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REVIEW

Sphingolipids in Tumor Metastases and Angiogenesis

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Abstract—This review article summarizes data on the involvement of sphingolipids (sphingosine-1-phosphate, sphingosine-1-phosphocholine, neutral glycosphingolipids, and gangliosides) in tumor metastases and angiogenesis.

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Sphingolipids represent the most chemically diverse class of lipids exhibiting a wide spectrum of biological activity. It covers hundreds of compounds sharing a common fragment, the sphingosine base. In animals and human cells sphingosine (trivial name of C_{18}-sphingenine; D-*erythro*-(2S,3R,4E)-2-amino-4-octadecene-1,3-diol) is the most common sphingoid. All sphingolipids represent metabolically related molecules.

Good evidence now exists that sphingolipids are involved in regulation of cell growth and apoptosis (including stimulation and inhibition of tumor growth). Several reviews have been published on the role of sphingolipids in the development [1-6] and therapy [5-12] of tumors. Metastases and angiogenesis are important biochemical and clinical aspects in tumor processes. However, a detailed review on tumor metastases almost lacks data on sphingolipids [13]. So in this review we analyze and discuss sphingolipid involvement in tumor metastases and angiogenesis and use of these data for chemotherapy.

It is known that tumor cells release (shed) membrane fragments containing sialosphingolipids (gangliosides) (together with other molecules). This phenomenon, known as shedding [14], is suggested to protect the tumor against the host immune system [15-17] and promote tumor metastases and angiogenesis [18].

The metastasizing process is determined by two principal stages: migration of tumor cells in extracellular medium and their adhesion to other tissue. Sphingolipids are actively involved in mechanisms underlying both stages. It is suggested that glycosylation and glycosphingolipid composition are crucial for the metastasizing process [19-21]. In metastases of R3230AC breast adenocarcinoma cells [22] and rat hepatoma [23], isoglobotetraosylceramide (GalNAcβ1-3Galα1-3Galβ1-4Glcβ1-Cer) was identified, and the authors [22] suggested that it might be a metastasis marker. This compound underlined high metastasizing potential of tumor cells to lung, and its blockade significantly reduced the metastasizing activity of these cells [22]. The latter suggests that this sphingolipid is mainly involved in the second stage of the metastasizing process. Later studies also revealed involvement of a phosphosphingolipid, sphingosine-1-phosphate (S1P), in the metastasizing process. S1P stimulated migration of breast cancer MDA-MB-231 cells and increased their metastasizing activity [24]. Effects of S1P are realized via specific G-protein coupled receptors S1P(1), S1P(2), and S1P(3) located on the surface of tumor cells; the resultant effect (stimulation or inhibition of cell migration) depends on S1P binding to a particular type of its receptor: binding to S1P(1) or S1P(3) causes stimulation of cell migration, whereas binding to S1P(2) inhibits cell motility [25-28]. This indicates involvement of S1P in the first stage of metastasis. Sphingosine-1-phosphocholine [29] also influences motility of tumor cells. However, it has been shown that autotaxin (endonucleotide pyrophosphatase/phosphodiesterase) hydrolyzes sphingosine-1-phosphocholine with formation of S1P, which is responsible for regulation of cell motility [30]. S1P inhibits the promoting effect of autotaxin on cell motility [31].

Sialoglycosphingolipids (gangliosides) play a crucial role in metastasis. Numerous studies have demonstrated

Abbreviations: S1P) sphingosine-1-phosphate; S1P(1), S1P(2), S1P(3)) receptors of sphingosine-1-phosphate.

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that tumor cells and metastases differ in ganglioside composition. For example, the ganglioside composition of subcutaneous tumor developed after subcutaneous injection of melanoma cells to rats was the same as in the initial melanoma cells, but in nodules of grown lung metastases ganglioside biosynthesis was inhibited and lactosylceramide (LacCer) accumulated [32]. Study of ganglioside profile of metastases formed in mouse liver after inoculation of human uveal melanoma SP6.5 cells into mouse spleen revealed significant increase in GM3 and reduction in GD3 and GD2 in metastases compared with initial SP6.5 tumor cells [33]. In all metastatic tumors of human brain of various origin (metastases from colon, kidney, lung, esophagus, pancreas, and mammary gland carcinomas) GM3 was the main ganglioside component. All metastases were characterized by the presence of ganglioside GT1b, which was not detected in carcinomas lacking brain metastases [34].

