をとる。水が循環していることを確認してから治療部位にコイルヘッド部の位置決めをして無理なく治療可能であることを確認する。治療部位が水平でコイルヘッド部の中央に来るように体位を取りベッドの上、治療部位の下にタオルなどを挿入する。治療する腫瘍をマーキングし、その中心を基準に患者様の腫瘍がコイルの中央にきちんと圧着されるように直行する照準線を描く。温度計のセンサーを腫瘍の直上に貼り付け、フィルム製被覆材で被覆固定する。腫瘍内温度を測定する際には 14G サーフローを局麻下に腫瘍中心部まで挿入、内筒を除去した後に消毒した温度センサーを挿入しテープで固定する。交番磁場治療装置の治療ヘッドの中心が腫瘍の直上に圧迫くした状態で固定する。温度記録を開始し、機器の電源を ON とする。赤いボタンを 2 個左から順に(まず準備、次いで加熱)押してボリューム(レベル)を右(時計回り)に回してはじめだけ 150-200V 位で開始し、腫瘍内温度が 41 度を越えたら後は 50-100V 位にして腫瘍内温度が 43.0 度くらい、体表温度が 42.5 度くらいになるようにボリューム(レベル)を調整して保持する。その条件では次第に温度が上昇するので、43 度を絶対に越えないように調整する。転移腫瘍が皮膚の直下の場合は腫瘍内の温度計測は必ずしも必要ない。30 分の加熱治療が終了したらボリューム(レベル)を左(反時計回り)に回して赤いボタンを 2 個右から順に(まず加熱、次いで準備)押して shut down する。CTI 治療室の扉を完全に開放する。次いで交番磁場治療装置の治療ヘッドを患者から離脱させ、治療部位に異常がないかを確認する。10 分間程度ポンプを運転して機器が完全に cool down されたことを確認してからポンプの電源、次いで壁の電源ブレーカーを OFF にする。

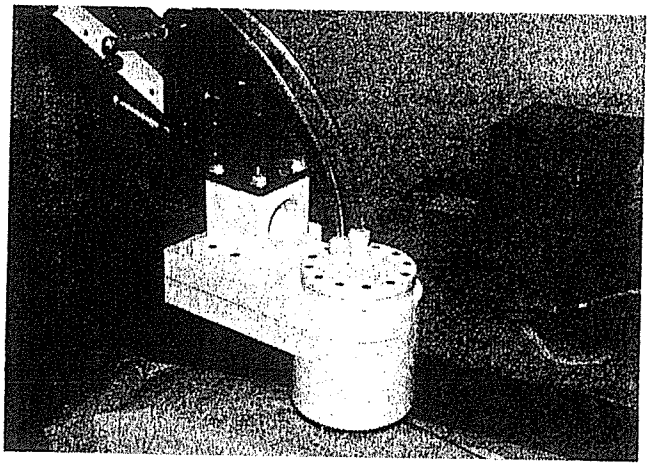

図 4　模擬腫瘍を用いた治療中の状態

D．考察

優れた基礎的研究成果も実際の臨床応用に適応させるためには種々の問題をクリアーしなければならないのは当然である。治療環境には安全で操作性の優れた治療装置の開発改良、治療装置を設置して患者の治療を行うことに適した治療室の設計・施工の 2 点が基本的に最も重要である。この点では本研究で述べたように十分に臨床応用に適した治療機器が開発改造できた。特に模擬腫瘍を用いた実験でも比較的低い出力で達成目的温度である 43℃に達し、30 分間問題なく維持できることが確認できた。この点から少数の症例の治療には既に十分な性能を持つことが確認できた。今後、改善が望まれるのは交番磁場治療装置の治療ヘッドが三次元的に回転する装置の開発が望まれる。また、より長時間の治療をするためには冷却水の流量を増加させることが望ましい。

一方、今回設計。施工した CTI 治療室は交番磁場治療装置から発生されるマイクロ波、電磁場を 40-50db 程度シールド可能な極めて高性能な治療室として設計されていることから現実的には全く、人体はもとより、他の精密電子機器に対して影響を与える可能性は無い。また、治療室内には治療用の木製ベッド(プラム診察台(木製)、

080-041-01)を設置してあるので異常発熱などで患者に障害を与える可能性は皆無であることが確認されている。

以上の治療環境が整備されたことを受けて治療手順を作成したが、模擬皮膚腫瘍を用いた検討で研修を積んだ医師であればこの手順に従って臨床例の治療に当たれば安全に効果的な治療を進められるものと考えている。今後は臨床例で注意深く治療を進め、経験を積み重ねることでさらに優れた治療プロトコールの設定とマニュアルの完成を目指したい。

E．結論

完全なシールドが達成されたCTI治療室を設置できた。CTI治療室には蛍光灯照明、冷暖房、煙熱感知器、電源(3相200V1口、100V 6口)、給排水装置完備、磁場防止ガラス使用観察窓装備。車椅子、ストレッチャーによる患者移送可能なバリアーフリー設計。治療用ベッドは木製。改造後の交番磁場治療装置も従来装置と同様の優れた加熱効果を持つことが試験運転で証明された。フレキシブルアームなどの操作性も比較的良好である。患者の治療に当たっては水平に規定された治療部位に垂直に装置の治療面を圧抵することが重要。今後は治療ヘッドが三次元的に回転する装置の開発が望まれる。少ない症例の治療では現状でも十分であるが、今後長時間の治療のためには冷却水の流量を増加させることが望ましい。以上、CTI治療環境が整備され、治療手順が整備されたことを受けて臨床例での治療を開始させることが可能と考えられた。

