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EXPRESSION OF SPECIFIC ANTIBODIES IN NON-LYMPHOID CELL TYPES AND THEIR IMPORTANCE FOR DEVELOPMENT OF NOVEL DIAGNOSTIC, PROPHYLACTIC AND THERAPEUTIC STRATEGIES

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Abstract: The titers of ganglioside GM3 and anti-GM3 antibodies were assessed in extracts from brain, pancreas and *in vitro*-cultures of normal cells, malignant cells and mixed culture of both cell types. Total lysates from both organs were prepared (controls) and equal volumes of them were passed through GSH-Agarose Columns for separation of bio-molecules with affinity to the reduced form of tripeptide Glutathione - GSH. Separate aliquots from the lysates of both organs were mixed with lysates of laboratory-incubated cells, containing additionally-inserted copy of tumor-suppressor gene scgn, coding hormone-like protein Secretagogin (SCGN) for separation of molecules with affinity to it. The amounts of GM3 and titers of anti-GM3 antibodies in each of the prepared samples were estimated by ELISA technique, after addition of the tested ganglioside, and/or of serum, previously proved as positive on anti-GM3 antibodies. Statistically significant frequency of statistically higher GM3 and anti-GM3 antibodies titers in the lysates from cultures of malignant cells and of the mixed cultures were established, compared to the lysate from the culture of normal cells. These results were in agreement with literature data about increased titers of these molecules in neoplastic cells and malignancies. Higher titers of GM3 and anti-GM3 antibodies in the samples containing molecules with

affinity to SCGN and GSH compared to the controls were assessed. The differences could be explained with decreased titers in equivalent volume of the respective biological material at the expense of other bio-molecules, not possessing such affinity. These data were in confirmation of literature findings about the ability of non-lymphoid cell types to produce immunoglobulins (antibodies). These features could suggest a possibility for development of novel strategies for diagnostic, prophylactic and therapy goals.

INTRODUCTION

Gangliosides are complex glycosphingolipids, which have been found to participate in the mechanisms of various intermolecular intra- and extra-cellular interactions in cascade mechanisms, known to underline the cellular functions of the separate cells, tissues and organs (Kolyovska *et al.,* 2015; Xu e*t al.,* 2010; Yu *et al.,* 2011). These cascade regulatory pathways have been found to be responsible for the differences between the cells but also vital and specific functions as growth, proliferation and differentiation (Zurita *et al.,* 2001). In this way, these molecules have been proposed to be important in the control of many biological processes (Xu *et al.,* 2010; Yu *et al.,* 2011; Zurita *et al.,* 2001). Besides in free form, each ganglioside has been found to exist in various bounded forms with different bio-molecules, depending on the functions in which it participates. The protective role of ganglioside GM3 against many malignancies (Bai and Seyfried; 1997; Iwabuchi *et al.,* 1998; Miura *et al.,* 2004; Toledo *et al.,* 2004; Watanabe *et al.,* 2002), neurodegenerative processes (Simpson *et al.,* 2004; Ohkawa *et al.,* 2011; Zaprianova *et al.,* 2010) and some endocrine diseases as diabetes (Inokuchi, 2014; Kabayama *et al.,* 2007; Kabayama *et al.,* 2005) has been proved. It is known to regulate membrane-transmitted signals and to modulate the functions of tumor suppressors. Its anti-angiogenic role in some solid malignancies has also been established.