Studies of various laboratories [35-37] revealed that reduced content of complex gangliosides is common for many types of transformed cells, especially for highly metastasizing tumor cells [38, 39]. For example, 3T3 fibroblasts transformed with SV-40 and RSV had lower level of ganglioside GD1a, and this change was more pronounce in the highly metastasizing subclone isolated from RSV-transformed cells [39]. Study of glycosphingolipid composition in rat breast cancer MTC subclones exhibiting different metastasizing potential revealed that nonmetastasizing MTLn2 cell line had significantly higher level of GD1a than MTLn3 cell line characterized by high lung metastasizing potential [40]. A similar tendency was also found for lowly metastasizing FBJ-S1 cells in comparison with FBJ-LL [41]. The decrease in GD1a level together with appearance of gangliosides GM3, GM2, GM1a, and GD1b was also found in highly metastasizing variant of Esb lymphoma compared with lowly metastasizing initial Eb cell line characterized by high content of GD1a, gangliotriaosylceramide and gangliotetraosylceramide [42]. However, there are some lymphomas exhibiting high metastasizing potential and containing higher level of complex gangliosides than their corresponding lowly metastasizing counterparts. For example, highly metastasizing MDAY-D2 cells contain GM3, GM2 and large quantities of GM1 and GD1a, whereas lowly metastasizing MDW4 cells are characterized by the decrease in total ganglioside content, disappearance of GM1, and accumulation of GM2 [43, 44]. A similar phenomenon was also found in a lowly metastasizing clone derived from mouse T-cell lymphoma [42]. Moreover, the increase in metastasizing potential of Kirsten sarcoma cells (transformed by fibroblast 3T3 virus) [45] and various variants of rat hepatomas [46] is accompanied by an increase in asialoganglioside level. In 3T3 fibrosarcoma cells high level of globosylceramide correlated with marked metastatic phenotype and expression of surface globosylceramide was 10 times higher in highly metastasizing cells than in their lowly metastasizing analogs [39]. Study of ganglioside composition of two human melanoma clones exhibiting different metastasizing capacities revealed that ganglioside profile of the lowly metastasizing cells corresponded to that of "parent" tumor whereas the highly metastasizing tumor cells were characterized by inhibition of ganglioside biosynthesis. In this highly metastasizing clone, ganglioside GM3 content (the only ganglioside found in these cells) and activity of GM3 synthase were reduced [32]. Study of changes in ganglioside composition in metastases *in vivo* using metastasizing and nonmetastasizing liver carcinomas revealed that total content of gangliosides was higher in carcinomas than in homologous normal tissues, but the ganglioside content in metastasizing carcinomas was significantly lower than in nonmetastasizing counterparts. Relative content of ganglioside GM3 in the non-metastasizing tumors reduced whereas in highly metastasizing tumor it increased [47]. Two human melanomas of various histogenesis, skin melanoma and uveal melanoma, were characterized by different ganglioside composition [48]. Skin melanoma contained GM3 (up to 75% of total ganglioside content), significant amounts of GD3, and also GD2, GM2, and 9-O-Ac-GD3. In uveal melanoma, GM3 represented more than 90% of total ganglioside content, and there was reduced content of gangliosides GD3 and GD2 and lack of GM2 and 9-O-Ac-GD3. The authors suggest that differences in metastasizing activity of these two types of melanomas (lowly metastasizing activity of uveal melanoma and highly metastasizing activity of skin melanoma) may be explained by differences in ganglioside expression and their composition [48].

Use of experimental models of spontaneous metastases of human M4Be melanoma to lung (seven clones of different metastasizing potential) revealed that higher metastasizing capacity *in vivo* corresponded to lower expression of ganglioside GD3 on the surface of the tumor cells [49, 50]. Incubation of highly metastasizing cell clones with ganglioside GM1 significantly suppressed their metastasizing activity [50].

Study of 10 cell lines of B16 melanoma (B16Lu1-B16Lu10) characterized by increasing metastasizing potential with respect to lung revealed increase in ganglioside content with increase in metastasizing potential. In the case of gangliosides GM2 and GM3, this dependence exhibited nearly linear behavior. This emphasizes the role of these gangliosides in regulation of the metastasizing potential of B16 melanoma cells [51]. *In vitro* treatment of mouse B16LuF1 melanoma cells with gangliosides GM2 and GM3 isolated from melanomas B16LuF5, B16LuF9, and B16LuF10 exhibiting higher metastasizing capacity to lung resulted in increase in metastasizing potential of these cells following their subsequent administration *in vivo* [52, 53]. The metastatic activity of kidney sarcoma RCC cells also depended on ganglioside GM3 expression [54].

