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Review

Altered sphingolipid metabolism induced by tumor hypoxia – New vistas in glycolipid tumor markers

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ABSTRACT

Uncontrolled growth of malignant cells produces hypoxic regions in locally advanced tumors. Recently we showed that tumor hypoxia-induced transcription of multiple genes involved in glycan synthesis, leading to expression of useful glycolipid tumor markers, such as gangliosides having *N*-glycolyl sialic acid. Our subsequent studies indicated that the ceramide portion of glycolipids, as well as their glycan moiety, was also significantly affected by hypoxia. Tumor hypoxia-induced marked accumulation of sphinganine (dihydrosphingosine) long-chain base, and significant reduction of unsaturated very long-chain fatty acids in the ceramide moiety. Mass-spectrometry, which yields information on both glycan- and ceramide moieties, is expected to be clinically useful in detecting such distinct molecular species of cancer-associated glycolipids having combined alteration in both glycan- and ceramide moieties.

1. Introduction

Cell-surface glycolipids are known to undergo drastic changes upon malignant transformation [1,2]. Glycolipids appearing in cancer cells are shown to serve as good tumor markers, and applied also as therapeutic targets of cancers [3,4]. The mechanisms underlying tumor-associated changes of glycolipids, however, are complicated and remain elusive. Some changes occur at the relatively early stages of carcinogenesis, while some other changes become apparent only in the advanced stages along with progression of cancers. In the stages of locally advanced cancers, uncontrolled growth of tumor cells produces hypoxic areas in expanded cancer cell nests. Tumor hypoxia affects various aspects of intracellular metabolisms of cancer cells, and recently it was disclosed to be one of the major mechanisms for induction of cancer-associated glycans in glycolipids and glycoproteins. Here we introduce the notion that tumor hypoxia affects not only the glycan moiety of glycolipids, but also their ceramide moiety.

2. Hypoxia and cancer-associated glycolipids

Recently we showed that tumor hypoxia leads to enhanced expression of some cell-surface glycans, that had been well known to be associated with cancers, such as sialyl Lewis X and sialyl Lewis A. This turned out to be due to induction of the transcription of the genes involved in the synthesis of these glycans by hypoxia as analyzed by DNA microarray [5]. Since then, it is becoming clearer year after year that tumor hypoxia affects a wide variety of genes involved in the expression of cell-surface glycoconjugates, and induces profound changes in the expression of glycolipids and glycoproteins in cancers.

For instance, it has long been known that gangliosides carrying *N*-glycolyl sialic acid increases in human cancers. Such gangliosides are sometimes called Hanganatziu–Deicher antigens [6,7]. Recently we have shown that hypoxia-induced up-regulation of a gene for the sialic acid transporter, *Sialin*, is closely related to the enhanced expression of gangliosides carrying *N*-glycolyl sialic acid (Fig. 1) [8]. Humans lack the gene for CMP-neuraminic acid hydroxylase, the enzyme required to synthesize *N*-glycolyl sialic acid (NeuGc), and most NeuGc in the human body is thought to

Abbreviations: CerS, ceramide synthase; CMH, ceramide monohexose; DHCer, Dihydroceramide; DHCMH, dihydroceramide monohexose; DHSph, dihydrosphingomyelin; HIF, hypoxia-inducible factor; HPLC–ESI-MS/MS, high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry; HRE, hypoxia-responsive element; LCB, long-chain base; MALDI-MS, matrix-assisted laser desorption mass spectrometry; NeuAc, *N*-acetyl sialic acid; NeuGc, *N*-glycolyl sialic acid; SPT, serine palmitoyl transferase; Sph, sphingomyelin; VLCFA, very long-chain fatty acid

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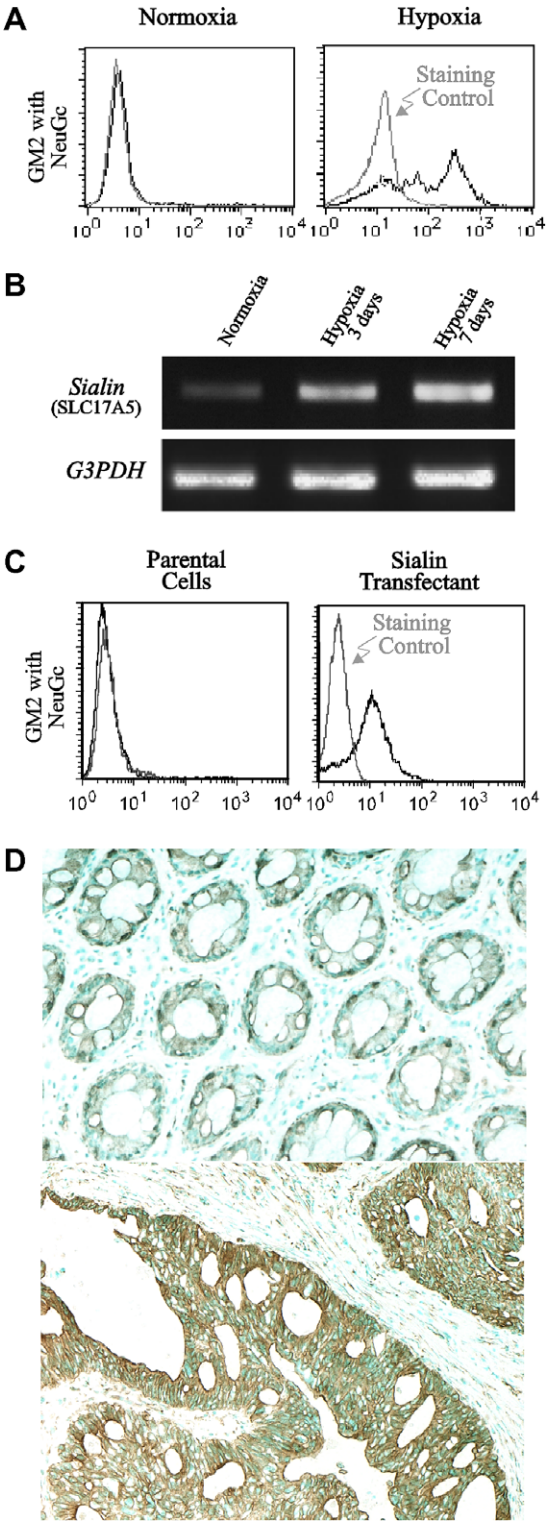