F．健康危険情報

(分担研究報告書には記入せずに、総括研究報告書にまとめて記入)

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なし

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H．知的財産権の出願・登録状況

(予定を含む。)

1．特許取得

なし。

２．実用新案登録

なし。

３．その他

なし

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表【雑誌】

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Ⅳ. 研究成果の刊行物・別冊

Diacylglycerol kinase α suppresses tumor necrosis factor-α-induced apoptosis of human melanoma cells through NF-κB activation

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Running title: DGKα suppresses melanoma cell apoptosis

Keywords: Diacylglycerol kinase; Apoptosis; Tumor necrosis factor-α; NF-κB; Melanoma

Abstract

We investigated the implication of diacylglycerol kinase (DGK) α (type I isoform) in melanoma cells, because we found that this DGK isoform was expressed in several human melanoma cell lines but not in noncancerous melanocytes. Intriguingly, the overexpression of wild-type (WT) DGKα, but not of its kinase-dead (KD) mutant, markedly suppressed tumor necrosis factor (TNF)-α-induced apoptosis of AKI human melanoma cells. In the reverse experiment, siRNA-mediated knockdown of DGK□ significantly enhanced the apoptosis. The overexpression of other type I isoforms (DGKβ and DGKγ) had, on the other hand, no detectable effects on the apoptosis. These results indicate that DGKα specifically suppresses the TNF-α-induced apoptosis through its catalytic action. We found that the overexpression of DGKα-WT, but not of DGKα-KD, further enhanced the TNF-□-stimulated transcriptional activity of an anti-apoptotic factor, NFκ-B. Conversely, DGKα-knockdown considerably inhibited the NF-□B activity. Moreover, an NF-□B inhibitor blunted the anti-apoptotic effect of DGK□ overexpression. Together, these results strongly suggest that DGKα is a novel positive regulator of NF-κB, which suppresses TNF-α-induced melanoma cell apoptosis.

1. Introduction

Melanoma is the most aggressive form of skin cancer and notoriously resistant to all current modalities of cancer therapy including chemotherapy [1-3]. A number of genetic, functional and biochemical studies suggest that melanoma cells become 'bullet proof' against a variety of chemotherapeutic drugs by reprogramming their proliferation and survival pathways during melanoma progression. In particular, the constitutive activation of nuclear factor-κB (NF-κB) is known to be an emerging hallmark of melanoma and plays a pivotal role in many aspects of melanoma tumorigenesis including protection from apoptosis [4-6].

NF-κB is a heterodimeric transcription factor that is predominantly composed

of 65 and 50 kDa subunits of the Rel family [5, 7, 8]. In resting cells, NF-□B is mainly retained in the cytoplasm by the IκB (inhibitor of NF-κB) family of proteins, which mask the nuclear translocation signal of the transcription factor. Upon cell stimulation, two defined serine residues in the N-terminus of IκB proteins are phosphorylated, thus triggering their ubiquitination and subsequent degradation by the 26S proteasome. Hereby, the NF-κB proteins are released for translocation to the nucleus and subsequent induction of a variety of κB-dependent genes.

A major apoptosis signaling pathway in melanoma cells relies on tumor necrosis factor (TNF)- α and the network associated with TNF receptor (TNFR)-1 signaling [4-6]. TNFR-1 is trimerized upon the binding of its ligand, TNF- α . Upon such a binding, TNFR-1 recruits TRADD (TNFR-associated death domain), TRAF2 (TNFR-associated factor 2) and FADD (Fas-associated death domain), resulting in the cleavage of procaspase-8 into its active form and subsequent apoptosis [9, 10]. However, unlike Fas, which mainly functions as a stimulator of the apoptosis cascade, signaling from TNFR-1 often results in inhibition of apoptosis through the efficient activation of NF-κB. Therefore, the balance between the pro- and anti-apoptotic signals within the TNFR-1 framework is central in dictating whether TNF

It is well recognized that a variety of lipid second messengers in low abundanc R-1 activation results in the TNF- α -dependent cell death in melanoma cells. e carry out specific tasks for a wide range of biological processes in eukaryotic cells. The cellular concentrations of such signaling lipids must be strictly regulated by the action of metabolic enzymes. Diacylglycerol (DAG) kinase (DGK) phosphorylates DAG to yield phosphatidic acid (PA) [11-14]. DAG is an established activator of conventional and novel protein kinase Cs, Unc-13, chimaerins and Ras guanyl nucleotide-releasing protein [15, 16]. PA has also been reported to regulate a number of signaling proteins such as phosphatidylinositol-4-phosphate 5-kinase [17-19], Ras GTPase-activating protein [20], Raf-1 kinase [21], atypical protein kinase C [22], mammalian target of rapamycin [23] and $p47^{phox}$ [24]. Thus, DGK can potentially participate in a diverse range of cellular events through modulating the balance between two bioactive lipids, DAG and PA.

