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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 antibody therapy for melanomas [16,17]. Furthermore, biological

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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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

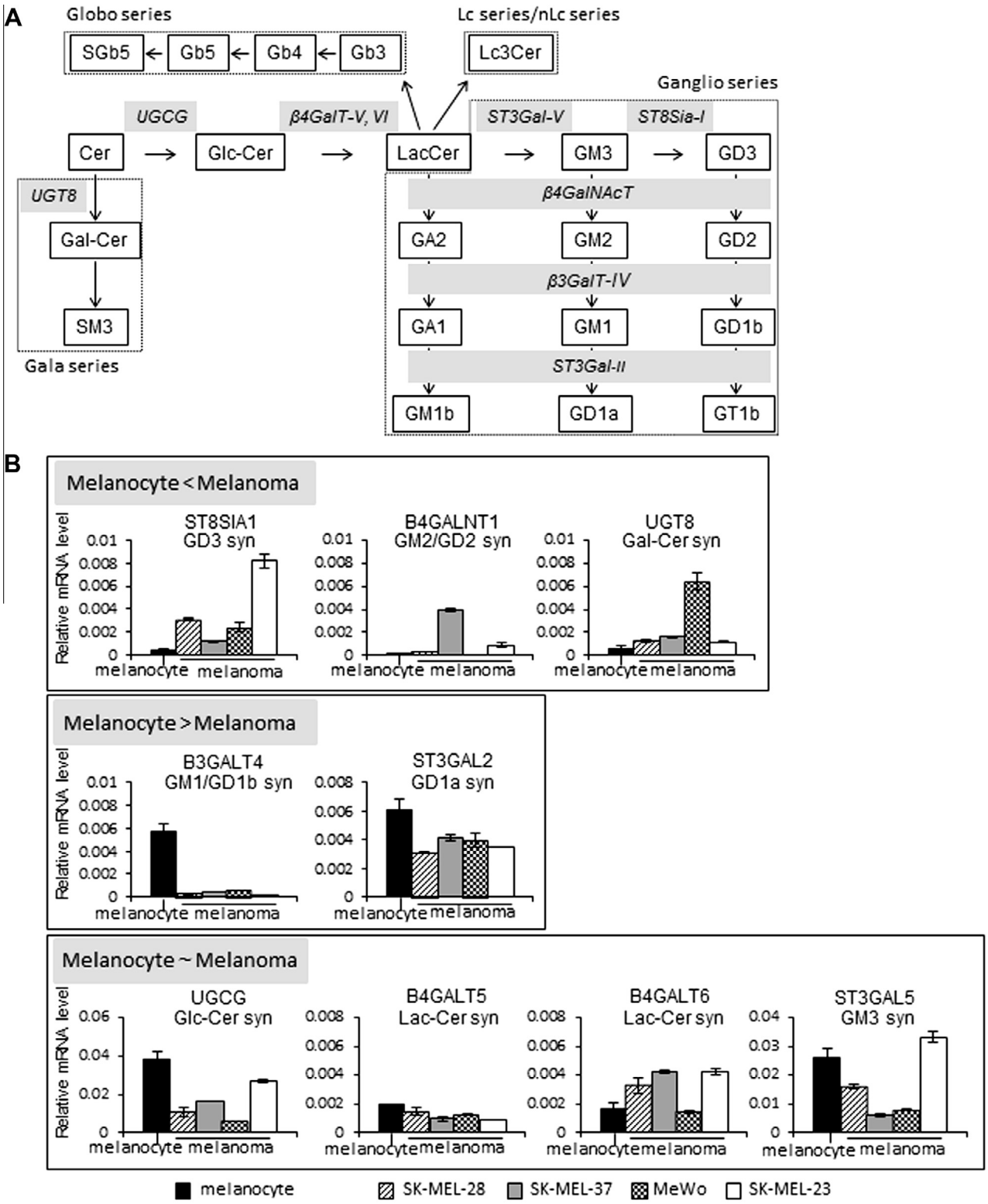

Fig. 1. Gene expression levels of glycosyltransferases in melanocytes and melanoma cell lines. (A) Synthetic pathway of glycosphingolipids. Nine glycosyltransferases tested here were shown in italic. (B) mRNA expressions of glycosyltransferases were analyzed by qRT-PCR in melanocytes and melanoma lines (SK-MEL-28, SK-MEL-37, MeWo, and SK-MEL-23).