The tripeptide glutathione (GSH) and the hormone-like protein secretagogin (SCGN) have been characterized as key molecules participating in different intra- and extra-cellular inter-molecular interactions (Gartner *et al.,* 2007; Hayes and McLellan, 1999; Jahren-Hodje *et al.,* 1997; Maj *et al.,* 2010). The role of protein SCGN in the coordinated action of oncogenes and tumor-suppressor genes and, hence, in the regulation and prevention of malignant transformation has been shown (Witcher *et al.,* 1995). Its role as neuroprotector, endocrine regulator and anti-diabetic substance has also been proved (Hayes and McLellan, 1999; Maj *et al.,* 2010) Calcium-dependent SCGN-TAU interaction, as well as co-induction of TAU, has been found in the islets of Langerhans and beta-cell-derived lines with high expression of the neuroendocrine-specific protein SCGN (Butner and Kirschner, 1991; Rogstam *et al.,* 2007). These effects of SCGN have been proposed to be related with its ability to bound to cytoskeleton components as microtubule proteins and cyclins (Oakata *et al.,* 1995). The last have been proved to connect with histones and histone-like nuclear proteins, in cascade regulatory pathways (Jaumot *et al.,* 1994). On the other hand, and in particular its reduced form (GSH) has been proved as one of the main regulatory

molecules in the processes of cell proliferation, differentiation, death, inflammatory and pro-inflammatory processes (Hayes and McLellan, 1999; Jahren-Hodje *et al.,* 1997).

Considering these data the current study aimed to determine the presence and titers of ganglioside GM3 and antibodies against it in extracts from *in vitro*-incubated cell cultures and anatomic organs, prepared by techniques, providing different proportions of specific molecules.

MATERIALS AND METHODS

The amounts of ganglioside GM3, as well as the titers of specific antibodies against it, were assessed in *in vitro*-, *in vivo*- and "*in vivo*+*in vitro*"-experimental models. The in vitro-models were presented by lysates from three types of laboratory-incubated cell cultures: of normal mouse cells from 3T3 embryonic line, of malignant cells from mouse myeloma line Ag853, but also mixed cultures of co-cultivated cells from both types. The in vivo-models were presented by extracts of rat brain and pancreas. The experimental models "*in vivo*+*in vitro*" were presented by lysates from the same anatomic organs, determined as containing molecules with affinity to protein SCGN after passing through GSH-Agarose Columns (by taking in consideration the presence of GST-tag in the used recombinant DNA-constructs), applying the protocol of Derrick Witcher *et al.* (1995). These samples were prepared by mixing separate aliquots from the lysates of both organs with isolated SCGN from prokaryotic *Escherichia coli* strains and eukaryotic malignant RIN-5F cells from rat insulinoma, both containing previously transferred tumor-suppressor gene *scgn* by transfection with appropriate recombinant DNA-constructs. In general, total extracts were isolated separately from RIN-5F malignant rat cells and from bacteria strains, respectively, both containing additionally-inserted copy of scgn gene by transfection with appropriate recombinant DNA-constructs, after which polyclonal anti-rat SCGN antibody was added to each one of the two types of lysates. After centrifugation and removing the supernatants, the pellets/precipitates (probably containing SCGN protein) were resuspended with PBS. The acquired suspensions were mixed together and the so prepared suspension mixture was separated in two equal parts. One of them was mixed with previously prepared total lysate from rat brain and the other – with total lysate from rat pancreas, respectively. The organ extracts were prepared by mincing of the isolated anatomic organs and subsequent treatment with lysis buffer. These two anatomic organs are known to actively express protein SCGN from most of the cells, composing each one of them.

For determination of the titers of GM3 and anti-GM3 antibodies in each of the tested samples, ELISA-method, published by Roxana Mitzutamari *et al.* (1994) with slight modifications was applied. For anti-GM3 antibodies, a solution of 1000 ng dry substance of the ganglioside GM3 (Sigma) in 100 ml of methanol were pipetted into 96-well micro-titre plates, containing the respective samples. For GM3, instead