Mammalian DGK is known to exist as a large protein family consisting of ten isoforms classified into five subtypes according to their structural features [11-14, 25]. These subfamilies can be characterized by the presence of a variety of regulatory domains of known and/or predicted functions, clearly indicating their distinct functions and regulatory mechanisms. The type I DGKs presently consisting of α [26, 27], β [28], and γ [29, 30] isoforms contain two sets of Ca^{2+}-binding EF-hand motifs at their N-termini [26, 31, 32]. The tissue- and cell-dependent expression patterns detected distinctively for these isoforms suggest that, even belonging to the same subfamily, each member exerts differentiated functions in particular types of cells. Moreover, we reported that the EF-hand motifs of the type I DGKs have properties distinct from each other with respect to affinities for Ca^{2+} and to Ca^{2+}-induced conformational changes [32]. Among the type I DGKs, DGK□ has recently been a subject of intensive investigation in T-lymphocytes and shown to be critically involved in the regulation of immune response [11-14]. However, physiological functions of type I DGK isoforms in melanoma cells have not yet been explored.

Here we found that, among type I DGKs (α , β , γ), only DGK α was expressed

in several human melanoma cell lines including AKI but not in noncancerous normal human epidermal melanocytes (NHEM). In order to search for melanoma-specific functions of DGK α, this isoform was overexpressed and, conversely, down-regulated in AKI melanoma cells by transfecting DGK α expression plasmids and small interfering RNA (siRNA), respectively. Interestingly, this study clarified that DGK α negatively regulates TNF- α -induced apoptosis in the human melanoma cells through activation of NF-κB.

2. Materials and Methods

2.1. Cell culture

AKI and MMAc human melanoma cell lines were generous gifts from Drs. T. Moriuchi and J. Hamada (Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan). G361, 70W, SK-mel-23 and SK-mel-118 human melanoma cell lines were kindly provided by Dr. A. N. Houghton (Memorial Sloan-Kettering Cancer Center, New York, NY). These melanoma cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Tokyo, Japan) containing 10% fetal bovine serum (Roche Diagnostics, Tokyo, Japan) and antibiotics (penicillin, 100 U/ml; streptomycin, 100 □g/ml; Invitrogen, Tokyo, Japan) at 37 °C in an atmosphere containing 5% CO_2. NHEM was purchased from Kurabo (Tokyo, Japan) and maintained in Medium 254 (Cascade Biologics, Portland, OR) containing Human Melanocyte Growth Supplement (bovine pituitary extract, 0.2%; fetal bovine serum, 0.5%; bovine insulin, 5 □g/ml; bovine transferrin, 5 □g/ml; basic fibroblast growth factor, 3 ng/ml; hydrocortisone, 0.18 □g/ml; heparin, 3 □g/ml; phorbol 12-myristate 13-acetate, 10 ng/ml; Cascade Biologics) at 37 °C in an atmosphere containing 5% CO_2.

2.2. Plasmids

The cDNAs encoding wild-type versions of pig DGK α [26], rat DGK β [33] and human DGK γ [30] were amplified by PCR and subcloned into pEGFP-C3 (Takara Bio-Clontech, Tokyo, Japan). The kinase-dead (KD) version of green fluorescent protein (GFP)-DGK α was generated replacing Gly-435 in its catalytic domain with Asp using the QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) [34]. Cells were transiently transfected with cDNAs using Effectene transfection reagent (Qiagen, Tokyo, Japan) according to the instructions from the manufacturer.

2.3. Antibody

Anti-human DGK□ polyclonal antibody was prepared as follows: the cDNA fragment encoding amino acids 1–134 of human DGK β was ligated with the pGEX-6P vector (GE Healthcare Bio-Sciences, Piscataway, NJ). The GST-tagged DGK□-1–134 fusion protein was bacterially expressed and purified using glutathione Sepharose 4B (GE Healthcare Bio-Sciences). Rabbits were immunized by intramuscular multiple injections of 200 μ g of the fusion protein emulsified with an equal volume of Freund's complete adjuvant (Wako Pure Chemicals, Tokyo, Japan). The serum obtained after the fourth injection was used. This antibody did not react with DGKs α and γ (data not shown). Anti-pig DGK α polyclonal (cross-reactive with the human enzyme) [35] and anti-human DGK γ polyclonal [30]

antibodies were prepared as described previously. Anti-NF-κB p65 mouse monoclonal (F-6), anti-phosho-Akt rabbit polyclonal (Ser 473), anti-actin rabbit polyclonal (C-11) and anti-GFP mouse monoclonal (B-2) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Akt mouse monoclonal antibody was purchased from BD Biosciences-Pharmingen (San Diego, CA). Anti-extracellular signal-regulated protein kinase (ERK) rabbit polyclonal and anti-phospho-ERK rabbit polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA).