3.2. UVB did not induce GD3 synthase gene expression in melanocytes

To investigate effects of environmental factor on expression levels of three glycosyltransferase genes, GD3 synthase, GM2/GD2 synthase and GM1/GD1b synthase were analyzed in UVB-irradiated melanocytes and SK-MEL-28 (Fig. 2A). This is because these three genes showed distinct differences in the expression levels between melanocytes and melanomas (Fig. 1B). GD3 synthase mRNA and GM1/GD1b synthase mRNA expression were reduced and GM2/GD2 synthase expression was slightly increased in

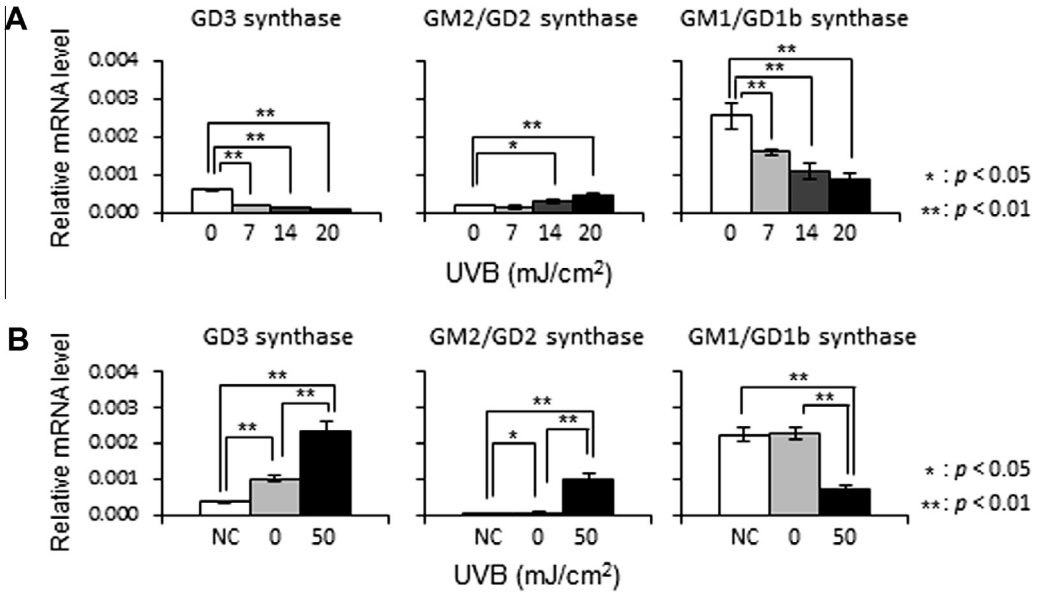

Fig. 2. mRNA expression levels of GD3 synthase, GM2/GD2 synthase and GM1/GD1b synthase in melanocytes treated with UVB or culture supernatants of UVB-irradiated HaCaT cells. (A) mRNA expression levels of GD3 synthase, GM2/GD2 synthase and GM1/GD1b synthase in UVB-irradiated melanocytes. Melanocytes (6×10^5 cells) were plated in 3.5-cm culture dishes and irradiated with UVB (0, 7, 14, 20 mJ/cm^2). Cells were collected at 24 h after irradiation and analyzed by qRT-PCR. (B) mRNA expression levels of the 3 glycosyltransferases in melanocytes treated with culture supernatants of UVB-irradiated HaCaT cells. HaCaT cells (1×10^5 or 3×10^6 cells) were plated in 6-cm culture dishes. Cells were irradiated with UVB (1×10^5 cells: 0 mJ/cm^2; 3×10^6 cells: 50 mJ/cm^2) and refed with Ham's F-10 medium containing supplements. Culture supernatant was collected at 24 h after irradiation. For second irradiation, this culture supernatant was used as medium after UVB irradiation of another HaCaT cells. Culture medium of melanocytes was replaced with culture supernatant of HaCaT cells and incubated for 2 days. Then, melanocytes were collected and analyzed by qRT-PCR. Cells cultured with fresh medium were used as negative controls (NC).

UVB-irradiated melanocytes. All these synthase expression levels were reduced after UVB irradiation in SK-MEL-28 (data not shown).