of ganglioside solution, serum, previously proved to contain specific anti-GM3 antibodies, was added to the tested samples. After air drying, the wells were blocked with BSA-PBS (Sigma) (1% bovine serum albumin in phosphate-buffered saline) for 1 hour. After six-fold washing with PBS, 100 µl from each one of the prepared lysates, described above, diluted 1:20 to 1:5000 in PBS, were added to each well and incubated overnight. Subsequently, the plates were washed six times with PBS. Binding was detected after 2 hours incubation period with BSA-PBS (Sigma) diluted (1/3200) peroxidase-conjugated goat anti-IgG antibodies (Bul Bio Ltd., NCIPD, Sofia). All the incubations were performed at 4°C. After six washes with PBS, colour development was achieved in a substrate solution, containing 15mM O-phenilendiamine and 0.015% H_2O_2 in 0.1M sodium acetate buffer (0,2 M CH_3COONa/0,2 M CH_3COOH; pH 5.0) at 20°C. The reaction was stopped after 30 minutes by addition of 50 µl of 1N H_2SO_4, and the optical density (OD) was read spectrometrically at 490 nm on ELISA-reader (TECAN TM, Sunrise, Austria). Non-specific antibody binding (OD value in a well not containing the specific molecule in the respective sample) was subtracted for each measurement. The data were considered strongly positive, when the mean OD exceeded 2 ± SD (standard deviation), compared with the controls. The standard error of mean varied between ± 0.01 and ± 0.1. For the best reliability the procedure was performed three times.

RESULTS

Statistically significant frequency of higher titers of ganglioside GM3 and anti-GM3 antibodies were observed in the samples from the neoplastic cells and mixed cultures, compared to these in the normal cells (**Figs. 1a, b**).

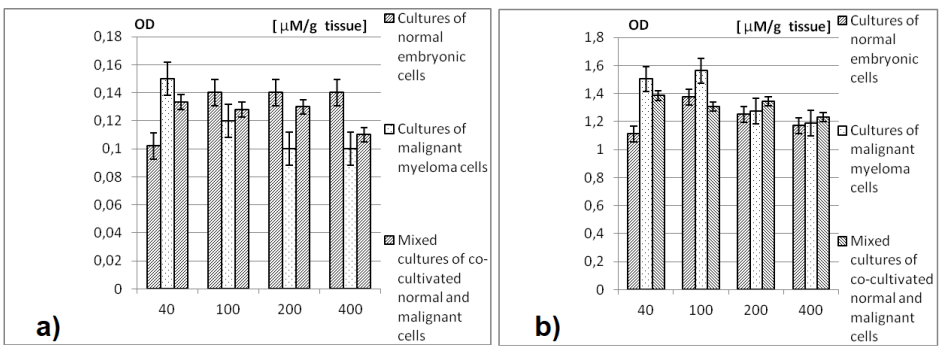

Fig. 1. Titers of ganglioside GM3 **(a)** and anti-GM3 antibodies **(b)** in lysates from in vitro-cultures of normal mouse embryonic cells from 3T3 fibroblast cell line, mouse malignant myeloma cells, and mixed of both cell types, OD – optical density.

Analogically, statistically significant frequency of higher titers of the same ganglioside and antibodies to it were established in the lysates containing molecules with affinity to SCGN and to GSH, respectively, compared to the control extracts, non-passed through columns and containing the full composition of biological molecules (**Figs. 3-6**). These differences could be explained with probably decreased titers in equivalent volumes of the respective biological material, at the expense of the presence of other molecules, not possessing such affinity

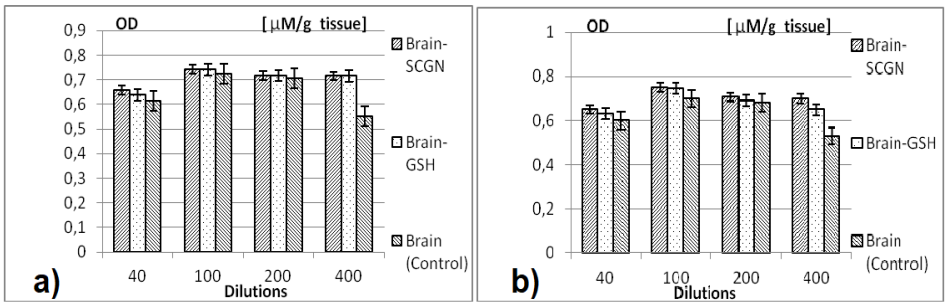

Fig. 2. Titers of ganglioside GM3 **(a)** and anti-GM3 antibodies **(b)** in lysate from rat brain: control sample, containing the full composition of molecules; containing molecules, possessing affinity to GSH; containing molecules, possessing affinity to protein SCGN, OD – optical density.