2.4. RNA interference

To silence the expression of human DGK α [27], the following oligonucleotides (iGENE Therapeutics, Tsukuba, Japan) were used: DGKα sense; 5'-CAAAGAUCCUCAAGGAUUUAGAGAU-AG-3', DGKα antisense; 3'-UA-GUUUCUAGGAGUUCCUAAAUCUCUA-5' (nucleotide 1658 – 1681 in the open reading frame). As a negative control, the following oligonucleotides targeting GFP, which is not expressed in mammalian cells, were used: GFP sense; 5'-ACGGCAUCAAGGUGAACUUCAAGAU-AG-3', GFP antisense; 3'-UA-UGCCGUAGUUCCACUUGAAGUUCUA-5'. The annealed oligonucleotide duplex, siRNA, (15 nM) was transfected into cells using HiPerFect transfection reagent (Qiagen) according to the instructions from the manufacturer.

2.5. Western blot analysis

Cells were transfected with cDNAs encoding various DGK constructs N-terminally fused with GFP or with siRNA targeting DGK α or GFP (control). After cells were harvested in lysis buffer (150 mM NaCl, 20 mM Tris-HCl (pH 7.2), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, Protease Inhibitor Cocktail (1 tablet/50 ml, Roche Diagnostics)), they were sonicated for 1 min and then centrifuged at 400 X g for 5 min at 4 °C to give cell lysates. Protein concentration was determined by using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Equal amounts of protein were resolved in SDS-PAGE (7.5% or 10% acrylamide). The separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Tokyo, Japan) and blocked with Block Ace (Dainippon Pharmaceutical). The membrane was incubated with anti-DGK α [35], anti-actin, anti-GFP, anti-Akt, anti-phospho-Akt or anti-NF-□B antibody in Block Ace for 1 h. When anti-ERK and anti-phospho-ERK antibodies were used, the membrane was blocked with blocking buffer (10 mM Tris-HCl, pH7.4, 150 mM NaCl, 0.1 % Tween-20 with 5 % nonfat dry milk) and then incubated with the antibodies in dilution buffer (10 mM Tris-HCl, pH7.4, 150 mM NaCl, 0.1 % Tween-20 with 5 % bovine serum albumin) for 12 h at 4 °C. The immunoreactive bands were visualized using peroxidase-conjugated anti-mouse, anti-rabbit or anti-goat IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and ECL (GE Healthcare Bio-Sciences). To measure the relative density of immunoreactive bands, images were scanned and analyzed by Image J software (National Institute of Health, Bethesda, MD).

2.6. Reverse transcriptase (RT)-polymerase chain reaction (PCR)

Total RNA was isolated from 70W, AKI, G361, MMAc, SK-mel-23, SK-mel-118 or NHEM cells using Isogen (Nippon Gene, Tokyo, Japan) according to the instructions from the manufacturer. Reverse transcription into cDNA was achieved

using the SuperScript III First-Strand Synthesis System (Invitrogen) according to the protocol from the manufacturer. PCR amplification was performed with Takara Ex Taq (Takara Bio) using gene-specific oligonucleotide primers as follows: human DGK□ forward primer, 5'-TCCCTTCTGGAGGGTGGTCGG-3' (nucleotides 413–433); human DGK α reverse primer, 5'-CTCCAGACCCAGCAGCACTAG-3' (nucleotides 694–674) [27, 34]; human DGK α forward primer, 5'-GCAAAGTATAATCCTGAAGGG-3' (nucleotides 127–147); human DGK□□reverse primer, 5'-CTGTCCATCATCCCTTCACGTT-3' (nucleotides 712–732) [36] (GenBank accession No. NM 004080); human DGK α forward primer, 5'-TCCCTGCTGGAGACGGGGAGG-3' (nucleotides 1021–1041); human DGK□□reverse primer, 5'-ATCCATCCCCAGGAGCACCAG-3' (nucleotides 1302–1282) [30, 34]. PCR conditions were as follows: 94 °C for 4 min; 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min; and 72 °C for 5 min. For normalization, human glyceraldehyde-3-phosphate dehydrogenase mRNA was simultaneously amplified (25 cycles). PCR products amplified were separated by agarose gel electrophoresis and stained with ethidium bromide.

2.7. Fluorescence microscopy

AKI cells were grown on poly-L-lysine-coated glass coverslips and transfected with either cDNAs encoding various DGK α constructs N-terminally fused with GFP or siRNA targeting DGK α or GFP. After 24 h, cells were incubated with or without 50 ng/ml of TNF- α (Strathmann Biotec, Hamburg, Germany) for 12 h. The cells were then fixed with 3.7 % formaldehyde in phosphate-buffered saline (PBS) for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min. Coverslips were then incubated with anti-NF-κB p65 antibody in 2% bovine serum albumin-PBS for 1 h, and further incubated with Alexa Fluor 594-conjugated secondary antibody (Molecular Probes, Eugene, OR) in 2% bovine serum albumin-PBS for 1 h. The coverslips were mounted using Vectashield (Vector Laboratories, Burlingame, CA). Cells were examined using an inverted confocal laser scanning microscope (LSM 510, Carl Zeiss, Tokyo, Japan). Images were scanned and analyzed by Image J software.