Fig. 1. Hypoxia-induced expression of ganglioside GM2 having *N*-glycolyl sialic acid. (Panel A) Flow-cytometric analysis of a clone of human cultured colon cancer cells Caco-2M using monoclonal antibody specific to NeuGc-GM2 (MK2-34) indicating prominent induction of the ganglioside by hypoxia. (Panel B) RT-PCR analysis of transcriptional induction of the gene for a sialic acid transporter, *Sialin*, by hypoxia in Caco-2M cells. (Panel C) Results of flow-cytometric analysis of Caco-2M cells indicating transfection of *Sialin* gene confers significant NeuGc-GM2 expression. (Panel D) Immunohistochemical staining of NeuGc-GM2 in a colon cancer tissue using specific monoclonal antibody, indicating the ganglioside serves as a good marker for advanced stage cancer cells. Advanced cancer cells acquire hypoxia-tolerance, and this accompanies sustained *Sialin* expression. (Upper panel) Non-malignant colonic epithelial cells. (Lower panel) Cancer cell nests.

be acquired from the external milieu, mainly of dietary origin [9,10]. Tumor hypoxia induces transcription of *Sialin*, and enhances incorporation of exogenous sialic acid. This leads to enhanced incorporation of NeuGc as well as *N*-acetyl sialic acid (NeuAc), and results in significant accumulation of unusual gangliosides carrying *N*-glycolyl sialic acid in cancers. Such gangliosides serve as surrogate markers for the presence of cell masses suffering from chronic hypoxia.

De novo synthesis of *N*-glycolyl sialic acid is based on oxidative hydroxylation of *N*-acetyl sialic acid catalyzed by CMP-NeuAc hydroxylase, a specific oxidase, expression of which is phylogenetically well controlled; it is present in mammals up to higher apes, but absent in humans [11–13]. As the conversion of *N*-acetyl to *N*-glycolyl sialic acid is based on oxidative hydroxylation, production of NeuGc through de novo synthesis may be suppressed under hypoxic conditions in mammals up to higher apes. In contrast, the expression of NeuGc rather increases under hypoxic conditions in humans, where its level is determined by its salvaging through the transporter Sialin, instead of de novo synthesis.

Effects of hypoxia are not limited to changes in sialic acid molecular species, but sometimes extend to the glycan backbone of gangliosides. Marked induction of GD3 expression and moderate induction of that of GM3 were observed in this cell line after hypoxic culture (Fig. 2A). This was accompanied by a prominent induction of ST8Sia-I transcription (Fig. 2B). The ST3GalV gene shows a delayed moderate induction, while genes for other glycosyltransferases show only minimal changes.

3. Ceramide composition and tumor hypoxia

During the course of study of tumor-associated gangliosides, we have noticed that significant changes occur at the ceramide moiety as well as carbohydrate moiety of the gangliosides during hypoxia. This was initially noticed from changes in the mobilities of ganglioside bands in TLC-immunostaining with specific anti-ganglioside antibodies after hypoxic culture. Broadening of the main band and appearance of additional slow-migrating band were frequently observed, which implied altered ceramide composition in gangliosides by hypoxia.

Analyses of sphingolipid molecular species by high-performance liquid chromatography–electrospray ionization-tandem mass spectrometry (HPLC–ESI-MS/MS) indicated a significant accumulation of dihydroceramide (DHCer) under hypoxia (Fig. 3A). A similar accumulation of DHCer moiety was detected also in the glycolipid and sphingomyelin (Sph) fraction (Fig. 3A). This was accompanied with the decrease in ceramide moiety with d18:1 long-chain base (LCB) (Fig. 3B). It is interesting to note that the ratio of sphingosine/sphinganine is reportedly decreased in certain tumors both in ceramide and Sph fractions in the literature [14,15].

Another notable finding was a sharp decrease in the amount of unsaturated very long-chain fatty acids (VLCFAs) in the ceramide moiety by hypoxia. C24:1 was the major unsaturated acyl chain in the given cells, and its amount exhibited a marked decrease by hypoxia, and tended to recover after reoxygenation. This was accompanied by an increase of saturated VLCFAs by hypoxia, which was most prominent with C22:0 species. Accordingly, the ratio of unsaturated/saturated VLCFAs showed a sharp reduction upon hypoxic culture, and tended to recover after reoxygenation in all tested sphingolipid fractions (Fig. 3C), reflecting impaired desaturase reaction for VLCFAs under hypoxic condition.

Subsequent RT-PCR analysis (Fig. 3D) indicated a significant enhancement of transcription of fatty acid 2-hydroxylase (*FA2H*),