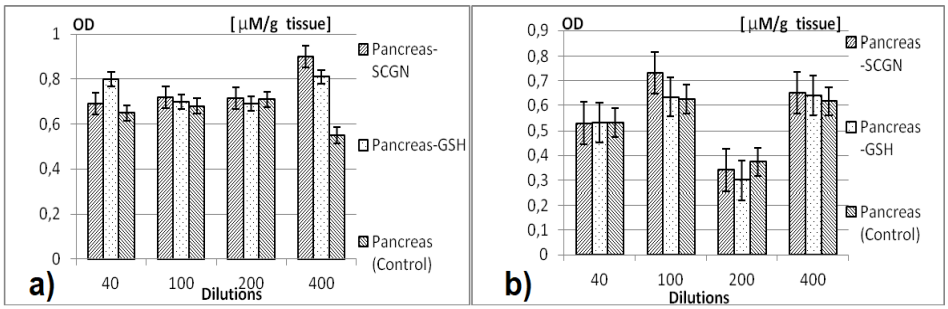

Fig. 3. Titers of ganglioside GM3 **(a)** and anti-GM3 antibodies **(b)** in lysate from rat pancreas: control sample, containing the full composition of molecules; containing molecules, possessing affinity to GSH; containing molecules, possessing affinity to protein SCGN, OD – optical density.

DISCUSSION

The results obtained confirmed the literature data about the proved role of GM3 and anti-GM3 antibodies as markers of malignancy (Bai and Seyfried, 1997; Choi *et al.,* 2006; Miura *et al.,* 2004; Nematpour *et al.,* 2018). A protective function of the increased amounts of this ganglioside in malignant cells has also been proposed by its accumulation during apoptosis (Watanabe *et al.,* 2002). On the other hand, these data were in support of the literature findings about the importance of this ganglioside for the insulin signaling pathways in the control of diabetes (Inokuchi, 2014; Kabayama *et al.,* 2007; Kabayama *et al.,* 2005). These data were in agreement with suggested neuro-protective function of this substance (Kolyovska *et al.,* 2015), supporting the explanation of its role in the control of neurodegenerative processes by the established relationship of disorder symptoms and mutations, connected with a lack of the enzyme GM3 synthase (Simpson *et al.,* 2004). These results were also in confirmation of the changes in the titers of GM3 and anti-GM3 antibodies in pathogenic and/or ageing processes in people and rats (Kolyovska *et al.,* 2015; Zaprianova et al., 2010). Also, the results were in agreement with literature data about a possibility of non-lymphoid cell types to express and produce immunoglobulins (IgG antibodies) in appropriate internal (genetic), epigenetic and/or external conditions (Bebbington, 1991), including such with malignant characteristics (Chen *et al.,* 2009). In support of the scientific literature, these features could be explained with the ability of immature cells to differentiate in lymphoid direction (Cho *et al.,* 1999), but also with the immunoglobulin characteristics and antibody properties of cell-produced proteins, performing different specific functions in the respective cell type (Nematpour *et al.,* 2018). Another possible explanation was the proved action of proteins, performing specific functions in the respective types of non-lymphoid cells, tissues and organs (for example, enzymes), as antibodies and/or immunoglobulin domains/chains (Ponomarenko *et al.,* 2006). In this way, the presented data suggested a possibility for development of novel strategies for diagnostic, prophylactic and therapeutic purposes. Similar studies on other types of cells, tissues and organs should be performed in the near future.