3.3. UVB-irradiated HaCaT cell culture supernatant induced gene expression of GD3 synthase and GM2/GD2 synthase in melanocytes

Whether keratinocytes, major sources of cytokines in skin, affect glycosyltransferase expression in melanocytes was examined. Melanocytes were treated with culture supernatants of HaCaT cells irradiated with UVB (50 mJ/cm^2 × 2 times), then expression levels of GD3 synthase, GM2/GD2 synthase and GM1/GD1b synthase genes were analyzed (Fig. 2B). GD3 synthase gene expression was slightly induced when melanocytes were treated with culture supernatant of unirradiated HaCaT cells. The induction of GD3 synthase gene expression was enhanced when cultured in the presence of supernatants of UVB-irradiated HaCaT cells. Culture supernatants of UVB-irradiated HaCaT cells also induced GM2/GD2 synthase gene expression and suppressed GM1/GD1b synthase gene expression in melanocytes. These expression patterns of glycosyltransferase genes were similar to those generally found in melanomas.

3.4. UVB-irradiated HaCaT cells secreted TNFα, IL-6, IL-1β and IL-8

Concentrations of six inflammatory cytokines, IL-12p70, TNFα, IL-10, IL-1β, IL-6 and IL-8, in culture supernatants of UVB-irradiated HaCaT cells were measured (Fig. 3A). Unirradiated cells expressed IL-6 (0.23 ng/ml) and IL-8 (1.09 ng/ml). Cells irradiated with UVB (50 mJ/cm^2 × 2 times) showed induction of TNFα (585.90 pg/ml) and IL-1β (193.65 pg/ml) and enhanced levels of IL-6 (23.16 ng/ml) and IL-8 (63.16 ng/ml). IL-12 and IL-10 were undetected in the supernatants of UVB-irradiated and unirradiated cells.

3.5. TNFα and IL-6 induced expression of GD3 synthase gene in melanocytes but not in SK-MEL-28

Expression levels of GD3 synthase, GM2/GD2 synthase and GM1/GD1b synthase genes in melanocytes were analyzed after treatment with TNFα, IL-6, IL-1β or IL-8, which are released from UVB-irradiated HaCaT cells (Fig. 3B). Approximately same or 2 to 3-fold concentrations with those in culture supernatants of UVB-irradiated HaCaT cells were used. TNFα (1.0 ng/ml) and IL-6 (20 ng/ml) induced the gene expression of GD3 synthase. IL-1β (0.4 ng/ml) also induced the gene expression of GD3 synthase with less intensity. IL-8 (200 ng/ml) did not show any effects on GD3 synthase gene expression. All four cytokines tested here did not change the expression levels of GM2/GD2 synthase and GM1/GD1b synthase genes.

Gene expression levels of GD3 synthase, GM2/GD2 synthase and GM1/GD1b synthase in melanocytes and SK-MEL-28 were analyzed after treatment with TNFα or IL-6 (Fig. 3C). GD3 synthase expression was induced by TNFα or IL-6 in melanocytes. The expression levels reached the comparable levels of SK-MEL-28 with 10 ng/ml of TNFα and GD3 expression was also clearly detected depending on the concentration of TNFα (data not shown), while it was not the case for increased concentration of IL-6. Either TNFα or IL-6 did not affect gene expression levels of GM2/GD2 synthase and GM1/GD1b synthase in melanocytes. None of three glycosyltransferase genes showed significant alteration in the expression levels by TNFα or IL-6 treatment in SK-MEL-28.

4. Discussion

Since early reports on ganglioside expression in human melanomas [8,7], a number of studies have been performed not only on the expression, but also on the synthesis and function of gangliosides in human melanoma cells [6]. Development of monoclonal techniques largely contributed in these studies [11,22,23]. Further-

