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UVB-irradiated keratinocytes induce melanoma-associated ganglioside GD3 synthase gene in melanocytes via secretion of tumor necrosis factor α and interleukin 6

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ABSTRACT

Although expression of gangliosides and their synthetic enzyme genes in malignant melanomas has been well studied, that in normal melanocytes has been scarcely analyzed. In particular, changes in expression levels of glycosyltransferase genes responsible for ganglioside synthesis during evolution of melanomas from melanocytes are very important to understand roles of gangliosides in melanomas. Here, expression of glycosyltransferase genes related to the ganglioside synthesis was analyzed using RNAs from cultured melanocytes and melanoma cell lines. Quantitative RT-PCR revealed that melanomas expressed high levels of mRNA of GD3 synthase and GM2/GD2 synthase genes and low levels of GM1/GD1b synthase genes compared with melanocytes. As a representative exogenous stimulation, effects of ultraviolet B (UVB) on the expression levels of 3 major ganglioside synthase genes in melanocytes were analyzed. Although direct UVB irradiation of melanocytes caused no marked changes, culture supernatants of UVB-irradiated keratinocytes (HaCaT cells) induced definite up-regulation of GD3 synthase and GM2/GD2 synthase genes. Detailed examination of the supernatants revealed that inflammatory cytokines such as TNFα and IL-6 enhanced GD3 synthase gene expression. These results suggest that inflammatory cytokines secreted from UVB-irradiated keratinocytes induced melanoma-associated ganglioside synthase genes, proposing roles of skin microenvironment in the promotion of melanoma-like ganglioside profiles in melanocytes.

1. Introduction

In malignant melanomas, a variety of gene mutations have been identified to be involved in the induction of malignant transformation of melanocytes. Among those genes, NRAS, p16/p14, BRAF, KIT, PTEN and TP53 are included [1,2]. Malignant melanomas are also caused by various environmental factors such as ultraviolet beam [3], although no particular factor can be identified in the majority of cases [4]. Since melanomas are resistant to current chemotherapy and radiation therapy, development of novel therapeutics and/or efficient ways to prevent its evolution has been long expected [1,5].

In malignant melanoma tissues, some kinds of sialic acid-containing glycosphingolipids, gangliosides have been identified as tumor-associated antigens [6]. In particular, ganglioside GD3 and GD2 were defined as melanoma-specific antigen based on biochemical analysis [7,8], and also on immunological approaches such as serological analysis of patients' sera [9,10], and generation of melanoma specific monoclonal antibodies (mAbs) [11]. Immunohistochemical analysis was also performed to examine ganglioside expression in tumor tissues [12]. Human mAbs reactive with melanoma gangliosides were generated using melanoma patients-derived B lymphocytes [13,14].

These melanoma-associated gangliosides have been utilized as tumor markers [15], or as targets of immunotherapy and/or

Abbreviations: UV, ultraviolet; FBS, fetal bovine serum; D-MEM, Dulbecco's modified Eagle's essential medium; RT-PCR, reverse transcription-polymerase chain reaction; IL-, interleukin; TNFα, tumor necrosis factor α; UVB, ultraviolet B.

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antibody therapy for melanomas [16,17]. Furthermore, biological functions of GD3 and GD2 in the malignant properties in melanoma cells have been demonstrated [18]. Expression of GD3 resulted in the increased cell proliferation and invasion activity [19]. It also enhanced cell adhesion to extracellular matrix in melanoma cells [20].

In this study, expression patterns of major glycosyltransferase genes related to the synthesis of those gangliosides were compared between melanocytes and melanomas to investigate mechanisms for melanoma-associated ganglioside antigens with focus on the events during transformation from melanocytes to melanomas. Then, effects of ultraviolet (UV) irradiation as a representative environmental factor probably involved in the evolution of melanomas on the expression of those glycosyltransferase genes were analyzed.

2. Materials and methods

2.1. Cell culture

HEMn-LP, a lightly pigmented human melanocyte line, was purchased from KURABO (Osaka, Japan) and cultured in Medium 254 supplemented with Human Melanocyte Growth Supplement™ (HMGS) (Life Technologies, Carlsbad, CA). When 80% confluency was reached, cells were cultured in Ham's F-10 medium supplemented with 7% fetal bovine serum (FBS), 1% penicillin–streptomycin, 1 mM N^6, 2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt, 0.1 mM 3-Isobutyl-1-methylxanthine, 1 μM Na_3VO_4, 50 ng/ml Phorbol 12-myristate 13-acetate (Ham's F-10 medium and penicillin–streptomycin; Life Technologies, FBS; Equitech-Bio, Kerrville, TX, USA, all others; Sigma–Aldrich, St. Louis, MO) [21]. After 3 or 4 days incubation, cells were used for these studies. All melanoma cell lines were provided by Dr. L.J. Old (Memorial Sloan-Kettering Cancer Center, New York) and cultured in Dulbecco's modified Eagle's essential medium (D-MEM) supplemented with 7.5% FBS. A human keratinocyte line (HaCaT cell) was provided by Dr. K. Sugiura (Nagoya University, Aichi, Japan) and cultured in D-MEM supplemented with 10% FBS.