Analysis of these data on ganglioside involvement in tumor metastasizing processes revealed that: 1) ganglioside composition of metastases differs from that of "parent" cells and is characterized by reduced content of complex gangliosides; 2) ganglioside profiles of lowly and highly metastasizing forms of cells from the same tumor significantly differ, and highly metastasizing cells are characterized by significant content of gangliosides GM3, GD3, and GM2 and their increased expression.

Injections of gangliosides into cells, organs, or tissues represent one of the methods used for studies of ganglioside effects on the metastasizing process. Treatment of mice with injections of a ganglioside mixture (twice a day) caused a two-fold increase in tumor volume, number of spontaneous metastases per mouse, and number of animals with metastases compared with untreated animals. Preincubation of tumor cells with the ganglioside mixture (before their injection into mice) increased metastatic nodules in lungs [55].

Another method used for studying the metastasizing process consists of inhibition or stimulation of expression of various enzymes involved in ganglioside metabolism. For example, it was demonstrated that mouse melanoma MEB4 cells synthesize GM3 as the main ganglioside. Inhibition of GM3 synthesis by specific glucosylceramide synthase inhibitor resulted in decrease in malignancy and metastasizing potential of these cells [56]. Suppression of the gene encoding GD3 synthase caused a similar result in F-11 cells [57]. Ganglioside functions in metastasizing processes were also studied using introduction of the GM2/GD3 synthase gene to Lewis mouse carcinoma cells (subclone P29) [58]. Usually cells of the P29 subclone exhibit lowly metastasizing activity during *in vivo* injections, but after transfection they have acquired high lung metastasizing capacity [58].

Various ganglioside components of cell membranes can influence a wide spectrum of biological functions of cell surface related to the metastasizing process. Correlation between invasive properties and characteristics of components of cell surface has also been studied using a number of metastasizing cell systems obtained *in vivo* and *in vitro*.

For example, addition of a ganglioside mixture to the cultivation medium caused almost three-fold increase in motility of tumor cells [55]. Insertion of genes encoding various enzymes of ganglioside metabolism or their inhibition in various tumor cells gave valuable information on the effects of gangliosides on cell invasiveness. Insertion of gene encoding cytosolic sialidase to cells of a highly metastasizing and invasive line of mouse B16-BL6 melanoma caused significant reduction in GM3 level. This was accompanied by decrease in metastases in lung, invasiveness, and motility of cells [59]. Using various clones of mouse colon adenocarcinoma, it was demonstrated that highly metastasizing clones NL17 and NL22 are characterized by decreased expression of sialidase and increased level of GM3 compared with lowly metastasizing NL4 and NL44. Transfection of the sialidase gene to the cells of the NL17 clone decreased ganglioside GM3 level accompanied by significant inhibition of lung metastases, invasiveness, and cell motility [60]. Study of ganglioside GM3 in B16 melanoma cell lines with low (B16-F1) and high (B16-F10 and B16-BL6) metastasizing potential revealed that GM3 content in B16-F1 cells was two times lower than in B16-F10 and B16-BL6 cells [61]. Inhibition of glucosylceramide synthase in Lewis lung adenocarcinoma cells caused significant decrease in ganglioside GM3 content. This also decreased cell attachment to model membranes and their migration *in vitro* and also attachment to laminin *in vivo* [62]. However, in the case of mouse and human urinary bladder tumors there was reversed interrelationship between invasive properties and ganglioside GM3 content: superficial tumors contained larger quantities of this ganglioside than invasive tumors. Cell clones transfected with GM3 synthase and expressing increased amounts of GM3 were characterized by decreased proliferation, metastases, and invasiveness. Exogenously added GM3 decreased invasive potential of human T-24 and KK-47 cell lines [63-65]. Study of gangliosides in human kidney RCC carcinoma cells revealed that the level of expression of disialosylgalactosylgloboside (DSGG) and monosialosylgalactosylgloboside in these cells correlated with metastases to lung and lymph nodes [66]. Study of adhesive capacity of various RCC lines to different tissues revealed that TOS-1 cells expression DSGG tightly bound to lung tissue and binding degree correlated with the level of DSGG expression. Other RCC cell variants lacking DSGG exhibited weaker adhesive properties [66]. In TOS-1 cell line the increase in GM3 and decrease in GM2 caused by transfection of the β(1,4)GalNAc transferase gene resulted in increased inhibition of cell motility and invasiveness [67]. Insertion of the α-2,8-sialyl transferase gene into rat glioma C6 cells increased *de novo* synthesis of ganglioside GD3 in these cells accompanied by their increased motility and invasiveness [68].