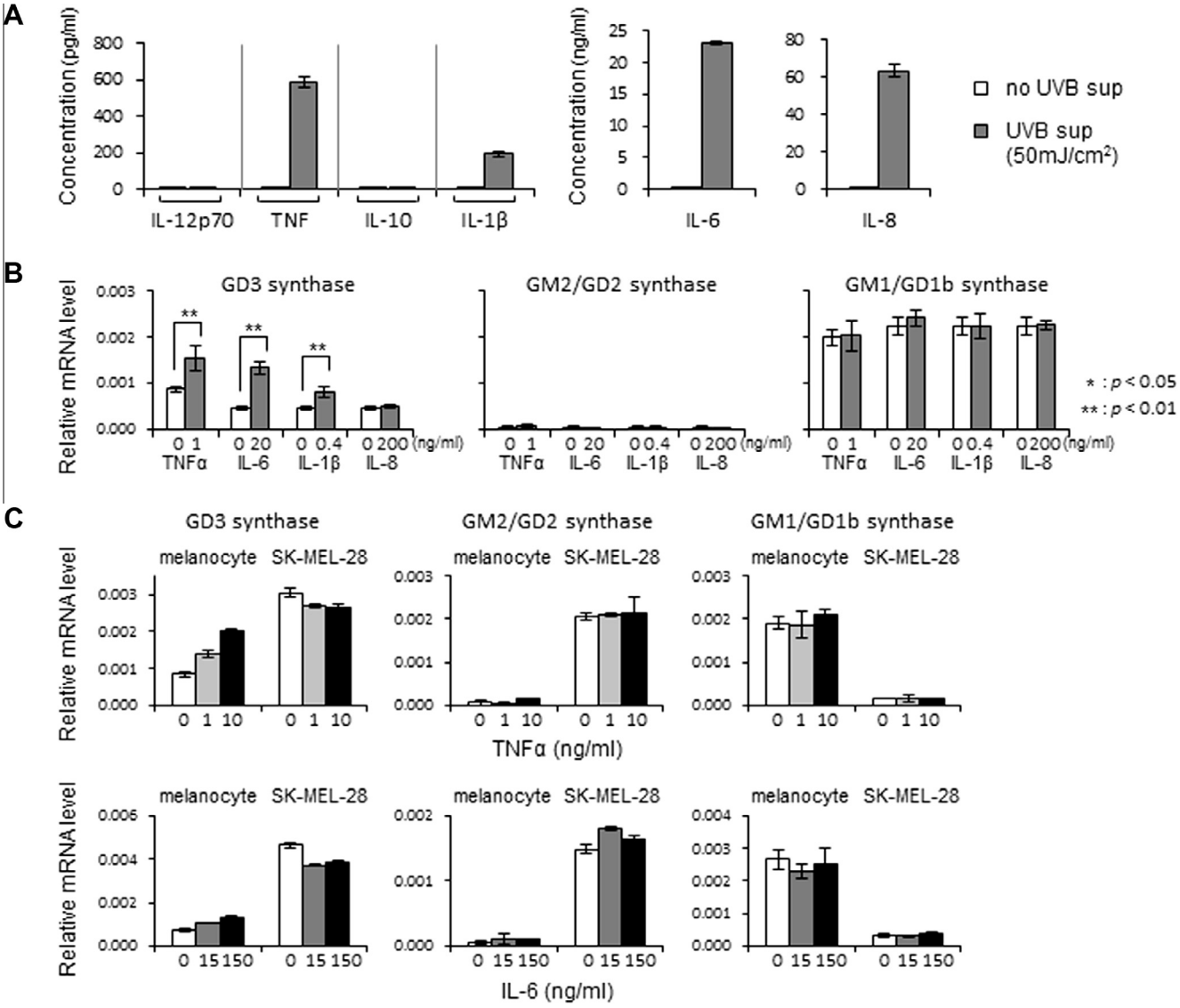

Fig. 3. Inflammatory cytokines are secreted from UVB-irradiated keratinocytes, and induced GD3 synthase and/or GM2/GD2 synthase genes. (A) Concentrations of cytokines in culture supernatant of UVB irradiated HaCaT cells. HaCaT cells (1×10^5 and 3×10^6 cells) were plated in 6-cm culture dishes. Cells were irradiated with UVB (1×10^5 cells: 0 mJ/cm^2, 3×10^6 cells: 50 mJ/cm^2) and refed with Ham's F-10 medium containing supplements. Culture supernatant was collected at 24 h after irradiation. For second irradiation, this culture supernatant was used as medium after UVB irradiation of another HaCaT cells. Culture supernatant was assayed with BD™ CBA kit for measurement of cytokine concentrations. (B) mRNA expression levels of glycosyltransferases in melanocytes after cytokine treatment. Melanocytes were incubated with TNFα (1.0 ng/ml), IL-6 (20 ng/ml), IL-1β (0.4 ng/ml), or IL-8 (200 ng/ml) for 2–3 days in the culture medium. Cells were collected and analyzed by qRT-PCR. (C) mRNA expression levels of glycosyltransferases in melanocytes and SK-MEL-28 after TNFα or IL-6 treatment. mRNA expression levels of GD3 synthase, GM2/GD2 synthase and GM1/GD1b synthase were analyzed by qRT-PCR in melanocytes and SK-MEL-28 after TNFα or IL-6 treatment. Melanocytes and SK-MEL-28 were incubated with TNFα (0, 1, 10 ng/ml) or IL-6 (0, 15, 150 ng/ml) for 2–3 days in the culture medium.