CONCLUSION

The established tendency for increased titers of GM3 and anti-GM3 antibodies in the lysates from the cultures of malignant cells and from the mixed cultures, compared to the lysates from the normal cells were in agreement with literature findings about the role of this ganglioside as a marker for malignancy. The tendency for its increased amounts, but also of the titers of specific antibodies to it in the samples of both organ extracts, containing selected molecules with affinity to GSH and to protein SCGN, respectively, compared to the control

samples, were probably due to the presence of many other molecules in the last, not possessing such affinity, in equal volumes of biological material. In this way, the current study suggested the role of ganglioside GM3 in the mechanisms of prevention of diabetes, and of many neurodegenerative changes, by molecules, which it interacts. A possibility for production of antibodies from non-lymphoid cells, tissues and organs in appropriate conditions was shown, including in result of probable initial lymphoid differentiation of low-differentiated immature cell sub-populations. In this way, a possibility for expression of immunoglobulin genes in non-lymphoid cell types, including in malignant cells, in appropriate conditions, was proved, which was in confirmation of literature data in this attitude and could suggest a possibility for development of novel diagnostic, prophylactic and therapeutic strategies.

DECLARATION OF INTEREST

The authors declare no existing conflict of interests.

AUTHOR CONTRIBUTION STATEMENT

IS provided incubation of prokaryotic and eukaryotic cells and their transfection with appropriate DNA-vectors, as well as preparation of lysates from cell cultures (in vitro-), from anatomic organs (in vivo-) and mixed ("in vitro+in vivo"-) experimental models, as well as collection of the data

VK developed and performed the described ELISA-technique and prove the presence of specific IgG anti-GM3 antibodies in human sera, as well as analyzing the data

SE confirmed the analogies in the biochemical activity of molecules from lysate of transfected prokaryotic Escherichia coli bacteria strains with these from the isolated analogically prepared lysate from transfected eukaryotic malignant rat insulinoma cells of line RIN-5F

RE allowed approach to the microbiology laboratory and helped the final edition of the manuscript

TM provided myeloid malignant cells from mouse myeloma line Ag853 and helped the final edition of the manuscript

DD provided medical diagnosis of patients with diabetes and neurodegenerative disorders, which experimental models are used in the current study

DM provided human sera from the patients, described above, for prove the presence of specific IgG anti-GM3 antibodies in them

REFERENCES

1. Bai H., T. Seyfried. Influence of ganglioside GM3 and high density lipoprotein on the cohesion of mouse brain tumor cells. *J. Lipid Res.,* 1997, 38(1), 160-172.
2. Bebbington, C. Expression of antibody genes in nonlymphoid mammalian cells. *Methods*, 1991, 2(2), 136-145.
3. Butner, K., M. Kirschner. Tau protein binds to microtubules through a flexible array of distributed weak seats. *J. Cell. Biol.,* 1991, 115(3), 717-730.