2.8. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

AKI or SK-mel-118 cells were grown on poly-L-lysine-coated glass coverslips and transfected either with cDNAs encoding various GFP-DGK constructs or with siRNA targeting DGK□. After 24 h, cells were incubated with or without 50 ng/ml of TNF-□ for 24 h. The cells were then fixed with 3.7 % formaldehyde in PBS for 10 min and permeabilized with 0.1 % Triton X-100 in PBS for 5 min. Cell apoptosis was detected by TUNEL assay using the In Situ Cell Death Detection Kit (Roche Diagnostics) according to the instructions from the manufacturer. The coverslips were mounted using Vectashield. Cells were examined using an inverted confocal laser scanning microscope (LSM 510). Random fields (at least ten fields per experiment) were analyzed, and TUNEL-positive cells were counted. In the case of GFP-DGK overexpression experiments, TUNEL-positive cells that expressed GFP were counted. A minimum of 1000 cells/sample was scored for apoptotic changes (TUNEL positive).

2.9. Fluorescence activated cell sorting (FACS)

AKI cells were grown on 60-mm dishes and transfected with siRNA. After 24 h, cells were incubated with or without 50 ng/ml of TNF-α for 48 h. Adherent and floating cells were then collected together and washed by ice-cold PBS. Cells were dehydrated in 75 % cold ethanol and stored on ice for 2 h. Then, cells were rehydrated in ice-cold PBS and treated with RNase A (50 μg/ml, Sigma-Aldrich) at 37 °C for 30 min. After the incubation, cells were rinsed twice in ice-cold PBS and resuspended in 2.0 ml PBS containing 50 μg/ml of propidium iodide (Sigma-Aldrich) at 4 °C for 2 h. Cell debris and fixation artifacts were gated out, and sub-G1, G1, S and G2/M populations were quantified with a FACScan Cell Sorter (BD Biosciences, Tokyo, Japan) using the CellQuest software (BD Biosciences).

2.10. Cell fractionation

Cytosol, membrane/organelle, nuclear and cytoskeletal fractions were isolated using the ProteoExtract Subcellular Proteome Extraction Kit (Merck Biosciences-Calbiochem, Tokyo, Japan) according to the instructions from the manufacturer.

2.11. Luciferase reporter assay

AKI cells were seeded in 12-well plates. Cells were co-transfected with pNF-□B-Luc Vector (Takara-Bio-Clontech), pSV-β-Galactosidase Control Vector (Promega, Tokyo, Japan) and with either cDNAs encoding various DGK constructs or siRNA targeting DGKα. After 24 h, cells were incubated with or without 50 ng/ml of TNF-α for 12 h. Lysates were collected in Glo Lysis Buffer (Promega) and mixed with Steady-Glo (Promega). Luciferase activity in the lysates was measured by Wallac 1420 ARVOsx multilabel plate reader (PerkinElmer, Tokyo, Japan) and was normalized to β-galactosidase activity.

3. Results

3.1. DGKα is expressed in melanoma cells but not in noncancerous melanocytes

Mammalian DGK is known to exist as a large protein family being composed of ten isoforms [11-14, 25]. However, the patho-physiological functions of DGK isoforms in melanoma cells remain to be clarified. Thus, as a first step, we examined the expression levels of type I DGKs (α, β, γ) in several human melanoma cell lines by RT-PCR and Western blot analyses. We found that, in all melanoma cell lines examined, DGKα mRNA and protein were clearly expressed (Fig. 1A and Table 1). The DGK□ protein was in particular enriched in AKI and SK-mel-118 cells (Fig. 1A). However, the expression of both mRNA and protein for other type I DGKs (β and γ) was not detectable (Table 1). In Western blot analysis of DGK□ (Fig. 1A), anti-DGKα antibody recognized a lower band (70-kDa) in addition to the expected band (80-kDa). Because intensities of both 80-kDa and 70-kDa bands were markedly decreased by siRNA specifically targeting DGKα (see Figs. 3A and 4A), we conclude that the lower band represents another form of DGK□ protein probably generated by proteolysis or alternative splicing. Because there appeared to be only one band of the GFP-tagged DGKα of the expected size (see Fig. 2C), alternative splicing is a more likely mechanism underlying the generation of the two bands. Interestingly, in noncancerous NHEM, the DGKα protein was not detected (Fig. 1B). Moreover, the DGK□ mRNA also failed to be detected in NHEM (Fig. 1C). These results

and that its expression is at least in part regulated at the transcriptional level.

3.2. DGK α suppresses apoptosis of human melanoma cells

To assess melanoma cell-specific functions of DGK α, this isoform was overexpressed or, conversely, down-regulated in AKI human melanoma cells. We examined the effects of DGK α overexpression/knockdown on apoptosis because of three reasons as follows: first, because we have already happened to find that when DGK α was overexpressed in COS7 cells, the cells appeared to become resistant to apoptosis induced by TNF-α (Sakane et al. unpublished data); second, because it was recently suggested using a DGK inhibitor R59949 that DGK α is involved in the apoptosis protection in T-lymphocytes [37, 38]; and third, because it is known that melanoma cells are generally more resistant compared to apoptosis than noncancerous melanocytes [4-6]. We first confirmed that TNF-α induced a ~4-fold increase in apoptosis 24 h after the addition of the cytokine as determined by TUNEL assay (Fig. 2, A and B). Interestingly, the overexpression of wild-type (WT) DGK□ (Fig. 2C) considerably suppressed the TNF-□-induced apoptosis of AKI cells almost to the basal level without TNF α (Fig. 2, A and B). However, although the expression level of a kinase-dead (KD) mutant of DGK α was almost the same as that of the WT enzyme (Fig. 2C), this mutant failed to affect the extent of the apoptosis (Fig. 2, A and B). Therefore, the catalytic activity of DGK α is essential for suppressing apoptosis. Moreover, in contrast to DGK α-WT, the overexpression of DGK β-WT and DGK γ-WT (Fig. 2C) slightly enhanced rather than suppressed the apoptosis (Fig. 2, A and B), indicating that this anti-apoptotic effect is α-isoform-specific.