2.2. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using RNeasy Plus Mini™ Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The first strand synthesis system for RT-PCR kits (iScript; Bio-Rad Laboratories, Hercules, CA) were used for cDNA synthesis. Real-time RT-PCR was performed with DyNAmo™ Flash SYBR Green qPCR Kit (FINNZYMES, Vantaa, Finland). Primers used in this study (Table 1) were designed with Probe Finder software (Roche Diagnostics, Basel, Switzerland).

2.3. Ultraviolet B (UVB) irradiation

Melanocytes and HaCaT cells were plated in culture dishes (BECTON DICKINSON, Franklin Lakes, NJ, USA). After 24 h, culture medium was replaced with PBS containing 7.5% FBS. Cells were incubated for 30 min, and exposed to a UVB irradiation (312 nm) using DNA-FIX™ (ATTO, Tokyo, Japan). Cells were refed with culture medium immediately after irradiation.

2.4. Measurement of cytokines

Culture supernatants from UVB-irradiated cells were collected after 24 h. Levels of cytokines (IL-8, IL-1β, IL-6, IL-10, TNFα and IL-12p70) were determined using BD™ Cytometric Bead Array (CBA): Human Inflammation Kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. Briefly, capture beads, culture supernatants and PE-conjugated antibodies were mixed, and incubated for 3 h. After washing, samples were acquired with Accuri™ C6 flow cytometer and analyzed with FCAP Array™ software (BD Biosciences).

2.5. Reagents

Recombinant human IL-8, IL-6, TNFα and IL-1β were purchased from Sigma–Aldrich.

2.6. Statistical analysis

Statistical significance of data was determined using Student's *t* test.

3. Results

3.1. GD3 synthase and GM2/GD2 synthase were upregulated and GM1/GD1b synthase was downregulated in melanoma cell lines

For nine glycosyltransferase genes involved in the synthesis of glycosphingolipids (Fig. 1A), expression levels were analyzed in melanocytes and four melanoma cell lines, SK-MEL-28, SK-MEL-37, MeWo, and SK-MEL-23 (Fig. 1B). Expression levels of GD3 synthase (ST8SIA1), GM2/GD2 synthase (B4GALNT1) and GalCer synthase (UGT8) genes were remarkably high in melanoma lines compared with melanocytes. In contrast, GM1/GD1b synthase (B3GALT4) showed extremely low expression in melanoma lines, while melanocytes exhibited fairly high levels. As for the other genes, differences in expression levels were not observed between melanocytes and melanoma lines. Results with another melanocytes derived from a moderately pigmented human melanocyte line (KURABO) showed very similar patterns (data not shown).

Table 1
Primer sequences for genes analyzed in this study.

Enzymes etc.	Genes	Forward	Reverse
Glc-Cer syn	UGCG	gctgccttacgtagcagaca	tcttggatgtgaagttccaaaata
Lac-Cer syn	B4GALT5	ttttgcaaccaaattggataag	ctcactccgccaaagaactc
Lac-Cer syn	B4GALT6	tctgattccaatgctccaga	atcgcacggttaaaaggttg
GM3 syn	ST3GAL5	ctgcctttgacatccttcagt	cgattgtggggacgttctta
GD3 syn	ST8SIA1	ggaaatggtgggattctgaag	tgacaaaggagggagattgc
GM2/GD2 syn	B4GALNT1	ccaactcaacaggcaactacaa	atgtccctcggtggagaac
GM1/GD1b syn	B3GALT4	tgctgcagttgttctctcaag	aagtttattgaggagcttgacacc
GD1a syn	ST3GAL2	gtccagaggtggtggatgat	cagcacctcattggtgttgt
Gal-Cer syn	UGT8	ttgtttatgtaggaggaatcctaacc	accatttacccatctttggaga
β-Actin	ACTB	ccaaccgcgagaagatga	ccagaggcgtacagggatag

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