4. Chen, Z., X. Qiu, J. Gu. Immunoglobulin expression in non-lymphoid lineage and neoplastic cells. *Am. J. Pathol.,* 2009, 174(4), 1139-1148.
5. Cho, S., T. Webber, J. Carlile, T. Nakano, S. Lewis, J. Zuniga-Pflucker. Functional characterization of B lymphocytes generated in vitro from embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.,* 1999, 96(17), 9797-9802.
6. Choi H., T. Chung, S. Kang, Y. Lee, J. Ko, J. Kim, C. Kim. Ganglioside GM3 modulates tumor suppressor PTEN-mediated cell cycle progression—transcriptional induction of p21WAF1 and p27kip1 by inhibition of PI-3K/AKT pathway. *Glycobiology*, 2006, 16(7), 573-583.
7. Gartner, W., G. Vila, T. Daneva, A. Nabokikh, F. Koc-Saral, A. Ilhan, O. Majdic, A. Luger, L. Wagner. New functional aspects of the neuroendocrine marker secretagogin based on the characterization of its rat homolog. *Am. J. Physiol. Endocrinol. Metab.,* 2007, 293(1), E347–E354.
8. Hayes, J., L. McLellan. Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. *Free Radic. Res.,* 1999, 31(4), 273-300.
9. Inokuchi, J. GM3 and diabetes. *Glycoconj. J.*, 2014, 31(3), 193-197.
10. Iwabuchi, K., S. Yamamura, A. Prinetti, K. Handa, S. Hakomori. GM3-enriched microdomain involved in cell adhesion and signal transduction through carbohydrate-carbohydrate interaction in mouse melanoma B16 cells. *J. Biol. Chem.,* 1998, 273, 9130-9138
11. Jahren-Hodje, J., M. Obin, X. Gong, S. Shang, T. Nowel, J. Gong, H. Abasi, J. Blumberg, A. Taylor. Regulation of ubiquitin-conjugating enzymes by Glutathione following oxidative stress. *J. Biol. Chem.,* 1997, 272(45), 28218-28226.
12. Jaumot, M., X. Grana, A. Giordano, P. Reddy, N. Agell, O. Bachs. Cyclin/cdk2 complexes in the nucleus of Hela cells. *Biochem. Biophys. Res. Commun.,* 1994, 203(3), 1527-1534.
13. Kabayama, K., T. Sato, F. Kitamura, K. Saito, N. Loberto, A. Pronetti, S. Sonnino, M. Konjo, Y. Igarashi, J. Inokuchi. Dissociation of the insulin receptor and caveolin-1 complex by ganglioside GM3 in the state of insulin resistance. *Proc. Natl. Acad. Sci. U.S.A.,* 2007, 104(34), 13678-13683.
14. Kabayama, K., T. Sato, F. Kitamura, S. Uemura, B. Kang, Y. Igarashi, J. Inokuchi. TNFα-induced insulin resistance in adipocytes as a membrane microdomain disorder: involvement of ganglioside GM3. *Glycobiology*, 2005, 15(1), 21-29.
15. Kolyovska, V., V. Pavlova, I. Iliev, Y. Dokova, D. Maslarov. Neurodegenerative changes in serum IgG antibodies to GD1a, GM1 and GM3 gangliosides in aging rats. *Am. J. Neuroprotection and Neurodegeneration,* 2015, 7(1), 72-74.
16. Iwabuchi K., S. Yamamura, A. Prinetti, K. Handa, S. Hakomori. GM3-enriched microdomain involved in cell adhesion and signal transduction through carbohydrate-carbohydrate interaction in mouse melanoma B16 cells. *J. Biol. Chem.,* 1998, 273(15), 9130-9138.
17. Maj, M., W. Gartner, A. Ilhan, D. Neziri, J. Attems, L. Wagner. Expression of TAU in insulin-secreting cells and its interaction with the calcium-binding protein secretagogin. *J. Endocrinol.,* 2010, 205(1), 25-36.
18. Mitzutamari, R., H. Wiegandt, G. Nores. Characterization of anti-ganglioside antibodies present in normal human plasma. *J. Neuroimmunol.,* 1994, 50(2), 215-220.
19. Miura, Y., M. Kainuma, H. Jiang, H. Velasco, P. Vogt, S. Hakomori. Reversion of the Jun-induced oncogenic phenotype by enhanced synthesis of sialosyllactosylceramide (GM3 ganglioside). *Proc. Natl. Acad. Sci. U.S.A.,* 2004, 101(46), 16204-16209.