Transfection of the GD3 synthase gene into lung cancer small cells resulted in expression of increased amounts of gangliosides GD2 and GD3; these cell clones also exhibited increased invasive activity [69]. Suppression of GD3 synthase in F-11 cells was accompanied by suppression of migration, invasiveness, and metastases [70].

Human melanoma cells express on cell surface relative high quantities of gangliosides GD3 and GD2; neuroblastoma cells express GD2 as the main cell ganglioside. Monoclonal antibodies specifically elaborated against the carbohydrate components of these gangliosides inhibit adhesion of melanoma and neuroblastoma cells to various extracellular matrix proteins (collagen, vitronectin, laminin, fibronectin); this underlines an important role of these gangliosides for the adhesion

process [71]. Subsequent studies [72, 73] revealed that initial adhesion of melanoma B16 cells to non-activated endothelial cells involves specific interaction between ganglioside GM3 expressed on the surface of the melanoma cells and endothelial cell lactosylceramide. This adhesion was inhibited by liposomes containing GM3 or LacCer or by treatment with antibodies against GM3 [72].

Cultivation of neuroblastoma LA-N1 cells characterized by tight adhesion to collagen in the presence of glucosylceramide synthase inhibitor caused inhibition of adhesion by 67% but stimulation of their migration capacity. Preincubation of these cells with GD2 ganglioside completely restored adhesion [74].

Cooperative inhibitory effect of ganglioside GM3 and tetraspanine CD9 on cell motility was demonstrated using several lines of tumor cells. Motility of colon carcinomas SW480, SW620, and HRT18 characterized by high level of CD9 expression was inhibited by exogenous administration of GM3 but not by GM1. Exogenous GM3 did not influence stomach cancer MKN74 cell line with decreased CD9 expression. Use of cell clones unable to synthesize GM3 provided further evidence for joint involvement of CD9 and GM3 in inhibition of cell motility. Insertion of the CD9 gene and stimulation of GM3 synthesis in these cells caused a sharp decrease in their motility, whereas in intact IdlD cells GM3 did not produce such an effect [75, 76].

Complex gangliosides may also influence the metastatic process. For example, ganglioside GT1b inhibited migration and adhesion of squamaous carcinoma cells to fibronectin [77, 78]. A lowly metastasizing variant of osteosarcoma FJB, FJB-S1, is characterized by a significant level of ganglioside GD1a expression, whereas the highly metastasizing FJB-LL cells have lower level of this ganglioside expression. Treatment of FJB-LL cells with gangliosides GD1a, GD1b, and GT1b caused 50% reduction in their migrating ability [41] and 30% reduction in their adhesion to vitronectin [79]. Artificial increase in GD1a expression in the FJB-LL clone of cells decreased their migrating ability and adhesion to vitronectin [79, 80]. Inoculation of osteosarcoma cells expressing GD1a (but not intact variants of FJB-LL cells) to mice caused formation of metastatic nodules in liver, kidneys, lungs, and adrenals [79]. Gangliosides GD1a and GM1b inhibited adhesion of mouse lymphosarcoma RAW117-H10 cells to sinusoidal endothelial liver cells, and the effect of GD1a was more pronounced than that of GM1b [81].

It has recently been demonstrated that processes of cell adhesion involve so-called glycosphingolipid-enriched microdomains (glycosynapses) [82]. It was found that the adhesion process in mouse B16 melanoma involves GM3-enriched microdomain located on the surface of melanoma cells [83]. This conclusion is based on the fact that more than 90% of melanoma cell ganglioside GM3 was detected in this microdomain. This microdomain also contains such signal molecules as c-Src, Ras, Rho, and FAK; this means that the GM3-enriched microdomain is the structural and functional unit for initiation of GM3-dependent cell adhesion and related signal transduction [83]. Similar data have also been obtained for kidney carcinoma RCC cells; in these cells DSGG involved in adhesion is clustered on the cell surface in the microdomain also containing c-Src, Rho A, and FAK [84]. In human mammary gland carcinoma MCF-7 cells glycosphingolipid-enriched microdomains accumulate gangliosides of the globo-series, ganglioside GM2, and also c-Src and FAK [85].