In the reverse experiment, we next silenced DGK α expression in AKI melanoma cells by transfecting siRNA that specifically targeted DGK□ mRNA. Successful silencing was confirmed by Western blotting 48 h after transfection (Fig. 3A). In marked contrast to the DGK α-overexpression (Fig. 2), the siRNA-mediated knockdown of DGK α significantly enhanced TNF-□-induced apoptosis (at 24 h) of AKI melanoma cells by ~2.5-fold in comparison with the mock- and the control siRNA-transfections (Fig. 3, B and C). Moreover, siRNA targeting DGK γ used as an additional control did not affect the apoptosis (data not shown). Therefore, these results more convincingly indicate that DGK α specifically suppresses the TNF-α-induced apoptosis of AKI melanoma cells. In the siRNA experiments, only ~5% of AKI cells was stained by TUNEL assay even in the presence of TNF-α (Fig. 3, B and C). This value is considerably lower than that (~20%) of the cells overexpressing GFP (Fig. 2, A and B). However, in any cases, apoptosis was induced in a TNF-α-dependent manner. We consider that this discrepancy may be due to the use of Effectene (for the overexpression experiments), which appeared to be more toxic to cells than HiPerFect (for the siRNA transfection) in preliminary experiments (data not shown). In this case, the overexpression of GFP alone also appeared to cause some damage to cells when challenged with TNF-α (not shown).

To validate the enhancing effect of DGK α siRNA on apoptosis, we FACS-analyzed AKI cells stained with propidium iodide. When AKI cells were treated with TNF-α for 48 h, the percentage of cells falling in the sub-G1 gate (apoptotic cells) increased from 10 to 28 (Fig. 3D). Compatible with the results obtained by the TUNEL method (Fig. 3, B and C), DGK α-silenced cells died more

quickly, with 42% of cells falling in the sub-G1 gate. Taken together, these results indicate that DGK α specifically suppresses AKI cell apoptosis induced by TNF- α.

We next asked whether these observations could be required in other melanoma cell lines. As shown in Fig. 4A, the DGK α protein was successfully depleted in SK-mel-118 melanoma cells when introduced with the DGK α -specific siRNA. SK-mel-118 cells treated with TNF- α for 24 h exhibited a ~7-fold increase in apoptosis compared to untreated cells. Similar to the results obtained with AKI cells (Fig. 3), the siRNA-mediated knockdown of DGK□ considerably enhanced TNF-□-induced apoptosis in SK-mel-118 cells (Fig. 4, B and C), suggesting that the negative regulation of apoptosis by DGK α commonly occurs in melanoma cells expressing this isoform.

3.3. Antiapoptotic effects of DGK α were mediated by NF-κB activation

We next sought to delineate how DGK α conferred the survival features on AKI melanoma cells. It is known that the main forces limiting melanoma cell death are survival signals derived from the activation of the phosphatidylinositol 3-kinase/Akt-, the Raf/extracellular signal-regulated protein kinase- and the NF-κB-pathways [6]. However, phosphorylation (activation) levels of Akt and ERK failed to be considerably affected by DGK α -knockdown in TNF- α -treated AKI cells (Fig. 5, A and B). Thus, we next investigated whether DGK α regulated the NF-κB transcriptional activity. To this end, we used a reporter construct with a promoter containing four tandem copies of NF-□B binding sites upstream of the firefly luciferase reporter coding region. We first confirmed that the transcriptional activity of NF-κB was augmented 12 h after the addition of TNF-□ (Fig. 5C). Intriguingly, the overexpression of DGK α -WT further enhanced the TNF-□-induced NF-κB activation compared with the vector control. In accord with the apoptosis suppression experiment (Fig. 2, A and B), the overexpression of DGK□-KD did not affect the NF-κB (luciferase) activity. Although the NF-κB activity was relatively low, essentially the same results were obtained using AKI cells 2 h after the addition of TNF- α (data not shown). Conversely, the DGK□-knockdown considerably inhibited the TNF- α dependent NF-κB activity (~30% inhibition) (Fig. 5D). In these experiments, we confirmed that the expression levels of NF-κB were not changed in AKI cells transfected with the DGK α cDNA constructs or the DGK α siRNA (data not shown). Taken together, these results indicate that DGK α positively controls the NF-κB activity.

Upon activation, NF-κB is known to translocate from the cytoplasm to the nucleus [7, 8]. This translocation step is essential for NF-κB activation and can be visualized using indirect immunofluorescence. Thus, to substantiate the effect of DGK α on NF-κB action, we next investigated subcellular localization of NF-κB in TNF- α -treated AKI melanoma cells. We confirmed using confocal microscopy that a nucleus/cytoplasm (N/C) ratio of NF-κB was markedly increased by the cell treatment with TNF- α for 12 h (Fig. 6, A and B). Compatible with the NF-κB (luciferase) activity assay (Fig. 5C), the overexpression of DGK□-WT considerably increased the TNF- α -induced nuclear localization of NF-κB when compared with the vector control (Fig. 6, A and B). On the other hand, the overexpression of DGK α -KD did not affect the NF-κB nuclear localization again.