20. Nematpour, F., F. Mahboudi, V. Khalaj, B. Vaziri, S. Ahmadi, S. Ebadat, F. Davami. Optimization of monoclonal antibody expression in CHO cells by employing epigenetic gene regulation tools. *Turk. J. Biol.,* 2018, 2017(41), 622-628.
21. Ohkawa Y., O. Yuhsuke, T. Orie, Y. Yoshio, F. Keiko, F. Koichi. Wisp2/CCN5 up-regulated in the central nervous system of GM3-only mice facilitates neurite formation in Neuro2a cells via integrin-Akt signaling. *Biochem. Biophys. Res.,* 2011, 411(3), 483-889.
22. Ookata, K., S. Hisinaga, J. Bulinski, H. Murofushi, H. Aizawa, T. Itoh, H. Hotani, E. Okumura, K. Tachibana, T. Kishimoto. Cyclin B interaction with microtubule-associated protein 4 (MAP4) targets p34cdc2 kinase to microtubules and is a potential regulator of M-phase microtubule dynamics. *J. Cell. Biol.,* 1995, 128(5), 849-862.
23. Ponomarenko, N., O. Durova, I. Vorobiev, A. Belogurov, I. Kurkova, A. Petrenko, G. Telegin, S. Suchkov, S. Kiselev, M. Lagarkova, V. Govorun, M. Serebryakova, B. Avalle, P. Tornatore, A. Karavanov, H. Morse, D. Thomas, A. Friboulet, A. Gabibov. Autoantibodies to myelin basic protein catalyze site-specific degradation of their antigen. *Proc. Nat. Acad. Sci. U. S. A.,* 2006, 103(2), 281-286.
24. Rogstam, A., S. Linse, A. Lindqvist, P. James, L. Wagner, T. Berggard. Binding of calcium ions and SNAP-25 to the hexa EF-hand protein secretagogin. *Biochem. J.,* 2007, 401(1), 353-363.
25. Simpson, M., H. Cross, C. Proukakis, D. Priestman, D. Neville, G. Reinkensmaier, H. Wang, M. Wiznitzer, K. Gurtz, A. Verganelaki, A. Pryde, M. Patton, R. Dwek, T. Butters, F. Platt, A. Crosby. Infantile-onset symptomatic epilepsy syndrome caused by a homozygous loss-of-function mutation of GM3 synthase. *Nat. Genet.,* 2004, 36(11), 1225-1229.
26. Toledo M., E. Suzuki, K. Handa, S. Hakomori. Cell growth regulation through GM3-enriched microdomain (glycosynapse) in human lung embryonal fibroblast WI38 and its oncogenic transformant VA13. *J. Biol. Chem.,* 2004, 279(33), 34555-34664.
27. Watanabe, R., C. Ohyama, H. Aoki, T. Takahashi, M. Satoh, S. Saito, S. Hoshi, A. Ishii, M. Saito, Y. Arai. Ganglioside GM3 overexpression induces apoptosis and reduces malignant potential in murine bladder cancer. *Cancer Res.,* 2002, 62(13), 3850-3854.
28. Witcher, D., M. De Waard, H. Liu, M. Pragnell, K.P. Campbell. Association of native Ca^{2+} channel β subunits with the α1 subunit interaction domain. *J. Biol. Chem.,* 1995, 270(30), 18088-18093.
29. Xu, Y., S. Barnes, Y. Sun, G. Grabowski. Multi-system disorders of glycosphingolipid and ganglioside metabolism. *J. Lipid Res.,* 2010, 51(7), 1643-1675.
30. Yu, R., Y. Tsai, T. Ariga, M. Yanagisawa. Structures, biosynthesis, and functions of gangliosides - an overview. *J. Oleo Sci.,* 2011, 60(10), 537-544.
31. Zaprianova, E., D. Deleva, B. Sultanov, V. Kolyovska. Serum ganglioside GM3 changes in patients with early multiple sclerosis. *Acta morphol. et anthropol.,* 2010, 15, 16-18.
32. Zurita, A., H. Maccioni, J. Daniotti. Modulation of epidermal growth factor receptor phosphorylation by endogenously expressed gangliosides. *Biochem. J.,* 2001, 355(Pt2), 465-472.