more, molecular cloning of glycosyltransferases enabled us to further understand mechanisms for the synthesis and expression of cancer-associated gangliosides [24,25]. In particular, genetic modulation of gangliosides using isolated cDNAs for ganglioside synthases strongly promoted functional analysis of those gangliosides [18,26,20]. However, little has been known about gangliosides in melanocytes, normal counterparts of melanomas. Results of quantitative RT-PCR (qRT-PCR) on glycosyltransferase genes obtained in this study well corresponded with already-known expression patterns of gangliosides in melanomas and melanocytes, i.e. melanoma-dominant expression of ST8SIA1 (GD3 synthase) and B4GALNT1 (GM2/GD2 synthase) genes. To our surprise, B3GALT4 (GM1/GD1b synthase) gene showed very contrastive expression levels between melanomas and melanocytes. It showed extremely high expression in melanocytes, while all melanoma cell lines uniformly exhibited low levels. These results well corresponded with our previous conclusion that disialyl gangliosides such as GD3 and GD2 promote malignant properties in cancer cells, while monosialyl gangliosides like GM1 exert reverse actions [27,19].

UV exposure is one of the most important environment factors that affect our bodies [28]. In particular, UVB (290–320 nm) from sunlight represents one of the most important extrinsic stimuli to affect our skin by inducing DNA damage, immune suppression, inflammation, cell death and cancers [29,30]. UVB can modulate signaling processes determining cell survival or death [31]. However, when cultured melanocytes were directly exposed to UVB, they showed minimal responses in terms of expression levels of ganglioside synthase genes (Fig. 2A). Then, we used a spontaneously immortalized keratinocyte line HaCaT [32], since skin

microenvironment seemed to be more important to regulate melanocyte fates and melanoma genesis [33], and HaCaT cells were proved to be able to represent physiological features of normal keratinocytes [31,32,34]. Consequently, culture supernatants of UVB-irradiated HaCaT cells brought about marked changes in the expression levels of ganglioside synthase genes in melanocytes as shown in Fig. 2B. Above all, ST8SIA1 underwent marked up-regulation in a dose-dependent manner (data not shown).

Among cells composing skin tissues, keratinocytes are its major component and play important roles in the protection of our bodies from exogenous stimuli, regulate immunologic and inflammatory responses in the skin, and maintain homeostasis in skin tissues [35]. Furthermore, it has been increasingly understood that keratinocytes regulate skin microenvironments by secreting various cytokines and chemokines to stimulate other surrounding cells such as fibroblasts, immune cells and melanocytes [33]. As for cytokines secreted from keratinocytes, a variety of cytokines and chemokines were identified in the culture supernatants of HaCaT cells and other keratinocytes [34], i.e. IL-1β, IL-6, IL-8, IFN-γ, G-CSF, MIP-1β and TNF-α etc. Especially, TNFα is an important component of the inflammatory cascade in skin [36].

In this study, TNFα was the most effective cytokine to up-regulate ST8SIA1 gene in melanocytes as shown in Fig. 3, suggesting its crucial role *in vivo*. Furthermore, it is intriguing that effects of supernatants from UVB-exposed keratinocytes on the expression of ganglioside synthase genes were on the direction to getting close to the patterns of melanoma cells, i.e. increase in ST8SIA1 and B4GALNT1 genes and reduction of B3GALT4 gene. Effects on B4GALNT1 gene (increase) and B3GALT4 gene (decrease) could not be observed by treatment with pure TNFα or IL-6 (Fig. 3C). Therefore, responsible factors for these effects in UVB-irradiated HaCaT cell supernatants should be identified by proteomic analysis.

Recently, critical roles of tumor microenvironments have been emphasized to consider therapeutic strategies for cancer (stem) cells [37]. Consequently, various combinatorial approaches have been proposed as a rational choice for melanoma treatment [38]. As described above, gangliosides have been proved to be involved in the enhancement of malignant properties in melanomas [19]. Moreover, our recent study revealed that GD3 plays a role in the convergence and synergism of adhesion- and HGF/c-MET-mediated signals [39]. Therefore, regulation of gangliosides and their synthetic enzyme expression in melanocytes might be critical during the promotion of melanomas from "activated melanocytes".

Chronic inflammation may cause carcinogenesis via various inflammatory cytokines and eventually via genetic mutation and epigenetic changes [40]. Protection of chemokine/cytokine from UVB is, therefore, rigorously being studied [41,42]. Molecular mechanisms by which UVB-induced cytokines and chemokines regulate ganglioside synthase genes, including transcriptional levels and epigenetic levels, remains to be investigated.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

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