Conversely, DGK□-knockdown considerably inhibited the TNF- α -dependent

NF-κB translocation (~30% inhibition) as judged by confocal microscopy (Fig. 6, C and D). We further studied the NF-κB translocation by a biochemical method, *i.e.* cell fractionation, in order to verify the inhibitory effect of DGK α -knockdown. As shown in Fig. 6E, TNF- α markedly induced the nuclear translocation of NF-κB. In accord with the results obtained by the cell biological method (confocal microscopy), the DGK α -knockdown weakly but reproducibly suppressed the TNF- α -dependent NFκ-B translocation to the nucleus (~30% less nuclear localization than that of control siRNA) at every time point (30 min, 2 h and 12 h) after TNF- α treatment. Therefore, using distinct approaches (cell biological and biochemical methods), essentially the same results were obtained. These results collectively indicate that DGK α positively regulates TNF- α -induced nuclear translocation and the subsequent activation of NF-κB.

To implicate the NF-κB transcriptional activity in the DGK α -mediated suppression of melanoma cell apoptosis, we evaluated the effect of a proteasome inhibitor MG-132, which blocks the activation of NF-κB [39-41]. This compound inhibits NF-κB activity through inactivation of proteasome, which degrades IκB [41]. We validated that MG-132 markedly decreased the NF-κB activity enhanced by DGK α overexpressed (Fig. 7A). Moreover, although the overexpression of DGK α -WT significantly suppressed TNF- α -induced apoptosis of AKI cells as given in Fig. 2, A and B, MG-132 completely blunted the anti-apoptotic effect of the DGK α overexpression (Fig. 7, B and C). Together, these results strongly suggest that the negative regulation of TNF- α -induced apoptosis by DGK α is mediated by a pathway that requires NF-κB action.

4. Discussion

Melanoma is the most aggressive form of skin cancer and its incidence has increased more than 6-fold over the past 50 years. In 2003, metastatic melanoma was estimated to have caused 7600 deaths in the United States and this disease was the second greatest cause of lost productive years among cancers [42, 43]. Although exact mechanisms remain to be explored, melanoma has proven to be highly resistant to conventional chemotherapy [1-3]. Thus far, the options of effective treatment have been limited. Hence, it is imperative to investigate new therapeutic targets for the treatment of melanoma in order to improve the dismal prognosis for this disease. In this regard, NF-κB has emerged as one of the key factors to facilitate melanoma tumorigenesis through negative control of apoptosis [4-6] and, consequently, as a target for the treatment of the disease [4]. Here, we show that DGK α is a negative regulator of TNF- α -induced melanoma cell apoptosis. Moreover, we present multiple lines of evidence that support a pivotal role for DGK α in NF-κB activation, leading to the suppression of apoptosis. In T-lymphocytes, DGK α was implicated in the suppression of apoptosis using a non-selective DGK inhibitor, R59949 [37, 38]. In the present study, however, we more directly and convincingly demonstrate the involvement of DGK α in apoptosis of melanoma cells using siRNA that specifically knocks down the isoform. Therefore, our work also shows for the first time that DGK α acts as a potent anti-apoptotic factor in not only T-lymphocytes but also cancer-derived, melanoma cells. It is thus possible that decreasing intracellular DGK α activity is an attractive strategy to modify the

outcome of this devastating disease.

DGK α was recently reported to be involved in apoptosis of T lymphocytes induced by T-cell receptor (TCR) stimulation [38]. Moreover, it was suggested that, in this event, DGK α negatively regulates TCR-mediated cell death through reducing Fas ligand release, which mediates apoptotic responses [37]. However, in AKI melanoma cells, cell treatments with high concentrations of anti-Fas antibody (0.5 – 5 μ g/ml) for 24 – 48 h barely induced apoptosis (data not shown), strongly suggesting that, unlike T-lymphocytes, the Fas ligand release is not the main pathway of apoptosis execution in melanoma cells. In the present study, we found that the NF-κB pathway, but not the Fas ligand release, plays a central role in the apoptosis suppression caused by DGK α in melanoma cells. Are these observations obtained for T-cells and melanoma cells completely contradictory to each other? Interestingly, NF-κB was reported to negatively regulate the TCR-mediated cell death [44], implying that DGK α may suppress apoptosis via NF-κB activation also in T-lymphocytes. Moreover, in T-lymphocytes, DGK□ enhanced expression of anti-apoptotic factor, Bcl-x_L [38], which is transcriptionally regulated by NF-κB [4-6], further supporting this notion. Thus, a possible explanation for the contradictory observations described above might be that, although DGK α commonly regulates NF-κB activity, distinct pathways downstream of NF-κB are utilized by different stimulations (TCR ligands and TNF- α) and/or in different cell lines (T-lymphocytes and melanoma cells).

