well as flow cytometry (Fig. 8).

Discussion

In the present study, successful generation of mAbs specific for 0-series gangliosides GM1b and GD1α using KO mice were demonstrated. These mAbs showed high specificity to GM1b or GD1α. We examined the expression levels of those gangliosides in 71 human cancer cell lines derived from various tissues using newly generated anti-GD1α or anti-GM1b mAbs. Glycosyltransferase gene *ST6GalNAc5* was expressed at relatively high levels in Y79 and HS cells compared with other cell lines with low or negative expression of those gangliosides, indicating well correlation between expression of GD1α and *ST6GalNAc5*. Anti-tumor activities of these mAbs as shown in CDC were of moderate levels, and ADCC of mAb MR30-3 (IgG) to Y79 with PBMC was also not very much strong, but definite, suggesting the possibility of these mAbs for application in cancer therapeutics.

Conclusion

The mAbs established in this study should be useful tools for the analysis of expression and functions of 0-series gangliosides in human cancer cell lines as well as in normal cells and tissues.