All these data clearly demonstrate ganglioside involvement in migration, invasiveness, and adhesion of tumor cells, and these activities depend on the structure of the glycolipid carbohydrate chain.

Glycosphingolipids are not only involved in the metastatic processes; some of them can also be employed for chemotherapy of metastases. These include α-galactosylceramide. This compound has been isolated from the sponge *Agelas mauritianus* [86]. Later it was shown that α-galactosylceramide inhibited metastases of melanoma B16 [87] and melanoma B16-BL6-HM [88] to liver; metastases of B16-BL6 melanoma cells to mouse lymph nodes [89]; metastases of M5076 sarcoma cells [90], colon adenocarcinoma [91] and EL-4 lymphoma [92] to mouse liver; pancreas cancer cell to hamster liver [93]. Long-term administration of this preparation also caused regression of liver metastases [91].

The study of mechanisms underlying the effect of α-galactosylceramide revealed that it acts as an immunostimulator [93], which activates T-helpers, increases cytokine secretion [94] (particularly IL-12 [90]), and stimulates NKT-cells producing γ-interferon [95, 96]. Studies revealed that α-galactosylceramide increases antitumor cytotoxicity of hepatic lymphocytes [97] and also invasiveness of NK1 and CD8 cells and macrophages [87]. Immune response to α-galactosylceramide involves a unique lymphoid system Vα14NKT, which inhibits melanoma metastases to mouse liver *in vivo* [98]. There is increasing evidence that α-galactosylceramide might be used as an anti-metastatic drug.

Metastasizing as well as tumor growth is accompanied by angiogenesis, i.e., formation of new blood vessels "spanning" tissue. Sphingolipids are actively involved in angiogenesis.

As it has already been mentioned above that ganglioside shedding from the surface of tumor cells promotes angiogenesis [18]. Released membrane vesicles also contain sphingomyelin; it is suggested that that latter is an active participant of angiogenesis [99]. However, the most important sphingolipid involved in the process of angiogenesis is S1P [100, 101]. S1P acts as an intercellular mediator and its effects are realized via G-coupled cell receptors. S1P is involved not only in metastasis (see

above) but also in angiogenesis [102]. For example, it was shown that S1P stimulates migration of umbilical vein endothelial cells, and this effect promotes angiogenesis [103]. Receptors of S1P also play an important role in tumor angiogenesis. Stabilization of newly formed vesicles requires S1P(1) receptor [104]. Another phosphosphingolipid, sphingosine-1-phosphocholine, also induces angiogenesis *in vivo* [105, 106], and its functioning requires GPR4 receptor [106]. Recently it has been shown that lactosylceramide influencing expression of VEGF (vascular endothelium growth factor) plays an essential role in angiogenesis [107].

Gangliosides play one of the key roles in metastasis and angiogenesis [18, 55, 108]. Tumor cells shed ganglioside-containing membrane vesicles from their surface. So blood serum of cancer patients has significantly higher amounts of gangliosides. These gangliosides influence tumor angiogenesis and metastases by regulating VEGF expression [109]. Addition of exogenous GD3 to the cultivation medium stimulated VEGF release from glioma cells [110]. The ratio of gangliosides GM3 and GD3 [111] also influences the process of angiogenesis because GM3 inhibits angiogenesis [109, 112] and more complex gangliosides (e.g., GD3, GM1, GM2, GD1a, and GT1b) activate it [106, 110, 111, 113-116]. Moreover, it is suggested that tumor gangliosides regulate tumor growth via angiogenesis [113]. Although gangliosides do not induce angiogenesis [117], they act synergistically with VEGF and fibroblast growth factor [106, 118], prostaglandin E1 [106, 113, 119], and copper ions [119].

Summarizing all the data presented in this review, one can see that sphingolipids are involved not only into regulation of cell growth apoptosis, not only in stimulation and inhibition of cell growth, but also in migration of tumor cells in the body, and their adhesion and invasiveness (i.e., in the process of metastasis and also in angiogenesis). Phosphosphingolipid S1P plays an important role in these processes; it promotes migration of tumor cells and gangliosides (mainly, GM3, GM2, and GD3) influencing metastatic potential of tumor cells, their invasiveness, and regulation of angiogenesis.

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