Because the inactive mutant, DGK□-KD, failed to affect the extent of NF-κB activation (Fig. 5C), DAG consumption or PA production plays an important role in the regulation of NF-κB activity. However, there are apparently no reports describing direct regulation of NF-κB activity by DAG or PA. Thus, it remains unclear how DGK α regulates NF-κB activity. Moreover, it is also not known how DGK□ activity is regulated when melanoma cells are stimulated with TNF- α. Because only DGK α among the type I DGK isoforms (DGKs α, β and γ) suppressed the TNF- α -induced apoptosis of AKI melanoma cells (Fig. 2), the properties of DGK α different from those of DGKs α and β may provide some cues. In this context, we noted three major different properties. First, type I DGK isoforms exhibited different affinities for Ca^{2+} [32]. Although DGK α is activated by 0.1 – 1 μ M of Ca^{2+}, DGKs β and γ are constitutively active and their activities are apparently insensitive to Ca^{2+} [32]. Because an increased concentration of Ca^{2+} in response to cell stimulation with TNF- α plays an important role in the suppression of apoptosis [45, 46], it is possible that Ca^{2+}-dependent DGK activation regulates NF-□B activity. Thus, the sensitivity of DGK α to Ca^{2+} may be the reason why this isoform specifically suppresses the apoptosis.

Second, only DGK α was reported to be phosphorylated and activated by c-Src tyrosine kinase, with which this isoform forms a complex [47, 48]. It is worthy to note that in several hematopoietic cell lines, the c-Src-mediated activation of DGK α is required for cell proliferation where apoptosis is suppressed [47]. Moreover, c-Src was recently reported to positively regulate TNF- α -mediated NF-κB activation in endothelial cells [49]. Thus, the association with c-Src may be critical for the activation of DGK α leading to the prevention of apoptosis.

Finally, subcellular localizations are different among the type I DGK isoforms. In thymocytes and peripheral T-lymphocytes, DGK α was translocated to the

nucleus by cell stimulation with concanavalin A or anti-TCR antibody [50]. Interestingly, DGKα overexpressed in AKI cells (Fig. 6A, N/C ratio: 0.70 ± 0.20) and other melanoma cells (data not shown) was partly localized in the nucleus even without cell stimulation. Moreover, endogenous DGKα displayed essentially the same distribution pattern (data not shown). On the other hand, DGKα was reported to be distributed to the plasma membrane in the brain and fibroblasts [33, 51]. We confirmed that, indeed, this isoform was exclusively located at the plasma membrane, being almost absent in the nucleus in AKI melanoma cells (Yanagisawa et al. unpublished data). Although DGKα is known to show cytoskeletal localization [29, 51], it was recently reported that, a few days after transfection, DGKα became to be localized in the nucleus in several cell lines such as CHO-K1, NIH3T3 and SH-SY5Y [52]. However, we confirmed that DGKα was detected mainly in the cell periphery and the cytoplasm with very little localization in the nucleus of AKI melanoma cells (Yanagisawa et al. unpublished data). Thus, it is possible to speculate that DGKβ and DGKγ failed to affect apoptosis because of their lack of significant nuclear localization.

As for nuclear localization signals of DGK, a region that is homologous to the phosphorylation-site domain of the myristoylated alanine-rich C-kinase substrate protein is known to be essential for the nuclear transport of DGK□ [53, 54]. However, DGK□ does not contain this motif. Recently, it was reported that DGK□ mutants having substitution of Cys-285 to Gly in the C1A domain or Cys-348 to Gly in the C1B domain failed to translocate into the nucleus in CHO-K1 cells [52]. Similarly, DGK□ mutants having the corresponding substitution (Cys-221 or Cys-284 to Gly) also showed significantly reduced nuclear localizations (N/C ratio: 0.14 ± 0.04 and 0.15 ± 0.04, respectively) (Yanagisawa et al. unpublished data), suggesting that the C1 domains play a pivotal role in the nuclear translocation of this isozyme. Interestingly, we also found that these DGKα mutants failed to affect NF-κB activity (Yanagisawa et al. unpublished data). Although we cannot deny the possibility that these mutations may affect DGKα functions other than the nuclear localization, the results support the notion that DGK□ localized in the nucleus may regulate NF-κB activity. Moreover, degradation of IκB, which occurs in the cytoplasm, was not affected by DGK□-silencing (data not shown), further supporting this hypothesis. However, at present, it is still unclear whether the three unique properties of DGKα as noted above are responsible for the regulation of NF-kB activity. Further studies are required to elucidate this issue.

Our study has highlighted DGKα as a novel regulator of NF-κB, acting as a potent anti-apoptotic factor in melanoma. However, the mechanisms linking the activities of DGKα and NF-κB remain unknown. Future studies exploring the mechanism by which DGKα controls NF-κB activity will help to elucidate in greater detail how the action of NF-κB, which regulates the transcription of wide array of genes, is precisely controlled. In addition to melanoma, many different neoplasms have been shown to express high levels of NF-κB [4-6]. Moreover, the blockade of NF-κB activity in several cancer cells was reported to be associated with the suppression of carcinogenesis and metastasis [55, 56]. Thus, knowledge obtained from future studies on the regulatory mechanisms of NF-κB through DGKα activity in melanoma cells can be extended to other neoplasms. Moreover, genetic and pharmacological manipulation of DGKα activity should be an attractive approach for therapy of not only melanoma but also many other cancerous diseases.