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

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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 DGKα 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-α-stimulated transcriptional activity of an anti-apoptotic factor, NF-κB. Conversely, DGKα-knockdown considerably inhibited the NF-κB activity. Moreover, an NF-κB inhibitor blunted the anti-apoptotic effect of DGKα overexpression. Together, these results strongly suggest that DGKα is a novel positive regulator of NF-κB, which suppresses TNF-α-induced melanoma cell apoptosis.

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

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-κB 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

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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 TNFR-1 activation results in the TNF-α-dependent cell death in melanoma cells.

It is well recognized that a variety of lipid second messengers in low abundance 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, DGKα 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 DGKα 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 DGKα, this isoform was overexpressed and, conversely, down-regulated in AKI melanoma cells by transfecting DGKα expression plasmids and small interfering RNA (siRNA), respectively. Interestingly, this study clarified that DGKα 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 μg/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 μg/ml; bovine transferrin, 5 μg/ml; basic fibroblast growth factor, 3 ng/ml; hydrocortisone, 0.18 μg/ml; heparin, 3 μg/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 DGKα [26], rat DGKβ [33] and human DGKγ [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)-DGKα 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 DGKβ polyclonal antibody was prepared as follows: the cDNA fragment encoding amino acids 1–134 of human DGKβ was ligated with the pGEX-6P vector (GE Healthcare Bio-Sciences, Piscataway, NJ). The GST-tagged DGKβ-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 μg of the fusion protein emulsified with an equal volume of Freund's incomplete adjuvant (Wako Pure Chemicals, Osaka, 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 DGKα polyclonal (cross-reactive with the human enzyme) [35] and anti-human DGKγ 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 DGKα [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.